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Rapid detection of high-risk *Enterococcus faecium* clones by matrix-assisted laser desorption ionization time-of-flight mass spectrometry

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ABSTRACT

We aimed to explore the potential of matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) for early identification of dominant *Enterococcus faecium* (Efm) clones involved in human infections. Well-characterized Efm isolates (n = 77), analyzed by pulsed-field gel electrophoresis and multilocus sequence typing(eBURST and BAPS [Bayesian analysis of population structure] algorithms), and belonging to different hospital (n = 53) and community (n = 24) phylogenomic groups, were tested. Mass spectra (Bruker) were analyzed by visual inspection and different chemometric tools. Discrimination between groups comprising isolates commonly found in hospitals (BAPS 2.1a, 3.3a1, 3.3a2) and community (BAPS 2.1b and 3.2) was achieved with >99% accuracy, while identification of sequence types belonging to different BAPS subgroups was associated with >95% correct predictions. Our work is a proof of concept with regard to the suitability of MALDI-TOF MS in the identification of high-risk Efm clones. Further studies including strains from a wider variety of clones and sources will strengthen the potential of the workflow here described.

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1. Introduction

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has recently emerged as a powerful bioanalytical tool in clinical and food microbiology, due to its ability to rapidly and unequivocally identify a wide variety of bacteria (Clark et al. 2013; Singhal et al. 2015; van Belkum et al. 2015). The detection of specific peaks corresponding to conserved ribosomal or abundant housekeeping proteins and peptides derived from whole bacterial cells is the base of the routine identification of different bacterial species by MALDI-TOF MS (Barbuddhe et al. 2008; Dieckmann and Malorny 2011; Sousa et al. 2014; Werner et al. 2012). The exploitation of this technique for bacterial discrimination at the subspecies level is more challenging, but relevant for epidemiological investigations of hospital outbreaks caused by multidrug-resistant strains, microbial forensics, or source tracking in food microbiology (Sandrin et al. 2013; Spinali et al. 2015).

E. faecium is a member of the gastrointestinal flora of mammals able to cause severe human infections (ECDC 2014). It is also one of the most common enterococcal species used in food industry and as a feed additive (animal probiotic). Analysis of the population structure of *E. faecium*, based on multilocus sequence typing (MLST) data and whole genomic sequencing, identified different clades that mainly differ in the genome size, host background, and the content of mobile genetic elements (Lebreton et al. 2013; Willems and van Schaik 2009; de Been et al. 2015). Due to the lack of suitability of eBURST to analyze the population structure of species with high recombination rates as *E. faecium* (Turner et al. 2007; Willems et al. 2012), MLST data of this species are also analyzed by Bayesian-based algorithm leading to BAPS (Bayesian analysis of population structure) groups (Tedim et al. 2015; Willems et al. 2012). For instance, the former clonal complex (CC) 17 identified by eBURST and comprising most of the hospital-acquired (HA) *E. faecium* infections analyzed in the clinical setting, is split into 3 lineages of independent origin, namely BAPS subgroups 3.3a1 (sequence type [ST] 18 lineage), 3.3a2 (ST17 lineage), and 2.1a (ST78 lineage) (Tedim et al. 2015; Willems et al. 2012). These clones have been mainly associated with severe HA infections and hospital outbreaks worldwide

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being considered "high-risk" clones in a clinical context as well as for probiotic purposes (Leavis et al. 2006; Willems et al. 2011). Acquisition of vancomycin-resistance by these *E. faecium* high-risk clones is of particular concern due to the few available therapeutic options. Vancomycin-resistant *E. faecium* (VREfm) strains from swine are frequently allocated into CC5, which has also occasionally been identified in both hospitalized patients and healthy humans (Freitas et al. 2011). These CC5 strains are currently split into BAPS subgroups 2.1b (ST5 lineage) and 3.2 (ST6) (Willems et al. 2012).

While the accurate identification of different *Enterococcus* species is achievable by MALDI-TOF MS (Eigner et al. 2009; Fang et al. 2012; Werner et al. 2012), their discrimination at the clonal level is still not demonstrated (Lasch et al. 2014). Currently, the identification of *E. faecium* high-risk clones is time-demanding and not accurate. Our aim was to evaluate the potential of a MALDI-TOF MS and chemometrics combined approach to depict relevant *E. faecium* clones globally associated with human infections.

2. Materials and methods

2.1. Epidemiological and clonal background of bacterial isolates

A total of 77 VREfm (69 *vanA* and 8 *vanB*) isolates from hospital (hospitalized patients, hospital wastewaters) and nonhospital (healthy humans, swine) origins were studied (Table 1). Clinical isolates were obtained from hospitalized patients in different cities, which were in most of the cases distantly located from sites where nonhospital isolates were collected. All isolates were authorized by the ethical committees of their respective hospitals. We included strains deeply characterized at both phenotypic and molecular levels, which represent all major *E. faecium* clonal lineages described to date. In our setting, most of the characterized strains of these lineages mainly correspond to vancomycin-resistant strains of the VanA-type (Novais et al. 2005; Freitas et al. 2009; Freitas et al. 2011; Freitas et al. 2016). The population structure of these isolates had been determined by MLST and analyzed by using BAPS software as previously described (Tedim et al. 2015; Willems et al. 2012). The collection included isolates of 17 STs belonging to BAPS 3.3a1 (n = 31; ST18, ST132, ST173, ST368, ST670); BAPS 3.3a2 (n = 15; ST16, ST17, ST202, ST209) and BAPS 2.1a (n = 7; ST78, ST80, ST117, ST192) subgroups; and BAPS 2.1b (n = 11; ST5, ST147, ST185) and BAPS 3.2 (n = 13; ST6). For a more easily reading of the manuscript data and to accomplish the requirements of our chemometric analysis, the sample analyzed will be designated from now on as "hospital group" (n = 53; BAPS 3.3a1, 3.3a2, and 2.1a; 13 STs, mostly human infections) and "community or nonhospital group" (n = 24, BAPS 2.1b and 3.2; 4 STs, mostly swine and healthy humans) as isolates of these BAPS groups were positively associated with hosts in these settings (Tedim et al. 2015). The isolates exhibited different pulsed-field gel electrophoresis (PFGE) profiles as well as variable antibiotic resistance and virulence patterns to provide maximal diversity (data not shown) (Freitas et al. 2009, 2011, 2016).

2.2. VREfm isolation, propagation, and resistance characterization

E. faecium isolates were plated on Brain Heart agar (Liofilchem, Italy) plates from frozen (−80 °C) cultures (Trypticase Soy Broth [Liofilchem] with 15% glycerol) and incubated overnight at 37 °C. After normal growth, purity, and vancomycin-resistance phenotype being checked, they were propagated to Mueller-Hinton (BioMérieux, France) agar plates (37 °C/exactly 18 h) for further preparation of bacterial extracts. Antibiotic susceptibility testing was performed for 13 antibiotics by the disc diffusion method following CLSI guidelines (CLSI 2015).

2.3. MALDI-TOF MS

A protocol suggested by the mass spectrometer manufacturer for clinical microbiology diagnostics (extraction procedure, matrix, sample:matrix ratio, and solvents) was adopted in order to grant maximal compatibility with established workflows and easier application in routine clinical microbiology laboratories (detailed described in Alatoom et al. 2011; Clark et al. 2013; Sousa et al. 2014). Microbial mass spectra were automatically acquired in a linear positive mode at a nitrogen laser frequency of 20 Hz (2000–20,000 Da) using a Microflex III instrument (Bruker Daltonics, Bremen, Germany). The experiments were performed under strictly controlled conditions (temperature, incubation time, growth medium) in quadruplicate using 4 distinct spots of the MALDI target (instrumental replicates) at least in 2 different days (biological replicates) with 2 different bacterial extracts. External mass calibration was performed with a mixture of proteins contained in the Bruker bacterial test standard.

2.4. Data analysis

MALDI-TOF peaks were directly compared with the reference spectra of the integrated database using MALDI Biotyper Software (MALDI BioTyper Version 3.0 Build 25—Bruker Daltonics) or edited and subsequently subjected to chemometric analysis. All mass spectra were edited using the FlexAnalysis software by applying the "smoothing" and "baseline subtraction" options. Zeroline and low S/N ratio spectra were discarded after visual inspection. Given the large amount of data generated by MALDI experiments, mean spectra for each isolate were generated from the instrumental and biological replicates and considered for further analysis. Spectra were preprocessed with vector-normalization (unit norm) in order to consider only relative differences between isolates by removing the influence of different sample amounts and/or equipment variations in the peaks intensity (Sousa et al. 2015). The chemometric analysis was performed with Matlab version 8.3 (MathWorks, Natick, MA, United States) and the PLS Toolbox version 7.5 for Matlab (Eigenvector Research, Wenatchee, WA, United States).

2.4.1. Peak identification

Mass spectra were analyzed with the peakfind function of the PLS Toolbox for Matlab (arguments, 20—number of points in Savitzky–Golay filter, 8—tolerance on the estimated residuals; peaks heights are estimated to be >tolerance*residuals) and 20—window width for determining local maxima to evaluate the intra- and intergroup variability of hospital and nonhospital groups (see detailed method in Sousa et al. 2015).

2.4.2. Multivariate analysis

MALDI-TOF mass spectra were analyzed by unsupervised and supervised chemometric methods. The selected unsupervised method for the discrimination of the isolates according to their STs was principal component analysis (PCA) due to the insufficient number of some of them to build a robust supervised model. The PCA scores were further used to a hierarchical cluster analysis (HCA) (Naes et al. 2002) using the agglomerative Ward algorithm. The purpose of HCA was the generation of a dendrogram highlighting the association between strains. Partial least squares discriminant analysis (PLSDA) was the selected supervised chemometric method for the classification of spectral data according to hospital and nonhospital clones and the different STs when represented by a significant number of isolates (Barker and Rayens 2003; Geladi and Kowalsky 1986; Sousa et al. 2015). All PLSDA models were calibrated considering 70% randomly selected samples and tested on the remaining 30%. The most appropriate number of latent variables is estimated from the calibration dataset and optimized using the leave-one-isolate-out cross-validation method in order to prevent overfitting (Naes et al. 2002). All presented model results are based exclusively on testing data and presented as rate of correct ST predictions (Sousa et al. 2015). Before modeling with PLSDA, the spectral dataset is subjected to mean-centering. Different PLSDA models were calibrated in order to discriminate between different clonal classifications.

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