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ORIGINAL ARTICLE

Use of MALDI-TOF mass spectrometry to detect nosocomial outbreaks of *Serratia marcescens* and *Citrobacter freundii*

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Abstract

MALDI-TOF mass spectrometry (MS) may be used as a rapid typing method for nosocomial pathogens. Here, we evaluated MALDI-TOF MS for discrimination of hospital outbreak-related clusters of *Serratia marcescens* and carbapenemase-producing *Citrobacter freundii*. Thirty-three *S. marcescens* isolates collected from neonatal intensive care unit (NICU) patients, and 23 *C. freundii* isolates including VIM-positive isolates from a hospital colonization outbreak were measured by Vitek MS. Consensus spectra of each isolate were clustered using SARAMIS software. Genotyping was performed by whole-genome sequencing (WGS). First, a set of 21 *S. marcescens* isolates from 2014 with seven genotypes including three monoclonal clusters was used for the evaluation of MALDI-TOF typing. MS clustering was largely in agreement with genotyping results when the similarity cut-off for clonal identity was set on 90%. MALDI-TOF cluster analysis was then investigated for the surveillance of *S. marcescens* in the NICU in 2017 and demonstrated the introduction of new strains into the hospital and nosocomial transmissions. MS analysis of the *C. freundii* outbreak in 2016 revealed a monoclonal cluster of VIM-positive isolates and the separation of epidemiologically non-related VIM-positive and negative isolates. Two additional VIM-positive *Citrobacter* isolates from food samples were closely related to the large monoclonal cluster. WGS confirmed the MS results. MALDI-TOF MS may be used as a first-line typing tool for *S. marcescens* and *C. freundii* to detect transmission events in the hospital because isolates of an identical WGS type were grouped into the same MS cluster.

Keywords *Serratia marcescens* · *Citrobacter freundii* · Typing · MALDI-TOF · Whole genome sequencing

Introduction

The order of *Enterobacterales* includes a number of species that are frequently associated with nosocomial outbreaks. The increase in multi-drug resistance (MDR) in enterobacterial species has become a major challenge for the management of hospital-acquired infections and colonizations [1]. To prevent the spread of ESBL and carbapenemase-producing *Enterobacterales*, the surveillance of resistant strains is an essential part of the hygiene management [2]. The prerequisite for this is the early as well as fast identification of suspect isolates and carriers. Moreover, the continuous surveillance of nosocomial pathogens is also relevant for non-MDR strains at high-risk wards such as neonatology intensive care units (NICUs) [3, 4].

Molecular typing of bacterial isolates is the key strategy to identify clusters that are due to the transmission of clonal strains. Classical genotyping methods such as

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pulse-field gel electrophoresis (PFGE) are now replaced by next-generation whole genome sequencing (WGS) [5, 6]. This approach provides a highly discriminatory power by the gene-by-gene allelic profiling of core genomes [7]. However, high-resolution genotyping methods are still quite expensive and time-consuming [7]. For the implementation of infection control measures, a timely identification of clinically relevant clones is requested. There is a need for quick and reliable methods that can be easily integrated into the routine workflow and do not cause increased costs for consumables and additional staff [8].

Today, the identification of bacterial species is performed by MALDI-TOF mass spectrometry (MS). Because the technique is available in most of the clinical laboratories, it would be highly attractive if MS could be used to identify clonal clusters of nosocomial pathogens that correlate to distinct genotypes [8, 9]. Several studies that compared MALDI-TOF and genotyping have produced conflicting results; therefore, the reliability of MALDI-TOF-based typing is controversially discussed [10–12]. Moreover, it has been reported that protein mass spectra of bacterial strains often do not correspond with their genotypes and that the variability of spectra due to measurement factors negatively affects typing results [10, 12, 13]. The aim of this study was to evaluate the application of Vitek MS combined with SARAMIS data analysis for its use as a first-line subtyping tool to recognize clonal clusters of *Serratia marcescens* and *Citrobacter freundii* isolates collected from colonization outbreaks at the Jena University Hospital. The discriminatory power of MALDI-TOF MS was compared to WGS analysis based on a gene-by-gene allele typing [14].

Methods

Bacterial strains and antimicrobial susceptibility testing

The study included 33 *S. marcescens* and 23 *C. freundii* isolates mostly recovered from rectal or pharyngeal swabs as part of the patient screening. Patient samples were streaked onto Columbia sheep blood agar, Drigalski lactose agar (Oxoid, Thermo Fisher Scientific, Wesel, Germany), and CHROMagar ESBL/KPC (Mast Diagnostica, Reinfeld, Germany) for MDR *Enterobacteriales* screening. Species identification was performed by Vitek MS (Shimadzu, bioMérieux, Nürtingen, Germany) using the Vitek IVD database. Antimicrobial susceptibility testing (AST) was performed using Vitek 2 and minimum inhibitory concentration (MIC) interpretation according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints (eucast.org/clinical_breakpoints; versions 4.0 to 7.1). For *C. freundii*, MICs were verified by microdilution using the Micronaut-S MDR MRGN-Screening plate (Merlin Diagnostika; purchased from Sifin Diagnostics, Berlin, Germany). Isolates were tested for carbapenemase family genes by the eazyplex®SuperBug CRE assay (AmplexDiagnostics, Gars-Bahnhof, Germany) according to the manufacturer's protocol.

MALDI-TOF MS

Samples were prepared from bacterial isolates grown on Columbia sheep blood agar plates for 18–24 h by a standard direct smear method. Single colonies were spotted onto the target and overlaid with α-cyano-4-hydroxycinnamic acid (CHCA) matrix (bioMérieux). The mass spectra were acquired with the Vitek MS machine using the Vitek MS target manager and a mass range *m*/*z* of 3000 to 20,000. Vitek MS Acquisition switcher (bioMérieux) was used to set the configuration of the machine from the IVD into the research modus. Launchpad® software version 2.9.3 (Shimadzu, bioMérieux) was used with standard instrument settings for spectra acquisition and peak detection. Once the spectra acquired by Launchpad® were processed, the resulting peak lists were transferred to a dedicated folder on the SARAMIS™ server. Data were automatically analyzed for species identification by the SARAMIS™ Premium software version 4.14 (bioMérieux) and the peak list files were saved in the *.txt format. For each experiment, all isolates were measured in three replicates. Calibration was performed using the *Escherichia coli* reference strain ATCC 8739. Fine tuning of the laser device was performed by the technical service of bioMérieux in a 14-day interval to ensure constant quality of mass spectra acquisition.

MS data analysis

Spectra were manually imported to the SARAMIS™ RUO database version 4.14 (bioMérieux) using the button "import spectra to spectra database" in the results mode of SARAMIS Premium software. Spectra were loaded into the SARAMIS database as peak lists. In SARAMIS, the terms "spectrum," "peak list," and "text file" are used as synonyms. For MALDI-TOF MS cluster analysis, the following parameters were evaluated: comparison of single spectra vs. consensus spectra, variation of the *m*/*z* range, use of different solid media for cultivation of strains prior MS acquisition, use of CHCA with and without formic acid for the preparation of strains, and definition of a similarity cutoff for clonal identity. After switching into the spectra mode of SARAMIS premium software, cluster analysis was performed according to the manufacturer's instructions (Vitek MS Plus

SARAMIS Premium user manual, bioMérieux). Imported spectra were moved into a separated folder of the database by using the search function of the software. Three single spectra were used to generate a consensus spectrum for each strain. Spectra with at least 110 peaks were used for further analysis. Consensus spectra only include the mass signals that have been recorded with high frequency in a batch of spectra. The frequency threshold was set to 100%, i.e., only the mass signals that were recorded in all three replicates were considered. Peaks were defined to be identical by applying a mass accuracy of 0.08% as the SARAMIS standard setting. Relative intensities of mass signals were averaged. A peak range from 3000 to 15,000 *m*/*z* values was chosen for final analysis. Consensus spectra were analyzed with a single link agglomerative clustering algorithm, applying the relative taxonomy analysis tool of SARAMIS premium software to show the resulting dendrogram with differences and similarities in relative terms (percent matching masses). As a standard setting, the mass signal intensity was not considered in the cluster analysis. For *S. marcescens*, the discriminatory power was determined by the Simpson's index of diversity (*D*) using the online calculator insilico.ehu.eus/mini_tools/discriminatory_power/ [14]. The index *D* should be ≥ 0.95 for optimal resolution [15]. WGS-based genotyping served as the reference method. Because the calculation is based on the ability to discriminate between epidemiologically unrelated isolates, *N* was defined as the number of genotypes based on WGS analysis and *S* was defined as the number of MALDI-TOF clusters that could be discriminated. The adjusted Rand coefficient was calculated to determine the overall concordance between MALDI-TOF MS and WGS, adjusted for chance agreement [15, 16]. The Wallace coefficient was calculated to estimate the additional information provided by one typing method over another [16, 17]. Rand and Wallace coefficient values were computed using the comparing partitions online tool (www.comparingpartitions.info).

WGS and data analysis

High-throughput WGS and subsequent data analysis were performed using a MiSeq instrument (Illumina, San Diego, CA, USA) as described recently [7]. The resulting reads were quality-trimmed and de novo–assembled using the Velvet algorithm integrated in the RidomSeqsphere$^+$ software version 3 (Ridom GmbH, Münster, Germany) [18]. For core genome (cg) MLST, we created an ad hoc cgMLST scheme for each species. To illustrate the clonal relationships between different isolates, minimum-spanning tree analyses were performed based on the determined allelic profiles using the RidomSeqsphere$^+$ software with the parameter "pairwise ignore missing values."

For long-read WGS (nanopore), DNA was isolated from bacterial cultures using NucleoBond (Macherey-Nagel, Düren, Germany) and fragmented with g-TUBE™ (Covaris, Brighton, UK). The sequencing library was prepared using the 1D ligation sequencing kit (Oxford Nanopore Technologies, Oxford Science Park, UK). Sequencing and base calling were performed using a MinION sequencer and Albacore (Oxford Nanopore Technologies). Demultiplexing/trimming was done with porechop (https://github.com/rrwick/Porechop) and genome assembly was performed with 50× coverage using CANU Assembler version 1.7 and the Albacore Basecaller version 2.2.5 [19]. Whole genomes of bacterial isolates were compared using BRIG [20].

MiSeq raw reads are deposited at European Nucleotide Archive (ENA) under study accession number PRJEB18796.

Results

In this study, we investigated MALDI-TOF MS for typing of two different clinical species and compared the results with WGS analyses that were used as reference method. An outbreak of *S. marcescens* at the NICU of the Jena University hospital from December 2013 to April 2014 with nine distinct genotypes including a major clonal cluster was used to define typing criteria for MALDI-TOF MS [3]. In a second step, MALDI-TOF MS cluster analysis was applied for the surveillance of *S. marcescens* isolates at the NICU in 2017. A nosocomial colonization outbreak from 2016 caused by *C. freundii* producing a VIM carbapenemase was investigated to verify the defined typing criteria [21]. Several VIM-positive isolates and third-generation cephalosporin-resistant but carbapenem-sensitive isolates collected in 2017 were used for further analyses.

S. marcescens

Figure 1a shows the distribution and relatedness of 21 NICU isolates (A1–A21) that were collected from 2013 to 2014 and analyzed by Illumina WGS using cgMLST and ignoring non-closed regions. This set of isolates included three monoclonal outbreak clusters (I–III). All isolates were third-generation cephalosporin-sensitive. This well-characterized collection consisting of eight different genotypes was used to evaluate the criteria for the application of MALDI-TOF MS cluster analysis to discriminate *S. marcescens* clones, as described in the "Methods" section. The highest level of correlation between MALDI-TOF MS and WGS was obtained when the following criteria were applied: use of fresh

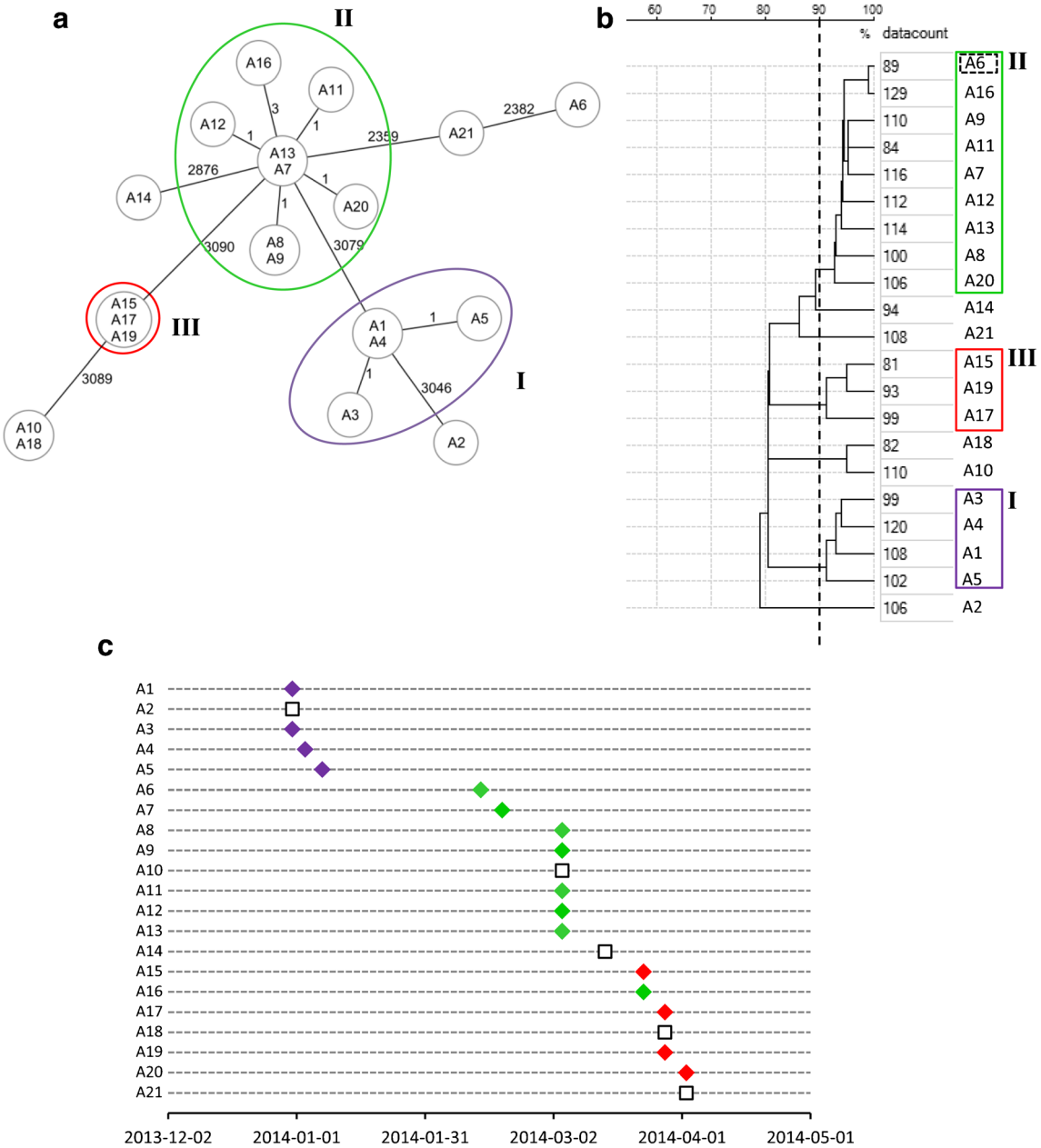

Fig. 1 WGS and MALDI-TOF MS-based typing of the 21 *S. marcescens* isolates from the NICU. **a** Minimum-spanning tree based on allelic profiles of 3126 target genes that were present in all isolates. Each circle represents an allelic profile, i. e., the genotype, and is named by the isolate(s). The number on connecting lines gives the number of differing alleles. Colored circles indicate clonal clusters. **b** Dendrogram of the MALDI-TOF consensus spectra using SARAMIS. Colored rectangles indicate clusters of isolates that show a mass spectrum similarity of ≥ 90%. **c** Time line of *S. marcescens* cases illustrating the date of first isolation in culture (diamonds). Colors represent the discriminated MALDI-TOF clusters

overnight cultures on Columbia sheep blood agar, direct smear target preparation with CHCA, choose of an *m/z* range from 3000 to 15,000, and generation of a consensus spectrum containing only peaks detected in three parallel single spectra of each isolate (reduction by 100%). Single spectra recorded in parallel on the same target showed a high variability and shared a fraction of peaks ranging from 43.8 to 67.5% (data not shown). Therefore, cluster analysis was performed using consensus spectra. Figure 1b displays the MALDI-TOF analysis of consensus spectra and differentiation of distinct clusters. Three main clusters could be distinguished with branching at 81 and 82% similarity. The discriminatory power value to differentiate the WGS-defined genotypes was 0.786, when a similarity cutoff of 82% was assumed. The major cluster contained all isolates from

the large monoclonal outbreak (cluster II) but also three genetically different strains (A6, A14, and A21). However, when the similarity cutoff was set at 90% (Fig. 1b, broken line), strains A14 and A 21 could be separated from the monoclonal cluster II, and overall, MALDI-TOF clustering corresponded to the distribution of genotypes, with only one exception (isolate A6). The discriminatory power value was increased to 0.964. The adjusted Rand coefficient for comparison of WGS and MALDI-TOF clustering was 0.881, indicating a good overall concordance. The Wallace coefficient was 0.826 (0.566–1, 95% CI), i.e., the probability that two isolates of the same MALDI-TOF type shared the same genotype is 83%. On the other hand, the probability that isolates of an identical genotype shared the same MALDI-TOF type was 100%. In Fig. 1c, the isolates were sorted by date of isolation, demonstrating the sequential emergence of three MALDI-TOF clusters according to WGS analysis. The major cluster (indicated in green) colonized eight patients; of these, five were tested positive on the same day (A8, A9, A11–A13).

The next step was to test MALDI-TOF analysis for the surveillance of *S. marcescens* in the NICU. Twelve isolates were collected from colonized patients in 2017. Figure 2a shows the MALDI-TOF cluster analysis of these isolates. Representative isolates of clusters identified in 2014 were included in the analysis as epidemiologically unrelated control strains. The new isolates could be discriminated into three MALDI-TOF clusters when a similarity cutoff of 90% was applied. The occurrence of isolates belonging to one cluster correlates with dates of isolation, as shown in Fig. 2b. Cluster I emerged in March 2017. The isolates of cluster II were recovered in summer, followed by cluster III in autumn. The sequential occurrence of clusters indicated that new *S. marcescens* strains had been introduced from time to time, and subsequently, nosocomial transmissions took place. To verify the MALDI-TOF results, representative isolates of each cluster (B1, B5, and B10) as well as the control isolates from 2014 (A1, A8, and A15) were analyzed by nanopore sequencing using the MinION system. This technology still has higher error rates compared to lllumina sequencing (99.5 to 99.9% assembly accuracy) [22]. The higher error rate of MinION is due to the nature of the technique (five bases produce one signal), and therefore, the error rate depends mostly on the quality of the base calling and assembly coverage. However, due to the long-read technology, allowing complete genome assembly, the entire genome can be compared directly, taking all sequencing data into account. WGS analysis confirmed the results of MALDI-TOF clustering because all six isolates tested were different from each other, showing only 95% similarity at most through the whole genome (Fig. 2c).

C. freundii

From February to April 2016, *C. freundii* producing a VIM carbapenemase was isolated from rectal swabs of several patients during the weekly screening for MDR *Enterobacterales* (Table 1, E1–E11). In one case, VIM-positive *C. freundii* was isolated in blood cultures from a patient with transient bacteremia (isolate E5). Because patients from different wards and without any contact were affected, food from the hospital's kitchen came under suspicion as contaminated source. Microbiological analysis of food samples was positive for green salad and chocolate pudding (Table 1, isolates E12 and E13, respectively). To clarify whether all isolates were of clonal origin, MALDI-TOF typing was performed as the first-step epidemiological analysis during this outbreak. As shown for *S. marcescens*, consensus spectra were used for cluster analysis because single spectra of the same isolate were variable and shared a fraction of 38.1 to 61.7%, when recorded in parallel (data not shown). A VIM-positive *C. freundii* isolate that was recovered 3 months before the outbreak (E14) and a third-generation cephalosporine-resistant but carbapenem-sensitive *C. freundii* isolate from 2016 (F1) were included in the analysis. The consensus MALDI-TOF spectra of all isolates from February to April 2016, including those recovered from food samples, showed a similarity of ≥ 90% (Fig. 3a). The single isolate from December 2015 was clearly different from this cluster with a similarity of only 75%. The carbapenem-sensitive control strain branched at about 80% similarity from the outbreak cluster. The results of MALDI-TOF consensus spectra analysis strongly indicated a colonization outbreak of a monoclonal carbapenemase-producing *Enterobacteriales* (CPE) strain. Moreover, MALDI-TOF was helpful to verify the infection source responsible for the outbreak. All results were subsequently confirmed by WGS-based typing using Illumina, exhibiting nearly identical (E11, differing in a single gen, only) or identical (E1–E10, E12, E13) cgMLST genotypes among the 13 outbreak isolates (Fig. 3b). All isolates of the monoclonal cluster harbored $bla_{\text{VIM-1}}$; the carbapenemase gene of the separated isolate E14 was identified as $bla_{\text{VIM-4}}$.

In 2017, several *C. freundii* isolates producing VIM were identified during the MDR *Enterobacterales* screening of the hospital's patients (Table 1, G1–G5). All isolates were collected from rectal swabs. They showed an antibiotic resistance pattern similar to the 2016 outbreak strain, although MICs for meropenem, gentamicin, and ciprofloxacin were increased (Table 1). The isolates G1 to G5 belonged to one MALDI-TOF cluster and were closely related to two reference isolates from the outbreak strain of 2016 (E1 and E12) when the similarity cutoff was set at 90% (Fig. 4a). This cluster was clearly

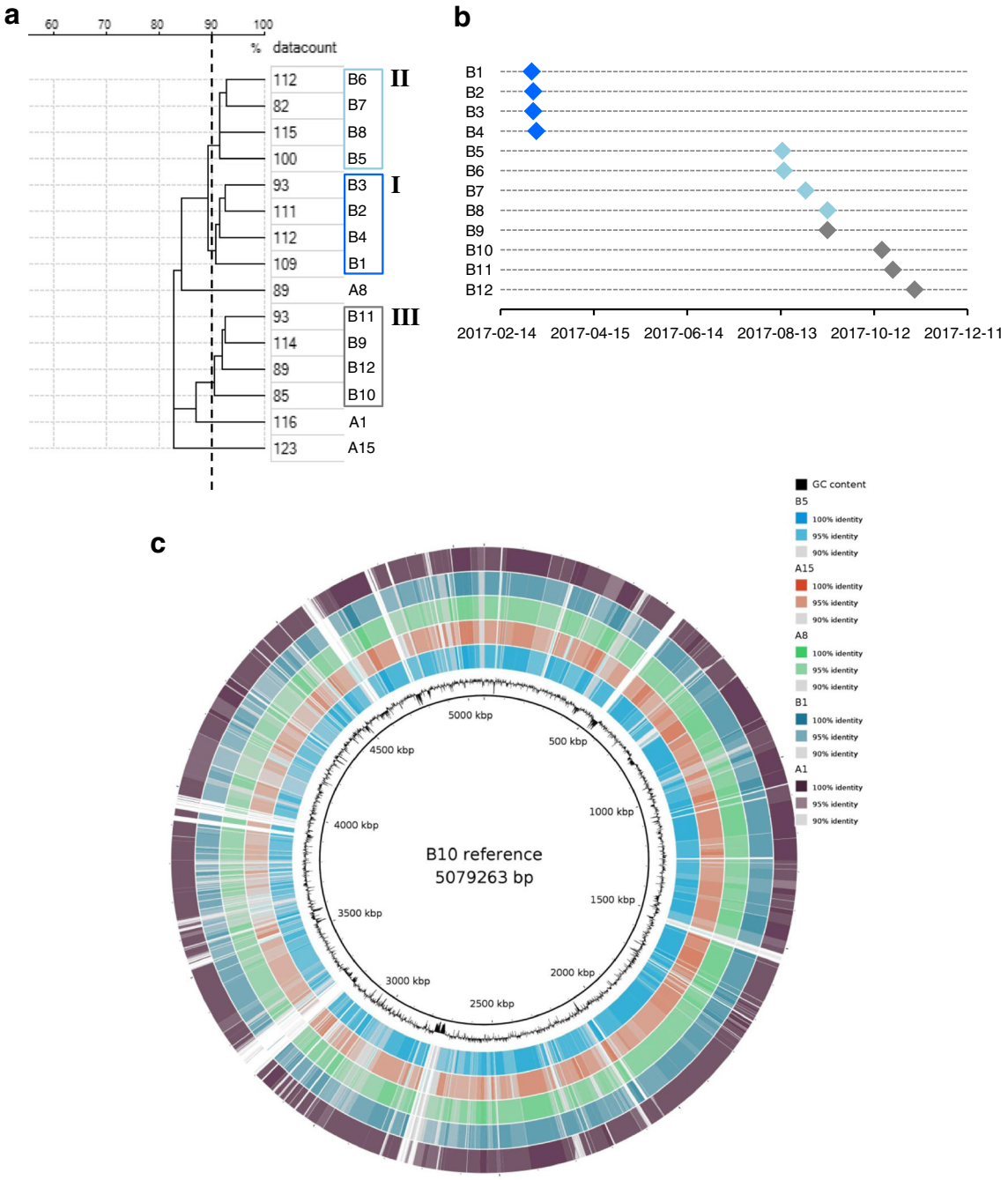

Fig. 2 MALDI-TOF MS-based typing of the *S. marcescens* isolates (B1–B12) from the NICU collected in 2017. **a** Dendrogram of the MALDI-TOF consensus spectra using SARAMIS. Colored rectangles indicate clusters of isolates that show a mass spectrum similarity of ≥90%. Isolates A1, A8, and A15 served as epidemiologically unrelated controls. **b** Time line of *S. marcescens* cases in 2017 illustrating the date of first isolation in culture (diamonds). Colors represent the discriminated MALDI-TOF clusters. **c** Whole genome comparison of representative isolates of different MALDI-TOF clusters using MinION sequencing and BRIG analysis. Strain B10 was used as reference genome and compared against the remaining five strains (A1, A8, A15, B1, B5). The innermost ring shows the GC content (black). Sequence data were assembled with CANU and each genome was completely closed to one contig with the exception of strain A8 that was assembled to 3 contigs covering in total more than 98% of the whole genome

separated from epidemiologically unrelated isolates, including the VIM-positive one from 2015 (E14) and several three third-generation cephalosporin-resistant but carbapenem-sensitive control isolates (F1, H1–H3; Table 1 and Fig. 4a). The results of MALDI-TOF clustering indicated that the outbreak strain reemerged in 2017 in the hospital. To verify this conclusion, nanopore sequencing was performed for selected isolates, including G1 as the first isolate in 2017. For comparison, E1, the first isolate from the 2016 outbreak, the food-borne isolate E12, and the unrelated control E14 were chosen. Figure 4b

Table 1 Characteristics of *C. freundii* isolates

Isolate number[a]	Date of isolation	VIM	Minimum inhibitory concentrations[b]						SXT
			TZP	CTX	CAZ	MEM	GEN	CIP	
E1	2016-09-02	+	> 64/4	> 64	> 64	4	2	≤ 0.25	≤ 1/19
E2	2016-14-02	+	> 64/4	> 64	> 64	4	2	≤ 0.25	≤ 1/19
E3	2016-02-24	+	> 64/4	> 64	> 64	4	2	≤ 0.25	≤ 1/19
E4	2016-03-02	+	> 64/4	> 64	> 64	4	2	≤ 0.25	≤ 1/19
E5	2016-03-02	+	> 64/4	> 64	> 64	4	2	≤ 0.25	≤ 1/19
E6	2016-03-07	+	> 64/4	> 64	> 64	4	2	≤ 0.25	≤ 1/19
E7	2016-03-09	+	> 64/4	> 64	> 64	4	2	≤ 0.25	≤ 1/19
E8	2016-03-09	+	> 64/4	> 64	> 64	4	2	≤ 0.25	≤ 1/19
E9	2016-03-12	+	> 64/4	> 64	> 64	4	2	≤ 0.25	≤ 1/19
E10	2016-04-05	+	> 64/4	> 64	> 64	4	2	≤ 0.25	≤ 1/19
E11	2016-04-05	+	> 64/4	> 64	> 64	4	2	≤ 0.25	≤ 1/19
E12	2016-04-02	+	> 64/4	> 64	> 64	4	2	≤ 0.25	≤ 1/19
E13	2016-04-04	+	> 64/4	> 64	> 64	4	2	≤ 0.25	≤ 1/19
E14	2015-11-06	+	> 64/4	8	4	1	> 16	> 4	> 4/76
F1	2016-01-09	–	> 64/4	4	4	≤ 0.25	≤ 1	> 4	> 4/76
G1	2017-03-02	+	> 64/4	64	64	8	2	1	≤ 1/19
G2	2017-06-16	+	> 64/4	64	64	8	4	1	≤ 1/19
G3	2017-09-03	+	> 64/4	64	64	8	4	0.5	< 1/19
G4	2017-09-06	+	> 64/4	64	64	8	4	0.5	≤ 1/19
G5	2017-12-15	+	> 64/4	64	64	8	4	1	≤ 1/19
H1	2017-06-02	–	> 64/4	2	4	≤ 0.25	≤ 1	2	≤ 1/19
H2	2017-12-15	–	> 64/4	64	64	≤ 0.25	≤ 1	1	≤ 1/19
H3	2017-11-07	–	> 64/4	64	64	≤ 0.25	> 16	> 4	≤ 1/19

[a] Isolate E5 was generated from a positive blood culture. Isolates E12 and E13 were collected from pudding and vegetable salad, respectively. All other isolates were collected from rectal swabs

[b] *TZP* piperacillin-tazobactam, *CTX* cefotaxime, *CAZ* ceftazidime, *MEM* meropenem, *GEN* gentamicin, *CIP* ciprofloxacin, *SXT* trimethoprim-sulfamethoxazole

demonstrates that, with the exception of E14, all isolates were similar to isolate E12, showing nearly 100% identity with over 99% genome coverage.

Discussion

The timely identification of carriers and clonal transmissions of nosocomial pathogens supports the immediate implementation of appropriate infection control measures [7]. The findings of this work show that MALDI-TOF MS analysis can contribute in identifying clonal clusters of the opportunistic nosocomial pathogens *S. marcescens* and *C. freundii*. Although MALDI-TOF MS did not reach the discriminatory power of WGS, we could demonstrate that it may be used as a first-line subtyping tool for the sensitive detection of potential transmission events because none of the isolates were falsely negative excluded from clusters that were detected with WGS.

S. marcescens is an important cause of outbreaks and blood stream infection in NICUs [23–25]. Infections are associated with a high mortality rate in neonates. *C. freundii*producing carbapenemases has been associated with colonization outbreaks and nosocomial infections in recent years [26, 27]. Because the influx of CRE into the hospital environment is an emerging threat worldwide, the epidemiological surveillance of such strains gets increasing importance [1]. In this work, SARAMIS-based cluster analysis of consensus spectra could identify a major clonal cluster of VIM-positive *C. freundii* responsible for a nosocomial outbreak.

For MALDI-TOF typing, the whole spectrum of peaks detected within a defined range was used. Typing was performed using consensus spectra of each isolate to reduce the intrinsic variability of single mass spectra which is caused by several measurement factors that negatively affect the reproducibility of mass spectra. These factors include the wear level of the laser, the laser shot setting, and the quality of sample spots on the target [8]. To sustain a constant quality of spectra acquisition, fine

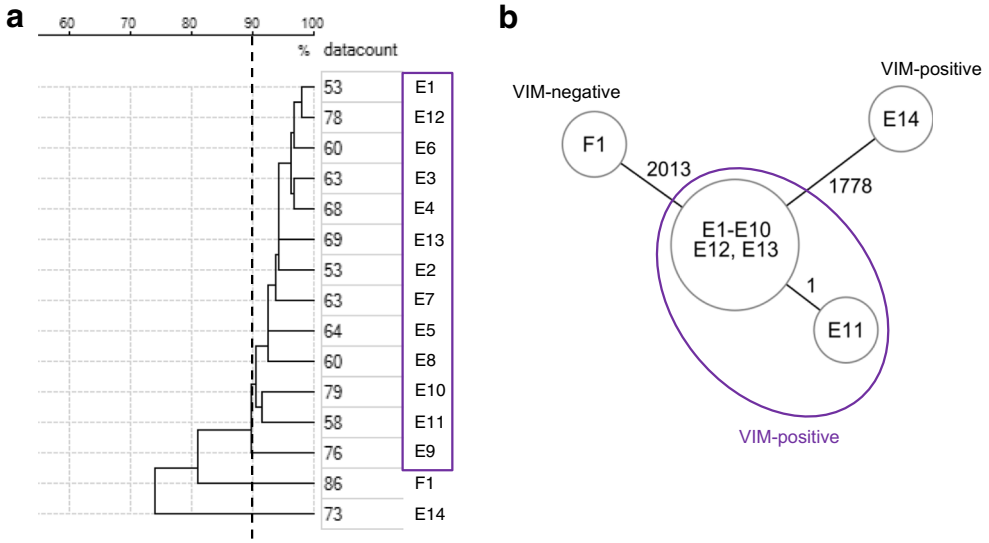

Fig. 3 MALDI-TOF MS cluster analysis of VIM-producing *C. freundii* isolates collected during an outbreak in 2016, compared to WGS analysis. **a** SARAMIS relative cluster analysis of MALDI-TOF consensus spectra. The rectangle indicated in purple (E1–E13) includes outbreak isolates showing a mass spectrum similarity of ≥ 90%. E14, VIM-positive control strain; F1, VIM-negative control strain. **b** Minimum-spanning tree displaying the relationship of the 15 *C. freundii* isolates based on allelic profiles of 2062 target genes that were present in all isolates. Each circle represents an allelic profile, i. e., the genotype, and is named by the isolate(s). The number on connecting lines gives the number of differing alleles

tuning of the laser device was performed every second week by the technical service unit of the company. Because the intrinsic variability of the spectra in peak intensities and peak locations is affected by their acquisition at different time or from different target spots, the comparison of independent data sets is not generally possible and reference strains of well-defined clones should be included in each analysis [8]. To reduce the influence of the spot quality, we used same-age bacterial cultures for all analyses. It has been suggested that the extraction of mass peaks that are specific for a defined clone and the identification of specific *m*/*z* peak shifts due to amino acid substitutions in defined proteins may be very useful to overcome the influence of intrinsic whole spectrum variability and improve the quality of MALDI-TOF typing results [12, 13, 28]. However, the representativeness of a superspectrum for a clonal cluster may depend on the number of isolates used for the identification of specific *m*/*z* signals or peak shifts and it will not always be possible to define superspectra of a high stringency for each clone. Therefore, in this study, the comparison of whole spectrum consensus spectra was used to keep the analytical approach user-friendly. From a technical point of view, consensus spectra-based MALDI-TOF typing may be most likely comparable with classical genotyping methods such as RAPD or PFGE and preferentially applicable in local settings [28, 29].

Technical reproducibility of mass spectra remains a critical problem and limits the resolution power for typing [8, 11]. It is also important to note that MALDI-TOF may not be applicable for typing of all relevant *Enterobacterales* species. A low discriminatory potential of MALDI-TOF MS at the infra-species level has been reported for *K. pneumoniae*, suggesting the need of optimized protocols for different species [10, 29, 30]. For the pathogens that were investigated in this study, a similarity cutoff of about 90% was reliable to give an indication for a clonal cluster whereas the branching of isolates at about 80% excluded a close relatedness. These criteria may help to rapidly identify monoclonal outbreaks of *S. marcescens* and *C. freundii*. However, the limited discriminatory power of the method was also present in this study because one *S. marcescens* isolate of a different genotype could not be discriminated from a large monoclonal outbreak cluster. Nevertheless, the strongest argument that MALDI-TOF may be a useful epidemiological tool comes from the finding that all major clusters responsible for clonal outbreaks could be identified. In the case of *C. freundii*, for the first time, a monoclonal outbreak of CRE at the Jena University Hospital was identified using MALDI-TOF analysis under real performance conditions. When VIM-positive *C. freundii* isolates were recovered from food samples of the hospital's kitchen, their identity with the patient isolates could be rapidly confirmed, resulting in immediate consequences for the hygiene management that included stop of production of pudding and salad, exchange of the mixer for quark and salad sauces, change of the providing companies for salad, and finishing the search for transmission routes by the medical staff [21].

An advantage of MALDI-TOF typing is that the results can be available within 24 h after first isolation of bacteria that are suspected to be of clonal origin. In comparison to WGS, the

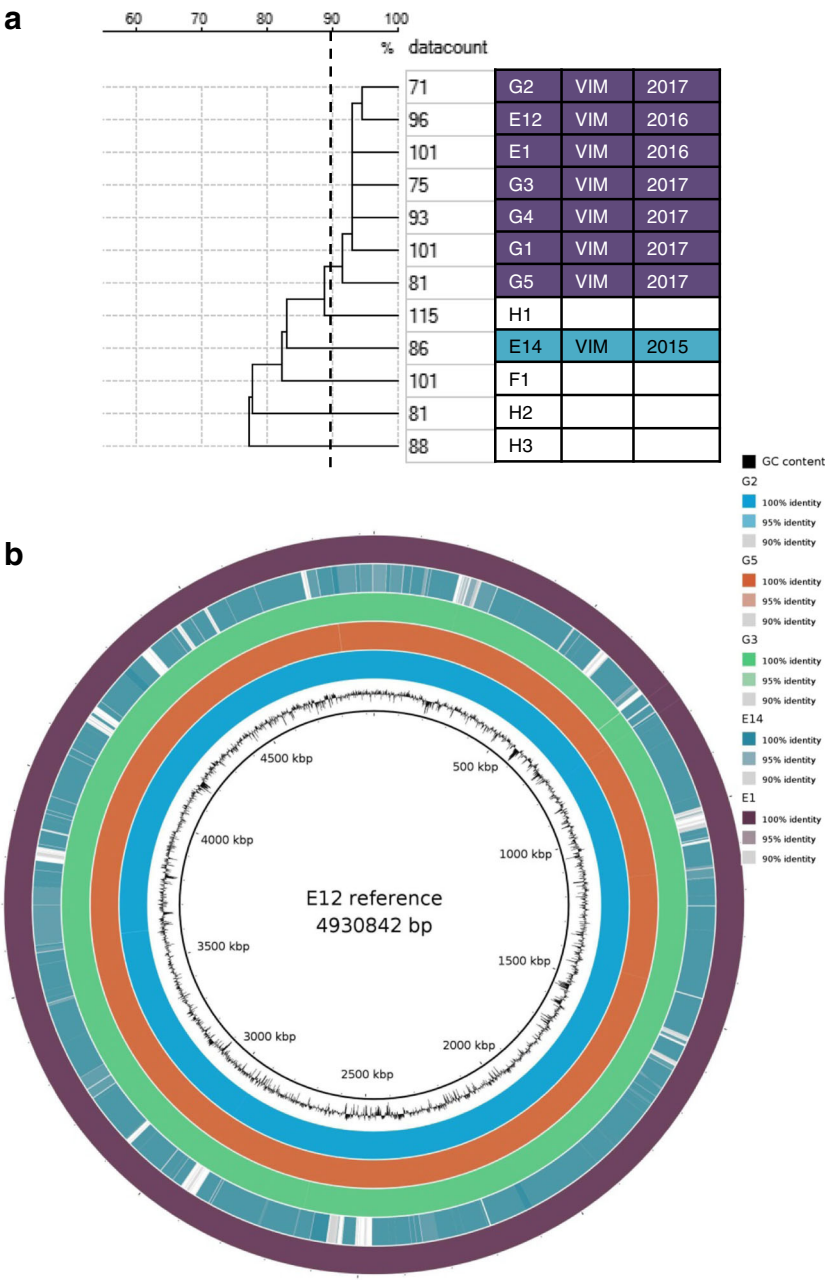

Fig. 4 Typing of VIM-producing *C. freundii* isolates collected in 2017 (G1–G5), compared with VIM-positive reference isolates E1, E12, and E14 recovered in 2016.VIM-negative isolates (F1, H1–H3) served as controls. **a** Dendrogram of the MALDI-TOF consensus spectra using SARAMIS. The cluster indicated in purple includes VIM-positive isolates from 2017 (G1–G4) and from the 2016 outbreak, showing a mass spectrum similarity of ≥ 90%. **b** Whole genome comparison of representative VIM-positive isolates from 2016 and 2017 using MinION sequencing and BRIG analysis. Strain E12 was used as reference genome and compared against the remaining five strains (G2, G3, G5, E1, E14). The innermost ring shows the GC content (black). Sequence data was assembled with CANU and each genome was completely closed to one contig

costs for consumables are low and the little effort does not require additional staff [7, 8, 28]. Regarding the limited number of outbreak-related isolates available for typing, further work is needed to extent the findings presented here.

In conclusion, the findings of this study suggest that consensus spectra-based MALDI-TOF typing has the potential to serve as a screening method for identification of clonal clusters of *S. marcescens* and *C. freundii*. Because isolates that showed an identical WGS type were grouped in the same MALDI-TOF cluster, the branching of consensus spectra at about 80% or lower can exclude a clonal relatedness of the isolates. A first-line epidemiological screening by MALDI-TOF MS may contribute to reduce the number of isolates that have to be analyzed by WGS, thereby saving costs. Further studies are underway that will evaluate the potential of MALDI-TOF typing for a continuous monitoring of appropriate *Enterobacterales* species at high-risk wards such as NICUs to provide first indication of nosocomial transmissions.

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Compliance with ethical standards

Ethical approval Because in this work a method for subtyping bacterial isolates was developed and the paper contains no patient data, research ethics committee approval was not required.

Conflict of interest The authors declare that there are no conflicts of interest.

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