Journal of Equine Veterinary Science 34 (2014) 833–836

Contents lists available at ScienceDirect

Journal of Equine Veterinary Science

journal homepage: www.j-evs.com

Short Communication

Application of Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry in Identification of Stallion Semen Bacterial Contamination

Martina Masarikova DVM [a,b,*], Miroslava Mrackova DVM [c], Marketa Sedlinska PhD [c]

[a] Department of Infectious Diseases and Microbiology, Faculty of Veterinary Medicine, University of Veterinary and Pharmaceutical Sciences Brno, Brno, Czech Republic
[b] CEITEC VFU, University of Veterinary and Pharmaceutical Sciences Brno, Brno, Czech Republic
[c] Equine Clinic, Faculty of Veterinary Medicine, University of Veterinary and Pharmaceutical Sciences Brno, Brno, Czech Republic

ARTICLE INFO

Article history:
Received 27 September 2013
Received in revised form 13 December 2013
Accepted 26 January 2014
Available online 1 February 2014

Keywords:
MALDI-TOF
Mass spectrometry
Stallion semen
Microflora

ABSTRACT

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) is one of the cutting-edge methods currently applied in medical bacteriologic diagnostics. The aim of the study was to prove the possibility of applying MALDI-TOF MS to identify bacterial contamination in the ejaculate of stud stallions, which may cause infections to reproductive organs of mares following artificial insemination with cooled semen. A partial aim was to determine changes in the total count of microorganisms in long-term storage of ejaculate after its treatment with gentamicin and also without antimicrobial medication. Aerobic cultivation on Columbia agar was used to examine 26 semen samples from 13 horses; 31 different species of bacteria were isolated, which were identified by MALDI-TOF MS. The most frequently detected species came from *Aerococcaceae*, *Staphylococcaceae*, and *Micrococcaceae* families. The results of our work confirm that MALDI-TOF MS is a quick alternative method for identifying bacterial species that may contaminate stallion semen.

1. Introduction

Artificial insemination (AI) with cooled semen in horses is a well-established and widely-used technique in all major horse breeding countries. Good result after AI is the reason why breeders strive to achieve the highest quality semen in the insemination doses (ID). The surface of a stallions' penis and foreskin is colonized by different kinds of bacteria, mostly commensals and nonpathogenic species. During sampling, these microorganisms get into the ejaculate as its microbial contamination that cannot be avoided even if all the conditions of aseptic and antiseptic handling are met [1–3]. This is why antimicrobial preparations are used as standard components in diluents for stallion semen. The most common is gentamicin, an aminoglycoside antibiotic [4]. Frequently, artificial insemination is not performed immediately, and therefore, the logical conclusion is that the risk of an infection to the mare is directly proportional not only to the presence of microorganisms and dose but also to the length of time between the sampling and the actual use of the semen. Readily available information about the bacteria species present in the semen would be very useful for its further processing and use. A fast new analytical technique of identifying bacteria on mass spectrometry of expressed ribosomal proteins could be used for this purpose. The most commonly used modification of mass spectrometry analyzing biomolecules is matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) [5–8].

* Corresponding author at: Dr. Martina Masarikova, Department of Infectious Diseases and Microbiology, Faculty of Veterinary Medicine, University of Veterinary and Pharmaceutical Sciences Brno, Palackého tř. 1/3, 612 42 Brno, Czech Republic.
E-mail address: martasekmm@seznam.cz (M. Masarikova).

0737-0806/$ – see front matter © 2014 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.jevs.2014.01.013

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry is based on ionization of bacterial proteins through crystallization matrix (cinnamic acid or benzoic acid derivate) after beaming by the spectrometer laser. Protein ions are electrostatically accelerated and fly through a vacuum tube toward the detector. The length of their flight responds to their mass and charge load. Time in nanoseconds, which elapses between the ions' pulse acceleration and signal received by the detector, can be accurately recalculated to molecular mass of each individual protein. For the analysis of mass spectra, the received data are transformed to a set of peaks, which is compared with all reference profiles stored in the machine's database. The spectrum of flight path lengths of all expressed proteins in the examined sample creates a specific fingerprint that is unique for each bacterial species [9,5].

The sensitivity and specificity of the method were repeatedly verified and published by a number of author teams [10,11], and identification of pathogenic organisms through MALDI-TOF MS is currently used in routine diagnostic protocols and veterinary microbiological laboratories. Because of the fact that only few experiences with applying mass spectromectry in veterinary microbiological diagnostics have been published so far, the aim of our work was to use this method to identify microorganisms contaminating stallion semen taken for the preparation of ID and represents a potential risk of urogenital infections in mares undergoing artificial insemination.

2. Materials and Methods

The examined set consisted of 13 ejaculate samples taken from 13 stud and non-stud stallions aged 3–17 years between April and July 2012. Each sample was diluted with standard commercially available diluents (EquiPro; Minitübe, Tiefenbach, Germany) and divided to part A, which was treated with gentamicin at a dose of 1 g/L (Genta-kel 05 inj. ad us. vet., Kela Laboratoria N.V., Hoogstraten, Belgium) and part B—control sample without gentamicin. Both parts were kept at the temperature of 4°C and then examined in intervals after 6, 12, 24, and 48 hours following sampling. One hundred microliters of each sample, from parts A and B, was applied to the surface of Columbia blood agar (CBA; Oxoid, Hampshire, UK) with a sterile glass L-shape hockey-stick spreader. The agars were incubated under aerobic conditions at 37°C. After 24 hours, the total count of microorganisms was taken, and each morphologically different colony was used for subcultivation on CBA. The final pure culture represented a basic material for identification by MALDI-TOF MS on MALDI Biotyper (Bruker Daltonic, Karlsruhe, Germany).

Statistic evaluation of changes in the total microorganism count in time was performed through nonparametric Wilcoxon test for pair data.

3. Results

Aerobic cultivation performed on 13 semen samples yielded the isolation of 31 different species of bacteria that the MALDI Biotyper identified as skin and mucosal commensals and environmental microorganisms. The specific species were *Acinetobacter lwoffii*, *Aerococcus viridans*, *Arthrobacter castelli*, *Arthrobacter gandavensis*, *Arthrobacter polychromogenes*, *Bacillus cereus*, *Bacillus pumilus*, *Brevibacterium paucivorans*, *Corynebacterium glutamicum*, *Corynebacterium stationis*, *Dermabacter hominis*, *Chryseobacterium indologenes*, *Klebsiella pneumoniae*, *Kocuria rosea*, *Kytococcus sedentarius*, *Microbacterium foliorum*, *Micrococcus luteus*, *Pantoea agglomerans*, *Pseudomonas fulva*, *Pseudomonas stutzeri*, *Raoultella ornithinolytica*, *Rothia nasimurium*, *Staphylococcus epidermidis*, *Staphylococcus equorum*, *Staphylococcus haemolyticus*, *Staphylococcus hominis*, *Staphylococcus pasteuri*, *Staphylococcus sciuri*, *Staphylococcus warneri*, *Stenotrophomonas maltophilia*, and *Streptococcus equi*. The most frequently occurring microorganisms came from *Aerococcaceae*, *Staphylococcaceae*, and *Micrococcaceae* families. Obligatory pathogen of the reproductive system, *K. pneumoniae*, was found in one stallion. Semen from individual horses contained from four to nine different species of bacteria, see Table 1.

Quantification of colony-forming units in 1 mL of semen showed that samples not treated with gentamicin contained a statistically higher total count of microorganism in each of the four readings (6, 12, 24, and 48 hours) ($P < .01$). The dynamics of microbial load in both medicated and nonmedicated samples showed a substantial shift >48 hours. After 2 days, the total microorganism count dropped significantly in all of the semen samples containing gentamicin ($P < .01$), although this drop was not so marked in the nontreated samples ($P < .05$), see Table 2.

Table 1
Detected bacterial species in equine semen and its frequency of isolation

Bacterial Species	Family	Number of the Positive Horses
Acinetobacter lwoffii	*Moraxellaceae*	1
Aerococcus viridans	*Aerococcaceae*	9
Arthrobacter castelli	*Micrococcaceae*	1
Arthrobacter gandavensis	*Micrococcaceae*	2
Arthrobacter polychromogenes	*Micrococcaceae*	1
Bacillus cereus	*Bacillaceae*	1
Bacillus pumilus	*Bacillaceae*	1
Brevibacterium paucivorans	*Brevibacteriaceae*	4
Chryseobacterium indologenes	*Flavobacteriaceae*	1
Corynebacterium glutamicum	*Corynebacteriaceae*	1
Corynebacterium stationis	*Corynebacteriaceae*	1
Dermabacter hominis	*Dermabacteraceae*	4
Klebsiella pneumoniae	*Enterobacteriaceae*	1
Kocuria rosea	*Micrococcaceae*	1
Kytococcus sedentarius	*Dermacoccaceae*	1
Microbacterium foliorum	*Microbacteriaceae*	1
Micrococcus luteus	*Micrococcaceae*	3
Pantoea agglomerans	*Enterobacteriaceae*	2
Pseudomonas fulva	*Pseudomonadaceae*	1
Pseudomonas stutzeri	*Pseudomonadaceae*	1
Raoultella ornithinolytica	*Enterobacteriaceae*	1
Rothia nasimurium	*Micrococcaceae*	1
Staphylococcus equorum	*Staphylococcaceae*	7
Staphylococcus haemolyticus	*Staphylococcaceae*	5
Staphylococcus hominis	*Staphylococcaceae*	1
Staphylococcus pasteuri	*Staphylococcaceae*	7
Staphylococcus sciuri	*Staphylococcaceae*	1
Staphylococcus warneri	*Staphylococcaceae*	2
Stenotrophomonas maltophilia	*Xanthomonadaceae*	4
Streptococcus equi	*Streptococcaceae*	1