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Identification of Bacteria Causing Subclinical Cow Mastitis: Comparative 16S DNA Sequence Analysis and Biotyping Using MALDI-TOF MS.

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ABSTRACT

Mastitis is one of the most common diseases affecting dairy cows and it causes major economic losses to the dairy industry. Identification of bacteria in udder quarters and cows is important for disease management in the herd and for targeting antimicrobial treatment. Current techniques employed for their detection include time-consuming and laborious phenotypic methods requiring costly equipment and consumables and highly trained staff. In this work we tested the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) as a tool for identification of bacteria causing subclinical mastitis for Algerian cows. Thirteen bacteria obtained from two dairy cows in the east of Algeria were analysed by MALDI-TOF MS and compared to the 16S rDNA gene sequencing as the reference molecular technique. Alternatively, rpoB gene was used as a secondary gene target when 16S rDNA did not allow species identification. Our results showed high reliability of the MALDI-TOF MS identification at the species level covering 100% of cases (13 isolates). In conclusion, MALDI-TOF MS may be regarded as a powerful tool for routine bacterial identification in veterinarian laboratory allowing a reduced cost and fast identification of mastitis-causing pathogens.

Keywords: Subclinical Mastitis, Bacteria, Identification 16S rDNA sequencing, MALDI-TOF MS.

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INTRODUCTION

Mastitis is one of the most common diseases affecting dairy cows causing major economic losses to the dairy industry [1]. It is still a main topic in veterinary research due to financial losses and food safety concerns over antimicrobial use [2]. This disease results from the inflammation of the mammary gland and is characterized by a marked reduction in the amount of milk production and in changes in levels of its specific components, reducing the overall milk quality. The severity of the inflammation can be classified into sub-clinical, clinical and chronic forms. While detection of clinical mastitis is relatively easy, subclinical mastitis is difficult to detect due to the absence of any visible clinical signs [3].

Reliable identification of the bacteria responsible for mastitis is important for disease management in the herd and for targeting antimicrobial treatment [4]. The traditional diagnostic test for identifying pathogens in milk is bacterial culture, which needs the use of an appropriate culture media and incubation time for colonies growth. Subsequently, bacterial species identification is achieved based on their phenotypic characteristics including colony morphology, serotyping and analysis of enzymatic profiles. Although Bacterial culture has been regarded as the gold standard for identifying mastitis pathogens, it still time consuming, requiring from 24 to 48 h [5], and either fails to identify some bacteria such as Gram-positive rods [6].

Genotypic identification methods have become widely used, and most of them are based on the polymorphism of the 16S rDNA genes. It offers a useful alternative, and nowadays, this technology is considered as the reference method for species identification, and many studies have shown its superiority to phenotypic methods for the identification of various groups of bacteria [7]. However, this method does not provide the complete solution in routine bacterial identifications. And it is still not commonly used in many veterinary or environmental laboratories, mainly due to cost and time constraints [8].There is still an urgent need for rapid and simple techniques for microbial identification [9].

In the last few years, Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (MS), whitch can be used to analyze the protein composition of a bacterial cell, has emerged as a new technology for bacterial species identification. By measuring the exact sizes of peptides and small proteins, which are assumed to be characteristic for each bacterial species, it is possible to determine the species within a few minutes either with whole cells, cell lysates, or crude bacterial extracts [7]. It is suitable for high-throughput, rapid and cost-effective diagnostics and can be considered as an alternative for conventional biochemical and molecular identification systems in a conventional microbiological laboratory [9-11]. This has been especially shown recently, by the identification of intramammary infections using bacterial extract [11, 12].

The aim of this work was to evaluate whole-cell MALDI-TOF MS identification of species causing subclinical mastitis in cows in Algeria.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Thirteen Bacteria were isolated from sub-clinical bovine mastitis cases during spring 2013 in two dairy farms in two east Algerian regions: pilot farm of El-Baaraouia, City of El Khroub in Constantine and from a private farm in the town of Djarma, City of El-Madher, Batna.

Sampling :

Milk samples were collected following the NMC guidelines [5]. Individual milk samples were taken from the four quarters of some lacting cow, just before the second milking time (afternoon). Milk samples were analyzed by the California Mastitis Test (CMT) as explained by Shalm [13], after cleaning of the udder using alcohol soaked cotton and expulsion of the first of milk jets. Positive milk samples were collected in sterile vials immediately, labeled and transported to the laboratory under strict conditions of refrigeration (4° C) for bacteriological analyses.

Culture and Isolation:

The isolation was made from a culture on Columbia agar supplemented with 5% sheep blood using an inoculum of 10µl of milk. The cultures were incubatedaerobically at 37 °C for 24 to 48h.

Bacterial identification:

Matrix-assisted laser desorption ionization–time of flight identification

For MALDI-TOF-MS identification, colonies were grown overnight on blood agar at 37 °C under aerobic conditions. A colony of a fresh culture was applied directly, by touching it with a sterile pipette tip, onto a ground steel MSP 96 target plate (BrukerDaltonics) as a thin film and air-dried. Subsequently, the sample was overlaid with 1 µl of matrix solution (a saturated solution of alpha-cyano-4-hydroxy-cinnamic acid (HCCA), in 50% HPLC acetonitrile, 2.5% Trifluoacetic Acid) and allowed to dry at room temperature. Isolates were tested in duplicate by MALDI-TOF MS. The MALDI target plate was introduced into a microflex LT MALDI-TOF mass spectrometer for automated measurement and controlled by the FlexControl 3.3 (Bruker®) program. The spectra were collected in a mass range between 2,000-20,000 m/z then analyzed using the Bruker Biotyper 3.0 software package and compared to reference spectra for identification. The results of the pattern matching process were expressed as log (score) values in the range 0–3; A score greater than 1.9 indicates a high-level identification of genus and species. A score greater than 1.7 indicates the identification of genus but not species, and a score lower than 1.7 indicates no identification of bacteria.

16S rDNA identification:

DNA extraction:

The genomic DNA extraction from strains was done using the EZ1 Qiagen Kit on the automate EZ1 XL (200 µl sample were grown on liquid broth LB and were incubated at 37 °C for 24 h with 100 µl elution volume),according to the manufacturer's instructions.

Table 1: Primers used in 16S rDNA sequencing

Primers	Sequences (5'-3')
fD1	AGAGTTTGATCCTGGCTCAG
rP2	ACGGCTACCTTGTTACGACTT
536F	CAGCAGCCGCGGTAATAC
536R	GTATTACCGCGGCTGCTG
800F	ATTAGATACCCTGGTAG
800R	CTACCAGGGTATCTAAT
1050F	TGTCGTCAGCTCGTG
1050R	CACGAGCTGACGACA

16S rDNA sequencing and sequence analysis:

The 16S ribosomal DNA was obtained with PCR amplification using the universal primers fD1 and rP2 [14]. The thermal cycling reactions consisted of an initial denaturation step of 10 min at 95 °C followed by 39 cycles (30 s of denaturation at 95 °C, 30 s annealing at 55 °C, and 1 min of extension at 72 °C) and a final extension step at 72 °C for 5 min.PCR products were analyzed on an ethidium bromide-stained 1.5 % agarose gel electrophoresis and purified with the QIAquick spin PCR purification kit (Qiagen). Sequencing reactions carried out with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions and using the primers 536F, 536R, 800F, 800R, 1050F, 1050R [15] listed in table 1 with the following program: an initial 5 min denaturation step at 96 °C followed by 25 cycles (30 s of denaturation at 96 °C, 20 s annealing at 54 °C, and 4 min of extension at 60 °C). The products of sequencing reactions were purified on the Sephadexplaq 5%, and then sequenced with the ABI 3130XL sequencer (Applied Biosystems). Partial sequences were combined into a single consensus sequence with ChromoasPro Software.The obtained sequences were compared with the GenBank database using BLAST software.

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RpoB gene sequencing

RNA polymerase beta-subunit gene (rpoB) sequencing was used as a secondary gene target when 16Sr RNA did not allow species identification. Partial rpoB gene sequencing was done using the primers 2491F (AACCAATTCCGTATIGGTTT) and 3554R (CCGTCCCAAGTCATGAAAC) as previously described [16].

RESULTS

16S rDNA sequencing and bacterial identification

The PCR primers amplified approximately 1,500 base pairs (bp) 16S rDNA fragment in all the 13 isolates, the amplified rDNA was directly sequenced. The sequences obtained were compared with GeneBank database using BLAST alignment software (http://www.ncbi.nlm.nih.gov/blast). Sequences were interpreted following the criteria described by [17].Briefly, identification was assessed by ≥ 98.7% similarity with one of the database sequences for secure identification of species.

The 16S rDNA sequences of the 13 strains have been submitted to the EMBL Nucleotide Sequence Database (http://www.ebi.ac.uk/embl/) under the accession numbers which are given in table 2.

MALDI-TOF MS

For MALDI-TOF MS analysis, reliable identification score > 1.9 at both genus and species level was observed in all bacterial isolates (see table 2). For twelve isolates it coincides with identification by 16S rDNA sequencing. For one case "isolate 8", the identification by both methods coincided at the genus level, it was identified as *Staphylococcus saprophyticus* by 16S rDNA sequencing and *St. xylosus* by MALDI-TOF MS the result was confirmed to be S. *xylosus* by partial rpoB gene sequencing .

The rpoB gene of strain 8 was submitted to EMBL under the accession number LM651923.

Table 2: Comparison of 16S rDNA Sequencing and MALDI-TOF MS results for the 13 isolates.

Strain	16S rDNA sequencing analysis			MALDI-TOF	
	Results (NCBI nr/nt database)	EMBL accession no.		Result	Score
B1	Staphylococcus cohnii	HG941657		Staphylococcus cohnii	2.223
B2	Micrococcus luteus	HG941658		Micrococcus luteus	2.149
B3	Staphylococcus hominis	HG941659		Staphylococcus hominis	2.188
C4	Staphylococcus haemolyticus	HG941660		Staphylococcus haemolyticus	2,228
B5	Staphylococcus hominis	HG941661		Staphylococcus hominis	2.222
B6	Staphylococcus hominis	HG941662		Staphylococcus hominis	2.137
C7	Escherichia coli	HG941663		Escherichia coli	2.16
C8	Staphylococcus xylosus	HG941664		Staphylococcus xylosus	2.163
B9	Micrococcus luteus	HG941665		Micrococcus luteus	2.215
C10	Escherichia coli	HG941666		Escherichia coli	2.149
B11	Staphylococcus haemolyticus	HG941667		Staphylococcus haemolyticus	2.189
C12	Staphylococcus equorum	HG941668		Staphylococcus equorum	2.016
B14	Staphylococcus hominis	HG941670		Staphylococcus hominis	2.101

DISCUSSION

Identification of bacteria in udder quarters and cows is important for disease management in the herd and for a better targeting of antimicrobial treatment [4]. Moreover, it represents an important part of control programs to reduce their spread within and between dairy herds in the case of contagious pathogens [18].

Current techniques employed for detection of bacteria causing sub-clinical mastitis include time-consuming and laborious phenotypic methods requiring the use of many equipments and consumables and highly trained staff.

In the present study, we have tested MALDI-TOF MS to identify species causing subclinical mastitis in dairy cows in the east of Algeria. Our results show high reliability of the employed technique, permitting the identification of 100 % of isolates at the species level.

Overall, five different species of coagulase negative staphylococci (CoNS) were identified. CoNS have become the most common mastitis-causing agents in many countries [19-23]. In routine mastitis diagnostics, CoNS are normally not identified to species level but treated as a uniform group [24]. An important advantage of MALDI-TOF MS, relies in its capacity to recognize correctly spectra of various species of CoNS as it was demonstrated in the studies of [9, 12, 25]

One strain (isolate 8) was identified as *St. xylosus* by MALDI TOF. Interestingly, 16S rDNA gene sequencing identified this isolate to be *St. saprophyticus* with 99.86% similarity with the accession number (EU855210.1) and as *S. xylosus* (LN554884.1) with 99.73 % of similarity. The result was confirmed to be S. *xylosus* by partial rpoB gene sequencing which in common with ribosomal RNA-encoding genes, is universal (Mollet *et al*. 1997) and it was shown in the study of Drancourt *et al*. (2002) [16] to be objective and accurate method of species identification of staphylococci. In their study, Heikens *et al*. (2005) [26] found that genotypic identification based on 16S rDNA sequencing has limited discriminating power for closely related Staphylococcus species and pairwise comparison of the 16S rDNA sequences showed difficulties to distinguish between *S. saprophyticus* and *S. xylosus.*Interestingly, in our study, MALDI-TOF MS identified this strain as *St. xylosus* with a high score of 2.163, showing the possible ability of this method to differentiate the species within this group.

In the present study, MALDI-TOF correctly identified all bacteria causing subclinical mastitis. This result is confirmed by other studies [11, 12, 27-29]. MALDI-TOF mass spectrometry presents another advantage, it was also used to rapidly discriminate antibiotic resistance in bacteria [30-35]. In fact, rapid and accurate discrimination between sensitive and resistant strains is essential for appropriate therapeutic management and timely intervention for infection control.

In this study, The 16s RNA gene sequencing provides a high discriminative power for differentiating subclinical isolates. Nevertheless, when we compared this molecular technique with MALDI-TOF MS in terms of time and cost–benefit, we noted that MALDI-TOF MS is time-saving, specimens can be prepared in a few seconds from plate cultures and a spectrum can be obtained within 2 min *vs* 24h for 16S RNA sequencing, and, remarkably, more cost-effective, as a result of inexpensive consumables and simple operating procedures that do not require specialized laboratory technicians. Also, it has been demonstrated that the cost of bacterial identification by MALDI TOF mass spectrometry was estimated to represent only 22%–32% of the cost of conventional phenotypic identification [36]. Our results confirm further studies [11, 12, 28, 37] indicating that MALDI-TOF MS is an efficient tool for species identification of bacteria causing intra-mammary infections, making from it a good alternative for conventional methods used in veterinary laboratories.

CONCLUSION

In conclusion, we showed that MALDI-TOF-MS is a powerful method for the identification of bacteria isolated from milk of cows affected by subclinical mastitis with high specificity and sensitivity. This confirms that this technique can provide a faster, cost-effective once the instrument is acquired and more reliable identification of microorganisms, which is essential for appropriate therapeutic management. Finally, other studies are required to test the suitability of MALDI-TOF for detecting bacteria directly in infected milk.

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