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Mycoplasmas & Mycobacteria: Minimalists at Work

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MYCOPLASMAS & MYCOBACTERIA: MINIMALISTS AT WORK

by

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Running Head: A COMPREHENSIVE LOOK INTO THE IMPACT, TRANSMISSION, AND TREATMENTS OF MYCOPLASMAS AND MYCOBACTERIA.

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Abstract:

Mycoplasmas and mycobacteria are two types of opportunistic pathogens whose prevalence and virulence have long been underestimated. As a broad overview, this paper will explore the current understanding of these bacteria as well as some common pathological outcomes they can induce. Chronic infections, such as contagious bovine pleuropneumonia and Johne's disease, can have extensive health and economic impacts on animal industries when improperly managed. Contamination of biopharmaceuticals and human variants of disease, are also matters of concern as more research is conducted in these areas. A more detailed understanding of these bacteria is beginning to emerge – a view which reveals mycoplasmas and mycobacteria as successful pathogens capable of adapting to their host environments and evading eradication efforts.

Keywords: mycoplasma, mycobacteria, Johne's disease, contagious bovine pleuropneumonia, bovine disease, minimalistic pathogens

Minimalists at Work

Mycoplasmas and mycobacteria are responsible for a wide variety of mammalian diseases which can have an extensive economic impact on animal industries when improperly managed. The smallest known self-replicating organisms are bacteria within the genus Mycoplasma. These bacteria can only survive in association with a host cell since their small size deters complete metabolic independence. (A quite common misconception is that the term "mycoplasma" is synonymous with "mycobacterium." Although mycoplasmas are bacteria, "mycobacteria" comprise a genus of bacteria found within the phylum of Actinobacteria (Ryan, 2004). Similar to mycoplasmas, mycobacteria are opportunistic pathogens which can cause serious disease.) Throughout this paper we will be exploring the current understanding of both of these minimalistic pathogens as well as their potential impact.

First discovered in 1898 mycoplasmas were initially thought to be viral for many years due to their minuscule size which allowed them to pass through the typical anti-bacterial laboratory filters (Razin, 2010). The erroneous nature of mycoplasmas was further perpetuated by the subsequent belief that they were actually L-forms, or mutant strains of bacteria, which are considered cell wall deficient (entirely or in-part) when compared to the wild type strain (Leaver, 2009). In the late 1960's, however, this long standing view was put to rest through the analysis of genomic data obtained via DNA hybridization and rRNA sequencing – which revealed no relationship to L-forms and further classified mycoplasmas as separate entities from any known species. Currently, it is understood that mycoplasmas are descendants of gram-positive walled eubacteria, which lost their cell wall over the long course of evolutionary history (Razin, 2010).

This degenerative evolutionary process drastically impacted the mycoplasma genome – which retained only the most fundamental necessities of a free-living organism. The smallest complete nucleotide sequence for a mycoplasma species (*Mycoplasma genitalium*) contains just over 580 Kilobases, with only 470 predicted coding regions for essential cellular function such as transcription, translation, DNA replication and repair, as well as some simple metabolic processes (Razin, 2010).

Mycoplasmas are found within the class of Mollicutes (meaning soft skin) in close association with Ureaplasma (Figure 1). The trivial names "mycoplasmas" and "mollicutes" are used interchangeably in reference to any species included in Mollicutes. For the purposes of this paper, however, these terms will denote specifically "mycoplasma" species to the exclusion of all others within this class. Mycoplasmosis, will be used denote the infection of a mammal or bird caused by an organism within the genus of Mycoplasma. Again, this classification is distinct from that of mycobacterium, although they are commonly confused due to the similarities of their name; Mycobacterium will be given a more in-depth review later on within this article.

The cell membrane, ribosomes, and circular DNA are the three basic components that make up the mycoplasma entity. Mollicutes are distinct in several ways – most notably their total lack of a cell wall. This contributes to the special sensitivity of mycoplasma species to osmotic shock and detergents, but it also confers resistance to beta-lactams and to antimicrobial treatments, such as penicillin, which target the cell wall (Bebear, 2005). In place of a cell wall, mycoplasmas exhibit a triple layered membrane that is both flexible and sturdy. The highly pleomorphic nature of mollicutes gives them an added advantage in adapting to environmental stresses. Although they do not have any intra-ctyoplasmic membranes, the outer-cytoplasmic membrane is comprised of lipoproteins and lipids appropriated from the

host (Held, 2008). The most prevalent shape is a sphere, although helical, pear-shaped, flask-shaped, and filamentous types have also been discovered (Razin, 2010). When grown *in vitro* they can also form a peculiar shape described best as a "fried-egg" colony (Figure 2).

Although their simplistic genome supports self replication, it precludes an extensive range of the metabolic pathways needed in the synthesis of substrates or highly complex cell organelles. The degree of this incapacitation is species specific. For instance, *Mycoplasma genitalium* and *Mycoplasma pneumoniae* lack all of the genes associated with the production of amino acids and therefore they are entirely reliant upon exogenous sources for survival, while other species can exhibit limited amino acid production (Razin, 2010). Mollicutes also are devoid of cytochromes and quinones, thus oxidative phosphorylation is excluded from their energy-yielding repertoire. Instead, they are forced to rely on fermentation pathways which produce relatively low ATP and a higher output of metabolic end-products, in some cases entirely exhausting the host tissues of the substrate being metabolized (Razin, 2010). Mycoplasmas also must take up cholesterol or other sterols directly from their hosts in order to sustain the cell membrane (Held, 2008). They grow and divide in a similar fashion to binary fission, although it is not uncommon for the synthesis of the cell membrane to lag behind the replication of the genetic material – resulting in multinucleated filaments (Razin, 2010). Typically these bacterium will range from 0.1 - 0.6 μm in diameter – that's 800 times smaller than a fine human hair, or 70 times smaller than average red blood cell in your body (Micrometer, 2014).

Their small size made them extremely hard to detect initially, and even today contamination of cell cultures by mycoplasmas presents a substantial problem. Upwards of 35% of all cell cultures are believed to be contaminated, although roughly 80% of cell cultures are never tested for the bacteria (Razin, 2010). The major sources of contamination stem from

the laboratory personal themselves, from the animal serum and trypsin used in cell preparation, as well as from environmental aerosols. Complete avoidance of the bacterial parasite is difficult, as the flexible cytoplasmic membrane allows the bacterium to pass through laboratory filters even as small as 0.45 μm in diameter and, as mentioned before, they are resistant to common antibiotics used for cell culturing purposes (Drexler, 2003).

The greater number of sub-cultivations a line of cells goes through, the greater the chance it has for mycoplasma contamination due to the increased likelihood of exposure. The variety of manifestations reported in infected cultures is extensive. Symptoms of contamination are often quite subtle at first, though in increasing number mollicutes can have an extensive impact on the host cell's growth, metabolism, function, and secretion of products – all factors which can distort data collection and compromise both the health of the cell, as well as the integrity of the experiment being conducted (Held, 2008).

Despite lacking the protection of a cell wall, mycoplasmas seem to be able to resist constituents of the host immune system. Some studies indicate that these pathogens possess a remarkable ability for rapid adaptation to changing environments (Razin, 2010). Reversible alterations to the expression of protein antigens on the surface of the bacterial membrane create an elusive disguise which protects the parasite from destruction, as indicated by the chronic nature of their disease (Razin, 2010). Mycoplasmas are prolific extracellular parasites – within three to five days a single mollicute can grow to 10^6 CFUs (colony forming units) per milliliter (Drexler, 2003). An estimated 100 to 1000 mycoplasmas can easily be found attached to a single infected cell (Drexler, 2003). In these large numbers mycoplasmas can exhibit a strong influence on the host cells. However, mycoplasmas can also act as intracellular parasites, meaning that they can actually penetrate and thrive within the host cell itself (Razin, 2010). This ability further aids in protecting the parasite against the attack of the host's

immune system as well as from antibiotic treatments. Intracellular activity has been shown in both *in vitro* and *in vivo* systems, and indicates that mycoplasmas are capable of suppressing or stimulating lymphocytes in a "non-specific, polyclonal manner" (Razin, 2000). In addition, these parasites are able to modify the "activities of macrophages and natural killer cells," and they can "trigger the production of a wide variety of up-regulating and down-regulating cytokines and chemokines" (Razin, 2005).

In general, there are four main types of animal disease that mycoplasmas are associated with, these include: respiratory tract infections, mastitis, arthritis and keratoconjunctivitis (Truscott, 2004). Most infections tend to follow a chronic course within the host, even though fulminant cases can occasionally emerge. Although the molecular action for pathogenicity of mycoplasma is still obscure, clinical signs indicate most damage stems from the inflammation and immune response of the host rather than due to direct toxicity of the bacterium (Razin, 2010). Another hypothesis with strong support suggests that oxidation damage from free radicals (superoxides) produced by the mycoplasma is the preliminary source of all detrimental effects seen within the host (Razin, 2010).

There are several species of Mycoplasmas which lead to respiratory disease, most notably *Mycoplasma mycoides* and *Mycoplasma bovis* (Truscott, 2004). *Mycoplasma mycoides* is the causative agent of contagious bovine pleuropneumonia (CBPP), a disease primarily affecting cattle and one that is still a significant problem in India and Africa (Truscott, 2004). Common clinical signs include loss of appetite, lowered milk production, fever, depression, purulent nasal and oral discharges, coughing, throat swelling, and dyspnea (Contagious, 2008). Some animals may exhibit epistaxis, or bleeding from the nostrils, and diarrhea has also been recorded in rare instances. In addition to the respiratory symptoms, younger calves (under six weeks of age) may also develop polyarthritis in the carpal and tarsal joints (Contagious, 2008).

In peracute cases mortality can be high – up to 80% in naive herds with some deaths occurring asymptomatically or while only showing a fever (Contagious, 2008). In herds facing previous exposure, the mortality and morbidity rates are lower with a higher number of chronic cases.

As the name indicates, *Mycoplasma bovis* also targets cattle and is largely responsible for bovine pneumonia. It is considered the "most pathogenic bovine mycoplasma in Europe and North America" (Laura, 2006). Along with the pulmonary lesions of the respiratory disease, mastitis, arthritis, lameness and abortion are also common outcomes. An estimated loss of $32 million per year can be attributed to this organism due to diminished carcass value and weight loss during infection; an estimated $108 million per year is lost from mastitis caused directly by *Mycoplasma bovis* (Laura, 2006).

Transmission of these species may occur by direct and indirect means. They can be shed in the urine, and although they are not known to transverse the placental barrier, they can also be shed with the placenta during birth – contaminating the environment and infecting the calf through oral transmission (Truscott, 2004). Strict culling and quarantines of infected and in-contact animals are the most effective methods of control. Vaccines are often used in endemic areas but their efficacy varies with the different strains of the bacterium – offering anywhere from 33% to 67% protective coverage (Contagious, 2008). Antibiotic treatments may help slow the progression of disease but will not eradicate the mycoplasma and are not effective in chronically infected cases – thus, antibiotic use is discouraged in favor of preventative measures (Contagious, 2008). *Mycoplasma pneumoniae*, a human variant of the disease, is known to cause hemolytic anemia and can result in life threatening side effects in addition to pneumonia (Kashyap, 2010).

Mastitis in conjunction with mycoplasmosis has been well defined. In addition to those mentioned above, numerous other species of mollicutes have been shown to produce it within

their host. Clinical signs generally appear within a few days and include: a sudden and very rapid onset – loss of up to 90% of total milk production between only two or three milking periods, firm and swollen udder but without heat, and the affected quarter(s) become hard and painful with enlarged supramammary lymph nodes (Truscott, 2004). The milk will become thick and purulent. Mollicutes can be found in the milk within 12 hours of onset and continue to be shed for several months following the initial infection (Truscott, 2004).

The economic impact of this can be quite severe and will depend upon the quantity of cows infected, the stage of lactation, and steps taken towards treatment. Again, antibiotics can help minimize the symptoms but will not eliminate the causative agent. After confirmation of mycoplasmosis via culture identification, culling and slaughter is recommended (Truscott, 2004). Horizontal transmission commonly occurs through mucus secretions (eyes and nose) as well as in the urine (Truscott, 2004). Mycoplasmas have shown surprising resilience in the environment and are capable of surviving several days on unsterilized surfaces, which also lends viability to the spread of the disease (Truscott, 2004).

Calves receiving mastitic milk from a cow infected with *Mycoplasma bovis* will generally develop arthritis. Arthritis can also arise through other means, including oral consumption of mycoplasmas or infection via joint injury (Truscott, 2004). This is not lethal, though severe polyarthritis can restrict animal movement causing malnutrition and dehydration. Concomitant pneumonia can occur, but more often than not regression of the swellings will naturally transpire over time without major cause for concern. Keratoconjunctivitis, or swelling and redness of the conjunctiva, is another common sequela of mycoplasmosis but can also occur as a stand-alone condition causing severe irritation or even blindness (Truscott, 2004).

The detection of different species of mycoplasmas can be difficult but improvements in nucleic acid amplification assays, such as quantitative real-time PCR and 16S rRNA sequencing, have helped in testing for these parasites and distinguishing them. These techniques are playing an increasing role in scanning biopharmaceutical products for mycoplasma contaminants, as well as in diagnosing bacterial infections in animals and humans (Razin, 2005).

Often mollicutes work in coordination with other, more aggressive bacterium to produce a greater virulent affect. Attacking when the immune system is already compromised gives them an advantage and exacerbates the symptoms of the diseases involved – impacting the recovery time and economic resources needed in diagnosing and treating the conditions. One such grouping of bacteria that share this synergistic tendency are the mycobacteria.

Mycobacteria (Figure 3) are aerobic, gram-positive and nonmotile (Ryan, 2004). In direct contrast to mollicutes, mycobacteria exhibit a thick, waxy cell wall which is highly resistant and contributes greatly to the robustness of this genus (Ryan, 2004). The cell wall is quite unique in that it consists of both a hydrophobic mycolate layer and a peptidoglycan layer (Ryan, 2004). These bacteria are rod shaped, can be slow or rapidly growing, and are typically between 1 to 10 μm long and about 0.4 μm wide (Ryan, 2004). There are many different species and subspecies which can be free living and are often found in water sources. Other varieties, including those for tuberculosis and leprosy, appear to be obligate parasites (Ryan, 2004). This genus is also responsible for many other types of pulmonary, intestinal and disseminating skin diseases affecting both people and animals.

Mycobacterium bovis causes tuberculosis (TB) in cattle and can spread to humans through direct contact, consumption of raw meats or dairy products, inhalation of aerosols, or contact with infected excrement (*Mycobacterium*, 2011). TB remains one of the deadliest infectious diseases in humans and when left untreated has a mortality rate of up to 50%

(*Mycobacterium*, 2011). Once relatively common, *Mycobacterium bovis* now accounts for only about 2% of the total TB cases in Americans thanks to routine pasteurization procedures and increased disease control efforts (*Mycobacterium*, 2011). The prevalence of this disease among U.S. cattle, however, is still cause for concern. Complete eradication of the disease is difficult to obtain in livestock herds due to wild maintenance hosts (such as white-tailed deer and badgers) and thus resurgences can periodically occur (Phillips, 2001). Economic losses can be substantial, especially in the dairy industry where spread of the disease is rapid in close quarters. Once an animal is found to have *Mycobacterium bovis* strict isolation and culling procedures must be adhered to (Phillips, 2001).

Paratuberculosis, or Bovine Johne's Disease (BJD), is akin to but distinct from tuberculosis. Caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP) this disease is a contagious, chronic and potentially fatal condition found mostly in ruminants, although some non-ruminant species have also been known to be infected (Collins, 2003). It is particularly common in dairy herds due to the frequency of potential exposure in the milking parlor. In fact, an estimated 68% of U.S. dairy herds contain at least one infected animal, while only 8% of beef herds are believed to be infected (Collins, 2001). It is also commonly reported in sheep goats and camelids, as well as captive elk, deer and bison populations. MAP is hardy and can withstand intense heat, cold or dry environmental conditions outside of the host for days at a time (Collins, 2001).

Most animals become infected shortly after birth, although clinical signs typically do not appear until several years later and are often associated with periods of stress, such as calving. The primary mode of transmission is through fecal-oral contact and infected feces which can contaminate the soil and water supplies for the whole herd. Some studies also suggest that in

late stages of the disease, vertical transmission from the cow to the calf can occur through colostrum or even *in utero* (22).

Infected animals will shed the organism for long periods of time causing entire herds to be contaminated long before clinical signs ever begin to manifest (22). Those signs include rapid weight loss, chronic diarrhea and overall cachexia in animals two to six years in age (Collins, 2003). There is no fever, and the animal continues to eat well although the diarrhea is unresponsive to treatment. These symptoms are broad and can occur in association with variety of other diseases, making detection of BJD a substantial problem for management. "Bottle jaw" or intermandibular edema, within the weeks following the onset of diarrhea gives a more noticeable indication of the causative disease but unfortunately it may not always develop. This swelling is due to severe protein deficiencies in the blood (Collins, 2003).

As BJD progresses, the ileum (the lower part of the intestine) becomes overridden by replicating mycobacterium. Macrophages from the Peyer's patches of gut-associated lymphoid tissue engulf the bacteria in an attempt to kill the invader, but for unknown reasons the mybocateria are able to withstand these attacks (perhaps due to their specialized cell wall) and they continue to multiply until eventually the macrophage is killed (Collins, 2003). As the bacteria flood the infected tissue the intestinal walls will actually thicken in what is known as granulomatous inflammation (Collins, 2001). Nutrient absorption declines and is eventually blocked altogether as the inflammation continues – essentially starving the animal in spite proper food consumption.

In endemic herds very few animals will develop clinical signs – most will either eliminate the infection in its early stages or become asymptomatic carriers but these carriers still fight lower production and poor nutrition uptake over their lifetime. If the clinical signs are expressed the disease can manifest itself as chronic or acute – causing fatalities from severe

dehydration and wasting (Figure 4) although generally animals are culled before the final stages of the disease (Collins, 2003). Slaughter value of an infected animal is typically decreased about 30% but in late cases the carcass may have become so malnourished it won't even pass meat inspection for human consumption.

Decreases in feed efficiency, lowered heard performance and increased overall culling rates are always cause for concern among producers, but the true price of this disease goes beyond that. Increases in veterinary, supplementation, and replacement costs can be extensive. There is also a decline in the gene pool of available replacements and marketing animals for sale. Investments in young stock that have been unknowingly infected can have difficulties breeding and are also a liability to producers wanting to sell breeding stock later on. In such a compromised condition, diseased animals are also at increased risk to other pathogens, such as mycoplasmas, which can exaggerate the decreases in production and cause other medical problems. In fact, mycoplasma-related pulmonary diseases have been strongly associated with Johne's disease due to this increased susceptibility in livestock.

Estimated annual losses in dairy production due to BJD are upwards of $200 million dollars, some even go as high as $250 million (Cost, 2011). One study suggested that the average total farm loss was $800.40 for every animal that was infected (which may or may not be showing clinical signs) while others varied significantly (Cost, 2011). Referring to Table 1 (The Cost of Johne's Disease) the cost of a cow that is experiencing clinical symptoms rises to over $1,500 per animal – filling in this table can provide producers with a more personalized outlook on the losses they might be facing if allowing this minimalistic pathogen to range unchecked.

While BJD has not been proven to affect humans, there have been similarities between Johne's disease and a human bowel inflammatory disease, known as Crohn's disease, which has

led some to believe that MAP may be the root cause. Isolation of MAP from the breast milk of Crohn's patients has been a large factor in this debate but at this time, experimental results have been variable and no definitive conclusion about this theory has been reached (Collins, 2003). Despite this uncertainly, simply the insinuation of a causative link between Johne's and Crohn's disease is a potential target of the media, and thus could result in a drastic decline in public opinion and consumer consumption of dairy products (Collins, 2003).

As with most mycobaterial disease, prevention is going to be the best treatment. Poor husbandry practices and sanitation of equipment are the biggest predisposing factors to the spread of MAP. Many states support a voluntary Johne's disease control program which provides affordable and achievable testing plans, these programs also outline isolation and biosecurity procedures for infected animals (Uniform, 2010). Diagnosis of the disease is generally divided into two main types: detection of antibodies (ELISA via serum or milk) and detection of MAP (fecal culture or PCR) (Cost, 2011). It only takes one test to show positive results, while both an ELISA analysis and fecal culture are required to classify a cow as "negative" for Johne's disease – because it can be challenging to identify animals in the earlier stages of infection repeated testing is recommended at least on an annual basis (Cost, 2011). Screening can be conducted at the herd level through ELISA and quantitative real-time PCR testing of bulk milk samples (Wilson, 2010). Although this method is not as sensitive or accurate as individual testing it can provide producers with an indication of how prevalent the presence of MAP is within the herd.

Once Johne's disease has been determined it is important to identify and immediately quarantine the infected animals from the rest of the herd, especially from the younger calves. Culling is strongly encouraged, but due to the prevalence of the disease in some herds that could be impractical. With the help of a veterinarian, producers may instead elect for a herd

biosecurity plan to maintain infected animals, while minimizing potential exposure to the rest of the herd (Uniform, 2010). This is generally implemented short term as culling and replacements of the animals' takes place due to the excessive costs and risk of MAP. Treatment is expensive and simply prolongs the life of the animal, but often does not cure the infection. Even under heavy treatment intermittent shedding of the organism can occur and thus infected hosts pose a continual danger in spreading the disease. More research is needed to understand how to better care for these animals and in efforts for finding a cure.

Eradication efforts for mycoplasmas and mycobacterial infections have been met with great difficulty due to the resilient and largely unclear mechanisms of these parasites. As more research uncovers the truth behind their methods work can begin to overcome these hurdles in developing a more effective treatment plan, or even a cure to entirely eliminate the presence these minimalistic parasites from their host. A coordinated effort between researchers and producers must be made to determine the prevalence and distribution of bacterial species through species-specific PCR. Improvements can emerge in serological testing and scanning efforts to identify the causative agent early in its developmental stages in anticipation of treatments which could undermine the bacteria and prevent chronic infection. Vaccination is a useful long term solution but current vaccines are often unsuccessful and expensive; thus more research and funding will be required in obtaining an effective vaccine in the future. Additional research should encompass associations between these parasites and zoonotic disease and of course, methods of treatment for human patients.

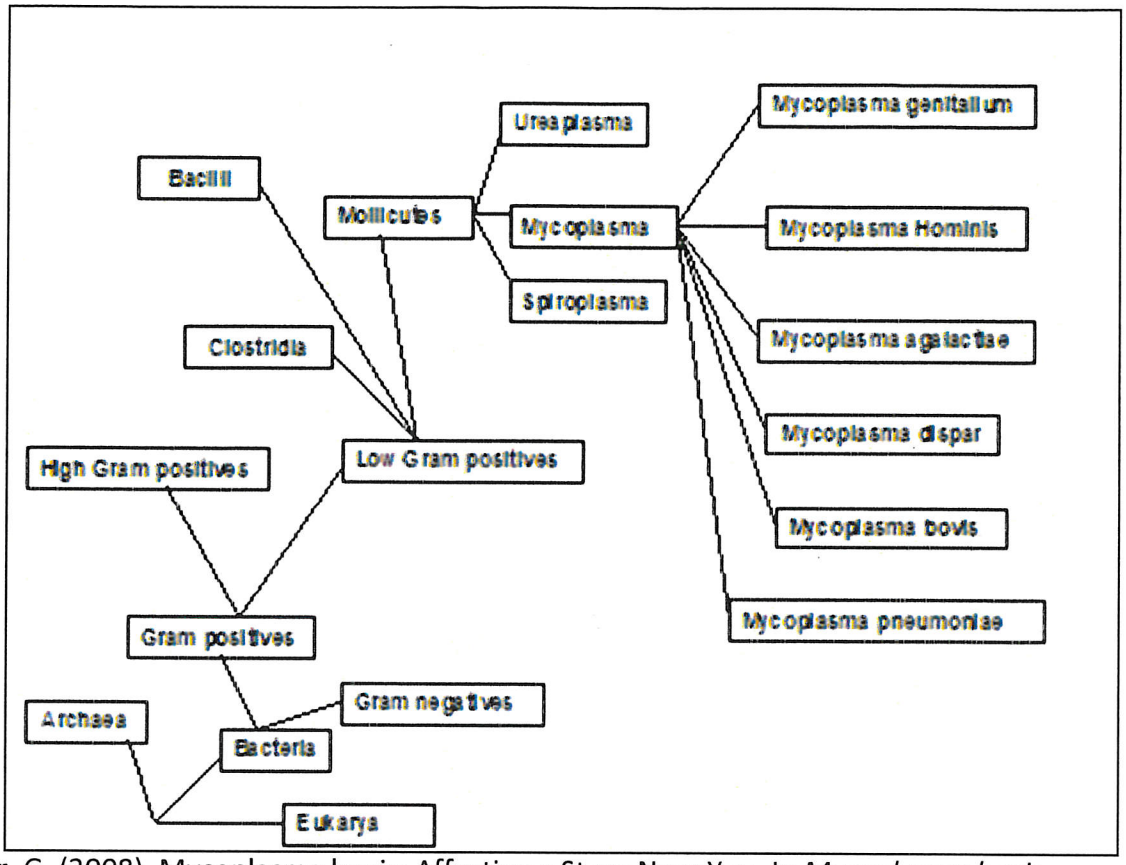
Educating the public and producers on strategies for keeping herds free of infection can help spread awareness about the prevalence of these pathogens, and teach the need for strict biosecurity and effective monitoring. More control and support programs could be utilized across the United States to offer guidance, incentives and support in disease control. Though

some of these goals may seem lofty, with determined, rational strategies of cooperation they are certainly attainable.

Although small in size, it is clear that mycobacteria and mycoplasmas make a large impact within the animal industry. The damages caused by these minimalistic pathogens have far reaching consequences when looking at the health of a given animal as well as the economic costs for the producer. Proper diagnosis and management can be difficult at times, but has become increasingly important as more is understood about their pathogenic presence both in humans and in the livestock industry. Research will continue as we search for better understanding, successful preventions and definitive treatments for these small, yet significant parasites.

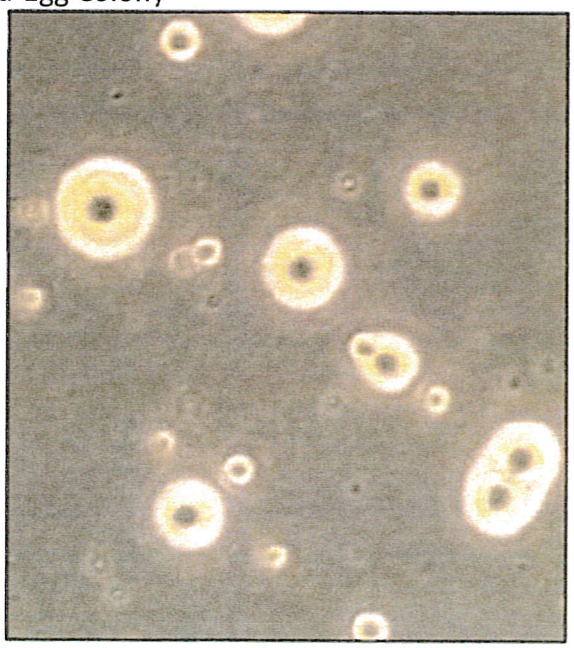
Figures

Figure 1: Mycoplasma Phylogenic Tree



Blumer, G. (2008). Mycoplasma bovis: Affecting a Store Near You. In *Mycoplasma bovis*. Retrieved 2014, from http://bioweb.uwlax.edu/bio203/s2008/blumer/Phylogeny.htm

Figure 2: Mycoplasma; Fried-Egg Colony



Testing for Mycoplasma by Culture Isolation. (2014). In *Sigma-Aldrich*. Retrieved 2014, from http://www.sigmaaldrich.com /biology/testing-for-mycoplasma.html

Figure 3: Mycobacteria

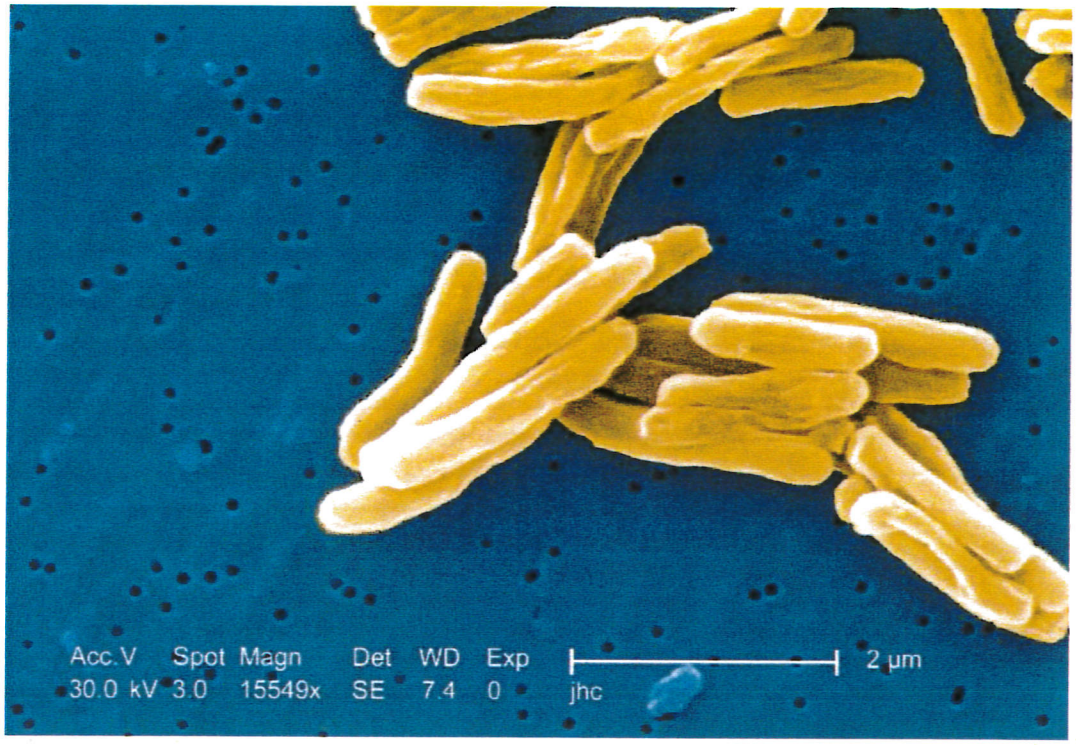


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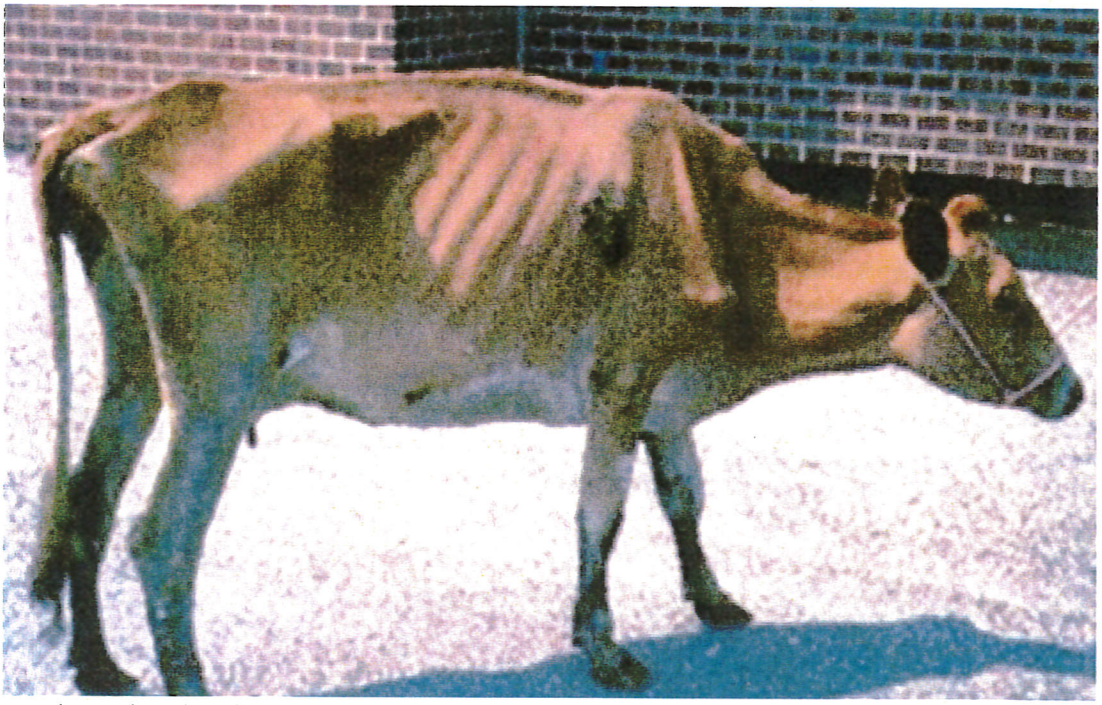
Figure 4: Johne's Disease; Late Clinical Stages



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Tables

Table 1: The Cost of Johne's Disease

		Example	Costs
A	No. of cows in your herd	1,000	
B	No. of cows clinically affected with Johne's disease in the last year	20	
C	Annual average rolling herd average (RHA) milk production (lbs./cow)	24,000	
D	Est. percent decrease in milk prod. for clinically affected cows (5%-15% expected)	10%	
	Lost Milk Production *(Multiply Row B by Row C by Row D)*	**48,000**	
E	Annual average milk price ($/cwt)	$12/cwt	
	Lost Milk Revenue *(Multiply Lost Milk Production by Row E and divide by 100)*		**$5,760**
F	Annual average replacement cost	$1,200	
G	No. of cows clinically affected with Johne's disease in the last year	20	
	Increased Replacement Costs *(Multiply Row F by Row G)*		**$24,000**
H	Average market price of a cull cow	$750	
I	Avg. market price of a JD Stage III cull cow	$550	
	Difference in Market Revenue *(Subtract Row I from Row H)*	**$200**	
J	No. of cows clinically affected with Johne's disease in the last year	20	
	Decreased Revenue from Sales of Clinically Affected Cows *(Multiply Difference in Market Revenue by Row J)*		**$4,000**
	TOTAL ANNUAL COST OF CLINICAL CASES *(Add Lost Milk Revenue, Increased Replacement Costs and Decreased Revenue from Sales of Clinically Affected Cows)*		**$33,760**
	TOTAL ANNUAL COST OF JOHNE'S DISEASE PER CLINICAL CASE *(Divide Total Cost of Clinical Cases by No. of Clinical Cows)*		**$1,688**

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