Theriogenology 80 (2013) 337–345

Contents lists available at SciVerse ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Microorganisms in cryopreserved semen and culture media used in the *in vitro* production (IVP) of bovine embryos identified by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS)

Dávila Zampieri [a], Vanessa G. Santos [a], Patrícia A.C. Braga [a], Christina R. Ferreira [a], Daniela Ballottin [b], Ljubica Tasic [b], Andréa C. Basso [c], Bruno V. Sanches [c], José H.F. Pontes [c], Bárbara Pereira da Silva [d], Fabiana Fantinatti Garboggini [d], Marcos N. Eberlin [a], Alessandra Tata [a,*]

[a] *ThoMSon Mass Spectrometry Laboratory, Institute of Chemistry, University of Campinas, Campinas, Sao Paulo, Brazil*
[b] *Biological Chemistry Laboratory, Institute of Chemistry, University of Campinas, Campinas, Sao Paulo, Brazil*
[c] *InVitro Brasil Ltda, Mogi Mirim, Sao Paulo, Brazil*
[d] *Microbial Resources Division, Chemical, Biological and Agricultural Pluridisciplinary Research Center (CPQBA), University of Campinas, Campinas, Sao Paulo, Brazil*

ARTICLE INFO

Article history:
Received 18 February 2013
Received in revised form 15 April 2013
Accepted 22 April 2013

Keywords:
Matrix-assisted laser desorption ionization mass spectrometry
Microorganisms
Bacteria
Semen
Culture media
In vitro production

ABSTRACT

Commercial cattle breeders produce their own herd offspring for the dairy and beef market using artificial insemination. The procedure involves sanitary risks associated with the collection and commercialization of the germplasm, and the *in vitro* production and transfer of the bovine embryos must be monitored by strict health surveillance. To avoid the spreading of infectious diseases, one must rely on using controlled and monitored germplasm, media, and reagents that are guaranteed free of pathogens. In this article, we investigated the use of a new mass spectrometric approach for fast and accurate identification of bacteria and fungi in bovine semen and in culture media employed in the embryo *in vitro* production process. The microorganisms isolated from samples obtained in a commercial bovine embryo IVP setting were identified in a few minutes by their conserved peptide/protein profile, obtained applying matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS), matched against a commercial database. The successful microorganisms MS identification has been confirmed by DNA amplification and sequencing. Therefore, the MS technique seems to offer a powerful tool for rapid and accurate microorganism identification in semen and culture media samples.

1. Introduction

Assisted reproduction technology strategies have been intensely developed and used over the last 50 years, and its application in the cattle reproduction is worldwide well established. However, there are severe sanitary risks associated with these techniques, which have the potential to spread infectious diseases [1]. The sources of pathogens can be many [2], such as the environmental contaminations associated with the collection and the cryopreservation of germplasm and if not monitored. The cryopreservation of semen and embryos does have the advantage of ensuring long-term storage of viable cells of germplasm, but at the same time, if not monitored, the risk of dissemination of some pathogens by embryo transfer and artificial insemination may increase [3–5]. In fact, the cryopreserved bovine semen has been found to be a potential source for

* Corresponding author. Tel.: +55 19 3521 3049; fax: +55 19 3521 3073.
E-mail address: alessandratata1@gmail.com (A. Tata).

0093-691X/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.theriogenology.2013.04.020

several microorganisms such as *Stenotrophomonas maltophilia*, *Pseudomonas putida*, *Pseudomonas aeroginosa*, *Enterobacter cloacae*, *Staphylococcus sciuri*, *Acinectobacter cacloaceticus*, *Pantoeau agglomerans*, and *Flavobacterium* spp. [6–8] that can seriously affect the motility of the sperm and the embryonic development [9]. It is important, therefore, to efficiently control the population of microorganisms during the entire assisted reproduction technology procedures and prevent the introduction of diseases into individual animals, herds, areas, or countries where they were not previously present. It is also important to note that testing the presence of microorganisms that survive in association with the germplasm is crucial for the health certification of embryos for international movements [1].

In this article, we focused on contaminations in bovine frozen semen and culture media dishes. Routine microbiological methods applied to microorganism identification have remained nearly unchanged over the last century [10]. These methods still employ classical approaches on the basis of sample streaking, colony growth using various culture media, and morphological and biochemical characterization of the isolated bacterial species [8]. Therefore, a tool for the fast identification of the pathogens in the frozen semen and in the culture media along with the *in vitro* fertilization (IVF) process may constitute an important quality control methodology for *in vitro* production (IVP).

Recently, matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) has emerged as a rapid and powerful tool for microbial species identification [11,12]. This approach is on the basis of the acquisition of microorganism's conserved proteins "fingerprints" directly from the crude bacterial extract or from the lysate supernatants of whole cells [13]. Interestingly, the protein profiles (represented mostly by ribosomal proteins) have been found to vary considerably and to properly characterize different microorganisms. By measuring the *m/z* profile of peptides and small proteins, which are characteristic for each bacterial species, it is possible to determine the genus and species within a few minutes when the analysis is started with whole cells, cell lysates, or crude bacterial extracts [14]. This methodology has been shown to be easier, faster, and more reliable than the classical protocols even when compared with more sophisticated DNA analysis–based technologies [15,16]. MALDI-MS–based microorganism identification is therefore being introduced in human laboratorial and clinical settings with reliable diagnostic results not only for genus, but also at species level in bacteria [17–24], fungus [25,26], algae [27], viruses [28–30], and protozoan [31]. Contaminations in different real matrices, such as blood [32], urine [33], cerebrospinal fluid, and biopsy [11] have been successfully identified by MALDI-MS, and, nowadays, this approach is already in use by clinical diagnostics laboratories [34–36].

In the veterinarian field, MALDI-MS has been recently used for the identification of bacteria from milk affected by cow subclinical mastis [37,38]. A recent review clearly summarized the application of MS-based techniques in a variety of fields [10].

We are currently involved in a collaborating effort with a large-scale bovine embryo production Brazilian company, and this research partnership has resulted in the development of MS techniques aimed to accomplish high sanitary excellence in bovine IVF [39,40]. We have tested the applicability of MALDI-MS for fast bacterial screening of frozen bovine semen and *in vitro* maturation media (IVMm), *in vitro* fertilization media (IVFm), and *in vitro* culture media (IVCm) employed in the IVF process. The isolated bacteria were successfully identified in a few minutes by the integrated pattern-matching algorithm of the mass spectra peaks list with the reference library. The microorganisms that achieved log score values <2.3 (score values >2.3 indicate a highly probable genus and species identification) were submitted to a DNA amplification and gene sequencing which confirmed the identification. The study therefore introduces the MALDI-MS as an efficient tool for the identification of bacteria for the routinely quality control of the semen before IVF process and for monitoring the quality culture media along all the steps of the bovine IVF procedure.

2. Materials and methods

Methanol and acetonitrile (ACS/HPLC grade) were purchased from Burdick and Jackson (Muskegon, MI, USA). Formic acid, 2,5-alfa-ciano-4-hydroxycinnamic (CHCA), trifluoroacetic acid, and ethanol were purchased by Sigma-Aldrich. Ultrapure water, purified by a Direct-Q water system (Millipore, Bedford, MA, USA), was used.

2.1. Sample collection and culturing

Cryopreserved bovine semen and IVFm, IVCm, and IVMm samples were collected from the commercial routine of In Vitro Brasil Ltda (Mogi Mirim, Sao Paulo, Brazil). The samples of cryopreserved semen were aliquots of commercial sealed straws collected after thawing and before the use for the bovine embryo IVP.

In vitro oocyte maturation and fertilization have been performed during 22 to 24 hours and the culture during 7 days in BD Falcon disposable cell culture dishes 35 × 10 mm in drops containing 90 μL of medium under mineral oil (Sigma, St Louis, MO, USA). Culture media composition and IVP procedures has been described elsewhere [41]. After each of the IVP steps, 50 μL of IVFm, IVCm, and IVMm were sampled, sent refrigerated to the microbiological laboratory, and rapidly incubated as described below. The samples have been cultured in brain heart infusion (BHI) broth, a highly nutritious general-purpose growth medium. If the presence of a specific microorganism must be screened, a selective growing can be performed by using a differential and selective medium.

A volume of 50 μL of semen were incubated in 10 mL of BHI broth (OXOID) at 37 °C. After 24 hours, more than one-third of them showed turbidity, which is indicative of microorganism growth. Then, the bacterial cultures were centrifuged at 5000 × *g* for 10 minutes and submitted to MS analysis, described as follows. For the isolation of single colonies, decimal dilutions of the bacteria pellet were plated on BHI/agar (2% wt/vol malt extract agar—OXOID)

dishes and incubated for 24 hours at 37 °C. The single colonies were scratched out from the Petri dishes and incubated again on BHI/agar (2% wt/vol malt extract agar—OXOID) dishes for 24 hours at 37 °C to increase the amount of pure microorganisms and to be submitted to DNA amplification and gene sequencing. The same sample amount (50 μL) of culture medium and culturing procedure was employed for the IVFm, IVCm, and IVMm samples.

2.2. Mass spectrometric analysis by protein extraction method and intact cell method

The bacterial pellet was dissolved in 1 mL of water and centrifuged (all centrifugation steps were performed at 13,000 × *g* for 2 minutes) in order to wash out the residue of BHI.

According to the protein extraction method (PEM), 1 mL of 75% ethanol solution was added to the pellet, and after centrifugation, the supernatant was removed by carefully pouring it from the microtube. A second centrifugation step was performed, and the remaining liquid was carefully removed with a pipette. The bacterial pellets were allowed to air-dry at room temperature for 15 minutes. A solution of 70% formic acid was added to lyse bacterial cells and to release the inner-cell proteins, which are predominantly ribosomal proteins that produce diagnostic ions in MALDI-MS fingerprinting. The 70% formic acid solution was added proportionally to the size of pellet to completely dissolve it. Subsequently, 100% acetonitrile was added to each sample in volumes equal to the 70% formic acid solution previously added, thus producing a bacterial extract in a (1:1) ratio of 70% formic acid and acetonitrile. A final centrifugation step was performed to separate the bacterial cell debris from the supernatant containing the ribosomal proteins used for the MALDI-MS identification.

To prepare the MALDI target plates, 1 μL of the supernatant containing the bacterial proteins was placed onto a steel target plate (MSP 96 polished-steel target; Bruker Daltonics, Bremen, Germany) and allowed to air-dry. The dried supernatant was overlaid with 1 μL of matrix solution, which consisted of α-cyano-4-hydroxy-cinnamic acid (CHCA) dissolved in 50% acetonitrile and 2.5% trifluoroacetic acid.

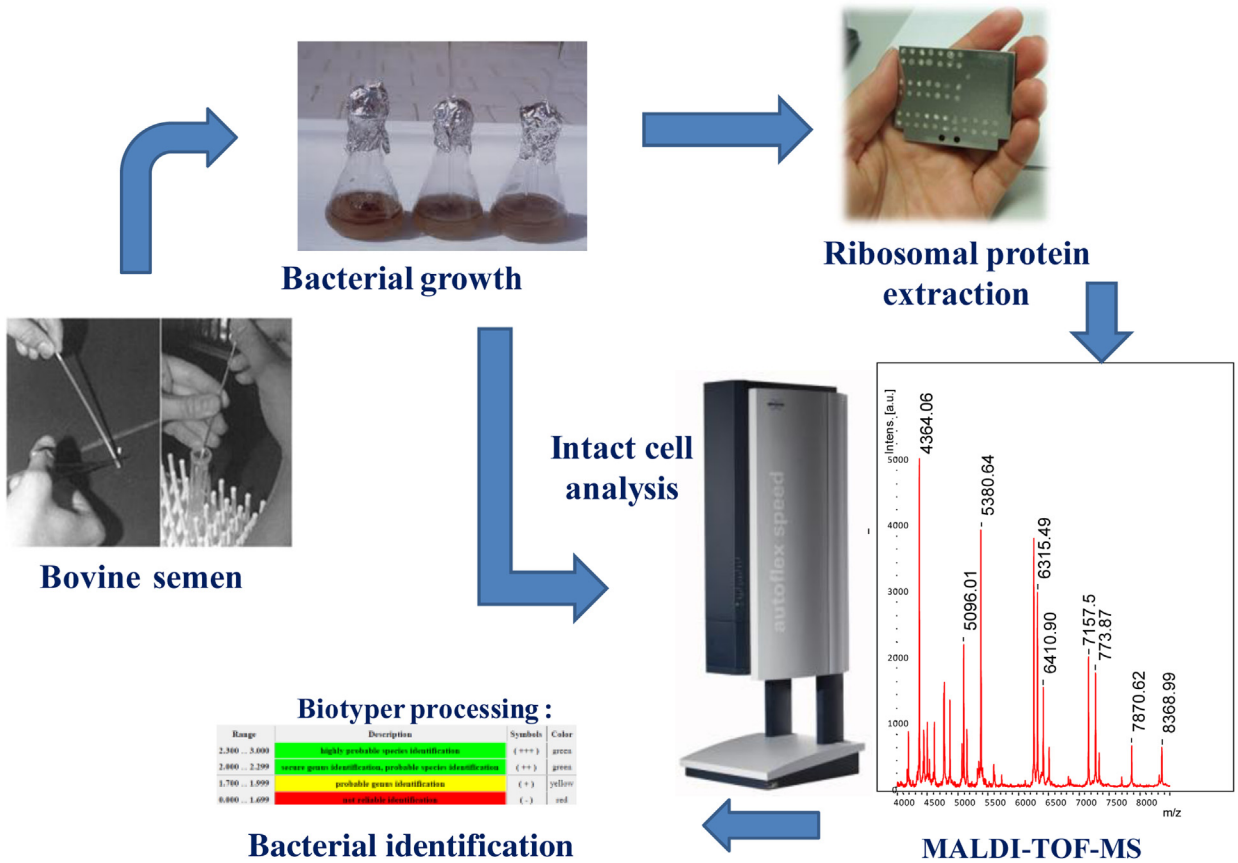
For the intact cell method (ICM), the washed bacteria pellet (~0.1 g) was diluted in 1 mL of water and acetonitrile (1:1 vol/vol). A volume of 1 μL of the obtained solution was directly spotted on the MALDI plate and the CHCA matrix was added as described previously.

The matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis was performed in a Bruker Microflex LT MALDI-TOF-MS operated in the linear mode and equipped with a 337-nm nitrogen laser using FlexControl 3.3 software (Bruker Daltonics). The mass spectra were collected within the mass range of *m/z* 2000 to 20,000. The instrument settings were as follows: ion source 1 at 19.50 kV, ion source 2 at 18.25 kV, lens at 6.49 kV, and an extraction delay time of 4 ns. The instrument was externally calibrated with the bacterial test standard supplied by Bruker Daltonics. The bacterial test standard is an *Escherichia coli* extract including the additional proteins RNase A and myoglobin. Three thousand laser shots were accumulated to generate each spectrum. The spectra were analyzed with the database library MALDI Biotyper 3.0 software (Bruker Daltonics). For each sample, the automatic analysis generated a peak list, which was used to match the reference library by the integrated pattern-matching algorithm. The software performs normalization, smoothing, baseline subtraction, and peak picking, creating a list of the most significant peaks of the spectrum. In general, the MALDI Biotyper pattern-matching algorithm considers the matches of the unknown sample spectrum against the reference database and the reverse matches of the main spectrum with the unknown spectrum. Finally, it also compares the relative intensities of unknown and database spectra. The result is given by means of a log score with a maximum value of 3.0. A log score of a maximum value of 3.0 represents the maximum product values (1000). It has been confirmed with statistical differences that log score values >1.7 correspond to reliable genus identifications, whereas log score values >2.0 indicate a secure genus and probable species identifications. Finally, log score values >2.3 represent a high probably genus and species identification [42,43]. In this study, only scores >2.0 were considered.

2.3. Molecular identification by DNA amplification and gene sequencing

All isolates were cultured on nutrient agar, and genomic DNA was obtained using the protocol described by Van Soolingen et al. [44]. Strains belonging to the *Bacillus brevis* group and *Bacillus aneurinolyticus* group were identified by 16S rRNA gene amplification by using specific detection primers and PCR. The sequences of forward detection primers BREV174F and ANEU506F were 5′-AGACCGGGA TAACATAGGGAAACTTAT-3′ and 5′-GAACCGCCGGGATGACC TCCCGGTC-3′, respectively, and the sequence of reverse primer 1377R was 5′-GGCATGCTGATCCGCGATTACTAGC-3′ [45]. PCR was performed in reaction mixtures containing 50 to 100 ng of genomic DNA, 2 U of *Taq* polymerase (Invitrogen), 0.2 μM of dNTP mix (GE Healthcare), and 0.4 μM each primer (forward and reverse) in a final volume of 50 μL. Amplification program consisted of an initial denaturation step at 94 °C for 30 seconds, followed by 25 cycles of 94 °C/1 minute, 58 °C/1.5 minutes, and 72 °C/1.5 minutes, with final extension of 72 °C/5 minutes, in an Eppendorf thermal cycler (Eppendorf).

Samples of the *Enterobacter cloacae* complex were identified by amplification and sequencing of a fragment of *rpoB* gene. PCR was performed in the same conditions as described above, using the primers Vic3[a] (forward): 5′-GGCGAAATGGCWGAGAACCA-3′ and Vic2[a] (reverse): 5′-GAGTCTTCGAAGTTGTAACC-3′ [46]. The thermal cycle consisted an initial denaturation step at 94 °C for 1.5 minutes, followed by 40 cycles of 94 °C/10 seconds, 55 °C/20 seconds and 72 °C/50 seconds, with final extension of 72 °C/5 minutes [47]. Amplified products were purified using GFX PCR DNA and Gel Band Purification kit (GE Healthcare) for subsequent sequencing using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Life Technologies) for an automated ABI sequencer 3500xL



Scheme 1. MALDI-MS bacterial identification in bovine semen general workflow. After thawing the semen straws, 50 μL are collected and refrigerated. Semen samples are seeded in BHI and cultured for 24 hours. If BHI turbidity is observed, samples can be directly spotted in the MALDI target plate after centrifugation (ICM strategy) or can be submitted to protein extraction before being spotted in the MALDI target plate (PEM strategy). All samples are covered with the organic matrix CHCA. MALDI-MS sample acquisition and database search are performed automatically. All the procedure takes 15 to 20 min/sample, so that the overall process of bacterial identification in semen samples can be performed in ~1 day.

(Applied Biosystems Life Technologies), according to the manufacturer's instructions. The sequencing was carried out using the same set of primers used for DNA amplification.

Partial *rpo*B sequences obtained from isolates were assembled in a contig using the phred/Phrap/CONSED program [48,49]. The identification was achieved by comparing the contiguous *rpo*B gene sequences obtained with *rpo*B sequence data from reference and type strains available in the public database GenBank (http://www.ncbi.nlm.nih.gov) using the BLASTn routine. The sequences were aligned with bacteria *rpo*B genes data retrieved from NCBI website using CLUSTAL X program [50] and analyzed with MEGA software v.4 [51]. Evolutionary distances were derived from sequence-pair dissimilarities calculated using the Kimura's DNA substitution model [52]. The phylogenetic reconstruction was done using the neighbor-joining algorithm [53], with bootstrap values calculated from 1000 replicate runs [54].

2.4. Experimental design

Before working on real contaminated semen samples, the applicability of MALDI-MS to the bacterial screening of bull sperm was tested. In this pilot experiment, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 strains were cultured on BHI broth at 38 °C for 24 hours. After growing, each bacterial pellet was diluted in 100 μL of water, and 50 μL of the obtained solution were added to 50 μL of bull semen. The spiked mix was carefully submitted to inactivation and proteins extraction as reported in the Materials and methods section. The *Escherichia coli and Staphylococcus aureus* were properly identified by Biotyper 3.0 with a high score (>2.3). This proved that the semen proteins do not interfere with the MS identification.

Thirty semen samples and 570 culture medium samples (204, 172, and 194 for IVFm, IVCm, and IVMm, respectively) collected at the commercial setting for bovine embryo IVP were examined in this study.

3. Results and discussion

This study tested the use of MALDI-MS–based bacterial identification in semen and bovine IVP media samples collected in a large-scale commercial setting (Mogi Mirim, SP, Brazil). Aliquots of semen samples (used in the

Table 1
Results of Biotyper software best two matches for microorganism identification in bovine semen samples by MALDI-MS by ICM.

Analyte name	Organism (best match)	Score value	Organism (second best match)	Score value
Sample 2 ICM	*Citrobacter freundii*	2.365	*Citrobacter freundii*	2.215
Sample 10 ICM	*Citrobacter freundii*	2.304	*Citrobacter freundii*	2.071
Sample 1 ICM	*Enterobacter kobei*[a]	2.493	*Enterobacter asburiae*[a]	2.426
Sample 3 ICM	*Enterobacter kobei*[a]	2.373	*Enterobacter asburiae*[a]	2.329
Sample 4 ICM	*Enterobacter kobei*[a]	2.449	*Enterobacter hormaechei*[a]	2.332
Sample 5 ICM	*Enterobacter kobei*[a]	2.436	*Enterobacter asburiae*[a]	2.302
Sample 9 ICM	*Enterobacter kobei*[a]	2.528	*Enterobacter asburiae*[a]	2.398
Sample 7 ICM	*Stenotrophomonas maltophilia*	2.482	*Stenotrophomonas maltophilia*	2.362
Sample 8 ICM	*Stenotrophomonas maltophilia*	2.455	*Stenotrophomonas maltophilia*	2.221

Only score values >2 have been considered as successful identification.
[a] Enterobacter cloacae complex.

commercial large-scale IVF setting) were collected after thawing, and culture media were collected after oocytes and embryos incubation. Samples were refrigerated and sent to the analytical laboratory at the University of Campinas (UNICAMP) to perform overnight fast culturing (24 hours) and the MALDI-TOF-MS analysis (few minutes per sample).

Initially, a pilot study was performed by experimental contamination of the semen samples with *Escherichia coli and Staphylococcus aureus*. The *Escherichia coli and Staphylococcus aureus* were properly identified by Biotyper 3.0 with a high score (>2.3) (data not shown). This proved that the semen proteins do not to interfere with the MS identification.

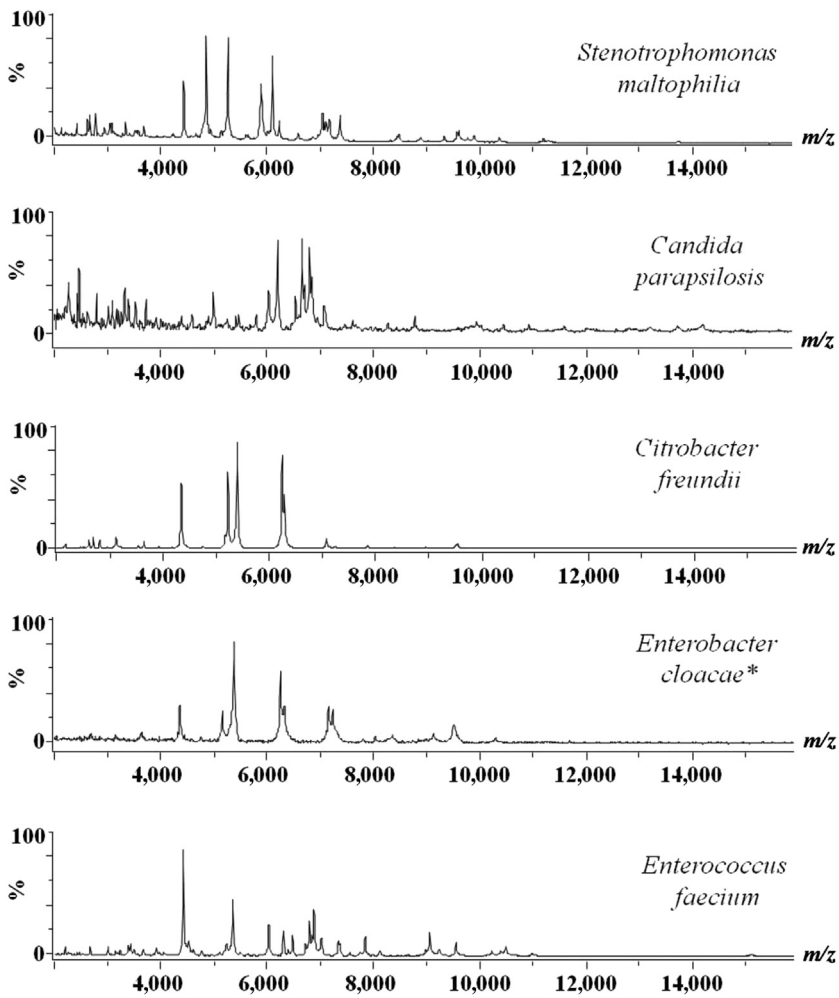


Fig. 1. MALDI-TOF-MS in the *m/z* range of 2000 to 16,000, showing abundant protein ions extracted from the microorganisms isolated from bovine semen used for bovine IVP.

Table 2
Results of Biotyper software best two matches for microorganism identification in bovine semen samples by MALDI-TOF-MS after extraction of the ribosomal proteins (PEM strategy).

Analyte Name	Organism (best match)	Score value	Organism (second best match)	Score value
Sample 1 PEM	*Enterobacter kobei*[a]	2.167	*Enterobacter asburiae*[a]	2.106
Sample 2 PEM	*Citrobacter freundii*	2.313	*Citrobacter freundii*	2.024
Sample 3 PEM	*Enterobacter kobei*[a]	2.206	*Enterobacter asburiae*[a]	2.201
Sample 4 PEM	*Enterobacter kobei*[a]	2.378	*Enterobacter hormaechei*[a]	2.289
Sample 5 PEM	*Enterobacter kobei*[a]	2.484	*Enterobacter cloacae*[a]	2.395
Sample 6 PEM	*Enterococcus faecium*	2.350	*Enterococcus faecium*	2.040
Sample 7 PEM	*Stenotrophomonas maltophilia*	2.302	*Stenotrophomonas maltophilia*	2.120
Sample 8 PEM	*Stenotrophomonas maltophilia*	2.361	*Stenotrophomonas maltophilia*	2.013
Sample 9 PEM	*Enterobacter kobei*[a]	2.159	*Enterobacter asburiae*[a]	2.137
Sample 10 PEM	*Citrobacter freundii*	2.317	*Citrobacter freundii*	2.029
Sample 11 PEM	*Candida parapsilosis*	2.396	*Candida parapsilosis*	2.379

[a] Enterobacter cloacae complex.

Subsequently, the 30 samples of frozen semen were cultured. After 24 hours of growing, 11 samples showed turbidity. After centrifugation and removal of the BHI growth medium, each pellet was aliquoted and submitted to sample preparation for MALDI-MS analysis for bacterial identification. The Scheme 1 shows the workflow of the bovine semen MALDI-MS analysis.

As Scheme 1 shows, two MS methods were used: ICM and ribosomal PEM. First, the capability of the MALDI-MS technique to directly identify the microorganisms without protein extraction was evaluated. This protocol was performed by diluting the bacteria pellet (0.1 g) in 1 mL of water and acetonitrile solution (1:1 vol/vol). One microliter of the obtained mixture was directly spotted on the MALDI plate. The direct MALDI-MS identification was successful in 9 of the 11 samples that showed turbidity, which were contaminated by *Stenotrophomonas maltophilia* (samples 7 and 8), *Citrobacter freundii* (samples 10 and 2), and *Enterobacter cloacae* complex (samples 1, 3, 4, 5, and 9). In all of them, Biotyper scores were >2.3, indicating a very high reliability of identification of the bacterial genus and the species (Table 1).

Note that in these cases, the high score and easy identification via ICM could be explained by considering that they are all gram-negative bacteria and their membrane is composed of a thin peptidoglycan layer (which is thinner than the the gram-positive bacteria), and the acidity of the matrix solution is sufficient to lyse the membrane of the bacteria and directly extract the ribosomal proteins. The identification by ICM failed for sample 11 (unreliable identification; confirmed to be *Candida parapsilosis* by PEM) and sample 6 (*Enterococcus faecium*, score <1.7). The reason for the failure can be accounted again by considering the membrane composition of the microorganism. The *Enterococcus faecium* is a gram-positive bacterium that has a membrane composed of a thick peptidoglycan layer. *Candida parapsilosis* is a fungal species of the yeast family and its membrane is composed of a complex structure of chitin, glucans, and other polymer chains. This type of membrane needs a harsher process to be broken, so that the PEM is effective by enabling proteins to be thoroughly extracted, giving more diverse and intense signals in the MALDI-MS.

The PEM allowed the identification of the cultured bacteria with a score values >2.0 in all the samples. For the 11 samples of semen presenting bacterial growth in this study, five microorganisms were identified: *Citrobacter freundii*, *Stenotrophomonas maltophilia*, *Enterobacter cloacae* complex, *Candida parapsilosis*, and *Enterococcus faecium*. Figure 1 shows the characteristic mass spectrum of each microorganism. Table 2 reports the Biotyper score values. Note that all the identified bacteria consistently matched with the most common bacterial microorganisms reported in the literature for bovine frozen semen [8–10,55]. Although the Biotyper database is able to detect the mixture of microorganisms [37], this was not observed. Therefore, if more than one microorganism may exist in the semen, the amount was not enough to interfere in the protein profiles obtained.

Our results of the ICM and PEM comparison are also consistent with those reported by Ferreira et al. that compared the intact cell and PEMs in bacteria contaminating urine and blood [56]. We suggest that the ICM should be initially applied to all samples. If reliable identification is not achieved, the more demanding PEM approach should be applied to increase the number of reliable identifications. The same experiments (applying both ICM and PEM methods) were also performed on isolated colonies. After isolation, the single colonies were cultured (to increase the amount of material) obtaining the same Biotyper results (data not shown).

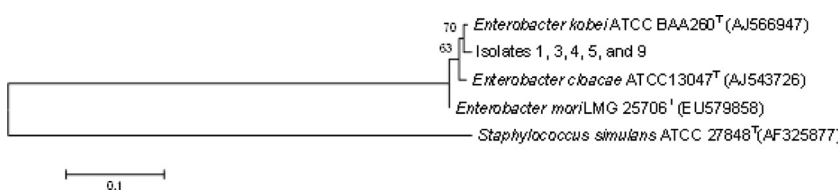


Fig. 2. Neighbor-joining phylogenetic tree from the analysis of *Enterobacter* sequences of partial *rpo*B genes. The numbers at the nodes are percentages indicating the levels of bootstrap support, based on a neighbor-joining analysis of 1000 resampled data sets. Only values >50% are shown.

Table 3
Microorganisms identified in IVMm, IVFm, and IVCm samples and corresponding score values of identification (scores >2.0 indicate confident genus and species identification).

Microorganisms identified	IVM	IVF	IVC	Average score values
Aneurinibacillus spp.	13/204	6/172	11/194	2.072
Brevibacillus spp.	16/204	11/172	19/194	2.019
Candida guilliermondii	0/204	2/172	0/194	2.352
Candida parapsilosis	0/204	2/172	0/194	2.396

Due to the restrictions of the MALDI Biotyper 3.0 software, a secure identification inside the *Enterobacter cloacae* complex was not possible. The *Enterobacter cloacae* complex is a group of six very closely related species with similar resistance pattern, (*Enterobacter asburiae*, *Enterobacter cloacae*, *Enterobacter hormoachei*, *Enterobacter kobei*, *Enterobacter ludwigii*, and *Enterobacter nimipressuralis*), and the Biotyper library is still not able to properly distinguish them. In samples 1, 3, 4, 5, and 9, the Biotyper software identified mostly *Enterobacter kobei* as the first best match and *Enterobacter asburiae* as the second best match with very close score values. To identify the exact *Enterobacter cloacae* species, the samples cultured from isolated colonies were submitted to DNA amplification and partial *rpo*B gene sequencing.

The usefulness of RNA polymerase beta-subunit encoding gene (*rpo*B) sequences as an alternative tool for universal bacterial genotypic identification has been investigated and proved to be very accurate among enteric bacteria [50,51]. The phylogenetic analysis of the *rpo*B sequences identified the isolates 1, 3, 4, 5, and 9 as *Enterobacter kobei* (Fig. 2), confirming the identification of the Biotyper software. When comparing the contiguous *rpo*B gene sequences obtained with *rpo*B sequences data from type strains available in GenBank (http://www.ncbi.nlm.nih.gov) using the BLASTn routine, *Enterobacter asburiae* was not identified as one of the best matches, since its similarity is 97%, and because of that this sequence was not included on the phylogenetic reconstruct.

For the culture media MALDI-TOF-MS analysis, just the PEM protocol has been performed due to its effectiveness. For this work, the week total amount of dishes used for oocytes maturation, IVF, and *in vitro* culture in the commercial setting were classified into two sets of samples, called “work A” and “work B.”

We incubated 204 IVMm samples, 172 IVFm samples, and 194 IVCm samples obtained from the IVP routine procedures along 3 weeks. Table 3 reports the microorganisms identified and the related averaging score. We observed the turbidity mainly in one set of samples (“work A” of the first week), which were entirely contaminated by two nonpathogenic environmental microorganisms, the *Aneurinibacillus* spp. and *Brevibacillus* spp. Figure 3 shows the corresponding MALDI spectra. Both *Aneurinibacillus* spp. and *Brevibacillus* spp. are two new genera recently proposed after the reclassification of *Bacillus brevis* genus [46]. They are associated with the soil, and their isolates have been found in the dairy environment [57].

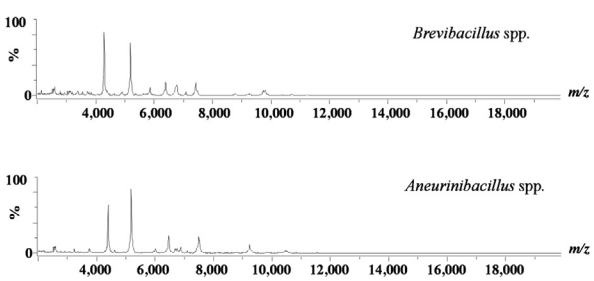


Fig. 3. MALDI-MS in the *m/z* range of 2000 to 20,000 for abundant proteins present in the extract of microorganisms cultured from IVMm, IVFm, and IVCm media.

The identification of the *Aneurinibacillus* spp. and *Brevibacillus* spp. was confirmed by 16S rRNA gene amplification by using specific detection primers and PCR. All isolates that needed a confirmation of the identification were submitted to PCR using both primers BREV174F and ANEU506F. The primers BREV174F and ANEU506F were designed to detect only members of the *Bacillus brevis* and *Bacillus aneurinolyticus* clusters, respectively, confirming the identification according to which primer the amplification occurred.

The samples of week 2 showed a contamination in two IVFm samples of “work B” with the same microorganism. In this case, the fungus *Candida guilliermondii* was identified with a score >2.3. This fungus is a pathogenic agent mostly present in human nails and associated with human onychomycosis infections. It has been first isolated [58] in ruminants in 1961, and further investigations have revealed that on cattle, *Candida guilliermondii* attacks primarily the urogenital tract, and it is the causative of several disorders, such as inflammatory diseases of the urogenital organs (uterus, seminal gland, epididymis, udder, kidney, etc.) and various other reproductive disorders [59]. In other two IVFm samples of work B of the third week, *Candida parapsilosis* was detected. As Table 2 shows, this fungus was also identified during the screening of the cryopreserved bovine semen (sample 11) with a score >2.3. This fungus of the yeast family has been reported as a pathogen causing abortion in cows [60]. Note that to avoid the spread of fungal infections in cows (which are notoriously the most dangerous), after the IVF step, two strong washes are routinely performed. In the first washing step, the granulosa cells and spermatozoa are removed by pipetting the zygotes in three droplets of IVCm. Before transferring to the culture plates, the zygotes are washed in two more droplets of the same media to ensure the removal of contaminations. In this study, we found that these washing steps are quite efficient; in fact, no contamination of fungi was observed in the *in vitro* culture media (see Table 3).

Therefore, although rare, microbial contamination of culture dishes occasionally occurs in the IVP routine. The results indicate that the contamination is mainly due to the collection of oocytes in farms, under open environment,

and to the microorganisms that may be present in the semen. If necessary, the proposed technique can be also applied to find out the source of contaminations that may occur at different levels of the IVP.

The application of a fast microbial identification technique can, therefore, be useful not only for the early detection of microorganisms but also for the administration of a more specific antibiotic in the samples that require it.

Microorganisms (mainly nonpathogenic) sometimes survive, but their detection and control can be monitored in a fast and reliable way by MALDI-MS in order to reach sanitary excellence and certification for bovine semen and embryo IVP production and commercialization.

3.1. Conclusions

The MALDI-MS technique was successfully used for the identification of microorganisms in the bovine semen and IVCm, IVMm, and IVFm used for embryo IVP. Even though the ICM method does not always allow obtaining results at species or subspecies level, this approach can be useful for an even faster microbiological screening of the cryopreserved semen and IVFm, IVMm, and IVCm samples.

Notable is the efficiency of the MALDI-MS technique, because the identification results have all been confirmed by DNA sequencing. Compared with the time-consuming sequencing and biochemical methods, MALDI-MS allows obtaining high confident results in very short time (15–20 minutes/sample). The time for the bacteria culturing can be significantly shortened by the use of incubators with culture media turbidity sensors, making the MALDI-TOF-MS approach even faster.

Even though the "gold standard" for microorganism identification is by microorganism DNA amplification and/or sequencing, our results as well as recent literature on human microbiology [61–63] show that MALDI-MS provides secure molecular identification of specific bacteria, making it an alternative accurate and fast method able to identify unknown bacteria based on profiles of proteins and peptides. This fast approach can be used to detect early contamination in semen and to allow sanitary excellence and certification to prevent economical losses and commercialization barriers in bovine IVP process. A significant biological question is how the microorganisms detected in semen and culture media influence the fertilization and/or embryo development. To answer this question, in the near future this research group will carry out a large-scale screening of microorganisms in semen and culture media.

Acknowledgments

Dr. Valeria Micheli, Dr. Giorgia Matteucci of University of Rome Tor Vergata, Diego M. Assis from Federal University of Sao Paulo, Biophysics Department, SP, Brazil, and Juliano L. Gonçalves of the University of Sao Paulo State (USP) of Pirassununga are gratefully acknowledged for the useful discussions. The authors thank the Brazilian science foundation "Fundaçao de Amparo à Pesquisa do Estado de São Paulo" (FAPESP) Number of Process 2011/06191-7 for financial assistance.

References

[1] Eanglesome MD, Garcia MM. Disease risks to animal health from artificial insemination with bovine semen. Rev Sci Tech 1997;16:215–25.
[2] Guerin B, Nibart M, Marquant-Le Guienne B, Humblot P. Sanitary risks related to embryo transfer in domestic species. Theriogenology 1997;47:33–42.
[3] Bailey JL, Ois Bilodeau JF, Cormier N. Semen cryopreservation in domestic animals: a damaging and capacitating phenomena. J Androl 2000;21:1–7.
[4] Karrow AM, Critser JK. Reproductive tissue banking: scientific principles. San Diego, CA: Academic Press; 1997.
[5] Mortimer D. Current and future concepts and practices in human sperm cryobanking. Reprod Biomed Online 2004;9:134–51.
[6] Bielanski A, Bergeronb H, Laub PCK, Devenish J. Microbial contamination of embryos and semen during long term banking in liquid nitrogen. Cryobiology 2003;46:146–52.
[7] Thibier M, Guerin B. Hygienic aspects of storage and use of semen for artificial insemination. Anim Reprod Sci 2000;62:233–51.
[8] D'Angelo M, Pavão DL, Melo GM, Rojas N, Souza RJ, Athayde C, et al. Acceptable microorganisms concentration in a semen sample for *in vitro* embryo production. Braz J Microbiol 2006;37:571–2.
[9] Bielanski A, Vajta G. Risk of contamination of germplasm during cryopreservation and cryobanking in IVF units. Human Reprod 2009;24:2457–67.
[10] Braga ACP, Tata A, Dos Santos VG, Barreiro JR, Schwab NV, Dos Santos MV, et al. Bacterial identification: from the agar plate to the mass spectrometer. RSC Adv 2013;3:994–1008.
[11] Seng P, Rolain JM, Fournier PE, La Scola B, Drancourt M, Raoult D. Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Future Microbiol 2010;5:1733–54.
[12] Bizzini A, Greub G. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry: a revolution in clinical microbial identification. Clin Microbiol Infect 2010;16:1614–9.
[13] Fenselau C, Demirev PA. Characterization of intact microorganisms by MALDI mass spectrometry. Mass Spectrom Rev 2001;20:157–71.
[14] Lay Jr JO. MALDI-TOF mass spectrometry of bacteria. Mass Spec Rev 2001;20:172–94.
[15] Mellmann A, Cloud J, Maier T, Keckevoet U, Ramminger I, Iwen P, et al. Evaluation of matrix-assisted laser desorption ionization-time-of-flight mass spectrometry in comparison to 16S rRNA gene sequencing for species identification of non-fermenting bacteria. J Clin Microbiol 2008;46:1946–54.
[16] Verroken A, Janssens M, Berhin C, Bogaerts P, Huang TD, Wauters G, et al. Evaluation of matrix-assisted laser desorption ionization-time of flight mass spectrometry for identification of Nocardia species. J Clin Microbiol 2010;48:4015–21.
[17] Claydon MA, Davey SN, Edwards-Jones V, Gordon DB. The rapid identification of intact microorganisms using mass spectrometry. Nat Biotechnol 1996;14:1584–6.
[18] Holland RD, Wilkes JG, Rafii F, Sutherland JB, Persons CC, Voorhees KJ, et al. Rapid identification of intact whole bacteria based on spectral patterns using MALDI-TOF-MS. Rapid Commun Mass Spectrom 1996;10:1227–32.
[19] Krishnamurthy T, Ross PL. Rapid identification of bacteria by direct matrix-assisted laser desorption/ionization mass spectrometric analysis of whole cells. Rapid Commun Mass Spectrom 1996;10:1992–6.
[20] Arnold RJ, Karty JA, Ellington AD, Reilly JP. Monitoring the growth of a bacteria culture by MALDI-MS of whole cells. Anal Chem 1999;71:1990–6.
[21] Arnold RJ, Reilly JP. Fingerprint matching of *E. coli* strains with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of whole cells using a modified correlation approach. Rapid Commun Mass Spectrom 1998;12:630–6.
[22] Wang Z, Russon L, Li L, Roser DC, Long SR. Investigation of spectral reproducibility in direct analysis of bacteria proteins by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 1998;12:456–64.
[23] Welham KJ, Domin MA, Scannell DE, Cohen E, Ashton DS. The characterization of micro-organisms by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 1998;12:176–80.
[24] Dai Y, Li L, Roser DC, Long SR. Detection and identification of low-mass peptides and proteins from solvent suspensions of *Escherichia coli* by high performance liquid chromatography fractionation and matrix-assisted laser desorption/ionization mass spectrometry. Rapid Commun Mass Spectrom 1999;13:73–8.

[25] Buskirk AD, Hettick JM, Chipinda I, Law BF, Siegel PD, Slaven JE, et al. Fungal pigments inhibit the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis of darkly pigmented fungi. Anal Biochem 2011;411:122–8.
[26] Seneviratne CJ, Wang Y, Jin L, Abiko Y, Samaranayake LP. Proteomics of drug resistance in *Candida glabrata* biofilms. Proteomics 2010;10: 1444–54.
[27] Wirth H, von Bergen M, Murugaiyan J, Rosler U, Stokowy T, Binder HJ. MALDI-typing of infectious algae of the genus *Prototheca* using SOM portraits. Microbiol Methods 2012;88:83–97.
[28] Yao ZP, Demirev PA, Fenselau C. Mass spectrometry-based proteolytic mapping for rapid virus identification. Anal Chem 2002;74: 2529–34.
[29] Zhou S, Wan Q, Huang Y, Huang X, Cao J, Ye L, et al. Proteomic analysis of Singapore grouper iridovirus envelope proteins and characterization of a novel envelope protein VP088. Proteomics 2011;11:2236–48.
[30] Franco CF, Mellado MC, Alves PM, Coelho AV. Monitoring virus-like particle and viral protein production by intact cell MALDI-TOF mass spectrometry. Talanta 2010;80:1561–8.
[31] Diaz ML, Solari A, Gonzalez CI. Differential expression of *Trypanosoma cruzi* I associated with clinical forms of Chagas disease: overexpression of oxidative stress proteins in acute patient isolate. J Proteomics 2011;74:1673–82.
[32] Prodhom G, Bizzini A, Durussel C, Bille J, Greub G. Matrix-assisted laser desorption ionization-time of flight mass spectrometry for direct bacterial identification from positive blood culture pellets. J Clin Microbiol 2010;48:1481–3.
[33] Ferreira L, Sanchez-Juanes F, Gonzalez-Avila M, Cembrero-Fuciños D, Herrero-Hernández A, González-Buitrago JM, et al. Direct identification of urinary tract pathogens from urine samples by MALDI-TOF (matrix-assisted laser desorption ionization time-of-flight) mass spectrometry. J Clin Microbiol 2010;48:2110–5.
[34] Croxatto A, Prodhom G, Greub G. Applications of MALDI-TOF mass spectrometry in clinical diagnostic microbiology FEMS. Microbiol Rev 2012;36:380–407.
[35] El Khechine A, Couderc C, Flaudrops C, Raoult D, Drancourt M. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry identification of Mycobacteria in routine clinical practice. PLoS One 2011;6:e24720.
[36] Wieser A, Schneider L, Jung J, Schubert S. MALDI-TOF MS in microbiological diagnostics-identification of microorganisms and beyond (mini review). Appl Microbiol Biotechnol 2012;93:965–74.
[37] Barreiro JR, Ferreira CR, Sanvido GB, Kostrzewa M, Maier T, Wegemann B, et al. Identification of subclinical cow mastitis pathogens in milk by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. J Diary Sci 2010;93:5661–6.
[38] Barreiro JR, Braga PAC, Ferreira CR, Kostrzewa M, Maier T, Wegemann B, et al. Non culture-based identification of bacteria in milk by protein fingerprinting. Proteomics 2012;12:2739–45.
[39] Ferreira CR, Gustavo HMF, Souza MFR, Catharino RR, Pontes HFJ, Basso AC, et al. Mass spectrometry fingerprinting of media used for *in vitro* production of bovine embryos. Rapid Commun Mass Spectrom 2009;23:1313–20.
[40] Ferreira CR, Saraiva SA, Catharino RR, Garcia JS, Gozzo FC, Sanvido GB, et al. Single embryo and oocyte lipid fingerprinting by mass spectrometry. J Lipid Res 2010;51:1218–27.
[41] Pontes JH, Melo Sterza FA, Basso AC, Ferreira CR, Sanches BV, Rubin KC, et al. Ovum pick up, *in vitro* embryo production, and pregnancy rates from a large-scale commercial program using Nelore cattle (Bos indicus) donors. Theriogenology 2011;75: 1640–6.
[42] Lartigue MF, Hery-Arnaud G, Haguenoer E, Domelier AS. Identification of *Streptococcus agalactiae* isolates from various phylogenetic lineages by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol 2009;47:2284–7.
[43] Barbuddhe SB, Maier T, Schwarz G, Kostrzewa M. Rapid identification and typing of Listeria species by matrix-assisted laser desorption ionization-time of flight mass spectrometry. Appl Environ Microbiol 2008;74:5402–7.
[44] Van Soolinger D, De Haas PEW, Hermans PWM, Groenen P, Van Embden JDA. Comparison of various repetitive DNA elements as genetics markers for strain differentiation and epidemiology of *M. tuberculosis*. J Clin Microbiol 1993;31:1987–95.
[45] Shida O, Takagi H, Kadowaki K, Komagata K. Proposal for two new genera, *Brevibacillus* gen. nov. and *Aneurinibacillus* gen. nov. Int J Syst Bacteriol 1996;46:939–46.
[46] Paauw A, Caspers MPM, Schuren FHJ, Leverstein-van Hall MA, Deletoile A. Genomic diversity within the *Enterobacter cloacae* complex. PLoS One 2008;3:e3018.
[47] Mollet C, Drancourt M, Raoult D. rpoB sequence analysis as a novel basis for bacterial identification. Mol Microbiol 1997;26:1005–11.
[48] Ewing B, Hillier L, Wendl M, Green P. Basecalling of automated sequencer traces using phred. I. Accuracy assessment. Genome Res 1998;8:175–85.
[49] Gordon D, Abajian C, Green P. Consed: a graphical tool for sequence finishing. Genome Res 1998;8:195–202.
[50] Thompson JD, Higgins DJ, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res 1994;22:4673–80.
[51] Tamura K, Dudley J, Nei M, Kumar S. MEGA4: molecular evolutionary genetics analysis. (MEGA) software version 4.0. Mol Biol Evol 2007;24:1596–9.
[52] Kimura M. A simple model for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 1980;16:111–20.
[53] Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 1987;4: 406–25.
[54] Felsenstein J. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 1985;39:783–91.
[55] Lee K, Kim I, Son D, Lee H, Lee D, Seo G, et al. Identification of bacteria derived from frozen bovine semen that resulted in contamination during *in vitro* fertilization. Theriogenology 1997;47. 375-375(1).
[56] Ferreira L, Sánchez-Juanes F, Muñoz-Bellido JL, González-Buitrago JM. Rapid method for direct identification of bacteria in urine and blood culture samples by matrix-assisted laser desorption ionization time-of-flight mass spectrometry: intact cell vs. extraction method. Clin Microbiol Infect 2011;17:1007–12.
[57] Sanders ME, Morelli L, Tompkins TA. Sporeformers as human probiotics: *Bacillus*, *Sporolactobacillus*, and *Brevibacillus*. Compr Rev Food Sci F 2003;2:101–10.
[58] Hauptman B. Med Vet 1961;17:22.
[59] Sutka P, Sutka K. Process for the production of immunobiological preparations applicable in the diagnosis preventions and /or treatment of *Candida guilliermondii* infections. United States Patent. 1987 Patent number 4678748.
[60] Foley GL, Schlafer DH. Candida abortion in cattle. Vet Pathol 1987; 24:532–6.
[61] Romero-Gómez MP, Gómez-Gil R, Paño-Pardo JR, Mingorance J. Identification and susceptibility testing of microorganism by direct inoculation from positive blood culture bottles by combining MALDI-TOF and Vitek-2 Compact is rapid and effective. J Infect 2012;65:513–20.
[62] Schubert S, Weinert K, Wagner C, Gunzl B, Wieser A, Maier T, et al. Novel, improved sample preparation for rapid, direct identification from positive blood cultures using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. J Mol Diagn 2011;13:701–6.
[63] Juiz PM, Almela M, Melción C, Campo I, Esteban C, Pitart C, et al. A comparative study of two different methods of sample preparation for positive blood cultures for the rapid identification of bacteria using MALDI-TOF MS. Eur J Clin Microbiol Infect Dis 2012;31: 1353–8.