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Genotypic analysis of nontuberculous mycobacteria isolated from raw milk and human cases in Wisconsin

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ABSTRACT

Nontuberculous mycobacteria (NTM) compose a group of mycobacteria that do not belong to the *Mycobacterium tuberculosis* complex group. They are frequently isolated from environmental samples such as water, soil, and, to a lesser extent, food samples. Isolates of NTM represent a major health threat to humans worldwide, especially those who have asthma or are immunocompromised. Human disease is acquired from environmental exposures and through consumption of NTM-contaminated food. The most common clinical manifestation of NTM disease in human is lung disease, but lymphatic, skin and soft tissue, and disseminated disease are also important. The main objective of the current study was to profile the farm-level contamination of cow milk with NTM by examining milk filters and bulk tank milk samples. Five different NTM species were isolated in one dairy herd in Wisconsin, with confirmed 16S rRNA genotypes including *Mycobacterium fortuitum*, *Mycobacterium avium* ssp. *hominissuis*, *Mycobacterium abscessus*, *Mycobacterium simiae*, and *Mycobacterium avium* ssp. *paratuberculosis* (*Mycobacterium paratuberculosis*). In tank milk samples, *M. fortuitum* was the predominant species in 48% of the samples, whereas *M. chelonae*/*abscessus* and *M. fortuitum* were the only 2 species obtained from 77 and 23% of the examined filters, respectively. Surprisingly, *M. avium* ssp. *hominissuis*, *M. paratuberculosis*, and *M. simiae* were isolated from 16.7, 10.4, and 4% of the examined milk samples, respectively, but not from milk filters. Interestingly, NTM isolates from human clinical cases in Wisconsin clustered very closely with those from milk samples. These findings suggest that the problem of NTM contamination is underestimated in dairy herds and could contribute to human infections with NTM. Overall, the study validates the use of bulk tank samples rather than milk filters to assess contamination of milk with NTM. Nontuberculous mycobacteria represent one type of pathogens that extensively contaminate raw milk at the farm level. The significance of our research is in evaluating the existence of NTM at the farm level and identifying a simple approach to examine the potential milk contamination with NTM members using tank milk or milk filters from dairy operations. In addition, we attempted to examine the potential link between NTM isolates found in the farm to those circulating in humans in Wisconsin.
Key words: nontuberculous mycobacteria, cow tank milk, milk filter, human cases

INTRODUCTION

Nontuberculous mycobacteria (**NTM**), also known as environmental mycobacteria due to their abundant existence in the environment, are opportunistic pathogens of public health concern, especially for immunocompromised individuals (Claeys and Robinson, 2018). Members of NTM are the causative agents of many human infections such as lymphadenitis in children and pulmonary, skin, and soft tissue infections (Nishiuchi et al., 2017). Although human-to-human transmission is rare, it is essential to identify potential sources and routes of exposure to NTM. Although the risk of NTM transmission through water and several foods is documented (Argueta et al., 2000), the prevalence and risk of NTM in animal products, such as milk, is understudied. Globally, NTM isolates have been detected in milk products from both high- and low-income countries (Yoo et al., 2012; Kendall and Winthrop, 2013; Panagiotou et al., 2014). Nontuberculous mycobacteria are considered emerging milk-borne pathogens worldwide. In Brazil, 21.7% of mozzarella cheese manufactured from raw buffalo milk was positive for NTM, demonstrating a risk for consumers (Jordão Junior et al., 2009). Another study, in Spain, reported the presence of viable *Mycobacterium avium* ssp. *hominissuis*

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in a powdered infant formula and mycobacterial DNA (*M. avium*, *Mycobacterium tuberculosis* complex, and other NTM mycobacteria) was detected in 15% of the examined dairy products (Sevilla et al., 2017). Finally, *Mycobacterium gordonae*, *Mycobacterium fortuitum*, *Mycobacterium senegalense*, and *M. avium* have been isolated from fresh milk sold in Nigeria, suggesting a high prevalence of NTM in dairy food (Agada et al., 2014). In Taiwan, the incidence of NTM infections increased from 2.7 to 10.2 cases per 100,000 between the years 2000 and 2008 (Lai et al., 2010). The incidence of NTM infection increased from 0.9 per 100,000 population in 1995 to 2.9 per 100,000 in 2006 in England, Wales, and Northern Ireland (Moore et al., 2010). The increase in NTM prevalence highlights the need to examine potential sources of infection and develop protocols to eliminate them. With the increasing popularity of raw milk consumption throughout the United States (allowed in 30 of 50 states; Costard et al., 2017), surveillance of NTM in milk and dairy herds is becoming of a paramount importance. This study was designed to help close this knowledge gap.

Previously, NTM have been detected in meat and cow milk (raw and pasteurized; Sgarioni et al., 2014), major food sources for humans. Water, soil, and food have been suggested as important NTM sources for infection transmission to humans (Shitaye et al., 2009; Sgarioni et al., 2014). The relationship between environmental sources of NTM and disease in humans has been studied for decades (Wolinsky, 1979), but mostly as association studies because of a lack of data on the genetic basis of the relationship. However, with the advancement of molecular techniques, several reports have suggested a stronger link between NTM in food samples and those circulating in patients (Yoder et al., 1999). In California, 25 isolates of NTM, including *M. avium*, *M. gordonae*, and *Mycobacterium simiae*, were recovered from 121 supermarket food items, mainly of plant origin (Argueta et al., 2000). Unfortunately, the published data regarding the prevalence of NTM in food of animal origin are scarce in the United States. In Brazil, NTM was found in 9 to 25% of raw milk samples and related dairy products (Franco et al., 2013). Similar levels were obtained from analyses of raw and processed meat samples from the Czech Republic (Shitaye et al., 2009). High levels of NTM contamination were also reported in raw milk samples analyzed in several countries from Europe and Africa (Kazwala et al., 1998; Konuk et al., 2007). Both PCR and genetic fingerprinting-based approaches (e.g., variable number of tandem repeats, DNA sequencing) were suggested to accelerate the detection of the prevalence and persistence of NTM in food samples (Bolanos et al., 2017). Here, we hypothesized that the diversity of NTM contamination is underestimated in dairy products, which should be of concern to public health authorities. Our study was conducted to develop a simple approach to examine the potential milk contamination with NTM on the farm level using tank milk and milk filters from dairy operations. In addition, we estimated the level of diversity among NTM present in raw milk and examined any potential link to the NTM isolated from patients in Wisconsin.

MATERIALS AND METHODS

Milk Sample Collection

A medium-size local dairy herd with 80 lactating cows was used for this investigation from January to May 2016. Milk filters and raw milk samples were collected from the bulk tank with a total of 85 samples for each (milk filters and tank milk samples = 170 samples). Milk filters are usually placed just before the bulk tank to remove gross foreign material from milk. The 85 raw milk samples (50 mL each) were collected using aseptic technique directly from the bulk tank that contains the milk that has passed through the milk filter. The milk filters and tank milk samples were maintained at 4°C during transport and prepared as described before (Slana et al., 2012). Briefly, each filter was placed separately in a stomacher bag with 100 mL of PBS (pH 7.0) containing 0.05% Tween 20 (**PBS-T**) and homogenized in a stomacher for 2 min. Sample homogenates were centrifuged (15 min at 2,500 × *g*) and pellets were resuspended in 10 mL of PBS-T for culturing.

Isolation of Mycobacteria from Filters and Milk Samples

Ten milliliters from milk samples and 1 mL from the processed filter homogenates were centrifuged (15 min at 2,500 × *g*) before the decontamination step. The obtained pellets and cream layers were resuspended in 10 mL of 0.75% 1-hexadecylpyridinium chloride (Sigma-Aldrich, St. Louis, MO) and incubated for 5 h at room temperature for decontamination. Samples were centrifuged again (15 min at 2,500 × *g*) and pellets were resuspended in 1 mL of PBS for inoculation onto Lowenstein Jensen (**LJ**) medium (LJ slants) and Middlebrook 7H10 agar (BD, Franklin Lakes, NJ) as described before (Dundee et al., 2001). Cultures were incubated in the presence of 5 to 10% CO_2 at 37°C for 90 d and inspected weekly for bacterial growth (Ghosh et al., 2012). One set of Middlebrook 7H10 plates was

supplemented with mycobactin J (**MJ**, 2 mg/mL), an iron-chelating cell wall component for the isolation of *M. paratuberculosis*, and 2 μg of vancomycin, 30 μg of amphotericin B, and 20 μg of nalidixic acid (**VAN**) per milliliter. All antibiotics were from Sigma Chemical Co. (St. Louis, MO) Colonies growing on *Mycobacterium*-specific media were characterized by Ziehl-Neelsen staining and 16S rRNA PCR. For Ziehl-Neelsen staining, wet smears from each suspected colony were prepared on a clean and dry glass slide and heat fixed over flame until dried. Slides were placed on a 90°C adjusted heat block, and carbol-fuchsin solution was added. After 5 min, slides were washed with distilled water, followed by an additional wash with 1% acid alcohol for discoloration. The slides were counterstained with methylene blue solution for 30 s and washed with distilled water before air drying and microscopical examination.

NTM Isolates from Human Cases

With the help of Wisconsin State Laboratory of Hygiene (Madison), we obtained 13 de-identified isolates of NTM that were collected from human patients with diagnosed NTM infections cultured from various sources including sputum, urine, blood, or neck tissues. All isolates were cultured as detailed above and their DNA was extracted for PCR amplification as detailed below.

Extraction of Genomic DNA

To obtain high-quality genomic DNA (**gDNA**), we followed a method previously developed in our group (Talaat et al., 1997). Briefly, mycobacterial cultures were pelleted by centrifugation, and the pellets were washed and resuspended in equal volume of Tris-EDTA buffer. Bacterial suspensions were placed at 80°C for 20 min to kill all living organisms. Tubes were allowed to cool at room temperature, and 10 μL of 100 mg/mL lysozyme was then added to each tube, followed by incubation at 37°C for 3 h with occasional mixing. A solution of 10% SDS and proteinase K (20 mg/mL) was added to each tube at a ratio of 88:12. Tubes were incubated at 65°C for 2 h followed by addition of 100 μL of 5 *M* NaCl at 65°C for additional 10 min. A 10% solution of cetyl trimethyl ammonium bromide (80 μL; Sigma-Aldrich) was added, followed by mixing and incubation at 65°C for another 10 min. Then, DNA was extracted with an equal volume of phenol:chloroform: isoamylalcohol (25:24:1 vol:vol:vol) and similarly with chloroform:isoamylalcohol (24:1 vol:vol) followed by precipitation with 0.6 volumes of ice-cold isopropanol and centrifugation (14,000 × *g*) at 4°C for 15 min. Pellets were washed with cold 75% ethanol and dried in a SpeedVac (Thermo Scientific, Wilmington, DE) for 5 min. The DNA was finally resuspended in 50 μL of sterile distilled water. Quality of the gDNA was verified by both NanoDrop (Thermo Scientific) machine and electrophoresis. To detect the presence of mycobacterial DNA in the collected samples, gDNA was extracted directly following our previously described procedure (Talaat et al., 1997).

Polymerase Chain Reaction

To confirm the identity of the mycobacterial colonies and gDNA extracted directly from the examined samples, we subjected them to PCR amplification targeting the 16S rRNA gene. A partial sequence (938 bp) of the 16S rRNA gene was amplified from all isolates and samples. Primers used for this purpose were designed by our group (Talaat et al., 1997); namely, AMT 36 (GCGAACGGGTGAGTAACACG) and AMT 37 (TGCACACAGGCCACAAGC). Each PCR amplification was 25 μL, which contained 1 *M* betaine, 50 m*M* potassium glutamate, 10 m*M* Tris-HCl pH 8.8, 0.1% Triton X-100, 2 m*M* magnesium chloride, 0.2 m*M* dNTPs, 0.5 m*M* each primer, 0.5 U of *Taq* DNA polymerase (Promega, Madison, WI), and 25 ng of genomic DNA. The amplification cycle consisted of an initial denaturation step of 94°C for 5 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min; and a final extension at 72°C for 7 min. Amplicons were evaluated by electrophoresis in 2% agarose gels prestained with ethidium bromide (0.5 mg/mL). Single band products of amplicons were purified from agarose gels and extracted using Wizard SV Gel and PCR cleanup system (Promega) as detailed before (Talaat et al., 1997).

Sanger DNA Sequencing and Phylogenetic Analysis

Following PCR amplification, purified PCR fragments were sequenced with BigDye Terminator v3.1 (Applied Biosystems, Foster City, CA) with AMT 36 and AMT 37 primers, according to the manufacturer's instruction. All sequences were analyzed with BLASTn algorithm on the NCBI web portal (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

A rooted tree was computed using MEGA7 software (Kumar et al., 2016) and neighbor-joining method to align all sequences of 16S rRNA and build a phylogenetic tree with the presence of reference sequences from several mycobacterial species deposited in GenBank (Tamura et al., 2013).

Whole-Genome Sequence Analysis and Phylogenetic Analysis

Selected isolates from farm tank milk samples and de-identified human isolates from the Wisconsin State Laboratory of Hygiene were submitted to the Wisconsin Biotechnology Center for whole-genome sequencing (**WGS**) as detailed before by our group (Hsu et al., 2011; Abdelaal et al., 2019). Genomic libraries for a total of 19 isolates were prepared and run on Illumina-MiSeq 2000 platform (Illumina Inc., San Diego, CA). Reads with an average length of 250 bp were assembled using SPAdes 3.14.1 (Bankevich et al., 2012) for de novo genome assembly. The assembled contigs were searched against the NCBI database using BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to ensure the identity of each isolate. CLC Bio Workbench version 8.5.1 (https://digitalinsights.qiagen.com/) was used, and we used standard genome corresponding to identification from BLASTn; namely, *M. avium* 104 (accession number: CP000479.1), *M. abscessus* ATCC19977 (accession number: NC_010397.1), or *M. fortuitum* CT6 (accession number: CP011269.1), to identify SNP; insertions/deletions (InDels) event with minimum variation frequency was set to 50% to count SNP. Consensus sequences for mapped reads were exported from CLC Bio workbench version 8.5.1 as FASTA format and aligned using the Harvest software package (Treangen et al., 2014) and the "parsnp" function to output a newick tree file that was visualized using MEGA X software.

Data Availability

The sequences of the 16S rRNA gene have been deposited in the GenBank database under the accession numbers reported in Table 1. All whole-genome sequences for mycobacterial isolates used in this study were deposited at the NCBI GenBank (BioProject no. PRJNA656902) with BioSample numbers from SAMN15806900 to SAMN15806920. See Table 2 for isolate names, BioSample numbers, and isolate sources.

RESULTS

Milk Contamination with NTM

We hypothesized that examination of milk filters and tank milk would provide a means of detecting mycobacteria contamination on the herd level. Although milk filters trap debris, typically in the range of 100 to 150 μm, they do not prevent pathogenic bacteria (~1–10 μm) from passing through. Culture-positive milk filters likely indicate that tank milk has been contaminated, but the reverse might not always be correct. Detecting

Table 1. Different nontuberculous mycobacteria (NTM) isolates (*M.* = *Mycobacterium*) detected from tank milk and milk filters[1]

Source	NTM isolate				
	M. fortuitum	*M. avium* ssp. *hominissuis*	*M. abscessus*	*M. simiae*	*M. avium* ssp. *paratuberculosis*
Tank milk	23	8	10	2	5
Accession number	MN880273–MN880298	MN880384–MN880391	MN880315–MN880324	MN880382–MN880383	MN880377–MN880381
LJ medium	8 (34.8)	4 (50)	1 (10)	—	—
Middlebrook medium	10 (43.5)	4 (50)	4 (40)	1 (50)	—
MJ Middlebrook medium	5 (21.7)	—	5 (50)	1 (50)	5 (100)
Milk filters	3	—	10	—	—
Accession number	MN880309–MN880311		MN880300–MN880308 and MN880272(F39)		
LJ medium	—	—	—	—	—
Middlebrook medium	—	—	—	—	—
MJ Middlebrook medium	3 (100)	—	10 (100)	—	—

[1]Numbers followed by parenthetical values are the total number of isolates for tank milk samples or milk filters and the percentage of each NTM isolate. The collected tank milk and milk filter samples were cultured on 3 selective media including Lowenstein-Jensen (LJ) medium, 7H10 Middlebrook medium, and 7H10 Middlebrook medium supplemented with Mycobactin J (MJ).

Table 2. Summary data of whole-genome sequence analysis of *Mycobacterium* (*M.*) species isolates from milk tank samples and human specimens in Wisconsin[1]

Sample ID	BioSample no.	Reference	Mapping %	Average coverage	No. of SNP	No. of syn SNP	No. of non-syn SNP	No. of intragenic SNP	No. of MNV	No. of replacements	No. of insertions	No. of deletions
W9	SAMN15806900	*M. avium* 104	82.59	309.81	36,650	23,739	9,130	3,781	1,540	93	473	475
W11	SAMN15806902	*M. avium* 104	82.10	454.27	32,550	21,895	7,580	3,075	1,769	107	482	446
W12	SAMN15806903	*M. avium* 104	72.86	295.34	23,236	16,297	5,455	1,484	1,969	94	398	391
W14	SAMN15806906	*M. avium* 104	86.50	412.08	37,411	24,799	8,961	3,651	1,811	106	538	532
W15	SAMN15806905	*M. avium* 104	77.23	243.02	32,625	21,864	7,755	3,006	1,836	106	491	446
T9	SAMN15806917	*M. avium* 104	67.17	48.15	37,938	24,744	9,115	4,079	1,898	113	566	598
T52b	SAMN15806914	*M. avium* 104	84.85	128.3	38,910	25,547	9,484	3,879	2,030	132	578	609
T62b	SAMN15806915	*M. avium* 104	87.69	153.53	38,656	25,324	9,426	3,906	1,981	133	585	624
T84	SAMN15806916	*M. avium* 104	88.81	79.08	37,150	24,194	9,133	3,823	1,655	120	564	587
T2	SAMN15806913	*M. avium* 104	88.1	97.99	47,909	26,229	9,100	3,569	2,204	136	631	595
W7	SAMN15806907	*M. abscessus* ATCC 19977	91.28	378.06	103,148	76,858	18,304	7,986	3,489	166	675	781
W8	SAMN15806908	*M. abscessus* ATCC 19978	98.74	425.4	65	23	32	10	3	0	7	6
T7	SAMN15806920	*M. abscessus* ATCC 19977	93.53	128.18	103,448	77,109	18,388	7,951	3,648	213	743	983
T29C	SAMN15806918	*M. abscessus* ATCC 19977	93.39	75.14	103,269	76,959	18,363	7,947	3,560	196	726	962
T84b	SAMN15806919	*M. abscessus* ATCC 19977	92.87	162.65	103,616	77,223	18,442	7,951	3,695	209	762	1007
W4	SAMN15806909	*M. fortuitum* CT6	79.30	296.11	152,070	114,505	27,137	10,428	8,386	403	1,259	1,295
W5	SAMN15806910	*M. fortuitum* CT6	83.60	336.8	67,506	13,599	49,214	4,693	3,625	170	635	594
W6	SAMN15806911	*M. fortuitum* CT6	68.40	238.6	71,979	55,018	13,881	5,080	4,271	192	730	685
T60a	SAMN15806912	*M. fortuitum* CT6	84.30	51.9	68,126	41,471	13,511	5,147	3,973	219	815	766

[1]Syn = synonymous; non-syn = nonsynonymous; MNV = multiple nucleotide variant.

mycobacterial DNA directly from milk filters and the examined milk samples resulted in a total of 48 positive milk filter samples out of 85 (56.5%) with almost the same percentage for the tank milk (44 samples out of 85, 51.8%).

Using culturing on mycobacteria-specific media, tank milk and milk filter samples were positive in 17 (20%) and 10 (11.7%) samples, including 48 and 13 mycobacterial isolates (Table 1), respectively, with a total of 61 independent isolates cultured from the examined dairy herd.

A total of 5 mycobacterial species were isolated from the tank milk samples and only 2 were obtained from the milk filters (Table 1), an indication of the validity of sampling tank milk to assess herd health and milk contamination with NTM. All isolates grown on the selected media were positive for acid fast staining.

Genotypes of NTM Present in Raw Milk

To confirm isolate identity, we amplified the 16S rRNA gene from all isolates. As expected, specific DNA bands of approximately 938 bp were observed on agarose gel for all positive samples, indicating that all isolates were members of the genus *Mycobacterium*. To identify the species of mycobacterial isolates, amplicons from all isolates were sequenced using Sanger sequencing (Talaat et al., 1997). DNA sequences from all isolates were analyzed using the BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) algorithm against all bacterial genomes present in the GenBank database (NCBI Resource Coordinators, 2016). From the tank milk samples cultured on LJ slants, 13 independent isolates were grown. Among these isolates, 8 belonged to the *M. fortuitum* group, 4 belonged to *M. avium* ssp. *hominissuis*, and 1 was identified as *M. abscessus* (Table 1). The other set of tank milk samples was cultured on 7H10 medium, and 19 isolates were grown. Among them, *M. fortuitum* was the predominant species (n = 10). Four isolates belonged to the *M. avium* ssp. *hominissuis* group, while 1 isolate was identified as *M. abscessus* and another was identified as *M. simiae*. In addition, when 7H10 medium was supplemented with MJ, 16 isolates were grown. Among them, 5 isolates belonged to each group for *M. avium* ssp. *paratuberculosis*, *M. abscessus*, and *M. fortuitum*, and 1 isolate was identified as *M. simiae*. For the milk filter samples, both LJ slants and 7H10 Middlebrook medium without antibiotics were heavily contaminated, a potential cause of isolation failure; however, all isolates were obtained from Middlebrook 7H10 medium supplemented with MJ and the antibiotics mixture (VAN). A total of 13 isolates were recovered from milk filters. Among them, 10 isolates belonged to the *M. abscessus* group, while

the remaining 3 isolates belonged to the *M. fortuitum* group (Table 1).

Phylogenetic Analysis of the NTM

To better examine the relationship between NTM isolates, we examined the evolutionary distances among isolates based on 16S rRNA sequences. As expected, clear phylogenetic clusters formed between members of the *M. abscessus/chelonae* complex and the *M. avium* ssp. *hominissuis/paratuberculosis* complex. No significant genetic distance was found between members of the *M. abscessus/chelonae* complex whether they were isolated from tank milk or milk filters, suggesting a common source of contamination. All isolates of *M. fortuitum* occupied a different cluster between the other 2 clusters. As expected, 2 isolates of *M. simiae* clustered independently, another confirmation of their identity and potential different source of transmission. However, the low bootstrapping percentage (<50%)

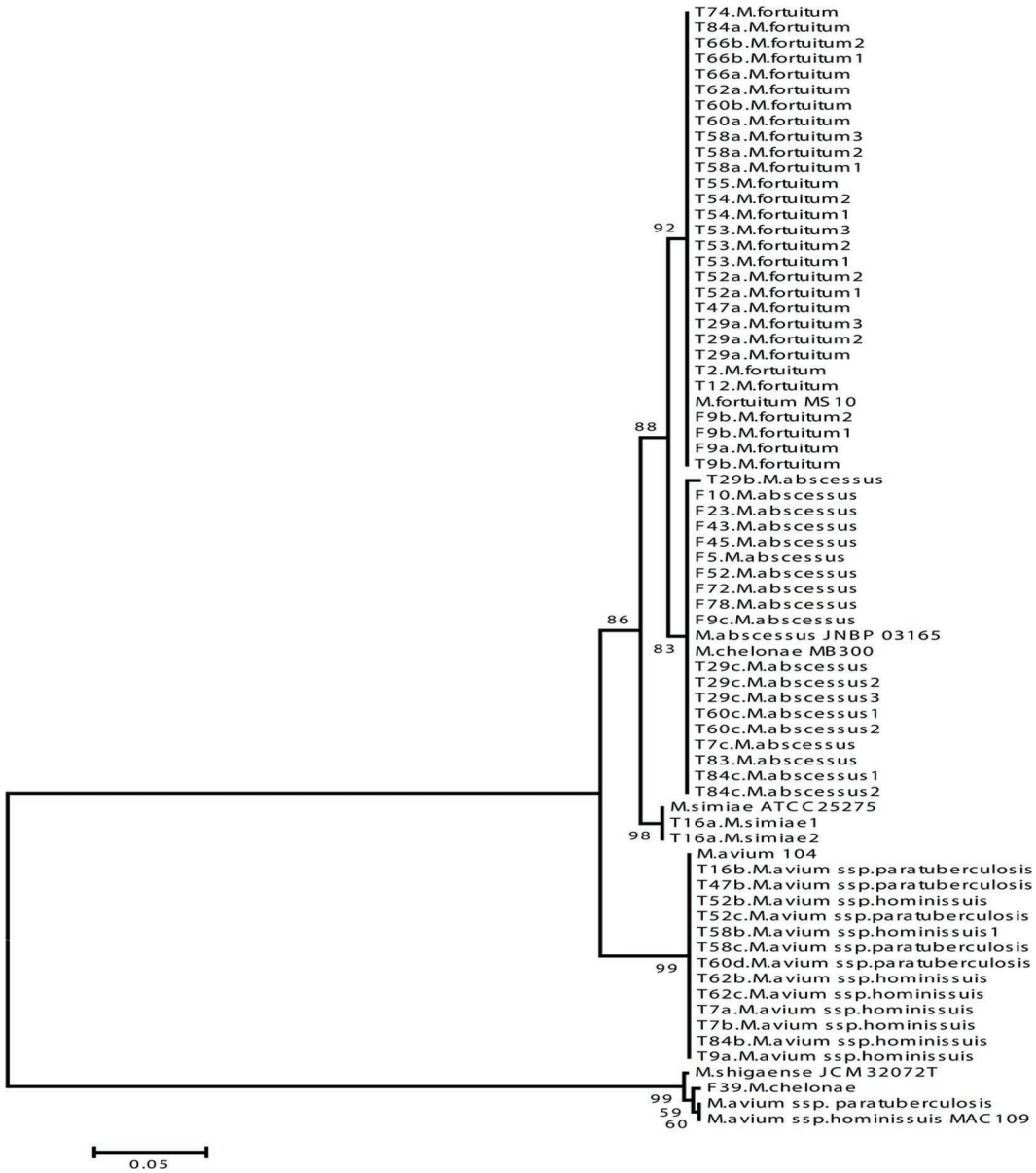

Figure 1. Evolutionary tree of all nontuberculous mycobacteria isolates from tank milk (identified as T samples) and milk filters (identified as F samples) isolated from a Wisconsin dairy herd. The tree is based on 16S rRNA sequences using the neighbor-joining method in MEGA7 software (https://www.megasoftware.net/). The tree is drawn to scale with a bar representing the phylogenetic distance. Numbers next to the branches represent percentages of 1,000 bootstrap test.

that distinguished the *M. simiae* from the *M. avium/paratuberculosis* complex could indicate their evolutionary relatedness (Figure 1).

In another analysis, we tested the phylogenetic relatedness between selected farm NTM isolates (n = 9) and those circulating in human patients (n = 10) from Wisconsin, using de-identified samples from the collection of the Wisconsin State Laboratory of Hygiene. In Table 2, we added a summary of the WGS of 19 NTM isolates from both milk and human samples. Single nucleotide polymorphisms for each isolate were compared with genomes representing the standard strains of *M. avium* ssp. *hominissuis*, *M. fortuitum*, and *M. abscessus* (Figure 2b). These isolates were the most commonly isolated mycobacteria from tank milk samples. Interestingly, the whole-genome sequences from all human isolates were clustered with isolates from milk samples, an indication of highly similar genotypes of NTM present in both milk samples and those circulating in humans infected with NTM (Figure 2). Moreover, the overall SNP 16S rRNA genotyping did not accurately identify 3 isolates of *M. fortuitum* (T2, T9, and T84), which WGS identified as *M. abscessus* for T2 and *M. avium* ssp. *hominissuis* for T9 and T84, respectively. On the other hand, T52b and T62b isolates that were identified by 16S rRNA as *M. avium* ssp. *hominissuis* were identified by WGS as *M. abscessus* and *M. fortuitum*, respectively. This finding further confirms the superiority of WGS for strain identification compared with other standard genotyping approaches.

DISCUSSION

Nontuberculous mycobacteria are a group of opportunistic mycobacterial species that do not belong to the *M. tuberculosis* complex (Brode et al., 2017). These mycobacterial species have been isolated from many environmental sources, such as water, soil, and food (Nishiuchi et al., 2017), implicating these sources as vehicles for NTM transmission to humans (Sgarioni et al., 2014). Previously, analysis of raw milk and dairy products indicated the presence of high levels of NTM in some developing countries (Kazwala et al., 1998; Konuk et al., 2007). Unfortunately, in tuberculosis-endemic countries (e.g., India, China), the problem of NTM is usually masked by problems associated with tuberculosis (Gopinath and Singh, 2010), suggesting an underestimation of NTM prevalence. Immunocompromised individuals are at high risk of contracting NTM infections, especially with the opportunistic members of the *M. avium* complex (Shojaei et al., 2011). The current study was conducted to examine the extent of NTM presence at the farm level, using a dairy herd in Wisconsin, where mycobacterial prevalence is low (Wisconsin Department of Health Services, 2019) compared with other locations (Gopinath and Singh, 2010). The milk filters are usually located just before the bulk tank and have pores of 65 to 75 μm to filter debris and fecal material from the milk (McKee et al., 2002). Microbial analysis of milk filters represents a first-choice sampling strategy to assess the overall microbial quality of milk, hence we compared the utility of milk filters with that of tank milk samples to assess mycobacterial presence in unpasteurized milk. Unexpectedly, our analysis of both sampling strategies indicated the superiority of bulk tank milk sampling over milk filters in both the number of isolates and diversity of isolated species. This result is likely due to high level of contamination that could not be trapped by milk filters.

One of the major findings obtained in this study is that *M. fortuitum* was the predominate NTM isolate from tank milk as shown in Table 1, while *M. chelonae/abscessus* was the prevailing isolate detected from milk filters. In an earlier study of NTM isolates, the *M. avium* complex, specifically *M. avium* ssp. *paratuberculosis* (*M. paratuberculosis*), represented the highest prevalence, followed by *M. fortuitum* (Gopinath and Singh, 2010). In this report, *M. paratuberculosis* was isolated from 5 tank milk samples but was not detected at all from any of the milk filters, most likely because of its low level in the examined herd. Similar studies reported the presence of *M. paratuberculosis* in raw milk samples worldwide (Grant et al., 2002; Ayele et al., 2005; Slana et al., 2008; Botsaris et al., 2010; Shankar et al., 2010).

Related studies indicated the presence of NTM in both raw and pasteurized milk in Brazil including *M. fortuitum*, *Mycobacterium marinum*, *Mycobacterium kansasii*, and *M. gordonae* (Leite et al., 2003). In other studies, *M. chelonae* and *Mycobacterium scrofulaceum* were also isolated from raw and pasteurized milk (Sgarioni et al., 2014). In Turkey, other members of the NTM group were detected in raw milk including, *Mycobacterium terrae*, *M. kansasii*, *Mycobacterium haemophilum*, and *Mycobacterium agri* (Konuk et al., 2007). In our study, 5 NTM species involved in a variety of human illnesses were detected including *M. fortuitum*, *M. chelonae/abscessus*, *M. paratuberculosis*, *M. avium* ssp. *hominissuis*, and *M. simiae*.

To better identify each isolate, we sequenced a large proportion of the 16S rRNA gene, which confirmed the identity of all mycobacterial isolates (Figure 1) and provided a tool for further analysis of such isolates in the context of other circulating mycobacterial species. Interestingly, phylogenetic analysis of milk isolate sequences and their counterparts from human cases of infection with NTM species circulating in Wisconsin patients indicated a close relationship between both groups of NTM (Figure 2) when whole genomes were

analyzed. Such analysis suggested a potential common source of NTM isolates, most likely potable tap water as indicated before (Honda et al., 2018) for *M. avium* complex but not for *M. paratuberculosis*, *M. abscessus*, and *M. fortuitum* complexes. It is possible that animal infections with such organisms are transmissible to human through the consumption of raw milk or dairy products made with unpasteurized milk. Results presented here should prompt examination of more milk and dairy products for mycobacterial contamination, especially with the increase of consumption of raw milk throughout the United States (Costard et al., 2017) and in low-income countries as well. Moreover, contradictory results from 16S rRNA genotyping and WGS of members of *M. fortuitum* and *M. avium* groups suggested the need to use more targets for genotyping (e.g., *rpoB* and *hsp65*) or the need to use WGS.

Overall, our study illustrates that examination of milk samples could help in the evaluation of mycobacterial diversity in a dairy herd, with bulk tank milk sampling being the superior strategy. The study validates the use of bulk tank samples over milk filters to assay milk contamination with NTM. Moreover, consumption of raw milk or unpasteurized dairy products constitutes a potential risk factor for contracting NTM infections in humans. The implementation of control measures that

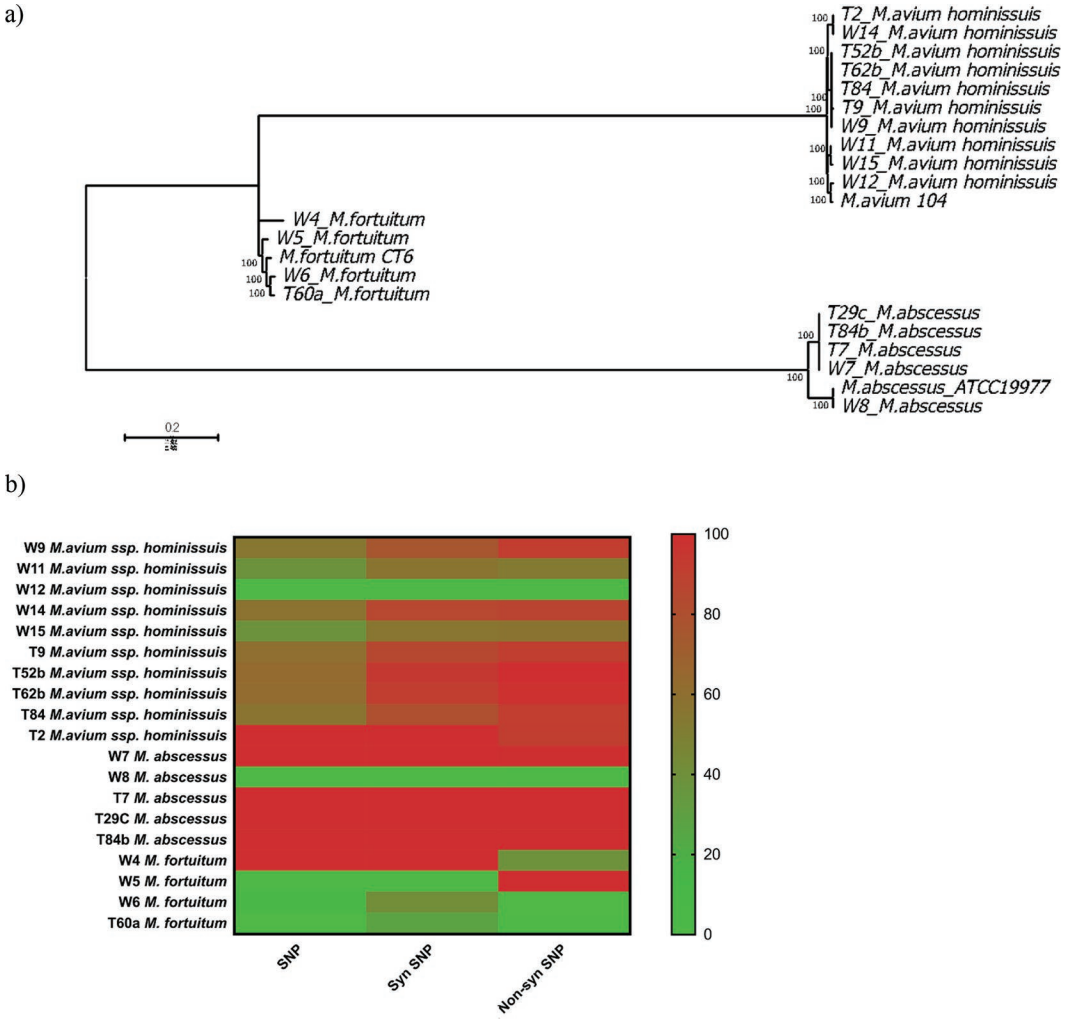

Figure 2. Evolutionary analysis of mycobacterial isolates from tank milk samples and human specimens. (a) Evolutionary tree based on whole-genome sequence analysis of 22 nontuberculous mycobacteria (NTM) isolates representing all groups detected in a Wisconsin dairy herd (designated with T) and human isolates from Wisconsin patients (designated with W). The unrooted tree is based on whole-genome sequences using Maximum Unique Match algorithm in the Harvest package and visualized by MEGA X software (https://www.megasoftware.net/). The nodes represent unique matches between bacterial genomes. The value on each branch is the posterior probability, showing the percentage support for the following node. (b) A heat map of NTM isolates displaying percentage of the total SNPs with synonymous (Syn) and nonsynonymous (Non-Syn) polymorphism when each isolate is compared with its own standard strain. The heat map was generated using GraphPad Prism version 7 (GraphPad Software Inc., La Jolla, CA).

reduce or prevent contamination of raw milk with NTM during and after milking is of a paramount importance to avoid human illness associated with NTM infection.

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