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Editor's Choice

UNDER THE MICROSCOPE

How does a *Mycobacterium* change its spots? Applying molecular tools to track diverse strains of *Mycobacterium avium* subspecies *paratuberculosis*

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Abstract

Defining genetic diversity in the wake of the release of several *Mycobacterium avium* subsp. *paratuberculosis* (MAP) genome sequences has become a major emphasis in the molecular biology and epidemiology of Johne's disease research. These data can now be used to define the extent of strain diversity on the farm. However, to perform these important tasks, researchers must have a way to distinguish the many MAP isolates/strains that are present in the environment or host to enable tracking over time. Recent studies have described genetic diversity of the *Mycobacterium avium* complex (MAC), of which MAP is a member, through pulsed-field gel electrophoresis, single sequence repeats, variable-number tandem repeats, genome rearrangements, single nucleotide polymorphisms and genomewide comparisons to identify insertions and deletions. Combinations of these methods can now provide discrimination sufficient for dependable strain tracking. These molecular epidemiology techniques are being applied to understand transmission of Johne's disease within dairy cattle herds as well as identify which strains predominate in wildlife.

Introduction

Within the past 5 years, there has been a spike in the scientific literature directed at defining genetic differences within the *Mycobacterium avium* complex (MAC). This is especially true for *Mycobacterium avium* subsp. *paratuberculosis* (MAP), a significant veterinary pathogen and member of the MAC. Much of the focus on MAP is warranted as it causes a disease of considerable economic impact to dairy industries worldwide termed 'Johne's disease'. Other members of the MAC include *Mycobacterium avium* subspecies *avium* (MAA), *Mycobacterium avium* subspecies *hominussuis* (MAH), *Mycobacterium avium* subspecies *silvaticum* (MAS) and a second species *Mycobacterium intracellulare*. All members of the MAC are genetically similar, although the small distinctions between *Mycobacterium avium* and *Mycobacterium intracellulare* have been well established (Boddinghaus *et al.* 1990; De Smet *et al.* 1996; Feizabadi *et al.* 1997; Thorel *et al.* 2001). In particular, DNA–DNA hybridization studies have long ago showed significant genetic similarity between MAP and MAA (Saxegaard *et al.* 1988; Yoshimura and Graham 1988; Hurley *et al.* 1989). More recently, a >98% nucleotide identity was shown to exist when comparing MAP and MAA genomes with each other (Bannantine *et al.* 2003). Preliminary data suggest that MAP and MAS may be even more similar than MAP and MAH (Paustian *et al.* 2005). However, not much is known about MAS, and comparatively little sequence information on MAS is present in public sequence databases.

In contrast, the genome sequences of MAH strain 104 and MAP strain K-10 have long been completed. The genome of MAP K-10 is present in NCBI's GenBank and has been published (Li *et al.* 2005), whereas MAH 104 has been completely sequenced and annotated, but has not been peer-reviewed. More recently, draft genomes for a series of MAP isolates from a variety of geographic

locations have been published. These include camel (Ghosh *et al.* 2012), human (Wynne *et al.* 2011) and sheep isolates (Bannantine *et al.* 2012). Furthermore, the complete genome of a human strain isolated from breast milk will soon be published (Li *et al.*, submitted). Importantly, it is the availability of these genomes that has spurred the increased research on genomic diversity amongst the MAC.

History of MAP Strain Typing

To gain perspective on where the field is now, it is necessary to reflect on its history. The genetic manipulation of MAP has proven to be more difficult than most other bacterial systems. Therefore, molecular strain typing studies have lagged behind other bacteria and even other mycobacteria such as *Myco. tuberculosis*. Nonetheless, owing to the importance of Johne's disease worldwide, researchers have continued to grind out progress, albeit slowly, much like the organism itself.

Before 1988, MAP was a species by itself. The proposal of a subspecies designation for MAP was based on early DNA–DNA hybridization studies (Saxegaard *et al.* 1988; Yoshimura and Graham 1988; Thorel *et al.* 1990). This reclassification was met with some resistance due to the distinct phenotype of MAP (Chiodini 1990), but phylogenetically, the subspecies designation was justified. There are no naturally occurring plasmids or extrachromosomal elements in MAP or other members of the MAC. Then, in 1989, the insertion sequence IS*900* was discovered (Collins *et al.* 1989; Green *et al.* 1989), and its characterization was the subject of several articles (Tizard *et al.* 1992; Doran *et al.* 1994, 1997) because it was initially considered to be a MAP-specific element. With this discovery, there was finally a way to distinguish MAP genetically instead of always depending on the mycobactin J requirement as the sole distinguishing phenotype for taxonomic classification. The initial efforts to type MAP strains using the IS*900* element also occurred at this time. Genomic DNA was extracted and subjected to *BstEII* restriction enzyme digestion with the resulting fragments separated out by electrophoresis on long agarose gels to detect distinct banding patterns (Collins *et al.* 1990; Whipple *et al.* 1990). Whilst sheep and cattle strains were long observed to be phenotypically different in growth rate and appearance, these experiments showed, for the first time, a genetic difference between these two classes. Furthermore, these experiments led to the first DNA strain typing method used for MAP, the IS*900* RFLP method.

MAP Genomic Diversity

With the advent of newer technologies, the genetic diversity amongst MAP isolates as well as in comparison with MAC strains has been thoroughly studied in the last decade. Some regions of the MAP genome have been discovered that are absent in MAH, and a few studies have examined these regions more closely (Sheridan *et al.* 2003; Stratmann *et al.* 2004). Sheridan and coworkers (Sheridan *et al.* 2003) examined the GS element of MAP, which was previously found using representational difference analysis (RDA) and was reported absent in *Myco. avium* subsp. *avium* (Tizard *et al.* 1998). This 6500-bp region was analysed in silico using bioinformatics tools, which predicted that coding sequences are involved in GDP-fucose biosynthesis and modification of the oligosaccharide moiety of GPL. Stratmann and coworkers also used the RDA technique to find a novel 7-kb ABC transporter operon located within a 38-kb segment that is flanked by an insertion sequence (Stratmann *et al.* 2004). Also located on this 38-kb island are several gene clusters thought to be involved in iron uptake. These investigators went further by demonstrating the location and expression of two coding sequences in the ABC transporter operon to support in silico findings. They found that both MptC and MptD were surface-located on the MAP bacilli. Since these studies, several other genomic insertions and deletions have been discovered that represent a significant source of MAP genetic diversity (Marsh *et al.* 2006; Alexander *et al.* 2009). These have been collectively named large sequence polymorphisms (LSPs), and some are quite useful in strain identification as discussed later.

Another significant source of diversity lies in mobile genetic elements. The genome sequence of MAP has revealed a total of 19 different insertion elements in the cattle strain K-10 (Li *et al.* 2005), and these elements are important factors contributing to the diversity of MAC members. Olsen *et al.* (2004) discovered the ISMpa1 element and observed that three copies were present in the MAP genome. The genome project designation of ISMpa1 is IS_MAP12, and sequence data analysis confirms that it is present in 3 copies in the K-10 genome (MAP0832c, MAP1287 and MAP2050). This element was found in all MAP isolates examined and in selected porcine isolates of *Myco. avium* subsp. *avium* (Olsen *et al.* 2004), but absent in other MAH isolates. Another study by Johansen *et al.* (2005) examined two insertion sequences, IS1311 and IS1245, which share 85% homology in an effort to clear up discrepancies in some published studies involving these elements. The authors discovered that IS1245 could mistakenly be observed in MAP when using a long IS1245 probe; however, they designed a shorter, more specific probe to show that the element is in fact not present in MAP. This discrepancy was attributed to the strong sequence similarity between IS1245 and IS1311, an element that is represented seven times in MAP. This study serves to further highlight that even the known differences between MAP

and MAA are subtle and can lead to confusion. A few MAP insertion elements, discovered only by genome sequencing, are present uniquely in MAP isolates and absent from MAH isolates and laboratory strains. One such element, ISMAP02, which has already been used to develop a novel PCR-based test for faecal samples (Stabel and Bannantine 2005) and colostrum (Pithua *et al.* 2010), is present in six copies in the genome.

The most widely known IS element is IS*900*, which is present in 17 copies in the K-10 genome and has been used for decades as a diagnostic target in addition to the strain typing method mentioned above. Sequencing of other MAP genomes has shown that the number of IS*900* elements is stable across strains. However, analysis of MAP vaccine strains has shown that IS*900* copy number can vary depending on the existence of duplicated regions or deletions in the specific strain analysed (Bull *et al.* 2013). Also, this element has more recently been shown to exist in non-MAP strains (Englund *et al.* 2002; Motiwala *et al.* 2004).

Strain Differences Applied to Strain Typing Methods

From repetitive DNA sequences (Bull *et al.* 2003; Amonsin *et al.* 2004) to amplified fragment length polymorphism (AFLP) and pulsed-field gel electrophoresis (PFGE) analysis (Motiwala *et al.* 2003; O'Shea *et al.* 2004; de Juan *et al.* 2005; Kiehnbaum *et al.* 2005; Sevilla *et al.* 2008), differences in the MAP chromosome have been identified and utilized for discriminatory subtyping of isolates. Many of these studies have used the genome sequence of the MAP bovine strain K-10 to aid in the identification of genetic regions of variability (Amonsin *et al.* 2004; Overduin *et al.* 2004; Semret *et al.* 2004; Paustian *et al.* 2005). This resource has continued to aid researchers as they seek to define additional genetic variations amongst MAP isolates, especially differences between the cattle and sheep isolates (Dohmann *et al.* 2003; Marsh *et al.* 2006) or differences amongst vaccine strains (Bull *et al.* 2013).

Cattle or C strains and sheep or S strains have long been shown to be phenotypically different. The C strains are generally more robust and grow faster than the fastidious S strains. Furthermore, S strains were thought to have a narrow host range compared with C strains, although camel isolates have recently been shown to be in the S strain lineage (Ghosh *et al.* 2012). Some pathogenic differences between these lineages have also been noted (Motiwala *et al.* 2006; Gollnick *et al.* 2007; Borrmann *et al.* 2011). Whittington *et al.* (2000, 2001) and Marsh *et al.* (1999) developed the IS1311 PCR amplification–restriction enzyme analysis and used it successfully to distinguish C strains from S strains on a genotype level. This technique could also be used to distinguish other subspecies of *Myco. avium*, but this assay was not able to further subdivide MAP isolates. Likewise, analysis of genomic LSPs quickly distinguishes C strains from S strains by the presence of $LSP^{A}20$ and absence of $LSP^{A}4$, but fails to further discriminate the S strains (Biet *et al.* 2012). However, PFGE was able to further divide the S strains into type I and type III (Stevenson *et al.* 2002; de Juan *et al.* 2006). Although the presence of unique strains has been suggested previously by IS*900* RFLP analysis (Pavlik *et al.* 1999), these isolates did not fall into either the type I (sheep) or type II (cattle) strains, and hence, a type III designation was first proposed by de Juan *et al.* (2005). IS*900* RFLP and SNP analysis of the *gyrAB* genes (Castellanos *et al.* 2007) and MAP1506 (Griffiths *et al.* 2008) also clearly divide MAP into the type II, type I and type III lineages, whereas MIRU-VNTR does not (Biet *et al.* 2012). In case of MAP1506, a single nucleotide polymorphism at nucleotide position 344 is used to differentiate type I and type III S strains. A 'G' nucleotide at this position signifies a type I strain, and an 'A' signifies a type III strain (Griffiths *et al.* 2008). In agreement with these findings, the genome-sequenced sheep strain S397 has an 'A' nucleotide at this position, and it was reported to be a type III strain isolated from the United States (Bannantine *et al.* 2012). The type I strains are more homogeneous, whilst the type III are considered more heterogeneous.

A recent study (de Juan *et al.* 2005) examined caprine isolates of MAP using three molecular typing techniques, PFGE, IS*900* RFLP analysis and IS1311 PCR amplification–restriction enzyme analysis. They found that PFGE analysis was more discriminatory than the other two methods, enabling a resolution of 13 different PFGE profiles amongst the 44 isolates evaluated. The variable-number tandem repeat method used by Overduin and coworkers had a lower discriminatory index (DI) than IS*900* RFLP; however, the method subdivided the most predominant RFLP type (37% of 250 isolates analysed were of the R01 type) into six subtypes, and thus, it provides a promising molecular subtyping approach (Overduin *et al.* 2004). If DNA quantities were not a factor between these methods, it would be interesting to directly compare VNTR and PFGE analysis using a standardize strain set to determine which technique displays the highest discriminatory capabilities. An indirect comparison of discrimination indices between typing methods can be inferred from published studies summarized in Table 1.

Techniques that both reveal genetic diversity and can discriminate amongst MAP isolates include short sequence repeat (SSR) analysis (Amonsin *et al.* 2004), variable-number tandem repeat analysis (Bull *et al.* 2003; Overduin *et al.* 2004), PFGE (de Juan *et al.* 2005), AFLP (O'Shea *et al.* 2004), microarray hybridization (Semret *et al.* 2004; Paustian *et al.* 2005; Bull *et al.* 2013), IS*900* RFLP and RDA (Dohmann *et al.* 2003). Each has their advantages and

Table 1 Summary of molecular typing studies and their resulting discriminatory indices (DI)

Method	Description	No. of isolates	DI range	No. of Studies
AFLP	Enzymes *Eco*RI and *Mse*I	33–104	0·711–0·920	3
IS900-RFLP	Single restriction enzymes	59–1008	0·440–0·856	8
IS900-RFLP	Multiple restriction enzymes	59–1008	0·636–0·970	4
PFGE	Single restriction enzymes	39–123	0·485–0·960*	4
PFGE	Multiple restriction enzymes	39–268	0·654–1·000†	3
PFGE + IS900-RFLP	Multiplex PFGE + IS900-RFLP	123	0·834	1
MIRU-VNTR	5–10 loci	38–123	0·316–0·925‡	10
MIRU-VNTR + IS900-RFLP	MIRU-VNTR (5–10 loci) + RFLP	59–123	0·779–0·997	5
MIRU-VNTR + PFGE	Multiplex PFGE	123	0·797	1
SSR	2–11 loci	33–268	0·69–0·969	10
SSR + MIRU-VNTR	2–11 SSR + 8–10 MIRU-VNTR loci	38–100	0·93–0·996	4
SSR + IS900-RFLP	11 SSR loci + single RE IS900-RFLP	84	0·973	1
SSR + MIRU-VNTR + IS900-RFLP	3–11 SSR + 8 MIRU-VNTR loci	57–77	0·950–0·983	2
SSR + PFGE	2 SSR loci + multiplex PFGE	268	0·820	1

*DI value is higher than others (0·485–0·594).
†DI value is higher than others (0·654–0·884).
‡DI value is lower than others (0·664–0·925).

disadvantages in terms of ease, discrimination and reproducibility. For example, microarray hybridization does not give high-resolution distinctions and is not practical without a considerable investment in constructing the DNA microarray. However, it can quickly identify the location of deletions (Marsh *et al.* 2006; Bull *et al.* 2013). IS*900* RFLP requires high-quality genomic DNA as the starting material, thus necessitating the culturing of isolates that are slow growing or may not grow at all. Similarly, SSR requires DNA sequencing and the ability to accurately read long poly G tracts, particularly at locus 2, which can be subjected to sequencing errors (Thibault *et al.* 2008; Fritsch *et al.* 2012), but it appears that despite these deficiencies, SSR typing has recently emerged as the most commonly used technique and the most discriminatory (Harris *et al.* 2006; Pradhan *et al.* 2011; Forde *et al.* 2012). Of the 11 discriminating loci originally discovered for MAP SSR, locus 1 is always included in published studies suggesting its importance. It was the only locus used by Motiwala *et al.* (2004), whereas most other studies included both loci 1 and 8 in some combination (Ghadiali *et al.* 2004; Corn *et al.* 2005; Motiwala *et al.* 2005; Sevilla *et al.* 2008; Douarre *et al.* 2011). The four most discriminating loci are 1, 2, 8, and 9 (Douarre *et al.* 2011). Despite the rise in SSR typing, it should be noted that historically, IS*900* RFLP has been the most used method (Collins 2010) and has been standardized for ease of interpretation (Pavlik *et al.* 1999).

Application of Molecular Epidemiology to Johne's Disease

The application of genotyping methods to Johne's disease enables the tracking of MAP isolates/subtypes on a single dairy farm or across multiple regionally located farms. This allows researchers to identify and track movements of the most successful/widespread isolates as well as obtain clues to prevent the on-farm transmission of MAP.

Several studies have used one or more of the typing methods described herein to survey MAP strains within a given region. For example, 17 sheep isolates from Spain showed considerable diversity amongst type III strains (Sevilla *et al.* 2008). They further showed through SSR and PFGE methods that multiple strain types are present on a single farm in 20 of 33 bovine farms analysed. They observed that new strains emerged, whilst others disappeared over a 6-year time frame from 2000 to 2005. Interestingly, the type III strains seem to be predominant in Spain, whilst type I strains appear more common in Australia, New Zealand and the United States. Two studies have examined MAP strain diversity in Germany using MIRU-VNTR analysis. Dairy cattle in this country were found to have primarily two MAP strains that are common in Europe (Stevenson *et al.* 2009; Douarre *et al.* 2011), which are MIRU-VNTR genotypes 1 and 2 present in 44 and 28% of the 91 isolates examined, respectively (Fernandez-Silva *et al.* 2012). The same is true for the second study, although genotype 1 was particularly dominant at 52% of all 71 bovine isolates examined amongst 14 herds in Germany (Mobius *et al.* 2008).

The SSR method was used to track strains shed in cattle faeces and present in tissues from three dairy herds over time in north-eastern United States to determine transmission dynamics (Pradhan *et al.* 2011). A total of 15 different strains amongst 142 were catalogued within and between the three herds. Seven strain types were

present on more than one farm, whilst the eight others were farm specific. One strain (SSR type 2) predominated with 89% of the infections in a single herd, whilst multiple strains infected animals simultaneously in the other herds. They further found that at least half of the low shedding cows had the same strain as that of supper shedding cows. This suggests that supershedding cows readily transmit MAP to their herd mates. This is an excellent example of how molecular epidemiology was applied to answer important questions about transmission within a farm.

Investigations with closely related groups of isolates suggest that the application of any one typing method alone may not provide sufficient discriminatory power (Ricchi *et al.* 2011; Fritsch *et al.* 2012). For example, SSR and MIRU-VNTR were used to subtype 84 type C MAP isolates that were collected from 10 herds in Italy during 2007 and 2010 (Ricchi *et al.* 2011). The investigators were able to group these isolates into 15 clusters with Simpson's discriminatory index value of 0·840 using SSR alone (3 loci) and only 11 clusters with DI 0·688 using MIRU-VNTR alone (10 loci). However, with the combination of SSR and MIRU-VNTR, they differentiated these isolates into 33 clusters, reaching the DI 0·952. These data suggest that the combination of methods could be a useful molecular tool, especially when performing molecular epidemiological investigations within herds or within a relatively well-defined geographic area. Consistent with this, SSR and MIRU-VNTR have also been applied together with IS900-RFLP, the most used typing technique, for ultrahigh resolution typing. A total of 67 MAP isolates from bovine, caprine, ovine, cervine, leporine and human origin were typed with a combination of these three techniques, providing a DI 0·983 (Thibault *et al.* 2008). However, we emphasize that direct comparisons of discriminatory indices from different studies should be considered with caution due to a selection bias of the sample panels.

A combination of SSR, MIRU-VNTR and IS900-RFLP was also used to investigate MAP transmission between wild-living red deer and farmed cattle with known shared habitats in Germany (Fritsch *et al.* 2012). A total 57 type C MAP isolates consist of 13 recovered from deer, 23 from 6 cattle herds with shared habitats and 17 from cattle herds 40 and 120 km away from the habitats. Seventeen genotypes were found from these isolates with DI 0·95 when the three methods were combined. Four of seventeen genotypes are shared between deer and cattle, especially genotype III*, which was dominant in cattle herd F and was also found in 50% of deer isolates originating from the neighbouring forest district, suggesting cross-species transmission. Based on these results, the authors speculated that MAP-contaminated grasslands were the result of spreading bovine faecal slurries and that this represented the primary cause of infection of wild-living deer in this region.

In summary, the selection of molecular tools for the epidemiological tracking of MAP is dependent on the specific objectives of the study. For instance, tracking MAP transmission within herds or amongst a homogeneous group of isolates requires high-resolution genotyping that can be achieved by the combined application of several techniques. Of the various techniques employed, a combination of SSR and MIRU-VNTR is generally the method of choice given the fact that there is no requirement for culture or isolation of high-quality genomic DNA, and these approaches are easily standardized (Fernandez-Silva *et al.* 2012; Fritsch *et al.* 2012). To provide additional discriminatory power or to confirm the clustering pattern of otherwise closely related strains, IS900-RFLP is often the next choice because this technique has been well standardized and widely employed. In some instances, approaches using PFGE and SNP analysis of specific genes can be also considered for this purpose.

Conclusions and Perspectives

More work has been completed in this field over the past 2–3 years, but much remains to be accomplished. There are still significant gaps in knowledge about how fast a strain is transmitted through a herd and the role wildlife plays in spreading MAP from one farm to the next farm. Only recently have molecular epidemiological studies emerged applying strain typing techniques beyond the laboratory to track MAP strains in farm settings. This is precisely where the future research focus should be. Effective control strategies for Johne's disease depend on a good understanding of strain presence and movement within dairy herds or sheep flocks. Furthermore, the question of transmission of MAP between livestock and wildlife species can now be traced (Motiwala *et al.* 2004; Forde *et al.* 2012). Using the molecular epidemiology techniques described, investigators can determine whether one strain of MAP predominates on a farm or whether multiple circulating strains exist at similar levels. Both types of examples have been reported (Pradhan *et al.* 2011). These methods can also determine whether supershedding cows are infected with a particular genotype of MAP or whether clinical animals shed multiple strains in their faeces (Harris *et al.* 2006).

Only recently have these genotyping tools been used to examine the molecular epidemiology of MAP on farm, but it appears early on that multiple genotyping methods will be needed to obtain the discrimination necessary for tracking strains in a farm setting. Because of this focus, researchers now have an excellent understanding of differences in the genomes of MAP isolates and species within

the MAC complex. All of the IS elements have been identified, and some have been found to be useful in subtyping isolates. There remains the tension between techniques that 'lump' isolates versus techniques that discriminate isolates, but it is our opinion to examine discriminatory ability and then determine whether the separations make biological sense.

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