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Semen as a source of *Mycoplasma bovis* mastitis in dairy herds

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ABSTRACT

Mycoplasma bovis infections are responsible for substantial economic losses in the cattle industry, have significant welfare effects and increase antibiotic use. The pathogen is often introduced into naive herds through healthy carrier animals.

In countries with a low prevalence of *M. bovis*, transmission from less common sources can be better explored as the pathogen has limited circulation compared to high prevalence populations. In this study, we describe how *M. bovis* was introduced into two closed and adequately biosecure dairy herds through the use of contaminated semen during artificial insemination (AI), leading to mastitis outbreak in both herds. Epidemiological analysis did not reveal an infection source other than semen. In both farms the primary clinical cases were *M. bovis* mastitis in cows inseminated with the semen of the same bull four weeks before the onset of the disease. One semen straw derived from the semen tank on the farm and other semen lots of this bull were positive for *M. bovis*. In contrast, semen samples were negative from other bulls that had been used for insemination in previous or later oestrus to those cows with *M. bovis* mastitis. Furthermore, cgMLST of *M. bovis* isolates supported the epidemiological results. To our knowledge this is the first study describing the introduction of *M. bovis* infection into a naive dairy herd via processed semen. The antibiotics used in semen extenders should be re-evaluated in order to provide farms with *M. bovis*-free semen or tested *M. bovis*-free semen should be available.

1. Introduction

Mycoplasma bovis infection causes substantial economic losses and welfare effects in the cattle industry (Nicholas and Ayling, 2003) and increases the use of antibiotics. The infection presents a variety of signs, the most common being respiratory disease, mastitis and joint infections. (Byrne et al. 2001). The prevalence of *M. bovis* varies in different countries and areas.

Animal contact is the main source of *M. bovis* infection. The major risks for infection are related to animal movement, animal purchase and animal fairs (Amram et al. 2013; Aebi et al. 2015). Other well known risks include artificial insemination (AI) (Wrathall et al. 2007), embryo transfer (Bielanski et al. 2000), contaminated equipment and environment (Piccinini et al. 2015), airborne transmission (Jasper et al., 1974) and contact with infected people (Madoff et al. 1979) or other animal species (Dyer et al. 2004; Ongor et al. 2008; Spergser et al., 2013).

A reproductive challenge study was able to induce *M. bovis* intramammary infection in nine cows following intra-amniotic or intra-arterial inoculation (Ruben, 1980). *Mycoplasma bovis* has been isolated in commercial semen (Amram et al. 2013), with *M. bovis* positive semen reported to cause alterations in the fertilization process leading to infertility (Eaglesome and Garcia, 1990) and pathological alterations in the reproductive organs after experimental intrauterine challenge (Hartman et al. 1964).

In countries with a low prevalence of *M. bovis*, transmission from less common sources can be better explored as the pathogen has limited circulation compared to high prevalence populations. *Mycoplasma bovis* was detected for the first time in Finland at the end of 2012 and has since spread among dairy herds. It has since been monitored because it is of national interest to control the infection. In this study, we describe how *M. bovis* was introduced into two closed and adequately biosecure dairy herds through the use of contaminated semen during artificial insemination (AI), leading to mastitis outbreak in both herds. As far as we know, our study is the first to demonstrate that semen used in AI can

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be the initial source of *M. bovis* infection on a farm.

2. Material and methods

2.1. Surveillance of M. bovis in Finland

M. bovis was detected in Finland for the first time in November 2012 in deep nasopharyngeal samples from pneumonic calves in a calf rearing farm. The calves in this rearing unit originate from several dairy farms. The infection was indicated to be recent. *Mycoplasma bovis* was not detected in a research project on bovine respiratory disease in calf-rearing units in 2002–2003 (Autio et al. 2007). Since 2003, suspected cases, such as indicated by respiratory disease and aborted fetuses, have been examined for *M. bovis* using culture and PCR. Mastitis pathogen testing of individual milk samples from clinical and subclinical mastitis has a long tradition in Finland. Roughly 150 000 samples were tested in 2015 (285 000 cows in Finland in 2015). Since early 2012, almost all mastitis diagnostic laboratories use multiplex PCR tests that target also *M. bovis* (Thermo Scientific PathoProof Mastitis Complete-16 assay, Thermo Fisher Scientific Ltd.). Practicing veterinarians have to report all *M. bovis* cases monthly to animal disease control authorities. During 2012–2015, altogether 20 Finnish dairy farms were infected with *M. bovis*, which represented 0.26% of all 7 600 Finnish dairy farms in 2015.

Since 2013 the organization Animal Health ETT maintains a voluntary *M. bovis* control program for cattle farms. The program includes regular veterinary health care visits, restricted animal movement, surveillance of signs and laboratory examination of subclinical and clinical mastitis samples with PCR and nasal swabs from calves. The data are entered into a centralized health care register (Naseva) for cattle herds. The register includes production data, health records and veterinary treatments. A total of 75% of all dairy farms belonged to the health register in 2017 (Animal Health ETT, personal communication).

2.2. Farms in the study

Farmers from 20 newly infected *M. bovis* dairy farms were interviewed and their herds were sampled. Epidemiological data were collected to assess the infection source. In two concurrently infected farms the epidemiological data did not suggest a typical infection source and the farms (X and Y) were investigated in more detail.

2.3. Herd data collection

Herd data were collected using the centralized health care register and by use of a questionnaire. A project veterinarian visited both farms twice. Information gathered from the registers included the number of cattle and cows, milk yield, mortality rate, treatments, dates of *M. bovis* mastitis, the number of cattle slaughtered, meat rejected, the number of milk samples, numbers of purchased and imported cattle, milk-recording system and laboratory results of nasal swab analyses related to the *M. bovis* control program.

Information gathered from the farmers included the housing type, milking system, bedding material, cattle movements, contract heifer rearing unit, imported or domestic embryos, health care visits, protective clothing, loading area, corporate truck, equipment of the hoof trimmer and AMS (automatic milking system) maintenance, vermin and bird control, the number of cattle slaughtered, meat rejected and participation in the *M. bovis* control program.

Distance to the closest cattle farm was determined from a national register. Insemination data (dates, bulls and lots) was gathered from the Finnish Animal Breeding Association (FABA).

2.4. Samples

We examined 98 deep-frozen semen straws, representing 32 lots from ten bulls (A–J) used in AI in herds X and Y. Four semen straws were taken from the liquid nitrogen tank from farm X, and the rest were obtained from the breeding company. The straws examined belonged either to the same lot used in the herds or the closest lot available.

Semen from bull A was collected and handled at a semen collection center and was examined according to OIE requirements. Collection of semen started at the age of 11 months. Based on the results of semen samples, conjunctival swabs, nasal swabs, preputial swabs (Transsystem, Copan Brescia, Italy), pre-ejaculate and semen samples were taken from the bull A at the age of 2 years 4 months. Pre-ejaculate and semen were placed in F-broth for mycoplasma culture.

A total of 15 and 20 calves were sampled on farms X and Y, respectively, 4 to 5 weeks after the primary infection. Nasal swabs (Transsystem) were collected from calves 1 week to 6 months old.

2.5. Culture of M. bovis

The straws (n = 58) were thawed in 37 °C water bath. Semen from the straw was inoculated in a tube containing 2.7 ml F broth prepared according to Bölske (1988). Inoculated F broth was diluted 10-fold up to 10^{-6} and incubated at 37 °C for 14 days. The growth and color change were monitored every other day, and samples suspected of containing mycoplasma were subcultured onto F-medium plates (Bölske, 1988). Plates were incubated at 5% CO_2, 37 °C for seven days, and inspected every second day under the microscope for mycoplasma growth. *Mycoplasma bovis* was identified using PCR.

2.6. DNA extraction and Mycoplasma bovis real time PCR

DNA was extracted from semen straws (n = 40) using a QIAamp Mini Kit (Qiagen, Hilden, German), following the manufacturer's protocol for blood and body fluids. DNA was eluted from the spin column with 50 µl of water. The protocols described by Sachse et al. (2010) were used for nasal swabs and broth cultures.

Semen, nasal swabs and broth cultures were examined using real time PCR targeting the *opp*D gene of *M. bovis* (CFX96 Touch Real-Time PCR Detection System, Bio-Rad Laboratories, CA, USA) as described previously (Sachse et al. 2010). Commercially available plasmid pUC19 was used as the internal amplification control according to Fricker et al. (2007), except that BHQ1 was used instead of TAMRA in the probe.

2.7. Whole genome sequencing

Whole Genome Sequencing (WGS) was used to compare genomes. A total of 11 *M. bovis* isolates were included in the study (Table 1): the isolate 11911 from the first mastitis case on farm X, the isolate 13775 from the first mastitis case on farm Y, the isolate 198 from semen lot 3 from bull A, seven isolates obtained from diseased cattle in Finland during 2012–2015, and one isolate from a pneumonic calf in Estonia. The isolates were selected to represent various herd types, clinical presentations and geographical distribution within Finland, including isolates from the first two *M. bovis* cases in Finland.

All isolates were purified three times before freezing and were stored in F-broth medium (Bölske, 1988) at −80 °C. For DNA extraction, the isolates were grown in 50 ml F-broth in closed tubes at 37 °C for 90 h. The bacteria were spun down at +4 °C, 19,800 g, for 30 min. The pellets were washed with sterile PBS and spun down again. The pellets were resuspended in 180 µl of sterile PBS. DNA was extracted using a QIAamp Mini Kit, following the manufacturer's protocol for blood and body fluids. DNA concentration was measured (Qubit dsDNA BR assay system, Invitrogen, Carlsdab, CA) and quality assessed in 0.8% gel electrophoresis.

WGS was done at the Danish Technical University, Department for Biotechnology and Biomedicine, Lyngby, Denmark. A Nextera XT kit (Illumina, San Diego, CA) was used according to the manufacturer's instructions to prepare the libraries for WGS. An Illumina MiSeq