Rapid and reproducible MALDI-TOF-based method for detection Vancomycin-resistant *Enterococcus faecium* using classifying algorithms

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Running Title: Vancomycin-resistant *E. faecium* discrimination using MALDI-TOF MS

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ABSTRACT

Vancomycin-resistant *Enterococcus faecium* has become a health threat over the last 20 years due to its ability to rapidly spread and cause outbreaks in hospital settings. Although MALDI-TOF MS has already demonstrated its usefulness for accurate identification of *E. faecium*, its implementation for antimicrobial resistance detection is still under evaluation. The reproducibility of MALDI-TOF MS for peak analysis and its performance for correct discrimination of vancomycin susceptible isolates (VSE) from those hosting the VanA and VanB resistance mechanisms was evaluated in this study. For the first goal, intra-spot, inter-spot -technical- and inter-day -biological- reproducibility was assayed. The capability of MALDI-TOF to discriminate VSE isolates from VanA VRE and VanB VRE strains was carried out on protein spectra from 178 *E. faecium* unique clinical isolates -92 VSE, 31 VanA VRE, 55 VanB VRE-, processed with Clover MS Data Analysis software. Unsupervised (Principal Component Analysis –PCA-) and supervised algorithms (Support Vector Machine -SVM-, Random Forest -RF- and Partial Least Squares-Discriminant Analysis -PLS-DA-) were applied. The reproducibility assay showed lower variability for normalized data ($p<0.0001$) and for the peaks within the 3000-9000 *m/z* range. Besides, 80.9%, 79.21% and 77.53% VSE vs VRE (VanA + VanB) discrimination was achieved by applying SVM, RF and PLS-DA, respectively. Correct differentiation of VanA from VanB VRE isolates was obtained by SVM in 86.65% cases. The implementation MALDI-TOF MS and peak analysis could represent a rapid and effective tool for VRE screening. However, further improvements are needed to increase the accuracy of this approach.

1. INTRODUCTION

Enterococcus faecium are Gram-positive, non-spore forming, facultative anaerobic cocci that can be found as part of the microbiota of the human gastrointestinal tract (1). Because of its genomic plasticity and its adaptation to harsh conditions, *E. faecium* has become a health threat due to its ability to rapidly spread and cause outbreaks in hospital settings (2) (3). Adding up to the intrinsic antibiotic resistance displayed by *E. faecium*, infections caused by strains with acquired resistance to certain antimicrobials are of special interest (4). In the past years, vancomycin resistance on *E. faecium* has been a major concern owing to its rise and rapid spread of high-risk clones among hospitalized patients (5). Therefore, *E. faecium* was included in the ESKAPE group of highly resistant microorganisms because of its ability to "escape" the action of conventional treatments (6). Vancomycin resistance is by far the biggest threat regarding enterococci, mainly *E. faecium*, due to its position as first line treatment for multidrug-resistant enterococcal infections (7) (8).

Susceptibility to vancomycin can be routinely determined in the clinical microbiology laboratory using two approaches: i) phenotypically, by the standard broth antimicrobial susceptibility testing microdilution method or by gradient diffusion, or ii) genotypically, by amplification of the *vanA*/*vanB* genes and subsequent analysis of the specific amplicons (9) (10). The first approach has a turnaround time of approximately 2 days. Although the implementation of molecular methods provides final results in 1-3 hours after isolation in culture, its cost in laboratory reagents is high.

While Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) usefulness in the microbiology laboratory for bacterial identification is settled, its implementation for antimicrobial resistance detection is not well standardized yet (11).

In the last years, MALDI-TOF MS has been proposed as a fast and cost-efficient method for the detection of some antimicrobial resistance mechanisms, such as β-lactamase activity (12), discrimination of methicillin resistant *S. aureus* (13) or the

detection of the *cfiA* gene in *B. fragilis* (14) (15). It will be of interest in the clinical microbiology laboratory the use of MALDI-TOF MS as a rapid approach for the differentiation of VRE from VSE, based on their mass spectra protein profile.

The main objective of this study was the development of a MALDI-TOF-based classifying algorithm for the discrimination of VRE from susceptible ones (VSE).

2. MATERIALS AND METHODS

2.1. Bacterial strains

A total of n=178 *E. faecium* strains were included in the study and considered as the "classification set" (92 VSE, 31 VanA VRE, 55 VanB VRE). The isolates were collected consecutively throughout the years 2017 to 2019 from clinical samples of patients admitted at Hospital General Universitario Gregorio Marañón in Madrid (HGUGM) –Figure 1-.

Strains were selected from blood cultures (n=95) and rectal swabs (n=83) -Table S1-. Other types of clinical samples were not included to avoid high variability. Clonality was clinically discarded by analyzing the date, patient location and department where the inpatients were located. Besides, no clinical outbreak was detected during the period of study. All strains were isolated from inpatient clinical samples, characterized and kept frozen at -80ºC for further analysis. For this study, samples were thawed and cultured overnight at 37ºC in Columbia Blood agar.

2.2. Antibiotic susceptibility testing for vancomycin

Antimicrobial susceptibility testing was performed with the automated microdilution method Microscan® System (Beckman-Coulter, CA, USA) using PM33 panels following the manufacturer's guidelines. Vancomycin and teicoplanin breakpoints were stablished as indicated by the EUCAST (2021) v. 11. The results obtained were confirmed by real-time PCR for the amplification of the *vanA* and *vanB* genes (9). Besides, confirmation of the presence of the vancomycin resistance genes was

141 confirmed a second time by the implementation of the commercial Xpert® *vanA/vanB*
142 cartridges (Cepheid, CA, USA).
143
144 **2.3. Identification of the isolates by MALDI-TOF MS**
145 Bacterial strains were analyzed by MALDI-TOF MS in an MBT Smart MALDI
146 Biotyper (Bruker Daltonics, Bremen) using the updated database containing 9957 Mass
147 Spectra Profiles (MSPs). A few bacterial colonies of each enterococcal isolate were
148 spotted onto the MALDI target plate. On-plate protein extraction was performed by
149 applying 1µl formic acid on each target spot and let dry at room temperature before
150 adding 1µl of HCCA matrix solution (Bruker Daltonics), following the manufacturer's
151 instructions. Spectra were acquired in positive mode in the range of 2,000 to 20,000 Da,
152 applying default settings (16).
153
154 **2.4 Spectra acquisition and pre-processing**
155 Each isolate was analyzed in two different spots from the MALDI target plate and
156 each spot was read twice, obtaining 4 spectra per strain (17). Protein spectra were
157 visually inspected with FlexAnalysis (Bruker Daltonics) and aligned with the genus-
158 specific peak at 4428 *m/z*, present in all isolates (18) (19). Outlier spectra and zero-lines
159 were discarded.
160 After the previous screening, protein spectra were processed with Clover MS
161 Data Analysis software (Clover Biosoft, Granada, Spain). For classification purposes,
162 peak matrices were generated in the range of 2,000 *m/z* to 20,000 *m/z*. For this goal,
163 pre-processing was performed as follows: Savitzky-Golay Filter (window length 11;
164 polynomial order 3) was applied for smoothing spectra and then baseline was removed
165 by the Top-Hat filter method (factor 0.02);
166 Processed spectra were aligned using the following method: replicates within the
167 same spot were aligned to create an average spectrum per spot (shift medium; linear
168 mass tolerance 2000 ppm). Then, average spectra from each replicated spot were

aligned, and thus one average spectrum per sample was obtained. Finally, average spectra from different isolates were aligned together.

2.5. Reproducibility study

A reproducibility assay was performed to determine the robustness of MALDI-TOF-MS-based bacterial classification. For this purpose, 20 different *E. faecium* isolates (VSE -n=10- and VRE strains -n=10, 5 VanA VRE and 5 VanB VRE-) were randomly selected and considered as the "reproducibility set" -Table S2-. For intra-spot reproducibility two spectra per spot were acquired, whilst for inter-spot reproducibility (or technical reproducibility) an average spectrum was built from each spot. Finally, each isolate was subcultured during three consecutive days and their average spectra were compared in order to evaluate the biological (inter-day) reproducibility of the method (Figure 2). This methodology has been described by Oberle et al., 2016 (17).

All spectra were pre-processed using Clover MS Data Analysis Software following the pipeline described in section 2.4. The post-processed spectra (smoothed and baseline removed) were aligned (shift medium; linear mass tolerance 2000 ppm) to obtain an average spectrum for each spot. This process was repeated to obtain a single average spectrum per day for each isolate.

Once the average Day 1 spectra from all isolates were aligned, a first assay was performed to identify all common peaks and establish their characteristic peak profiles. Peak finding was carried out by applying a threshold filter (0.01), so peaks with less than 1.0% of the maximal intensity recorded were discarded. Group-specific peaks were then searched in the reproducibility set by the mass position method (constant mass tolerance 0.2 Da; linear mass tolerance 500 ppm) at the shot-, spot- and day-level.

The coefficient of variation (% CV) of the intensities registered for each of these common peaks was calculated from raw spectra and from spectra normalized with the TIC method in two different ways: i) normalizing by TIC the peaks previously found in raw spectra (pTIC) and ii) normalizing first the entire spectra by TIC and then finding the

common peaks (TICp). Results from both methods and from raw data were compared for intra-spot, technical and biological reproducibility.

In addition, Pearson correlation coefficient (p) was applied to protein spectra from VRE and VSE isolates to measure how biological variation could affect the discrimination of these two groups and how reproducibly the discrimination could be performed.

This peak study also included the calculation of arithmetic and post-alignment means and the subsequent comparison between them. The arithmetic means for spots and days were calculated directly from the intensity peaks values of each shot. On the other hand, the post-alignment means were calculated automatically with Clover MS Data Analysis software after replicated average spectra were aligned. Both mean values were compared at spot- and day-level. The assay was repeated without normalization and for both TIC methods. Thereby, the automatic alignment and replicate software process could be compared with the ideal arithmetic model in the three normalization cases. Once the Saphiro-Wilk and Levene test was applied to verify the normal distribution and the homoscedasticity of the data, a Student's t-test was performed to verify if the null hypothesis of equality of means was fulfilled for the three normalization methods.

2.6. Classification of *E. faecium* isolates based on their MALDI-TOF MS protein spectra

Protein spectra from *E. faecium* isolates were acquired as described in section 2.4. Clover MS Data Analysis Software was applied to differentiate 1) susceptible from resistant *E. faecium* strains and 2) isolates hosting *vanA* and *vanB* resistance genes. For this purpose, three different methods were evaluated: i) a "full-spectrum method" where the matrix obtained included all peak intensities from the spectrum separated by 0.5 Da regardless of their intensity. Their intensities were then normalized by Total Ion Current (TIC) normalization. The other two methods used a peak matrix generated by a "threshold method" where only peaks with intensities above 1.0% of the maximum peak

intensity (0.01 factor) were chosen. The difference in the last two methods was the order in which TIC normalization was applied: ii) before (TICp) or iii) after (pTIC) searching peaks by the threshold method.

The three peak matrices described above were used as input data for three different supervised machine learning algorithms: Partial Least Squares Discriminant Analysis (PLS-DA), Support Vector Machine (SVM) and Random Forest (RF). These algorithms were first tested for the discrimination VSE and VRE isolates and, secondly, for the differentiation of VRE isolates hosting *vanA* and *vanB* genes.

Internal validation of the results provided by each algorithm was assayed using k-fold cross validation (k=10) as previously described (20). Briefly, data was randomly split into 10 data subsets of the same size. The algorithms were trained with 9 of them and the remaining subset was used as a test set for internal validation. This process was iterated 10 times (one per each of the 10 subsets) and the accuracy rate of the classification was recorded (21).

Furthermore, a search for potential biomarkers was performed to use group-specific protein peaks as markers for the correct classification of VanA and VanB VRE and VSE strains. For this purpose, 178 average spectra (one per sample) from Day 1 were considered as input data for the Biomarker Analysis application within Clover MS Data Analysis software. The threshold method was applied as explained above and peaks were merged with 0.5 Da and 300 ppm as constant and linear mass tolerance, respectively.

2.6. Ethics statement

The Ethics Committee from the hospital Gregorio Marañón (CEIm) (code no. MICRO.HGUGM.2020-002) approved this study. Bacterial isolates -not human products- were analysed. Therefore, all the conditions to waive the informed consent have been met.

3. RESULTS

3.1. Identification by MALDI-TOF MS

All isolates (n=178) included in this study were correctly identified as *E. faecium* by MALDI-TOF MS with score ≥2.0. Identifications for this microorganism were consistent along the top 10 identifications provided by MALDI-TOF MS since this is a common pathogen well represented in the updated commercial library.

3.2. Reproducibility Study

When the different methods for peak finding described before were applied to the Day 1 spectra from the "classification set", a total of 18 common peaks were found in all *E. faecium* (VSE and VRE) protein spectra with intensities above 1.0% of the maximum peak intensity (Table 1). The CV means of the intensities from these 18 common peaks were compared with the peaks present in the spectra from the reproducibility set at intra-spot-, inter-spot- and inter-day-levels.

Comparing the means of the CVs in the reproducibility set, it showed lower CV values at intra-spot level than at inter-spot and inter-day level. For not normalized raw data the average CVs were $CV_{intra\text{-}spot}$ = 15.35, $CV_{inter\text{-}spot}$ = 29.29 and $CV_{inter\text{-}day}$= 31.25. The same pattern was shown for the TICp ($CV_{intra\text{-}spot}$ = 8.46, $CV_{inter\text{-}spot}$ = 20.88 and $CV_{inter\text{-}day}$ = 20.66) and pTIC methods ($CV_{intra\text{-}spot}$ = 7.99, $CV_{inter\text{-}spot}$ = 19.91 and $CV_{inter\text{-}day}$ = 19.30) methods (Figure 3). Besides, data normalization allowed the reduction of CV values at the three levels ($p<0.0001$). Differences between both normalization methods (TICp and pTIC) varied between 0.47 for $CV_{intra\text{-}spot}$ and 1.36 for $CV_{inter\text{-}day}$, demonstrating that both allowed for reduced CV values at intra-spot -44.80% and 47.94% for TICp and pTIC, respectively-, inter-spot -28.71% and 32.0%- and inter-day level -33.89% and 38.24%-. Reduced CV values were consistently recorded for VSE and VRE isolates alike (Table S3).

The variability of the 18 common peaks found in all *E. faecium* isolates showed that CV values were lower for peaks between 3000 and 9000 *m/z* at intra-spot (Figure

4A), inter-spot (Figure 4B) and inter-day level (Figure 4C), especially when data were normalized, showing different means ($p<0.05$) versus the 2000 and 3000 *m/z* range (Table S4). The 5974.6 m/z peak showed higher CV values at the three levels but the region between 6000-9000 *m/z* showed lower variation again, although the CVs were higher for inter-spot and inter-day reproducibility (Figures 4B and 4C). These results support the fact that the central *m/z* region of the spectrum is the most reliable for peak analysis (22).

Pearson Correlation coefficient was applied to inter-day reproducibility, showing a mean of 0.94 factor for all samples (Table S2). This value was higher for VRE ($p=0.95$) than for VSE isolates (0.93). Within the VRE group, the p value for isolates hosting the *vanA* mechanism was 0.98 versus 0.92 for the isolates the *vanB* resistance gene.

Arithmetic mean, as a representative value of peak intensity, and post-alignment mean were calculated and compared (Table 2). For not normalized data, the arithmetic mean was higher than post-alignment for inter-spots and inter-day, unlike what happened for the TICp normalization method. Regarding pTIC normalization, the arithmetic and post-alignment means were identical. Besides, the homoscedasticity of the data and their normal distribution were checked by the implementation of the Levene and Saphiro-Wilk tests respectively. The t-Student test was then performed to check if the null hypothesis of equality of means could be accepted among arithmetic and post-alignment means. In all possible comparisons p-values obtained were $p>0.05$ (Table S5), showing that even when a post-alignment was performed, the intensity values were not affected. The fact that both means in all methods did not show statistical differences, proved the high reproducibility of the assays based on protein spectra analysis.

3.3. Classification of the isolates using Machine Learning

Three peak matrices were generated (Full spectrum method and Threshold methods TIC-p and p-TIC) with data from the “classification set” were used as input data to test the capacity of the algorithms to discriminate VRE (VanA + VanB) from VSE

isolates (Table S6). The 10-fold cross validation results for SVM algorithm showed the best accuracy with 80.9% and F1 Score (the harmonic mean of the sensitivity and the accuracy of the model) of 80.5% for Full spectrum-TIC method (Table 3A).

The same procedures were also applied for the discrimination of VanA from VanB VRE strains (Table S7). In this approach, PLS-DA algorithm with TICp method provided 86.65% correct classification (Figure 5). This algorithm achieved 89.09% of predictive value for identifying VanB strains in a 10-fold cross validation (Table 3B).

The biomarker analysis revealed the presence of two potential resistance biomarkers at 6891.33 *m/z* and 5095.01 *m/z* (Table 4). These two peaks showed AUC values greater than 0.8 in its Receiver Operating Characteristic (ROC) curve (Figure S1). The 5095.01 m/z peak allowed the discrimination of the VRE strains from the VSE strains (Figure S2). This peak was present in 82 of the 86 VRE strains in this study. Also, the AUC for peak at 6891.33 m/z allowed the discrimination of VRE isolates hosting the VanA resistant mechanism from those carrying the VanB one. Its intensity was higher in all VanA *E. faecium* isolates tested with an AUC of 0.831 and a CV value of 22.99% (Figure S2). These results were obtained by applying a threshold (0.01 factor) after a TIC normalization using the 178 pre-processed samples of the classification set.

4. DISCUSSION

The application of MALDI-TOF MS coupled with data analysis has shown to be a reproducible methodology -CV values ≤ 20.88 for normalized data- that allowed the discrimination of VRE (VanA + VanB) from VSE in 80.9% of the cases using SVM and the correct differentiation of 86.6% of the *E. faecium* VanB isolates from the VanA isolates by PLS-DA. Specific peaks for the discrimination of the studied isolates have been found: the 5095.01 *m/z* peak was present in 82/86 VREs. Although this peak has already been described as a biomarker for VRE isolates (23) its real meaning is currently under debate. Brackmann et al. (2020) recently reported the sequencing of the 5095.01 *m/z* protein and identified the protein hiracin, a secretory protein encoded by the *hirJM79*

337 gene, whose role in vancomycin resistance mechanism remains unknown (24). Although
338 our study supports the value of the 5095.01 *m/z* peak as a marker for VRE isolates,
339 caution should be exercised when using this peak for VRE presence until further studies
340 unravel its correlation with vancomycin resistance.

341 The 6891.33 *m/z* peak was found as a biomarker for VanA isolates. Both the AUC
342 (0.831) and the CV values (22.99%) indicate the uniqueness and reproducibility of this
343 marker. Although this peak had been related before with different clonal complexes and
344 sequence types (25), its correlation with VanA VRE isolates had not been reported so far
345 (26).

346 The reproducibility study carried out with 20 *E. faecium* isolates (10 VSE, 5 VanA
347 VRE and 5 VanB VRE) demonstrated that the lowest CV values for peak intensities were
348 obtained for normalized data ($p<0.0001$), regardless of the order in which normalization
349 and peak finding is performed. Intra-spot reproducibility showed the highest rate of
350 reproducibility (CV values ranging from 7.99 to 8.46) and, although the CV values for
351 inter-spot and inter-day reproducibility ranged between 19.91 and 20.88 for inter-spot
352 variability and between 19.30 to 20.66 for inter-day reproducibility. Similar CV values -
353 6.5-17%- have been reported in a study that evaluated the technical reproducibility of
354 MALDI-TOF MS for quantitative protein profiling (27). Therefore, this methodology is
355 considered reproducible and feasible for peak analysis, especially in the range of 3000-
356 9000 *m/z*. Besides, 13 of the 18 most representative common peaks for all *E. faecium*
357 and also both biomarker peaks for VRE vs VSE and VanA vs VanB differentiation are
358 located within this spectrum range. The implementation of the methodology described in
359 this study could provide standardization for data comparison with other studies analyzing
360 antimicrobial resistance with MALDI-TOF MS.

361 Previous studies have shown the ability of MALDI-TOF MS to differentiate among *E.*
362 *faecium* vancomycin-resistant high-risk clones, clonal complexes and sequence types
363 with different success rates (18, 25, 28). Differentiation between VRE and VSE isolates
364 has also been reported. Griffin et al. reported 88.45% correct discrimination using SVM

and 88.24% with the implementation of the Genetic Algorithm (23). This algorithm also allowed the discrimination 92.4% VanA VRE from VSE isolates in the study developed by Nakano et al. (26). In our case, the application of SVM, RF and PLS-DA algorithms provided 80.9%, 79.2% and 77.5% correct classification of VRE and VSE isolates using the full spectrum method. Besides, discrimination between VanA and VanB VRE isolates was achieved in 86.6% of the cases by applying the TIC-p method. Discrepancies in the peaks used for discrimination of the different *E. faecium* groups were detected with the two previous studies: none of the peaks included in the study by Nakano et al. were found relevant in our models; besides, only the 5095.01 *m/z* peak (5094.7 *m/z* in Griffin et al.) was common to both studies for the discrimination of VRE from VSE. In our predictive models, the 6603 *m/z* peak proposed by Griffin et al. for the discrimination of VanA from VanB VRE was not considered discriminative. Instead, the 6891.78 *m/z* peak served this purpose in our study.

Although further studies are requested in order to clarify the role of the 5095.01 *m/z* peak for the routine detection of VanB VRE isolates, we propose the detection of this peak in combination with the 6891.78 *m/z* peak for the detection of suspected VanA VRE, for the differentiation of VRE using MALDI-TOF MS. Despite VanB VRE isolates being more prevalent in our setting, VanA VRE isolates have been reportedly correlated with hospital infections in different European countries (8). Thus, both biomarker peaks could be used for rapid screening of VRE isolates with MALDI-TOF MS.

One of the limitations of this study is the lack of genomic background for the analyzed isolates. Only the genes encoding vancomycin resistance were targeted and its presence or absence confirmed by molecular methods. Although this information was useful for the development of predictive models, more comprehensive information about our VRE isolates could help obtain higher discrimination power from the applied algorithms and, besides, explain the misclassifications from the current models.

Another limitation of the study is that clonality of the isolates was only analyzed within a clinical approach. HGUGM is a tertiary hospital where clinical departments are in

393 different wards and contact among them is sporadic -as each department has its own
394 medical staff- so an outbreak affecting different departments is unlikely. The theory of
395 non-clonality is also supported by Griffin et al., since the 5095.01 *m/z* peak was also
396 found in their study, performed in a different continent (23). Although more accurate
397 molecular methods are needed to rule out that the *E. faecium* isolates analyzed in this
398 study belong to the same clone, our results support the importance of a previously
399 described peak for differentiating VRE from VSE isolates and add a new specific
400 biomarker for the discrimination of VanA VRE strains.

401 The classification accuracy of the applied algorithms has shown to be <90% in all
402 cases. Therefore, further studies with well-characterized isolates sourcing from different
403 geographic origins are needed to confirm the results obtained in this work and improve
404 them if possible.

405 In conclusion, MALDI-TOF MS has demonstrated acceptable discrimination of *E.*
406 *faecium* isolates beyond species assignment. Although further refining is requested and
407 isolates from different clones and origins have to be included in predictive models in
408 order to understand how they are discriminated by MALDI-TOF, protein profiling could
409 become a suitable tool for rapid detection of VRE in clinical microbiology laboratories. Its
410 implementation could be key for the control of VRE isolates in hospital settings.

411

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REFERENCES

1. Vu J, Carvalho J. 2011. Enterococcus: review of its physiology, pathogenesis, diseases and the challenges it poses for clinical microbiology. Frontiers in Biology 6.
2. Arias CA, Murray BE. 2012. The rise of the Enterococcus: beyond vancomycin resistance. Nature reviews Microbiology 10:266-78.
3. Cattoir V, Leclercq R. 2013. Twenty-five years of shared life with vancomycin-resistant enterococci: is it time to divorce? The Journal of antimicrobial chemotherapy 68:731-42.
4. Miller WR, Murray BE, Rice LB, Arias CA. 2020. Resistance in Vancomycin-Resistant Enterococci. Infect Dis Clin North Am 34:751-771.
5. Cattoir V, Giard JC. 2014. Antibiotic resistance in Enterococcus faecium clinical isolates. Expert review of anti-infective therapy 12:239-48.
6. Rice LB. 2008. Federal funding for the study of antimicrobial resistance in nosocomial pathogens: no ESKAPE. J Infect Dis 197:1079-81.
7. Chiang HY, Perencevich EN, Nair R, Nelson RE, Samore M, Khader K, Chorazy ML, Herwaldt LA, Blevins A, Ward MA, Schweizer ML. 2017. Incidence and Outcomes Associated With Infections Caused by Vancomycin-Resistant Enterococci in the United States: Systematic Literature Review and Meta-Analysis. Infect Control Hosp Epidemiol 38:203-215.
8. Lopez M, Cercenado E, Tenorio C, Ruiz-Larrea F, Torres C. 2012. Diversity of clones and genotypes among vancomycin-resistant clinical Enterococcus isolates recovered in a Spanish hospital. Microbial drug resistance 18:484-91.
9. Mirzaei B, Farivar TN, Juhari P, Mehr MA, Babaei R. 2013. Investigation of the Prevalence of vanA and vanB genes in vancomycin resistant enterococcus (VRE) by Taq Man real time PCR Assay. Journal of Microbiology and Infectious Diseases 3:192.
10. Hricova K, Stosova T, Kucova P, Fiserova K, Bardon J, Kolar M. 2020. Analysis of Vancomycin-Resistant Enterococci in Hemato-Oncological Patients. Antibiotics 9.
11. Seng P, Drancourt M, Gouriet F, La Scola B, Fournier PE, Rolain JM, Raoult D. 2009. Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Clin Infect Dis 49:543-51.
12. Oviano M, Gomara M, Barba MJ, Revillo MJ, Barbeyto LP, Bou G. 2017. Towards the early detection of beta-lactamase-producing Enterobacteriaceae by MALDI-TOF MS analysis. The Journal of antimicrobial chemotherapy 72:2259-2262.
13. Schuster D, Josten M, Janssen K, Bodenstein I, Albert C, Schallenberg A, Gajdiss M, Sib E, Szekat C, Kehl K, Parcina M, Hischebeth GTR, Bierbaum G. 2018. Detection of methicillin-resistant coagulase-negative staphylococci harboring the class A mec complex by MALDI-TOF mass spectrometry. International journal of medical microbiology : IJMM 308:522-526.
14. Oviano M, Bou G. 2019. Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry for the Rapid Detection of Antimicrobial Resistance Mechanisms and Beyond. Clin Microbiol Rev 32.
15. Rodriguez-Sanchez B, Cercenado E, Coste AT, Greub G. 2019. Review of the impact of MALDI-TOF MS in public health and hospital hygiene, 2018. Euro

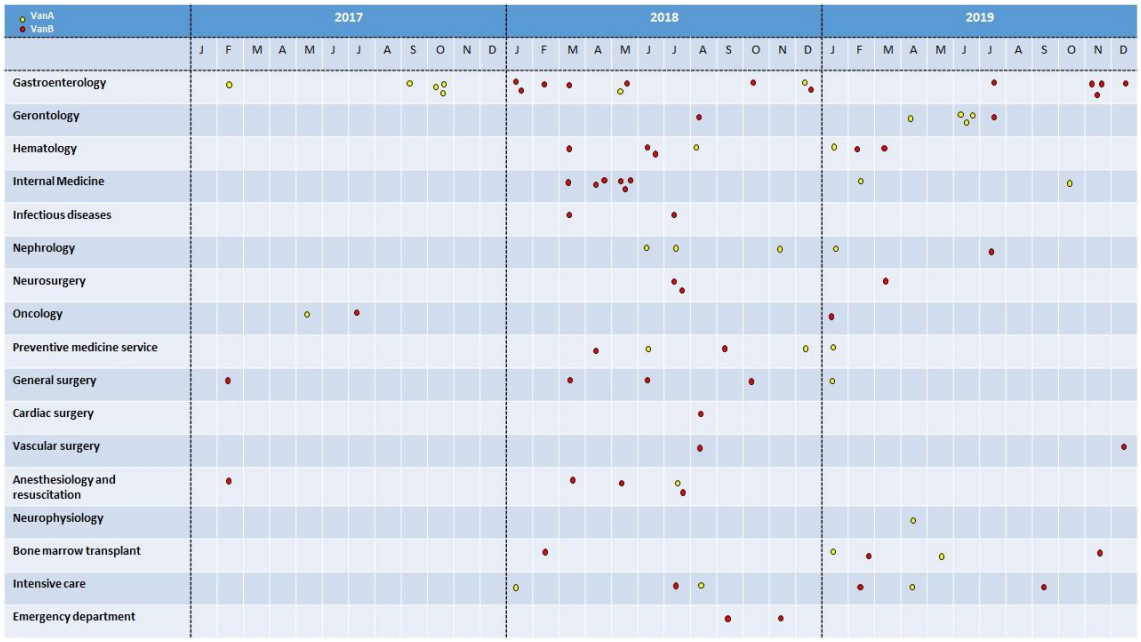
surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin 24.

16. Rodriguez-Sanchez B, Marin M, Sanchez-Carrillo C, Cercenado E, Ruiz A, Rodriguez-Creixems M, Bouza E. 2014. Improvement of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry identification of difficult-to-identify bacteria and its impact in the workflow of a clinical microbiology laboratory. Diagn Microbiol Infect Dis 79:1-6.
17. Oberle M, Wohlwend N, Jonas D, Maurer FP, Jost G, Tschudin-Sutter S, Vranckx K, Egli A. 2016. The Technical and Biological Reproducibility of Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) Based Typing: Employment of Bioinformatics in a Multicenter Study. PLoS One 11:e0164260.
18. Lasch P, Fleige C, Stammler M, Layer F, Nubel U, Witte W, Werner G. 2014. Insufficient discriminatory power of MALDI-TOF mass spectrometry for typing of Enterococcus faecium and Staphylococcus aureus isolates. J Microbiol Methods 100:58-69.
19. Quintela-Baluja M, Bohme K, Fernandez-No IC, Morandi S, Alnakip ME, Caamano-Antelo S, Barros-Velazquez J, Calo-Mata P. 2013. Characterization of different food-isolated Enterococcus strains by MALDI-TOF mass fingerprinting. Electrophoresis 34:2240-50.
20. Zvezdanova ME, Arroyo MJ, Mendez G, Guinea J, Mancera L, Munoz P, Rodriguez-Sanchez B, Escribano P. 2020. Implementation of MALDI-TOF Mass Spectrometry and Peak Analysis: Application to the Discrimination of Cryptococcus neoformans Species Complex and Their Interspecies Hybrids. J Fungi (Basel) 6.
21. Weis CV, Jutzeler CR, Borgwardt K. 2020. Machine learning for microbial identification and antimicrobial susceptibility testing on MALDI-TOF mass spectra: a systematic review. Clin Microbiol Infect 26:1310-1317.
22. Timm W, Scherbart A, Bocker S, Kohlbacher O, Nattkemper TW. 2008. Peak intensity prediction in MALDI-TOF mass spectrometry: a machine learning study to support quantitative proteomics. BMC Bioinformatics 9:443.
23. Griffin PM, Price GR, Schooneveldt JM, Schlebusch S, Tilse MH, Urbanski T, Hamilton B, Venter D. 2012. Use of matrix-assisted laser desorption ionization-time of flight mass spectrometry to identify vancomycin-resistant enterococci and investigate the epidemiology of an outbreak. Journal of clinical microbiology 50:2918-31.
24. Brackmann M, Leib SL, Tonolla M, Schurch N, Wittwer M. 2020. Antimicrobial resistance classification using MALDI-TOF-MS is not that easy: lessons from vancomycin-resistant Enterococcus faecium. Clin Microbiol Infect 26:391-393.
25. Freitas AR, Sousa C, Novais C, Silva L, Ramos H, Coque TM, Lopes J, Peixe L. 2017. Rapid detection of high-risk Enterococcus faecium clones by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Diagn Microbiol Infect Dis 87:299-307.
26. Nakano S, Matsumura Y, Kato K, Yunoki T, Hotta G, Noguchi T, Yamamoto M, Nagao M, Ito Y, Takakura S, Ichiyama S. 2014. Differentiation of vanA-positive Enterococcus faecium from vanA-negative E. faecium by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry. Int J Antimicrob Agents 44:256-9.

27. Pang RTK, Johnson PJ, Chan CML, Kong EKC, Chan ATC, Sung JJY, C.W. PT. 2004. Technical evaluation of MALDI-TOF mass spectrometry for quantitative proteomic profiling matrix formulation and application. Clinical Proteomics Journal 1:12.

28. Holzknecht BJ, Dargis R, Pedersen M, Pinholt M, Christensen JJ, Danish Enterococcal Study G. 2018. Typing of vancomycin-resistant enterococci with MALDI-TOF mass spectrometry in a nosocomial outbreak setting. Clin Microbiol Infect 24:1104 e1-1104 e4.

Figure 1. Distribution of the *E. faecium* isolates analysed in this study by hospital departments and date. VanA *E. faecium* isolates are shown with yellow dots and VanB isolates by red dots.



562 **Figure 2.** Graphic representation of 12 replicates spectra for each isolate: two spectra
563 are acquired per spot; two spots are compared for three consecutive days. Intra-spot
564 reproducibility level comparing spectra from shoots is represented in blue; technical
565 reproducibility level comparing average spectra of each spot is represented in green and
566 biological reproducibility level comparing the three average spectra per isolate during
567 three consecutive days are represented in pink. Arrows mean alignment and average
568 processing in Clover MS Software to obtain an average spectrum.
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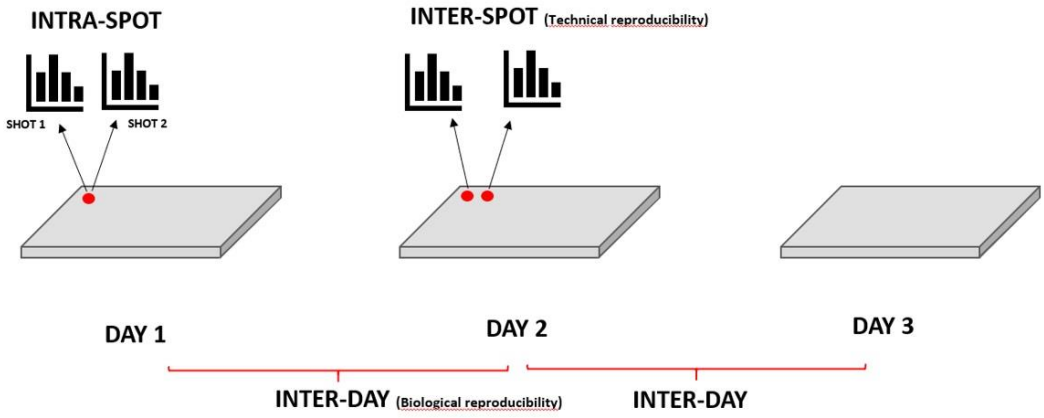
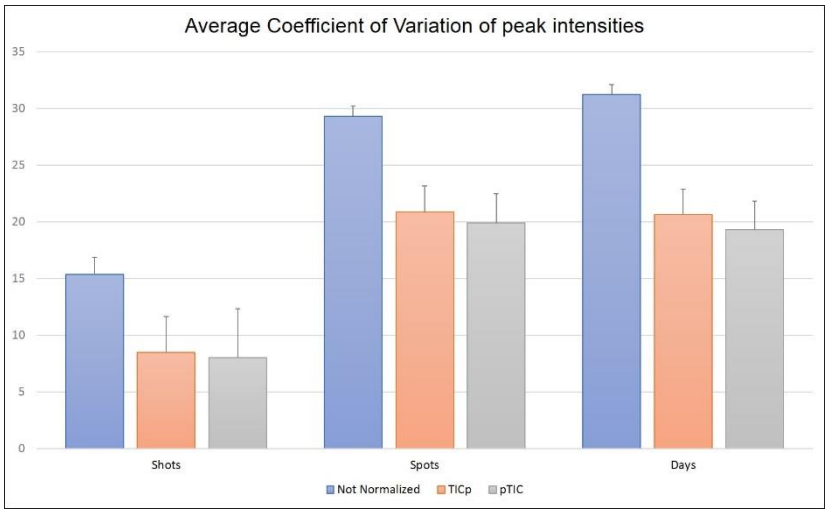


570

571 **Figure 3.** Coefficient of Variation (CV) means for the intensity of the 18 common protein
572 peaks of *E. faecium* analyzed at the intra-spot, inter-spot and inter-day levels using raw
573 data (Not Normalized) and both normalizations methods (TICp and pTIC).



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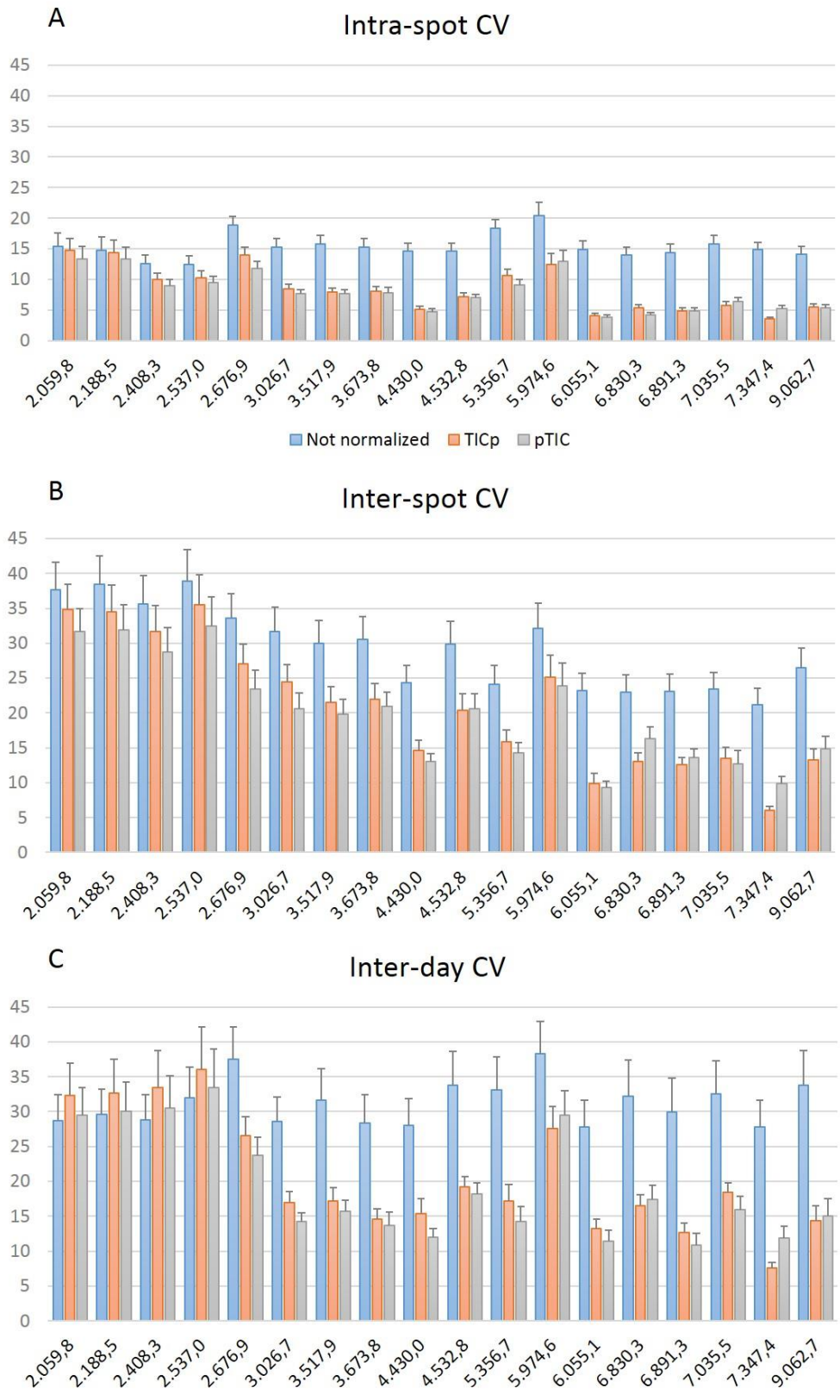
575 **Figure 4.** Comparison of the average Coefficient of Variation for the peak intensity of the

576 18 common peaks analyzed at intra-spot (A), inter-spot (B) and inter-day (C) levels.



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578 **Figure 5.** Distance plot of PLS-DA machine learning algorithm for the discrimination of

579 VanA from VanB VRE isolates using the TICp method.

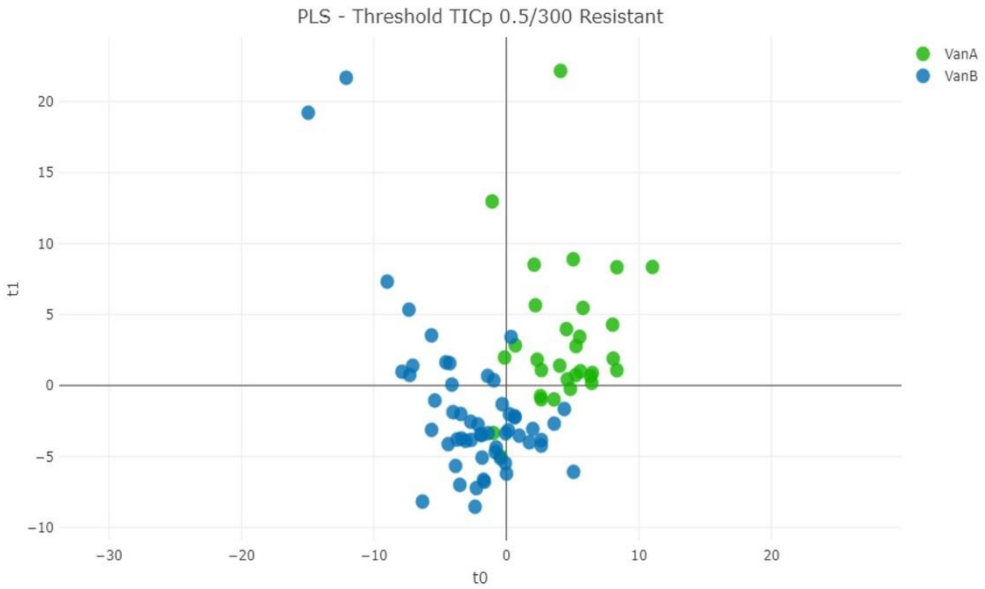

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Table 1. List of common peaks (n=18) found in average spectra of the VRE and VSE isolates included in the classification set. CV= Coefficient of Variation of intensity. Mean in intensity units.

Mass (*m/z*)	Measurements		
	Appearance (%)	CV (%)	Mean
2059.79	178/178 (100)	53.42	4361.20
2188.53	178/178 (100)	56.61	5269.55
2408.35	178/178 (100)	49.04	3316.45
2537.05	178/178 (100)	53.06	3527.45
2676.89	178/178 (100)	51.89	2170.47
3026.71	178/178 (100)	47.65	1270.16
3517.85	178/178 (100)	56.14	1327.19
3673.77	178/178 (100)	50.43	3344.71
4430.01	178/178 (100)	46.26	17437.72
4532.78	178/178 (100)	57.94	2296.61
5356.72	178/178 (100)	56.15	8719.83
5974.65	178/178 (100)	68.28	2531.38
6055.07	178/178 (100)	50.30	4187.74
6830.35	178/178 (100)	71.56	3020.35
6891.33	178/178 (100)	59.00	5252.80
7035.47	178/178 (100)	65.96	2656.59
7347.39	178/178 (100)	55.31	10326.29
9062.75	178/178 (100)	66.94	2820.45

Table 2. Arithmetic and post-alignment means at spot- and day-level without normalization and with the two different normalization methods applied (TICp and pTIC).

	Not Normalized		TICp		pTIC	
	Arithmetic	Post-alignment	Arithmetic	Post-alignment	Arithmetic	Post-alignment
Inter-Spot Mean	3825.44	3792.14	0.0005	0.0005	0.056	0.056
Inter-Day Mean	3974.07	3955.49	0.0005	0.0005	0.056	0.056

Table 3. Discrimination of VSE from VRE isolates and, within the latter group, differentiation between the strains hosting the *vanA* and *vanB* resistance genotypes. The actual classification is shown in columns and the predictive classification in rows. **A)** Results from SVM algorithm using Full spectrum-TIC method. Accuracy: 80.9%; F1 score: 80.46%; Sensitivity: 81.4% Specificity: 80.43%; Positive Predictive Value (PPV) or Precision: 79.55%; Negative Predictive Value (NPV): 82.22%. **B)** Scores from PLS algorithm using the TIC-p method. Accuracy: 86.05%; Predictive Value for *vanA*: 80.65% Predictive Value for *vanB*: 89.09%.

ACTUAL CLASSIFICATION	**PREDICTED CLASSIFICATION**	
A) SVM Full Spectrum	**VRE**	**VSE**
VRE	70	16
VSE	18	74
B) PLS Threshold TIC-p	***vanA***	***vanB***
vanA	25	6
vanB	6	49

Table 4. Biomarker peaks for the detection of VRE isolates and the discrimination of strains hosting the vanA mechanism.

Peak *m/z*	**AUC (≥0.8)**	**Appearance (Total samples)**	**Positive Category**	**Coefficient of Variation (CV)**
5095.01	0.814	123/178	82/86 (Resistant)	61.63%
6891.78	0.831	86/86	31/31 (vanA)	21.99%