Identification of *Actinobacillus seminis* as the cause of abortion in sheep by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and whole genome sequencing

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Abstract

Actinobacillus seminis is a gram-negative bacterium that affects reproductive organs, causing epididymitis, low fertility, and occasional abortions in sheep and goats. The virulence factors and the pathogenicity mechanisms of *A. seminis* have not been clearly elucidated yet. The aim of this study is to report a laboratory investigation performed on a sheep farm in the Basilicata region (southern Italy) following cases of abortion. Four samples of aborted fetuses taken from two sheep were analyzed to investigate the main pathogens causing abortions by bacterial isolation. In addition, serum samples belonging to the sheep have also been analyzed. Analysis by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) of the isolated colonies identified protein spectra referable to *A. seminis*. This result was also confirmed through the use of genome sequencing. Therefore, these data could explain cases of abortion that occurred on the farm and would confirm that MALDI-TOF MS technology is a rapid, accurate, and inexpensive method for identifying bacteria.

Introduction

Actinobacillus seminis is a Gram-negative bacterium of the *Pasteurellaceae* family that is a natural inhabitant of the preputial mucosa of sheep and goats. However, *A. seminis* is also the causal agent of epididymitis and orchitis, occasional abortions in ovine and goats, low rates of fertility, and consequent economic losses to the sheep industry (Al-Katib *et al.*, 2009). Other bacteria, such as *Brucella ovis, Histophilus somni, Haemophilus* spp., *Corynebacterium pseudotuberculosis ovis, Chlamydia abortus*, are responsible for ovine epididymitis. These infections normally do not show any visible lesion to the epididymis, making it difficult to suspect, just by clinical examination, the presence of the disease. The virulence factors and the pathogenicity mechanisms of *A. seminis* have not been clearly elucidated yet. This microorganism is transmitted mainly by venereal means; however, *A. seminis* has been isolated from rams without a history of previous mating, and transmission may occur in other ways. In fact, one of the most accepted alternative transmission methods is lamb infection during the release of pregnant ewes (Moustacas *et al.*, 2013).

A. seminis infections usually progress asymptomatically during the early stages, being diagnosed only when the disease is established. In rams, these bacteria can cause an infection that resembles ovine brucellosis due to *B. ovis*, which is characterized by epididymitis and orchitis that are associated with subfertility or infertility. Histologically, the initial lesion of the epididymis is sim-

ilar to that of *B. ovis*. However, in aborted fetuses, it is very difficult to discriminate macroscopically the lesions due to these two bacteria (dos Santos *et al.*, 2014). For these reasons, conventional serological tests are used for screening and monitoring infected flocks, while definitive diagnosis requires isolation of the organism and subsequent differentiation from *B. ovis* (Santos *et al.*, 2019). In 2013, the Food and Drug Administration authorized the use of matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) technology for the identification of pathogenic microorganisms responsible for infections and is currently in use in veterinary laboratories (Thompson, 2022).

Therefore, this study describes cases of abortion of sheep that occurred on a farm in southern Italy attributable to *A. seminis*, isolated from organs of aborted sheep fetuses and identified by MALDI-TOF analysis and whole genome sequencing (WGS).

Materials and Methods

Case Report

In January 2023, four samples of fetuses and ovine fetal membranes arrived at the Experimental Zooprophylactic Institute of Apulia and Basilicata from a farm in the Basilicata region, in southern Italy, in which abortions had been underway for approximately 10 days.

In the flock, consisting of approximately 160 animals, there were 10 adult rams, all of reproductive age, of which one was suffering from epididymitis. Previously, serological positivity for *Brucella* had been found in the same company, but never confirmed by bacterial isolation.

The organs from the fetuses and the serum samples taken from the two sheep were diagnosed for infectious abortigenic causes, in particular for *Brucella* spp., *Salmonella* spp., and *Chlamydia* spp. *Brucella* research was conducted on organ pools (brain, liver, spleen, lung, and contents of the fourth stomach) of a single fetus, according to the method described in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals of the World Organization for Animal Health (WOAH, founded as OIE) (Manzulli *et al.*, 2022).

In addition to previous research, each organ homogenate was plated on 5% blood agar plates and incubated at 37°C under aerobic, anaerobic, and microaerophilic conditions (5% CO_2). The isolated colonies were analyzed by MALDI-TOF technology.

Sample preparation for matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis

The identification of all strains grown on media was carried out as previously described (Manzulli *et al.*, 2021). Briefly, isolates were picked with a toothpick and spread onto a 96-well steel plate. Afterward, 1 µL of α-cyano-4-hydroxycinnamic acid was added to each sample and the plate thus prepared was appropriately analyzed using a Microflex LT/SH™ mass spectrometer (Bruker Daltonics, GmbH, Bremen, Germany), which was operated in linear positive mode covering a mass to charge ratio (m/z) between 2000 and 20.000. Each spot of the target plate was hit with a pulsed nitrogen laser beam operating at 337 nm, with a frequency equal to 60 Hz. The instrument was calibrated using Bruker Bacterial Test Standard (Bruker Daltonik GmbH, Bremen, Germany) in the molecular weight range between 2 and 20 kDa.

The data were automatically processed by the MBT Compass 4.1.70 software (Bruker Daltonik GmbH, Bremen, Germany), and the mass spectra were compared with those of known microbial isolates from the commercial libraries (Bruker Daltonics GmbH, Bremen, Germany). For the MALDI-TOF mass spectrometry analysis, the results are expressed with log(score) values between 0 and 3.0, indicative of the matching between the sample spectrum and the main spectra profiles of the reference database. In agreement with the manufacturer's protocols, a log(score) between 1.7 and 2.0 indicates that identification could be reliable only at the genus level, while a log(score) >2.0 indicates that identification could be reliable at the species level of the organism. A log(score) <1.7 indicates that the identification of the bacterial genus is not possible.

Whole genome sequencing and bioinformatic analysis

Genomic DNA of *A. seminis* strain was purified automatically using DNA/RNA Purification Platform MagMAX™ CORE Nucleic Acid Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. DNA quality and concentrations were assessed by Qubit Fluorometer 3.0 using Qubit dsDNA HS Assay (Thermo Fisher Scientific, Waltham, MA, USA). WGS was performed, and sequencing reads were processed as previously reported (Castellana *et al.,* 2024). The draft genome sequence is deposited in NCBI GenBank, accessible through BioProject PRJNA1161487. Species identification was performed by both ribosomal multilocus sequence typing (rMLST, www.pubmlst.org, accessed on 26th August 2024) and pairwise genome comparisons (ANIblast, www.https://jspecies.ribohost.com/jspeciesws/#analyse, accessed on 26th August 2024), using assembly sequence as input. Gene and functional annotations throughout the genome were obtained by implementing BAKTA v1.9.4 (database v5.1). The computational screening of antibiotic resistance and virulence genes and the presence of plasmid was predicted using the ABRicate v0.8.1 tool within the Galaxy ARIES (Knijn *et al.,* 2020) Platform (https://aries.iss.it). The circular genomic map was drawn using PROKSEE server (Stothard *et al.*, 2005).

Results

On 5% blood agar plates, after 48-72 hours of incubation, grey colonies of 2-3 mm in diameter, slightly convex, surrounded by a weak halo of hemolysis, grown in both aerobic and microaerophilic conditions, were isolated. No growth was observed under anaerobic conditions.

Observation by optical microscope after Gram staining revealed Gram-negative coccus-rod-shaped microorganisms, which were subsequently tested for catalase and cytochrome oxidase with positive results in both cases. Culture isolation for *Brucella* spp. and serological test for *Salmonella* spp. and *Chlamydia* spp. resulted negative.

Further analysis by MALDI-TOF spectrometry of the isolated bacterial strains identified protein spectra referable to *A. seminis* with a log(score) of 2.10 (Figure 1). The species identified was confirmed by both rMLSTv (29 out of 53 exact locus matches) and ANIBlast comparative analysis (98.95% similarity with *A. seminis* ATCC 15768 reference genome). Additionally, the BAKTA tool identified 2986 coding sequences, of which 530 were predicted as hypothetical proteins, 42 tRNA, 1 tmRNA, 2 rRNA, and 19 non-coding RNA. The annotation performed by Prokka (Seemann *et al.*, 2014) in the Proksee server (Figure 2) resulted similar to that

made with the BAKTA tool. The genome sequence analyzed had a size of 2.4 MB and exhibited a guanine-cytosine content of 40.57%. No known antimicrobial resistance genes have been identified, while two genes have been predicted as "virulence-associated", being mildly similar (sequence coverage >90% and similarity <85%) to *gmhA/lpcA* (phosphoheptose isomerase) and *lpxC* (UDP-3-O-(R-3-hydroxymyristoyl)-N-acetylglucosamine deacetylase) from *Haemophilus influenzae Rd KW20*.

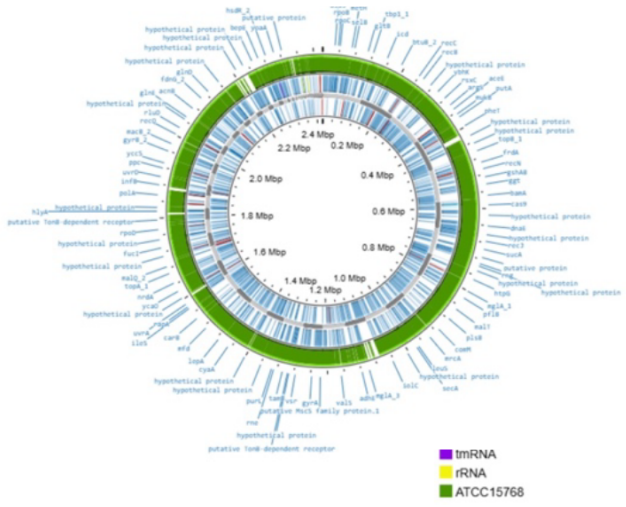

Figure 2. Genome visualization and annotation using Proksee. The blue ring showed coding sequences (n=2296) identified via genome annotation using Prokka; tRNA (n=41) were indicated in red; rRNA (n=2) were indicated in yellow; tmRNA (n=1) was indicated in purple. The green ring indicated the sequence similarity BLAST against *Actinobacillus seminis* ATCC15768.

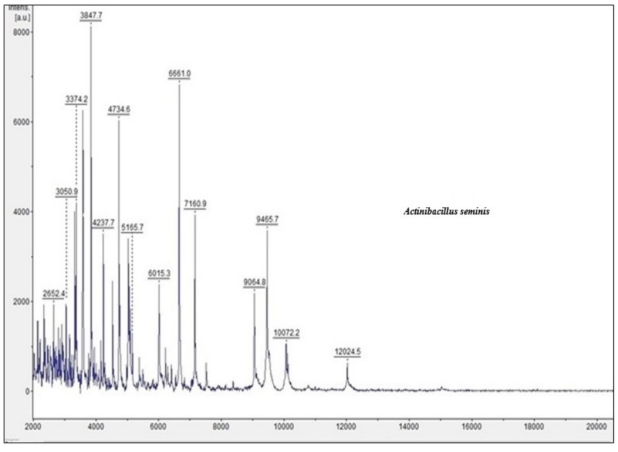

Figure 1. Matrix-assisted laser desorption/ionization-time of flight mass spectra of *Actinobacillus seminis* strain in the mass range of 2 to 20 kDa.

Discussion

A. seminis is well recognized as a common cause of ovine epididymitis and infertility, but in recent years, it has also been associated with cases of abortion in sheep (Al-Katib *et al.*, 2009). We reported a laboratory investigation performed on a sheep farm in the Basilicata region (southern Italy) during cases of abortion. The isolation of *A. seminis* from fetuses and ovine fetal membranes, in combination with the diagnosis of the bacterial infectious nature of the abortion cases described here, would also justify the presence of epididymitis within the flock. Interestingly, based on the information in our possession, this is the first deposition of the *A. seminis* genome in Italy, isolated from aborted fetuses in sheep.

These data suggest that differential diagnosis of *A. seminis* from other major abortifacient pathogens is important to understand the involvement of this pathogen in abortions and its epidemiological role in sheep livestock farming. Furthermore, this study may help to develop better diagnostic tests and treatment methods for this pathogen.

Conclusions

In conclusion, the identification of *A. seminis* using MALDI-TOF MS has highlighted how this approach can represent an "unconventional" method for the identification of bacteria to be used in the veterinary field as a screening method in routine applications. In addition, the WGS of bacteria can help increase the number of deposited genomes, which will aid the scientific community in further investigating the pathogenic power of the bacterium, which is still little studied to date.

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