

Mastitis associated with *Mycobacterium smegmatis* complex members in a Swiss dairy cattle herd: compost bedding material as a possible risk factor

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Animal welfare has assumed an important aspect for dairy milk product producers and consumers (Barkema et al., 2015). Therefore, freestall barns are becoming very popular in Switzerland and other European countries, allowing free movements and social interactions between cows (Chaplin et al., 2000). Freestall cubicle systems are most commonly used because they prevent accidental treading on injuries and grant individual animal resting. In addition to the housing system and dimension, the bedding material is crucial (Burgstaller et al., 2016). Dried manure, straw, sawdust and chopped woodchips are the most popular bedding material used in freestall barns. Nowadays compost bedded pack barns for dairy cows, consisting of a large bedded pack area and a feed alley with slatted floor, are cost effective and reduce the surface needed to house livestock (Black et al., 2014). Composted biodegradable waste represents a valid alternative to previous mentioned bedding materials, whereas lower mastitis prevalence and low somatic cell count in bulk tank milk compared to other housing systems have been described (Barberg et al., 2007). Bacterial contamination is decreased by heat treatment of the biodegradable waste for three days at 70°C and subsequent treatment with *Lactobacillus* spp. (Burgstaller et al., 2016). This inactivates weed seeds and, moreover, prevents infections of the mammary gland since bedding material may represent a primary source of environmental pathogen causing mastitis in dairy cows (Barberg et al., 2007). Hence, occurrence of clinical mastitis is related to the total bacterial load on the teat and in the bedding material (Hogan et al., 1989). The mentioned findings are related to the most common etiologic agents of bovine mastitis and little is known about rarely isolated pathogens like rapidly growing mycobacteria (RGM). RGM are aerobic, non-motile, opportunistic acid-fast bacteria normally present in water and soil (Brown-Elliott und Wallace, 2002). To date, three major groups are commonly identified among the RGM: *M. fortuitum*-, *M. chelonae-abscessus*- and *M. smeg-*

matitis complex (Brown-Elliott und Wallace, 2002). In veterinary medicine, members of the *M. smegmatis* complex are currently recognized as etiological agents of bovine mastitis (Richardson, 1970; Machado et al., 2015). Although there are numerous reports of infections caused by non-tuberculous mycobacteria (NTM) in veterinary literature, isolation of these pathogens is usually challenging due to their fastidious growth on culture and misinformation among practitioners. To date, the most accessible and accurate identification method for members of the *M. smegmatis* complex is based on molecular techniques including 16S rRNA and *rpoB* gene sequence analysis (Adékambi und Drancourt, 2004). Previous work from Adékambi *et al.* demonstrates the usefulness of the sequence-bases characterization of emerging non-tuberculous mycobacteria (Adékambi und Drancourt, 2004). Since 16S rRNA gene sequencing is commonly considered the first-line method for identification of unusual NTM isolates, large amount of free accessible data in this regard are available today, despite Adékambi *et al.* demonstrated the insufficient discriminatory power for large clinical NTM collections. Generally accepted differentiation criteria are to date missing and common 16S rRNA gene sequence similarity averages are not applicable to mycobacteria (Adékambi und Drancourt, 2004).

This report describes the isolation of two members of the *M. smegmatis* complex from two cows of the same holding showing milk alterations. Different molecular methods for identification of NTM to species level are illustrated. Higher mastitis rate in a Swiss herd composed by 60 Holstein dairy cows with free stabling was noticed after compost bedded pack change in summer 2016. The bedding material consisted of sawdust and woodchips mixed with organic biodegradable waste. The general proportion of woodchips to biodegradable waste varied between 50/50 during winter and 1/3 digestate to 2/3 woodchips during summer. Composting material

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was originating from a local company, which elaborates biodegradable garden and household catering waste. These are commonly heat treated for ten to fourteen days at 55°C and directly sold to farmers. The bedding mixture is then aerated once to twice daily, typically during milking. This procedure allows the incorporation of manure and urine into the compost, resulting in a dry laying surface for the cows. In the present study, the bedding material contained more humidity than the routinely used compost and mould growth was observed. After this change, 10% of the herd developed acute mastitis with different clinical signs and aetiological agents. While in two cases mastitis was caused by *Staphylococcus*- and *Streptococcus*-species and was successfully treated, a third cow was euthanized after ineffective topical therapy with penicillin and third generation cephalosporins. Milk samples from two additional Holstein cows were collected and analysed because of considerable alteration of early milking milk. The two animals showed no clinical signs or induration of the udder. Cows had a milk yield of 40 L per day at time of analysis. Considering the higher incidence of mastitis among the herd after the last compost change, samples were collected prior to its removal.

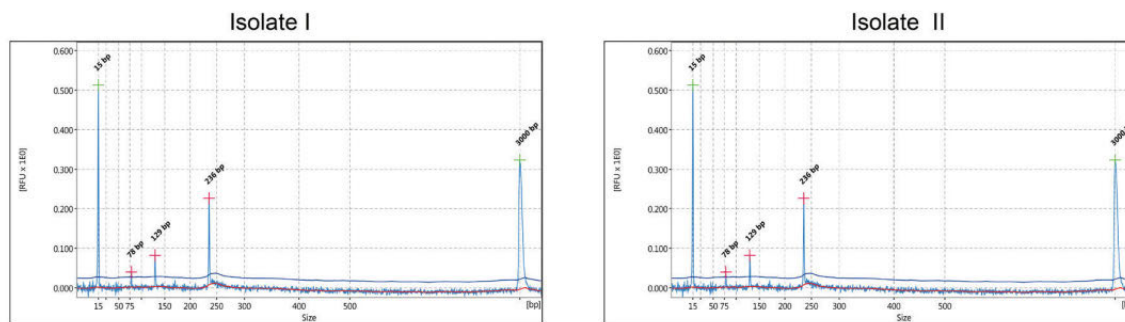
Laboratory examinations

Bacteriological examinations of the milk samples were performed according to standard procedures (Anonymous, 1999). Growth of smooth to mucoid, white colonies was observed after three days of incubation on sheep blood agar at 37°C. Gram staining revealed poorly staining, slender gram-positive rods, while after Ziehl-Neelsen distinct acid fast bacilli were visible. Based on these results, further bacteriological analyses were performed. Two grams of compost specimen was homogenized in 20 ml NaCl (0.9%) using a rotating-blade macerator system (T18 Digital Ultra-Turrax IKA, Staufen, Germany) and subsequently centrifuged 15 min at 3000 × g. After discarding the supernatant, the sediment was decontaminated according to the method described by Ghielmetti and co-workers (Ghielmetti et al., 2017). Briefly, 0.25 ml of decontaminated inoculum was transferred to a BBL MGIT liquid media tubes (Becton, Dickinson & Company, Allschwil, Switzerland) and incubated at 37°C. Three subcultures on 7H10 agar slants (BD) at intervals of three to four days were necessary in order to obtain a pure mycobacterial culture. DNA from cultured mycobacteria was extracted through mechanical cells lysis using a TissueLyser II (Qiagen, Hilden, Germany) and enzymatic digestion with Proteinase K overnight. Automated DNA purification was performed using the QIAcube instrument in accordance with the QIAamp cadior Pathogen Mini Kit protocol (Qiagen). DNA concentration in the final eluate was measured by

reading the absorbance at 260 nm using a NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific, Reinach, Switzerland), diluted to a concentration of 10 ng/μl and stored at -20°C until use. Molecular species identification was performed by partial sequencing of the 16S rRNA gene with universal primers (Lane, 1991) combined with sequencing of the Adékambi region of the *rpoB* gene (Adékambi et al., 2003). DNA sequencing was performed at Microsynth (Balgach, Switzerland). Resulting 16S rRNA and *rpoB* sequences were assembled using CLC Genomics Workbench 7.5.1 (Qiagen) and BLAST similarity searching for multiple sequence alignment was performed (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Further characterization of the isolates belonging to the *smegmatis* complex has been achieved by PCR restriction analysis (PRA) of the *hsp-65* gene as described by Brown et al. (Brown et al., 1999). Restriction fragments were analysed by capillary electrophoresis, using the OM1700 separation method (Ghielmetti et al., 2017). Control strains included *M. smegmatis* ATCC 19420TM and *M. goodii* Brown et al. ATCC BAA-955TM.

Results

Sequencing of 16S rRNA revealed *M. porcinum* species from compost with an identity score of 1363/1363 bp compared to *M. porcinum* strain ATCC 33776, *M. goodii* species for isolate I from the first cow with an identity score of 1366/1371 bp compared to *M. goodii* strain ATCC 700504 and *M. smegmatis* species for isolate II from the second cow with an identity score of 1366/1366 bp compared to *M. smegmatis* strain ATCC 19420. This allowed the identification of two out of three isolates, namely *M. porcinum* and *M. smegmatis*, whereas the identity of isolate I remained uncertain and was classified as a member of the *M. smegmatis* complex according to Brown et al. (Brown et al., 1999). Sequencing of the Adékambi region of the *rpoB* gene, known to be more discriminatory compared to 16S rRNA (Enrico Tortoli, personal communication) confirmed the identity of isolate II as *M. smegmatis* with an identity score of 761/761 bp compared to *M. smegmatis* strain ATCC 19420, as well as *M. porcinum*, identity score of 761/761 bp compared to *M. porcinum* strain CIP 105392. However, the identification of isolate I at species level remained uncertain after sequencing of the *rpoB* gene, since to closest strain identified using BLAST, *M. goodii* strain X7B accession No. CP01215, showed an identity score of 751/767. PRA of the *hsp-65* gene, using two selected commercial restriction endonucleases, *BstEII* and *BsaHI* enabled the characterisation of isolate I among the *M. smegmatis* complex. Exhibiting a three *BsaHI*-derived PRA band pattern (82, 100 and 259 bp) as shown in Figure 1, isolate I was classified as *M. goodii*.

BstEII = *Eco91I*

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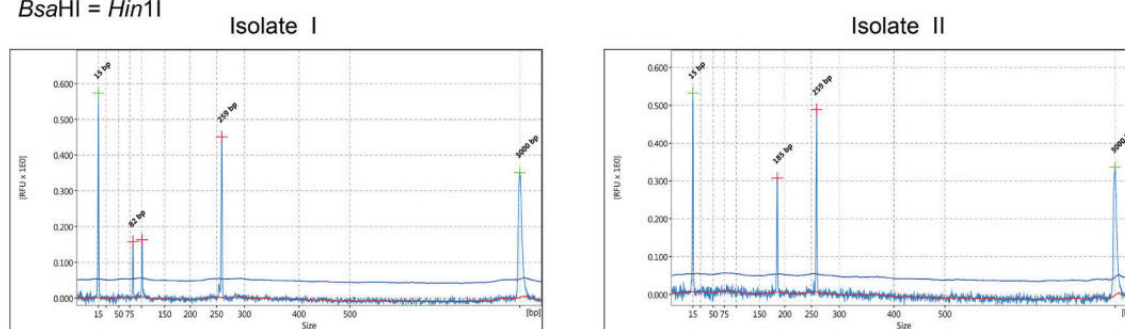
BsaHI = *Hin1I*

Figure 1: Differentiation of the *smegmatis* group members by PRA. For each isolate a 493 bp segment of the *hsp-65* gene was analysed using *BstEII* and *BsaHI* restriction endonucleases according to Brown B.A. *et al.* 1999. Restriction fragments size was determined by capillary electrophoresis, using the OM1700 separation method with a QX DNA high-resolution cartridge. QX 15 bp – 3 kb alignment marker peaks are displayed for each run.

ii, while isolate II, exhibiting a two *BsaHI*-derived PRA band pattern (185 and 259 bp) was confirmed to be a *M. smegmatis sensu stricto* according to Brown *et al.* (Brown *et al.*, 1999).

Discussion

About one hundred and fifty species of NTM have been reported worldwide, more than 60% of these are to date known to be pathogenic to humans or animals (Biet und Boschiroli, 2014; Tortoli, 2014). Unequivocal identification of these members among the genus *Mycobacterium* is often a challenging task due to their high genetic similarity compared to other microorganisms (Kabongo-Kayoka *et al.*, 2017). However, this effort should not be underestimated since patient management, therapy and measures at herd's level are dependent from the isolated pathogen. The discriminatory power of 16S rRNA gene sequencing alone was found to be insufficient for accurate identification of the isolated NTM at species level. Therefore, in the present study, a stepwise algorithm combining sequencing of 16S rRNA and *rpoB* in addition to PRA of the *hsp65* gene was adopted. These techniques allowed the identification of three NTM species, two of which described to be closely related and

appertaining to the *M. smegmatis* complex. Despite the fact that mastitis in cattle caused by NTM is an uncommon finding, in our opinion, numerous cases in large animals remain undiagnosed or misdiagnosed (Richardson, 1970; Machado *et al.*, 2015). Previous cases of udder infections reported in the literature proved the pathogeny of *M. smegmatis*. Although modern identification techniques like those described in the present study enable the identification of another close related pathogen: *M. goodii*.

The present study demonstrates that compost bedding material represent a source of RGM and may be associated with a higher incidence of NTM's linked mastitis. Therefore, more substantial investigations concerning bovine mastitis in association with compost bedded freestall barns holding systems are needed, since this housing trend is increasing. Considering that inactivation of *Mycobacteria* requires laborious procedures compared to other microorganisms, their maintenance in the environment and in the milking system represent a potential infection source for other cows or for raw milk products consumers.

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