

# Mycoplasmaology: the big issues

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## Abstract

Important diseases like contagious bovine and caprine pleuropneumonia, avian mycoplasmosis, atypical pneumonia and contagious agalactia are caused by small degenerate wall-less organisms called mycoplasmas which evolved from gram positive bacteria about 2.5 billion years ago. The smallest of them are close to the theoretical limit for free-living existence containing less than 500 genes. However, they have acquired sophisticated means to survive both in the environment and in the host. Recent discoveries of their ability to survive as biofilms and within cells have changed our understanding of these degenerate bacteria. Despite increased knowledge over the last few decades, control of many mycoplasma infections seems further away than ever due to antibiotic resistance and our inability to develop effective vaccines. This review discusses some of the important issues confronting mycoplasmaologists.

## New mycoplasmas, new diseases?

It is not well known that some of the most important diseases of livestock are caused by mycoplasmas. Four are listed by the World Association for Animal Health (OIE) because of their socio-economic impacts and comprise: contagious bovine pleuropneumonia (CBPP), contagious agalactia, contagious caprine pleuropneumonia (CCPP) and avian mycoplasmosis. Others include enzootic pneumonia, a costly world-wide disease of pigs, bovine mycoplasmosis, which is characterised by respiratory disease, mastitis and arthritis, and community-acquired atypical pneumonia in humans. New outbreaks of mycoplasma disease in desert tortoises and big horn sheep have caused concern for the future of these endangered species.

Mycoplasmas, or more correctly mollicutes, are the smallest self-replicating organisms on the planet and composed of wall-less, triple-layered membranes surrounding a matrix of nucleic acid and ribosomes. However once thought of as simple and possibly the precursors of more complex life they are actually highly sophisticated bacteria with elaborate defence mechanisms; in fact, they have undergone degenerative evolution from gram positive lactobacillus-clostridial ancestors beginning some 2.5 billion years ago with the loss of the cell wall. Further huge gene loss occurred over the next few millennia until about 0.1 billion years the mycoplasma branch was created leading to the smallest of all mollicutes, the human pathogen *Mycoplasma genitalium* which consists of less than 500 genes: about a quarter of their original size and 1/10<sup>th</sup> of the size of *E. coli*.

The size of these organisms has generated interest in the minimum cell concept: "how many genes are essential for life?" with some estimating this figure at about 250. Attracted by this question, Craig Venter and his team in the USA attempted to put together a synthetic mycoplasma armed with the complete genome sequence of *M. genitalium* in the process discovering that at least 20% of the genes were not essential. After showing that they could successfully transplant genomes, they painstakingly assembled the genes of *M. mycoides* subsp. *capri* and inserted them into a closely related carrier mycoplasma.

Within a few subdivisions, the new mycoplasma, *M. laboratorium* was created [1].

Because of their lack of cell wall and proof-reading genes, mycoplasmas have high mutation rates which can be measured by sequencing genes such as the 16S rDNA gene which acts as a reliable biological clock. From this information, it seems that mycoplasmas were created around 0.1 billion years ago. Of course, evolution continues and the creation of new species can be tracked possibly triggered by social changes such as the domestication or changes in animal husbandry of livestock species.

The *Mycoplasma mycoides* cluster consists of 5 important ruminant pathogens which show immunological, genetic and biochemical similarity. However, *M. mycoides* subsp. *capri* shows great variation in the sequence of the 16S rDNA gene suggesting a long period of evolution whereas *M. mycoides* subsp. *mycoides*, the cause of CBPP, is remarkably homogenous indicating more recent emergence. Using next generation sequencing and Bayesian analysis, Dupuy *et al.*, [2] were able to date the most recent common ancestor of *M. mycoides* to approximately 1700 probably evolving following infection of cattle by the goat pathogen *M. m. capri*. A similar event may have been seen with genetically homogeneous *M. c. capripneumoniae*, the cause of CCPP, from the closely related but genetically diverse *M. c. capricolum* although this is yet to be proven.

The emergence of another bovine pathogen, *M. bovis*, may also have derived from the closely related small ruminant mycoplasma, *M. agalactiae*, as European strains of *M. bovis* show closer genetic similarity to the type strain of *M. agalactiae* than to its bovine counterpart [22]. The raised virulence of *M. mycoides*, *M. c. capripneumoniae* and *M.*

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*bovis* could reflect their recent colonisation of the bovine host and the lack of adaptation by host and pathogen.

It is however unclear how mycoplasmas cause pathological effects in their host although it should be remembered that the vast majority of them are commensals residing harmlessly in the mucosal surfaces. It is believed that a combination of factors, metabolic products, membrane components, nutrient competition with host cells and immunomodulation, may contribute to host damage. However, a great deal of genetic effort is linked to defence mechanisms such as antigenic variation and biofilm formation enabling the mycoplasmas to avoid the host immune system.

Despite the complete sequencing of over 20 mycoplasma species, these wall-less bacteria remain a mystery. Only about 59% of the putative genes identified in *M. m. mycoides* have been assigned a function with about 30% being unique to this mycoplasma and 15% having no known function [4].

### Are mycoplasmas tougher than they look?

Very little is known about how mycoplasmas initiate infections or cause disease in their host. In other bacterial species, it is known that biofilm formation, where a layer of adherent cells surrounded in a gel-like polysaccharide matrix exists, is an important step in disease. The most important and widely studied property of biofilms is their vastly increased resistance to antimicrobials and host defences. Compared with planktonic cells, biofilms are commonly 10-1000 times more resistant [5]. Unattached bacteria can be cleared by host defences and are susceptible to antibiotics. However, adherent biofilm cells are resistant to antibiotics, antibodies and phagocytes.

Despite historical reports of ruminants becoming infected from pasture contaminated with mycoplasma [6], environmental survival has not been considered a major risk factor for mycoplasma infections. Mycoplasmas grown planktonically do not survive for more than a few days. However, studies in the last decade have shown that not only can mycoplasmas, when grown under adverse conditions such as on solid surfaces, persist for longer than previously thought but that they may be more virulent in this state, perhaps requiring fewer organisms to initiate infection [7]. First identified at the Veterinary Laboratories Agency in the UK, biofilm formation was shown to occur *in vitro* in the vast majority of mycoplasma species and not just restricted to pathogens. In addition to increased survival times, biofilm-grown mycoplasmas were considerably more resistant to detergents, oxidative stress, heat and desiccation [8]. Furthermore, [9] showed that the avian pathogen, *M. gallisepticum*, was far more resistant to antibiotics such as tetracycline and gentamycin when grown as a biofilm; this is probably due to the lower growth rates seen in bacteria in biofilm as well as the reduced diffusion of antibiotics through this matrix. The existence of biofilms *in vivo* was demonstrated when structures resembling biofilm towers were reported in tracheal explants infected with the mouse pathogen, *M. pulmonis* [10].

Genome analysis has shown that mycoplasmas lack all the known regulatory systems, such as quorum sensing, that control biofilm formation in other bacterial species so they probably rely on chance events that enable rearrangements in variable surface antigens [10]. Striking images of proteomic analysis of biofilm-grown mycoplasmas show the expression of several proteins such as elongation factor Tu and pyruvate dehydrogenase that are thought also to play a role in binding of *M. pneumoniae* to host cells [3].

The formation of biofilms by mycoplasmas may help explain why

infections are often untreatable and chronic, erupting from time to time as biofilms hidden in immune-privileged tissue sites dissociate and cause new infection sites. Furthermore, the discrepancy between laboratory determined antibiotic susceptibility data, which use free living bacteria and not biofilm-grown bacteria, and the poor experience of veterinarians treating livestock must also be partly attributable to the existence of biofilm *in vivo*.

### Are mycoplasmas intracellular?

With the exception of the blood-borne haemotropic mycoplasmas, recently reclassified as mollicutes including *M. haematoparvum*, *M. haemocanis* and *M. suis*, it is generally believed that mycoplasmas are extracellular parasites. However, it was 25 years ago that the human mycoplasmas, *M. penetrans* and *M. genitalium*, were first shown conclusively to enter host cells [11] and since then many others including important animal pathogens like *M. gallisepticum*, *M. agalactiae* and *M. bovis* have also been shown to be intracellular. It should not be too much of a surprise as it is known that the humoral host response is rarely effective in combatting mycoplasma infections; the cell mediated immune system, necessary to destroy infected cells, plays a significant role in their elimination.

Hegde *et al.* [12] showed that not only can *M. agalactiae* invade cultured ruminant host cells but can persist there and escape in a viable state. Furthermore, following experimental udder infection, *M. agalactiae* was detected in the cytoplasm of mammary duct epithelium and macrophages providing the first proof of this mycoplasma's ability to translocate across the epithelium. The closely-related bovine pathogen *M. bovis*, has also been shown to invade, persist and multiply in primary embryonic calf turbinate cells [13].

The mechanism by which mycoplasmas enter cells is not well known but the lack of a cell wall should make the process relatively easy as mycoplasmas can readily fuse with host cell membranes [14]. The terminal organelle, shown in some but not all mycoplasmas, is thought to play a major role following intimate contact with the host cell surface possibly involving dramatic rearrangement of the cytoskeleton [11]. Recent studies with *M. pulmonis*, suggest that mycoplasmas may suppress host cell endosomal and autophagic systems to facilitate intracellular infection [15]. The increase in intracellular mycoplasma numbers, reported by these workers, could be the result, equally, of increased endocytosis of extracellular mycoplasmas or intracellular multiplication.

The intracellular phase represents a protective niche for pathogens and contributes to their avoidance of the host's immune defence and antimicrobial agents. It also enables their dissemination to new and safer niches where they can escape and develop new areas of infection when circumstances permit leading to persistent chronic infections. The number of mycoplasmas now shown to have an intracellular phase increases regularly as workers turn their attention to them with new tools like confocal microscopy and it must surely be a matter of time before all pathogenic mycoplasmas are shown have this invasive capability.

### Mycoplasma vaccines: do they work?

Vaccines against mycoplasma diseases like contagious bovine pleuropneumonia and contagious agalactia have been used for many years though they are rarely completely effective. In the early 1970s attempts were made to develop a vaccine for bovine respiratory disease (BRD) caused by *M. bovis* and *M. dispar* and early trials

were encouraging before project was abandoned for lack of funding. However, one of the biggest obstacles to the development of a vaccine against such diseases has been the lack of a reliable and reproducible animal model. This is partly due to the multifactorial nature of BRD and the inherent variability in susceptibility of cattle to mycoplasmas. Sourcing sufficient calves from a single herd that has not already been exposed to *M. bovis* is also a challenge. In addition, there are differences in the virulence of *M. bovis* isolates so selection of an appropriate challenge strain is crucial.

A variety of commercial vaccines, including autogenous preparations, are available in the USA, but few data exist on their effectiveness and they are not licensed for use outside the USA. While many experimental vaccines have proved ineffective or even damaging, a *M. bovis* vaccine inactivated by saponin was shown to be safe, immunogenic and protective against challenge by a heterologous strain [16]. Results show that this vaccine works optimally when given to young calves on arrival at the farm when infection rates are low and can significantly reduce mortality and treatment costs [17]. In one beef unit of about 130 calves in northern England, mortality fell from 24 per cent over one winter to 8 per cent the following winter after vaccination; treatment costs also fell from €29 to €14 per calf. Failure to vaccinate the calves the following year, however, led to a significant rise in mortality. In other calf operations results, have not been so encouraging though this has often been due to inadequate antigen concentrations or where other respiratory pathogens are prevalent.

Generally speaking, immunity induced by current inactivated mycoplasma vaccines such as that for porcine enzootic pneumonia caused by *M. hyopneumoniae* is transient and protection provides only small improvements in lung pathology [18]. The widely-used vaccines for the small ruminant disease, contagious agalactia, are even more modest in their achievements indeed some studies have failed to show that they work at all [19].

Live mycoplasma vaccines, on the other hand, have been shown to be far more effective: more immunogenic and protective and cheaper to produce. The T1/44, V5 and rabbit- adapted vaccines for CBPP have been used extensively in Africa, Australia and China to control and eventually eliminate the disease in parts or all the respective country. Despite its unfair recent poor reputation, T1/44 vaccine was highly successful in East Africa in the 1970s when vaccine coverage was high. Its failure today is mainly the result of poor coverage and modifications made to it such as the unnecessary creation of the streptomycin-resistant strain and its formulation as a bivalent vaccine with rinderpest [20]. A live vaccine produced by in vitro passage of a virulent strain of *M. agalactiae* in Turkey gave the best results in a recent trial of currently used preparations [19]. Attention should be switched to the development of live vaccines particularly for *Mycoplasma bovis* infections where they can be included in already existing multivalent vaccines. Today safety issues can be addressed easily using methods preventing reversion to virulence.

## Conclusion

Despite their very small size, mycoplasmas are highly successful pathogens of animals and man. Infections are rarely fatal enabling their survival and persistence increasing their chance of infecting new hosts. They were considered highly host specific because of their fastidiousness brought on by highly restrictive nutritional requirements but non-cultural detection methods like PCR and sequencing have found them in a wider range of host species [21]. Also, given the opportunity brought about by changes in agricultural management, mycoplasmas

can adapt rapidly to new hosts because of high mutation rates as was seen in the infection of gazelles and other antelope species by the caprine pathogen *M. capricolum* subsp. *capripneumoniae*, the cause of CCPP [22]. Rosengarten *et al.* [12] warned that “recent outbreaks and epidemiological studies predict that the incidence of human and animal diseases might increase which indicates the urgent need to develop new approaches to prevention and technology.” Over a decade and half on their warning remains pertinent.

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