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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 fecal 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, IS*900* 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 substreaked 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.