Multiple locus variable number tandem repeat analysis of *Mycoplasma bovis* isolated from local and imported cattle

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*Abstract*

*Mycoplasma bovis* is an important and emerging pathogen of cattle. In this study, multiple locus variable number tandem repeat (VNTR) analysis was used to differentiate *M. bovis* type strain PG-45 and 68 *M. bovis* field isolates, including 34 isolates from calves imported to Israel from Australia, Lithuania, and Hungary in the period 2006–2011, 32 isolates from mastitic dairy cows in Israel in the period 2000–2011, one isolate from the pneumatic lungs of a calf in Israel in 2010 and one isolate from frozen bull semen in Israel in 2008. A total of 33 VNTR types were distinguished, including three, eight and 10 different VNTR types among isolates from calves imported from Australia, Hungary and Lithuania, respectively, and 17 VNTR types among isolates from dairy cows in Israel. The VNTR types in isolates from Lithuanian calves were not identified among isolates from Israeli dairy cows. VNTR type XX, present in the Hungarian group, was identified in one Israeli mastitis-associated isolate. A cluster of 16 *M. bovis* isolates from Israeli dairy cows possessed the same VNTR type III as three Australian isolates from a single shipment of calves in 2006. The other clusters of isolates contained *M. bovis* strain 883, isolated from a mastitic cow, strain 72236, isolated from a calf with pneumonia, two isolates from calves imported from Australia to the same farm 3 months previously and four isolates from calves in quarantine imported to Israel from Australia in 2009–2010. Multiple locus VNTR analysis is a useful tool for understanding the movement and spread of strains of *M. bovis* within and across international boundaries.

*Introduction*

Bovine mycoplasmosis caused by *Mycoplasma bovis* may be manifested as pneumonia, mastitis, arthritis and/or otitis media (Gonzalez and Wilson, 2003; Nicholas and Ayling, 2003). *M. bovis* has an economic impact on the cattle industry in the USA (Maunsell and Donovan, 2009) and in Europe, where it is estimated to cost more than €150 million1 per year (Nicholas and Ayling, 2003).

*M. bovis* was first isolated in Israel from an outbreak of bovine mastitis in 1964 (Bar-Moshe, 1964) and has been increasingly implicated in bovine morbidity and mortality, especially bovine respiratory disease (BRD), in the last decade. Until 2008, *M. bovis* was isolated only sporadically from cases of mastitis (0–3 positive herds per year during 2004–2007; unpublished data). However, an outbreak of mastitis due to *M. bovis* affected 18 herds in 2008 and *M. bovis* was isolated from cases of mastitis in six, seven and four herds in 2009, 2010 and 2011, respectively (unpublished data). In addition, outbreaks of BRD from which *M. bovis* has been isolated have occurred in shipments of imported calves in quarantine in Israel. According to the Israeli Veterinary Services, 96,565 and 108,722 calves were imported to Israel from several European countries and Australia in 2010 and 2011, respectively.

Molecular typing and fingerprinting of bacterial pathogens are integral to epidemiological investigations and disease monitoring. Multiple locus variable number tandem repeat (VNTR) analysis (MLVA) has been described for molecular typing of *M. bovis* (Pinho et al., 2012) and modified by Spergser et al. (2013). The aim of the
present study was to use MLVA to type *M. bovis* field isolates from Israeli dairy cows with mastitis and from calves imported to Israel from Australia, Lithuania and Hungary.

**Materials and methods**

**Sampling and isolation of Mycoplasma bovis**

Milk samples from dairy cows with clinical or subclinical mastitis were collected by farmers according to the recommendations of the National Mastitis Council Milk Quality Monitoring Committee (Hogan et al., 1999). After fore-stripping, the teats were pre-dipped with an approved teat disinfectant for 30 s, dried with an individual disposable paper towel and scrubbed for 15 s with 70% alcohol. From each quarter, 5–20 ml milk were collected into sterile labelled tubes and stored at 4 °C until cultured within 24 h. 

Nasopharyngeal swabs or synovial fluid were collected from live animals at the quarantine station and on-farm. Lung samples were collected at postmortem examination at the Kibron Veterinary Institute (KVI). To clarify a possible epidemiological link between artificial insemination and the 2008 *M. bovis* mastitis outbreak, an isolate of *M. bovis* from frozen semen maintained at the Israeli Company for Artificial Insemination and Breeding (Hafetz Haim, Israel) was included in the study. 

The mode of entry for imported calves originating from Australia was by sea and calves were maintained in quarantine stations located in southern Israel (Eilot or Tzofar). Calves imported from Lithuania and Hungary entered Israel via air and were kept in quarantine stations located either in Haifa (North) or Ramle (Centre). Samples from quarantine stations were transported to KVI on the day of sampling and cultured within 24 h. No transport medium was used. Postmortem specimens and clinical samples were collected at the farms of the animals in quarantine, from a frozen semen sample of a bull (n = 1) and from a nasopharyngeal swab of a calf with no clinical signs (n = 1; a calf on-farm imported from Australia) (Fig. 1).

**Multiple locus variable number tandem repeat analysis of Mycoplasma bovis isolates**

The number of allelic profiles, obtained by different VNTRs, ranged from 2 to 7. Compilation of the allelic profiles of the 10 VNTRs resulted in 35 final VNTR types among the 69 isolates of *M. bovis* included in this study (Fig. 1). Isolates from calves imported from Australia, Hungary and Lithuania had three, eight and 10 VNTR patterns, respectively, whereas the isolates from dairy cows in Israel belonged to 17 VNTR types (Fig. 1).

On the basis of polymorphism identity among final VNTR types, *M. bovis* isolates could be divided into two main groups, designated A and B (Fig. 1). Group A included 27 isolates (Lithuania: n = 12; Hungary: n = 8; Australia: n = 1; Israel: n = 5; type strain PG45), with 21 VNTR types. Group B included 42 isolates (Lithuania: n = 1; Hungary: n = 3; Australia: n = 9; Israel: n = 29), with 14 VNTR types (Fig. 1).

The VNTR types in isolates from Lithuanian calves were not identified among the isolates from Israeli dairy cows. VNTR type XX, present in the 2010 Hungarian isolate F127, was identified in only one Israeli mastitis-associated isolate (510) from 2011 (Fig. 1). A cluster of 16 Israeli *M. bovis* isolates (group B) had 100% polymorphism identity to three Australian isolates (2E, H and 1254) from a single shipment of calves in 2006. Ten of 12 *M. bovis* isolates from the mastitis outbreak in 2008 were associated with this cluster (Fig. 1).

The other cluster of isolates within group B contained *M. bovis* isolate 883 from a mastitic cow and isolate 72236 from a local Israeli calf with pneumonia, which had 100% polymorphism identity to two isolates (314 and 612) from calves imported 3 months earlier onto the same farm from Australia. All of these isolates grouped with four Australian isolates (58234, 41569, 2583/3 and 63307) from quarantined calves in 2009–2010. Two clusters, namely VNTR types III and IV, differed by only one marker, TR427. *M. bovis* strain 13, which was representative of a group of five *M. bovis* isolates from frozen semen of five different bulls, demonstrated VNTR type XXIX, which was not identified in any *M. bovis* isolates in this study. These five *M. bovis* type XXIX semen isolates and three *M. bovis* isolates with VNTR type III were typed using ISMbov3 and ISMbov4 probes (Lysnyansky et al., 2009). All semen isolates showed the same genotype by IS-typing, which differed from the final IS-genotype obtained for three *M. bovis* strains isolated from dairy cows in 2008; both ISMbov3 and ISMbov4-related types differed between the two groups (data not shown).

**Discussion**

In Israel, calf pneumonia is the most frequent manifestation of *M. bovis* infection, whereas mastitis due to *M. bovis* was infrequent until 2008, when an outbreak affected 18 dairy farms. Most dairy herds in Israel maintain a ‘closed herd’ policy, which means that the farms use artificial insemination and raise their own replacement cows, but rarely introduce new cows from other farms. However, sometimes feedlots with high turnover of livestock are located in close proximity to the dairy herds, which might result in introduction of *M. bovis* to naive herds. Our study demonstrated...
Fig. 1. Dendrogram showing the genetic relationships among *M. bovis* isolates by multilocus variable number tandem repeat (VNTR) analysis. AU, Australia; L, Lithuania; H, Hungary; I, Israel; BRD, Bovine respiratory disease complex; A–L: The same letter indicates that *M. bovis* strains were isolated from the same shipment or on the same farm.
VNTR identity between \textit{M. bovis} isolates from Israeli dairy cows and calves imported from Australia, suggesting the possibility of the introduction of strains from imported animals into local dairy herds (Fig. 1).

The presence of the same VNTR type (type III) in 10/12 \textit{M. bovis} isolates from the outbreak of mastitis in dairy cows in Israel in 2008 points to a common source of infection. However, no epidemiological connection among affected herds has been identified and therefore the source of infection and mode of spread of this strain among dairy herds is unknown. In contrast, 6/7 \textit{M. bovis} isolates from mastitis cases during the period 2000–2007 possessed other VNTR types. The two other VNTR types identified during the 2008 mastitis outbreak were type XXVII, which differs from type III by TR-30, and type XXVIII, which differs from type III by TR147, TR148, TR31, TR35 and TR427.

The VNTR and IS-typing related data obtained for \textit{M. bovis} strain 13, isolated from frozen semen, indicate a lack of evidence for \textit{M. bovis} transmission via artificial insemination during the mastitis outbreak of 2008, although typing of a single isolate is not proof that this mode of transmission can be excluded. IS-typing of \textit{M. bovis} isolates from herds with outbreaks of mycoplasmal mastitis or pneumonia in Switzerland using IS\textit{Mbov1} and IS\textit{Mbov2} probes demonstrated that \textit{M. bovis} strains were similar within herds, but markedly divergent between herds (Aebi et al., 2012).

An additional example of possible introduction of \textit{M. bovis} to a dairy herd is an outbreak of BRD followed by mastitis that occurred on one Israeli farm in 2010; 190 calves from Australia were brought to the farm 3 months before the outbreak. MLVA typing grouped together \textit{M. bovis} isolates from the farm (883 and 72236 isolated from mastitic milk and pneumatic lungs, respectively; 314B and 612 isolated from calves imported from Australia) and \textit{M. bovis} isolates from Australian calves in quarantine (58234, 41569, 2583/3 and 63307). Although no calves introduced to the farm, nor cattle from nearby farms, were sampled before the outbreak, there is indirect evidence for a connection between the BRD/mastitis event and the introduction of calves originating from Australia: (1) \textit{M. bovis} had not been identified on the farm previously; (2) this was the first introduction of imported calves to the farm; (3) a group of lactating cows on the farm developed severe BRD, whereas imported calves had inapparent infection or relatively mild clinical signs, suggestive of introduction of \textit{M. bovis} to a naïve herd; and (4) isolates from the farm had the same VNTR type (IV) identified in four previous \textit{M. bovis} isolates from Australian calves in quarantine (2009–2010).

Transmission of \textit{M. bovis} from replacement animals that are introduced to dairy farms has a substantial impact at the herd level (Jasper, 1981; Bushnell, 1984; Gonzalez and Wilson, 2003). Clinical mastitis due to \textit{M. bovis} has been reported in imported cows in Sudan (Abbas, 1996) and Greece (Filioussis et al., 2005). Circumstantial evidence suggests that purchased cattle have been the source of several \textit{M. bovis} mastitis outbreaks (Bicknell et al., 1978, 1983; Gunning and Shepherd, 1996). In dairy herds in Australia, the prevalence of \textit{M. bovis} in milk samples by PCR was 43\% \((n = 183\) herds examined) in Victoria and 62\% \((n = 167\) herds examined) in Queensland (Ghadersohi et al., 1999). \textit{M. bovis} was detected by PCR and culture in quarter milk samples from 40/52 (77\%) cows with persistently high somatic cell counts (Ghadersohi et al., 1999). The prevalence of antibodies against \textit{M. bovis} in serum samples from 100 dairy cattle in North Queensland using a monoclonal blocking ELISA was 60\% (Ghadersohi et al., 2005).

The results of this study showed that cohorts of \textit{M. bovis} isolates from calves imported into Israel from Lithuania, Hungary and Australia had a marked diversity of VNTR types and that no genotypes related to the country of origin could be identified. The cohort of \textit{M. bovis} isolates from Australian calves was less diverse (only three VNTR types), but these isolates were from a smaller number of shipments \((n = 5)\) from Australia, whereas other isolates were derived from 12 Lithuanian and nine Hungarian shipments. In addition, Australian calves are imported to Israel by ships and are confined together during shipping, which may promote the selective spread of certain \textit{M. bovis} strains.

Using MLVA, 12/13 \textit{M. bovis} isolates from Lithuania and 8/11 \textit{M. bovis} isolates from Hungary were clustered in group A. Similarity by MLVA among \textit{M. bovis} isolates from several European countries was recently reported by Pinho et al. (2012), who suggested that this reflects the extensive trade among countries in the European Union.

Conclusions

This study shows that MLVA-typing may provide a useful tool for molecular tracking of \textit{M. bovis} strains in order to understand the movement and spread of strains within and across international boundaries. In general, VNTR-types present in isolates from animals originating in European countries differed from those found in Israeli and Australian cohorts. However VNTR identity between \textit{M. bovis} strains isolated from Israeli dairy cows with mastitis and from calves imported from Australia suggests an epidemiological link.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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