GP

Application Note

Analysis of an Outbreak of VIM-Producing *Citrobacter freundii* by Fourier-Transform Infrared (FT-IR) Spectroscopy using the IR Biotyper® system

Keywords:
Strain typing
Outbreak analysis
Infrared spectroscopy
FT-IR
Clonal relationships

Instrumentation and Software:
IR Biotyper

Introduction

The incidence of carbapenemase-producing *Enterobacteriaceae* (CPE) has increased in recent years, leading to a global public health issue. CPE infections can be very difficult to treat due to their resistance to last resort antibiotics, and they can easily pass this resistance to other bacteria. The spread of CPE within hospital settings therefore poses a serious threat to an increasing number of patients.

Typing of bacterial isolates is crucial to identify transmission routes and the reservoir in the healthcare environment, in order to apply proper infection control measures. For this purpose, techniques to infer clonal relationship between bacterial isolates are required. Several DNA-based methods, such as pulsed-field gel electrophoresis (PFGE), multi-locus sequence typing (MLST), repetitive element polymerase chain reaction (rep-PCR) and, lately, whole genome sequencing (WGS), have been successfully used, but they are not affordable for most laboratories, as they are expensive, laborious and time-consuming, and too slow for real-time routine surveillance.

Fourier-transform infrared (FT-IR) spectroscopy has recently been described as an alternative method for bacterial typing, thanks to its ease of use combined with high discriminatory power in recognizing clonal relationships between bacterial isolates. In this study the IR Biotyper system (Bruker Daltonics) was used to identify a previously well-characterized

Authors: Miriam Cordovana

outbreak of Verona integron-encoded metallo-β-lactamase (VIM)-producing *Citrobacter freundii*. VIM is one of the "Big Five" carbapenemases, together with *Klebsiella pneumoniae* carbapenemase (KPC), imipenemase (IMP), New Delhi metallo-β-lactamase (NDM), and class D oxacillinase-48 (OXA-48) family.

In Europe – Italy and Greece in particular – the spread of KPC, VIM and NDM has been widely reported in *K. pneumoniae*, while only few studies have reported *C. freundii* harbouring metallo-β-lactamase (MβL) or KPC.[1] Microbiological screening of carbapenem resistance mediated by CPE carrying-plasmids is needed to maintain and enforce infection control measures, especially when considering that *K. pneumoniae* is the most common carbapenemase-producing species and transfer of respective resistance encoding plasmids may involve all species of *Enterobacteriaceae*.

FT-IR spectroscopy for microbiology

Methods for human bacterial pathogen strain typing need to be fast, reliable, inexpensive, and time efficient. These requirements may be met by vibrational spectroscopic techniques, such as FT-IR. Traditionally used in chemistry to determine the molecular composition of a wide range of sample types, FT-IR spectroscopy is a technique that quantifies the absorption of infrared light by molecules present in the sample, such as lipids, nucleic acids, carbohydrates, lipopolysaccharides, and proteins. This results in the generation of a specific FT-IR spectrum, which reflects the overall chemical composition of the specimen.

Applied to bacterial cells, this technique enables a high-resolution analysis of the bacterial surface in a non-destructive manner, and hence an evaluation of similarity between different isolates at strain level.

Investigating clonal relationships between *C. freundii* isolates with the IR Biotyper

The IR Biotyper, based on FT-IR spectroscopy, measures the vibrations of carbohydrate constituents present in many molecules, such as glycoproteins. The resulting FT-IR spectrum is a highly specific fingerprint allowing the classification of microorganisms at the subspecies level (Figure 1).

This new application of FT-IR spectroscopy was used at Policlinico Sant'Orsola-Malpighi, Operative Unit Microbiology and Virology in Bologna, Italy, to evaluate the efficacy of the IR Biotyper for typing of a previously well-characterised cluster of VIM-producing *C. freundii*.

Materials

The study used seven VIM-producing *C. freundii* isolates belonging to an outbreak previously characterized by MLST[1], six VIM-producing isolates not related to the outbreak (different period of time – 2012 until 2019, different hospitals), two KPC-producing *C. freundii* isolates, one OXA-48-producing *C. freundii* isolate, and one *C. braakii* negative control isolate.

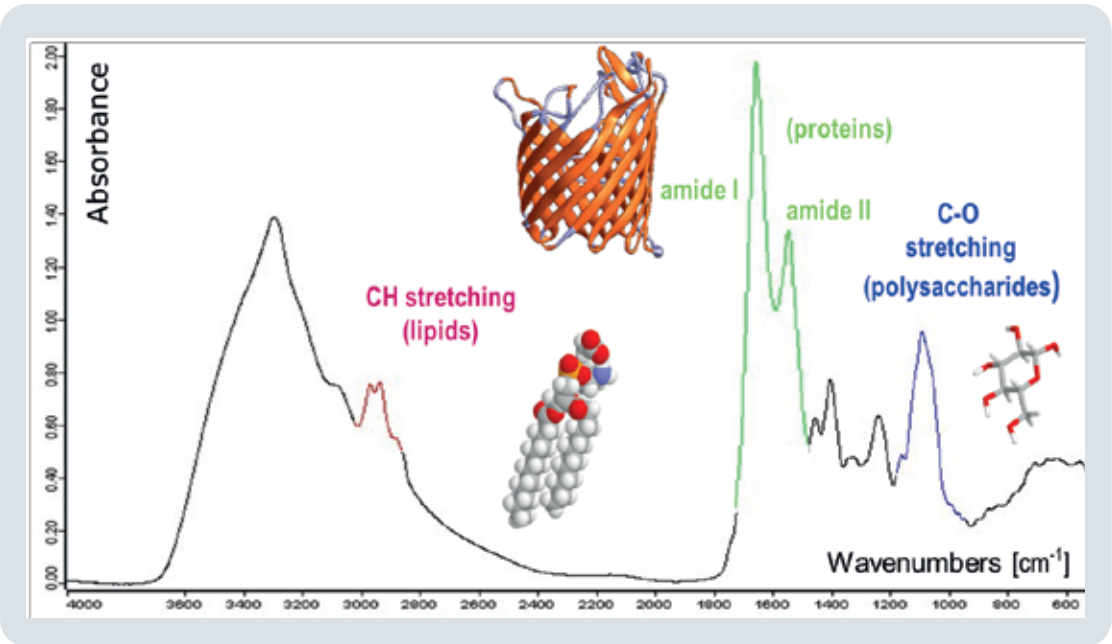

Figure 1: The IR Biotyper analyses IR spectra in the wavelength range as indicated by the blue coloured spectrum area. Spectra are then further analysed for specific subspecies characteristics.

The seven VIM-producing *C. freundii* isolates were collected from June 1 to June 15, 2012, isolated from rectal swabs of patients hospitalised in the same ward in the Bologna metropolitan area. The carbapenemase production was investigated by phenotypic (disk-diffusion synergy test with inhibitors) and genotypic (PCR for *bla* genes and sequencing) methods. The investigation of genetic relationship between the *C. freundii* isolates, assessed by MLST based on seven housekeeping genes (*aspC, clpX, fadD, mdh, arcA, dnaG, lypS*[2]), showed that the strains exhibited identical allele profiles. The control strains were also carbapenemase-producing isolates collected from surveillance samples, investigated by phenotypic methods.

Methods

In general, cultivation conditions like the agar medium used, the temperature and the incubation time, depend on the organism. These conditions must be kept as standardized as possible to take advantage of the high selectivity of the FT-IR method.

In this study all strains were cultivated under highly standardized conditions on Sheep Blood Agar for 24 hours at 37 °C. Three independent biological replicates were analysed on different days to establish the cut-off values for clustering. The technical variance was evaluated by three technical replicates of each biological replicate. Infrared absorption spectra were acquired from 15 µl of a bacterial suspension in ethanol solution spotted and air dried onto the IR Biotyper silica 96 spot target. The spectra were automatically processed by the IR Biotyper software, in the wavelength range corresponding to the carbohydrate region (1300-800 cm^{-1}). The IR Biotyper workflow is described in Figure 2. The similarity of spectra was assessed by an explorative data analysis tool which uses certain mathematical processing steps to calculate the distance between spectra. As a recommended default setting for these operations we used Euclidean distance, an average linkage algorithm, which generally provides a good starting point for data analysis. The results of the explorative data analysis were displayed as a scatter plot (Figure 3) or dendrogram (Figure 4).

Results

A new, standardised method

In this study, the IR Biotyper proved to be an effective tool to investigate clonal relationships between different *C. freundii* isolates, providing results consistent with the reference DNA-based methods. It successfully identified, retrospectively, a VIM-producing *C. freundii* outbreak cluster, which has been clearly differentiated from six other not clinically related VIM-producing isolates (not belonging to the outbreak), and from three other carbapenemase-producing isolates and one control isolate. All isolates belonging to the VIM outbreak were clearly clustered together as

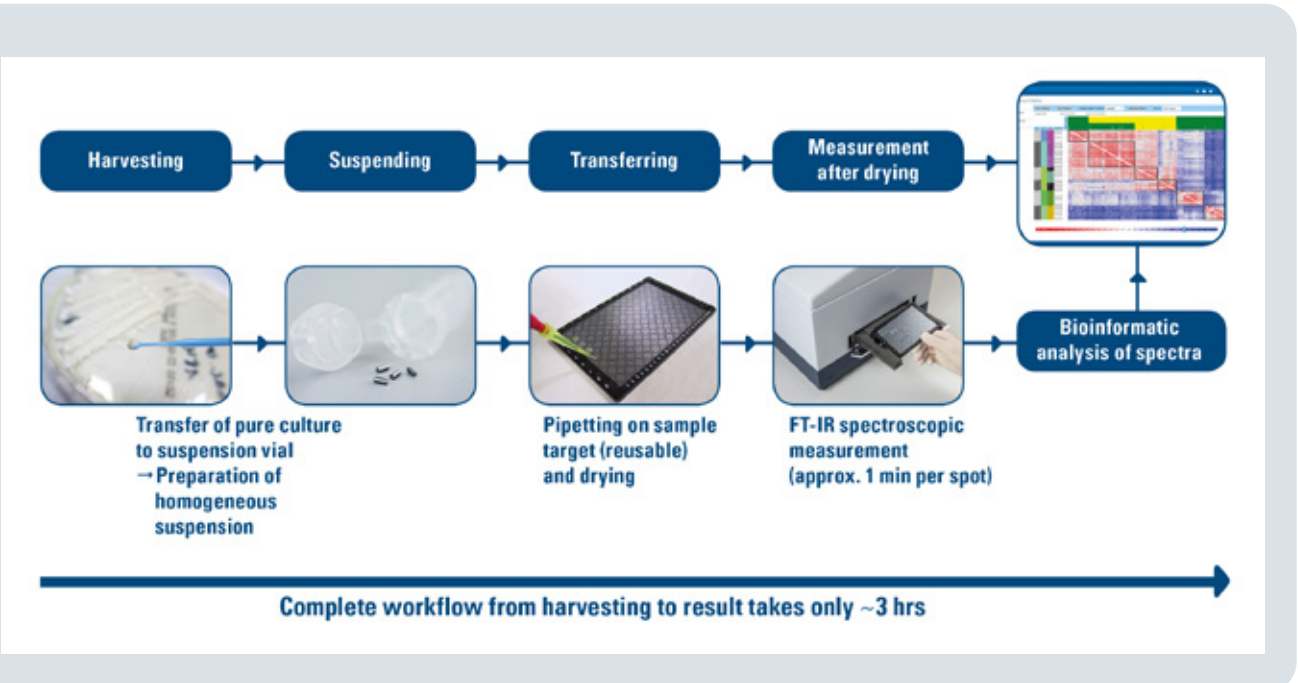

Figure 2: The IR Biotyper workflow. A bacterial suspension is prepared in an ethanol solution using a fresh overnight bacterial culture (standardized growth conditions), and 15 µl of the suspension is spotted in 3-4 technical replicates onto the IR Biotyper target. Once completely dried (15-20 minutes at 37 °C), the target is inserted into the IR Biotyper system for spectra acquisition, followed by an automated data analysis by the IR Biotyper software using different statistical methods.

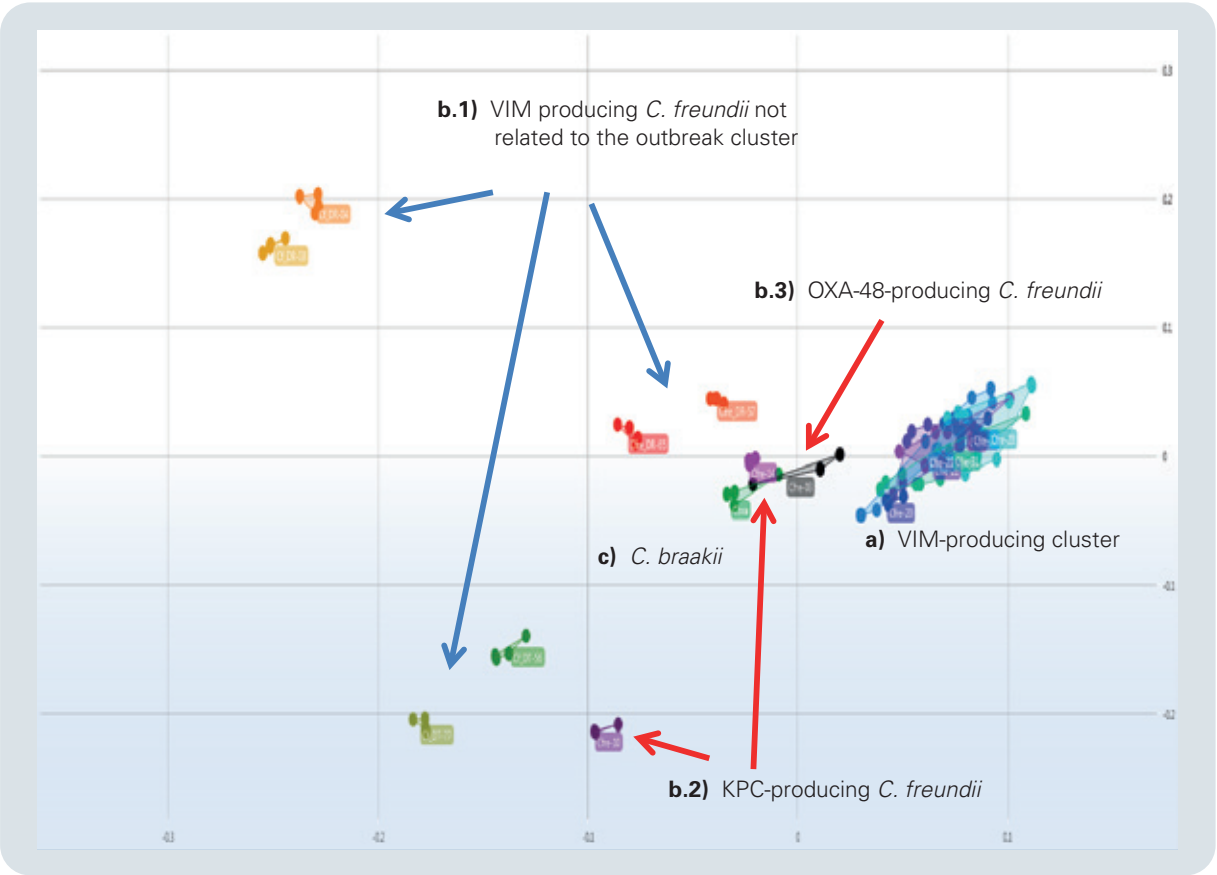

Figure 3: 2D PCA plot showing the clustering of the clonally related C. freundii isolates (CF-31, C-23, C-22, CF-28, C-21, C-19 and C-20) by the IR Biotyper. The software automatically assigns a different colour to each isolate. The dots represent the isolates, corresponding to the single measurements. The links between the dots belonging to the same isolate express the variance of the measurements of the technical replicates. In this plot, the clonally related isolates overlap each other, proving their strong relatedness, while the control isolates are well separated both from the cluster, and from each other.

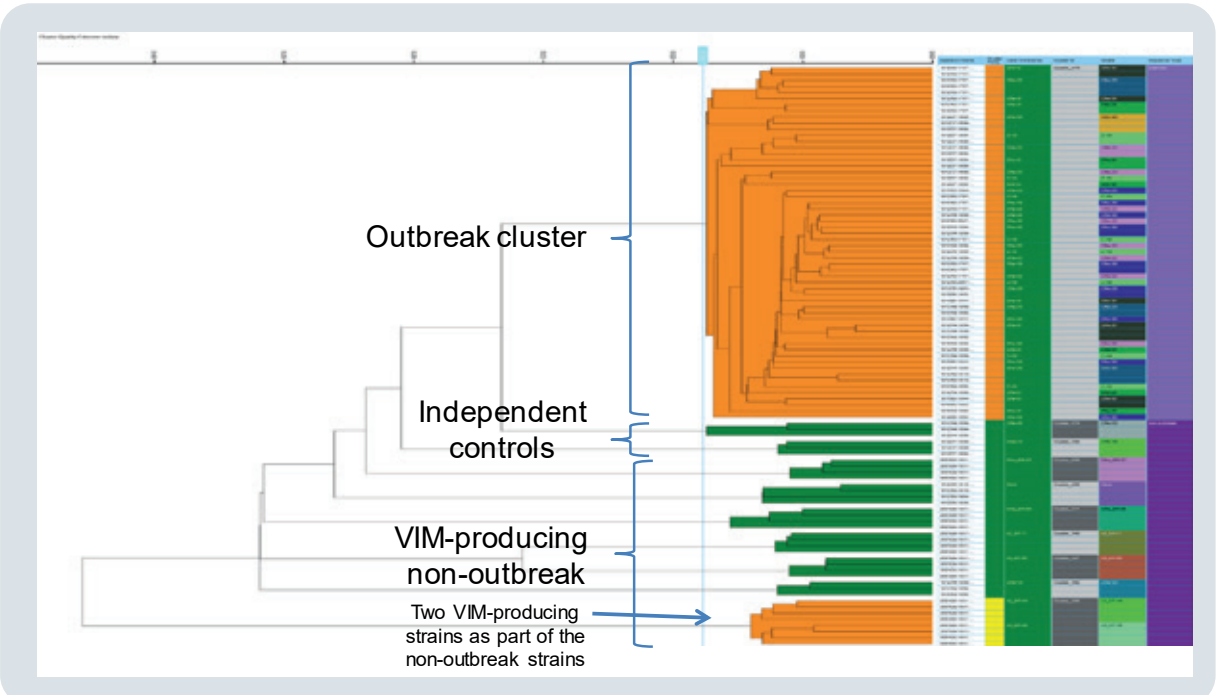

Figure 4: Dendrogram of all VIM isolates.

identified by the IR Biotyper software displayed in figure 3. Some of the spectra of different isolates in the outbreak cluster are more closely related than the replicates of the same outbreak isolate, showing a very high similarity within the outbreak cluster. The VIM cluster perfectly matched with sequencing results (MLST) and a clear differentiation was found between the VIM outbreak cluster and the other four isolates (Figure 3). Moreover, two of the six VIM-producing strains, which do not belong to the outbreak, are clustered closely together indicating a very close clonal relationship and therefore a potential transmission of this clone (see figure 4). This shows the potential of the IR Biotyper system in routine practice for a prompt and early detection of a new or beginning outbreak.

While further studies are required to assess robustness and reproducibility of the method at multiple global sites, the IR Biotyper offers the opportunity to become a standardized screening tool since the technical procedure is very simple and short, taking ~2 hours for the 16 described samples, including sample preparation and isolate measurements, and the operator variability is minimal.

Conclusion

In comparison to currently used molecular typing methods, the IR Biotyper represents several advantages that could enable a "quantum leap" for epidemiological investigations, which are currently mainly restricted to a few dedicated reference laboratories, with dedicated trained personnel, because molecular-based methods are costly and require high technical expertise.

In contrast to the molecular typing methods, the IR Biotyper fits with the needs of routine clinical microbiology laboratories, opening new perspectives in infection control practice, by enabling bacterial epidemiological surveillance not only retrospectively, but also in real time. The potential to track the spread of bacterial isolates in a very short time frame and in a cost-effective way would support the timely adoption of rigorous hygiene measures and can help to reduce the burden of hospital acquired infections.

The speed, ease-of-use, and cost-effectiveness of the IR Biotyper could play a major future role in enabling real-time routine surveillance, by detecting the dissemination and transmission routes of multidrug resistant isolates within hospital settings.

Learn More

For more information about Bruker's IR Biotyper system, visit the website
https://www.bruker.com/applications/microbiology/strain-typing-with-ir-biotyper/overview.html

or download the brochure
https://www.bruker.com/fileadmin/user_upload/8-PDF-Docs/Separations_MassSpectrometry/Literature/Brochures/1862836_IR_Biotyper_01_2019_eBook.pdf

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[2] Bai L, Xia S, Lan R, Liu L, Ye C, Wang Y, et al. Isolation and characterization of cytotoxic, aggregative *Citrobacter freundii. PLoS One* 2012; 7:e33054.

Not for use in clinical diagnostic procedures.
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Bruker Daltonics GmbH & Co. KG

Bremen · Germany
Phone +49 (0) 421-2205-0

Bruker Scientific LLC

Billerica, MA · USA
Phone +1 (978) 663-3660

info.md@bruker.com - www.bruker.com/microbiology