Relative frequency of 4 major strain types of *Mycobacterium avium* ssp. *paratuberculosis* in Canadian dairy herds using a novel single nucleotide polymorphism-based polymerase chain reaction

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ABSTRACT

Johne's disease is a worldwide concern, as it causes huge economic losses. The etiological agent, *Mycobacterium avium* ssp. *paratuberculosis* (MAP), has limited genetic diversity, impeding efforts to understand transmission and distribution of strain types. Whole-genome sequencing was previously performed on a representative set of MAP isolates from Canadian dairy herds and 9 divergent clades were identified. Four clades were of particular interest, as they were either MAP types rarely reported in North America, or they represented a substantial proportion of isolates recovered from dairy farms in Canada. One clade included type I/III isolates, whereas the remaining clades included type II isolates. Variant sites in the MAP genome are often separated by thousands of base pairs, limiting use of single nucleotide polymorphism (SNP)-based genotyping on a single genomic region. Therefore, a SNP-PCR assay was developed to facilitate interrogation of 5 SNP in 2 distant regions of the genome, linking them together in a single PCR reaction for subsequent Sanger sequencing. This high-throughput assay enabled discrimination of 602 MAP isolates from 264 herds (from all 10 provinces). More than 1 isolate was cultured from 133 herds, 14 of which included multiple subtypes. A previously identified dominant type included 87% of isolates, whereas the Bison type was more widespread than previously reported. The latter type and isolates from a second clade of interest were overrepresented in Québec and Saskatchewan, respectively. In conclusion, the distribution and relative frequency of MAP subtypes within Canadian dairy herds were assessed using a novel SNP-based typing assay. These findings will contribute to understanding the clinical relevance and transmission dynamics of MAP in this population and elsewhere.

Key words: Johne's disease, paratuberculosis, strains, distribution, Canada

INTRODUCTION

Mycobacterium avium ssp. *paratuberculosis* (MAP) is the causative bacterium of Johne's disease (JD), a chronic incurable enteritis in ruminants. Economic losses due to JD are a strong motivator for dairy producers to control the disease; consequently, several regional control programs have been implemented to prevent MAP infection at cow and herd levels (Geraghty et al., 2014; Wolf et al., 2014). Additionally, a potential association between MAP and Crohn's disease in humans warrants increased control efforts (Barkema et al., 2010; Behr, 2010).

The evolving field of molecular epidemiology is driven by technologies that can improve the discriminatory power of molecular typing techniques and enable an increasing number of genetic targets to be interrogated (Stevenson, 2015). The choice of genetic targets, however, is critical; unstable loci, such as repetitive elements, are prone to homoplasy and can confound the true relationship of isolates (Ahlstrom et al., 2015). Additionally, these targets contain minimal biologically relevant information, impeding efforts to elucidate genetic roles in phenotypic differences. Conversely, SNP are almost exclusively biallelic, with minimal chance of reversion. As a single SNP will (usually) only differentiate isolates into 2 types, multiple SNP are required to achieve desired levels of discrimination. Alternatively, a few SNP can be used to infer deeper phylogenetic branches before a more discriminatory genotyping method is employed (Keim et al., 2004; Comas et al., 2009). Unlike repeat-based genotyping, SNP-typing provides the phylogenetic context in which functional effects of SNP shared by all isolates within a clade can be explored. In previous studies, SNP were used to differentiate MAP strain types I, II, and III (Whittington et al., 2001; Castellanos et al., 2010a), although types I and III are rare in Canadian dairy cattle (Ahlstrom et al., 2016), limiting their use.

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Whole-genome sequencing (WGS) is a powerful tool to investigate genetic diversity within organisms with minimal genetic diversity, including MAP. Traditional sequence-based approaches (e.g., multilocus sequence typing) often fail to distinguish between subspecies of *M. avium*, let alone within MAP strain types (Turenne et al., 2007). However, recent studies using WGS on a large number of MAP isolates detected previously unidentified SNP within and between the broad strain types I, II, III, and Bison (Ahlstrom et al., 2016; Bryant et al., 2016; Leão et al., 2016). Therefore, this represented an opportunity to select informative SNP within a strain type that can differentiate a population of MAP into subtypes of interest. Furthermore, a SNP-based assay recently differentiated a global population of MAP isolates into 14 major subtypes, highlighting the utility of typing MAP based on an informative set of SNP (Leão et al., 2016).

Herein, we used an alternative method to differentiate MAP isolates within a more geographically localized population of dairy farms, which can provide key epidemiological evidence for understanding and controlling MAP transmission on a national scale. A recent WGS analysis of 182 MAP isolates from Canadian dairy herds identified 9 divergent MAP subtypes, including 1 type III isolate and Bison type (type B) isolates from 3 provinces (Ahlstrom et al., 2016). Additionally, a dominant and secondary clade, both belonging to the broader type II strain type, represented 86 and 4% of all isolates, respectively. Isolates were selected to maximize the number of herds represented; thus, the actual number of strain types at a herd and provincial level remained unknown. Understanding which strains and subtypes are circulating in the Canadian dairy industry, as well as their relative frequencies in different provinces, is important information that can guide control efforts. Slow progression of JD and lack of animal movement data in Canada have impeded efforts in understanding MAP transmission dynamics. However, source tracing (between- and within-herd levels) can be improved using appropriate strain typing tools with background knowledge on distribution of major strain types identified in the population of interest. Therefore, the objectives of our study were to (A) develop a high-throughput SNP-PCR assay to differentiate 4 MAP subtypes of interest and (B) determine relative frequencies of MAP subtypes in all 10 Canadian provinces.

MATERIALS AND METHODS

MAP Isolates

A collection of MAP isolates was generated from environmental manure samples and individual cow fe-

cal samples, as previously described by Ahlstrom et al. (2016). Samples originated from all 10 Canadian provinces and were collected as part of regional JD control efforts. All samples were cultured using the TREK ESP Culture System reagents (TREK Diagnostics, Cleveland, OH), with the exception of Québec samples where the BACTEC MGIT 960 ParaTB culture system (Becton, Dickinson and Company, Franklin Lakes, NJ) was used. For this, IS900 positive culture broth (Vary et al., 1990) was subsequently plated onto 7H11 agar (Becton, Dickinson and Company) supplemented with 2 mg/L of mycobactin J and OADC (oleic acid-albumin-dextrose-catalase; Becton, Dickinson and Company). Plates were incubated for 4 to 8 wk at 37°C, after which single MAP colonies were sub streaked onto a new 7H11 plate. After 4 to 8 wk of incubation, DNA was extracted from MAP cells using a modified version of the Qiagen DNeasy blood and tissue kit (Qiagen, Mississauga, ON, Canada), as described (Ahlstrom et al., 2014).

SNP Selection

Previous WGS was done on 182 Canadian and 26 global MAP isolates (Ahlstrom et al., 2016). The reads are available in the sequence read archive (accession number SRP067719 and SRR060191; <http://www.ncbi.nlm.nih.gov/sra>). A total of 9,670 SNP were identified and used for phylogenetic inference. The Canadian isolates belonged to a total of 9 divergent clades, 4 of which were chosen for SNP analysis in our study. These included 1 type III and 3 type II subclades (type B, dominant, and secondary) representing approximately 0.5, 3, 86, and 4% of 182 Canadian isolates, respectively. The concatenated SNP alignment was viewed in Geneious (version 7.1.7; Biomatters, Auckland, New Zealand; Kearse et al., 2012) and all SNP differentiating the 4 clades of interest were identified. At least 1 global isolate was represented in all clades, with the exception of the secondary clade that did not have any representative global isolates. The reference genome position of each SNP was determined and visualized using Civi (Overmars et al., 2015).

Single nucleotide polymorphisms differentiating clades of interest that were separated by <400 bp from another discriminatory SNP were identified. Two regions, A and B (each under 400 bp in length), together contained informative SNP that differentiated the 4 clades of interest. Region A contained 2 SNP that differentiated isolates belonging to type B and the secondary clade, and region B contained 2 SNP that differentiated isolates belonging to the dominant type as well as type I/III. Region B contained an additional confirmatory SNP for type I/III/B isolates.

Table 1. Primer sequences used in polymerase chain reaction analysis

Primer name	Primer sequence (5' to 3')
RegA-F	TGGTGAGCATGGCCAGATG
RegA-R	ACTTCGCGTTCAGGTCGCCCAGGATTAGTACCGG
RegB-F	ACTAATCCTGGGCGACCTGAACGCGAAGTTACTGA
RegB-R	GGGTGTTTCAATCCGAGTGG

Primer Design and Thermocycling Conditions

The distant SNP-linking PCR assay was created to amplify both regions A and B and subsequently link them together to create a single amplicon. Four MAP isolates, each representing a distinct subtype of interest, plus 1 isolate that did not belong to any of the 4 subtypes, were used as positive controls to test the SNP assay and optimize PCR parameters. Four primers were designed using Primer3 (Version 2.3.4; Untergasser et al., 2012) in Geneious to target both regions and produce 2 amplicons each <400 bp in length. A 15-bp tail was added to the reverse primer of region A that was complementary to the 5' end of region B. Similarly, a 14-bp tail was added to the forward primer of region B that was complementary to the 3' end of region A. Primer sequences are shown (Table 1).

Internal primers were added in lower concentrations to promote amplification of the single, linked amplicon (rather than 2 separate amplicons). The PCR mastermix (Qiagen, Germantown, MD) contained 10 pmol of RegA-F, 10 pmol of RegB-R, 5 pmol of RegA-R, 5 pmol of RegB-F, 200 μ M (each) deoxynucleoside triphosphate, 5 U of Taq DNA Polymerase, and 2 mM MgCl₂. In addition, 1 μ L of DNA was added to the final mastermix volume of 19 μ L. An initial denaturation step of 95°C for 5 min was followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 45 s, with a final elongation step (72°C for 7 min). A no-template control was included in each PCR run.

Sanger Sequencing and Sequence Analysis

Samples were prepared for Sanger sequencing by diluting 1 μ L of the PCR product into 11 μ L of distilled H₂O containing 3.2 pmol of RegA-F and were submitted to the University of Calgary Core DNA Services Centre. Chromatograms were aligned and visualized in Geneious to assign alleles at each locus. Over- and underrepresentations of specific MAP types per province was tested using the Chi-squared test or Fisher's exact test (one-sided, significance threshold $P \leq 0.05$) in R (version 3.2.4; R Core Team, 2016). Additionally, all MAP isolates for which WGS was performed and sufficient DNA was available were included as controls.

RESULTS

In our study, DNA was available for 602 MAP isolates originating from 264 herds (Table 2). The maximum number of isolates per herd was 15, whereas 155 herds contributed only 1 isolate. The number and location of SNP differentiating each subtype, as well as regions A and B, is shown (Figure 1). Region A contained 2 discriminatory SNP (1 synonymous and 1 resulting in an alternative start codon), whereas region B contained 3 SNP (1 synonymous, and 2 intergenic), including 1 confirmatory type I/III/B SNP (Table 3).

The WGS data from all 152 isolates that were subjected to both WGS and the SNP-PCR confirmed the PCR-based subtype results. Additionally, SNP4 in region B confirmed all type III and type B isolates. The total number of MAP isolates and herds per subtype per province is shown (Table 4). The dominant, secondary, Bison, and type III subtypes included 87.2, 2.4, 3.0, and 0.2% of all isolates and 89.4, 3.4, 3.4, and 0.4% of all herds, respectively. A total of 7.3% of isolates and 8.7% of herds were a subtype other than the 4 targeted in our study. The dominant subtype was identified in all 10 provinces, whereas secondary, Bison, and type III subtypes were identified in 5, 3, and 1 province, respectively. Québec was the only province in which all 4 subtypes were identified. Furthermore, in this province, we noted a significant overrepresentation

Table 2. Number of *Mycobacterium avium* ssp. *paratuberculosis* isolates and number of herds included from each Canadian province, including average and maximum isolates per herd

Province	Isolates	Herds	Average isolates per herd	Maximum isolates per herd
Alberta	319	119	2.7	13
Ontario	66	29	2.3	13
Quebec	62	41	1.5	15
Saskatchewan	51	24	2.1	4
New Brunswick	40	12	3.3	15
Prince Edward Island	37	17	2.2	15
British Columbia	12	7	1.7	3
Newfoundland	6	6	1.0	1
Manitoba	5	5	1.0	1
Nova Scotia	4	4	1.0	1
Total	602	264	2.3	—

Figure 1. Circular *Mycobacterium avium* ssp. *paratuberculosis* (MAP) genome displaying SNP differentiating each of the 4 subtypes. The outer ring indicates genomic coordinates and concentric rings indicate SNP differentiating type III (green), Bison type (orange), secondary (red), and dominant (blue) types, from outermost to innermost concentric ring, respectively. The number of unique SNP differentiating each type totaled 1,649, 142, 94, and 42, respectively. Regions A (reference bases 2,214,324–2,214,468) and B (reference bases 2,934,167–2,934,541) are indicated by a rectangle and the joined PCR amplicon labeled with primers (black arrows) and SNP positions is illustrated. Color version available online.

of type B isolates ($P = 0.029$) and herds ($P = 0.019$) harboring type B isolates. Additionally, Saskatchewan herds had an overrepresentation of the secondary type ($P = 0.034$).

Three subtypes were present in 1 herd in Alberta, whereas 2 subtypes were identified in 13 additional herds (Table 5). Only 1 of the 4 subtypes was present

in the remaining 93 herds in which more than 1 isolate was tested.

DISCUSSION

The WGS data from Canadian MAP isolates (Ahlstrom et al., 2016) were used to design a PCR assay to differentiate specific subtypes of interest from a larger collection, which was then applied to 602 isolates representing 264 dairy farms. This novel SNP-PCR amplified and linked 2 distantly located polymorphic regions of the MAP genome for subsequent Sanger sequencing. The use of SNP as the basis for discrimination is important for studying the molecular epidemiology of MAP, as these markers provided a much more reliable foundation to identify distinct MAP subtypes than the commonly used typing schemes based on repetitive elements (Ahlstrom et al., 2015). The relatively limited amount of genetic diversity in MAP has historically restricted the utility of sequenced-based genotyping; however, targeting and concatenating polymorphic regions in a single reaction provided accurate molecular typing information to elucidate distribution of important MAP subtypes in Canadian dairy farms.

The subtypes investigated in our study were selected based on the previously determined population structure of MAP in Canada (Ahlstrom et al., 2016). Parts of the genome other than regions A and B contained alternative discriminatory SNP; had these SNP been targeted instead, they would have given the same results. Increasing affordability of WGS will permit additional geographical regions to be analyzed, providing SNP data that could be used to customize PCR to accommodate additional MAP subtypes of interest. It is noteworthy that linkage of distant regions is not limited to 2 and this technique could be expanded to incorporate several additional canonical SNP. Other strategies are available to link amplicons (e.g., Gibson assembly), but the single-step PCR reaction is a major advantage of the current approach. Furthermore, a high-resolution melt analysis could be done to eliminate the sequencing step,

Table 3. Nucleotide identity of 5 SNP differentiating the 4 *Mycobacterium avium* ssp. *paratuberculosis* subtypes

Item	Region A (145 bp)		Region B (375 bp)		
	SNP1	SNP2	SNP3	SNP4	SNP5
Coordinate	2,214,379	2,214,445	2,934,204	2,934,492	2,934,514
SNP effect	Synonymous	Alternative start codon	Synonymous	Intergenic	Intergenic
Type I/III	C	C	G	C	C
Bison	T	C	C	C	C
Dominant	C	C	C	T	T
Secondary	C	T	C	T	C
Other	C	C	C	T	C

Table 4. Number of *Mycobacterium avium* ssp. *paratuberculosis* (MAP) isolates and number of herds that belonged to each MAP type in all 10 Canadian provinces

Province	Dominant		Secondary		Bison		Other		Type III	
	Isolates	Herds	Isolates	Herds	Isolates	Herds	Isolates	Herds	Isolates	Herds
Alberta	277	114	8	3	12	4	22	7	—	—
Ontario	63	28	—	—	1	1	2	1	—	—
Quebec	54	35	1	1	5 ¹	4 ¹	1	1	1	1
Saskatchewan	44	20	3	3 ¹	—	—	4	3	—	—
New Brunswick	33	8	—	—	—	—	7	4	—	—
Prince Edward Island	33	13	1	1	—	—	3	3	—	—
British Columbia	9	6	—	—	—	—	3	2	—	—
Newfoundland	6	6	—	—	—	—	—	—	—	—
Manitoba	4	4	—	—	—	—	1	1	—	—
Nova Scotia	2	2	1	1	—	—	1	1	—	—
Total	525	236	14	9	18	9	44	23	1	1

¹Indicates overrepresentation by province ($P \leq 0.05$).

similar to previous approaches used to differentiate the *Mycobacterium avium*-*intracellulare* complex and MAP types I, II, and III (Castellanos et al., 2010a,b).

A recent SNP-PCR assay based on PCR and restriction enzyme digestion was developed to differentiate a diverse collection of MAP isolates from 8 host species and 17 distinct geographical locations (Leão et al., 2016). Although this is a valuable tool to differentiate major global MAP types, it would likely not provide sufficient discriminatory power to identify all subtypes of interest within Canada. In that regard, customized typing schemes specific to a region of interest can be more valuable than a generalized approach.

The relative frequency of each subtype in Canadian dairy cattle was consistent with results obtained from the WGS analysis of a limited number of isolates (Ahlstrom et al., 2016). The presence of type B isolates in Alberta, Ontario, and Québec was observed previously; however, additional herds harboring this type were

identified in our study. Overrepresentation of type B in Québec highlighted the potentially large contribution of this type in JD in this region; consequently, further epidemiological analyses into potential risk factors or phenotypes attributed to this type could provide insights regarding this overrepresentation. Similarly, overrepresentation of the secondary clade in Saskatchewan could benefit from such an analysis to elucidate potential risk factors for this type.

Approximately 7% of isolates were identified as other, meaning they did not belong to the type I/III, B, secondary, or dominant clade. We inferred that they could belong to any of the other 5 known Canadian clades that were not targeted in the PCR, or they could represent new divergent subtypes not yet identified. These isolates could be further evaluated either through a modified SNP-PCR assay to determine if they belong to a previously identified subtype (and in what proportions), or through WGS.

Co-circulation of multiple MAP subtypes within a herd occurred in this and previous studies (Motiwala et al., 2004; Harris et al., 2006; Pradhan et al., 2011; Sohal et al., 2014). Purchasing cattle from multiple locations is a known risk factor for JD (Wolf et al., 2016), which also increases the number of MAP subtypes within a herd. The number of distinct MAP isolates within a herd can indicate the number of introduction events, though in the current study the amount of within-herd MAP diversity was likely underestimated, as the dominant clade is known to include many diverse subclades (Ahlstrom et al., 2015, 2016).

Furthermore, using this SNP-PCR in other countries would identify relative frequencies of these types outside of Canada. The secondary clade was not observed in a diverse set of 141 global MAP isolates (Ahlstrom et al., 2016), suggesting it may be less abundant in other regions. Characterization of a larger set of isolates from

Table 5. Province, number of isolates, and *Mycobacterium avium* ssp. *paratuberculosis* types in herds with more than one type

Province	No. of isolates	Types
AB ¹	6	Secondary, dominant
AB	5	Other, dominant
AB	5	Bison, other, dominant
AB	4	Secondary, dominant
AB	4	Bison, dominant
AB	4	Other, dominant
AB	3	Other, dominant
AB	3	Secondary, dominant
AB	2	Other, dominant
ON	5	Other, dominant
QC	13	Secondary, dominant
SK	3	Secondary, dominant
SK	2	Other, dominant
BC	2	Other, dominant

¹AB = Alberta; ON = Ontario; QC = Québec; SK = Saskatchewan; BC = British Columbia.

the United States and elsewhere could clarify the distribution of this type and others. Assessment of MAP strains circulating in US dairy herds is also relevant for Canadian JD control strategies, as Canada regularly imports cattle from the United States (Canadian Dairy Information Centre, 2016). Therefore, this PCR assay could also be modified to target specific subtypes of interest in other geographical locations.

Future research, such as *in vitro* assays, could be performed to identify phenotypic differences between MAP types in their survival, growth rates, and virulence capabilities. Although only 1 SNP targeted in this study (SNP2) had a clear functional effect (alternative start codon), other SNP could be investigated for potential functional consequences. Identification of distinct, phylogenetically supported subtypes (achieved using the current SNP-PCR) is first required to identify closely and more distantly related isolates for any *in vitro* or *in vivo* comparative studies.

CONCLUSIONS

This study demonstrated the development and use of a novel SNP-PCR targeting 4 major MAP types of relevance in Canada to assess their distribution and relative frequencies. Analysis of >600 MAP isolates from all 10 Canadian provinces supported the conclusion that >85% of MAP isolates belonged to a dominant type and that, although less frequent, other subtypes are also widely distributed. This PCR can be used to monitor prevalence of major strain types in Canada and elsewhere, as an understanding of the relative frequency of MAP subtypes in a population is necessary to properly evaluate the relevance of shared subtypes at various spatial scales.

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