Genomic and phenotypic variability of *Mycobaterium avium* subspecies

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ABSTRACT

Introduction: *Mycobacterium avium* complex consists of *M. intracellulare* and the subspecies of *M. avium* subsp. *avium*, *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *hominissuis*. Despite their taxonomic relationship, these subspecies are organisms with distinct phenotypes, ranging from environmental bacteria that cause infections in immuno-compromised hosts to pathogens targeting birds and ruminants. The reasons for the variable pathogenicity and host range of *M. avium* subspecies are not known. We hypothesize that genotypic differences between *M. avium* subsp. *avium* and *M. avium* subsp. *paraturberculosis* can explain different pathogenic outcomes. **Methods**: We used tri-genomic comparisons to look for DNA fragments unique to each subspecies. We also used an acute model of mouse infection to determine different phenotypic outcomes in response to infection with different *Mycobacterium* subspecies. **Results**: Through tri-genomic comparisons we identified genetic regions of interest that may contain genes to explain phenotypic or pathogenic differences among subspecies. In an 8 week course infection, mice infected with *M. avium* subspecies *avium* had the highest bacterial burden in their spleens and livers. At the same time, mice infected with *M. avium* subspecies *paraturberculosis* had the lowest bacterial burden. **Discussion**: Differences in the genomic sequences of the *M. avium* subspecies suggests that these sequences encode pathogenic factors. Consequently, this study shows that the sequencing of *M. avium* subspecies genomes can be useful for predicting and explaining variation in pathogenesis.

KEYWORDS

Mycobacterium avium, Tri-genomic comparison, Shotgun sequencing

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INTRODUCTION

The genus *Mycobacterium* has over 130 well-characterized species. The most characteristic feature of these species is their thick, hydrophobic cell wall containing mycolic acid, which makes them very resilient and naturally resistant to many antibiotics. It is also responsible for the acid-fast staining property most commonly used to identify the mycobacterium. Most *Mycobacterium* species are strictly saprophytic and not known to cause disease in humans or animals; however, some species are pathogens. Among the pathogenic mycobacteria are *Mycobacterium tuberculosis* and *Mycobacterium leprae*, etiologic agents of tuberculosis (TB) and leprosy respectively. Whilst there has been a decline in the total number of leprosy cases, TB remains a threat to public health on a global scale. Despite an attenuated vaccine having been available for more than 50 years, approximately 2 million people

die from *M. tuberculosis* infections every year and one-third of the world's population is latently infected with *M. tuberculosis* (*1*).

After *M. tuberculosis*, the most commonly isolated mycobacterial pathogens in clinical laboratories are organisms of the *M. avium* complex (MAC). MAC consists of *M. intracellulare* and the subspecies *M. avium* subspecies avium (MAA), *M. avium* subspecies *paratuberculosis* (MAP) and *M. avium* subspecies *hominissuis* (MAH) (2,3). Together these organisms account for over 70 percent of non-tuberculosis mycobacterial disease in the United States (4,5) and for more than 95 percent of non-tuberculosis mycobacterial disease among persons with HIV/AIDS (6).

M. avium appears to comprise two broad ecotypes of organisms: host-associated bacteria and environmental organisms. Host-associated subspecies include MAA and MAP. MAA is a poultry pathogen causing pulmonary TB in birds. MAP is an obligate pathogen of ruminants causing Johne's disease, which is characterized by chronic enteritis. Johne's disease is a significant economic problem in the cattle industry. A United States Department of Agriculture report from April 2008 suggests that as many as 70% of US dairy herds are infected. Although the condition is easy to identify once symptoms arise (diarrhoea, weight loss and decreased milk production), it is challenging to diagnose in the early, asymptomatic stages of infection. Healthy, but infected, animals transmit the organism through milk and feces, invisibly spreading the infection to younger animals. Recent studies have shown that MAP can survive pasteurization and has been identified in off-the-shelf milk from retail grocery stores in the US and the European Union (7). Recent reports also implicated MAP in cases of Crohn's disease in humans, a condition characterized by intestinal pathology that is reminiscent of Johne's disease in cattle (8,9). People with Crohn's disease have been shown to be seven times more likely to have MAP infection than those who do not have Crohn's disease (8). These observations have led to the hypothesis that MAP causes Crohn's disease in susceptible hosts. To date, despite all of the efforts to address the role of MAP in this context, the hypothesis that MAP is the cause of Crohn's disease remains neither proven nor refuted (10).

MAH is the environmental strain of *M. avium*. MAH is ubiquitous in the environment and can be isolated from fresh and saltwater, municipal water systems, pools, house dust and soil (11,12). MAH is also known to cause opportunistic infections in humans, including lymphadenitis in children, disseminated *M. avium* disease in immuno-compromised patients and pulmonary disease in immuno-competent adults (13,14). The most important risk factor for pulmonary *M. avium* infection in patients without HIV infection is underlying lung disease such as cystic fibrosis or chronic obstructive lung disease (11). MAH is the most commonly isolated clone of *M. avium* from humans (3); this

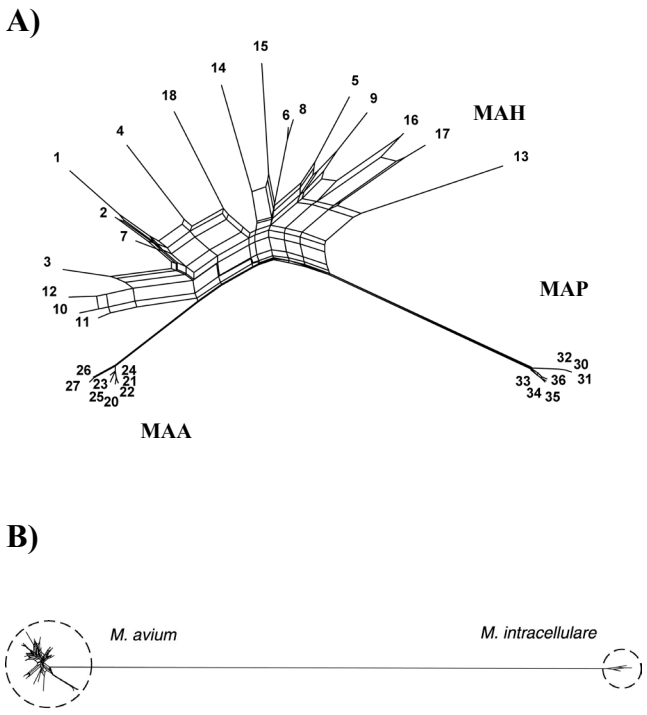

Fig. 1. Phylogenetic Representation of *M. avium*. (A) Unrooted Phylogenetic representation of *M. avium* subspecies based on 10 genes generated in SplitsTree4. The host-associated pathogen lineages are present as two independent branches that extend outward from the complex web containing exclusively strains of MAH. (B) Phylogenetic analysis of *M. avium*, based on six genes, using *M. intracellulare* as an out-group. As one can see, *M. intracellulare* remains quite distant from *M. avium*. (This Fig. was taken from "*Mycobacterium avium subsp. paratuberculosis* and *M. avium subsp. avium* are independently evolved pathogenic clones of a much broader group of *M. avium* organisms" by Turenne *et al.*)

may be due to the fact that MAH is ubiquitous in the environment and we are exposed to it more readily than host-associated subspecies that are not commonly found in the environment.

Multilocus sequence analysis, performed on 56 strains of *M. avium* and based on 10 housekeeping genes shared among all the subspecies of *M. avium*, confirms the classification scheme presented (Fig. 1) (2). As seen in Figure 1A, the two independent branches extending outward from the complex web consist exclusively of environmental strains, meaning that there is relatively small variation among host-associated pathogens. A follow-up study recently published by Alexander et al. demonstrated that MAP has evolved independently in a bi-phasic process from MAH, characterized by insertion of novel DNA followed by deletion events leading to genomic down-sizing (15). Whether this is the case for MAA remains to be seen. Despite this genetic model,

the reasons for the variable pathogenicity and host range of *M. avium* subspecies are not known. As a result, we decided to conduct a series of experiments to further characterize *M. avium*.

Our hypothesis is that phenotypic differences between subspecies of *M. avium* are encoded in their genomes, such that comparative genomic analysis can help explain the variance among these closely-related organisms. In recent years, genome projects have been completed for two of the three major subspecies of *M. avium*: MAP K-10 (type strain of MAP) and *M. avium* 104 (type strain of MAH). The K-10 genome is 4.83 Mb long, and contains 4,350 open reading frames. *M. avium* 104 has an additional 700 kb of DNA, for a total genome size of 5.48 Mb. Both genomes have a G+C content of approximately 69% and comparison of orthologous genes reveals 99% sequence identity. Because there are three major subspecies of this organism, bi-genomic comparisons between these two strains provide an incomplete portrait of the genomic diversity of these subspecies. As a result, we decided to sequence the type strain of the remaining subspecies, *M. avium* subspecies avium ATCC 25291 as a first step towards a set of genomic studies.

The genome project for MAA ATCC25291 is in its final stage. A shotgun library has been generated and 40% of the gaps in it have been closed. Through tri-genomic comparisons, we observed genomic variation compatible with the bi-phasic model of evolution. We showed that whereas host-associated pathogens had smaller genomes than environmental strains, they possess approximately 200 kb of extra DNA unique to each pathogen.

We also examined the phenotype of *M. avium* subspecies by injecting a representative of each subspecies intraperitoneally in a neutral, non-natural host: the C57BL/6 mouse. In a neutral host, neither subspecies has a replication advantage, which allows a comparison of pathogenicity. The murine model is a well-characterized model for mycobacterial infection, and has been used to study *M. tuberculosis* for many years. Mycobacterial burden in the livers and spleens of infected mice were examined one, four and eight weeks post-infection. We showed that although *M. avium* subspecies behave differently in the host, they do not have a common pathogenic profile. This is the first study comparing the relative virulence of all three subspecies.

MATERIALS AND METHODS

GENOME SEQUENCING

Whole-genome shotgun sequencing is currently the most widely used approach for whole genome sequencing. This approach has been used successfully to completely sequence both microbial and mammalian genomes. We employed shotgun sequencing to determine the complete genome sequence of *M. avium* subsp. avium ATCC 25291. In the shotgun sequencing method, all the DNA of the organism of choice is isolated, randomly fragmented, size selected and cloned to produce a random library in E. coli. The clones are then sequenced using the chain termination method. By following this procedure for several copies of the same long DNA strand, overlapping fragments are created. Finally, computer programs align these overlapping sequences and determine the original sequence. Once a sufficient number of sequences are generated, the sequences are assembled into continuous DNA assemblies of the consensus sequence from the shorter individual clone sequences. In practice, gaps in the genome are likely to occur due to repeat areas and unclonable regions in the genome. The last step in the shotgun sequencing method is to close the gaps.

SUBSPECIES	STRAIN NAME	INOCULUM DOSE
M. AVIUM SUBSPECIES *HOMINISSUIS*	*M. AVIUM* 104	4 X 10^7 CFU
M. AVIUM SUBSPECIES *AVIUM*	ATCC 25291	10^7 CFU
M. AVIUM SUBSPECIES *PARATUBERCULOSIS*	K10	4 X 10^6 CFU

Table 1. Strain name and inoculum dose of *M. avium* subspecies injected intraperitoneally into C57BL/6 mice.

Our collaborators at the University of Minnesota generated the shotgun library and we took the responsibility of closing the gaps. The gaps of the MAA genome were bridged by generating polymerase chain reaction (PCR) products across the gaps. Using *M. avium* MAP-K10 and *M. avium* 104 genomes, we assembled the DNA fragments and identified the gaps, after which we linked the gaps by PCR. The PCR products were sequenced by Genome Quebec. The sequences were then inserted into the genome, linking the DNA fragments.

DNA EXTRACTION AND PCR CONDITIONS

Bacterial DNA (*M. avium* subsp. avium ATCC 25291) was extracted using ribolyzer protocol. Primers were designed in Primer3 (http://primer3.sourceforge.net/). All PCRs were performed in a final reaction volume of 50 µl and contained 100 ng of DNA template, 2.5 mM MgCl2, 1× Taq buffer with (NH4)2SO4 (Fermentas), 5 µl of 50% acetamide (Sigma), 0.2 mM dNTPs, 0.5 µM of each primer and 1.5 U of Taq DNA polymerase (Fermentas). PCR was performed using an Applied Biosystems Gene Amp 2700 PCR System under the following conditions: 94 oC for 3 min; 30–35 cycles of 94 ˚C (30 sec), 55 ˚C (30 sec) and 72 ˚C (1.5 min); 72 ˚C for 5 min, then held at 4 ˚C. A higher annealing temperature was necessary with some PCRs to minimize non–specific amplification. We verified the amplification of PCR

products through electrophoresis with 1.5% agarose gels. In cases where PCR product was not obtained, we increased the MgCl2 concentration to 3.5 mM. As a positive control, we also amplified the hsp65 gene (~1.5 kb) with every PCR reaction.

MICE AND BACTERIAL CULTURE

We used germ-free, 8-10 week old male C57BL/6 mice which were purchased from Harlan Labratories and maintained in the McGill University Health Centre animal facility. Bacterial strains used in our experiments included *M. avium* MAP K-10, *M. avium* 104 and *M. avium* ATCC 15291. The strains were all grown in Middlebrook 7H9 medium containing ADC supplement (Difco Laboratories, Detroit, Ml) plus 0.04% of Tween 80 (Sigma, St Louis, MO) and Mycobactin J (Allied Monitor) at 37 °C.

INTRAPERITONEAL MURINE MODEL OF INFECTION

Because these organisms have different natural hosts, we chose a neutral, non-natural host: the C57BL/6 mouse. We challenged 20 C57BL/6 mice intraperitoneally with ~107 CFU of each *M. avium* subspecies (Table 1). The livers and spleens from the infected mice were collected at one, four and eight weeks post-infection. We sacrificed six animals per group per time-point, then homogenized, serially diluted and plated the tissue in Middlebrook 7H10 medium with OADC supplement (Difco) plus Mycobactin J (Allied Monitor) and PANTA (Becton Dickinson Diagnostic Instrument Systems, Sparks, Md.), which is a mixture of antibiotics intended to suppress the growth of contaminating organisms. The cultures were incubated at 37 °C and the colony forming units (CFU) were quantified after three weeks in the case of MAH and MAA, and 8 weeks in the case of MAP. Only plates containing 25 to 250 CFU were considered.

RESULTS

M. AVIUM SUBSPECIES ARE GENOTYPICALLY DIFFERENT

To determine the genomic variability of *M. avium* subspecies, we decided to do a tri-genomic comparison. Since full genome sequences for only two of *M. avium* subspecies are available, we, along with collaborators at the University of Minnesota, decided to sequence the genome of the remaining subspecies. Our colleagues in Minnesota generated the shotgun library and we took the responsibility of closing the gaps, as described below.

CLOSING THE GAPS IN MAA GENOME.

Initially, there were 401 gaps in the MAA ATCC 25291 genome. We bridged 160 gaps through PCR. There was a tremendous amount of variation in the gap sizes. The largest gap closed was ~1500 base pairs and the smallest gap was 0, which means there

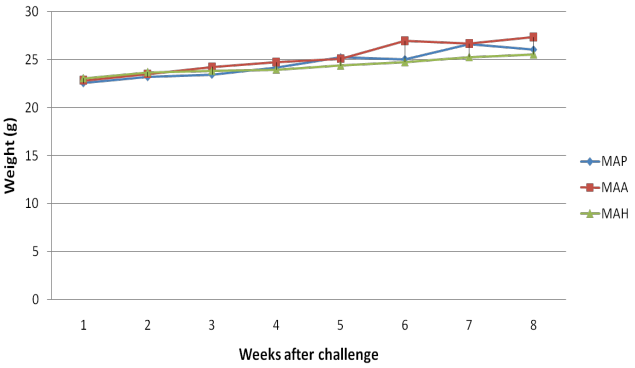

Fig. 2. Weight of Infected Mice over Time. Weight change in response to infection with different subspecies of *M. avium*. The mice in all groups gained weight and did not show any clinical sign of illness.

was no gap separating the adjacent contigs, which are sets of overlapping DNA segments from a single genetic source. The average gap length, however, was 153 base pairs. Since there were 401 gaps and 160 of them were closed, the efficiency of PCR in bridging the gaps was 40%. The sequences I bridged have been deposited in GenBank database on 22-Jan-2009. This project is ongoing. Given the average gap length is 150 base pairs, we estimate the full genome sequence of MAA contains approximately 4.9 Mb.

PRELIMINARY TRI-GENOMIC COMPARISON: GENOMIC VARIABILITY IN DIFFERENT M. AVIUM CLONES IS RELATIVELY GREAT.

To determine the degree of genomic variability of *M. avium* subspecies, we compared the three genomes. Early work based on the comparison of the two completely sequenced genomes of *M. avium* MAP-K10 and *M. avium* 104 and the incomplete sequenced genome of MAA ATCC 25291 revealed that these organisms share a core set of approximately 4000 genes with 99% identity at the DNA sequence level. However, there is also significant variability between them, as revealed by the presence of ~850 kb of DNA unique to *M. avium* 104, ~260 kb of DNA unique to *M. avium* MAP K-10 and ~200 kb of DNA unique to MAA ATCC 25291.

M. AVIUM SUBSPECIES ARE PHENOTYPICALLY DIFFERENT

To determine the relative virulence of the three sequenced strains of *M. avium*, we injected groups of C57BL/6 mice intraperitoneally with ~107 CFU of each *M. avium* subspecies. To ensure that we injected the right dose into each group, extra stocks were made, serially diluted and plated. The results can be seen in Table 1. As one can observe, there was some variation in the bacterial inocula. The initial differences were taken into account when we compared the bacterial burden at different time points by normalizing the CFU values.

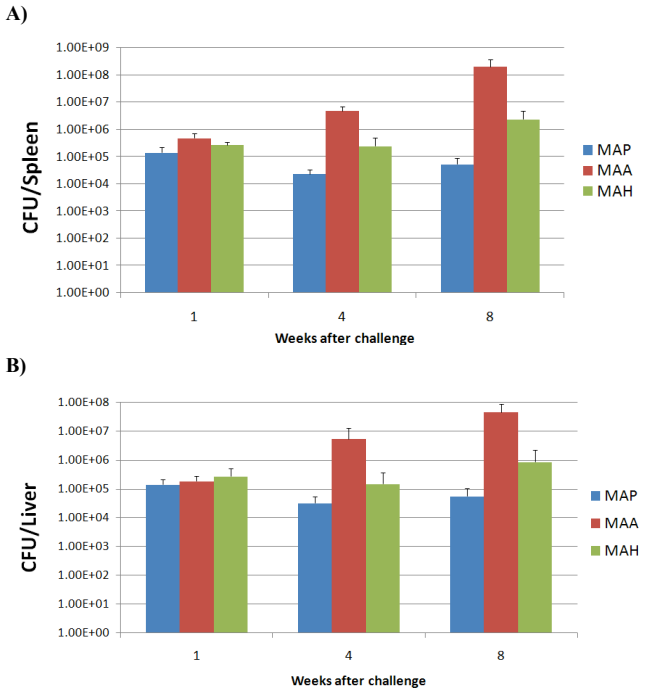

Fig. 3. Bacterial Burden in the Spleen and Liver of Infected Mice. Bacterial burden in the spleen (A) and liver (B) of mice infected with MAP, MAA and MAH. Bacterial burden is approximately the same for all groups at week 1 before diverging in subsequent weeks. MAP results are normalized to make up for its lower inoculum dose.

PHYSIOLOGIC RESPONSES TO M. AVIUM INFECTION: MICE INFECTED WITH DIFFERENT SUBSPECIES OF M. AVIUM DID NOT SHOW ANY CLINICAL SIGN OF ILLNESS OR GROWTH RETARDATION.

We monitored the animals' weights weekly as a marker of pathology since weight-loss or growth retardation is a global indicator of illness. Weight change over time in response to infection with different *M. avium* subspecies is shown in Fig. 2. The mice from all groups gained weight and did not show any clinical signs of illness. Mice infected with MAH gained 2.5 g, the ones infected with MAA gained 4.5 g and finally mice infected with MAP gained 3.5 g. Paradoxically, the mice infected with host-associated strains (MAA and MAP) appeared to gain more weight than the mice infected with the environmental strain (MAH). From previous experiments we know that C57BL/6 mice infected with PBS gain about 2.5 g over 8 weeks.

SUBSPECIES OF M. AVIUM HAVE DIFFERENT PATHOGENIC PROFILE IN ACUTE MODEL OF INFECTION.

To determine the relative virulence of *M. avium* subspecies, we quantified the bacterial burden from spleens and livers of infected mice at different time points. We expected that the mice infected with the most virulent subspecies would have the highest bacterial burden measured by the number of CFU. Given that MAA and MAP are obligate pathogens and MAH is an environmental strain, we expected that the mice infected with MAH would have the lowest bacterial burden compared to the pathogenic clones. The results are shown in Fig. 3.

We observed similar trends in both the spleens (Fig. 3A) and livers (Fig. 3B). At week 1, spleen and liver bacterial burden were approximately the same for all groups. However, by week 4, CFU values started to diverge. Over 8 weeks, the bacterial burden in the spleen and liver of mice infected with MAH was relatively stable. There was a drastic increase in the spleen and liver bacterial burden of the group infected with MAA, from ~105 CFU at week 1 to ~108 CFU at week 8. Unexpectedly, the bacterial burden of the group infected with MAP decreased over time even though MAP is considered one of the pathogenic strains of *M. avium*.

Looking at the CFU values, it was not possible to distinguish host-associated subspecies from the environmental strain. MAA, a causative agent of avian-TB, showed excellent proliferative capacity inside the host. MAP, a causative agent of Johne's disease in cattle, showed a poor proliferative capacity. The environmental strain was somewhere in between. Our results show that different pathogenic subspecies apparently behave quite differently in a standardized model of in vivo bacterial replication.

DISCUSSION

M. avium consists of a genetically related yet diverse group of bacteria in terms of environmental niches, host types and disease phenotypes. The basis of this variation is unknown. In order to explain this variability, we decided to compare the genomes of the three major subsets of *M. avium*. Since there are three major *M. avium* subsets and the genome projects for only two of the subsets are available, our first step was to initiate the genome project for the representative of the third subset, MAA ATCC 25291. Through collaboration with the University of Minnesota, we generated a shotgun library and bridged 40% of the gaps in the library. Tri-genomic comparisons revealed that although all the subspecies share about 4000 genes and are classified as single species, the genetic variability is significant. The host-restricted subsets, MAA and MAP, have smaller genomes and each contain approximately 200 kb DNA unique to them. Given that MAP and MAA are pathogens and MAH is an environmental strain, the genes present in the extra fragments of DNA are excellent virulence factor candidates that may account for the phenotypic heterogeneity of *M. avium* strains.

We also examined the virulence of *M. avium* subspecies in C57BL/6 mice. It was expected that the pathogenic clones would distinguish themselves from the environmental strain by a

common pathogenic profile. Unexpectedly, in an acute model of murine infection, it was not possible to distinguish pathogenic subspecies of *M. avium* based on weight of infected mice or bacterial burden.

The infected mice from all groups gained weight and did not show any signs of illness. If the mice were infected with *M. tuberculosis*, which is a known mycobacterial pathogen, growth retardation would be expected at the bacterial burdens we observed. Therefore, we can conclude that *M. avium* subspecies are not very virulent. It is worth noting that in the case of MAA, the bacterial burden was very high (~108 CFU in spleen at week 8) and the animals still did not show any sign of illness or weight loss. In *M. tuberculosis* infections, bacterial burdens never rose above 106 CFU in the spleen, as by that time all the mice had died. This shows that although MAA proliferates rapidly inside the host, it is not particularly virulent.

Although no one has ever compared all three subspecies of *M. avium* in vivo, it has been shown that MAA replicates better inside the host and hence is more virulent than MAH. In the present study, MAA was shown to replicate more rapidly in vivo, whereas MAH was shown to replicate more slowly (Fig. 3). This result is consistent with the findings reported by Young et al. that MAA is more virulent than MAH (16). In vitro studies comparing MAA and MAH have shown that MAH replicates more rapidly within murine macrophages and, unlike MAA, induces a strong inflammatory cytokine production, particularly tumor necrosis factor-alpha (16,17). It has been hypothesized that the rapid proliferation of MAA in vivo may result from its ability to suppress host responses, including inflammatory cytokines. In contrast, the induction of inflammatory cytokines by MAH may result in a more effective host control of infection. However, these in vitro observations need to be confirmed in vivo to determine whether strong cytokine production in vivo correlates with a decreased capacity to replicate inside the host. Future experiments to test this hypothesis would consist of immunological assessment one week after infection, when the bacterial burden is the same for all subspecies. This experiment would help clarify whether MAA replicates better in the host due to its ability to subvert host immune responses.

In contrast to MAA, MAP burden in the spleen and liver decreased over 8 weeks (Fig. 3). Unpublished data from our lab shows that following intravenous injection of 106 CFU of MAP into C57BL/6 mice, there is a persistent infection for up to 6 months. After combining the results, it appears that MAP is more of a stealth pathogen that replicates very slowly inside its host and, as a result, is able to persist for a long time. We believe that this is why MAP has infected 70% of dairy herds.

Our results in the present study show that the acute model of murine infection holds promise to study MAA virulence factors, but is unlikely to serve as a good model to understand MAP-specific virulence. Using the acute model of murine infection, we can start to screen MAA mutants for their virulence and identify the virulence factors. We can start with open reading frames identified as potential virulence factor genes through the trigenomic comparison.

Subspecies of *M. avium* behave differently inside the host and have different approaches to pathogenesis. MAA proliferates rapidly inside the host without generating a deleterious immune response. In contrast, MAH presented a reduced capacity to replicate inside the host, likely associated with the induction of a strong cytokine response (16,17). MAP did not proliferate as well as other subspecies in this model, but from other studies in the lab, appears to survive the longest of these three subspecies within the host. Very little is known about the biological differences between environmental, opportunistic and host-restricted pathogenic members of *M. avium*, including their virulence mechanisms.

However, we have begun to identify genetic regions of interest that may contain candidate genes that confer phenotypic or pathogenic differences among them, helping guide future functional studies. We also showed that acute models of murine infection, while not very helpful in screening MAP knock-outs, can be useful to screen MAA knock-outs for loss-of-virulence.

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