BIOCHEMICAL AND EPIDEMIOLOGICAL ANALYSIS OF *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* AND INVESTIGATION OF ITS RELATIONSHIP TO CROHN'S DISEASE IN HUMANS

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Title

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

ABSTRACT

Uzoigwe, Jacinta C., Ph.D., Program of Biochemistry; College of Science and Mathematics, North Dakota State University, March 2011. Biochemical and Epidemiological Analysis of *Mycobacterium avium* Subspecies *paratuberculosis* and Investigation of its Relationship to Crohn's Disease in Humans. Major Professor: Prof. S. Derek Killilea.

Background: Crohn's disease is a chronic inflammatory disease of the intestine in humans, with no known cause. Johne's disease is a chronic intestinal disease of ruminants caused by *Mycobacterium avium subspecies paratuberculosis* (MAP), and has some features similar to Crohn's disease. Although MAP has been purported to play an etiologic role in Crohn's disease, this causal link is still under debate. **Objective:** The overall aim of this project is to analyze MAP strains from different hosts (cattle, sheep and humans) and regions in North Dakota by biochemical and epidemiological methods, in order to better understand the pathogenesis and epidemiology of MAP strains and the relationship between MAP and Crohn's disease. The specific aims of this research are the following: **Aim 1.** Investigate the epidemiological evidence for MAP as a cause of Crohn's disease. **Aim 2.** Conduct a comparative causality study to investigate whether MAP or other enteric pathogens cause Crohn's disease. **Aim 3.** Evaluate the occurrence of MAP infections in cattle in North Dakota, 1995-2005. **Aim 4.** Analyze MAP strains from symptomatic and asymptomatic cattle. **Aim 5.** Investigate the biochemical variations of rapid and slow growing MAP strains. **Aim 6.** Evaluate MAP strains from low shedders and high shedders. **Methods:** MAP isolates were analyzed by biochemical methods of gas chromatography, high performance liquid chromatography and mass spectrometry. In addition, extensive literature review was performed to (1) determine the epidemiologic causal link between MAP and Crohn's disease and (2) determine whether MAP or other enteric pathogens

cause Crohn's disease. **Results:** Results from our study indicated the availablity of epidemiologic evidence supporting the causal role of MAP in Crohn's disease. It was also demonstrated that MAP is the most implicated organism in the etiology of Crohn's disease when compared to other infectious agents. Investigation of the occurrence of MAP infection in North Dakota showed an increase in number of MAP cases reported, with seasonal trends. Biochemical typing of MAP strains from symptomatic and asymptomatic cattle indicated that the symptoms status of isolates was significantly associated with mass spectra patterns and shedder status ($p < 0.05$). However, the association between symptoms status and HPLC and GC patterns was not significant ($p > 0.05$). Investigation of biochemical variations of rapid and slow growing MAP strains showed associations between the biochemical variability of MAP strains and their growth rate and presence of symptoms in the source cattle. Evaluation of MAP strains of different shedding characteristics by univariate logistic regression revealed that the shedder status of isolates was significantly associated with growth rate of isolates, symptom status, and source regions, but not with mass spectra patterns of isolates. **Conclusion:** Overall, this study strengthens the theories of strain sharing, intraspecies and interspecies transmission, and supports an association between MAP and Crohn's disease. In addition, the understanding of the biochemical variation among MAP isolates will help in the future design of diagnostics, therapeutics and vaccines for Johne's and Crohn's diseases.

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LIST OF ABBREVIATIONS

AIEC..... Adhesive-invasive *Escherichia coli*

ATCC.....American Type Culture Collection

CARD.....Caspase activation recruitment domain

CDC..... Centers for Disease Control and Prevention

CFU.....Colony forming unit

CMV..... Cytomegalovirus infection (CMV

Bruker BioTOF.....Reflectron Electrospray Ionization Time-of-Flight

BHI.....Brain heart infusion

CD.....Crohn's disease

DNA.....Deoxyribonucleic acid

EBV.....Epstein-Barr virus

EDTA.....Ethylenediaminetetraacetic acid

ELISA.....Enzyme-linked immunosorbent assay

ESI-TOF.....Electrospray Ionization Time-of-Flight

FAMES.....Fatty acid methyl esters

GC.....Gas chromatography

HPC..... Hexadecylpyridinium chloride monohydrate

HEY..... Herrold's egg yolk

HEYM.....Herrold's egg yolk medium

HEYMJ.....Herrold's egg yolk medium with mycobactin J

HHV 6.....Human Herpesvirus Six

HPLC..... High performance liquid chromatography

LIST OF ABBREVIATIONS

IgG..... Immunoglobulin G

MALDI.....Matrix-assisted laser desorption/ionization

MALDI-TOF.....Matrix-assisted laser desorption/ionization-Time-of-Flight

MAP.....*Mycobacterium avium* subspecies *paratuberculosis*

MNV.....Murine norovirus

MOSS.....Monitoring and surveillance systems

MS.....Mass spectrometry

IBD.....Inflammatory bowel disease

IL.....Interleukin

O.D.....Optical density

PBS.....Phosphate buffered saline

PCR.....Polymerase chain reaction

PFGE.....Pulsed field gel electrophoresis

RNA.....Ribonucleic acid

SDL.....State diagnostic laboratories

UC.....Ulcerative colitis

USDA.....U.S. Department of Agriculture

VJDCP.....Voluntary Johne's Disease Control Program

GENERAL INTRODUCTION

Mycobacterium paratuberculosis (MAP) causes a chronic inflammatory bowel disease in cattle and many other ruminant animals, referred to as Johne's disease (or paratuberculosis), which has many similarities with Crohn's disease. Crohn's disease is a chronic inflammatory disease of the gastrointestinal tract in humans. A controversial theory to date is the causal link between MAP and Crohn's disease. Despite the advances made in understanding human Crohn's disease, the etiology of this disease still remains unresolved. Clinically, Johne's disease is characterized by progressive weight loss leading to emaciation and often intermittent and constant diarrhea. It is reported that MAP is present in the milk, feces and meat of infected cattle.

The cell wall lipid component of MAP strains has been analyzed by use of biochemical techniques. However, to fully understand the pathogenesis, virulence and epidemiology of MAP strains, as well as the association between Johne's disease and Crohn's disease, it is important that more research be geared toward understanding the biochemical profiles of MAP strains with different growth, symptom, and shedding characteristics.

Our studies were divided into a number of sections that focused on epidemiological models of disease causation, the major philosophical doctrines about causation, the established epidemiological criteria for causation, and the currently known epidemiological evidence of *M. avium* subsp. *paratuberculosis* as a possible cause of Crohn's disease. The several lines of evidence that determine whether MAP or other enteric pathogens cause Crohn's disease, was examined. The trends and risk factors for MAP shedding in cattle in North Dakota were evaluated. The characteristics of MAP (e.g growth rate and shedding

status) that contribute to their virulence and pathogenity were also investigated. Furthermore, the association between presence of symptoms and growth rate of MAP strain was examined.

Overall, the data generated in our studies will improve our understanding of the differences in disease progression and pathogenicity of MAP isolates, as well as the relationship between MAP and Crohn's disease. These studies will also provide information that can be used to guide the future design of controls, diagnostics, therapeutics and vaccines for Johne's and Crohn's diseases.

Explanation of the dissertation organization

This dissertation consists of both biochemical and epidemiological studies and, is organized into six journal manuscripts. Paper 1 is a literature review of the epidemiological evidence for Mycobacterium avium subspecies paratuberculosis (MAP) as a cause of Crohn's disease. Paper 2 is another literature review that describes a comparative causality study of whether MAP or other enteric pathogens cause Crohn's disease. The remaining papers in this dissertation involve orginal research work, and each paper has its own introduction, materials and methods, results, discussion, conclusion and references. Paper 3 examines the occurrence of MAP infection in cattle in North Dakota, 1995-2005. Paper 4 describes the biochemical typing of MAP strains from symptomatic and asymptomatic cattle. Paper 5 investigates the biochemical variations of rapid and slow growing MAP strains. Paper 6 presents the analysis of MAP strains of different shedding characteristics. Finally, the general conclusions resulting from these studies are presented.

PAPER 1

EPIDEMIOLOGICAL EVIDENCE FOR *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* AS A CAUSE OF CROHN'S DISEASE

ABSTRACT

Mycobacterium avium subspecies *paratuberculosis* is the causative agent of Johne's disease, a chronic enteritis in ruminants including cattle, sheep, goats, and farmed deer. Recently, this bacterium has received an increasingly wide interest because of a rapidly growing body of scientific evidence which suggests that human infection with this microorganism may be causing some, and possibly all, cases of Crohn's disease. Recent studies have shown that a high percentage of people with Crohn's disease are infected with *M. avium* subspecies *paratuberculosis*; whether the association of this bacterium and Crohn's disease is causal or coincidental is not known. Crohn's disease is a gastrointestinal disease in humans with similar histopathological findings to those observed in the paucibacillary form of Johne's disease in cattle. The search for risk factors in Crohn's disease has been frustrating. However, epidemiologists have gathered enough information that points to an association between *M. avium* subsp. *paratuberculosis* and Crohn's disease. This paper reviews epidemiological models of disease causation, the major philosophical doctrines about causation, the established epidemiological criteria for causation, and the currently known epidemiological evidence of *M. avium* subsp. *paratuberculosis* as a possible cause of Crohn's disease.

INTRODUCTION

Mycobacterium avium subspecies *paratuberculosis* is a pathogenic bacteria in the genus Mycobacteria. It is often abbreviated as *M. paratuberculosis*, *M. avium* subsp. *paratuberculosis* or MAP. MAP causes paratuberculosis or Johne's disease, a chronic granulomatous gastroenteritis in ruminants (Harris and Lammerding). Johne's disease occurs worldwide and is primarily a disease of domesticated ruminants, including cattle (both beef and dairy), sheep, goats, and farmed deer (Tiwari et al. 2006; Kennedy and Benedictus, 2001). The host range for Johne's disease has been reported to include wild ruminant species, such as deer (Chiodini and Van Kruiningen, 1983; Buergelt and Ginn, 2000; Cook et al. 1997; Manning et al. 2003), as well as non-ruminants, such as wild rabbits (Greig et al. 1997; Beared et al. 2001a), their predators, including foxes and stoats (Beared et al. 2001b), and primates, such as mandrills and macaques (McClure et al. 1987; Zwick et al. 2000). The disease is characterized by profuse and intractable diarrhoea, severe weight loss and diagnostic changes in the lining of the small intestine (Grant, 1997; Collins et al. 2000). Crohn's disease is a chronic inflammatory disease of the intestines in humans (Hanauer, 1996). The disease primarily causes ulcerations of the small and large intestines, although it can affect the digestive system anywhere from the mouth to the anus. Common symptoms of Crohn's disease include severe bouts of watery or bloody diarrhoea, cramping, abdominal pain, fever, weight loss, and bloating (Hanauer, 1996). Morphological changes in Crohn's disease include chronic inflammation involving all layers of the intestinal wall (transmural involvement), thickening of involved segments, with narrowing of lumen, linear ulceration of the mucosa, submucosa oedema with elevation of the surviving mucosa, producing a characteristic cobblestone appearance.

Crohn's disease in humans has long been suspected of having a mycobacterial cause (Harris and Lammerding, 2001; El Zaatari et al. 2001; Hulten et al. 2000; Quirke, 2001). This proposition was first advanced by Dalziel, (1913). According to Clarke (1997), the histopathology of Johne's disease ranges from the more common pluribacillary or lepromatous form to the less common paucibacillary or paucimicrobial tuberculoid form like leprosy in humans. Due to the histopathological features of Crohn's disease closely resembling those found in animals with the paucibacillary form of Johne's disease, it has been suggested that the two diseases shared the same aetiology (Grant, 1997; Collins, 2000; Greenstein, 2003; Moss et a. 1992). The objectives of this paper were: (i) to review the epidemiological evidence involving the potential association of MAP with Crohn's disease in humans, and (ii) to determine if causation of Crohn's disease can be inferred based upon the evidence reviewed.

Epidemiology of Johne's disease

Mycobacterium avium subsp. *paratuberculosis* (MAP) is a member of the *M. avium* complex (Thorel et al. 1990). *M. avium* strains are widely distributed in the environment as well as in birds, animals, and humans (Hermon-Taylor, 2001; Primm et al. 2004; Wolinski and Rynearson, 1968). *M. avium* strains do not usually cause disease unless the host is debilitated or immunocompromised. By contrast MAP is a specific pathogen with the ability to cause chronic inflammation of the intestine (Johne's disease) in many species (Chiodini et al. 1984; Cocito et al. 1994; Harris and Barletta, 2001; Manning and Collins, 2001). MAP is a well recognized cause of disease and economic loss in dairy herds, and most control programmes have been designed for the dairy industry (Goodger et al. 1996;

Johnson-Iferulundu and Kanneene, 1999). It is estimated that nearly 40% of United States dairy herds are infected with MAP and that losses to the dairy industry may exceed $1.5 billion per year (Jones, 1989; Stabel, 1998). MAP is most commonly transmitted via the faecal–oral route (USDA, 1999; Reed et al. 2006). However, it can also be transmitted in the semen of bulls, in milk (or colostrum), and in utero across the placenta to the newborn calf (Tiwari et al. 2006). Moreover, it has been suggested that MAP can exist within the tissues of animals for years without causing clinical disease (Cetinkaya et al. 1996). Subclinically or clinically infected animals shed MAP in faeces and milk, enabling dissemination to susceptible calves, the environment, and in retail milk (Millar et al. 1996). MAP in milk may survive pasteurization (Millar et al. 1996). In the United Kingdom, the United States, and the Czech Republic, MAP has been cultured from 1.6% to 2.8% of units of retail pasteurized cow's milk (Millar et al. 1996; Grant et al. 2002; Ayele et al. 2005; Ellingson et al. 2005), and it has been suggested that live organisms might be transmitted to humans by this route.

Epidemiology of Crohn's disease

Crohn's disease occurs throughout the world, with a prevalence of 161–319 cases/100 000 people in Canada (Bernstein et al. 2006). It is most prevalent in Europe and North America (Scientific Committee on Animal Health and Animal Welfare, 2000). The disease affects between 400 000 and 600 000 people in North America alone (Loftus et al. 2002). Prevelance estimates for Northern Europe have ranged from 27–48/100 000 (Bernstein et al. 2006). The incidence of Crohn's disease in North America has been estimated at 6/100 000 per year, and is thought to be similar in Europe, but lower in Asia

and Africa (Hatt and Kaufman, 1988; Moum et al. 1996). The incidence of Crohn's disease in industrialized parts of the world has been reported to be increasing (Calkins and Mendelofff, 1986; Loftus et al. 1998; Hermon-Taylor et al. 1994; Armitage et al. 2001). The disorder occurs most frequently among people of European origin, is 3–8 times more common among Jews than among non-Jews (Podolsky, 2002). However, this excess risk is not evident in the Jewish population of Israel (Niv et al. 1999). Although the disorder can begin at any age, its onset most often occurs between 15 and 30 years of age (Loftus, 2004; Card et al. 2003; Shivananda et al. 1996; Lapidus et al. 1997). Satsangi et al. (1997) reported that parents, siblings or children of people with Crohn's disease were 3–20 times more likely to develop the disease than the general population. Twin studies show a concordance of greater than 55% for Crohn's disease (Tysk et al. 1988; Orholm et al. 2000; Thompson et al. 1996). Mutations in a gene called NOD2/CARD15 are associated with Crohn's disease (Ogura et al. 2001; Fielding, 1986; van Heel, 2000), and with susceptibility to certain phenotypes of disease location and activity (Cuthbert et al. 2002). The NOD2/CARD15 susceptibility does not apply to Chinese (Leong et al. 2003), Japanese (Inoue et al. 2002), Korean (Lee et al. 2005), Tunisian (Zouten-Mekki et al. 2005) or Turkish (Uyar et al. 2006) patients with Crohn's disease. A susceptibility locus for Crohn's disease has been mapped to chromosome 16 (Hugot et al. 2001). Three independent studies reported that mutations within the NOD2/CARD15 gene were strongly linked to Crohn's disease in Europeans (Ogura et al. 2001; Hugot et al. 2001; Hampe et al. 2001). However, Greenstein (2003) reported that the presence of a gene that is associated with an increased susceptibility to Crohn's disease does not preclude the possibility that the disease may be caused by an infectious agent. Another study (Inoue et al. 2002),

suggested the possibility of genetically identifiable subpopulations having different tendencies to develop Crohn's disease when exposed to the same infectious agent. Recent studies have identified an association between inflammatory bowel disease (IBD) and mutations in yet another gene termed NRAMP1 (also known as SLC11A1) (Kojima et al. 2001). This gene has been reported to be associated with both Crohn's disease and ulcerative colitis.

Epidemiological models for causation

Epidemiology is the scientific inquiry into the causation of disease; it is the search for the risk factors that cause the effect or the disease (Parascandola and Weed, 2001). In this search, various models or theories for causation have been developed over the years in an attempt to explain the interaction of risk factors and their effect on disease; Models are purposely simplified representations of that interaction (Rothman and Greenland, 2005). The various models of causation include: epidemiological triad/triangle (Last, 2001; Torrrence, 1997), web of causation (Krieger, 1994), wheel of causation (Mausner and Bahn, 1986) and Rothman's causal pie (Rothman and Greenland, 2005).

Epidemiological triangle/triad

This model makes the agent a component of causation along with the host and environment (Fig. 1.1). The model implies that all components are equally important in disease causation and that a change in any one of them would change the frequency of disease. The model applies to both infectious or noninfectious diseases. For instance, in Johne's disease the agent would be the bacterium, MAP; host factors include non-immune,

weakened resistance, poor nutrition, age, gender; and environmental factors include animal stocking density, poor environmental conditions (such as temperature, humidity, wind velocity, precipitation, poor housing as in crowded conditions, poor ventilation, and bad sanitation).

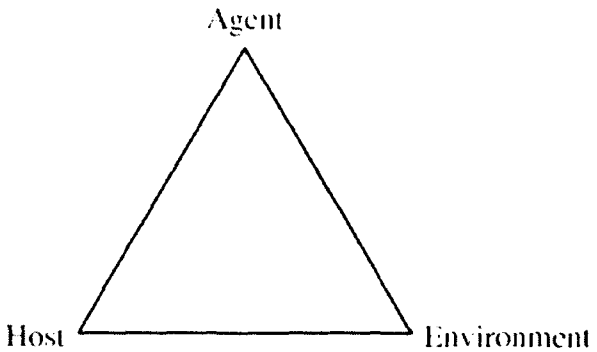

Figure 1.1: Epidemiological triangle/triad (Torrence, 1997).

Wheel of causation

The wheel model places genetic factors in the core of the wheel and varies the size of the host and environmental components depending on their influence in the particular disease process (Krieger, 1994). Surrounding the host is the total environment divided into the biological, physical, and social environments (Fig. 1.2). These divisions, of course, are not true divisions –there are considerable interactions among the environment types. Although it is a general model, the wheel of causation does illustrate the multiple aetiological factors of human infectious diseases (Mausner and Bahn, 1986). According to Jantchou et al. (2006), many environmental factors for IBD have been investigated, including infectious agents, diet, drugs, stress and social status. Among these factors, MAP, oral contraceptives and antibiotics could play a role in Crohn's disease (Jantchou et al. 2006; Sicilia, 2001; Chiodini and Rossiter, 1996; Dumonceau et al. 1996; El Zaatari et

al. 1995). Sicilia et al. (2001) reported that the pathogenesis of IBD probably involves an interaction between genetic and environmental factors: cigarette smoking, appendectomy and oral contraceptives are the factors most frequently linked to its aetiology.

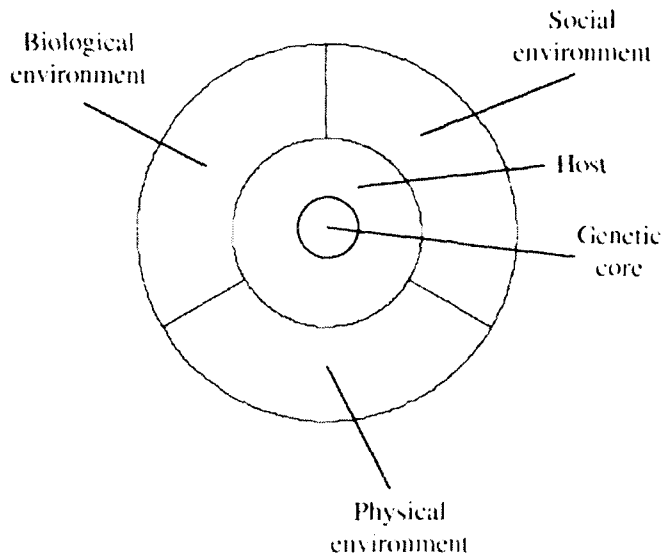

Figure 1.2: Wheel of causation (Mausner and Bahn, 1986).

Web of causation

This model refers to the 'web' of interconnected factors which lead to disease. The web of causation merely reflects the fact that there is a complex mixture or a 'web' of factors that can cause disease (Krieger, 1994). Many aetiological factors for Crohn's disease have been suggested, including autoimmune, genetic, dietary components plus various infectious agents including MAP (Chiodini and Rossiter, 1996; Dumonceau et al. 1996; El Zaatari et al. 1995).

Causal pie model

The main causal model used by epidemiologists today is Rothman's 'pies' (Rothman and Greenland, 2005). The idea is that a sufficient causal complex (a pie) is represented by the combination of several component causes (slices of the pie) (Fig. 1.3). A set of component causes occurring together may complete the 'pie ', creating a sufficient cause and thus initiating the disease process. Rothman and Greenland (2005) define both 'necessary' cause and 'sufficient' cause. A 'sufficient' cause is one that always results in disease (Rothman and Greenland, 2005), while a 'necessary' cause is one that must be present but might not be the cause of a disease to develop. In other words, a necessary cause is a component cause that is a member of every sufficient cause (Timmreck, 2002; Greenland, 1987; Gordis, 2000; Elwood, 1988; Lilienfeld and Stolley, 1994; Last, 1988; Kleinbaum et al. 1982). Rothman (1982) defines 'a cause of a disease event as an event, condition, or characteristic that preceded the disease event and without which the disease event either would not have occurred at all or would not have occurred until a later time'. If disease does not develop without the factor being present, then the causative factor is termed 'necessary'. If the disease always results from the factor, then the causative factor is termed 'sufficient'. In reference to Rothman's causal pie model, the possibility exists that MAP is a 'necessary' but not a 'sufficient' cause of Crohn's disease. As a necessary cause, MAP is required to be present to trigger the inflammatory reaction seen in Crohn's disease. However, not being a sufficient cause means that MAP cannot cause Crohn's disease alone but acts in concert with immune dysfunction and genetic susceptibility in order for Crohn's disease to occur (Chiodini and Rossiter, 1996; El Zaatari et al. 1995). Therefore, not every one with the presence of MAP in the intestine would suffer from

Crohn's disease. Moreover, the failure to detect MAP in some cases of Crohn's disease may not necessarily indicate that MAP is absent. Low specificity and sensitivity of the test, among other factors, may explain the inability to detect MAP in some patients (Jeyenathan et al. 2006). It has been reported that the sensitivity of nucleic-acid based tests is influenced by the bacterial burden, as exemplified by the compromised sensitivity of PCR-based assays for sputum smear-negative tuberculosis (Forbes, 1997; Ieven and Goossens, 1997; Noordhoek et al. 2004). By extension, assays that reliably detect abundant MAP organisms in livestock with Johne's disease may not provide sufficient sensitivity to study human Crohn's disease (Jeyenathan et al. 1994).

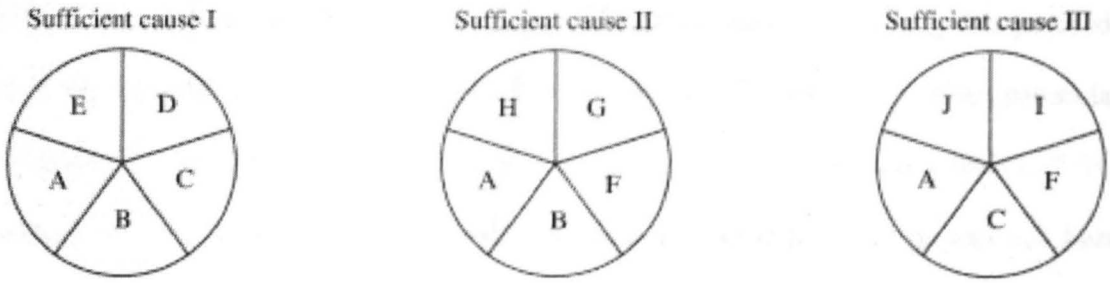

Figure 1.3: Causal pie model (Rothman and Greenland, 1998). This illustration shows a disease that has three sufficient causal complexes, each having five component causes. A is a necessary cause since it appears as a member of each sufficient cause. B. C, and F are not necessary since they fail to appear in all three sufficient causes.

The major philosophical doctrines about causation

The two major philosophical doctrines that have influenced modern science include inductivism and refutationism.

Inductivism

This doctrine holds that science proceeds from observation to theory, beginning with observations derived from experiments, and extrapolating from these to general laws (Last, 2001; Weed and Gorelic, 1996; Susser, 1991; Rychetnik et al. 2004; Bernard, 1949; Bacon, 1994). Bacon's vision of the 'true induction' comprises three interrelated stages: (i): Observation and Experiment (ii) Classification and Concept Formation and (iii) Eliminative Induction and Causal Inference (Bacon, 1994). The traditional view of science is that induction – the formation of a hypothesis based on observation – is cardinal to the scientific method. However, Hume (1978), the deductivists and others (Hendel, 1955) argued that a hypothesis that is derived by induction is flawed because it can be refuted by the first observation that proves an exception. Deduction refers to reasoning that proceeds from the general to the particular and relies on general theory to infer particular conclusions (Last, 2001). A century after Hume, Mill (1950a) proposed a canon of five methods to infer causes from their effects incorporating some of the ideas that had been proposed earlier by Bacon (1937). The canons of Mill (1950b) have evolved into inferential criteria that are in use today.

Refutationism or falsificationism

This theory is a rival account of the processes involved in scientific research to inductivism. While inductivism holds that science proceeds from observation to theory, beginning with observations derived from experiments, and extrapolating from these to general laws, falsificationism suggests that science proceeds in the opposite direction, beginning with scientific theories or 'conjectures', and then conducting experiments and

eliminating those theories that are falsified by results (Rothman, 1988; Pearce and Crawford-Brown, 1989; Kuhn, 1970; Susser, 1988). Karl Popper, one of the most influential philosophers of science of the twentieth century (Susser, 1986), followed Hume in rejecting induction, claiming that it is always possible to produce a theory to fit any set of observations (Buck, 1975). According to Karl Popper 'Our belief in a hypothesis can have no stronger basis than our repeated unsuccessful critical attempts to refute it' (Popper, 1972). Popper and other scientists believed that causation is established through a process of conjecture and refutation, and that science advances only by disproofs (Buck, 1975; Page et al. 2003; Platt, 1964). Popper insisted strictly on deduction, allowing the sole capability of science to be the falsification of prior hypotheses (the so-called hypothetico-deductive method), rejecting any place for verification (Rothman, 1988).

Relationship between association and causation

Association is an identifiable relationship between an exposure and disease. Association implies that exposure might cause disease (Rothman and Greenland, 2005; Krieger, 1994). Epidemiologists infer causation based upon the association and several other factors (Jantchou et al. 2006; Sicilia et al. 2001; Chiodini and Rossiter, 1996). Causation implies that there is a true mechanism that leads from exposure to disease (Torrence, 1997; Jantchou et al. 2006; Chiodini and Rossiter, 1996; Vineis and Kriebel, 2006; Gordis, 2004; Rothman, 2002; Kundi, 2006). However, the presence of an association does not necessarily mean that the relationship is causal (Parascandola and Weed, 2001; Torrence, 1997; Chiodini and Rossiter, 1996).

Deriving causal inferences

The variation among the viewpoints of epidemiologists with regard to causality is rooted in the variation among philosophical viewpoints. However, Hill's criteria provide interpretive guidelines for evaluating epidemiological evidence. Hill established the following classic operational causal criteria: strength of association, consistency, specificity, temporality, biological plausibility, dose–response effect, coherence, experimental evidence, and analogy (Hill, 1965; Hill, 1971; Hofler, 2005; Phillips and Goodman, 2001). According to Hill (1965), not all of these guidelines will be applicable in all situations and that there may be times when we wish to conclude that a putative cause–effect relationship is real even when some of the criteria are not met. According to Rothman (1986), only the criterion of temporality is a sine qua non for causality. If the putative cause did not precede the effect, that indeed is indisputable evidence that the observed association is not causal. Other than that one condition, there is no necessary or sufficient criterion for determining whether an observed association is causal (Rothman, 1986). This conclusion is in accordance with the view of Hume, Popper, and others that causal inferences cannot attain the certainty of logical deductions (Hume, 1978; Kuhn, 1970; Susser, 1988). There is no explicit consensus about what constitutes sufficient evidence to establish causation from association.

Established epidemiological criteria for causation

The established epidemiological criteria for causation are meant to be guidelines in assisting judgement as to whether an association is causal or not. Criteria of causation refer to a set of criteria used to assess the strength of a relation between a cause and an effect,

and provide a way of reaching jugdements on the likelihood of an association being causal. The most widely cited list of causal criteria, originally posed as a list of standards, is attributed to Hill (1965), who adapted them from the U.S. Surgeon General's 1964 report on smoking and health (United States Department of Health, Education and Welfare, 1964). Most of these lists stem from the canons of inference described by Mill (1950b) and the rules given by Hume (1978). The widely adopted criteria that have been refined by several scientists (Torrence, 1997; Krieger, 1994; Pearce and Crawford-Brown, 1989, Susser, 1986; Fletcher et al. 1996) include: (i) strength of association; (ii) consistency of effect; (iii) specificity of effect; (iv) temporality; (v) biological gradient or dose response; (vi) biological plausibility.

Strength of association

Strength of association refers to the extent to which a supposed cause and effect are related and should not be confused with statistical significance (Pearce and Crawford-Brown, 1989). The most common measure of strength of association is relative risk or rate ratio (Pearce and Crawford-Brown, 1989). Other measures of association in epidemiology include the odds ratio, a correlation coefficient and attributable risk (Pearce and Crawford-Brown, 1989). According to Chamberlin et al. (Chamberlin et al. 2001), technical advances have allowed the identification and/or isolation of MAP from a significantly higher proportion of Crohn's disease tissues than from controls. These methodologies include: (i) improved culture techniques; (ii) development of MAP-specific polymerase chain reaction assays; (iii) development of a novel in situ hybridization method; (iv) efficacy of macrolide and anti-mycobacterial drug therapies; and (v) discovery of Crohn's disease-specific

seroreactivity against two specific MAP recombinant antigens (Chamberlin et al. 2001). Several studies (Naser et al. 2004; Mishina et al. 1996) reported that 50% of Crohn's disease patients and 22% of ulcerative colitis patients were MAP positive and MAP was not cultured from the non-IBD patients. Some researchers suggest that all of inflammatory bowel disease (IBD) may be due to MAP (Naser et al. 2004; Mishina et al. 1996). Chiodini et al. (1984), described the isolation of a slow-growing, mycobactin-dependent Mycobacteria species from the intestinal mucosa of Crohn's disease patients but not from control tissue.

Consistency of effect

This epidemiological criteria refers to the fact that an association is found in many studies despite different circumstances, research designs, or time-periods (Krieger, 1994). Relationships that are demonstrated in multiple studies are more likely to be causal than those that are not. Several studies conducted at different times by different research methods have reported on the isolation of MAP from patients with Crohn's disease (Chamberlin et al. 2001; Hermon-Taylor et al. 2000; Hulten et al. 2001; Ikonomopoulos et al. 2000; McFadden et al. 1987; Sechi et al. 2005; Sechi et al. 2001). MAP has been found in Crohn's disease patients by genetic probes (including both DNA, and RNA) (Jeyenathan et al. 2006). The insertion element IS900, found at 14 to 18 copies per genome has been shown to be genomically specific for MAP (Sechi et al. 2001) and, has been widely used as a target for PCR (Chamberlin et al. 2001; Mishina et al. 1996; Turenne et al. 2006; Autschbach et al. 2005; Bull et al. 2000; Bull et al. 2003; Chiodini et al. 1986; Hermon-Taylor et al. 1990; Moss et al. 1991; Ryan et al. 2002).

Specificity of effect

Specificity describes the precision with which a factor will predict the occurrence of a specific disease; it adds plausibility to the causal claim but, if absent, does not detract from it (Susser, 1988). Routine culture of MAP from Crohn's disease patients' tissues is difficult because when present MAP is commonly in spheroplast form (cell wall deficient), which does not thrive in standard culture conditions (Chamberlin et al. 2001; Chiodini et al. 1986). It has also proved difficult to detect MAP in Crohn's disease tissues by other methods: the mycobacterial cell wall Ziehl–Neelsen (ZN) staining techniques, first described in 1882 (Ziehl, 1882; Neelsen, 1883) have not shown MAP in humans because MAP exists in the cell-wall-deficient form (Greenstein, 2003); serology studies have been beset by problems of nonspecificity because of antigen cross reactivity (Fiocchi, 1998), although more recent studies have reported a specific high immune reactivity to recombinant MAP antigens in Crohn's patients (Naser et al. 1999; El-Zaatari et al. 1999). These difficulties reflect the fact that MAP microorganisms when present in Crohn's disease are few in number, relative to bovine cases of MAP infection (Johne's disease) (Sanderson et al. 1992). An assay for MAP in Crohn's disease must be able to specifically detect small numbers of organsisms with tissue, near or below the threshold of microscopic detection (Jeyenathan et al. 2006). Molecular methods have been used to determine the prevalence of MAP in cases of Crohn's disease (McDonald, 2001). An important limitation of studies looking for novel pathogens is that information about the sensitivity and specificity of assays applied is generally lacking (Jeyenathan et al. 2006). In separate studies, it has been shown that IS900 element is genomically specific for MAP (Turenne et al. 2006) and that IS900 sequences from a heterogenous collection of MAP are invariant

(Semret et al. 2006). According to Sechi et al. (2001), MAP has been identified by in situ hybridization to the MAP-specific IS900 gene in tissue specimens of Crohn's disease. However, despite these favourable considerations, the IS900-based in situ probe was prone to non-specific hybridization, compromising the utility of IS900-based in situ hybridization and indirect in situ PCR (St. Amand et al. 2005). Jeyanathan et al. (2006), reported that the alternative means of increasing specificity and sensitivity involves the use of rRNA-specific oligonucleotide probe in situ hybridization. Probes targeting rRNA provided excellent specificity resulting in forms that were morphologically consistent with ZN-positive organisms on adjacent sections. Ryan et al. (2002), reported the detection of MAP DNA in 40% of Crohn's cases where microdissected granulomas were examined. However, only half of the granuloma positive cases had corresponding whole tissue sections that were positive for MAP. The greater detection rate of MAP in laser capture microdissection (LCM) isolated granulomas compared with whole tissue sections may have been attributable to better targeting of MAP DNA in granulomas – PCR may suffer loss of sensitivity because of the potential dilutional effect of the large quantities of non-target DNA found in whole tissue sections. Failure to detect MAP in some studies may have been attributable to inefficient amplification of long sequences (>250 bp) (Riggio et al. 1997).

Temporality

Temporality refers to the necessity that the cause precedes the effect in time (Fletcher et al. 1996). Causation is not possible without the cause occurring before the effect (Rothman, 1982). Data exist that indicate that temporal sequence criteria have been

fulfilled for the association between Crohn's disease and MAP (Van Kruiningen et al. 1986; Van Kruiningen et al. 1991). In a study by Van Kruiningen et al. (1986), a goat was infected with MAP organism taken from a human patient with Crohn's disease and showed progression to Johne's disease. A 1991 report found that 24-day-old specific pathogen-free Leghorn-Cochin chicks could be infected by multiple exposure routes using the same MAP strain ('Linda') (Van Kruiningen et al. 1991).

Dose–response relationship

A dose–response effect is present when the effect increases with the dose or level of exposure. In a study conducted by Schwartz et al. (2000), the intestinal mucosal layer from patients with IBD had high numbers of bacteria compared with people without Crohn's disease, however, there was no correlation between the numbers of bacteria present and either the degree of inflammation or the use of anti-inflammatory agents or sulfasalazine compounds (Schwartz et al. 2000). This study suggests that a demonstration of dose–response criterion may not be applicable to a relationship between MAP and Crohn's disease. The pivotal event that convinced a totally skeptical gastroenterological community to accept that *Helicobacter pylori* was the aetiological factor in peptic ulcers was the cure rate that was achieved when the putative *H. pylori* infection was treated with appropriate antibiotics (Greenstein, 2003). Similarly, Greenstein (2003) suggested that the failure to cure IBD with anti-MAP antibiotics is the main impediment to convincing a sceptical gastroenterological community that MAP is zoonotic. Possible reasons that could account for this inability to cure patients with Crohn's disease include, the use of the wrong antibiotics and lack of satisfactory performed studies that are prospective,

randomized, double blinded and placebo controlled, that have been performed using acknowledged satisfactory anti-MAP antibiotics (Greenstein, 2003). Recently, Greenstein et al. (2007), demonstrated that methotrexate and 6-mercaptopurine inhibit MAP growth in vitro. However, the dosages of methotrexate and 6-mercaptopurine in clinical use have not been titrated according to standard antibiotic conventions (Greenstein et al. 2007).

Biological plausibility

A hypothesized effect is biologically plausible if it makes sense in the context of current biological knowledge (Rothman and Greenland, 2005). By the 1930s, Johne's disease was found to be caused by an odd bacteria named *Mycobacteria paratuberculosis*. This organism is from the same family of bacteria which cause tuberculosis and leprosy. Current concepts regarding the cause of Crohn's disease emphasize a dysfunction of the immune system resulting in a prolonged and intense process of inflammation (Danze et al. 1996; Hodgson, 1998; Sartor, 1995; Strober and Neurath, 1996; Van Hogezand and Verspaget, 1996). The damage to the bowel appears to be due to this inflammatory process (Sartor, 1995; Strober and Neurath, 1996; Van Hogezand and Verspaget, 1996). MAP is thought to produce disease by over-stimulating the immune system. The bacterium lives inside the cells of the host, where it divides only once about every 2–12 h. By way of contrast, other bacteria in the gut such as *Escherichia coli*, *Salmonella spp.*, *Shigella spp.*, divide about once every 20 min. There are no toxins or poisons produced by MAP. Disease happens when the immune system recognizes the 'foreign' proteins of the bacteria, even inside a living cell and mounts a furious attack (Danze et al. 1996; Hodgson, 1998; Sartor, 1995). The immune 'attack' focuses on the infected cells in the mucosal layer of the

digestive system and results in massive inflammation, as well as ulcers, diarrhoea and weight loss (Schwartz et al. 2000; Danze et al. 1996; Hodgson, 1998; Sartor, 1995).

CONCLUSION

This paper has attempted to highlight current scientific evidence in regard to fulfilling the epidemiological criteria for a causal association between MAP and Crohn's disease. We were able to demonstrate that data exist that show that the MAP Crohn's disease phenomenon has fulfilled at least four (strength of association, consistency of effect, temporality and biological plausibility) of the six epidemiological causal criteria outlined by Hill. In summary, the current epidemiological evidence strongly supports the conjecture that Crohn's disease is caused by MAP especially for those who believe in the theory of inductivism. Several studies that demonstrated scientific evidence, including temporality, necessary to infer a causal association between MAP and Crohn's disease were highlighted. For the followers of Popper who believe in falsification/deductivism, whether enough observations or experiments have been conducted to falsify the MAP/Crohn's disease phenomenon is a matter of personal judgement. Moreover, there are people who believe that studies can falsify a theory only to a certain degree.

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PAPER 2

DOES *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* OR OTHER ENTERIC PATHOGENS CAUSE CROHN'S DISEASE? A COMPARATIVE CAUSALITY STUDY

ABSTRACT

Crohn's disease is defined as a chronic inflammatory disease of the intestine in humans characterized by transmural inflammation and formation of granuloma. Despite decades of research work, the exact cause of Crohn's disease has not been identified. It is proven that Crohn's disease is genetic; however, there are several lines of evidence that suggests that Crohn's disease also has a microbial element. Therefore, an important question is which microbes would have a role in the etiopathogenesis of Crohn's disease. Broadly, there are two groups of organisms considered: (1) normal flora and (2) pathogens, such as *Mycobacterium avium subspecies paratuberculosis* (MAP), Adherent-invasive *Escherichia coli* (AIEC), *Listeria monocytogenes*, *Yersinia species*, Herpesviruses, Measles virus, and Norovirus. We were interested in determining whether any of these candidates has met any or some of the criteria set by Rothman and others for causality. In a previous study, we conducted an extensive review of epidemiologic evidence of MAP as a cause of Crohn's disease. In the present study, we ask the question of whether there is epidemiologic evidence which supports other enteric microbes as possible causes of Crohn's disease. The answer to the question was obtained by subjecting other intestinal microbes to the same treatment applied to MAP. While MAP fulfilled 4 criteria, other intestinal pathogens fulfilled less than 4 of the established epidemiological criteria. In general, this study supported and strenghtened the hypothesis that MAP is the most commonly implicated organism in the etiology of Crohn's disease. The study provided a better understanding of the causal roles of microbes in Crohn's disease, and would help to provide better therapeutic interventions.

INTRODUCTION

Crohn's disease is recognized as a chronic inflammation of the wall of the intestine in humans (Dalziel, 1913; Greenstein, 2003). Together, Crohn's disease and ulcerative colitis are known as inflammatory bowel disease (Hanauer, 1996). Crohn's disease can affect any area of the gastrointestinal tract, from the mouth to the anus, but it most often begins at the terminal ileum, the junction between the small intestine and the large intestine. The disease, most commonly diagnosed in people between the ages of 20 and 30, is characterized by periods of active inflammation, called flare-ups, and periods of reduced or absent symptoms, called remission. All layers of the intestine may be involved in Crohn's disease as the inflammation often spreads deep into the layers of affected tissues, and normal healthy intestine can be found between sections of diseased bowel. The inflammation can lead to various symptoms of Crohn's disease, which most commonly include chronic diarrhea, abdominal pain, weight loss, fatigue and fever (Hanauer, 1996; Hanauer and Meyers, 1997). It is reported that some people with Crohn's disease also have problems outside of the gastrointestinal tract that may include inflammation of the eyes, skin, or joints, and liver problems (Hanauer and Meyers, 1997; Wagtmans et al. 1997). Under normal situations, the immune response in human intestine is characterized by a balance between pro-inflammatory and anti-inflammatory factors. In patients with Crohn's disease, the balance between these two factors in the intestinal mucosa is altered, thereby shifting the normal equilibrium toward a chronic inflammatory state. It is reported that once the cascade of inflammation begins, most of the subsequent pathophysiologic events are related (van Deventer, 1999; Hanauer et al. 1998; Sands, 2000). Many studies (van Deventer, 1999; Hanauer et al. 1998; Sands, 2000) have shown that the inflammatory

process in Crohn's disease is characterized by increased production of pro-inflammatory cytokines, especially tumor necrosis factor (TNF-alpha). Investigators reported that TNF-alpha is present in excess in the mucosa of patients with Crohn's disease, and that increases in TNF-alpha are associated with the release of other pro-inflammatory cytokines, such as interleukin 1, 6, and 8 (Hanauer et al. 1998). In Crohn's disease, the ongoing (chronic) intestinal inflammation may lead to complications (e.g intestinal strictures and fistulas) and may necessitate the removal of sections of intestine (Hanauer et al. 1998). It is known that inflammation is part of the body's immune response, and an immune response is usually triggered by antigens. Up to date, no specific trigger has been found to cause the inflammatory response seen in Crohn's disease. Thus, despite decades of relevant research, the exact cause of Crohn's disease is still unknown.

Several theories regarding the cause of Crohn's disease have been purported, but research has never confirmed a specific causal agent. There is substantial evidence that Crohn's disease has a genetic component. According to investigators, at least 30 distinct genes have been identified to influence the risk of developing Crohn's disease, including but not limited to genes involved in innate pattern recognition receptors (e.g. NOD2/CARD15, TLR4, CARD9), autophagy (e.g ATG16L1, IRGM, LRRK2), maintenance of mucosal integrity integrity (IBD5, DLG5, PTGER4, ITLN1, DMBT1, XBP1) and the promotion of the secondary immune response (HLA-region, TNFSF15/TL1A, IRF5, PTPN2, PTPN22, NKX2-3, IL-12B, IL-18RAP, MST1 (Van Limbergen et al. 2009). The first Crohn's disease susceptibility gene identified was a gene known as the caspase recruitment domain (CARD) 15 gene, which encodes the nucleotide oligomerisation domain (NOD) 2 receptor, a pattern recognition receptor (Hugot et al.

2001; Ogura et al. 2001). Studies have shown that the NOD2/CARD15 protein is an intracytoplasmic receptor that binds bacterial peptidoglycan-derived muramyl dipeptide via its leucin-rich repeat (LRR) region (Inohara et al. 2003; Inohara and Nunez, 2001). It is reported that mutations in CARD15 gene lead to defective recognition of muramyl dipeptide and a decreased clearance of intestinal bacteria (Inohara et al. 2003; Inohara and Nunez, 2001). Investigators have shown that autophagy plays a role in innate immunity and eliminates pathogens in vitro and in vivo, but when abberant as a result of mutations, contributes to chronic inflammatory diseases such as Crohn's disease (Delgado et al. 2009). Sanders (2005) demonstrated the presence of Crohn's-like lesions after compromise of mucosal integrity in the presence of an intact immune system, through altered expression of mucosal adhesion molecules, such as cadherins and tight junction proteins, which highlights the importance of the mucosal barrier in the development of Crohn's disease. Van de Vosse and Ottenhoff (2006) showed that mutations in *IL-12B* impair IL-12 and IL-23 responses and predispose individuals to infections caused by mycobacteria and salmonella.

It is clear that Crohn's disease has a genetic component. However, the mutation in Crohn's disease genes alone is not enough to trigger Crohn's disease. Specific triggering agents are just as important, including microbial or other environmental factors. Investigators have shown that various environmental factors may be involved in the development of Crohn's disease, but microbes are most consistently implied (Hertogh et al. 2008). Numerous studies indicated that bacteria from the intestinal lumen seem to be important in the development of mucosal inflammation in Crohn's disease patients. The two main hypotheses on the etiopathogenesis of Crohn's disease focus on whether (i) the

normal intestinal microflora are responsible for Crohn's disease by acting as a persistent antigenic stimulus in genetically susceptible hosts (Sartor, 1997), or (ii) that there is an as yet unidentified specific microbial pathogen causing Crohn's disease (Blaser, 1997). There are several lines of evidence that suggests that Crohn's also has a microbial element. These include: (i) most people with variants of Crohn's genes do not get Crohn's (Hugot, 2007); (ii) animals knocked out for Crohn's susceptibility genes, such as NOD2, do not have bowel disease (Wehkamp, 2005); (iii) most Crohn's susceptibility genes encode proteins responsible for innate immunity to bacteria (Parkes, 2007); and (iv) observed clinical benefit with antibiotic treatment (Selby, 2007). Therefore, an essential question is which microbes would have a role in the etiology and pathogenesis of Crohn's disease. Generally in this study, there are two categories of organisms considered: (1) normal flora and (2) pathogens, such as MAP, AIEC, *Listeria monocytogenes*, *Yersinia enterocolitica*, herpesviruses, measles virus and norovirus. We were interested in investigating whether any of these candidates has met any or some of the criteria set by Rothman and others for causality.

Comparison of common microbes implicated in etiopathogenesis of Crohn's disease using the established epidemiological criteria for causation

The established epidemiologic criteria are recognized as a set of criteria that serve as a guideline in the determining whether an association is causal or not (Hill, 1965). It is reported that, in theory, causal criteria could be used to either refute or predict causal effects (Weed, 1997). There are widely adopted criteria, which have been refined by several scientists (Hill, 1965; Torrence, 1997; Fletcher et al. 1996) that include: (i) consistency of effect; (ii) biological plausibility; (iii) strength of association; (iv)

temporality; (v) specificity of effect; and (vi) biological gradient or dose response. The criteria of "consistency of effect" describes the fact that if similar associations are found in different studies in different populations, the more likely the causal role of the factor. For the criteria of biological plausibility, association between the risk factor and the outcome is biologically plausible if it makes sense in the context of current biological knowledge (Rothman and Greenland, 2005). The criteria "strength of association" refers to the extent to which a supposed cause and effect are related. The larger the relative effect, the more likely the causal role of the factor (Pearce and Crawford-Brown, 1989). One of the ways to determine which bacteria might be likely candidates in Crohn's disease is to identify microorganisms that are present in diseased tissues but not in controls. Temporality refers to the necessity that exposure precedes the disease (Fletcher et al. 1996). According to Rothman (1982), causation is not possible without the cause occurring before the effect. The results presented herein will review application of six epidemiologic causal criteria outlined by Hill, to the relationship between other enteric microbes and Crohn's disease. "Specificity of association" refers to the fact that an exposure leads to a single or characteristic effect. It is easier to support causation when associations are specific, but this may not always be the case, since many exposures cause multiple diseases (Susser, 1988). According to Susser (1988), specificity adds plausibility to the causal claim, but if absent does not detract from it.

In the present study, it was observed that the criterion of "specificity of effect" was not fulfilled by any of the considered microbes, while the criterion of "dose-response" was not applicable to all the organisms based on available evidence. Thus the remaining discussion will be based on the other four established epidemiological criteria by Hill

(1965). Table 2.1 shows a comparison of the considered microbes in relation to fulfilling the six established epidemiological criteria by Hill (1965), while Table 2.2 shows the number and percentage of experimental evidence used to determine whether or not the various microbes has fulfilled any of the six established epidemiological criteria.

Table 2.1: Comparison of the various microbes in relation to fulfilling the six established epidemiological criteria.

	MICROBES	**Strength of associa-tion**	**Biological plausibility**	**Consistency of effect**	**Tempor a-lity**	**Specificity of associatio n**	**Dose-response relation-ship**
I	NORMAL INTESTINAL FLORA	√	√	√	unknown	absent	Not applicable
II	PATHOGENS						
1	MAP	√	√	√	√	absent	Not applicable
2	AIEC	√	√	√	Absent	absent	Not applicable
3	*Listeria monocytogenes*	absent	√	√	unknown	absent	Not applicable
4	*Yersinia species*	√	√	√	unknown	absent	Not applicable
5	Herpes viruses (EBV, CMV and HHV6)	√	Disputed	√	unknown	absent	Not applicable
6	Measles virus	absent	Disputed	√	unknown	absent	Not applicable
7	Norovirus	unknown	√	√	√	unknown	Not applicable

Key:
√ = experiment conducted and criteria fullfilled;
Disputed = experiment conducted but contradicting results;
Absent = experiment conducted but criteria was not fulfilled;
Unknown = no experiment conducted.

Table 2.2: Number and percentage of experimental evidence used to determine whether or not the various microbes has fulfilled any of the six established epidemiological criteria.

	MICROBES	Strength of association	Biological plausibility	Consistency of effect	Temporality	Specificity of association	Dose-response relationship
I	NORMAL INTESTINAL FLORA	1 (5.5%)	1 (4.2%)	6 (14.3%)	unknown	absent	Not applicable
II	PATHOGENS						
1	MAP	4 (25.0%)	7 (29.2%)	9 (23.6%)	3 (75.0%)	absent	Not applicable
2	AIEC	5 (31.3%)	3 (12.5%)	5 (13.2%)	Absent	absent	Not applicable
3	*Listeria monocytogens*	absent	2 (8.3%)	2 (5.3%)	unknown	absent	Not applicable
4	Yersinia species	6 (37.5%)	5 (20.8%)	1 (2.6%)	unknown	absent	Not applicable
5	Herpes viruses (EBV, CMV and HHV6)	1 (6.3%)	Disputed	13 (34.2%)	unknown	absent	Not applicable
6	Measles virus	absent	Disputed	6 (15.8%)	unknown	absent	Not applicable
7	Norovirus	unknown	6 (25.0%)	2 (5.3%)	1 (25.0%)	unknown	Not applicable
	Total	16 (100%)	24 (100%)	38 (100%)	4 (100%)		

Key:
Disputed = experiment conducted but contradicting results;
Absent = experiment conducted but criteria was not fulfilled;
Unknown = no experiment conducted.

Normal intestinal flora

The human intestine normally harbors a large and dynamic bacterial community. In most cases the bacteria are harmless and even protective. However, there is a substantial body of evidence implicating the resident flora in the pathogenesis of chronic intestinal

inflammation. The three established epidemiological criteria fulfilled by normal intestinal flora include: consistency of effect, biological plausibility and strength of association,

Consistency of effect: Multiple investigators have reported a possible link between commensal bacteria and Crohn's disease (Fiocchi, 1998). It is reported that not only the abundance but also the composition of the mucosa-associated flora is abnormal in Crohn's disease. Data from literature suggest that the mucosal flora in Crohn's disease patients is abnormal even before the onset of inflammation (Bourlioux et al. 2003). Studies have shown that some *E. coli* strains can be damaging, and some have been identified in Crohn's disease that bind excessively to the intestinal walls and can penetrate the lining. Studies have demonstrated the importance of the enteric microflora in the development of inflammatory bowel disease (IBD) in rodents with engineered susceptibility (Elson et al., 2005; Kim et al., 2005). Research has also demonstrated an abnormal mucosa-associated flora, considered to interact most closely with the innate immune system, in people with IBD (Kleessen et al., 2002; Swidsinski et al., 2005).

Biological plausibility: Many investigators have shown that the interaction of commensal bacteria with the intestinal immune system is an important factor in the development of Crohn's disease. It is reported that the study of isolated commensal bacteria's effects on the mucosal immune response is essential for an improved understanding of pathophysiological mechanisms in Crohn's disease (Llopis and colleagues, 2008). Llopis and co-workers investigated the immune responses to signals from the commensal *Escherichia coli* ATCC 35345 and the probiotic *Lactobacillus casei* DN-114 001 in Crohn's disease mucosa. Their results indicated that live *L. casei* significantly decreased secretion of TNF-alpha, IFN-gamma, IL-2, IL-6, IL-8, and CXCL1

by CD mucosa, but the effect was not reproduced by *L. casei* DNA. In addition, live *L. casei* downregulated expression of IL-8, IL-6, and CXCL1 and did not modify expression of IL-23p19, IL-12p35, and IL-17F. In contrast, it was observed that *E. coli* significantly upregulated expression of all these cytokines. These researchers concluded, based on combination experiments, that Live *L. casei* can prevent and counteract the proinflammatory effects of *E. coli* on Crohn's disease inflamed mucosa by specific downregulation of key proinflammatory mediators. In the present study we report that the criteria of biological plausibility have been accomplished by AIEC and Crohn's disease based on existing data.

Strength of association: Available data have shown that more intestinal flora were detected on the intestinal mucosal surface of Crohn's disease patients than on those of controls. Kleessen et al. (2002) investigated IBD tissues for different bacterial population groups harbouring the mucosal surface and/or invading the mucosa. Their results showed that more bacteria were detected on the mucosal surface of IBD patients than on those of non-IBD controls. In addition, it was discovered that colonic ulcerative colitis specimens were colonized by a variety of organisms, such as bacteria belonging to the gamma subdivision of Proteobacteria, the Enterobacteriaceae, the Bacteroides/Prevotella cluster, the Clostridium histolyticum/Clostridium lituseburense group, the Clostridium coccoides/Eubacterium rectale group, high G + C Gram-positive bacteria, or sulphate-reducing bacteria, while Crohn's disease samples harboured mainly bacteria belonging to the former three groups. They concluded that pathogenic events in Crohn's disease and ulcerative colitis may be associated with different alterations in the mucosal flora of the ileum and colon (Kleessen et al. 2002). Lodes and colleagues (2004) conducted a study to

identify commensal bacterial proteins that could contribute to the pathogenesis of IBD, using serological expression cloning. These investigators found increased concentrations of circulating IgG antibodies to flagellins in patients with Crohn's disease but not ulcerative colitis or controls. All these findings suggest that the criteria of "strength of association" have been fulfilled by normal intestinal flora.

Dose reponse relationship: Numerous studies have reported on the use of antibiotics and probiotics to manipulate intestinal bacterial flora for therapeutic purposes in Crohn's disease patient (Gionchetti et al. 2006; Balfour, 2007). However, since no titration of dosages of the antibiotics has yet been performed, we report that the established dose-reponse relationship criteria have not been fulfilled by normal intestinal flora.

Intestinal pathogens

Numerous intestinal pathogens have been considered in the search for microbes associated with occurrence or exacerbation of Crohn's disease, including bacteria (MAP, AIEC, *Listeria monocytogenes*, *Yersinia enterocolitica*) and viruses (herpesviruses, measles virus, noroviruses).

Mycobacterium avium subspecies *paratuberculosis*

An important suspect for Crohn's disease is *Mycobacterium avium* subspecies *paratuberculosis* (MAP), described as an obligate intracellular bacteria that is known to cause paratuberculois (Johnes disease) in ruminant animals. In a previous study, we (Uzoigwe et al. 2007) reported that the MAP Crohn's disease phenomenon has fulfilled at least four (strength of association, consistency of effect, temporality and biological

plausibility) of the six epidemiologic causal criteria outlined by Hill. These four epidemiological criteria fulfilled by MAP are briefly discussed below.

Consistency of effect: Several investigators (Mishina et al. 1996; Chamberlin et al. 2001; Hermon-Taylor et al. 2000; Hulten et al. 2001; McFadden et al. 1987; Sechi et al. 2005; Karp et al. 2007) have reported the isolation of MAP from patients with Crohn's disease. Karp and coworkers (2007) evaluated new knowledge and data involving MAP in Crohn's disease by examining relevant publications in the literature. Over 145 new clinical and laboratory studies supported an association between MAP and Crohn's disease. There have been several reports of a significantly higher frequency of MAP in intestinal tissue obtained from patients with Crohn's disease, by use of several different methods of analysis (Karp et al. 2007, Autschbach et al. 2005). Several different methods of analysis have included similar findings. Autschbach et al. (2005) reported that IS900 PCR detection rate was significantly higher in Crohn's dsease tissue samples (52%) than in ulcerative colitis (2%) or nIBD (5%) specimens ($p<0.0001$). Sechi et al. (2001) reported on the presence of cell wall-deficient *Mycobacterium avium* subsp. *paratuberculosis* in 35 of 48 paraffin-embedded tissue specimens from 33 patients with Crohn's disease, using the method of in situ hybridization with IS900 as a probe. Naser and co-workers (2004) reported the culturing of *Mycobacterium avium* subspecies *paratuberculosis* from the peripheral blood in 50% of patients with active Crohn's disease.

Biological plausibility: The mechanism of tissue damage by MAP in Crohn's disease has been reported. It is reported that MAP does not produce any toxins or posions. The damage to the intestine is attributed to the inflammatory process (Danze et al. 1996; Hodgson, 1996; Sartor, 1995). It has been shown that MAP produces disease in humans by

over-stimulating the immune system (Schwartz et al. 2000; Danze et al. 1996). Investigators reported that the immune system recognizes the "foreign" proteins of the bacteria, even inside a living cell and mounts a furious attack that results in massive inflammation, ulcers, diarrhea and weight loss (Schwartz et al. 2000; Danze et al. 1996; Hodgson, 1996; Sartor, 1995).

Strength of association: Numerous investigators (Mishina et al. 1996; Sechi et al. 2005; Chamberlin et al. 2001; Chiodini et al. 1984) reported the identification and/or isolation of MAP from a significantly higher proportion of Crohn's disease tissues than from controls. Sechi et al. (2005) demonstrated that twenty five patients (83.3%) with Crohn's disease and 3 control patients (10.3%) were IS900 PCR positive ($p = 0.000001$; Odds ratio 43.3). These researchers showed that Mycobacterium avium subspecies paratuberculosis grew in cultures from 19 Crohn's patients (63.3%) and from 3 control patients (10.3%) ($p = 0.00001$; Odds ratio 14.9) (Sechi et al. 2005).

Temporality: Studies using animal models have shown that exposure to MAP preceded the development of Crohn's disease. Van Kruiningen et al. (1986), reported that a goat infected with the MAP organism taken from a human patient with Crohn's disease, showed progression to Johne's Disease. In another study, it was demonstrated that twenty-four day-old specific pathogen-free Leghorn-Cochin chicks could be infected by multiple exposure routes using the same MAP strain ("Linda") (Van Kruiningen et al. 1991). Mutwiri and coworkers (2001) demonstrated that infection of six-week-old beige/scid mice with MAP, triggers significant intestinal pathophysiologic changes consistent with chronic inflammation. Vaughan and colleagues (2005) reported the development of a paratuberculosis infection model in a breeding group of adult and juvenile New Zealand

white laboratory rabbits following oral administration of three doses of the MAP strain, CLIJ623, on three occasions. These researchers monitored the disease progression in the rabbits for more than 2 years, using culture, post-mortem tissue bacteriological culture and histopathology. Their results showed that of the 4 adult and 16 juvenile orally dosed rabbits, MAP organisms were recovered bacteriologically from two and three animals, respectively, using the BACTEC™ radiometric culture system (Vaughan and colleagues, 2005)

Specificity of association. We reported earlier that MAP does not demonstrate specificity of association in regard to Crohn's disease (Uzoigwe et al. 2007), due to failure to detect MAP in some studies (Riggio et al. 1997; Chiba et al. 1998a; Kanazawa et al. 1999). Sibartie et al. (2010) conducted a study to examine the influence of MAP on T-cell proliferation and cytokine responses in patients with inflammatory bowel disease, and their results showed an increased proliferation of T cells and an altered cytokine response, which suggested that prior exposure to MAP and engagement of the immune system is seen in patients with Crohn's disease. They concluded that their results does not imply causation but does support further examination of MAP as an environmental modifying factor in Crohn's disease. By extension, other enteric pathogens implicated in Crohn's disease could not fulfill the criteria of "specificity of association", since the specific agent(s) that may cause of Crohn's disease have not yet been confirmed.

Dose-response relationship. It is reported that the intestinal mucosal layer from patients with inflammatory bowel disease had high numbers of MAP bacteria compared with people without Crohn's disease, but no correlation was found between the numbers of bacteria present and either the degree of inflammation or the use of anti-inflammatory

agents or sulfasalazine compounds (Schwartz et al. 2000). According to Greenstein et al. (2007), methotrexate and 6-mercaptopurine inhibit MAP growth in vitro. However, the dosages of methotrexate and 6-mercaptopurine in clinical use have not been titrated according to standard antibiotic conventions. These studies suggest that a demonstration of dose response criterion may not be applicable to the relationship between enteric microbial agents and Crohn's disease.

Adhesive-invasive *Escherichia coli*

Some *E. coli* strains, particularly the adherent-invasive *E. coli* (AIEC) pathovar, have been increasingly implicated in the etiology and pathogenesis of Crohn's disease. Based on available evidence, the three established criteria fulfilled by AIEC include: consistency of effect, biological plausibility and strength of association.

Consistency of effect: Numerous investigators (Darfeuille-Michaud, 2002; Bringer et al. 2006; Rolhion et al. 2007) have demonstrated an association between Crohn's disease and a strain of *E. coli* known as adherent-invasive *E. coli* (AIEC) strain LF82. Bringer et al. (2006) reported that the Crohn's disease-associated adherent-invasive *Escherichia coli* strain LF82 replicates in mature phagolysosomes within J774 macrophages. Rolhion et al. (2007) showed that OmpC and the σE regulatory pathway are involved in adhesion and invasion of the Crohn's disease-associated *Escherichia coli* strain LF82. Meconi et al. (2007) demonstrated that adherent-invasive *Escherichia coli* (AIEC) LF82 isolated from a Crohn's disease patient induced granulomas in vitro. Martinez-Medina et al. (2009) conducted a study about the ecological parameters of AIEC, and their results reinforced the

implication of AIEC in Crohn's disease. These findings indicate that AIEC Crohn's disease phenomenon satisfy the criteria of "consistency of effect".

Biological plausibility: It is reported that AIEC LF82 virulent bacteria, but not nonpathogenic *E. coli* K-12, were able to persist in the gut of CEABAC10 transgenic mice and to induce severe colitis with reduced survival rate, marked weight loss, increased rectal bleeding, presence of erosive lesions, mucosal inflammation, and increased proinflammatory cytokine expression (Carvalho et al. 2009). A study conducted by Darfeuille-Michaud (2002), showed some of the mechanisms underlying tissue damage by AIEC in Crohn's disease. Darfeuille-Michaud (2002), reported that a pathovar of *E. coli*, designated adherent-invasive *Escherichia coli* (AIEC) colonize the intestinal mucosa by adhering to intestinal epithelial cells, and are also recognized as true invasive pathogens with the ability to invade intestinal epithelial cells via a macropinocytosis-like process, and to survive and replicate intracellularly after lysis of the endocytic vacuole. These researchers additionally showed that within macrophages, AIEC strains survive and replicate extensively without inducing host cell death and induce the release of high amounts of TNFalpha cytokine. They further reported that all these virulence properties possessed by AIEC designate it as a possible pathogen with the potential to induce persistent intestinal inflammation in Crohn's disease, by crossing and breaching the intestinal barrier, moving to deep tissues, and continuously activating macrophages. Rolhion et al. (2007) reported that OmpC and the σE regulatory pathway are involved in adhesion and invasion of the Crohn's disease-associated *Escherichia coli* strain LF82.

Strength of association: Several studies have shown that AIEC could be isolated from intestinal tissue of Crohn's disease patients but not from control tissue. Kotlowski et

al. (2007) reported a higher prevalence of *E coli* from the B2+D phylogenetic group in tissues of patients with ulcerative colitis and Crohn's disease compared to controls. These researchers collected a total of 84 biopsies from 15 controls, 13 patients with Crohn's disease and 19 patients with ulcerative colitis. The DNA extracted from each biopsy sample was subjected to RISA analysis, and their results identified bands (~450 bp) that were consistently present in approximately 70% of patients but in <30% of controls (Kotlowski et al. 2007). Several studies have revealed a high prevalence of invasive strains associated with the ileal mucosa of patients with Crohn's disease compared to that for controls, supporting a putative role of *E. coli* invasiveness in the pathogenesis of Crohn's disease (Boudeau et al. 2000; Masseret et al. 2001). A study conducted by Darfeuille-Michaud et al. (2004) showed a high prevalence of adherent-invasive *Escherichia coli* associated with ileal mucosa in Crohn's disease compared to controls. These researchers reported isolation of the AIEC reference strain LF82 from ileal specimens of 21.7% of Crohn's disease patients compared to 6.2% of controls (Darfeuille-Michaud et al. 2004). Another study (Martin et al. 2004) demonstrated that mucosa-associated *E. coli*, which accounted for 53% of isolates, were more common in Crohn's disease (43%) than in noninflamed controls (17%), and that intramucosal *E. coli* were found in 29% of Crohn's disease patients compared to 9% of controls. Their study supported a role for mucosally adherent bacteria in the pathogenesis of Crohn's disease and colon cancer (Martin et al. 2004). In this study, based on exisiting data, we report that the criteria of strength of association have been fulfilled for AIEC and Crohn's disease.

Temporality: Studies have shown that the presence of AIEC is increased in Crohn's disease patients. It is also reported that in patients with inflammatory bowel diease, AIEC

can adhere to intestinal epithelial cells and induce the secretion of IL-8, but there is no evidence that these *E. coli* strains are invasive (Rolhion and Darfeuille-Michaud, 2007). According to Rolhion and Darfeuille-Michaud (2007), the absence of invasiveness of AIEC strains, could imply a possibility of not inducing the formation of granulomas or any other lesion, when applied to pathogen free animal models. Based on this finding, we report that the temporal sequence criteria have not been fulfilled for the association between Crohn's disease and AIEC.

Specificity of effect: While some studies have reported the presence of AIEC in Crohn's disease patient's intestinal mucosa, some other studies have failed to detect this *E. coli* strain in Crohn's disease tissue. Walmsley et al. (1998) reported the absence of *Escherichia coli*, *Listeria monocytogenes*, and *Klebsiella pneumoniae* antigens within inflammatory bowel disease tissues. Due to the inability to detect AIEC in some studies, this study reports that the criteria of specificity of effect have not been fulfilled.

Dose-response relationship. Several antibiotics with the ability to penetrate macrophages have been used as a target for the AIEC strain, which replicate within macrophages. Although investigators reported that azithromycin, ciprofloxacin, rifampicin, sulfamethoxazole, tetracycline and trimethoprim were all effective against *E. coli* within macrophages (Subramanian et al. 2008), there are no data indicating that the dosages of these antibiotics have been titrated according to standard antibiotic conventions. Thus, in the present study, we conclude that demonstration of dose response criterion may not be applicable to the relationship between AIEC and Crohn's disease.

Listeria monocytogenes

Listeria monocytogenes, a Gram-positive, rod-shaped, facultative intracellular organism, has been implicated in the etiopathogenesis of Crohn's disease, and has been shown to fulfill three established epidemiological criteria, including consistency of effect, biological plausibility, and strength of association.

Consistency of effect: The bacteria *Listeria monocytogenes* have been proposed as candidate causative agents of Crohns disease (Liu et al. 1995). Liu et al. (1995) reported that *Listeria* spp., *E. coli*, and streptococci, but not measles virus, play a role in the pathogenesis of Crohn's disease. Hugot et al. (2003) reported that Crohn's disease is the result of an excessive response to pathogenic psychrotrophic bacteria in some genetically predisposed people. The cold chain hypothesis suggests that Crohn's disease results from an increased and chronic exposure to psychrotrophic bacteria (microbes which are able to survive at low temperatures), such as *Yersinia spp* and *Listeria spp.* (Hugot et al. 2003).

Biological plausibility: Investigators reported that the consumption of refrigerated food containing low levels of psychotropic, pathogenic bacteria causes an over-active immune response, resulting in Crohns Disease (Hugot et al. 2003). Nakane et al. (1988) reported that a lower level of TNF-alpha is produced endogenously in mice that received *L. monocytogenes* infection and that it plays an essential role in the host defense against *L. monocytogenes* infection. Thus, the criteria of biological plausibility have been accomplished by *Yersinia species*.

Strength of association: Investigators have shown that *Listeria monocytogenes* have not been isolated from the lesions of Crohn's disease patients at higher rates than in controls (Chen et al. 2000). These researchers reported that *L. monocytogenes* DNA was

detected in 13.0% patients with CD, 17.9% patients with UC and 25.6% non-IBD control patients or in 29 of 274 (10.6%) endoscopic biopsies. They concluded that *Listeria monocytogenes* DNA was detected in the intestine of both patients with IBD and in non-IBD control patients, which probably reflect the widespread presence of this organism in the environment, and does not support a direct role for *L. monocytogenes* in the pathogenesis of IBD. Thus, the criterion of "strenght of association" is believed not to be fulfilled by *Listeria monocytogenes*.

Temporality: There are no data available which show that exposure to *L. monocytogenes* preceded the development of Crohn's disease. Hence, fulfillment of the temporality criteria for *L. monocytogenes* is reported as unknown.

Specificity of effect: While previous studies have demonstrated the presence of Listeria antigens in intestinal tissue of Crohn's disease patients (Liu et al. 1995), some other studies (Chiba and coworkers, 1998; Walmsley, 1998) reported that their data does not support the etiologic significance of *L. monocytogenes* in Crohn's disease. Thus the criterion of specificity of effect is regarded as not accomplished by *Listeria monocytogenes*.

Dose-response relationship: Similar to other bacterial agents implicated in Crohn's disease, the criteria of dose-response relationship could not be applied to *L. monocytogenes*, since there are no experimental data on titration of drugs used for treatment of Crohn's disease.

Yersinia species

The genus *Yersinia* consist of Gram-negative coccobacillus-shaped group of bacteria, that includes three species *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica* which are pathogenic for humans and rodents. *Yersinia pestis* is the etiologic agent of plague, while *Y. pseudotuberculosis* and *Y. enterocolitica* are intestinal pathogens that cause commonly self limited enteritis called yersiniosis and mesenteric adenolymphitis in humans. These enteropathogenic *Yersinia* strains, *Y. pseudotuberculosis* and *Y. enterocolitica*, have also been suggested to play a causal role in Crohn's disease. In the present study, *Yersinia species* have been shown to fulfill three established epidemiological criteria, including consistency of effect, biological plausibility and strength of association.

Consistency of effect: Many authors have independently found Yersinia species in Crohn's disease lesions, and have reported an association between Yersinia species and Crohn's disease (Kallinowski et al. 1998; Lamps et al. 2003). Several lines of evidence support a link between enteropathogenic *Yersinia* species and Crohn's disease, including: (i) *Y. enterocolitica* and *Y. pseudotuberculosis* have been found in Crohn's disease lesions (Kallinowski et al. 1998; Lamps et al. 2003; Swidsinski et al. 2002); (ii) investigators have shown that Crohn's disease may occur after a Yersiniosis (Saebo et al. 2005); (iii) the clinical manifestations of Crohn's disease and Yersiniosis are both characterized by ileitis or ileocolitis with formations of granulomas and in some cases by reactive arthritis; and (iv) studies have shown an increase in responsivity of mononuclear cells isolated from mesenteric lymph nodes of Crohn's disease patients to *Y. enterocolitica* when compared to other bacterial agents such as *E. coli*, *Salmonella agona*, *Candida albicans* or *Chlamydia*

trachomatis (Ibbotson et al. 1992). The presence of granulomata is a characteristic of infections where the causative organisms are capable of exisiting intracellularly and is a feature found in mycobacterial and yersinial diseases. According to the cold chain hypothesis, Crohn's disease is assumed to result from chronic exposure to bacteria able to grow at low temperature called psychrotrophic bacteria, which includes *Yersinia spp* and *Listeria spp.* (Hugot et al. 2003). In this study, the criterion of consistency of effect is considered fulfilled, since the association between the enteropathogenic *Yersinia* strains and Crohn's disease has been demonstrated in multiple studies.

Biological plausibility: It is reported that mice invalidated for CARD 15/NOD2 are characterized by an abnormal response after oral infection by *Yersinia pseudotuberculosis* (Meinzer et al. 2008). Studies have shown that enteropathogenic *Yersinia* strains display a tropism to lymphoid tissue (Brubaker, 1991). Many researchers (Autenrieth and Firsching, 1996; Clark et al. 1998) demonstrated that the enteropathogenic *Yersinia* species bind to and invade M cells within the follicle-associated epithelium overlying the lymphoid follicles of the Peyer's patches. After their entry into Peyer's patches, the bacteria induce the host immune response which is characterized by an inflammation with infiltration of immune cells, particularly neutrophils and macrophages (Handley et al. 2004). Since there are data describing the mechanism of tissue damage by *Yersinia species*, the criteria of biological plausibility is said to be accomplished in this study.

Strength of association: Lamps and colleagues (2003) reported that *Yersinia* DNA is detected in bowel and mesenteric lymph nodes from patients with Crohn's disease at higher rates than in controls. This finding indicates that this criterion has been fulfilled by Yersinia species.

Temporality: It is known that the Nucleotide oligomerisation domain 2 (NOD2) is a component of the innate immunity involved in the homeostasis of Peyer's patches in mice, and NOD2 mutations have been associated with Crohn's Disease (Hugot et al. 2001; Ogura et al. 2001). Meinzer et al. (2008) investigated the role of Nod2 during *Y. pseudotuberculosis* infection. Their results showed that mice invalidated for CARD 15/NOD2 are characterized by an abnormal response after oral infection by *Yersinia pseudotuberculosis* (Meinzer et al. 2008). These researchers concluded that Nod2 contributes to the susceptibility to *Y. pseudotuberculosis* in mice. A study conducted by Scandivanian researchers (Saebo et al. 2005) showed that at the population level, Crohn's disease is more frequent in the years following a recorded *Yersinia* infection. However, in contrast to the bacteria MAP, there are no specific studies indicating that exposure to Yersinia species preceded the development of Crohn's disease using animal models. Thus in this study, the fulfillment of the criterion of temporality is reported as unknown.

Specificity of effect: Some authors have reported the detection of enteropathogenic Yersinia species in Crohn's disease patient is intestinal tissues (Meinzer et al. 2008), and that individuals with Yersinia infection are more likely to progress to Crohn's disease (Saebo et al. 2005). However, some other investigators (Persson et al. 1976) reported that acute yersiniosis never progesses to Crohn's disease. Due to contradictory results, the criterion of specificity of effect is reported as not fulfilled by Yersinia species.

Dose-response relationship: This criterion is not applicable to Yersinia species, since there are no data on titration of dosages of drugs used for treatment of Crohn's disease.

Herpes viruses

Research suggests that herpes viruses might play a significant role in the pathogenesis of Crohn's disease. The two established epidemiological criteria fulfilled by herpesviruses include: consistency of effect and strength of association.

Consistency of effect: Many authors reported the presence of herpes virus DNA in intestinal tissue of Crohn's disease patient (Wakefiled et al. 1992; Ruther et al. 1998). A study conducted by Wakefield and coworkers (1992) have shown a high prevalence of herpesviruses (such as cytomegalovirus, human herpes virus 6, and Epstein Barr virus) infection in Crohn's disease patients. Ruther et al. (1998), used in-situ hybridization to investigate intestinal mucosa for viral agents such as HSV I + II- and Epstein-Barr virus DNA, and found these DNA in the cell nuclei in the surface and glandular epithelia of the affected mucosa of the small intestine and the colon. Their findings indicated that viruses may exacerbate these inflammatory bowel diseases (Ruther et al. 1998). Studies have shown that cytomegalovirus infection (CMV) infection is related to inflammatory bowel disease, either as a precipitating factor or as a coincidental infection (Cottone et al. 2001; Berk et al. 1985; Orvar et al. 1993; Farmer et al. 1973; Bernades, 1980; Kaufman et al. 1999; Rachima et al. 1998). Cottone et al. (2001) reported that cytomegalovirus infection is a cause of severe refractory ulcerative and Crohn's colitis. The presence of Epstein Barr virus (EBV) in intestinal tissues from Crohn's disease patients has been demonstrated by many investigators using PCR (Wakefield et al. 1992), immunohistochemistry or in situ hybridization (Spieker and Herbst, 2000; Yanai et al. 1999). Yanai et al. (1999) investigated the possible pathological role of EBV in inflammatory bowel disease (IBD), by testing for the presence of EBV in the colon in IBD patients. Their results showed a

limited presence of EBV-infected cells in the diseased areas of IBD colonic specimens, indicating that EBV infection may be related to such diseases. Since the association between Crohn's disease and herpesvirus infections has been well described in multiple studies, the criteria of consistency of effect is reported as fulfilled by herpesviruses.

Biological plausibility: Studies have shown Interleukin 12 (IL-12), is a potent proinflammatory heterodimeric, macrophage-derived cytokine that plays an essential role in rodent models of inflammatory bowel disease (IBD), and may also contribute to the pathogenesis of human disease (Monteleone et al. 1997). It is reported that EBI3 encodes a protein that shares 27% amino acid sequence identity with the p40 component of IL-12, and is considered a molecule belonging to the interleukin (IL)-12 family (Devergne et al. 1996). In view of the homologies between EB13 and IL-2, it was reasonable to consider EBI3 in the pathogenesis of inflammatory bowel disease. Gehlert et al. (2004) examined EB13 expression in mouse intestine, and reported an increased expression level of the EBI3, in ulcerative colitis as compared to Crohn's disease. In contrast to the above studies, Christ et al. (1998) showed that EBI3 expression is observed at low levels in normal human intestine and Crohn's disease and is significantly up-regulated within macrophage-like cells in the context of active ulcerative colitis but not active Crohn's disease. Based these contradictory findings, we report in this study that the criteria of biological plausibility is under dispute.

Strength of association: Researchers (Wakefield et al. 1992) examined the prevalence of herpesvirus DNA in inflammatory bowel disease tissue and their results showed that there was a high prevalence of CMV (81%), HHV6 (76%), and EBV (76%) DNA in ulcerative colitis tissue compared to Crohn's disease tissues (CMV 66%, HHV6

45%, EBV 55%). It was also shown that control tissue had a relatively low frequency of CMV (29%) and EBV (19%) DNA but a prevalence of HHV6 DNA similar to that of ulcerative colitis (86%). Since herpesvirus was isolated from a higher proportion of Crohn's disease tissues than from controls, we report that criterion of strength of association has been fulfilled by Crohn's disease.

Temporality: There are no available data indicating that animal models infected with herpesvirus, subsequently developed Crohn's disease. Thus the fulfillment of the criteria of temporality by herpesvirus is reported as unknown.

Specificity of effect: While some studies have reported that identification of herpesviruses in Crohn's disease intestinal tissues (Wakefield et al. 1992), some other studies have reported their absence (Sura et al. 2010). Sura et al. (2010) recently found equal evidence of HHV-6 in patients and controls by multiple methods, which suggests that this virus is ubiquitous and probably not a cause of Crohn's disease. In this study, we report that the criteria of specificity of effect have not been accomplished by herpes viruses.

Dose-response relationship: Similar to bacterial agents implicated in Crohn's disease, the criteria of dose-response relationship is not applicable to viral agents like herpesvirus, since there are no experimental data on titration of dosages of antiviral drugs used for Crohn's disease patients.

Measles virus

Measles virus, both wild-type and vaccine-attenuated, acquired either in utero, or as a consequence of postnatal infection has been suggested as a risk factor for Crohn's

disease. The only epidemiological criteria fulfilled by measles virus is the consistency of effect. The criteria of biological plausibility is said to be disputed.

Consistency of effect: Several investigators reported the detection of measles virus particles, protein or RNA in tissues from patients with Crohn's disease, using direct electron microscopy, immunohistochemistry and in situ hybridization techniques (Wakefield et al. 1993; 1997; Miyamoto et al. 1995; Daszak et al. 1997; Lewin et al. 1995; Ekbom et al. 1994; Thompson et al. 1995). Since the isolation of measles virus from Crohn's disease tissue has been demonstrated in multiple studies, the measles virus-Crohn's disease phenomenon is reported to satisfy the criteria of "consistency of effect".

Biological plausibility: The biologically plausible hypothesis linking measles virus exposure with the subsequent development of Crohn's disease is still disputed. One group of investigators reported that the measles virus is able to persist within the mesenteric endothelium, and its presence induces an inflammatory reaction characteristic of Crohn's disease, which suggests a biological plausibility between measles virus and Crohn's disease (Wakefield et al. 1995). In contrast, another group of researchers (Haga et al. 1996) reported the absence of measles viral genomic sequence in intestinal tissues from Crohn's disease by nested polymerase chain reaction. Additionally, the data relating to the humoral immune response against measles virus in patients with Crohn's disease are still under debate. Lavy et al. (2001) reported that the presence of IgG antibodies to measles virus was higher in patients with Crohn's disease than in patients with ulcerative colitis or controls. These researchers also demonstrated that exposure to measles in childhood was more frequent in Crohn's disease patients than in their controls, the difference being statistically significant ($p < 0.05$) in relation to community controls. These observations supported a

role for measles infection in Crohn's disease (Lavy et al 2001). Whereas one group of investigators found measles virus specific IgG antibodies in the majority of subjects with Crohn's disease (Lavy et al 2001), other groups could not detect any immunological evidence of persistence of measles virus or any differences compared with control groups (Balzola et al. 1997; Touze et al. 1995; Fisher et al. 1997). Since Crohn's disease has not been linked persistently with measles virus, the biological plausibility for the measles virus to cause Crohn's disease, as has been proposed, is considered "disputed".

Strength of association: Robertson and Sandler (2001) reported the use of well-accepted Bradford-Hill criteria to evaluate the possible causal association between measles and IBD. Their results indicated that although the association may be biologically plausible, the literature lacks consistency, specificity, strength, and dose response. Their study does not support an association between measles virus and IBD (Robertson and Sandler, 2001).

Specificity of effect: Epidemiological results have demonstrated an increased risk of Crohn's disease in children with perinatal exposure to the measles virus (Ekbom et al. 1994). However, subsequent investigators have failed to detect measles-virus DNA in the intestinal mucosa of Crohn's Disease patients (Iizuka et al. 1995; Robertson and Sandler, 2001). Haslam et al. (2000) reported that in utero or perinatal exposure to seasonal environmental factors (e.g measles virus), are unlikely potential actiological agents in the later development of Crohn's disease.

Dose-response relationship: Similar to bacterial agents implicated in Crohn's disease, the criteria of dose-response relationship is not applicable to measles virus, since

there is no experimental data on titration of dosages of antiviral drugs used for Crohn's disease patients.

Norovirus

Noroviruses (commonly Norwalk-like viruses or Winter Vomiting Disease) are part of a group of viruses from the family Caliciviridae that are responsible for the majority of nonbacterial gastroenteritis, an inflammation of the stomach and intestinal lining in humans, with its most common symptoms of vomiting and diarrhea (Glass et al. 2009). Recent studies have proposed that noroviruses are associated with Crohn's disease. In this study, three established epidemiological criteria have been fulfilled by norovirus, including consistency of effect, biological plausibility, and temporality.

Consistency of effect: The association of novovirus and Crohn's disease has been demonstrated in many studies, suggesting that the criteria of "consistency of effect" have been fulfilled by murine norovirus (MNV). Previously, researchers have found that mice with an ATG16L1 gene variant developed a condition similar to Crohn's in humans, but this mutation wasn't enough to trigger the disease (Cadwell et al. 2008). A recent study by Cadwell et al. (2010) demonstrated that a murine noroviral (MNV) infection and a Crohn's disease susceptibility gene combine to cause inflammatory disease in the mouse gut. According to these scientists, mice with the ATG16L1 gene mutation only developed Crohn's disease if they also are infected with MNV norovirus (Cadwell et al. 2010). To test whether the mouse norovirus was involved, these scientists orally infected the mice. A week later, the mutant mice displayed Paneth cell abnormalities. Infected control mice, in contrast, did not. Paneth cells weren't directly infected with virus, but their gene expression

patterns were altered. These findings show that a combination of the virus plus the gene mutation, but neither alone, causes abnormalities in the intestine (Cadwell et al. 2010). Khan et al. (2009) demonstrated that the norovirus infection may be associated with exacerbations of inflammatory bowel disease (IBD). They reported that when norovirus accompanies IBD it is more likely to be associated with hematochezia than when the infection occurs in the absence of IBD (Khan et al. 2009).

Biological plausibility: Many authors have shown or proposed the underlying mechanisms of tissue damage by infection with noroviruses in Crohn's disease. Several investigators reported that murine norovirus (MNV) is immunmodulatory and may alter disease phenotypes in mouse models of inflammatory bowel disease (Chase et al. 2008; Cadwell et al. 2010). McCartney et al. (2008) reported that MDA5−/− dendritic cells have a defect in cytokine response to murine norovirus-1 (MNV-1). Studies have shown or proposed that infection with certain viruses and an increase in production of the inflammatory molecule type 1 interferon are causes of Crohn's disease (Todd, 2010). Virgin et al. (2009) reported that an intracellular receptor, interferon-induced helicase C domain 1 (IFIH1) or MDA5, is required to suppress mouse norovirus infection It is reported that, although the receptor interferon-induced helicase C domain 1 (IFIH1) is widely expressed, the mice norovirus preferentially infects dendritic cells and macrophages of the immune system (McCartney et al. 2008). The above studies indicate the Crohn's disease and norovirus hypothesis is biologically plausible.

Temporality: Cadwell et al. (2008) infected *ATG16L1* mice with a particular strain of murine norovirus, called MNV CR6. After 7 days, inflammation showed up in a type of intestinal cell called Paneth cells, and the inflammation was similar to that found in

individuals with Crohn's disease in their intestinal Paneth cells. However, when identical *ATG16L1* mice were raised in the absence of murine norovirus in a "germ-free" environment, they showed no evidence of Paneth cell abnormality. In addition, when these researchers tested strain of norovirus, it did not have this effect, and the *ATG16L1* mice stayed healthy. Thus, it is demonstrated that the criteria of temporality have been accomplished by norovirus.

Strength of association: In contrast to MAP, there are no experimental results which show that norovirus has not been isolated from a significantly higher proportion of Crohn's disease tissues than from controls. Thus the criterion of "strength of association" has not been fulfilled by norovirus and, is reported as "unknown".

Specificity of effect: The murine norovirus has not consistently been isolated from intestinal tissues of patients with Crohn's disease. Therefore, in the present study, we suggest that the criterion of "specificity of effect" has not been accomplished by norovirus, and is reported as "unknown".

Dose-response relationship: Studies have shown that the immunosuppressant rapamycin, which induces autophagy in cultured cells, improved symptoms in a patient with refractory Crohn's disease (Massey et al. 2008). However, the dosages of rapamycin have not been titrated according to standard antibiotic conventions. Therefore in this study, the criterion of dose-response relationship is reported as "not applicable".

CONCLUSION

A study has been conducted investigating whether any of the enteric microbial candidates has met any or some of the criteria set by Rothman and others for causality. The candidates examined in this study include: MAP, AIEC, *Listeria monocytogenes*, Yersinia species, Herpes viruses (EBV, CMV and HHV6), measles virus and norovirus. While MAP has previously been reported to fulfill four out of the six epidemiological criteria for causation, the present study demonstrates that data exist that shows that enteric microbes (e.g Normal intestinal flora, AIEC and *Yersinia species*) and Crohn's disease phenomenon has fulfilled at least three (consistency of effect, biological plausibility and strength of association) of the six epidemiologic causal criteria outlined by Hill. Norovirus satisfied three (biological plausibility, consistency of effect and temporality) of the six epidemiologic causal criteria. *Listeria monocytogenes* fulfilled two (consistency of effect and biological plausibility), Herpesviruses also fulfilled two (consistency of effect and strength of association), while measles virus has fulfilled at least one (strength of association and biological plausibility) of the six epidemiological criteria. In addition, this present study suggests that in reference to Rothman's 'pie" model, the existing data are not enough to describe any of the enteric microbial agents, as "sufficient" causes of Crohn's disease. Furthermore, while MAP has been reported as a "necessary" cause in Crohn's disease, other enteric pathogens cannot currently be classified as a "necessary" cause in Crohn's disease, due to the presence of conflicting and unconvincing existing data. Based on available data, it is difficult to determine if the causal criteria bar has been applied in a similar manner to MAP and Crohn's disease as compared to other enteric organisms and Crohn's disease. More studies that support or refute the causal relationship between other

enteric organisms and Crohn's disease need to be conducted in order for this determination to be made. With increasing concern about the transmission of infectious diseases from animal to man, attention has refocused on MAP as a candidate organism implicated in the etiology of Crohn's disease. Suffice to say that, currently, there is more epidemiological evidence that supports the causal link between MAP and Crohn's disease compared to any other enteric organisms. It is possible that the bar for the causal criteria between MAP and Crohn's disease has been raised higher than it has been applied in other currently scientifically acceptable causal relationships. However, additional studies are required to further support the role of other enteric microbial agents in Crohn's disease. Additional research is warranted to confirm or disprove this hypothesis of a microbe or microbes as causes of Crohn's disease.

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PAPER 3

OCCURRENCE OF *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* INFECTION IN CATTLE IN NORTH DAKOTA, 1995-2005

ABSTRACT

Background: *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is a bacterium that causes Johne's disease, also known as paratuberculosis in cattle and other animals. MAP is found in milk and is suspected of having a link to Crohn's disease. **Objective**: The objective of this study was to evaluate trends and risk factors for *Mycobacterium avium* subspecies *paratuberculosis* (MAP) shedding in cattle in North Dakota. **Methods:** The North Dakota State University Veterinary Diagnostic Laboratory records of fecal culture-positive MAP cases diagnosed from 1995 to 2005 were examined. Epidemiological data on clinical history, age, sex, breed, herd size and county of origin were extracted. Additionally, data on producers enrolled in North Dakota's Voluntary Johne's Disease Control Program were obtained from the North Dakota State Veterinarian's office. Data were analyzed using Geographic Information Systems Arc Info 9.1 software, Epi Info version 6 and SAS version 9.2. **Results:** Of the 53 counties in North Dakota, 42 (79%) reported MAP infection (range 1 - 86, median 6) in both beef (n=204) and dairy cattle (n=175). Also, there was a correlation between distribution of cases by county and distribution of producers participating in the ND Johne's Disease Voluntary Control Program: participating counties had more MAP cases reported than counties without participants. The number of MAP cases reported increased during the study period with seasonal trends, as shedding was higher in winter and spring than summer and fall. Chi-square and logistic regression analyses indicated that large herds, female, beef animals and animals greater than four years of age were more likely to be categorized as high shedders of MAP than herds without these attributes.

INTRODUCTION

Johne's disease or paratuberculosis is chronic, non treatable granulomatous enteritis caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP) that affects both domestic and wild ruminants (Daniels et al. 2003). In recent years, the incidence of Johne's disease is reportedly increasing in many countries worldwide, including the United States (US), with considerable economic losses primarily reported in the dairy herds (Johnson-Ifearulundu et al. 1999; McKenna et al. 2006; Nielsen and Ersboll, 2006; Raizman et al. 2007; Roussel et al. 2005). Various studies have documented major economic losses in dairy and beef cattle herds resulting from decreased production, weight loss, lower slaughter value and premature culling of infected cattle (Judge et al. 2005; McKenna et al. 2006; Olsen et al. 2002; Raizman et al. 2007; Roussel et al. 2005). Major obstacles to control of Johne's disease include identifying subclinically infected animals (Daniels et al. 2003) and the wide host range in domestic and wild animals (Hosmer and Lemeshow, 2000; Manning and Collins, 2001; Olsen et al. 2002; Sechi et al. 2004; Stabel et al. 2002; Uzoigwe et al. 2007). Studies have shown that only one in 20 infected animals show overt clinical signs of Johne's disease, thus making early detection and culling difficult (Riemann and Abbas, 1983).

The negative economic impact caused by decreased animal productivity and welfare is often masked, and may appear insufficient to justify large investments in control programs by individual farmers, livestock industries or government (van Scaik et al. 1996). MAP has also become a public health concern (Biet et al. 2005; Hermon-Taylor et al. 2000; Uzoigwe et al. 2007) with some studies reporting that DNA from MAP has been found in up to 69% of patients with Crohn's disease (Taylor et al. 2000; Uzoigwe et al.

2007). Whether this association is causal or merely coincidental is not yet fully understood. While this association remains unproven and contentious, public perceptions of a causal link represents one of the most important economic risks to the milk and meat industries (Manning, 2001).

In the US, one study (CAST, 1996) reported that up to 35% of herds were infected with MAP, causing an estimated $1.5 billion annual loss to the cattle industry (Ott et al. 1999). Because of the economic importance and public health concerns about MAP, many states within the US have instituted Johne's disease certification programs to eradicate the disease. The USDA-US Voluntary Johne's Disease Herd Status Program (1998), and later USDA-Uniform Program Standards for the Voluntary Bovine Johne's Disease Control Programs (2006) (USDA-APHIS, 2006), were established to standardize control programs in the US. The North Dakota Voluntary Johne's Disease Control Program (VJDCP) is a sequel of the initial effort started in 2001 to screen the state cattle herds. Farmers participating in the VJDCP routinely submit fecal samples along with a disease history form to state diagnostic laboratories (SDL) for diagnosis of MAP (Bulaga and Collins, 2000). The compilation and dissemination of information from SDL provides information from which technical decisions are made for control programs. This information is useful to monitor the effectiveness of the control program, and is the basis for making adjustments to the Voluntary Johne's Disease Control Program. In general, examination of laboratory data and relating it to epidemiological information from the field is essential for strengthening national disease monitoring and surveillance systems (MOSS). Review of laboratory information is helpful for explaining changes in the disease seen in the field. The objective of this study was to evaluate trends and risk factors of MAP shedding in North Dakota (ND) cattle from 1995 to 2005, using

data from the North Dakota State University Veterinary Diagnostic Laboratory (NDSUVDL) in Fargo, ND.

MATERIALS AND METHODS

Study design

A retrospective case series of veterinary medical records of fecal culture-positive MAP infections in cattle diagnosed at the NDSU-VDL from 1995 to 2005 were examined. Suspected fecal samples were processed as follows: conventional fecal culture on Herrold's egg yolk (HEY) agar slants was performed as previously described (Whitlock and Rosenberger, 1990), with brief modifications. Briefly, one gram of feces was added to 20 mL of sterile distilled water, tubes were shaken for 30 minutes and then allowed to stand undisturbed for 30 minutes. Five mL of supernatant were added to a decontaminant mixture containing 25 mL of 0.9% hexadecylpyridinium chloride monohydrate and 30 mL of brain heart infusion broth, and the samples were allowed to decontaminate overnight at 98.6°F (37°C). Samples were centrifuged at 900 xg for 30 minutes and the supernatant discarded. Pellets were re-suspended in 1mL of antibiotic brew containing 50% BHI broth with 100 ug/mL vancomycin, 100 pg/mL nalidixic acid and 50 ug/mL amphotericin B. Tubes were vortexed and incubated overnight at 98.6°F. HEY slopes were inoculated with 0.25 mL of the suspension. Each sample was cultivated in duplicate on HEY with mycobactin J and one tube without mycobactin as the culture medium. Tubes were incubated at 98.6°F and observed at two-week intervals for 16 weeks. Isolation of a slowgrowing, acid-fast organism with colonial morphology typical of MAP on HEY with mycobactin, but not on HEY without mycobactin, was considered a positive culture. Animals were classified as high or low shedders if MAP colonies from cultured feces took less or more than two weeks, respectively.

Epidemiological data on clinical history, age, sex, breed, shedder status, herd size, season and county of origin of each case were extracted. Additionally, data on producers enrolled in VJDCP were obtained from the North Dakota State Veterinarian's office.

Data analyses

Information presented in the individual animal data did not have complete entries. Only those with 65% or more of complete entries or those with variables of interest were used in the analysis. Descriptive statistics of cattle with MAP infection were summarized using SAS[b] and Epi Info version 6. The Geographic Information System (GIS) software[c] was used to display the spatial distribution of bovine Johne's disease cases reported in the state by county, and the distribution of producers enrolled in VJDCP by county.

In the statistical analysis, the MAP shedding status of infected cattle was used as the outcome variable. The VDL laboratory protocol classifies an animal as a high or low fecal shedder based on whether MAP colonies took less or more than two weeks, respectively, to appear. Seasons were classified as winter (December to February), spring (March to May), summer (June to August) and fall (September to November). Age and herd size were dichotomized using their medians as the cut-off (above as high and below as low). Submission patterns were reviewed according to months, seasons, years and their geographical origin.

The cumulative incidence (CuI) was calculated from $CuI = 1\text{-}e^{-I}$, where e is the natural logarithm, and I is incidence rate (IR). IR was calculated from the number of new positive cases diagnosed during each year divided by population at risk at the start of the year. The population at risk was calculated from simple moving averages of beef and dairy cattle census of 1992, 1997, 2002 and projections made for 2005.

All listed independent variables of biological relevance to epidemiology of MAP were individually assessed by univariate logistic regression analysis (Hosmer and Lemeshow, 2000). The variables assessed were age, sex, county of origin, breed, herd size, number affected and season. All variables with a P-value of ≤ 0.20 from the univariate analyses were offered to the multivariate logistic regression for model building. Multivariate logistic model with the MAP shedding status as outcome was constructed using forward stepwise selection procedure, while

deviance was assessed using Akaike Information Criteria. Interaction between key variables was assessed using the same strategy. The adjusted odds ratios were estimated with 95% confidence intervals (95% CI). The Wald and likelihood ratio test and the Hosmer and Lemeshow Goodness-of-Fit test were used to assess the model fit.

RESULTS

In total, 562 of the 1,684 (33.4%) fecal samples from 42 of the 53 counties in ND submitted from 1995 to 2005 to NDSU-VDL, were diagnosed as culture-positive to MAP. Positive case reports varied from 1 to 86 (median=6) in the counties. Of the data with completed entries, 47.1% of the cases were dairy (n=197), while 52.9% (n=221) were beef breeds. Only the Holstein dairy breed was represented (100%; n=197). The beef breeds were Angus (49.3%; n=109); Gelbvieh (16.3%; n=36); cross breeds (9.0%; n=20); Shorthorn (5.4%; n=12); Limousin (4.1%; n=9); Simmental (4.1%; n=9); Brahman (1.8%, n=4); Charolais (1.8%; n=4); Hereford (1.8%; n=4); Black Maine, Corrient, Longhorn and Tarentaise-cross each represented 0.9% (n=2); and others constituted 3% (n=6). Breeds classified as "others" included: bucking bull (1), Normande (1) and Saler (1). Because of smaller numbers of individual cattle breeds involved, it was not possible to run analysis on their influence on fecal shedding; instead they grouped together according to their use (dairy or beef).

Figure 3.1 shows the spatial distribution of MAP cases per county in ND (n=467) from 1999 to 2005. The 1995-1998 (n=95) data were excluded due to missing county information in their entries.

Figure 3.2 shows the distribution by county of producers participating in the VJDCP as of March 22, 2007 (n=173). A total of 346 (91.8%) positive cases were females and 31 (8.2%) were males; 185 case records did not specify gender. Of the 562 culture-positive cattle, 225 (40%) had age information missing. Twenty-five (4.4%) were <2 years, 83 (14.8%) were 3-4 years and 229 (40.7%) were recorded as adults.

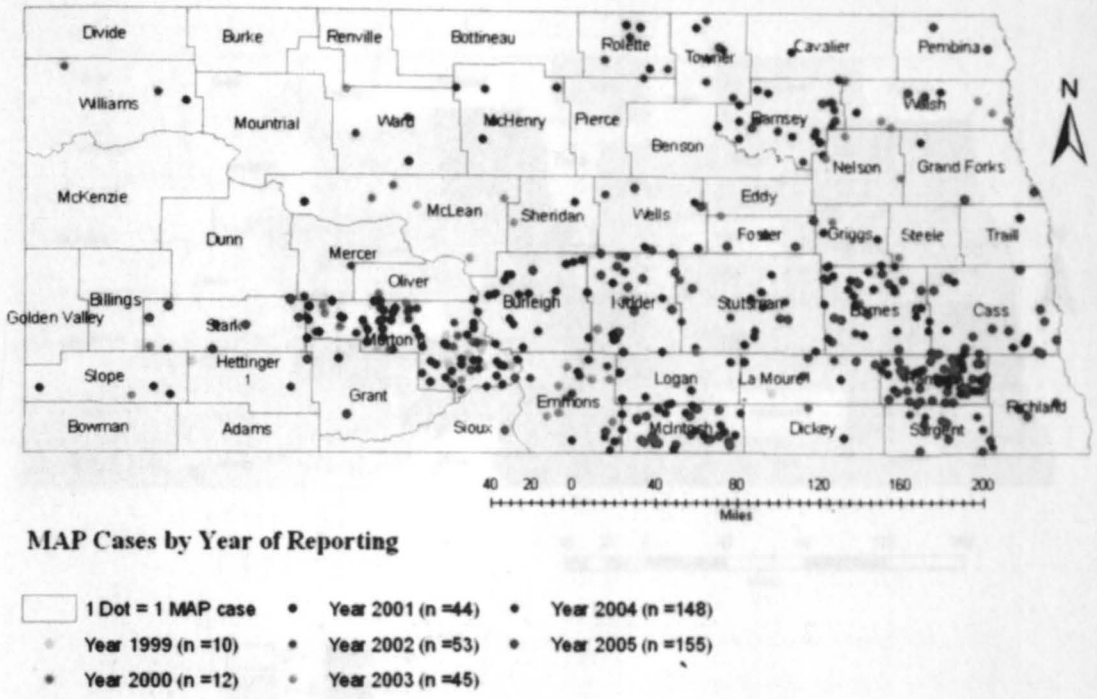

Figure 3.1: Spatial distribution by county of fecal culture- positive cases of *Mycobacterium avium* subspecies *paratuberculosis* infection in cattle in North Dakota, 1999 - 2005 (n=467).

Variations in the case reports by cattle type and year of reporting, 1995 to 2005, are given in Figure 3.3. As observed, the proportion of positive cases between the beef (52.9%, n=221/418) and dairy breeds (47.1%, n=197/418) were comparable although the breed entries for 144 other animals were missing. Eighty-eight percent (n=494/562) of MAP cases were reported from 2001 to 2005 following launch of the Voluntary Johne's Disease Control Program in 2001, while 58% of cases (n=327/562) were reported in 2004 and 2005, showing a very marked increase in the last two years.

Figure 3.4 shows the cattle type cumulative incidence of MAP per 10^6 cattle initially at risk in ND by year of reporting.

Figure 3.5 shows a monthly relationship of culture-positive results to total submissions over the same period.

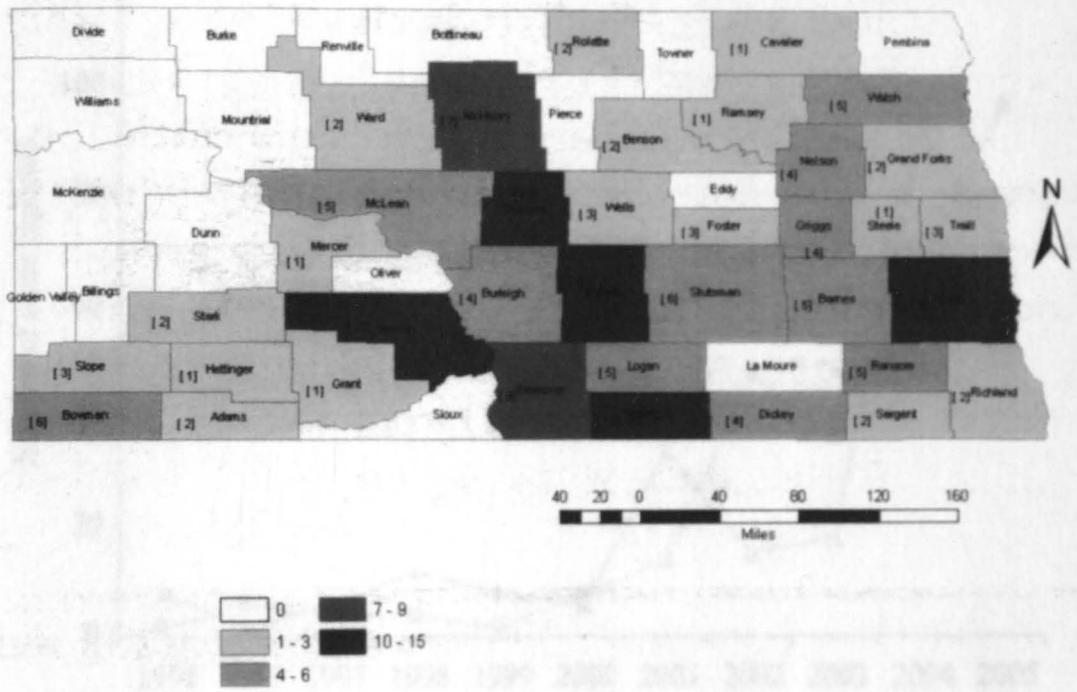

Figure 3.2: Distribution by county of producers participating in the North Dakota Voluntary Johne's Disease Control Program as of March 22, 2007 (n=173).

Figure 3.6 describes the variation in the monthly trend in the mean number of culture-positive MAP cases reported from start of the program in 2000 to 2005 (n=508, detailed information missing [n=54] for 1995-1999).

Trends show the highest mean number of positive MAP cases were diagnosed during early spring and late winter (38 and 25%, respectively) compared to summer and fall (19 and 18%, respectively; P=0.0091; Table 1). Further examination of submissions revealed higher fluctuations in the number of cases during May-July and November-December, showing yearly influence.

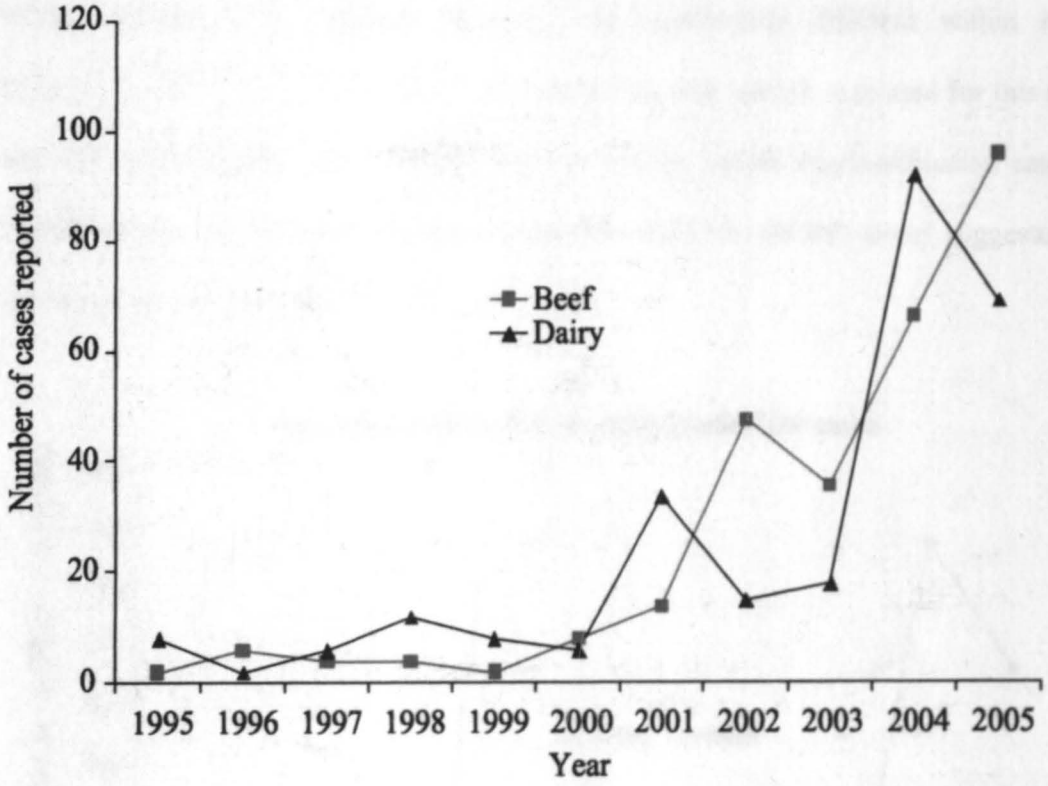

Figure 3.3: Spatial distribution by county and year of reporting of fecal culture-positive cases of *Mycobacterium avium* subspecies *paratuberculosis* infection in cattle in North Dakota, 1999 - 2005 (n=467).

At the univariate level, shedding status among MAP-infected cattle was found to be significantly associated with herd size, gender, type (dairy/beef) and season ($P<0.05$; Table 1), while age category was not ($P>0.05$). Details of the results from the tabular and univariate logistic regression analyses are given in Table 3.1. The final multivariate logistic regression model retained only two of the original variables, cattle type (dairy/beef) and season. Of these variables, the interpretation of their parameter estimates and estimated odds ratios suggested a strong association with shedding of MAP. In the final model, beef cattle were associated more strongly with high shedding of MAP than dairy cattle (χ^2_{2df} = 5.8, OR=2.13; 95% CI=1.3-3.5; P=0.016), despite higher case reports from the dairy

breeds (Figure 3.2). Shedding variation was significantly different within season (χ^2_{3df}=11.6, OR=3.8; 95% CI=1.2-2.3; P=0.009). The total sample size used for this model was 215, concordance was relatively high at 61.5%, actual misclassification rate was 24.4% and the Hosmer and Lemeshow Goodness-of-Fit test for this model suggested that the model fit was adequate (χ^2 =1.37, P=0.5029).

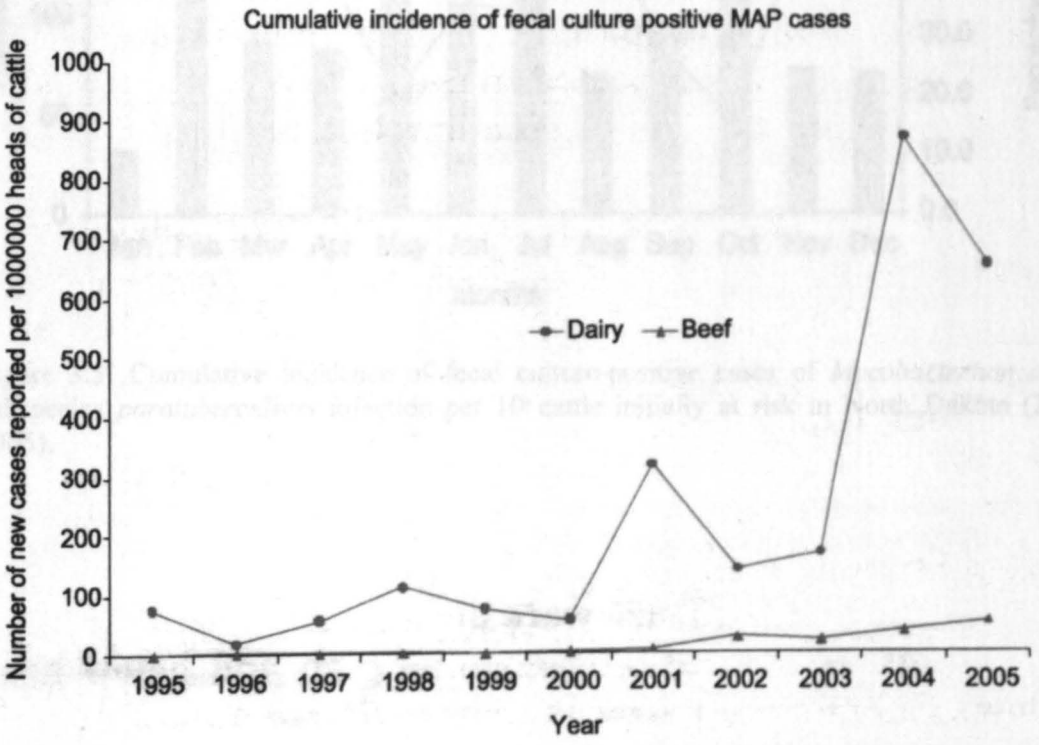

Figure 3.4: Distribution by breed and year of reporting, of fecal culture-positive cases of *Mycobacterium avium* subspecies *paratuberculosis* infection in cattle in North Dakota, 1995 - 2005 (n=562).

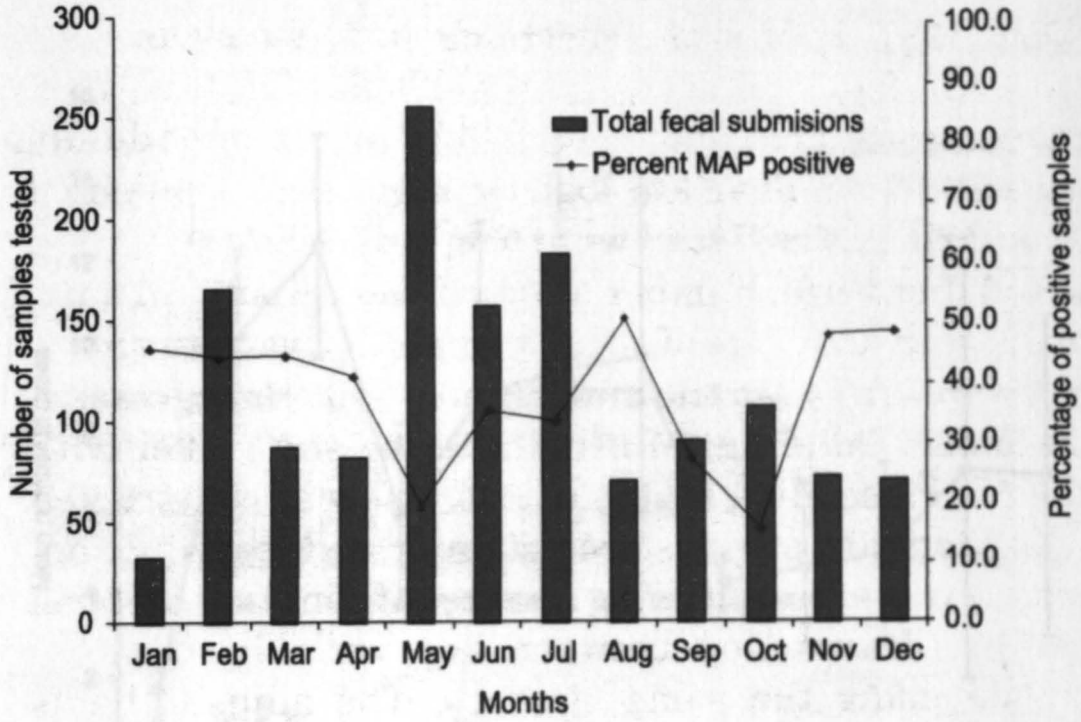

Figure 3.5: Cumulative incidence of fecal culture-positive cases of *Mycobacterium avium* subspecies *paratuberculosis* infection per 10^6 cattle initially at risk in North Dakota (2000-2005).

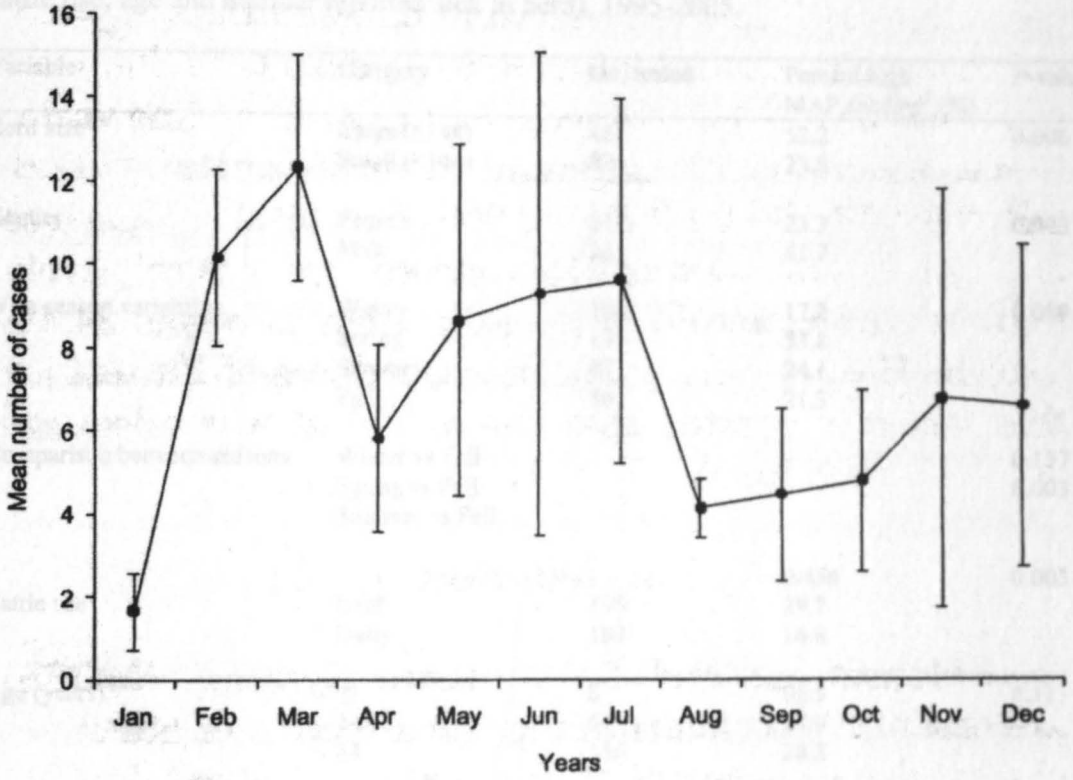

Figure 3.6: Mean number of fecal culture-positive cases of Mycobacterium avium subspecies paratuberculosis infection in cattle in North Dakota by month, 2000 to 2005 (n=508).

Table 3.1: Univariate logistic regression analysis of associations between shedding status (high or low) of MAP infected cattle and various risk factors (herd size, gender, season, cattle use, age and number reported sick in herd), 1995-2005.

Variable	Category	No. tested	Percent high MAP shedder[1] (%)	*P*-value
Herd size[2]	Large (≥144)	46	52.2	0.006
	Small (<144)	42	23.8	
Gender	Female	317	23.3	0.043
	Male	24	41.7	
With season variability	Winter	163	17.2	0.009
	Spring	133	33.8	
	Summer	87	24.1	
	Fall	80	21.3	
Comparison between seasons	Winter vs Fall			0.157
	Spring vs Fall			0.003
	Summer vs Fall			
			0.436	0.003
Cattle use	Beef	195	29.7	
	Dairy	169	16.6	
Age (years)	<1	8	62.5	0.111
	2-3	61	27.9	
	≥4	156	28.2	
Number individuals Reported sick in herd[3]	≤20	116	34.5	0.969
	≥20	76	34.2	

[1]High shedders: defined as high shedders if the MAP colonies from cultured feces took less than two weeks to show visible colonies.
[2]Herd size: herds with >144 heads of cattle considered large and ≤ 144 small.
3Number of individuals reported sick in herd: number farmer reported with similar signs at the time of diagnosis.

DISCUSSION

Monitoring and Surveillance Systems (MOSS) are essential for determining the direction or effectiveness of disease control or eradication strategy. Review of laboratory data from an ongoing control program is one of the MOSS tools used. This present study evaluated the status of MAP infection in cattle in ND, using information retrieved from the veterinary medical records of NDSU-VDL. The North Dakota Voluntary Johne's Disease Control Program is one of many similar ongoing programs in the US (USDA-APHIS, 2006), and is based upon early detection and removal of infected animals. To our knowledge, this is the first study to use laboratory data to assess the progress of the bovine Johne's disease control program in ND.

This study gives insight into the quality of data captured and limitations of using such data for disease monitoring purposes. The observed results could have been biased by lack of standardization of sample submissions because it was voluntary. Furthermore, it could have been influenced by the continued improvements in the laboratory diagnostic protocols. Differences in cattle type (dairy/beef) submissions could have been influenced by other factors, such as variations in the farmers' interest. The sampling was open to both beef and dairy. The state informed producers that they would be compensated by the federal government for fecal and serologic testing if they enrolled, and were also informed of the herd health benefits of controlling Johne's disease. Despite this, it is known that dairy cattle are more likely to be closely monitored than beef breeds. Likewise, producers are more likely to collect and submit samples when cattle are gathered, hence a seasonality bias.

This study demonstrated widespread distribution (42 of 53 counties) of MAP infection in ND (Figure 3.1), suggesting existence of probable reservoirs and an endemic situation in the state. The appearance of an upward trend in the number of case reports could not be directly linked to increases in the rate of new infection without considering other factors, such as increased producer awareness and recruitment of new participants. Also, there was a correlation between distribution of cases by county (Figure 3.1) and distribution of producers participating in VJDCP (Figure 3.2), with participating counties having more MAP cases reported than counties without participants, suggesting producer bias. In addition, it was evident that the lowest percent positive submissions occurred in 2000, which was prior to the introduction of VJDCP. The majority of cases (88%, n=494/562) were reported between 2001 and 2005, following introduction of the VJDCP in 2001, suggesting that submissions changed with participation. This observation highlights the importance of VJDCP participation as a way of increasing Johne's disease testing. From the available data, it is not possible to explain the appearance of an upward trend in the case reports. There are two possibilities: either there is a producer bias, where there is increased interest and participation, or simply that there is a true increase in the disease prevalence.

Early identification of MAP clinical shedders remains difficult (Whitlock and Buergelt, 1996), partly due to low sensitivity of the tests used and the presence of subclinically infected cows in the herds, both of which may substantially increase risk of infection. Currently, available tests to detect MAP-infected animals produce many false negatives, particularly in subclinically infected or low-MAP fecal-shedding animals, thus making their interpretation and utilization in control programs challenging (Tiwari et al.

2006). In the NDVJDCP serial testing was adopted, with either the whole herd or percentage of the herd being screened first with ELISA, and then followed by fecal culture of positive samples.

It has been suggested that the prolonged pre-clinical phase of lifelong MAP infections and the poor performance of diagnostic tests make identification of clinical shedders difficult. As a result, the susceptible population is exposed to subclinically infected cows, which are the main risk factor in the spread of the disease (Wells and Wagner, 2000).

The spatial-temporal distribution of MAP cases (not shown) showed cases clustered in the southwestern part of the state, while the more recent cases were reported mainly in southeastern ND. The direction of movement of the disease across the state is possibly linked to increased producer awareness and submissions from newly recruited participants. Interestingly, case reports and cumulative incidence of MAP infection in dairy breeds peaked every three years (1995, 1998, 2001 and 2004). This observation was more pronounced in dairy than beef cattle. It was not possible to establish whether this increment was an actual or an apparent increase in case-detection rate. However, from trends in our results, a three-year MAP infection cycle appears to exist in dairy herds in ND. Producers received incentives (testing) annually, and therefore monetary incentives should not have influenced the three-year trend.

The main limitation of this study was lack of baseline information on the reference population. In addition, no information was provided on the sampling criteria, although we were told that any herd was eligible for entry into the program.

Because results of this study do not conclusively define whether new cases resulted from more farmers responding to the program or an actual increase in number of cases, an annual review of control strategies in ND is highly recommended. The comparable distribution of case reports between beef (52%) and dairy breeds (48%) in ND (Figures 3.3 and 3.4) suggest the two breed groups were equally susceptible; however, when considering the reference population, the MAP cumulative incidence was higher in dairy than beef breeds.

The occurrence of infection in both cattle production systems supports the notion that MAP can cause serious economic losses in both dairy and beef cattle (Benedictus et al. 1987; Johnson-Ifearulundu et al. 1999; Roussel et al. 2005). However, the significantly higher MAP shedding rates in infected beef than dairy cattle (Table 3.1) could be linked to poor hygiene conditions of beef farms (Roussel et al. 2005), calves suckling infected cows, common beds or feeding.

In this study infection rates were higher in older animals (71%) than younger ones (29%). Also, larger herds were observed to have more heavy MAP shedders than smaller ones. This agrees with 1996 Dairy National Animal Health Monitoring System's (NAHMS) study (Johne's disease on U.S. dairy operations. USDA: APHIS:VS, CEAH, National Animal Health Monitoring System 1997; Fort Collins, Colorado), which reported that larger herds (>300 cows) were more likely to be infected than smaller herds (<50 cows). In the present study, the presence of high shedders in larger-sized herds could be due to increased close physical contact between individual animals, leading to more effective transfer of infection to non-infected animals in large herds. Also, sanitary practices to decrease the risk of new MAP infections are difficult to implement around beef

cattle compared to dairy cattle (Goodger et al. 1996; Johnson-Ifearulundu et al. 1998). While sanitary practices may be mandatory to control mastitis in dairy operations (Goodger et al. 1996; Johnson-Ifearulundu et al. 1998), that is not the case for beef herds, making these particular groups more vulnerable to infection than dairy cattle.

Additionally, at a univariate level, there was a significant difference in the proportion of female cattle (93%) that were infected compared to males (7%), however, high shedding was observed more in males than females ($P=0.043$). This could be attributed to differences in the submission rates. There are fewer bulls than cows in both beef and dairy herds, and most dairies use artificial insemination so they have few bulls. This may account for the difference in proportion of infected females as compared to males.

Although in this study the shedding status was higher in animals four years of age or older, the differerence was not statistically significant ($P>0.05$). This is contrary to several earlier studies in which high shedding was associated with increasing age. Several studies (Chiodini, 1996; Nielsen and Ersboll, 2006; Wells and Wagner, 2000) reported that cattle infected with MAP have a long prepatent period in which no shedding of the organism occurs, which is followed by intermittent then continuous shedding, increasing in volume as the disease progresses.

Seasonality in the shedding pattern of MAP was also detected, with higher shedding in colder months (winter and spring) compared to warmer months (summer and fall; Figure 3.4 and Table 3.1), in spite of most samples being submitted in fall. This agrees with other studies that found higher MAP incidence during the winter months (Crossley et al. 2005; Harris and Barletta, 2001). A likely explanation for the difference in incidence

and shedding status (high or low) between spring and summer could be the effects of over-wintering on body condition and/or calving season stress and lactation. Stress compromises the immune system and results in higher levels of disease in many mammalian species in the breeding season (Nelson and Demas, 1996). Other researchers (Judge et al. 2005), have downplayed this winter and spring stress hypothesis, and attribute increased MAP case reports to increased case detection as a result of increased attention paid to cattle during spring rather than an actual increase in MAP prevalence. This could explain observations in this study. The high proportion of MAP infection in winter and spring is also in agreement with the seasonal variation of other enteric animal diseases (Scott et al. 2006).

The main limitations of this study were lack of control of quality of data recorded (missing variables), limited access to management and ecological data and lack of information on the reference population. Access to these data could have provided vital information to better explain the host-environment-agent interactions during spring. This study could have been further limited by the low sensitivity (38%) of the fecal culture test used, as well as possible improvements in the laboratory protocols as the lab focused more on the program. Rapid and more sensitive procedures (with a sensitivity of 93 to 96%) using immunomagnetic bead separation and real-time PCR for diagnosis of clinical and subclinical Johne's disease (paratuberculosis) from milk and/or fecal samples from cattle and American bison have been reported (Khare et al. 2004). These methods (Khare et al. 2004), have been reported to be very useful and cost-effective compared to bacteriological culture, which is constrained by time, labor, and expense under diagnostic laboratory conditions.

CONCLUSION

This study demonstrated widespread occurrence of MAP and documented some risk factors for MAP shedding in dairy and beef cattle in ND. Counties with producers participating in ND Johne's Disease Voluntary Control Program reported more MAP cases than counties without participants. The study also provided information on the progress of the control programs for bovine Johne's disease in ND, helping to identify some critical areas for further research and intervention. This study further justifies the retrospective examination of laboratory data to strengthen and add value to national disease monitoring and surveillance efforts. It also recognizes serious limitations of this data and recommends that new efforts be made to improve the value of data for state and national reporting.

Endnotes

[a]Sigma, USA, St. Louis, MO 63103

[b]SAS-Institute, Inc, Gary, NC 27513

[c]ArcInfo™8, ESRI, 380 New York Street, Redlands, CA 92373

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PAPER 4

TYPING OF *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* STRAINS FROM SYMPTOMATIC AND ASYMPTOMATIC CATTLE

ABSTRACT

Background: *Mycobacterium avium subspecies paratuberculosis* (MAP) is an intracellular bacterial pathogen that causes Johne's disease, also known as paratuberculosis. This disease is a chronic inflammation of the intestine of cattle, sheep and other ruminant animals. Crohn's disease is a form of chronic inflammatory disease of the intestines in humans. An increasing human health concern is the potential link between Johne's and Crohn's disease; however, this hypothesis is still under debate. The typical symptoms of paratuberculosis include slowly progressive wasting and diarrhea. It is reported that both symptomatic and asymptomatic cattle shed MAP in feces. **Objective:** The goal of this study was to analyze MAP strains isolated from symptomatic and asymptomatic cattle, as well as humans, using various biochemical typing techniques and to investigate how variations in bacterial and non-bacterial factors could predict if an animal would be symptomatic or not. This study is essential in order to gain a better understanding of the pathogenicity and virulence of MAP strains, as well as the relationship between MAP and Crohn's disease. **Methods**: In total, 120 (one hundred and eighteen bovine MAP isolates and additional two human clinical) isolates were analyzed by methods of high performance liquid chromatography (HPLC), gas chromatography (GC), and mass spectrometry (MS). Statisitcal analysis including chi-square and logistic regression was done using Epi Info version 3.5.1. The symptom status of animals was used as the outcome variable and was binary (asymptomatic and symptomatic). **Results:** At the univariate logistic regression analysis, the symptoms status of isolates was significantly associated with mass spectra patterns (OR=3.7, 95% CI= 1.7, 8.2; p <0.00097), growth rate (OR= 11.0, 95% CI= 4.5, 26.8; p <0.0001), and shedder status (OR= 3.4, 95% CI= 1.6,

7.3; $p = 0.00127$). However, the association between symptoms status and HPLC and GC patterns was not significant ($p > 0.05$). **Conclusion:** Overall, the study demonstrated that bacterial and non-bacterial factors of MAP strains could predict if the source animal would be symptomatic or asymptomatic. These data could play a significant role in the design of future diagnostics, therapeutics and vaccines for Johne's and Crohn's diseases.

INTRODUCTION

Mycobacterium avium subspecies paratuberculosis (MAP) is a causative agent of paratuberculosis (or Johne's disease), described as chronic enteritis of cattle, sheep, goats and other ruminant animals (Chiodini et al. 1984; Sweeney et al. 1996). It reported that MAP is an economically important bacterial pathogen that causes considerable economic losses in domestic livestock (Manning and Collins, 2001). Studies have also implicated MAP in cases of Crohn's disease in humans (Naser et al. 2004), an inflammatory disease in which patients suffer from chronic enteritis and intestinal pathology that is similar to Johne's disease in cattle. Although several lines of evidence (Dalziel, 1913; El Zaatari et al. 2001; Naser et al. 2004; Clancy et al. 2007; Uzoigwe et al. 2007) support the causal role of MAP in Crohn's disease, this causal link has not yet been proven. The typical symptoms of Johne's disease are slowly progressive wasting (emanciation) and diarrhea, which is intermittent at first, becoming progressively more severe until it is constantly present in cattle. Besides diarrhea and weight loss, infected animals show decreased productivity, increased infertility and susceptibility to other infections, which may eventually lead to animal death (Merkal et al. 1975). Researchers have shown that cows become infected early in life through ingestion of MAP from a contaminated environment, referred to as transmission via fecal-oral route, and infected animals may remain in the asymptomatic or subclinical stage of the disease for several years (2-5 years), following initial exposure of the bacteria (Larsen et al. 1975). It is known that paratuberculosis can exist undetected in a herd for years, unless the animals are tested (Koets et al. 2002). Studies have shown that a phase of multiplication of MAP begins in the walls of the small intestine, during the early phase of infection, and depending on the resistance of the animal, this infection is

eliminated or the animal remains infected as a healthy carrier (Larsen et al. 1975). In only a few animals carriers, the multiplication of MAP at a later phase leads to the extension of intestinal lesions, interference with intestinal metabolism and development of overt clinical disease. Koets et al. (2002) reported that both peripheral and intestinal cell-mediated responses are decreased in symptomatic animals compared to asymptomatic animals. Many authors (Abbas et al. 1983; Whitlock et al. 1986) reported that the proportion of animals in the asymptomatic categories outnumber symptomatic cows in most herds with paratuberculosis.

As seen with most living bacteria, cells of MAP are surrounded by a cell wall responsible for providing their shape, as well as protection to the cell against physical and chemical factors (Hett and Rubin, 2008; Brennan, 2003; Chimote and Banerjee, 2008). The thick waxy nature of the mycobacterial cell wall makes it a highly impermeable outer surface, which enables mycobacteria to survive in the presence of antibiotics and harsh environmental conditions (Hett and Rubin, 2008; Brennan, 2003). The mycobacterium cell wall partly resembles a Gram-positive wall, but is unusual in having a layer of lipid (mycolate esters) (Daffé and Draper, 1986), which is very tightly connected to the peptidoglycan and arabinomannan inner layers of the cell wall (Minnikin 1982). Mycolic acids are recognized as high-molecular-mass 3-hydroxy, 2-alkyl-branched fatty acids found in the cell wall of all *Mycobacterium* species (Minnikin 1982). The lipid component of MAP cell wall, specifically the mycolic acid component has been analyzed by use of various biochemical typing techniques. It is reported that the major slowly growing pathogenic mycobacteria and *Mycobacterium gordonae* has been identified by high-performance liquid chromatography of their mycolic acids (Butler and Kilburn, 1988).

Many authors (Butler et al. 1991; 1996; 1999) have reported the analysis of mycolic acid using high-performance liquid chromatography (HPLC) and *p*-bromophenacyl bromide derivatizing reagent for UV detection. Parrish et al. (2007) demonstrated a rapid, standardized method for determination of *Mycobacterium tuberculosis* drug resistance and susceptibility by use of high-performance liquid chromatography (HPLC)-based quantitation of mycolic acids. Lefmann et al. (2004) reported the use of matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) after base-specific cleavage of PCR amplified and in vitro-transcribed the 16S rRNA gene (rDNA) for the identification of mycobacteria.

Previous works reported that the bacterial cell wall composition is not static and several factors such as physiological and/or environmental conditions affect the chemical composition of mycobacterial cell wall (He and De Buck, 2010). The chemical components and the assembly of the different macromolecules that make up the bacterial cell wall are modified during cell growth and morphogenesis. It is reported that mycobacteria have β-lactamases, enzymes associated with cell wall monitoring and remodeling (Flores et al. 2005a ; 2005b; McDonough et al. 2005; Wang et al. 2006). Toriyama et al. (1978, 1980) reported the effect of growth temperature on mycolic acid composition and synthesis. Their results showed that an increase in growth temperature of *Mycobacterium phlei* leads to an enhanced mean chain length of mycolic acids (Toriyama et al. 1978, 1980). According to Laval et al. (2001), examination of the MALDI mass spectra demonstrated that the chain lengths of the various mycolates correlated with the growth rate of mycobacterial strains. Shin and colleagues (2006) conducted a study on the the phenotypic screening of a library and identified disruptions of genes involved in iron.

tryptophan, or mycolic acid metabolic pathways that displayed unique growth characteristics. These researchers identified potential virulence determinants representing novel functional classes that could be necessary for mycobacterial survival during infection and could provide suitable targets for vaccine and drug development against Johne's and Crohn's diseases (Shin et al. 2006).

Molecular typing methods have been used by researchers to detect genetic variation between different isolates of a bacterial species. Numerous molecular techniques have repeatedly been used to analyze the mycobacteria organisms, including PFGE (Sevilla et al. 2007) and RFLP (Whittington et al. 2000). Pulsed-field gel electrophoresis (PFGE) is a molecular technique in which the entire genome can be represented as a distinct pattern of DNA restriction fragments (Hughes et al. 2001). Mira et al. (2002) reported that DNA rearrangements are responsible for genomic diversity in microbial systems and usually contribute to the fitness of a pathogen in specific microenvironments. Previous researchers (Oliveira et al. 2003) reported that examining restriction fragment length polymorphism in the *hsp65* gene showed greater variability and suggested that there are distinct lineages of strains that infect animals and strains that infect humans. A study by Wu et al. (2006) using comparative genomic hybridization to analyze the content of the *M. paratuberculosis* genome identified large regions that could explain its differential virulence compared to that of the closely related *M. avium* subsp. *avium*. Shin et al. (2006) reported that genes may be involved in different virulence mechanisms. According to Djonne et al. (2005), variation in virulence between different isolates of a bacterial species can be caused by genetic variation that is detected by phenotypic or genotypic characterization.

It is known that the mycobacterium cell wall mycolic acid is not static, but varies among MAP isolates. Likewise, studies using molecular typing techniques have shown that the genetic composition of MAP isolates varies. The goal of this study was to examine the differences in the mycolic acid compositions of MAP isolates from symptomatic and asymptomatic cattle, in order to obtain a better understanding of the various factors affecting the manifestation of symptoms in infected animals, as well as the differences in virulence and pathogenicity of MAP organisms. In addition, this study investigated whether the symptom status of animal is associated with growth rate, shedder status and mass spectra patterns of MAP isolates. The study also compared the biochemical profiles of isolates from Crohn's and Johne's disease.

MATERIAL AND METHODS

Growth and identification of bacteria isolates

A total of 120 [one hundred and eighteen bovine MAP isolates and additional two human clinical (ATCC 43015, 43544)] isolates, were used in this study. The human MAP isolates were purchased from the American Type Culture Collection (ATCC), Manassas, VA. The bovine MAP (66 asymptomatic and 52 symptomatic) isolates were obtained from the North Dakota State Veterinary Diagnostic Laboratory (ND-VDL), Fargo, North Dakota. The MAP isolates were grown on Herrold's egg yolk medium (HEYM) slants with mycobactin J (HEYMJ). Bacteria from HEYMJ slant were inoculated into Middlebrook 7H9 liquid media (Becton, Dickinson, Sparks, Md) supplemented with ADC (albumin, dextrose, catalase) enrichment (Sigma Chemical Co., St. Louis, Mo.), and mycobactin J (Allied Monitor Inc., Fayette, Mo.), and incubated at 37°C for up to 16 weeks. Based on results of culture on Herrold's egg yolk medium (HEYMJ) slants, all isolates were classified in two categories based on growth rate categories: group 1 (slow growth or growth in > 2 weeks) and group 2 (rapid growth or growth in < 2 weeks).

The identity of all MAP isolates from the cultures was confirmed using the IS900 insertion sequence. PCR analysis for IS900 was carried out by using the primers and PCR conditions described by Ayele et al. (2005). Briefly, 50 µl of sterile distilled water was inoculated with a single colony of a positive culture. DNA was extracted from the bacterial suspension by heating it at 100°C for 20 min and then centrifuged at 16,000 x g for 2 min; 2 µl of the supernatant was added to the amplication mixture. The reaction mixture was analyzed using a Perkin-Elmer 480 thermal cycler. Electrophoresis was performed in a 2%

agarose gel in 1X Tris-borate-EDTA buffer. The gels were stained in ethidium bromide (5 μg/ml) and visualized by UV light transillumination.

Gas chromatography (GC) analysis

Preparation of fatty acid methyl esters (FAMES): MAP cell wall mycolic acid was extracted and esterified by use of protocol previously described by Minnikin et al. (1980). In brief, one-milliliter aliquot of the bacterial culture (OD_{540} of 1.3 – 1.4 that corresponds to 3 x 10^9 - 5 × 10^9 CFU/ml of cells) was transferred to a new tube and centrifuged at 3,500 x g for 20 min. The cell pellets was esterified by mixing with 2 mls of transesterification reagent, methanol/toluene/H_2SO_4 in the ratio of 30:15:1. The samples were heated at 85°C for 16-18 hours in an oil bath. Then, 2 mls of chloroform: hexane (4:1) was added to the cooled sample. This was followed by addition of 1 ml of 0.3 M phosphate buffer (42.57 g of Na_2HPO_4 and 12.0 g of NaOH per liter of distilled water (pH 11 to 12). Samples were centrifuged (3,000 x g, 10 min, 4°C), and the lower organic layer containing the fatty acid was transferred to a clean tube. The extracts were evaporated to dryness under a stream of nitrogen at 37-42°C and stored at 0°C.

Analysis of fatty acid methyl esters by GC: The dried sample of fatty acid methyl ester was resuspended in 100 μl of chloroform. The fatty acid methyl esters, mycolic acid cleavage products were analysed on HP-5 column (30m by 0.25 mm [inside diameter] and 0.32 μM df (Agilent J&W Scientific, Folsom, CA, USA). The column was inserted in a GC-2014 Schimadzu gas chromatograph (Shimadzu Scientific Instrument, Inc. Lanexa, KS), equipped with a flame-ionization detector, an autosampler (AOC-20i autoinjector Schimadzu) and a computer software system (micronpc Client Pro585). The column was

then programmed at 225°C to 300°C at 10°C/min and maintained at 300°C for 30 min. The injector and detector temperature was 300°C. The carrier gas was helium with a flow rate of approximately 1.96ml/min. The pressure was maintained at 225.8 kPa. The peaks on the chromatograms were identified by matching peak retention times with those of fatty acid methyl ester standards (Ultra Scientific Analytical Solutions, North Kingstown, RI, USA). Each sample was analyzed three times with identical results.

HPLC and mass spectrometry analysis

Preparation of bromophenyl esters of mycolic acids: Fatty acids are extracted, derivatized and analyzed by a modification of the methods previously dscribed elsewhere (Butler and Kilburn, 1988). Bacteria were harvested by centrifugation (3,800 × g, 20 min, 4°C) and washed twice with middlebrook 7H9 broth. Bacterial pellets were resuspended in an appropriate amount of the same medium to obtain an OD_{540} of 1.3 – 1.4, which corresponded to $3 \times 10^9 - 5 \times 10^9$ CFU/ml^{-1}. Three milliliters of this suspension was transferred to a clean screw cap culture tube (13 by 100 mm) and centrifuged (3,800 × g, 20 min, 4°C). The cells were finally resuspended in 3 mls of sterile distilled water and autoclaved for 30 min at 121°C and 15 lb/in^2. The autoclaved supension of cells were centrifuged (3,800 × g, 20 min, 4°C) and the water was removed with a capillary pipette to ensure that a minimal amount of supernatant fluid was left with the cells. Fatty acids, mycolic acids and alcohols were liberated from each strain sample by saponification using 25% (w/v) KOH in ethanol/water (1: 1 vol/vol) at 85°C. The extract was then treated with chloroform, acidified with concentrated HCl, dried, and subsequently treated with potassium bicarbonate and dried again. For each strain, an aliquot of the mycolic acids

extract was transformed to *p*-bromophenacyl derivatives, by treatment with 100 µl p-Bromophenacyl/crown ether mix (Pierce Chemical Co.), followed by heating at 85°C for 20 min. Sample was filtered and the supernatant evaporated under vacuum.

Analysis of p-bromophenacyl esters of mycolic acids by HPLC-MS: About 10 mg of dried mycolic acid bromophenacyl ester was redissolved in 2 ml of chloroform (GC-MS grade) and serially diluted to a 1 µM concentration and 10 µl of the solution were analyzed by HPLC and Mass spectrometry. For analysis using HPLC, the *p*-bromophenacyl derivatives of the mycolic acids were separated in a HPLC system (Waters Associates) equipped with an UV/visible detector measuring absorbance at 254 nm. A reverse-phase C18 column (Nova-Pack 60A, 4 µm, 3·9x75 mm; Waters Associates) was used in the system. The column was equilibrated with 91 % methanol/9% chloroform. After injection of the sample, the gradient was changed linearly to 30% methanol/70% methylene chloride. The total run time was 30 min. at a total flow rate of 1 ml min^{-1} (Butler et al. 1986). A high-molecular-mass standard (Ribi; ImmunoChem Research) was used as a standard. The HPLC patterns were interpreted by comparison with standard reference chromatograms of known mycolic-acid-containing mycobacteria (Butler et al. 1986; Butler and Guthertz, 2001). For sample analysis by use of mass spectrometry, 10 µl of 1 µM concentration of the solution the mycolic acid bromophenacyl esters in chloroform was analyzed by use of BioTOF Electrospray Ionization Time-of-Flight (ESI-TOF) Mass Spectrometer, associated with Bruker Daltonic Data Analysis (Bruker Daltonics BioTOF Billerica, MA, USA). Each sample was analyzed in triplicate with identical results.

Statistical analysis

The results of this study were analyzed as described by Niemann et al. (1999). Data were analyzed using the software package Epi Info version 3.5.1. Categorical variables were compared by using χ^2 test or Fisher's exact test. Univariate logistic regression test was performed to determine whether changes in HPLC profiles, GC profiles, mass spectra patterns, growth rate and shedder status were associated with changes in symptom status of the animals.

RESULTS

Table 4.1 shows the relationships between symptom (symptomatic or asymptomatic) status of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infected cattle and other variables (HPLC, GC and mass spectrometry patterns of isolates, shedder status and Growth rate status of animals).

Table 4.1: Univariate logistic regression analysis of associations between symptom (symptomatic or asymptomatic) status of MAP infected cattle and other variable factors (HPLC, GC, mass spectrometry patterns of isolates (OR = 3.7, 95% CI = 1.7, 8.6), shedder status (OR = 3.4, 95% CI = 1.6, 7.3) and growth rate status of animals (OR = 11.0, 95% CI = 4.5, 26.8).

Variable	Category	No of isolates	Isolates from asymptomatic cattle (%)	Isolates from symptomatic cattle (%)	df	P value
GC patterns	GC1	73	58.9	41.1	1	0.4
	GC2	45	51.1	48.9		
HPLC patterns	HP1	64	57.8	42.2	1	0.7
	HP2	54	53.7	46.3		
Mass spectrometry Patterns	MS1	47	74.5	25.5	1	0.00097
	MS2	71	43.7	56.3		
Growth rate	Slow growers	55	83.6	16.4	1	<0.00001
	Rapid growers	63	31.7	68.3		
Shedder status	Low shedder	65	69.2	30.8	1	0.00127
	High shedder	53	39.6	60.4		

Figures 4.1-4.6 show examples of the results of gas chromatography analyses of MAP isolates from symptomatic and asymptomatic cattle. The GC results revealed the presence of identified and unidentified peaks. The peaks with retention times of approximately 1.40, 2.07, 3.33, 9.34 min and 16.25 min corresponding to $C_{16:0}$, $C_{18:0}$, $C_{20:0}$ and $C_{24:0}$ methyl esters respectively were observed. Other unidentified peaks with retention times of 3.07, 3.65, 4.36, 4.59, 5.91, 6.13, 6.85, 9.09, 10.3, 12.13, and 16.81 min were also

observed. Again, we observed that some isolates possessed C_{24} fatty acid (tetracosanoic acid), while some others do not. Based on that observation, we classified the GC results into two groups: GC1 (absence of C_{24} mycolic acid) and GC2 (presence of C_{24} mycolic aicd). In general, the GC results did not show any significant differences ($p>0.05$) between the slow and rapid growing bovine MAP isolates.

Figures 4.7 and 4.8 shows results for two human symptomatic MAP isolates.

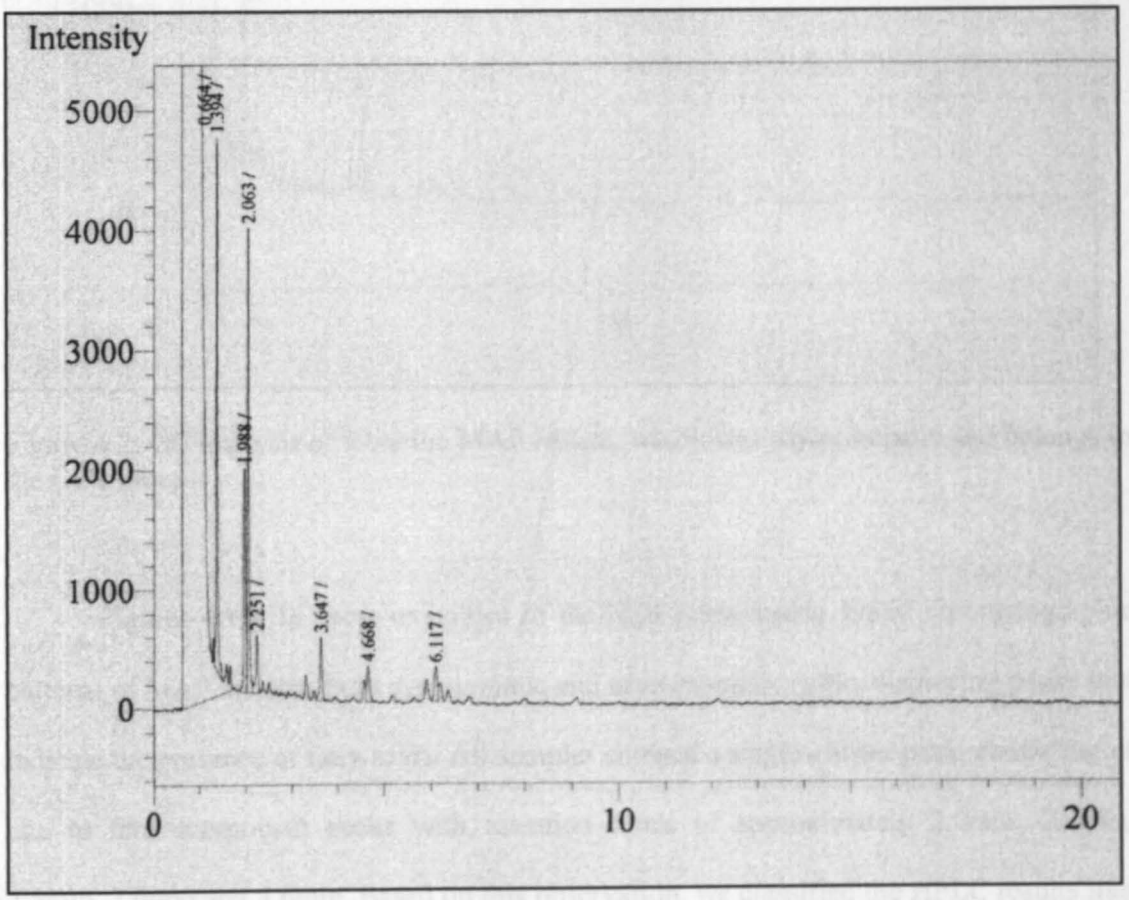

Figure 4.1: GC analysis of a bovine MAP isolate, which was asymptomatic and belongs in the GC1 group.

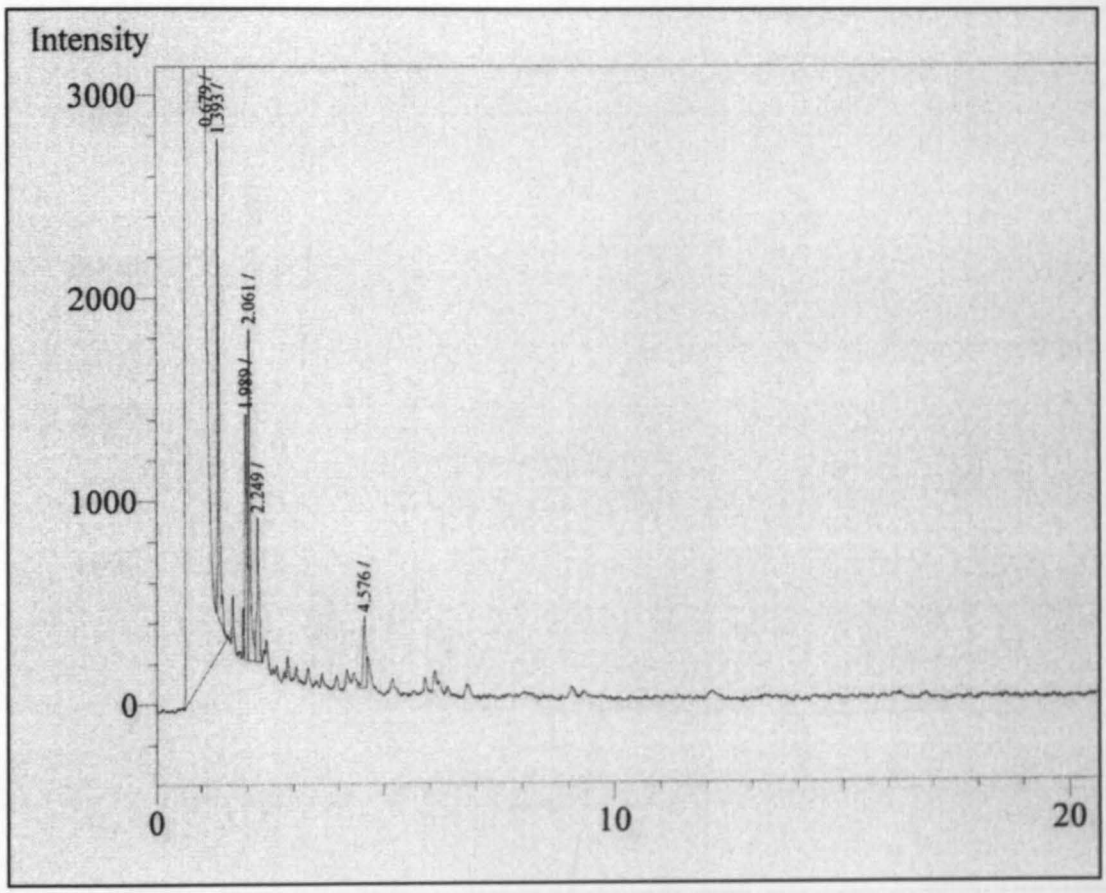

Figure 4.2: GC analysis of a bovine MAP isolate, which was asymptomatic and belongs in the GC1 group.

Figures 4.9-4.16 show examples of the high performance liquid chromatographic patterns of MAP isolates from symptomatic and asymptomatic cattle, displaying peaks that indicate the presence of fatty acids. All samples showed a single-cluster peak, consisting of one to five component peaks with retention times of approximately 2.0min, 2.3min, 2.5min, 2.6min and 3.6min. Based on this observation, we classified the HPLC results into two groups: HPLC1 (with ≤ 3 component peaks) and HPLC2 (with >3 component peaks).

Figures 4.17 and 4.18 shows results from two human symptomatic MAP isolates.

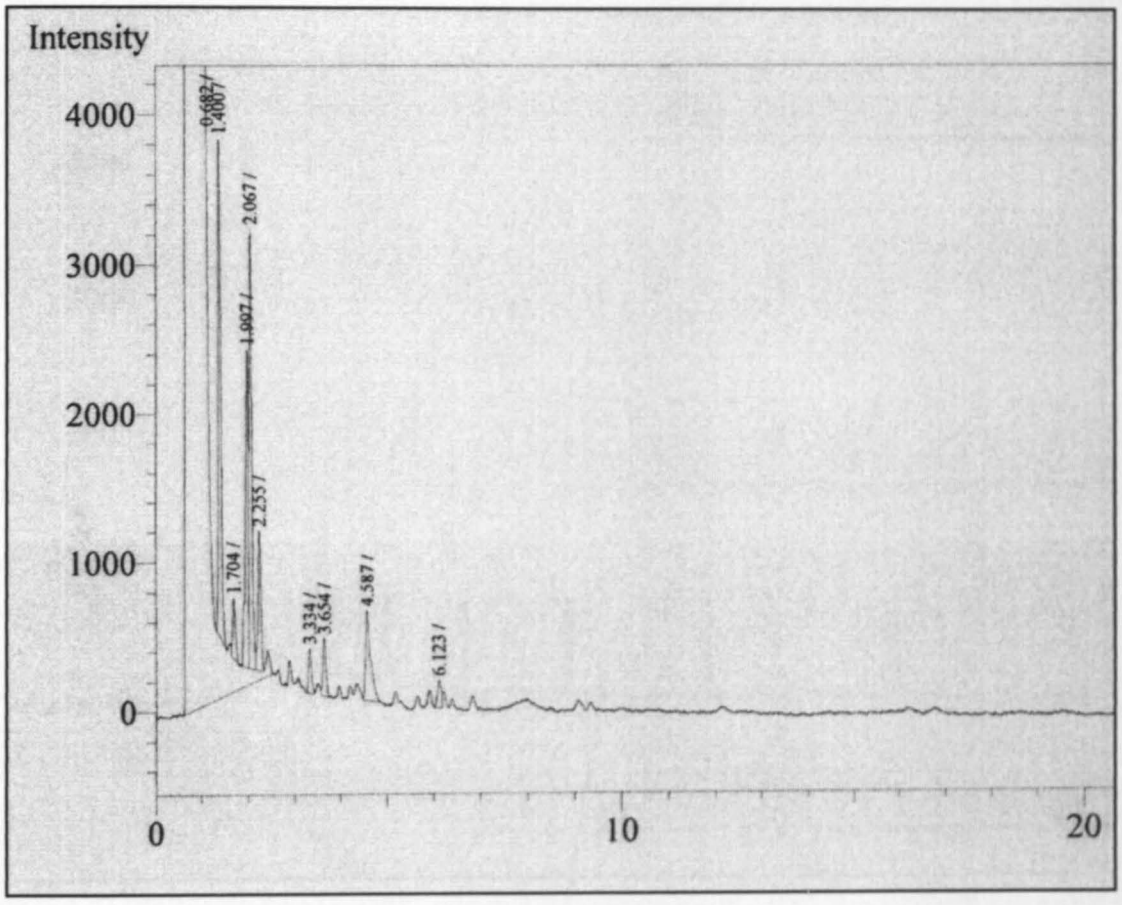

Figure 4.3: GC analysis of a bovine MAP isolate, which was symptomatic and belongs in the GC1 group.

Figures 4.19-4.24 show examples of the results of the mass spectra analysis of MAP isolates from asymptomatic and symptomatic cattle, which were classified into two groups: MC1 (having short carbon chain lengths) and MC2 (having long carbon chain lengths).

Figures 4.25 and 4.26 show the mass spectral results of the human symptomatic MAP isolates.

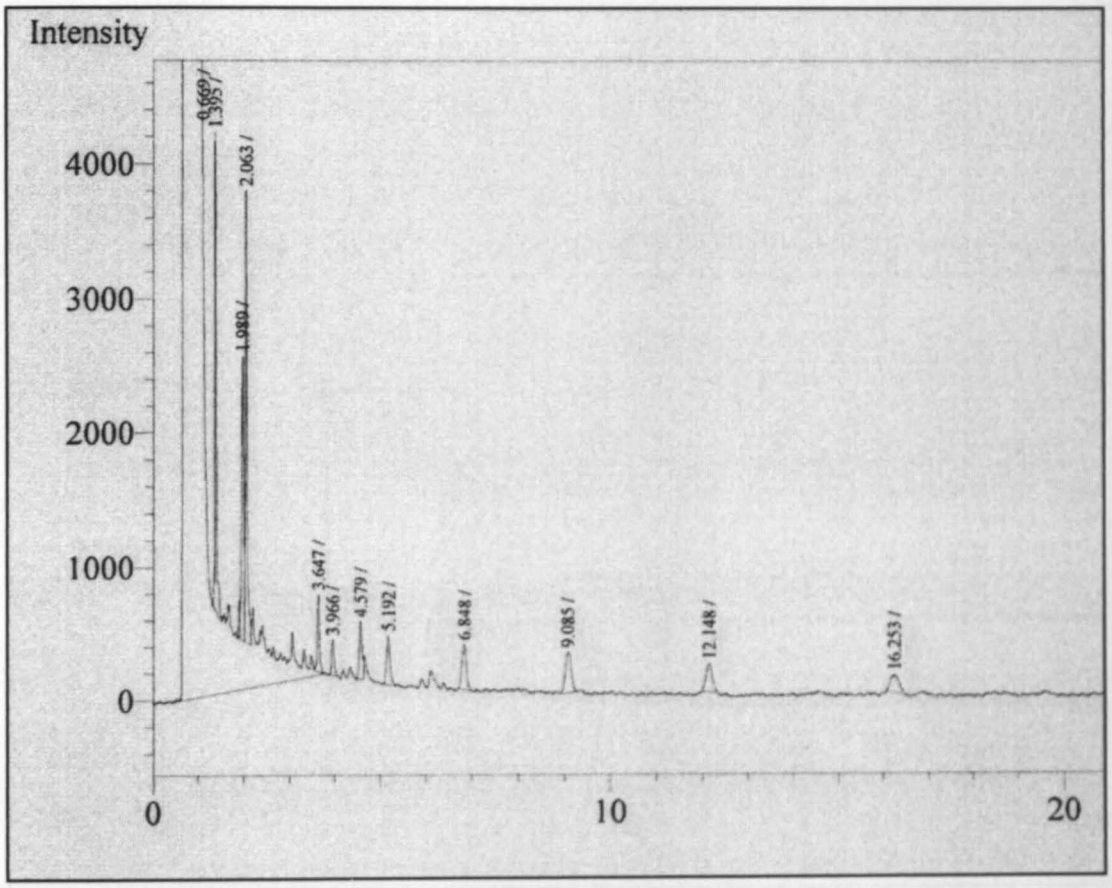

Figure 4.4: GC analysis of a bovine MAP isolate, which was asymptomatic and belongs in the GC2 group.

Figure 4.27 shows the bar chart representing the sources of MAP isolates (cattle and humans) and the percentage of mass spectra group (MC1 or MC2) belonging to each source.

Figure 4.28 shows the relationship between the symptom status of isolates and their shedder and growth rate status. There is a significant difference between asymptomatic and symptomatic MAP isolates. Each bar represents the percent of MAP isolates belonging to each category: shedder status (low and high) and growth rate status (slow and rapid growers).

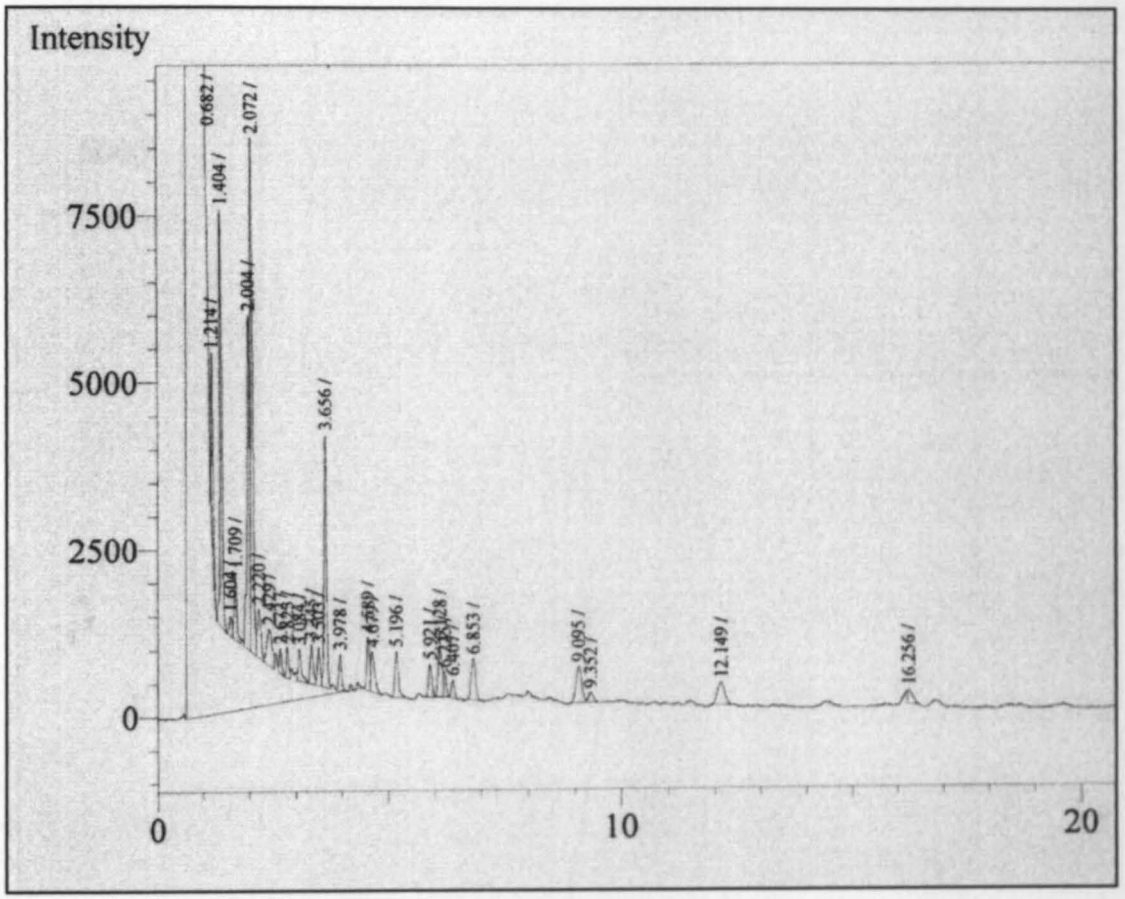

Figure 4.5: GC analysis of a bovine MAP isolate, which was symptomatic and belongs in the GC2 group.

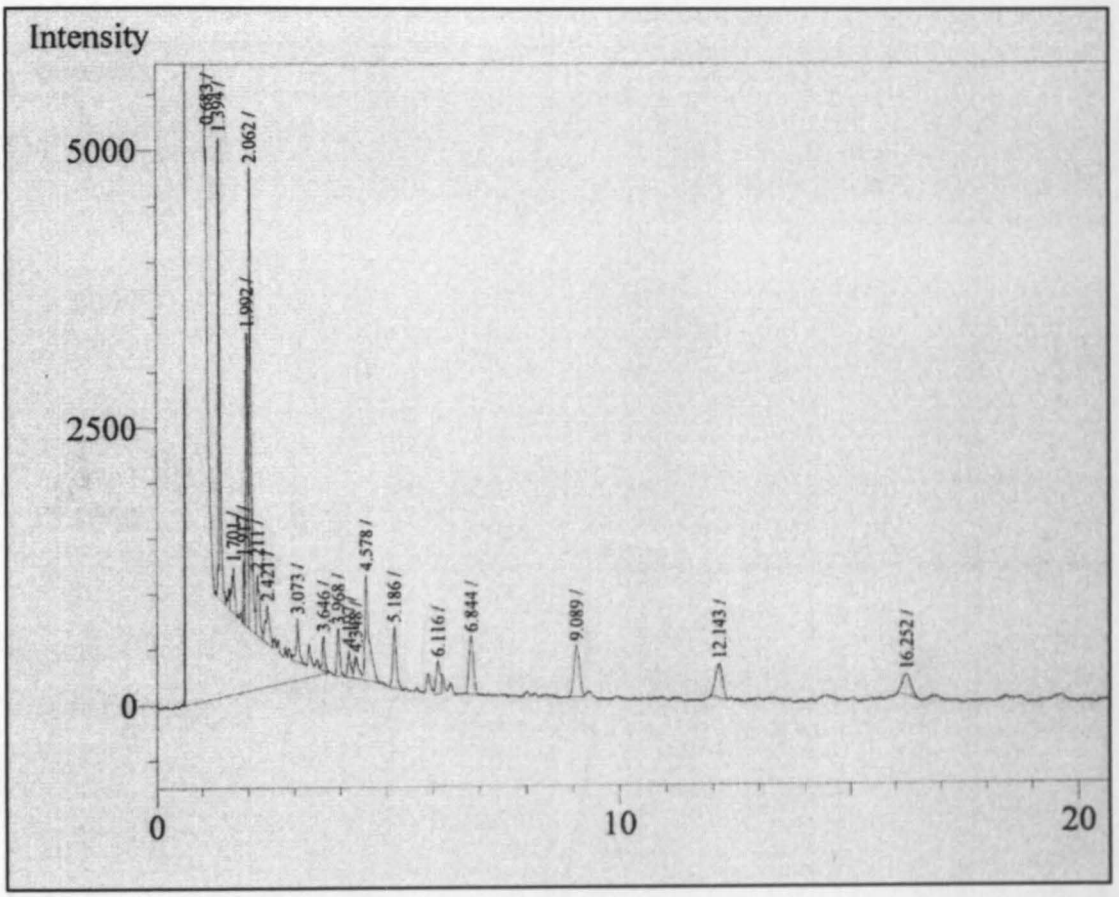

Figure 4.6: GC analysis of a bovine MAP isolate, which was symptomatic and belongs in the GC2 group.

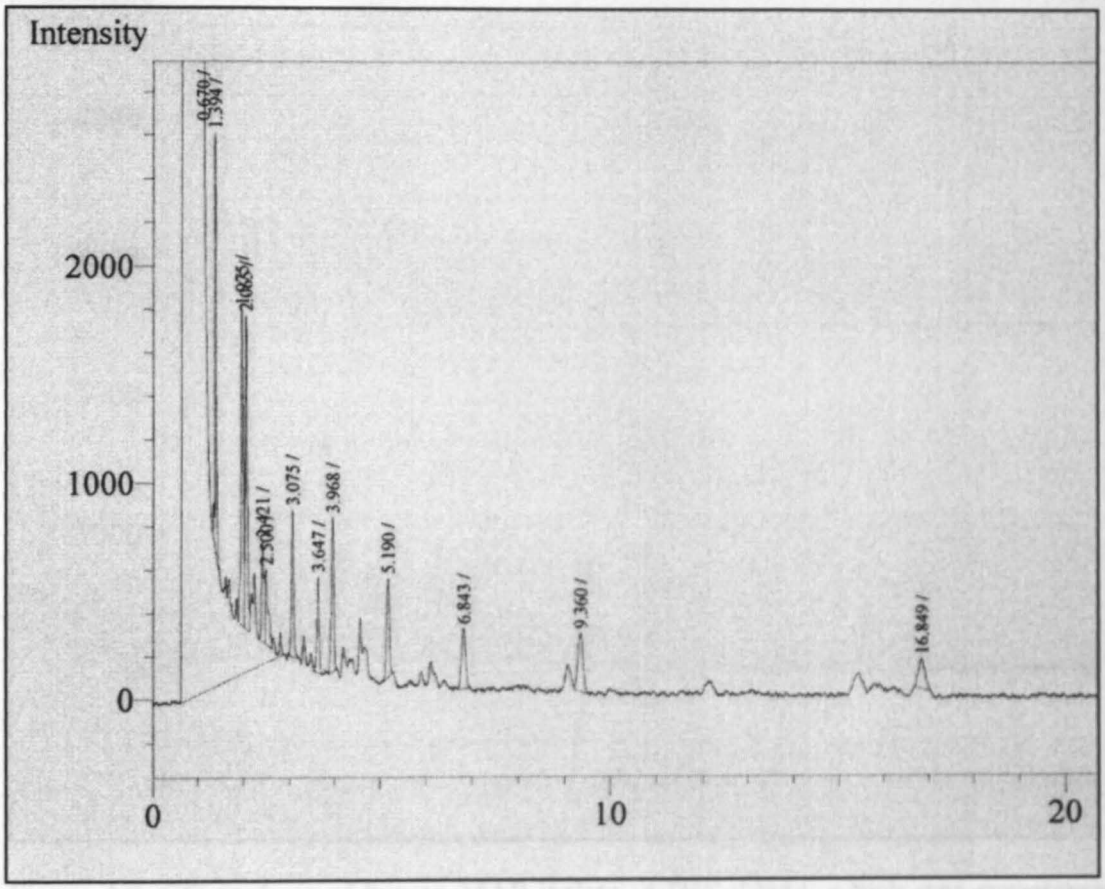

Figure 4.7: GC analysis of human MAP, ATCC 43015, which was symptomatic and belongs in the GC2 group.

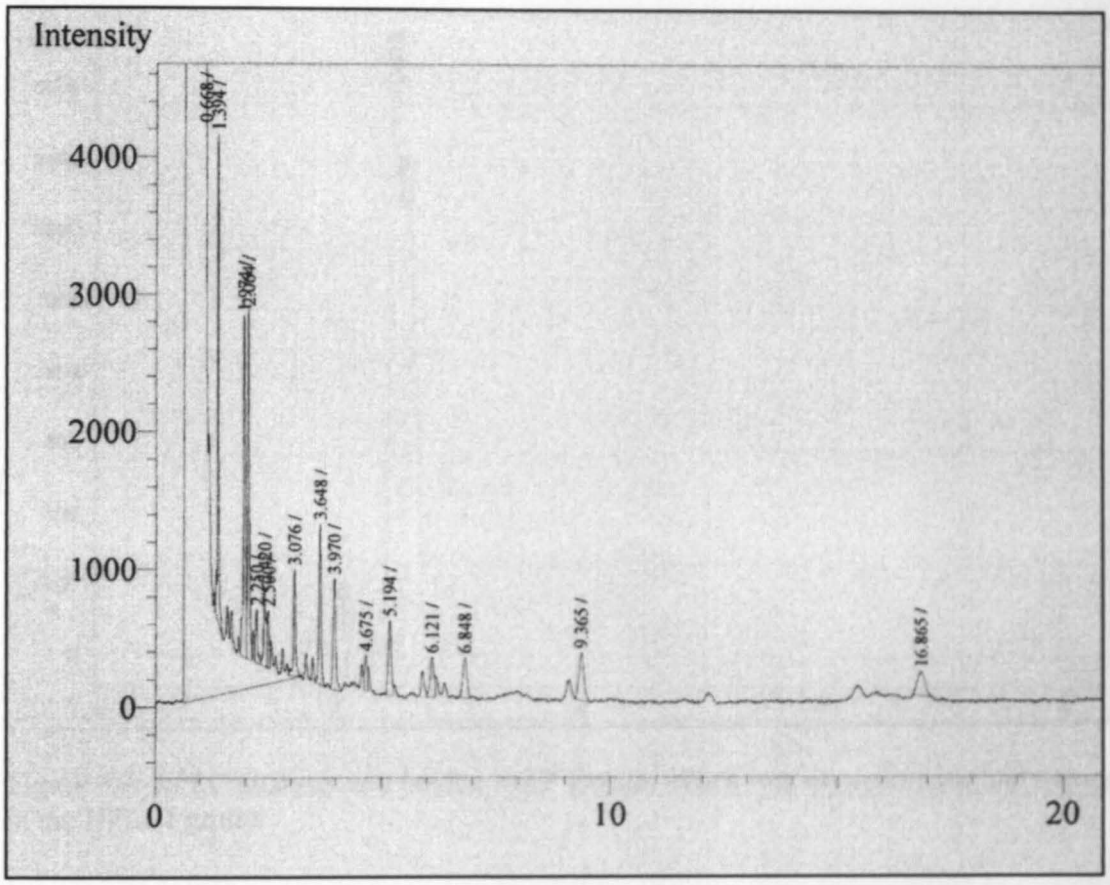

Figure 4.8: GC analysis of human MAP isolate, ATCC 43544, which was symptomatic and belongs in the GC2 group.

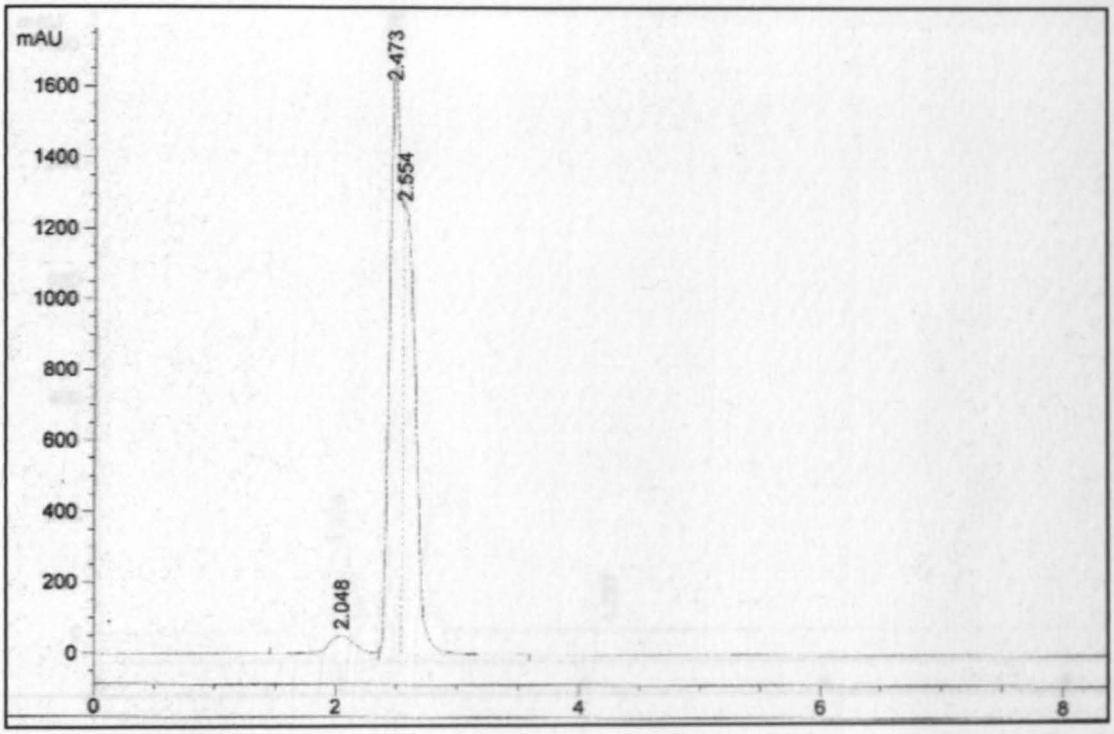

Figure 4.9: HPLC analysis of a bovine MAP isolate, which was asymptomatic and belongs in the HPLC1 group.

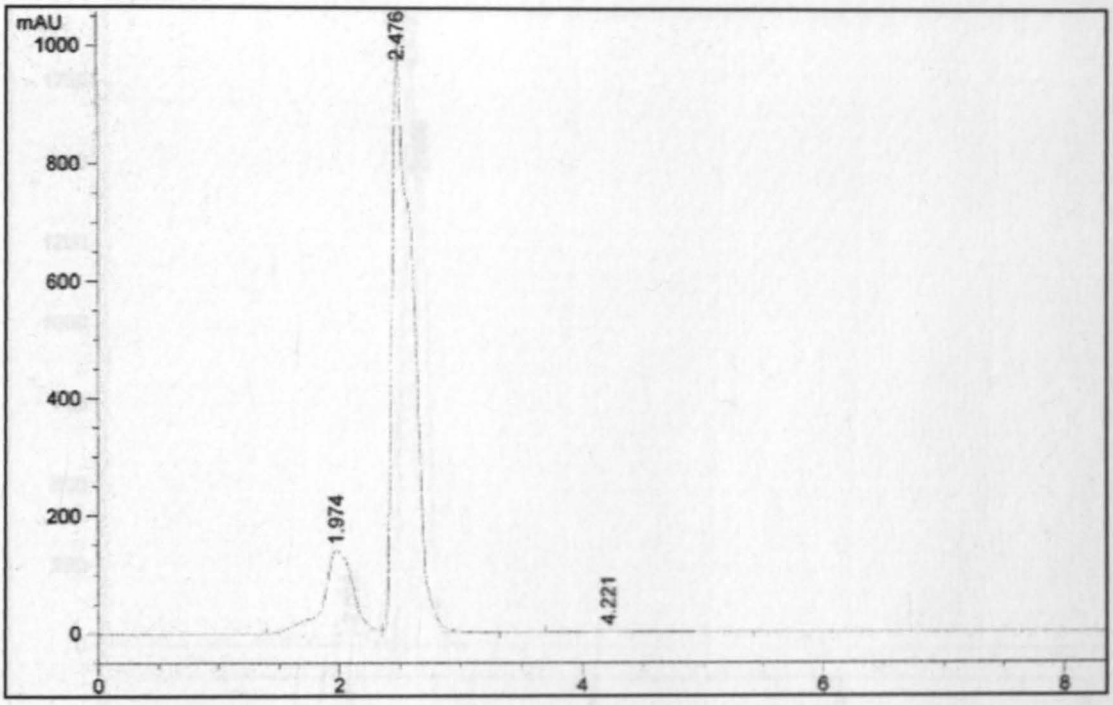

Figure 4.10: HPLC analysis of a bovine MAP isolate, which was asymptomatic and belongs in the HPLC1 group.

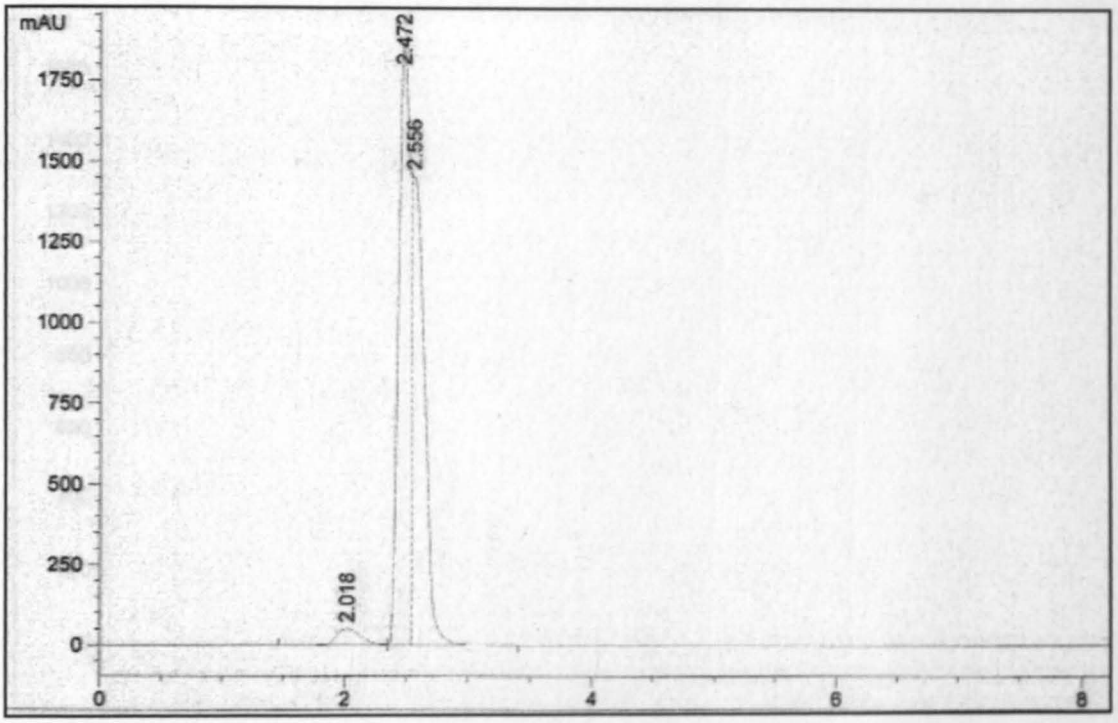

Figure 4.11: HPLC analysis of a MAP isolate, which was symptomatic and belongs in the HPLC1 group.

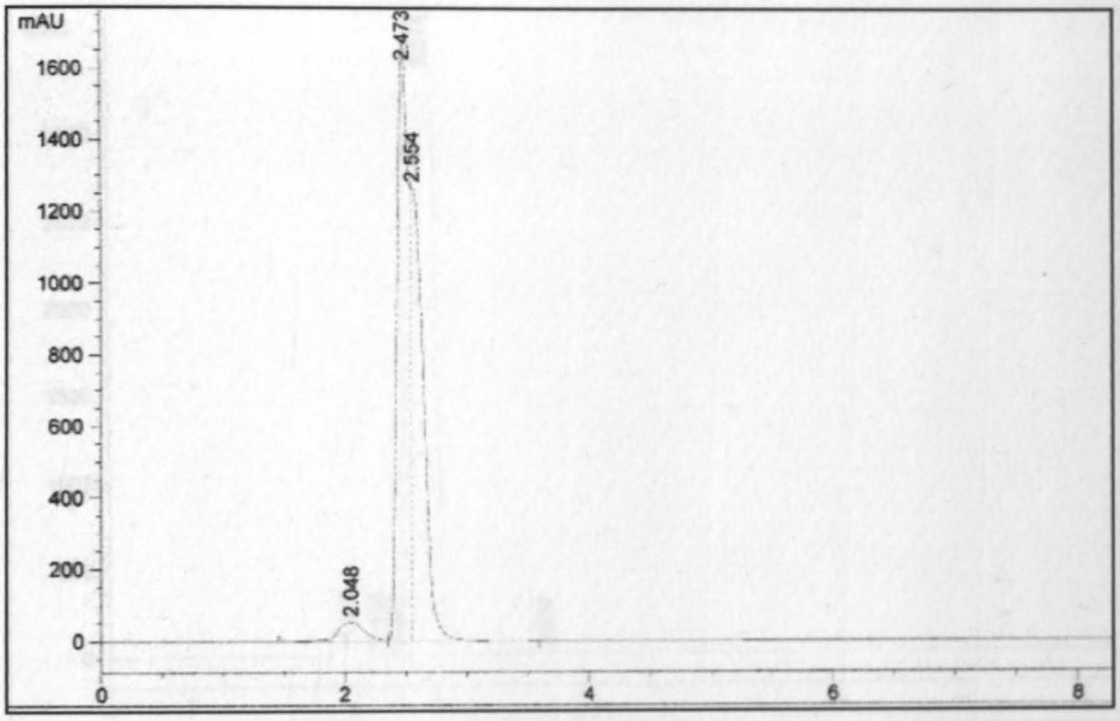

Figure 4.12: HPLC analysis of a bovine MAP, which was symptomatic and belongs in the HPLC1 group.

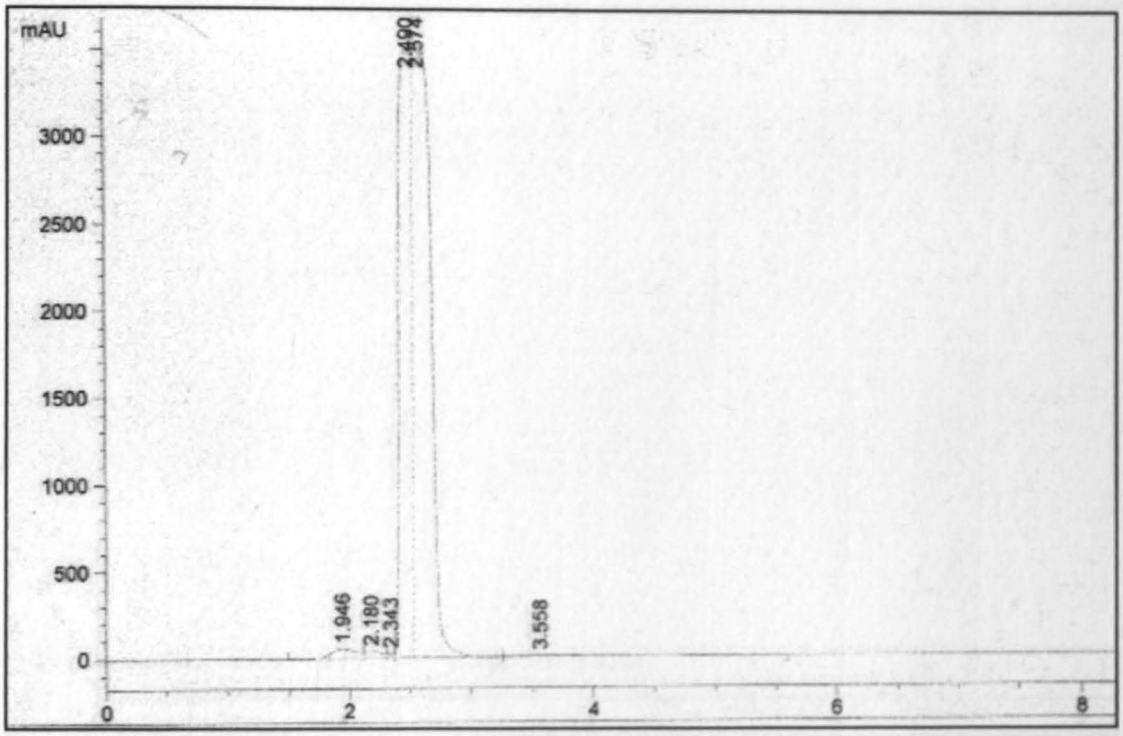

Figure 4.13: HPLC analysis of a bovine MAP isolate, which was asymptomatic and belongs in the HPLC2 group.

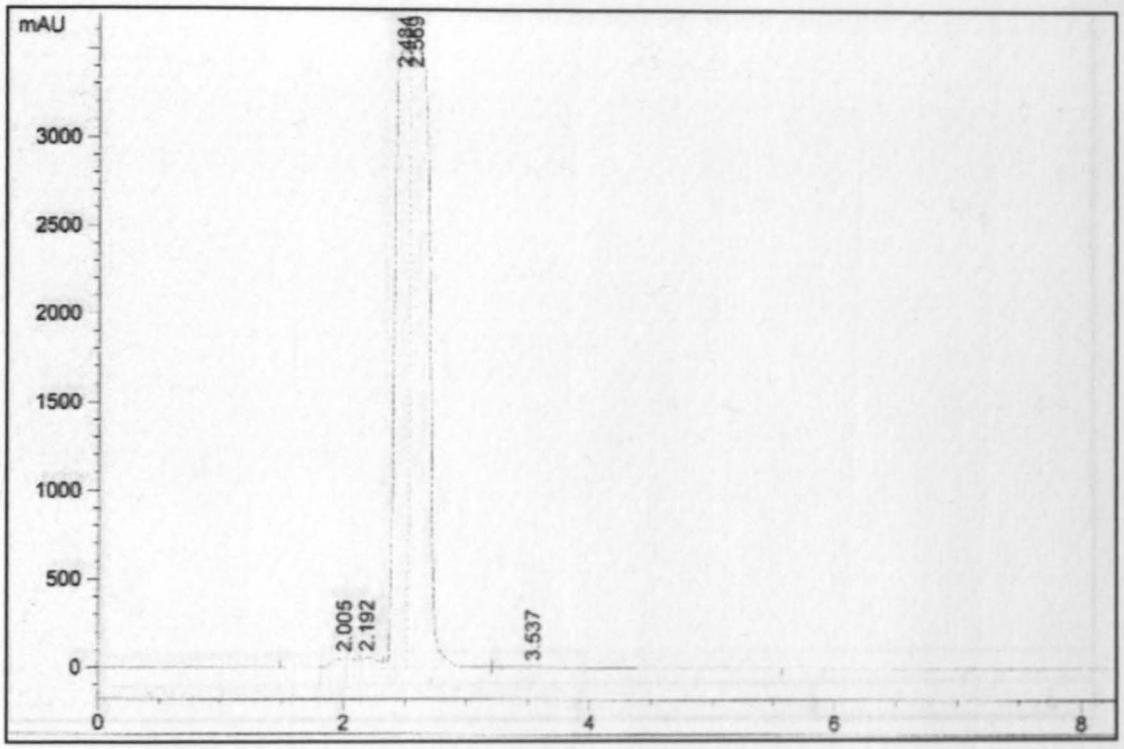

Figure 4.14: HPLC analysis of a bovine MAP isolate, which was asymptomatic and belongs in the HPLC2 group.

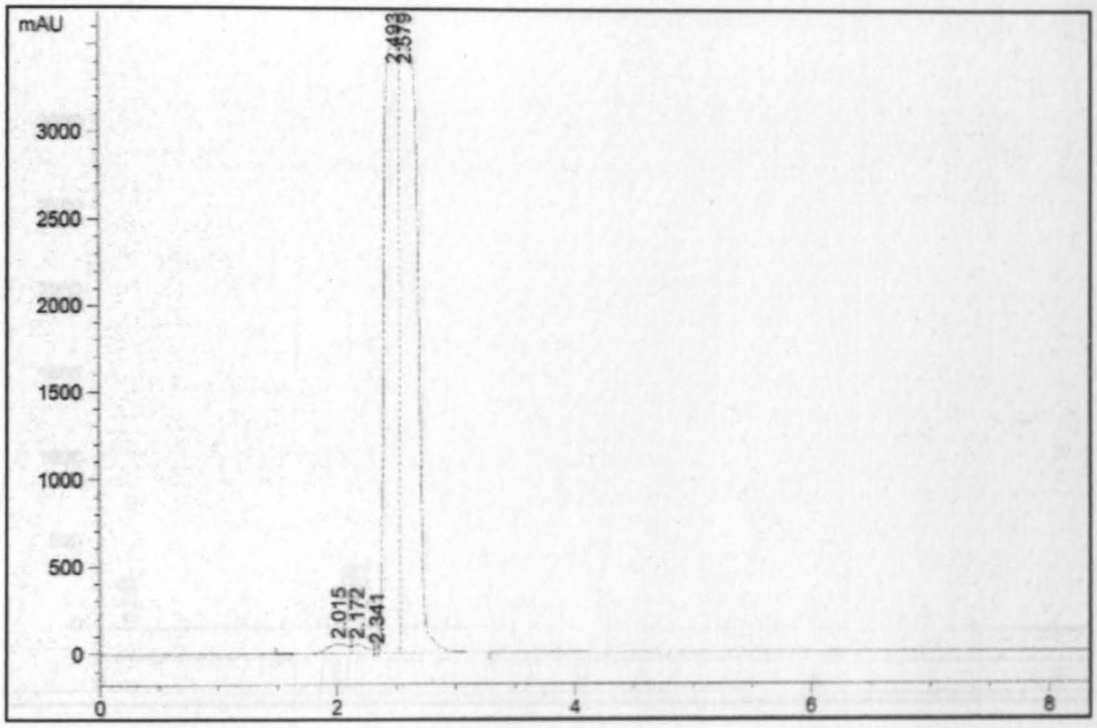

Figure 4.15: HPLC analysis of a bovine MAP isolate, which was symptomatic and belongs in the HPLC2 group.

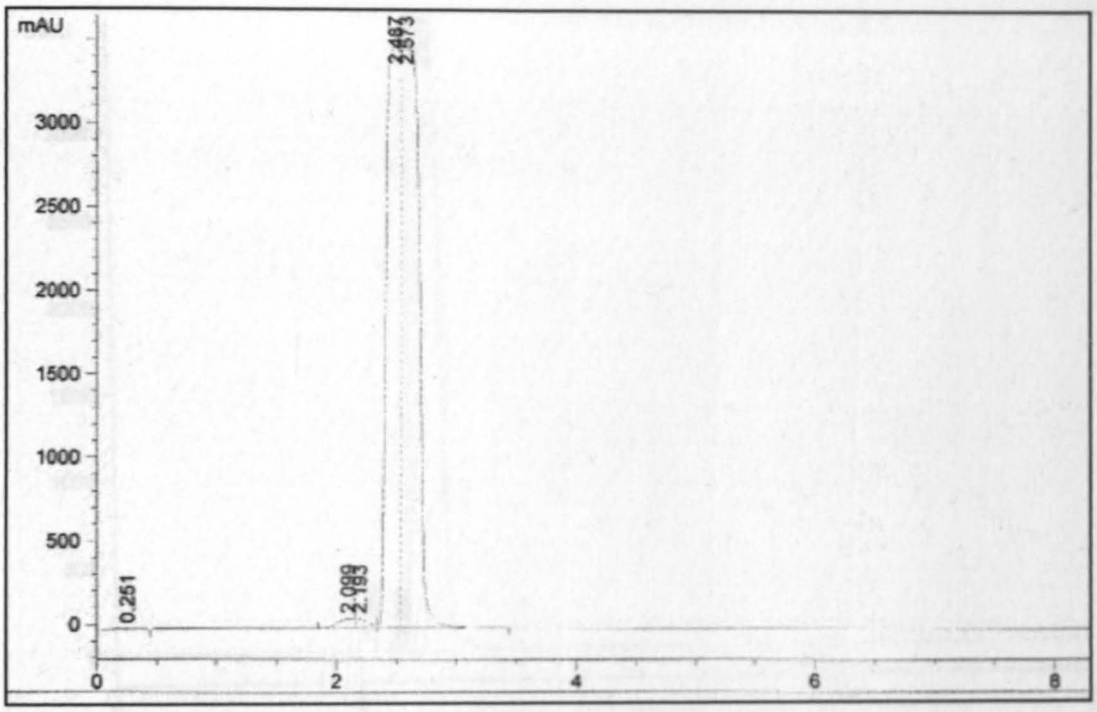

Figure 4.16: HPLC analysis of a bovine MAP isolate, which was symptomatic and belongs in the HPLC2 group.

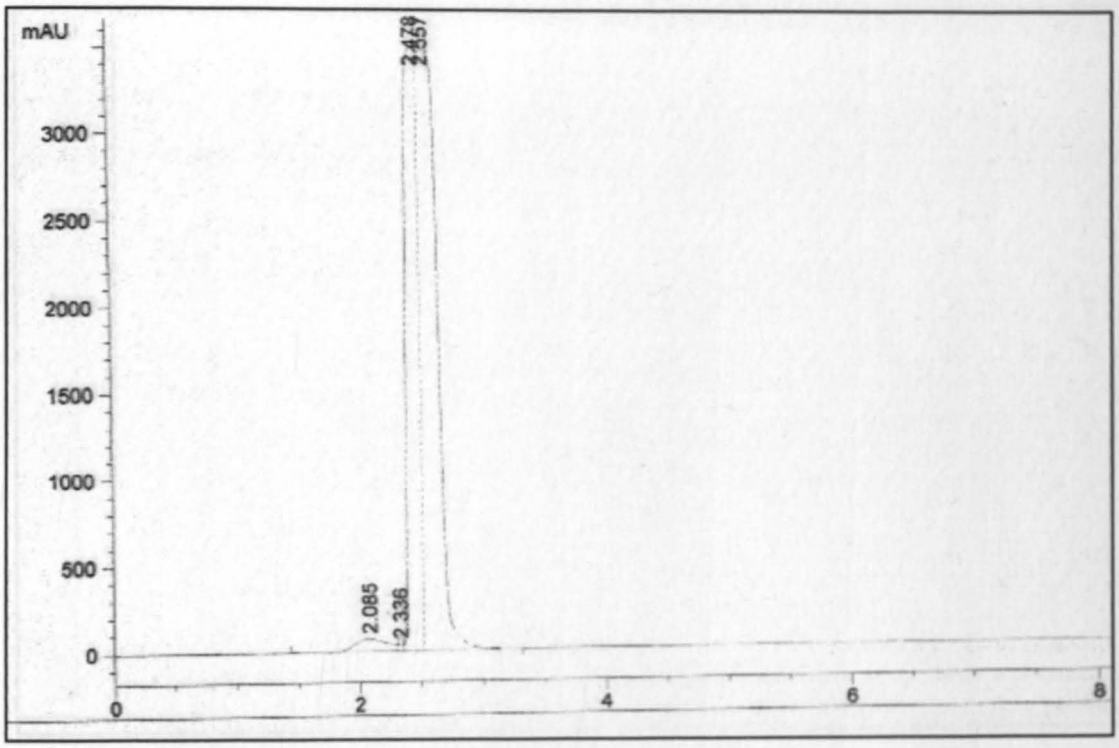

Figure 4.17: HPLC analysis of human MAP isolate, ATCC 43015, which was symptomatic and belongs in the HPLC2 group.

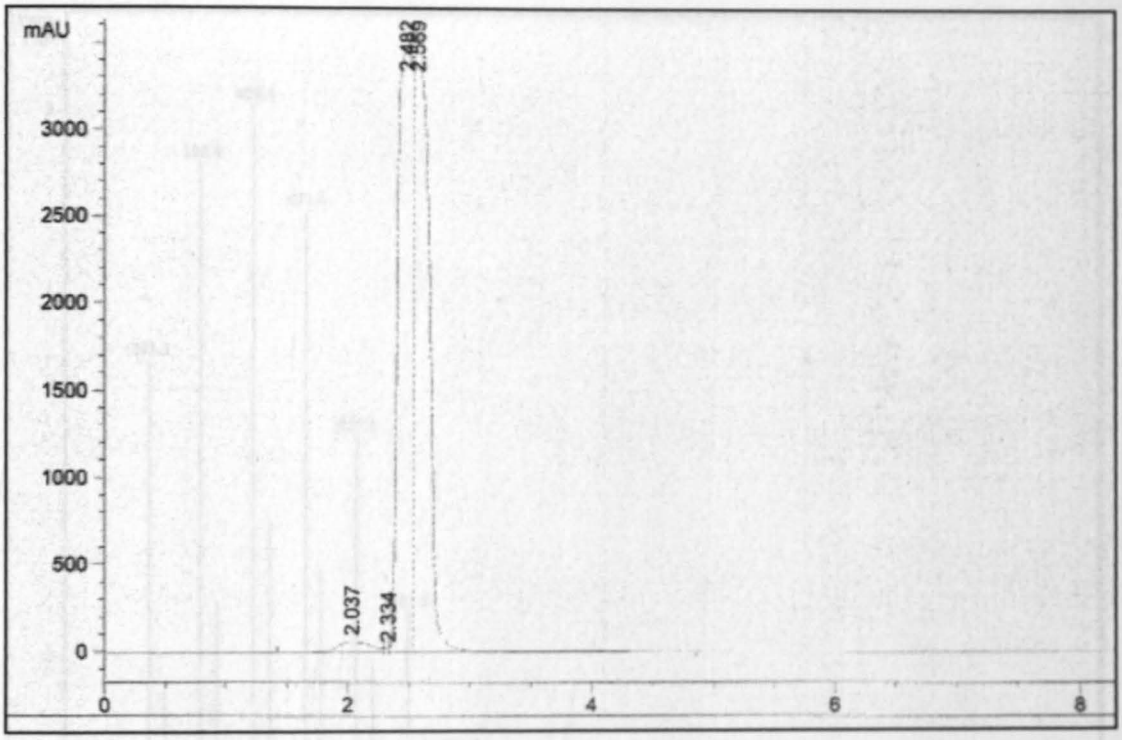

Figure 4.18: HPLC analysis of human MAP isolate, ATCC 43544, which was symptomatic and belongs in the HPLC2 group.

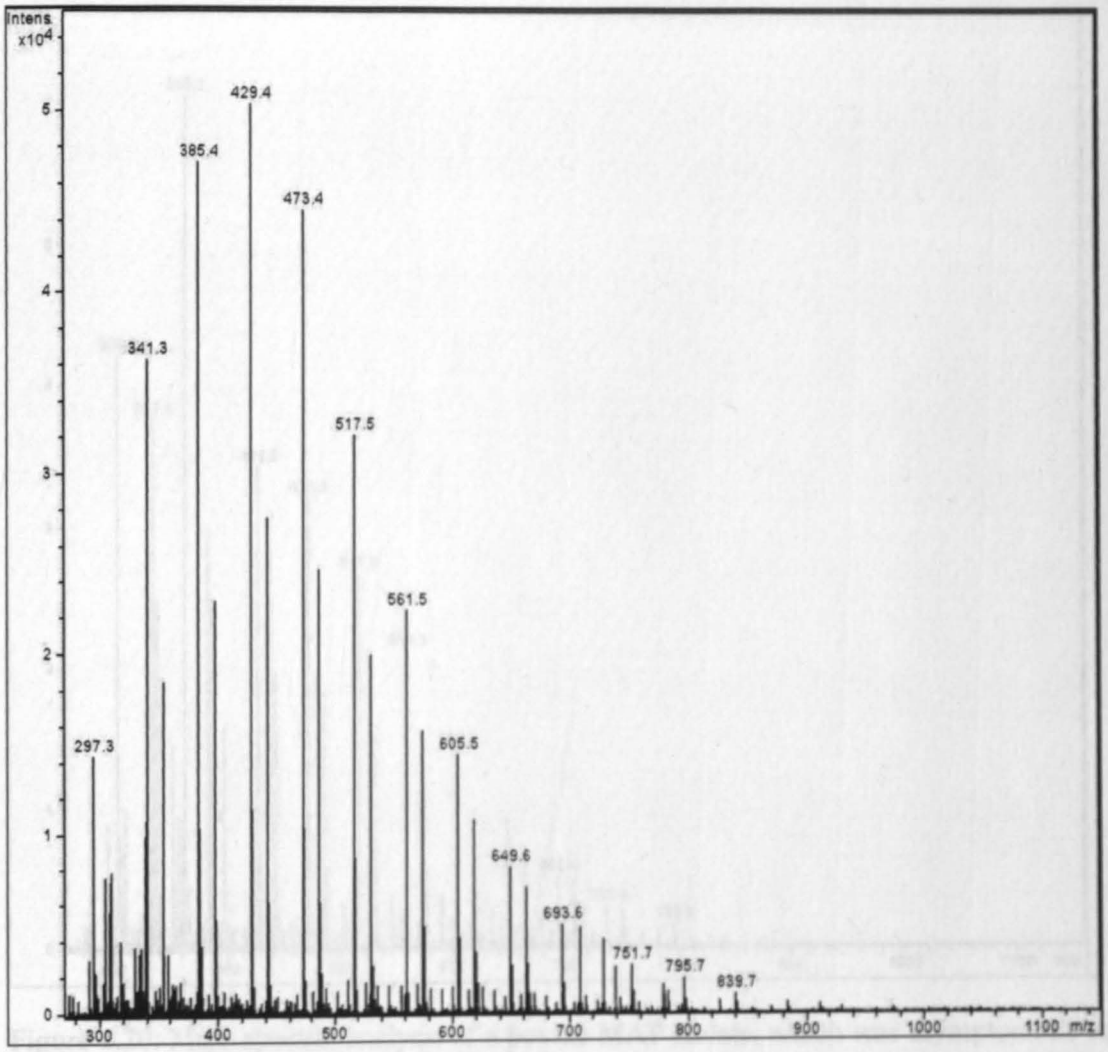

Figure 4.19: Mass spectral analysis of bovine MAP isolate, which was asymptomatic and belongs in the MC1 group.

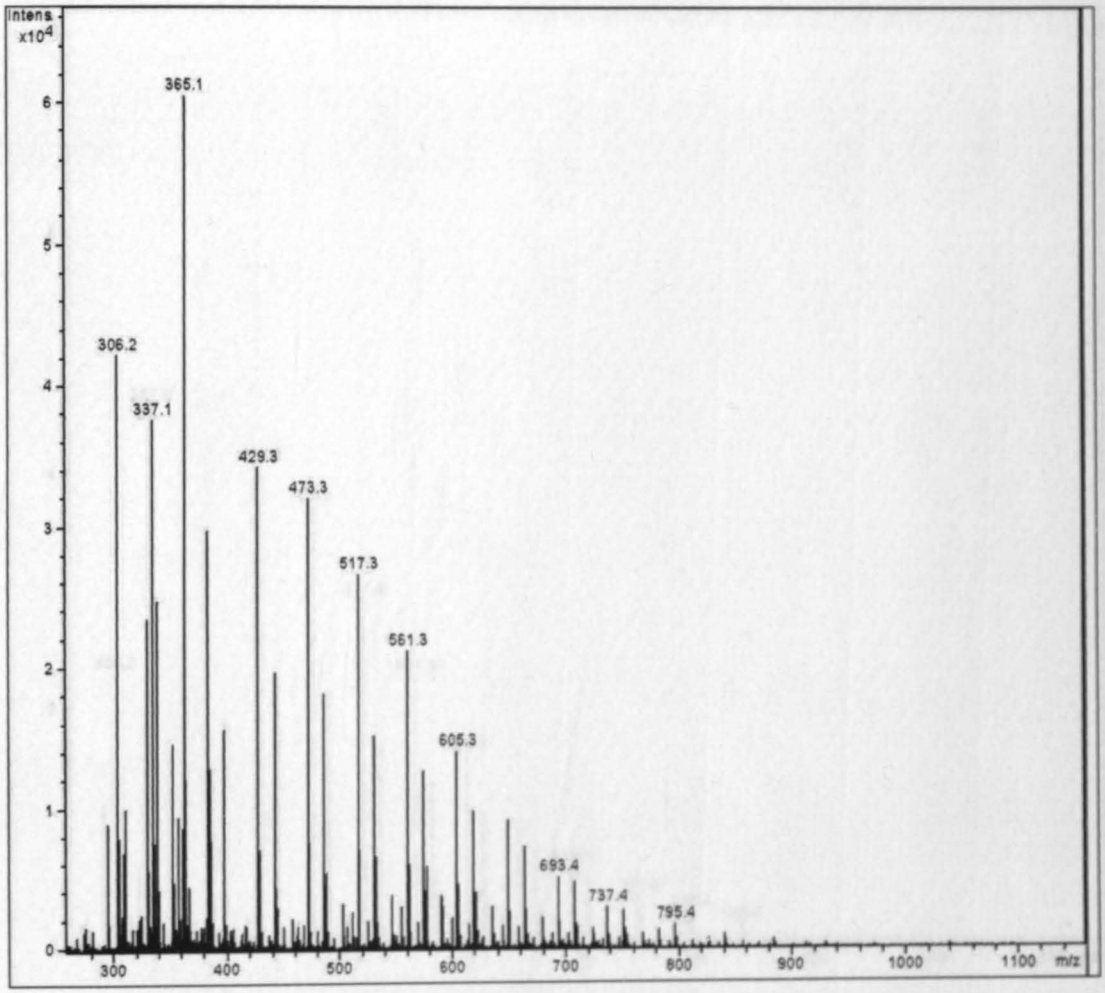

Figure 4.20: Mass spectral analysis of a bovine MAP isolate, which was asymptomatic and belongs in the MC1 group.

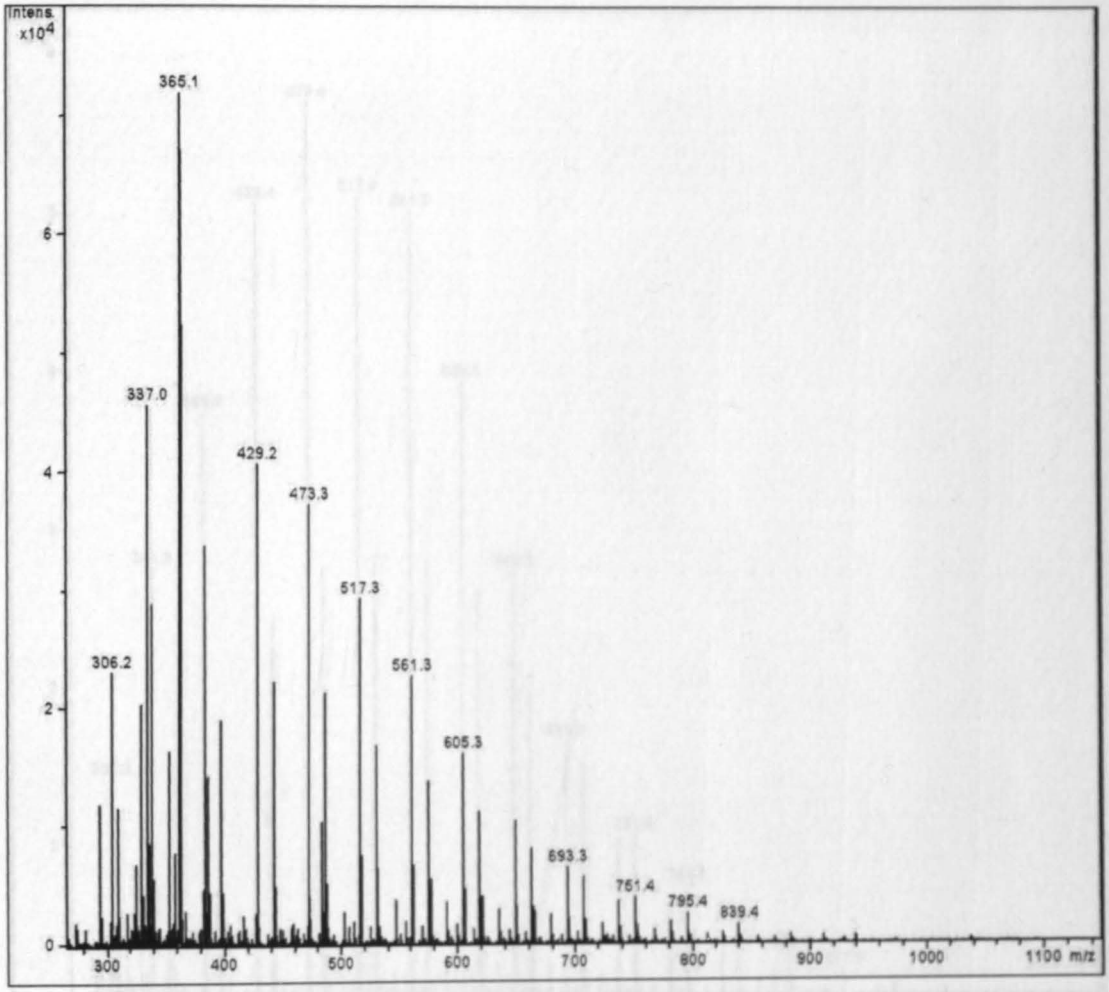

Figure 4.21: Mass spectral analysis of a bovine MAP isolate, which was asymptomatic and belongs in the MC1 group.

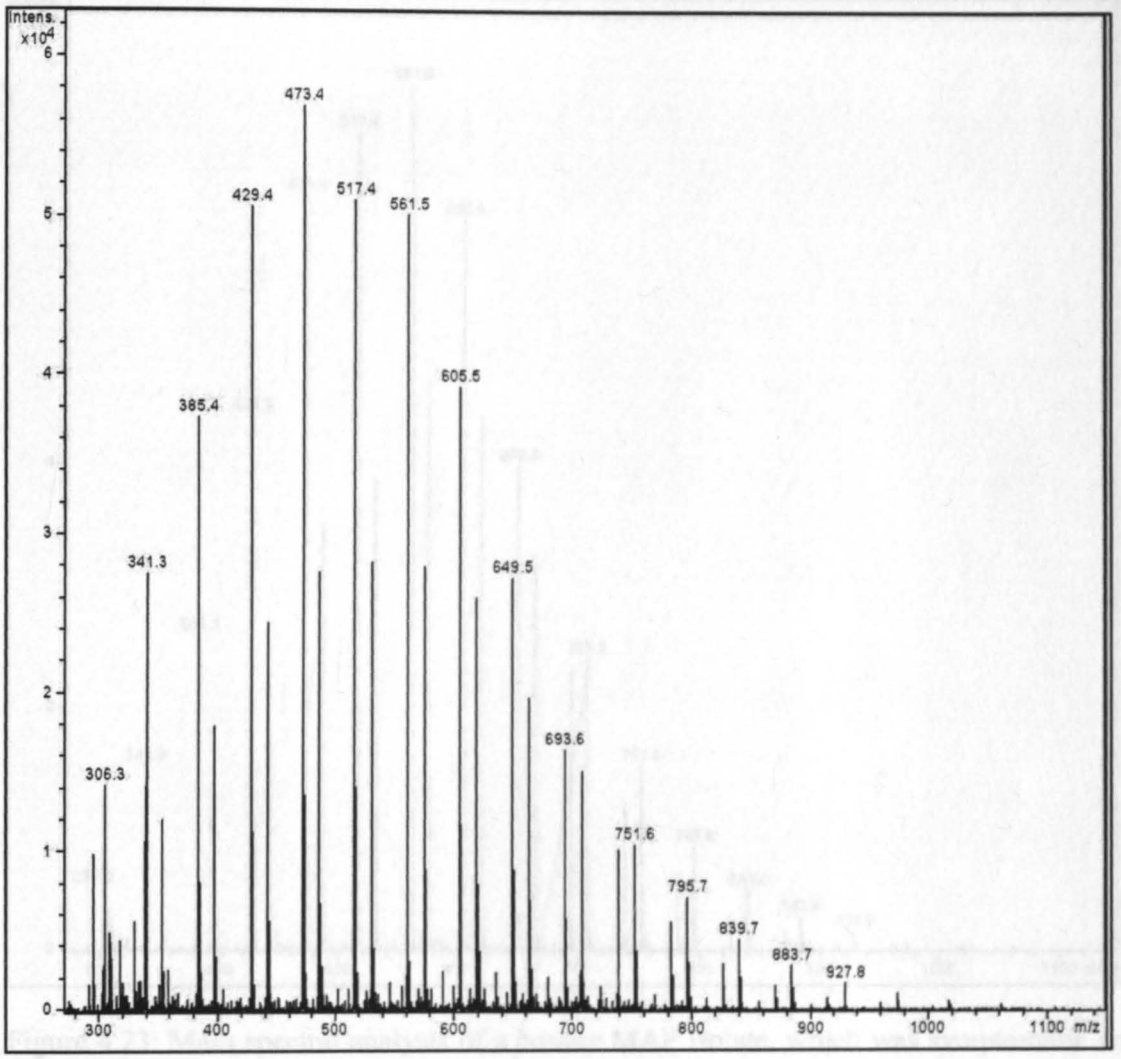

Figure 4.22: Mass spectral analysis of a bovine MAP isolate, which was symptomatic and belongs in the MC2 group.

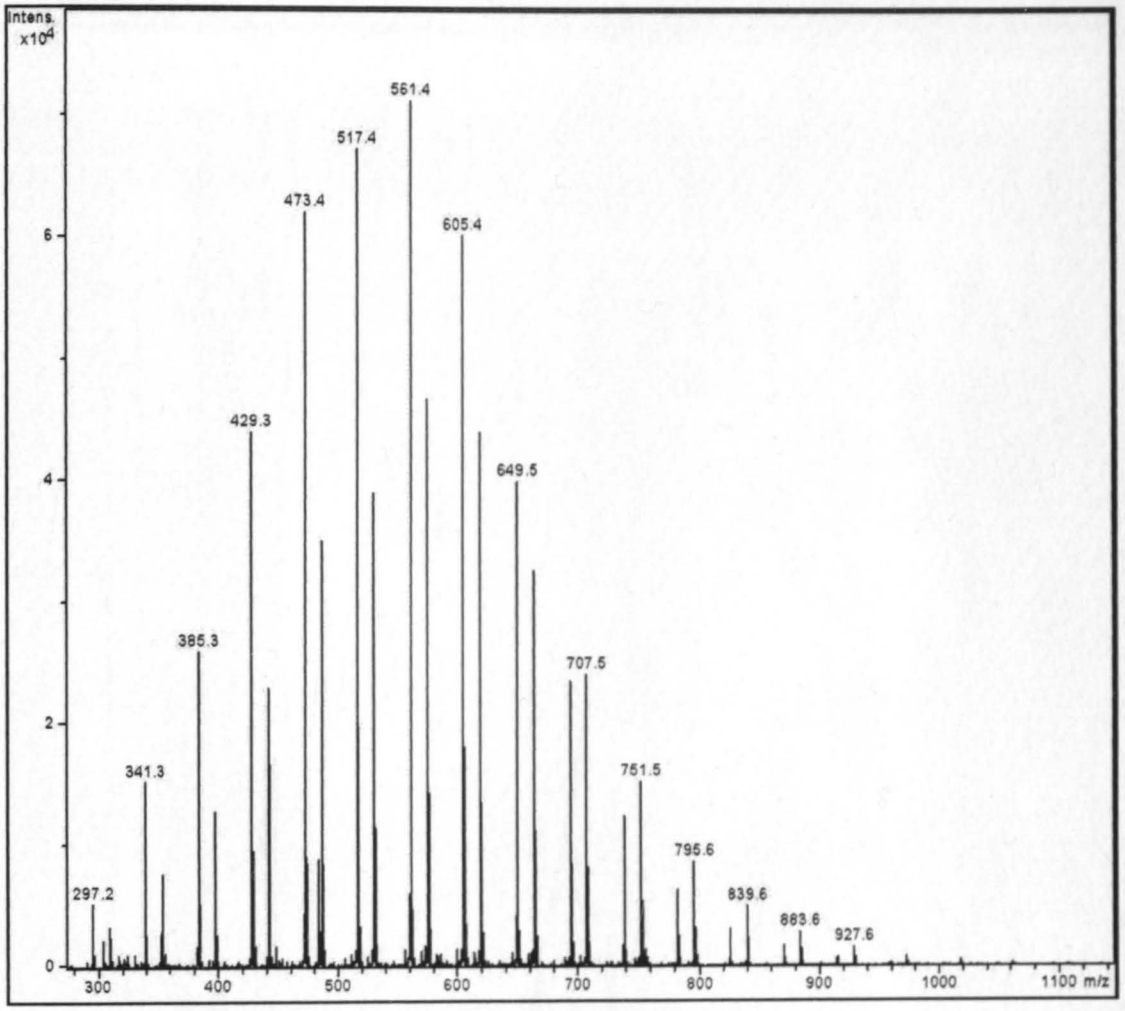

Figure 4.23: Mass spectral analysis of a bovine MAP isolate, which was symptomatic and belongs in the MC2 group.

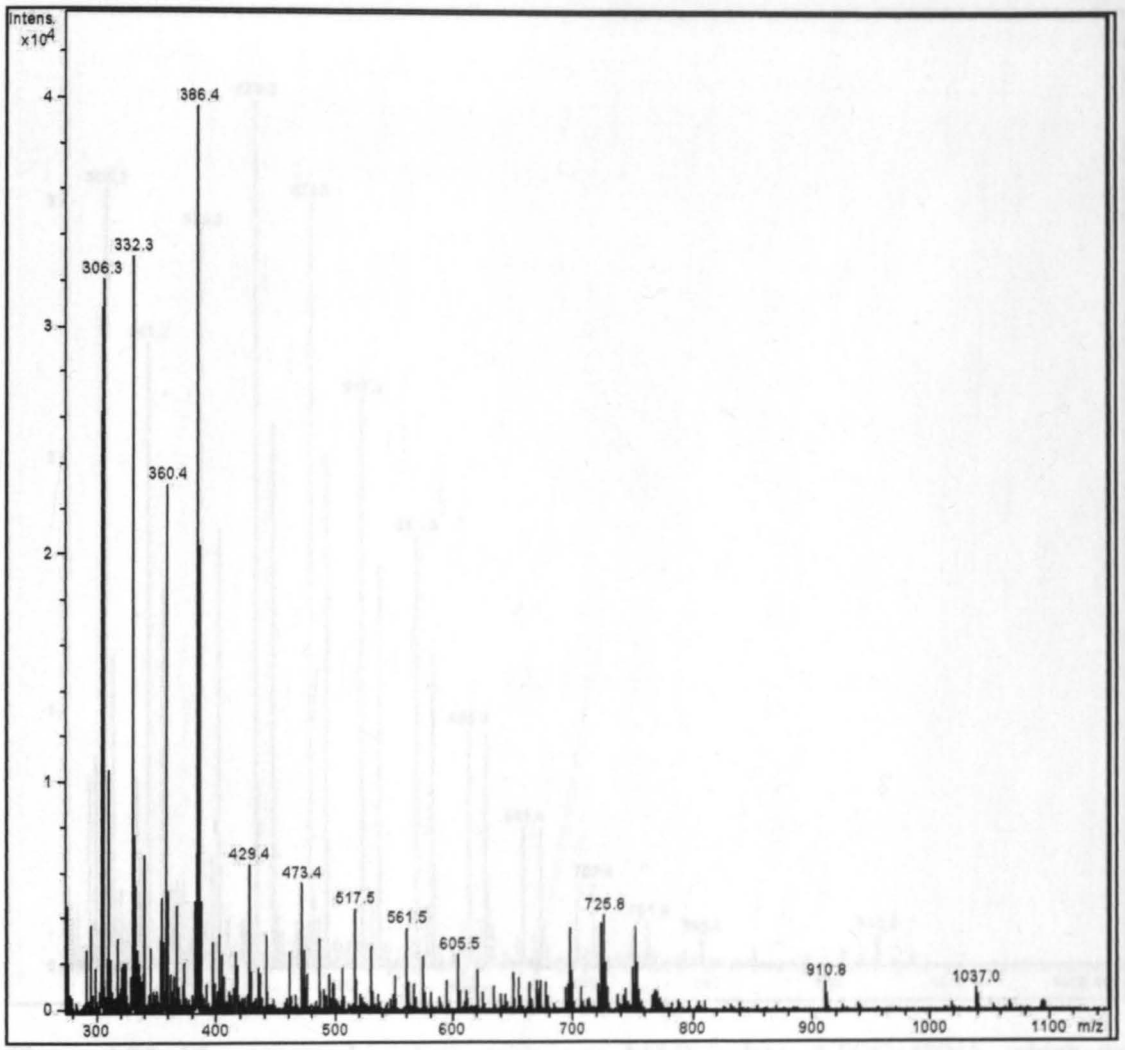

Figure 4.24: Mass spectral analysis of a bovine MAP, which was symptomatic and belongs in the MC2 group.

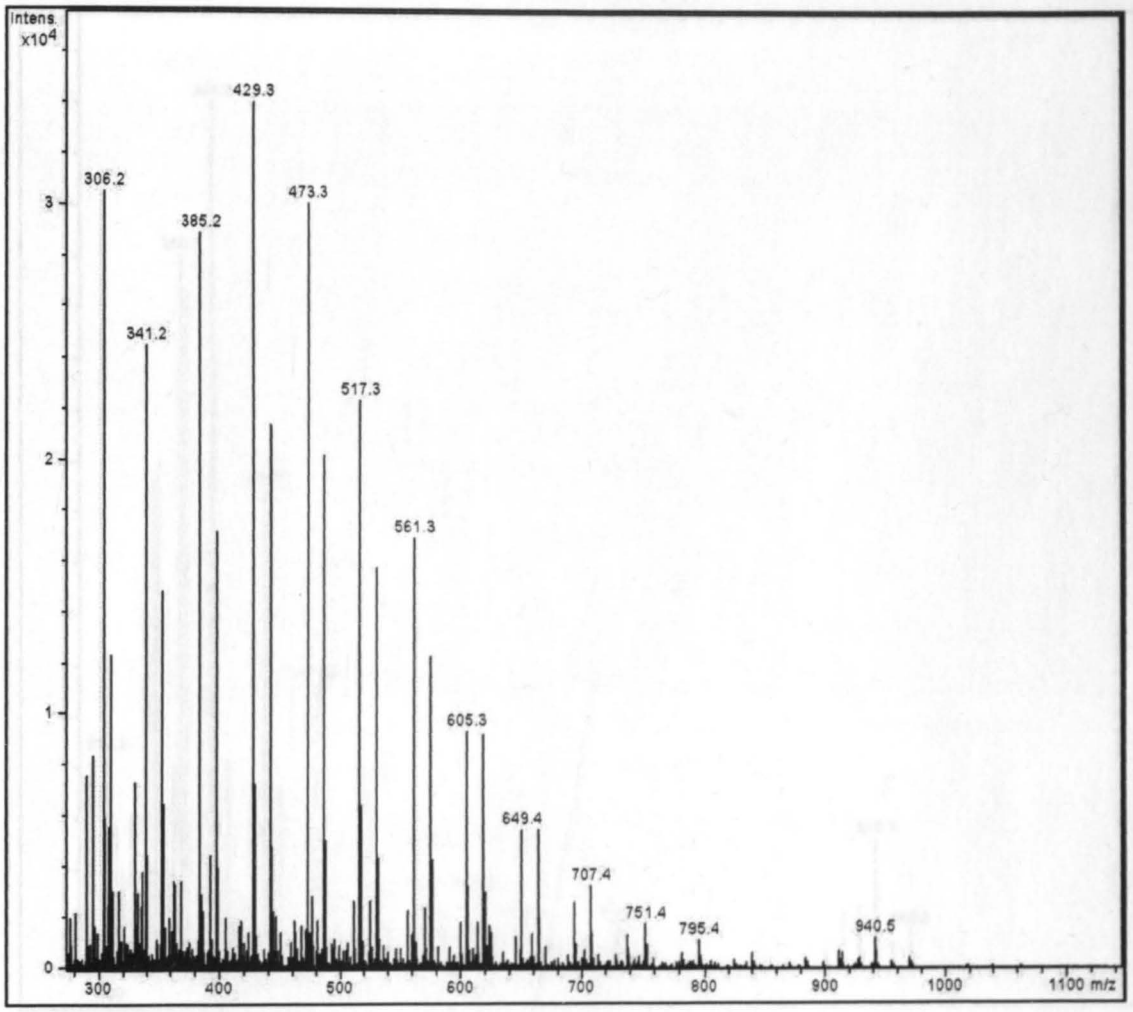

Figure 4.25: Mass spectral analysis of human MAP isolate, ATCC 43015, which was symptomatic and belongs in the MC2 group.

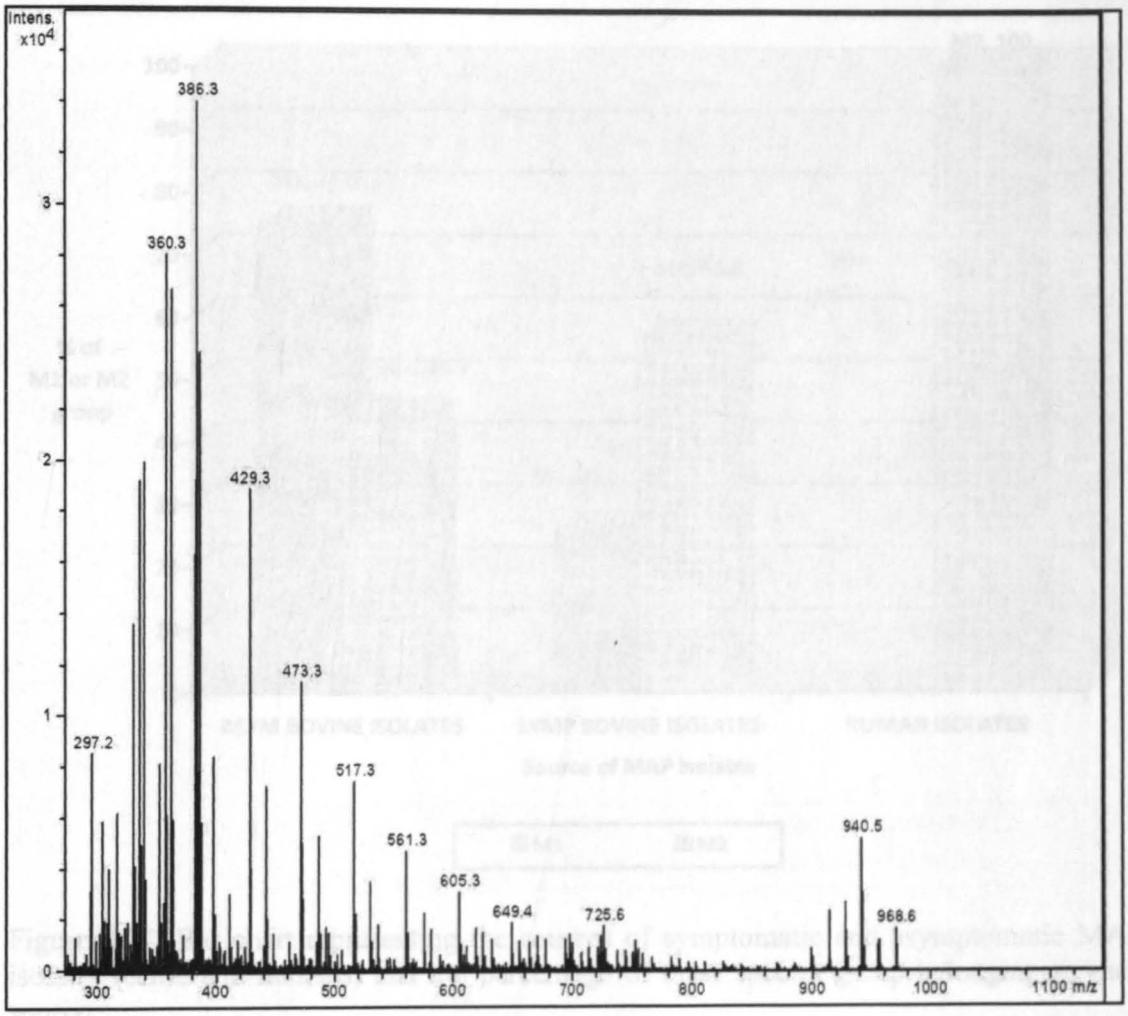

Figure 4.26: Mass spectral analysis of human MAP isolate, ATCC 43544, which was symptomatic and belongs in the MC2 group.

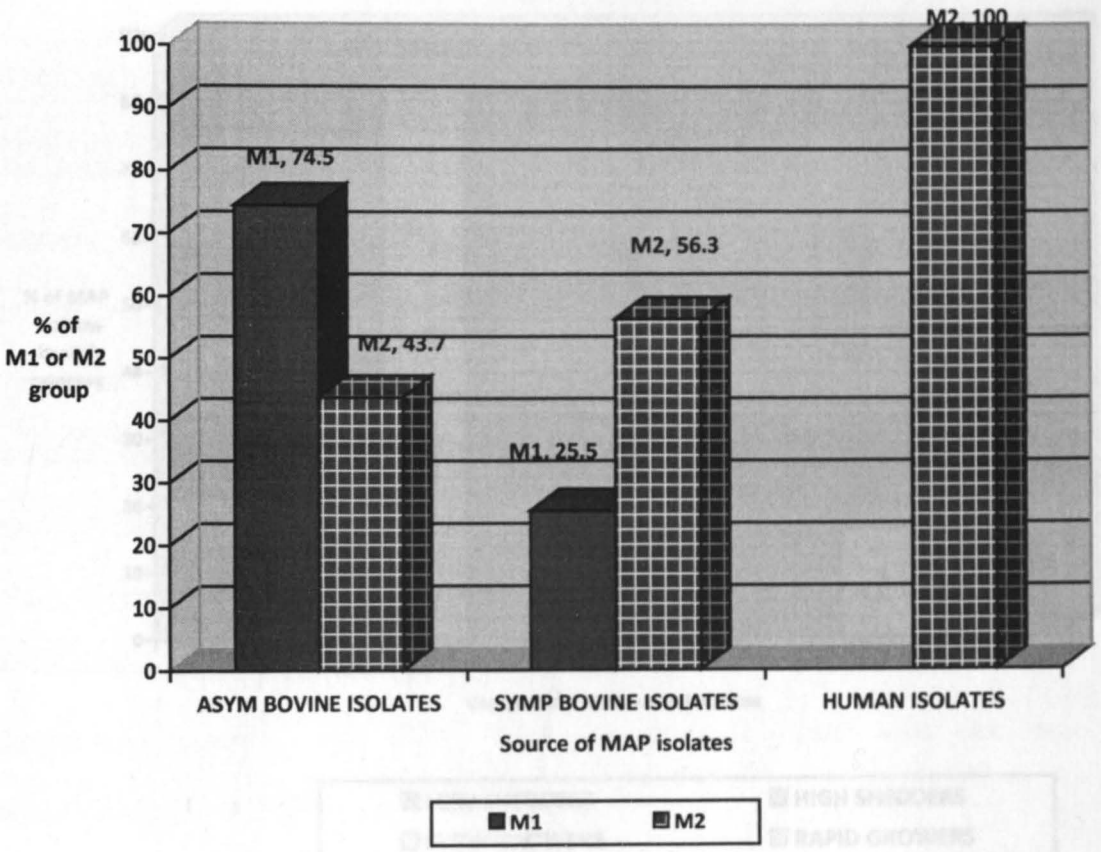

Figure 4.27: Bar chart representing the sources of symptomatic and asymptomatic MAP isolates (cattle and humans) and the percentage of mass spectra group belonging to each source.

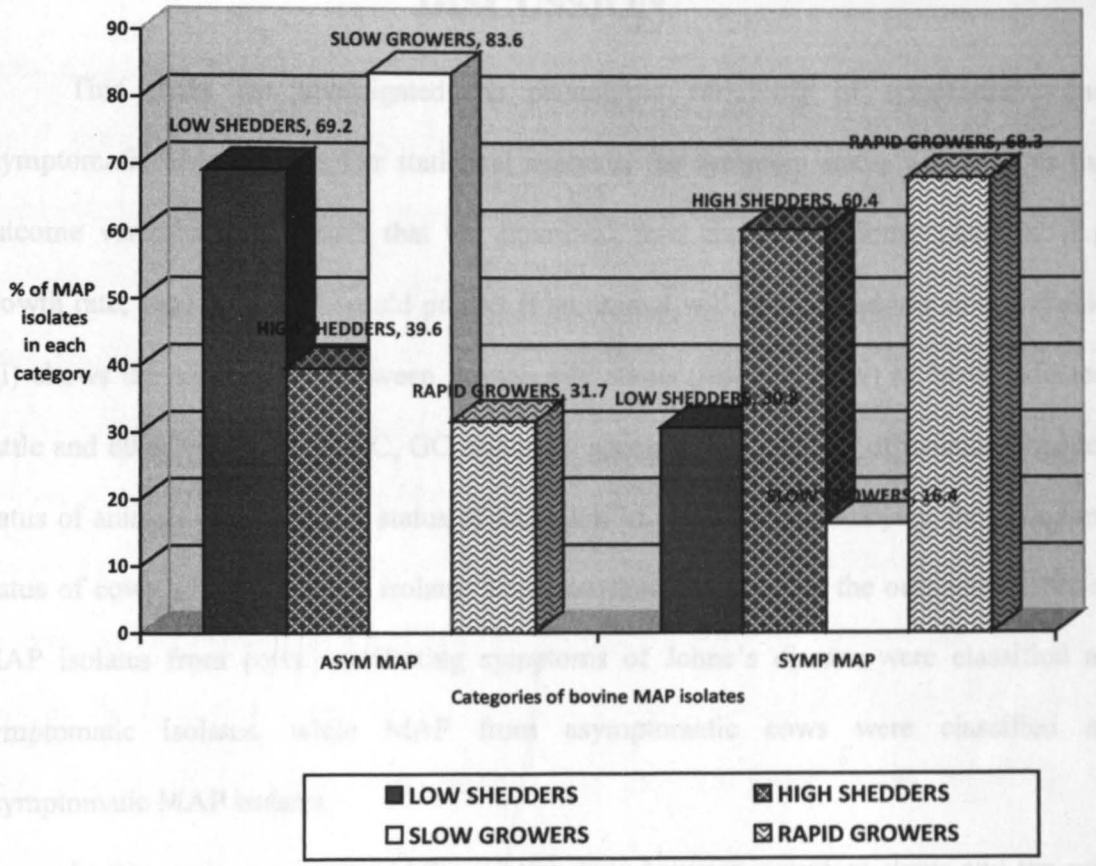

Figure 4.28: Bar chart of the relationship between symptom status of MAP isolates and the shedder and growth rate status of isolates.

DISCUSSION

This study has investigated the phenotypic variability of symptomatic and asymptomatic MAP strains. For statistical analysis, the symptom status was used as the outcome variable. This means that we examined how changes in some variables (e.g growth rate, shedder status) would predict if an animal will have symptoms or not. Table 5(i) shows the associations between growth rate status (rapid or slow) of MAP infected cattle and other variables (HPLC, GC and mass spectrometry patterns of isolates, shedder status of animals and symptom status of animals). In the statistical analysis, the symptom status of cows where the MAP isolates was recovered was used as the outcome variable. MAP isolates from cows manifesting symptoms of Johne's disease were classified as symptomatic isolates, while MAP from asymptomatic cows were classified as asymptomatic MAP isolates.

In this study, we examined the relationships between symptom status and the cell wall lipid of MAP isolates, by use of HPLC, GC and mass spectrometry analysis of mycolic acid. Univariate logistic regression shows that the symptoms status of isolates was significantly associated with mass spectra patterns (OR=3.7, 95% CI= 1.7, 8.6; p <0.00097), growth rate (OR= 11.0, 95% CI= 4.5, 26.8; p <0.0001), and shedder status (OR= 3.4, 95% CI= 1.6, 7.3; p = 0.00127). However, the association between symptoms status and the HPLC and GC patterns was not significant ($p > 0.05$). All the MAP isolates showed a single-cluster peak by HPLC analysis. Similar findings were reported by Dei et al. (1999), whose studies show that within the cluster of peaks obtained by both *M. avium* and *M. paratuberculosis*, they could not find a consistent difference typical of *M. paratuberculosis*. The present study provided further confirmation of the fact that MAP

isolates either individually or classified into two group (symptomatic and asymptomatic) could not be distinguished by HPLC.

Previous studies (He and De Buck, 2010) reported that bacterial cell wall composition is not static and several factors such as physiological and/or environmental conditions affect the chemical composition of mycobacterial cell wall. In our study, the mass spectra results of mycolic acid of MAP isolates showed several ions ranging from m/z 271 to 1071, which were pooled into two categories: (i) the low mass region consisting of ions ranging from m/z 271 to 900 and (ii) the high mass region that consists of peaks greater than 900. MAP isolates were classified into two groups: MS1 (consisting of peaks in only the low mass region) and MS2 (consisting of peaks in both low and high mass region). Our results indicated that 40 (56.3%) of symptomatic isolates produced a series of long chain carbon lengths mycolic acid (M2 pattern), compared to 31(43.7%) of asymptomatic isolates. On the contrary, 35 (74.5%) of asymptomatic isolates produced a series of short chain carbon lengths mycolic acid (M1 pattern), compared to 12 (25.5%) of symptomatic isolates. These findings indicate that, the low mass region of the spectra is correlated with asymptomatic cow isolates, while the high mass region of the spectra is correlated with symptomatic cow isolates. Statisticaly analysis by univariate logistic regression shows that the symptoms status of isolates was significantly associated with mass spectra patterns (OR=3.7, 95% CI=1.7, 8.6; $p < 0.00097$), and with growth rate (OR= 11.0, 95% CI= 4.5, 26.8; $p < 0.0001$) and shedder status of isolates (OR= 3.4, 95% CI= 1.6, 7.3; $p = 0.00127$). It is reasonable to believe that the rapid growth rate may have resulted in the shedding of high numbers of MAP isolates in feces. We further observed that 43 (68.3%) and 9 (16.4%) of symptomatic isolates grew in less than and more than two weeks

respectively, while 46 (83.6%) and 20 (31.7%) of asymptomatic isolates grew in more than and less than two weeks, respectively. Based on these findings, we reasoned that the presence of longer carbon chain length mycolic acid and increased growth rate may provide additional advantage to the organism, such as enhanced capacity to cause disease. Our results bear close resemblance to those of previous works (Laval et al. 2001). These researchers (Laval et al. 2001) conducted a study using MALDI mass spectra and demonstrated that the chain lengths of the various mycolates correlated with the growth rate of mycobacterial strains. Although slow growers, such as *Mycobacterium tuberculosis* and *Mycobacterium ulcerans*, produced a series of odd carbon numbers (C74–C82) of α-mycolic acids, rapid growers synthesized both odd and even carbon numbers (Laval et al. 2001).

CONCLUSION

Collectively, the results of our investigation showed that there was a significant association between symptoms status of isolates and mass spectra patterns, growth rate and shedder status, but not between symptoms status and the HPLC and GC patterns of isolates. Furthermore, our data supported the hypothesis of strain sharing, intra-species and inter-species transmission. Additionally, our findings demonstrate the potential power of mass spectrometry to illustrate biochemical variation in MAP cell walls. Since previous studies reported that bacterial cell wall composition is not static and several factors such as physiological and/or environmental conditions affect the chemical composition of mycobacterial cell wall, this observation could be further applied to our present study. Thus, it is important to note that bacterial cell wall mycolic acid analysis using biochemical methods may yield different results when analyzed under a different set of conditions. Overall, we are hopeful that new findings from this study should provide a better rationale for the future design of therapeutics for Johne's and Crohn's diseases, and should lead to a better understanding of the association between MAP and Crohn's disease

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PAPER 5

INVESTIGATION OF BIOCHEMICAL VARIATIONS OF RAPID AND SLOW GROWING *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* STRAINS

ABSTRACT

Background: *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is an organism that causes paratuberculosis (or Johne's disease) in cattle and other ruminants and one purported to have a causal link with Crohn's disease in humans. Previous works have shown that the growth rates for MAP are slow, with generation times of more than 24 hours. **Aim:** The purpose of this study was to examine for associations between the biochemical variability of MAP strains and their growth rate and presence of symptoms in the source cattle. Additionally, to obtain insights into the pathogenic mechanisms involving MAP in Crohn's disease, we compared the phenotypic characteristics of MAP strains isolated from Crohn's disease patients with those from cattle with Johnes disease. **Methods**: Mass spectrometry of 122 [one hundred and eighteen culture-positive bovine, three human (ATCC 43015, 43544, 49164), and one sheep] MAP isolates were performed. MAP isolates were classified as slow growers if the colonies appeared after 2 weeks and rapid growers if they grew in $\leq$ 2 weeks. Epi Info version 3.5.1 was used to analyze for associations between the biochemical variability of MAP strains and their growth rate or presence of symptoms in source cattle using Chi-square and logistic regression. **Results:** Different mass spectra patterns were detected and categorized in two groups: [M1 (short carbon chain lengths) and M2 (long carbon chain lengths)]. A significant difference ($P< 0.05$) was observed between growth rate and symptom manifestation with 82.7 % of rapid growers having symptoms in source cattle compared to only 17.3 % of slow growers. Also, univariate logistic regression showed a significant association between growth rate and presence of symptoms in source cattle (OR=11.0, 95% CI= 4.5, 26.8; $p <0.0001$), as well as between growth rate and presence of long chain length mycolic acid (M2) (OR= 4.4,

95% CI= 2.0, 9.8; p = 0.0002). Furthermore, the mass spectra 1(MS1) patterns of the sheep isolate were similar to those of bovine MAP strains. **Conclusion:** Data from this study provided an insight into the biochemical diversity among rapid and slow growing MAP strains and associations with manifestation of symptoms in source cattle. The similarities in mass spectrometry profiles between bovine, sheep and human MAP isolates could possibly explain the theory of strain sharing, intra-species and interspecies transmission, and particularly, the similarity between bovine and human MAP isolates may further support an association between Johnne's disease and Crohn's disease. The association between growth rate of MAP strains and presence of symptoms in source cattle, or between growth rate and lipid profiles provided a better understanding of the differences in disease progression and pathogenicity of MAP isolates. This study provides useful data to assist in future management of animal farming operations and possibly in future design of diagnostics and therapeutics for both Johnne's disease and Crohn's disease.

INTRODUCTION

Mycobacterium avium subspecies *paratuberculosis* (MAP) is recognized as an organism that causes Johne's disease (paratuberculosis) in ruminant animals, including cattle. Studies have shown that Johne's disease can vary in severity from inapparent infection to intractable clinical disease that eventually leads to severe emaciation and death (Chiodini et al. 1984). A controversial theory is that MAP also plays a causal role in Crohn's disease, a chronic inflammatory disease of the intestinal wall in humans, with unknown etiology. Molecular evidence (Clancy et al. 2007) and epidemiological evidence (Uzoigwe et al. 2007) have suggested a role for MAP in Crohn's disease. It is reported that milk is a potential source of human exposure to MAP bacteria (Chiodini, 1989; Chiodini et al. 1984). Several laboratory tests have been developed to aid in diagnosis of MAP infection; however, the cultivation of MAP from fecal specimens is recognized as a definitive and most frequently used diagnostic test in cattle (Chiodini et al. 1984; Colgrove et al. 1989), and is also regarded as the standard for comparison for nearly all other tests (de Lisle et al. 1980). It is reported that most Johne's disease control programs use fecal culture test as the basis for making management decision (Thoen and Baum, 1988).

A recognized feature of the genus mycobacteria is their slow growth rate on solid culture medium, which in addition, strongly varies in different species of the genus. Studies show that members of the slow growing mycobacteria include all highly pathogenic species such as *M. tuberculosis*, *M. leprae* and *M. paratuberculosis* causing tuberculosis, leprosy and paratuberculosis, respectively, while members of the fast growing mycobacteria are nonpathogenic and opportunistic species, such as *M. smegmatis* (Lewin and Sharbati-Tehrani, 2005). MAP is a slowly growing organism (>24-h generation time).

that depends on addition of the siderophore mycobactin J to permit in-vitro growth on solid media, and with a strong tendency to form large clumps (Grant et al. 2003). Studies have shown that MAP is a slowly growing mycobacterium, requiring up to 20 weeks, to produce colonies on solid medium (Whipple et al. 1991). It is reported that the two properties of MAP (slow growth and mycobactin-dependence) are not linked. Researchers have demonstrated that, while the addition of mycobactin J to culture broth permits growth of bovine strains of MAP (also called MAP Cow), the ovine strains (MAP Sheep) are probably not mycobactin-dependent (Turenne et al. 2007). MAP isolates could be categorized into various clusters, based on growth characteristics and other factors. Many authors (Collins et al. 1990; Stevenson et al. 2002; de Juan et al. 2005; Pavlik et al. 1999) have categorized MAP isolates into into three groups (types I, II, and III) using growth characteristics by culture and molecular characterization by PFGE and IS900-RFLP. It is reported that Type II MAP strains are considered slow growers that were originally described in cattle, followed by isolation from a wide variety of hosts and geographical locations, while Types I and III groups are extremely slow growing strains, that were first isolated in sheep (Collins et al. 1990) and susequently isolated in few cattle and goats (Whittington et al. 2001; de Juan et al. 2005; 2006). Castellanos et al. (2010) reported the molecular characterization of *Mycobacterium avium* subspecies *paratuberculosis* Types II and III isolates by a combination of MIRU-VNTR loci. A study conducted by Stevenson et al. (2002) utilized the methods of pulsed-field electrophoresis (PFGE) and IS900-restriction fragment length polymorphisms, to divide MAP isolates into two distinct types, with type I comprising very slow-growing, mostly pigmented isolates forming smooth and uniform colonies mainly obtained from sheep and other small ruminants and type II

comprising slow-growing, nonpigmented isolates forming rough and nonuniform colonies exhibiting a very broad host range. Dohmann and colleagues (2003) reported the characterization of genetic differences between *Mycobacterium avium* subsp. *paratuberculosis* Type I (sheep) and Type II (bovine) isolates. They concluded that both types of *M. avium* subsp. *paratuberculosis* strains originated from a common progenitor (Dohmann et al. 2003).

The growth rate variation of MAP could be the results of many factors. Previous authors have shown that many factors may contribute to growth rate variation of mycobacterial species, such as genetic differences between strains, sample storage conditions, medium difference, and clumping of the mycobacteria (Shin et al. 2007; Lambrecht et al. 1988; Hoal-van Helden et al. 2001). Investigators (Lambrecht et al. 1988; Hoal-van Helden et al. 2001) reported that clumping increases the growth rate of mycobacteria both in vitro and in vivo. According to Hoal-van Helden et al. (2001), clumping of the mycobacteria resulted in more vigorous growth in human monocyte-derived macrophages (MDM), suggesting that inoculum size could affect disease progression. According to Whipple et al. (1987), strain variation may explain why there are differences in the progression of disease among herds infected with *M. paratuberculosis*. Shin et al. (2007) reported that MAP subgroup II strains grew faster than subgroup I strains, especially at higher inocula ($>10^3$ CFU). They concluded that this growth rate difference between laboratory-adapted and low-passage clinical isolates could possibly be explained by the genetic differences between strains, by sample storage conditions, or by greater clumping at high inocula. Janagama et al. (2010) carried out transcriptional and proteomic profiling of MAP strains under iron-replete or -deplete conditions, and reported

that iron-sparing response, a phenotypic characteristic of *Mycobacterium avium* subsp. *paratuberculosis*, is strain dependent.

An essential component of cell wall of mycobacteria, and other members of the family *Corynebacterineae* are long-chain -alkyl, and -hydroxy fatty acids, refered to as mycolic acids (Brennan and Nikaido, 1995). Research has shown that the structure of the mycolic alkyl chains is affected by the nature of the carbon source (Sokolovská et al. 2003; Wick et al. 2002). Several studies (Glickman et al. 2000; Liu et al. 1996; Yuan et al. 1998; Dubnau et al. 2000) have demonstrated that the amounts and composition of mycolic acids affect the growth rate, virulence, permeability and colony morphology of *Mycobacteria tuberculosis*. According to Glickman et al. (2000), colonial morphology of pathogenic bacteria is often associated with virulence, and virulence is correlated with the formation of serpentine cords. Glickman et al. (2000) showed that a mycobacterial gene, *pcaA*, is required for cording and mycolic acid cyclopropane ring synthesis in the cell wall of both BCG and *M. tuberculosis*. They further demonstrated that mutants of *pcaA* fail to persist within and kill infected mice despite normal initial replication. Their results indicate that a novel member of a family of cyclopropane synthetases is necessary for lethal chronic persistent *M. tuberculosis* infection and define a role for cyclopropanated lipids in bacterial pathogenesis. Rao et al. (2006) examined the role of *Mycobacterium tuberculosis* bioactive cell envelope lipids in tuberculosis pathogenesis. Their results showed that trans-cyclopropanation of mycolic acids on trehalose dimycolate (TDM) suppresses *Mycobacterium tuberculosis*-induced inflammation and virulence, and *M. tuberculosis* mutant lacking *trans*-cyclopropane rings was hypervirulent in mice (Rao et al. 2006). Riley (2006) suggested that the relative composition and structural differences in TDM among

different mycobacteria influence the host animal biologic response. TDM is composed of a nonreducing sugar trehalose covalently linked to mycolic acids (Riley, 2006). Beaman and Moring (1988) reported that the specific composition of C54 mycolic acids was very different in virulent log-phase cells compared with less-virulent stationary-phase cells, and the avirulent mutant lacked a C54:3 mycolate that was prominent in the virulent log-phase parent strain (GUH-2P). These results suggest that certain mycolic acids are associated with virulence (Beaman and Moring 1988).

Although previous studies have characterized MAP based on phenotypic properties (e.g growth rate, pigmentation, lipid profile etc.) and molecular assays (e.g. PFGE, IS900 RFLP, IS1311 PCR-REA, SSR typing, VNTR typing etc.), more studies are still required in order to completely understand the virulence and pathogenicity of MAP organisms.

The aim of this study was to examine for associations between the biochemical variability of MAP strains and their growth rate and presence of symptoms in the source cattle. Additionally, to gain a better sense of the pathogenic mechanisms involving MAP in Crohn's disease, the present study compared the phenotypic characteristics of MAP strains isolated from Crohn's disease patients with those from cattle with paratuberculosis. Ultimately, the data obtained in this study will help in the future management of animal farming operations, and possibly in the future development of vaccines, new drugs and treatment methods for Johne's and Crohn's disease.

MATERIAL AND METHODS

Bacterial strains, fecal samples and growth condition

A total of 122 [118 bovine, 1 sheep and 3 human (ATCC 43015, 43544, 49164)] MAP isolates were used in this study. The bovine and sheep MAP isolates were obtained from the North Dakota State Veterinary Diagnostic Laboratory, Fargo, North Dakota, while the 3 MAP isolates from Crohn's disease patients (ATCC 43015, 43544, 49164) were purchased from the American Type Culture Collection, Manassas, VA. Fecal samples of cattle infected with MAP were cultured by modifications of methods described elsewhere (Whipple et al. 1991). Briefly, the fecal samples were decontaminated with 0.75% HPC (hexa-decyl-pyridinium chloride: cetylpyridinium chloride) and cultured on two Herrold Egg Yolk Media (HEYM) with and without Mycobactin J and incubated at 37°C. Results were examined weekly for up to 16 weeks of incubation. Positive cultures were confirmed by PCR analysis (IS900) of the growing colonies, following the protocols described previously (Ayele et al. 2005). In addition, freezer stocks of MAP isolates (ATCC 43015, 43544, 49164) were revived in 7H9 broth (Becton, Dickinson, Sparks, Md.). In this study, MAP colonies that appear in $\leq$ 2 weeks were classified as rapid growers, while those colonies that appear in $>$ 2 weeks were clustered as slow growers.

Bacteria counts

Preparation of single-cell suspensions and measurement by spectrophotometer: A single colony of pure mycobacterium culture was inoculated into a 5-ml volume of Middlebrook broth, and single-cell suspensions of each strain were prepared as previously described (Lambrecht et al. 1988), with slight modifications. Briefly, mycobacterial cells

grown in mycobactin-supplemented Middlebrook 7H9 broth at 37°C for 14–16 weeks. were harvested by centrifugation at 10,000 x g for 15 min and washed thrice in 10 mM phosphate-buffered saline (PBS) (pH 7.2). Cell pellets were homogenized by vortexing for 30 seconds to minimize clumping of cells. Cell pellets were resuspended in PBS and the mycobacteria cell suspension was adjusted to a spectrophotometer OD_{540} of 1.3 - 1.4.

Quantification of viable bacteria: An important foundation from several types of microbiological research is the quantification of viable bacteria. The number of viable mycobacterial cells in each single-cell suspension was determined by standard plate counting as a reference method. Briefly, one milliliter of the undiluted stock cell suspension (OD_{540} of 1.3-1.4) was added to 9.0 ml of 10 mM PBS (pH 7.2). Tenfold serial dilutions were made in 10 mM PBS (pH 7.2), with vortexing between each dilution step. One hundred microliters from each dilution was plated onto each of three 7H10 agar plates supplemented with 10% OADC (oleic acid-albumin-dextrose-catalase) and 2 µg/ml of mycobactin J. Colony counts (CFU) were determined after the incubation of plates at 37°C for 16 weeks. OD_{540} of 1.3 - 1.4 equates roughly to $3 \times 10^9 - 5 \times 10^9$ CFU/ml of MAP cells.

Grouping of isolates according to intensity of infection: The intensity of infection was determined using the colony forming units per tube (CFU/tube). Following 16 weeks of incubation, the number of MAP colonies was in the range of 0–200 CFU and were grouped as follows: low shedders (1-50 CFU/tube) and heavy shedders (greater than 50 CFU/tube).

Mass spectrometry analysis

Extraction and derivatization of mycolic acids: MAP cell wall mycolic acid was extracted and esterified by use of protocol previously described by Minnikin et al. (1980). In brief, one-milliliter aliquot of the bacterial culture (OD_{540} of 1.3 – 1.4 that corresponds to 3 x 10^9 - 5 × 10^9 CFU/ml of cells) was transferred to a new tube and centrifuged at 3,500 x g for 20 min. The cell pellets were esterified by mixing with 2 mls of transesterification reagent, methanol/toluene/H_2SO_4 in the ratio of 30:15:1. The samples were heated at 85°C for 16-18 hours in an oil bath. Then, 2mls of chloroform: hexane (4:1) was added to the cooled sample. This was followed by addition of 1 ml of 0.3 M phosphate buffer (42.57 g of Na_2HPO_4 and 12.0 g of NaOH per liter of distilled water (pH 11 to 12). Samples were centrifuged (3,000 x g, 10 min, 4°C), and the lower organic layer containing the fatty acid was transferred to a clean tube. The extracts were evaporated to dryness under a stream of nitrogen at 37-42°C and stored at 0°C.

Analysis of methyl mycolates by mass spectrometry: The purified methyl mycolates were analysed using BioTOF Electrospray Ionization Time-of-Flight (ESI-TOF) Mass Spectrometer, associated with Bruker Daltonic Data Analysis (Bruker Daltonics BioTOF Billerica, MA USA). About 10 mg of the mycolic acid methyl esters were dissolved in 2 ml of methanol and serially diluted to a 1 µM concentration and 10 µl of the solution was injected into the mass spectrometer. Each sample was analyzed three times with identical results.

Statistical analysis

The results of the present study were analyzed as described by Niemann et al. (1999). Data were analyzed with the use of software package CDC's Epi Info program (version 3.5.1). Chi-square and logistic regression were used to analyze for associations between the biochemical and genetic variability of MAP strains and their growth rate or presence of symptoms in source cattle.

RESULTS

Table 5.1 shows the relationships between growth rate status (rapid or slow) of MAP infected cattle and other variables (PFGE profiles of isolates, mass spectrometry patterns of isolates, shedder status of animals and symptom status of animals).

Table 5.1: Univariate logistic regression analysis of associations between growth rate (slow or rapid) status of MAP infected cattle and other variable factors (mass spectrometry patterns of isolates (OR = 4.4, 95% CI = 2.0, 9.8), symptom status (OR = 11.0, 95% CI = 4.5, 26.8) and shedder status (OR = 2.6, 95% CI = 1.2, 5.4).

Variable	Category	No of isolates	Percent slow growers (%)	Percent rapid growers (%)	df	P value
Mass spectrometry Patterns	M1	47	68.1	31.9	1	0.00014
	M2	71	32.4	67.6		
Symptoms	Asymptomatic	66	69.7	30.3	1	<0.00001
	Symptomatic	52	17.3	82.7		
Shedder status	Low shedder	65	56.9	43.1	1	0.012
	High shedder	53	34.0	66.0		

Figures 5.1-5.6 show examples of the results of the mass spectral analysis of slow and rapid growing bovine MAP isolates, which were classified into two groups: M1 and M2 based on the differences in carbon chain lengths, with M1 groups having short carbon lengths, and M2 groups having long carbon chain lengths.

Figures 5.7 shows the mass spectral result of the sheep MAP isolate.

Figures 5.8-5.10 shows the mass spectral results of the three human clinical isolates (ATCC 43015, 43544, 49164)].

Figure 5.11 shows the bar chart representing the sources of slow and rapid growing *Mycobacterum avium* subspecies *paratuberculosis* isolates (cattle, humans and sheep) and the percentage of mass spectra group (M1 or M2) belonging to each source.

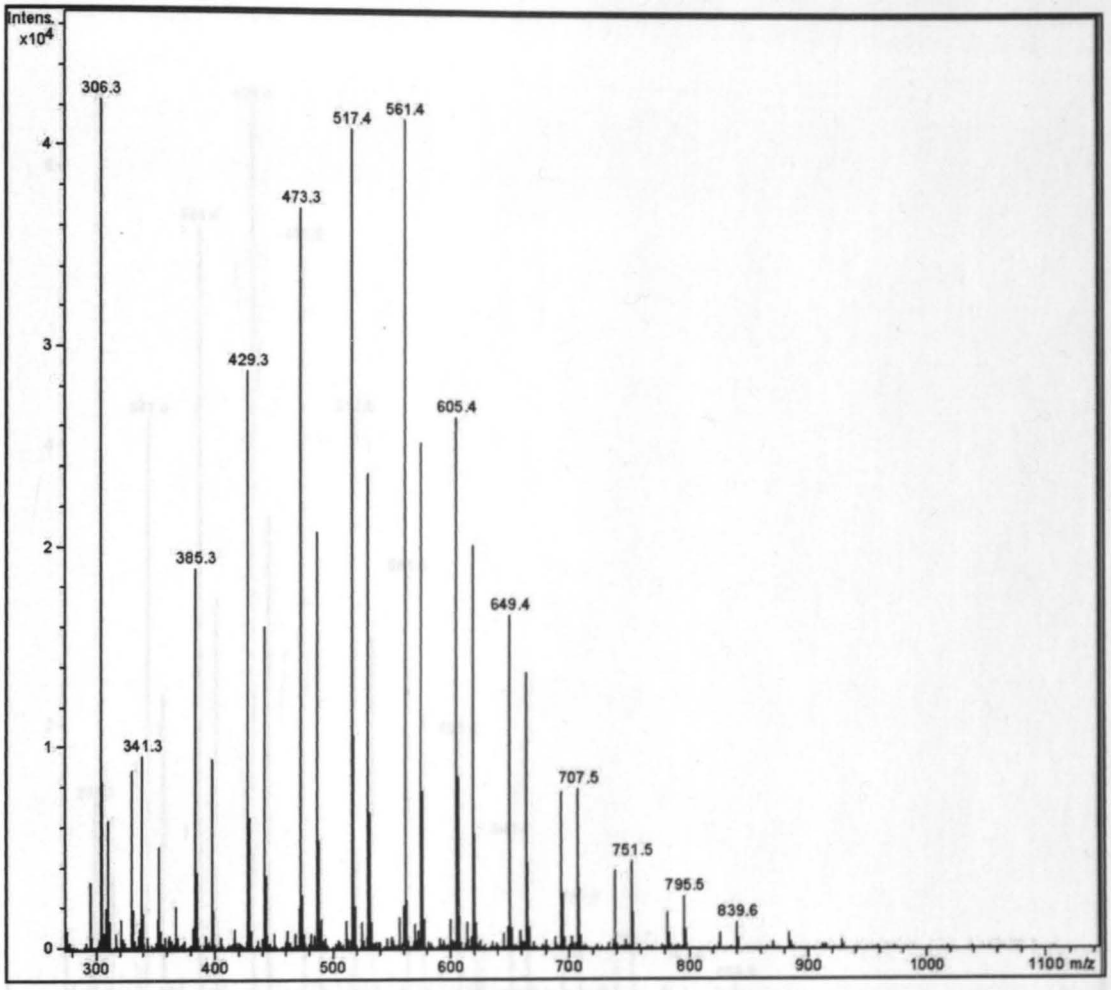

Figure 5.1: Mass spectral analysis of MAP isolate, which was slow growing and belongs in the M1 group.

Figure 5.12 shows the relationship between the growth rate of bovine MAP isolates and their shedder and symptom status. There is a significant difference between slow growers and rapid growers of MAP strains. Each bar represents the percent of MAP isolates belonging to each category: shedder status (low and high) and symptoms status (symptoms or no symptoms).

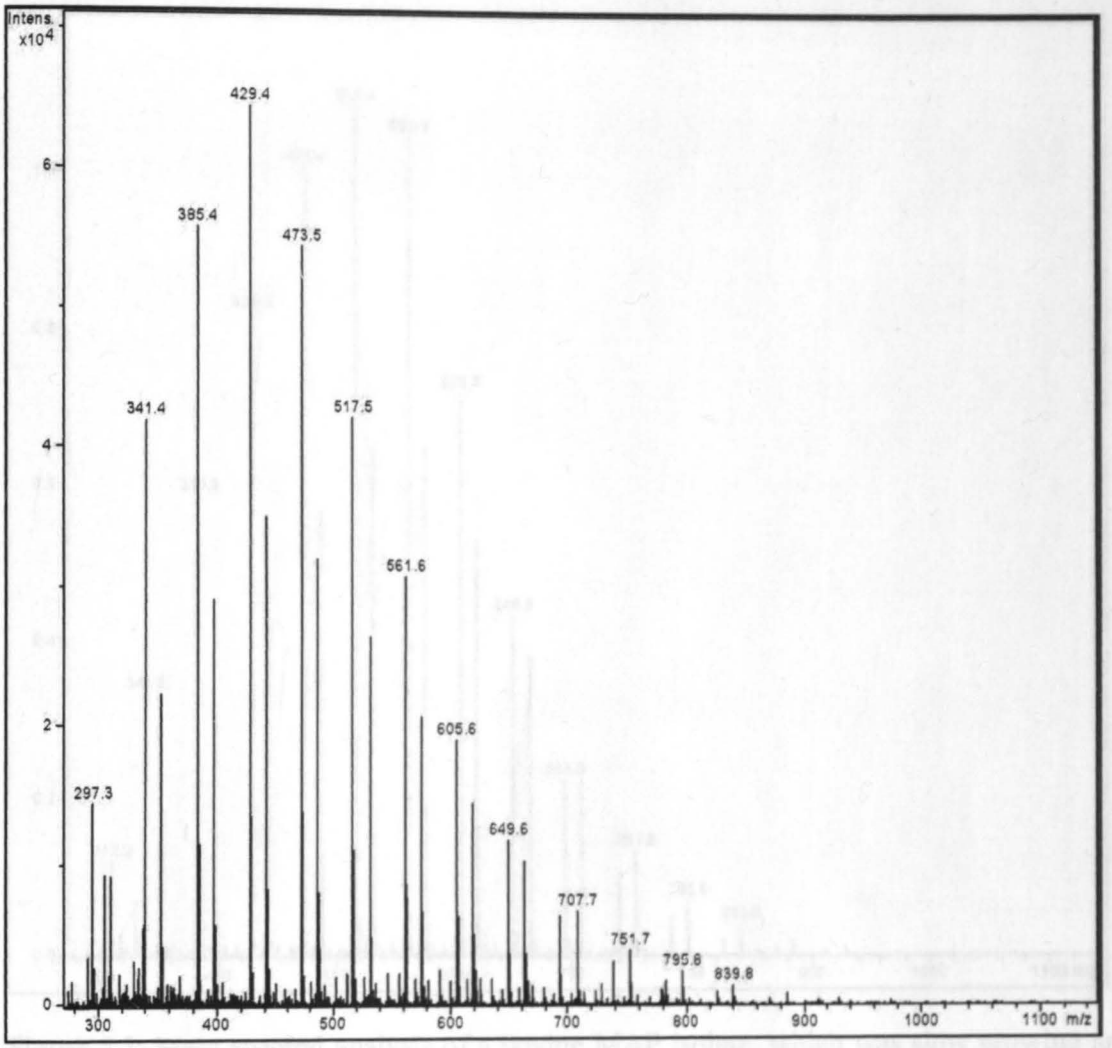

Figure 5.2: Mass spectral analysis of a bovine MAP isolate, which was slow growing and belongs in the M1group.

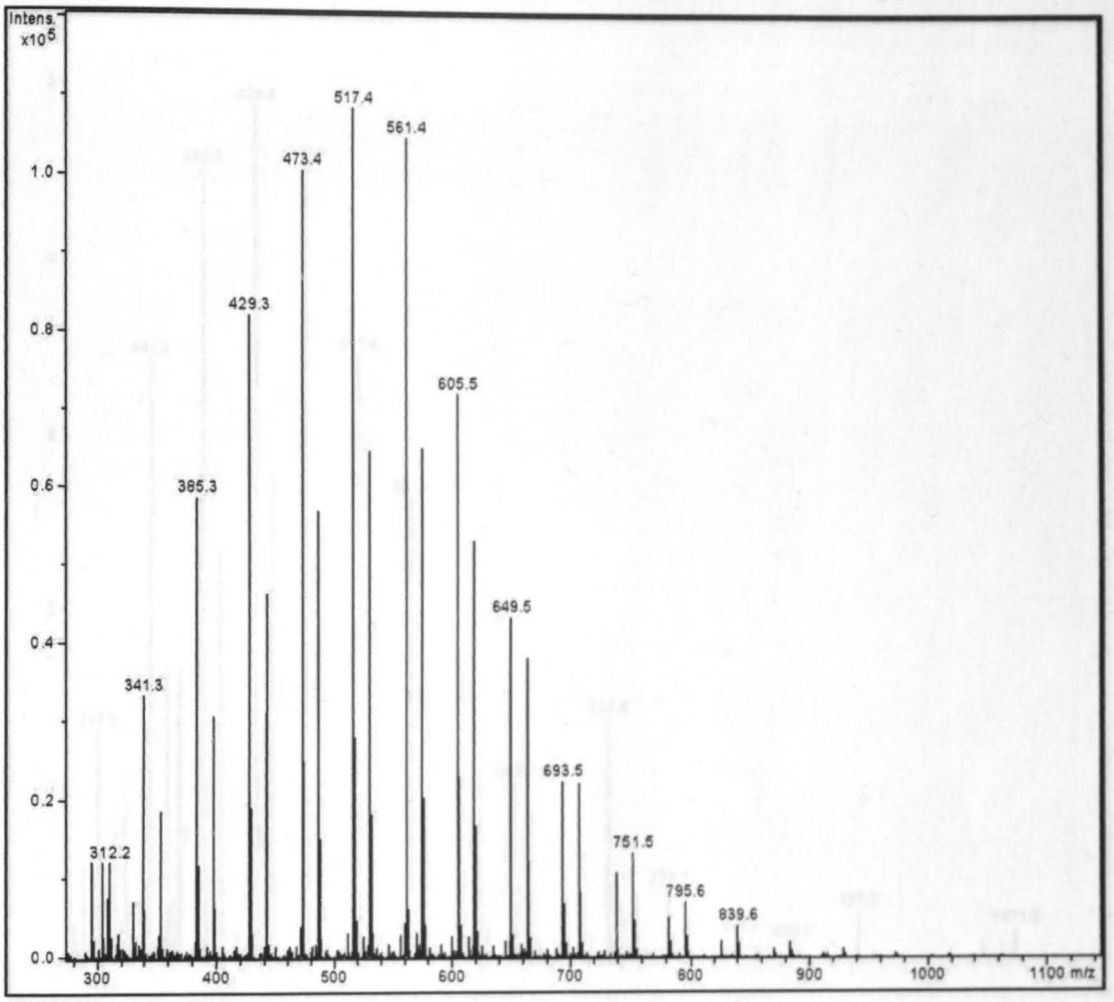

Figure 5.3: Mass spectral analysis of a bovine MAP isolate, which was slow growing and belongs in the M1 group.

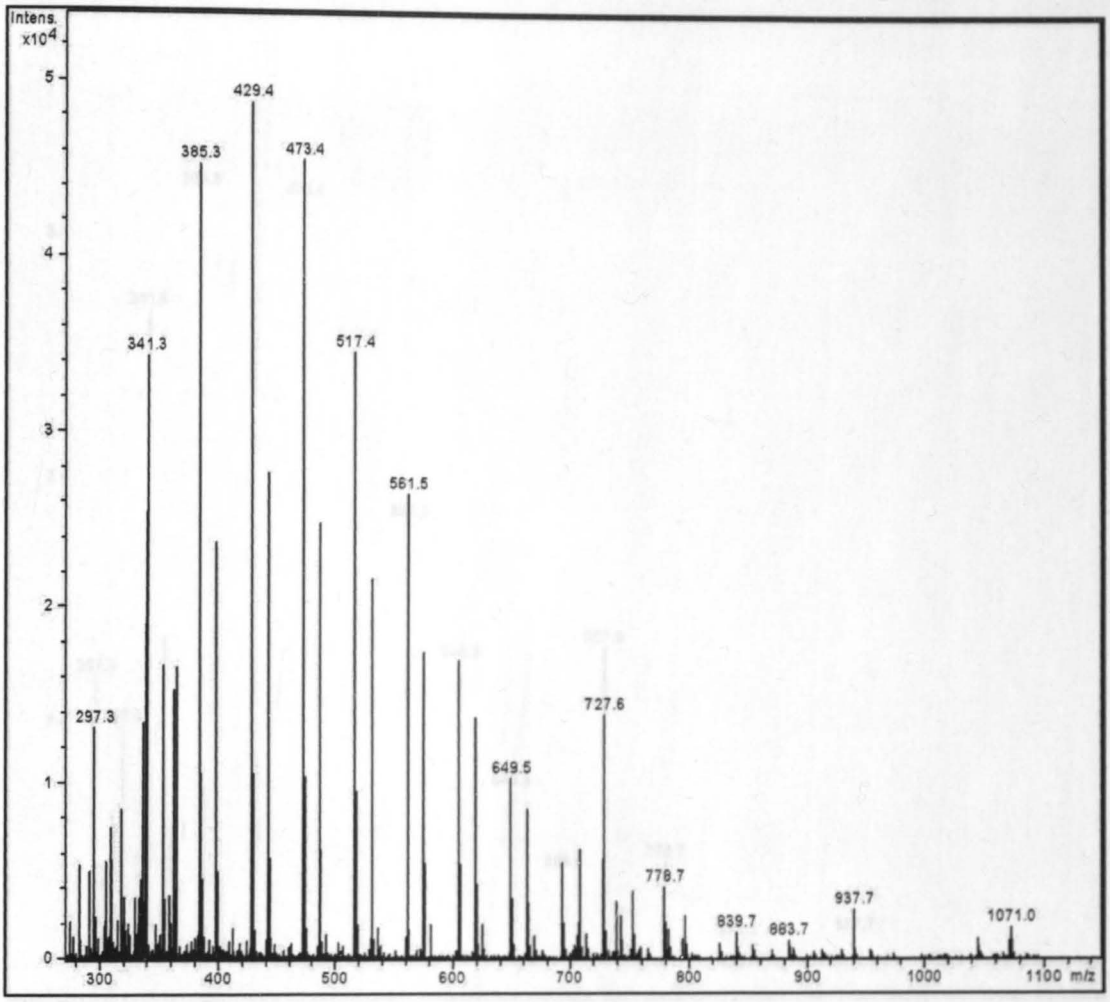

Figure 5.4: Mass spectral analysis of a bovine MAP isolate, which was rapid growing and belongs in the M2 group.

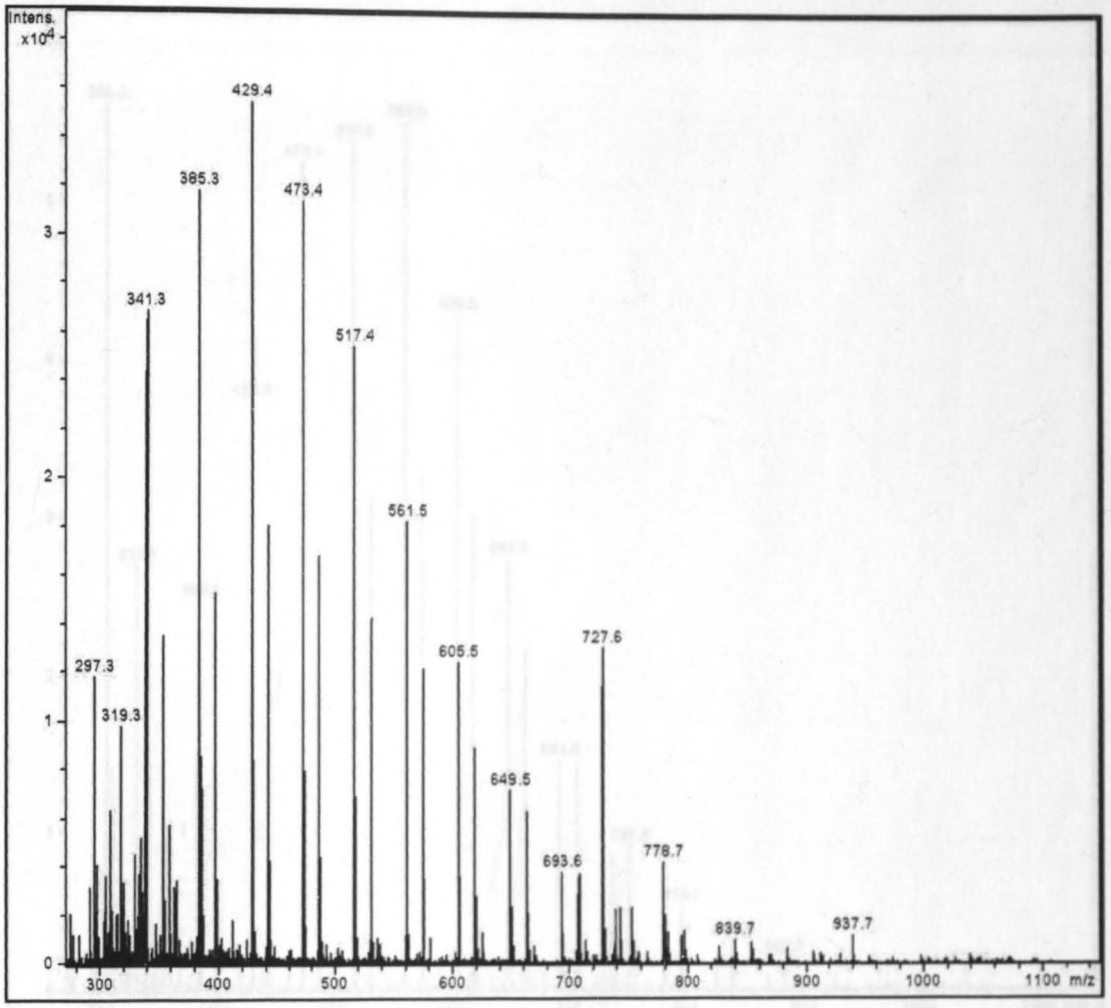

Figure 5.5: Mass spectral analysis of a bovine MAP isolate, which was rapid growing and belongs in the M2 group.

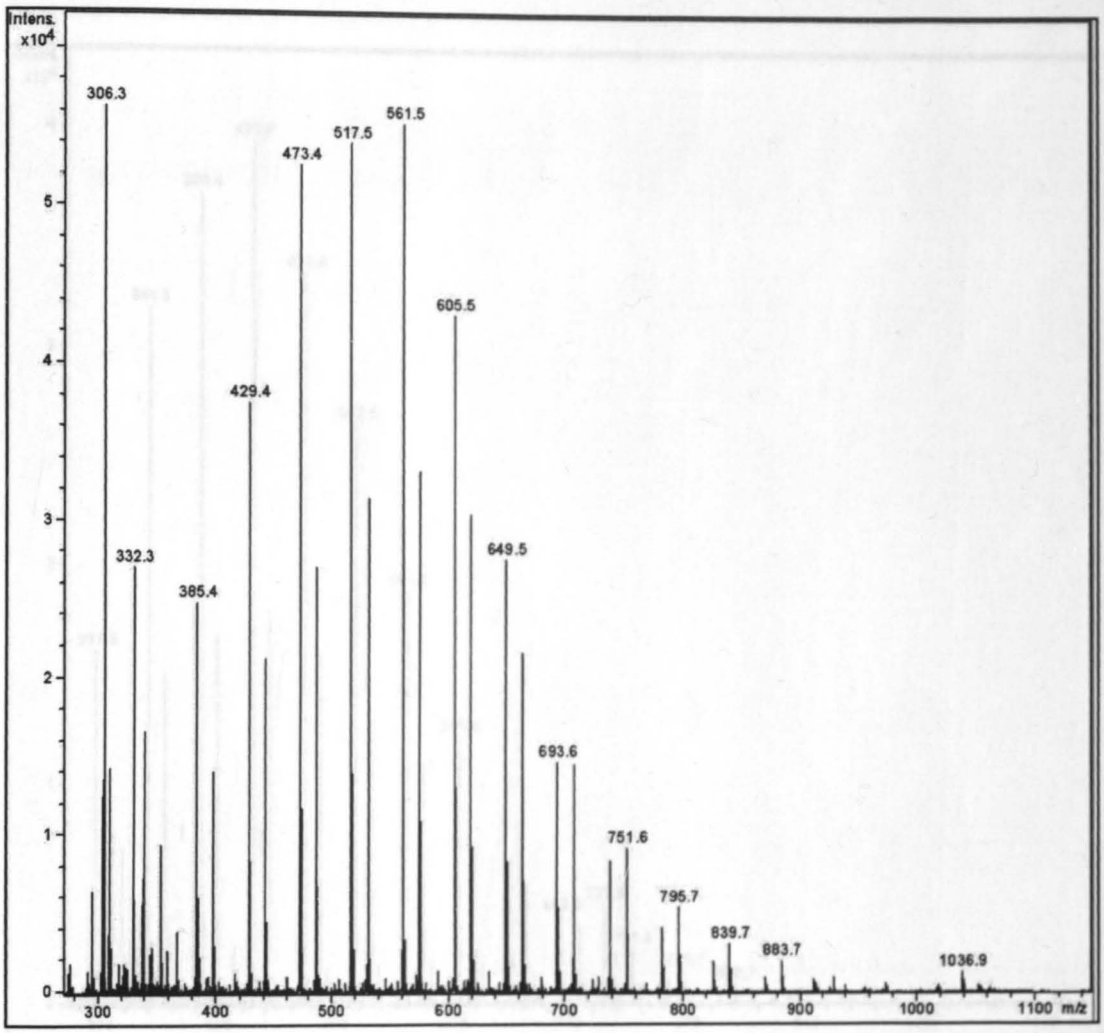

Figure 5.6: Mass spectral analysis of a bovine MAP isolate, which was rapid growing and belongs in the M2 group.

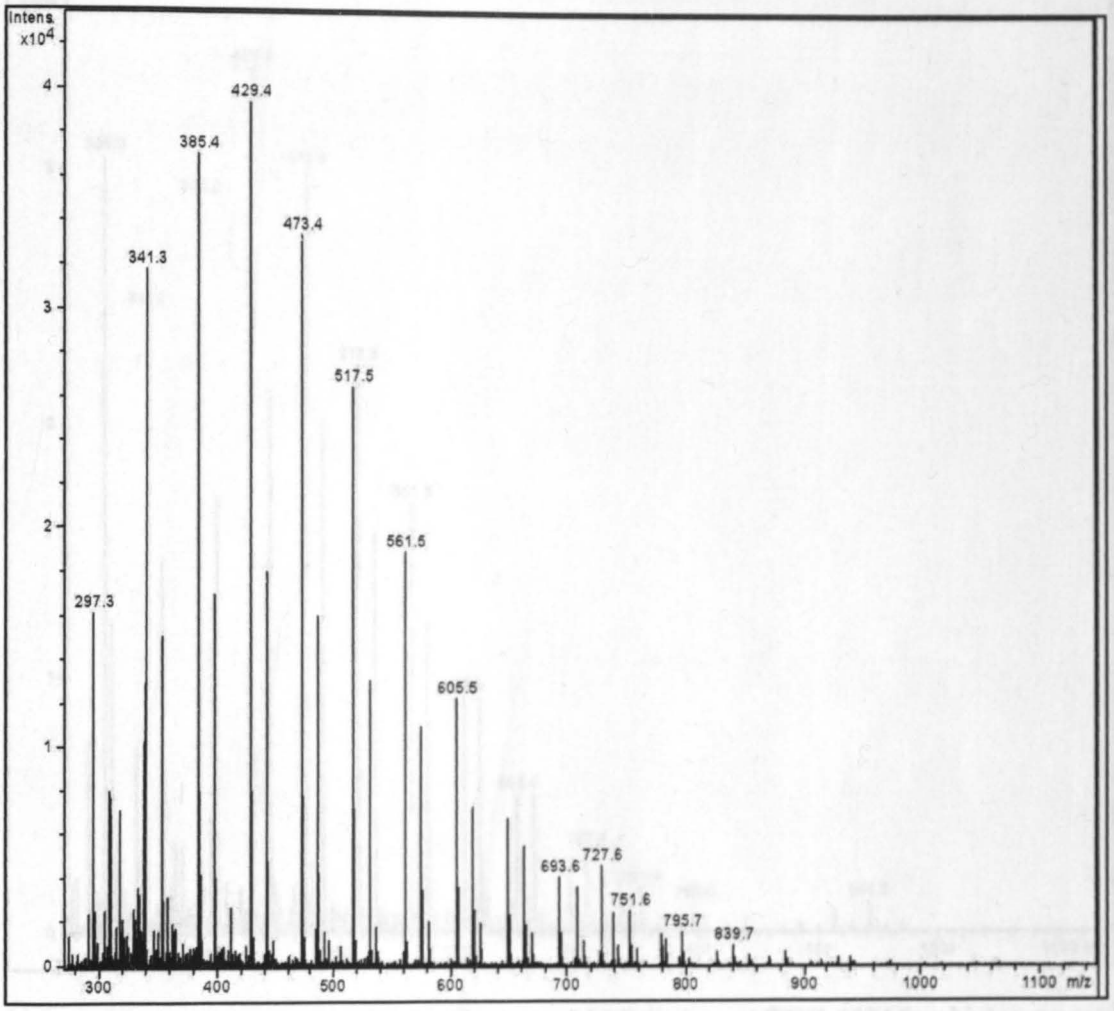

Figure 5.7: Mass spectral analysis of a sheep MAP isolate, which was slow growing and belongs in the MC1 group.

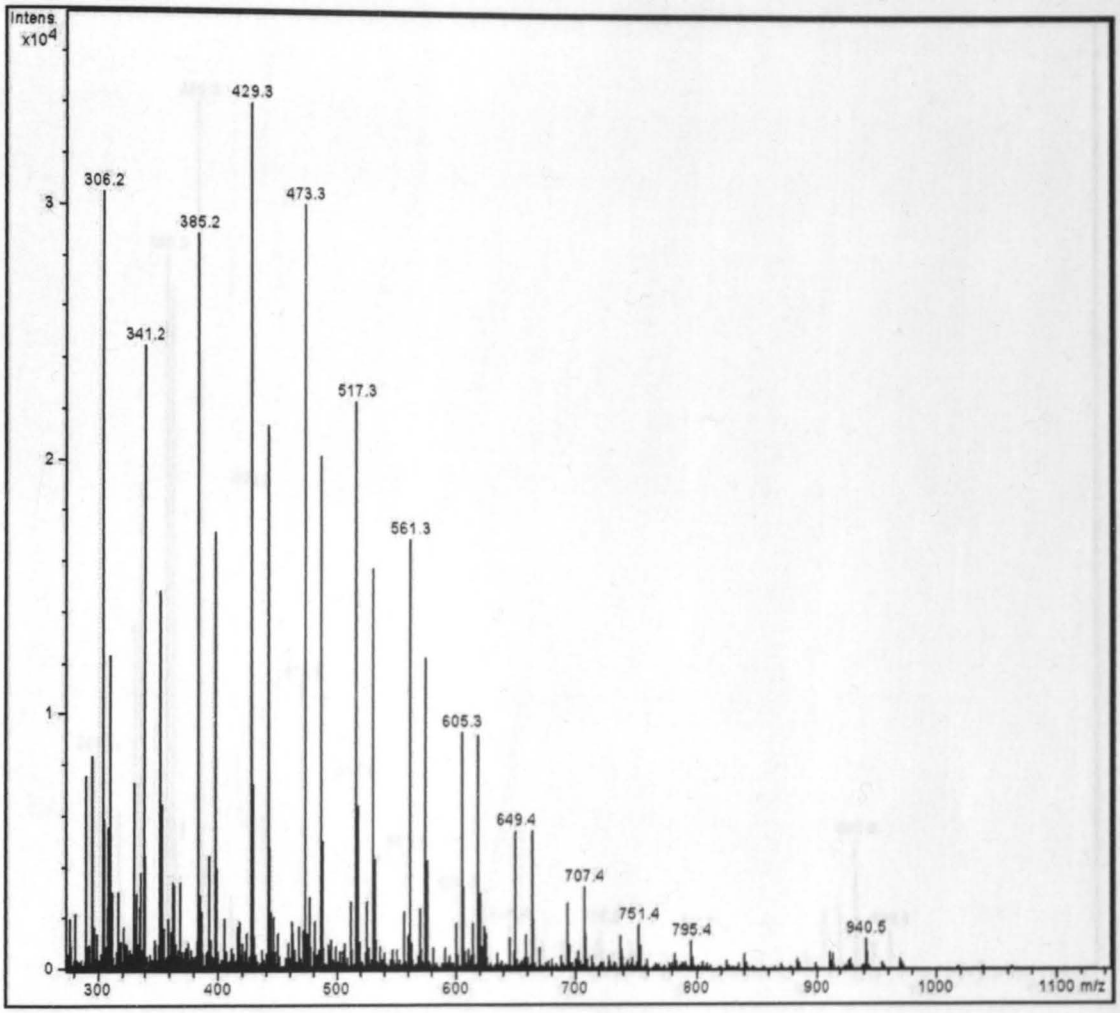

Figure 5.8: Mass spectral analysis of human MAP isolate, ATCC 43015, which was rapid growing and belongs in the MC2 group.

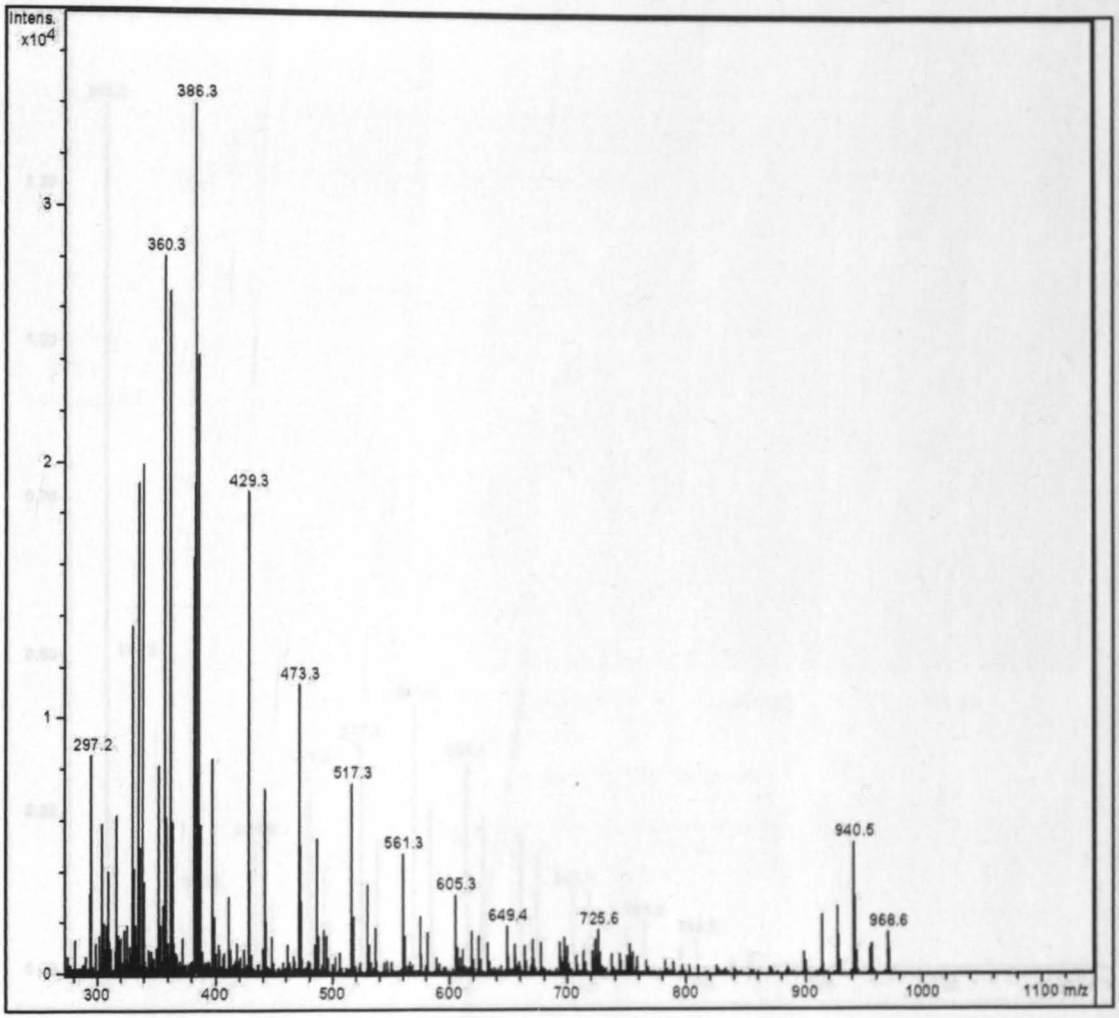

Figure 5.9: Mass spectral analysis of human MAP isolate, ATCC 43544, which was rapid growing and belongs in the MC2 group.

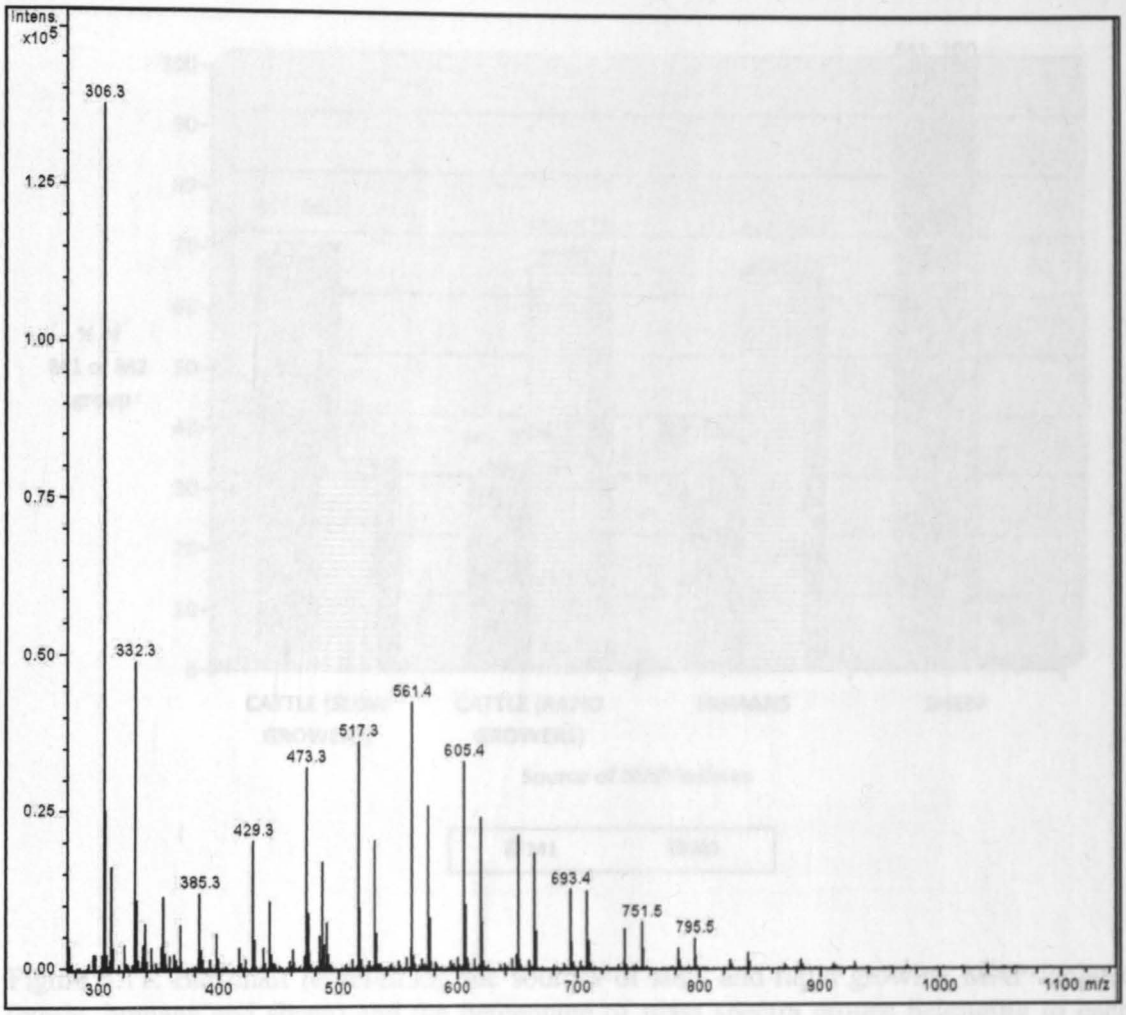

Figure 5.10: Mass spectral analysis of human MAP isolate, ATCC 49164, which was slow growing and belongs in the MC1 group.

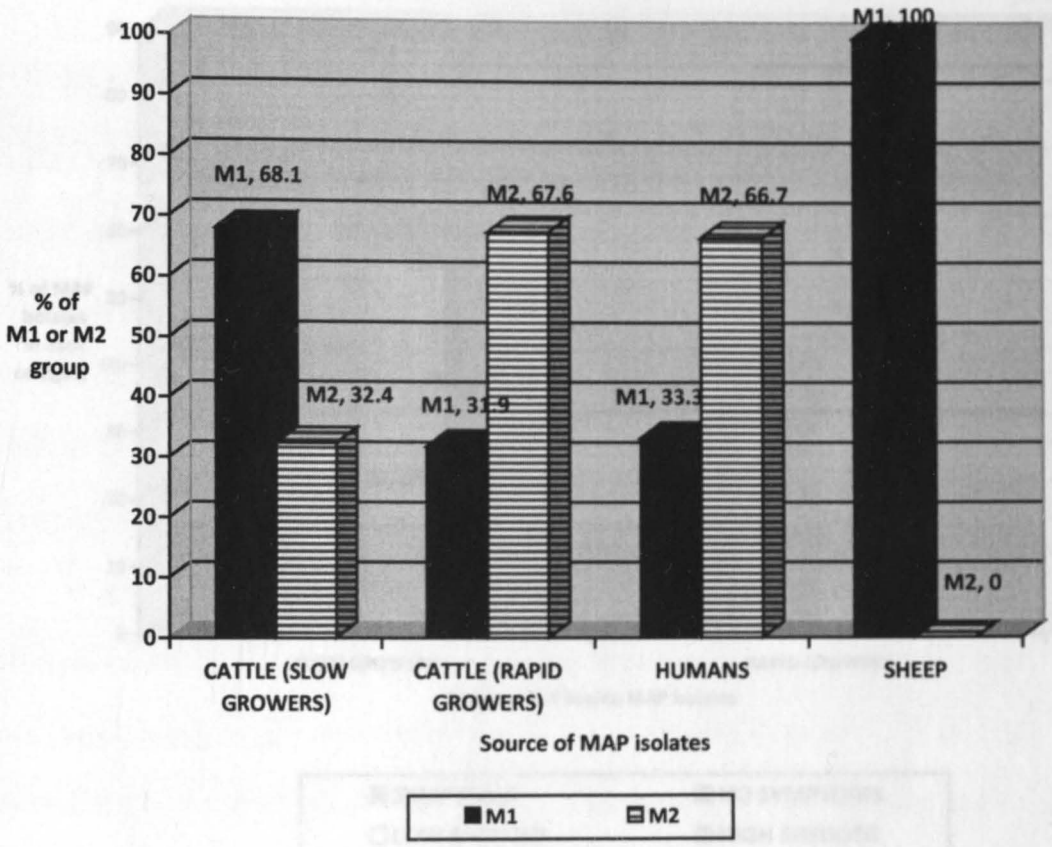

Figure 5.11: Bar chart representing the sources of slow and rapid growing MAP isolates (cattle, humans and sheep) and the percentage of mass spectra groups belonging to each source origin.

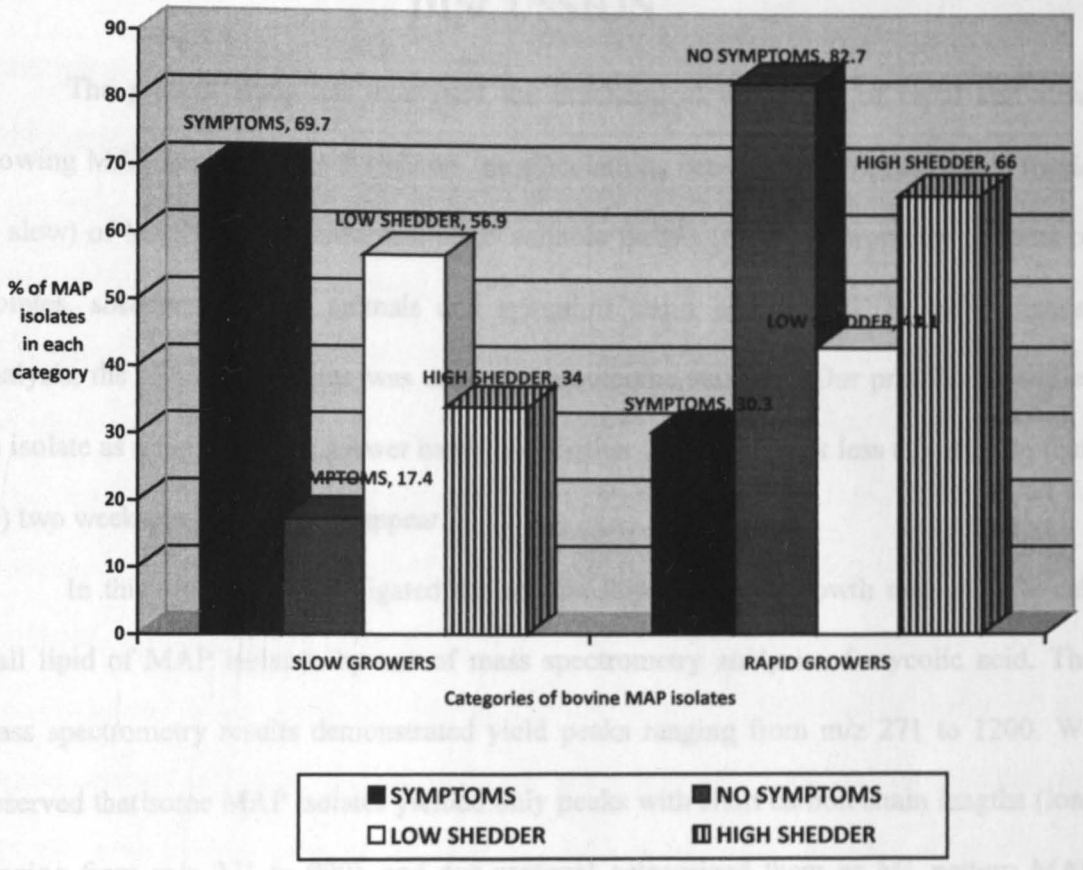

Figure 5.12: Bar chart of the relationship between growth rate of MAP isolates and the shedder and symptom status of the isolates.

DISCUSSION

The present study has examined the biochemical variability of rapid and slow growing MAP strains. Table 5.1 shows the associations between growth rate status (rapid or slow) of MAP infected cattle and other variable factors (mass spectrometry patterns of isolates, shedder status of animals and symptom status of animals). In the statistical analysis, the MAP growth rate was used as the outcome variable. Our protocol classifies an isolate as a rapid or slow grower based on whether the isolate took less ($\leq$) or more than ($>$) two weeks, respectively, to appear.

In this study, we investigated the relationships between growth rate and the cell wall lipid of MAP isolates, by use of mass spectrometry analysis of mycolic acid. The mass spectrometry results demonstrated yield peaks ranging from m/z 271 to 1200. We observed that some MAP isolates yielded only peaks with short carbon chain lengths (ions ranging from m/z 271 to 900), and our protocol categorized them as M1 pattern MAP group, while some other MAP isolates produced peaks with both short and long carbon chain length (ions ranging from m/z 271 to 1200), and these were classified as M2 pattern MAP group. Chi square analysis of our data revealed a significant difference ($p = 0.00014$) between mass spectrometry patterns and growth rate of isolates. We observed that the majority (68.1%) of slow growers produced a series of short chain carbon lengths mycolic acid (M1 pattern), compared to 31.9 % of slow growers. Likewise, most (67.6%) of rapids growers produced a series of long and short chain carbon lengths mycolic acid (M2 pattern), compared to 32.4 % of slow growers. Univariate logistic regression showed that short carbon chain length mycolic acid was significantly associated with slow growth rate, while increase in carbon chain lengths was associated with rapid growth rate (OR= 4.5,

95% CI= 2.0, 9.8; p = 0.0001). These findings suggest that changes in cell wall composition, especially the presence of long carbon lengths mycolic acid could affect the rate of growth of MAP bacteria, which in turn could affect disease progression.

Our results bear very close resemblance to those of previous studies (Laval et al 2001), where the chain lengths of the mycolates correlated with the growth rate of mycobacterium isolates. Laval and colleagues (2001) analyzed mycolic acids, major and specific long-chain fatty (C70-C90) acid components of the mycobacterial cell envelope, using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Their results showed that the chain lengths of the various mycolates correlated with the growth rate of mycobacterial strains, with slow growers, such as *Mycobacterium tuberculosis* and *Mycobacterium ulcerans*, producing a series of odd carbon numbers (C74-C82) of alpha-mycolic acids, and rapid growers synthesizing both odd and even carbon numbers. Additionally their results show that the main chain of oxygenated mycolic acids from slow growers were four to six carbon atoms longer than the corresponding alpha-mycolic acids, whereas rapid growers elaborated oxygenated homologues possessing the same chain lengths as their alpha-mycolic acids (Laval et al. 2001). Our data are also in agreement with those of previous investigators (Daffé and Draper, 1998; Brennan and Nikaido, 1995) that reported that the composition and amount of mycolic acids affect the growth rate, colony morphology and permeability and virulence of *M. tuberculosis*.

In addition, the present study observed a significant difference (P = 0.012) between growth rate and symptom manifestation with 82.7 % of rapid MAP growers causing symptoms in source cattle compared to only 17.3 % of slow growers. Also, univariate

logistic regression showed a significant association between growth rate and presence of symptoms in source cattle (OR=11.0, 95% CI= 4.5, 26.8; p <0.0001), between growth rate and shedder status (OR = 2.6, 95% CI = 1.2, 5.4), as well as between growth rate and presence of long chain length mycolic acid (M2) (OR= 4.4, 95% CI= 2.0, 9.8; p = 0.0002). Additionally, while 69.7% of slow growing MAP isolates were found in asymptomatic cattle, only 30.3% of rapid growers were found in asymptomatic cattle.

The association between long chain mycolic acid, rapid growth rate and presence of symptoms in cattle partly suggests that the genetic differences between strains could possibly be a reason for differences in growth rate, virulence, pathogenesis and progression of disease among MAP infected cattle. Our results are in agreement to those of previous studies (Whipple et al. 1987). According to Whipple et al. (1987), strain variation may explain why there are differences in the progression of disease among herds infected with *M. paratuberculosis*. Lewin and Sharbati-Tehrani (2005) reported that growth rate of mycobacteria is associated with pathogenicity, and slower-growth is related to greater pathogenicity. Valway et al. (1998) demonstrated that a strain of *Mycobacterium tuberculosis* (CDC155) have a greatly faster doubling time in the lungs of mice than the long established Erdman strain. Based on this finding, it was reasoned that strain CDC1551 has a very high virulence for mice and that this could indicate that it has a high virulence for humans, which in turn, would further explain its unusually high transmissibility.

In contrast to our results, some studies show that growth rate is not an indicator of virulence. Thus, while some studies have associated the growth rate of mycobacteria with pathogenicity (Whipple et al. 1987; Lewin and Sharbati-Tehrani, 2005; Valway et al. 1998), some other studies have rejected this idea (North et al. 1999). North et al. (1999)

investigated whether the ability of *M. tuberculosis* CDC1551 to multiply in the lungs of mice over a relatively short period of observation was associated with the ability to cause disease, and reported that the growth rate of mycobacteria in mice as an unreliable indicator of mycobacterial virulence.

In addition, in the present study, to obtain insights into the the association between Johne's and Crohn's disease, we investigated if the strains of MAP isolated from Crohn's disease could share similar phenotypic characteristics with those involved in Johnes disease. We observed that all the human MAP isolates possessed the MS2 mass spectrometry pattern. Furthermore, the mass spectrometry patterns of the sheep MAP isolate were similar to those of bovine MAP strains, and belonged to the group MS1. The similarities in the mass spectrometry patterns of bovine, sheep and human MAP isolates could possibly explain the theory of strain sharing, intra-species and interspecies transmission, and particularly, the similarity between bovine and human MAP isolates may further support an association between johnne's disease and Crohn's disease. Scanu et al. (2007) reported that MAP is a candidate pathogen in the causation of a proportion of cases of irritable bowel syndrome as well as in Crohn's disease. These researchers reported a significant association ($P = 0.0018$) between *Mycobacterium avium* subsp. *paratuberculosis* infection and the consumption of hand-made cheese (Scanu et al. 2007). According to Motiwala et al. (2006), evidence of strain sharing between bovine and human MAP isolates is of special interest since it implies the existence of a potential animal reservoir for Crohn's disease.

CONCLUSION

By using mass spectrometry, we were able to detect biochemical differences among one hundred and twenty-two [118 bovine strains of MAP belonging to two groups (rapid and slow growers), 3 human (ATCC 43015, 43544, 49164), and 1 sheep] isolates. Based on the similarities between bovine, sheep and human MAP isolates, the present study is considered to support the hypothesis of interspecies (e.g from sheep to cattle, cattle to sheep or cattle to humans), and intraspecies transmission (from cattle to cattle or sheep to sheep). Our data could further support an association between Johne's disease and Crohn's disease, based on similarities in biochemical profiles between bovine and human MAP isolates. Overall, the present study has provided a better understanding of some factors that may influence the bacteria growth rate (e.g lipid profiles), as well as the association between Johne's and Crohn's disease. Our study has shed more light on MAP biochemical features, and should fuel new developments in the control and treatment of Johne's and Crohn's disease. Future studies should include the identification of other bacterial and non-bacterial factors that may influence bacterial growth rate and also affect disease progression in MAP infected animals and humans.

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PAPER 6

EVALUATION OF *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* STRAINS HAVING DIFFERENT SHEDDING CHARACTERISTICS

ABSTRACT

Background: *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is bacteria pathogen that causes paratuberculosis, also known as Johne's disease, a chronic inflammatory disease of the intestine of ruminant animals, and is suggested to play a causal role in human Crohn's disease. However, this theory is still controversial. Previous works have shown that differences in cattle shedding levels existed among culture-positive MAP strains. **Objectives**: The goal of the present study was to evaluate associations between the shedding levels of MAP isolates and other factors, such as phenotypic features (e.g lipid profiles of isolates, growth rate of isolates, symptom status of source cattle and farm sources of isolates). **Methods**: The technique of mass spectrometry was used to analyze MAP isolates (n= 121) from cattle with different shedding status. MAP isolates were classified as low shedder isolates if there are < 50 CFU/tube and heavy shedder isolates if there are > 50 CFU/tube. Statistical analysis was done using Epi Info version 3.5.1. The shedder status of isolates was used as the outcome variable. **Results:** Univariate logistic regression analysis showed that the shedder status of isolates was significantly associated with growth rate of isolates, symptom status, and source regions, but not with mass spectra patterns of isolates. **Conclusion:** In general, the results of this study provided more insight into the biochemical diversity among MAP isolates from low and high shedder host cattle, which may be useful in the management of animal farms and in future evaluation of vaccines, diagnostics and therapeutics for paratuberculosis and possibly Crohn's disease.

INTRODUCTION

Mycobacterium avium subspecies *paratuberculosis* (MAP), is an acid-fast bacterium and the causative agent of paratuberculosis, also known as Johne's disease, a chronic gastroenteritis mainly affecting cattle and other ruminants (Chiodini et al. 1984; Sweeney et al. 1996), and widely distributed in different parts of the world (Kennedy and Benedictus, 2001). Animals with paratuberculosis shed viable MAP in their faeces and milk (Ellingson et al. 2005). Fecal shedding of MAP occurs in the absence and presence of clinical signs of disease. It is reported that both subclinically and clinically affected cows can shed off fecal material thus contaminating the environment and spreading the infection to newborn calves by fecal-oral route (Sweeney et al. 1996). Subclinical carriers excrete variable numbers of MAP in the feces. In most cases, large numbers of organisms are excreted as clinical disease develops. Studies show that MAP survives for long periods in the environment (Whittington et al. 2004; Rowe and Grant, 2006). According to Pavlik et al. (1999), the rapid spread of Johne's disease is caused by the trade in subclinically infected animals and infection in early calfhood.

Previous works have shown that differences in MAP shedding levels existed among culture-positive cattle and that the higher the numbers of MAP colonies detected by culture, the greater the risk of spread of the disease. Fecal culture is considered the gold-standard of diagnosis of MAP infection in cattle, with fecal-positive individuals classified as low shedder (1-9 CFU/tube), moderate shedders (10-50 CFU/tube) or heavy shedders (greater than 50 CFU/tube) (Crossley et al. 2005). According to Whitlock et al. (2005), cattle are classified as low, moderate or high shedders based on the visible colonies of MAP on the surface of solid media, specifically, Herrold's egg yolk media (HEYM).