Identifying Antibiotic Resistance Through Direct Detection of Intact Enzymes from Bacterial Lysates using LC-MS/MS: Exploring MRSA and Carbapenem Resistance

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ABSTRACT

Purpose: We sought to identify and characterize enzymes conferring antibiotic resistance for rapid and accurate detection using LC-MS/MS approaches for intact protein analysis.

Methods: LC-MS/MS is used to detect and characterize both intact and digested proteins from bacterial cell lysates of multiple species.

Results: The mature form of several different types of these enzymes have been identified across multiple species. High resolution and accurate mass analysis has allowed confident detection of different resistance markers, often with the ability to distinguish specific variants, in a fairly simple LC-MS/MS workflow.

INTRODUCTION

Antibiotic-resistant bacteria have been spreading at alarming rates over the last several decades, and have been considered to be urgent threats globally[1-3]. Two common types frequently associated with high rates of morbidity and mortality are MRSA and five main groups of carbapenemases[4]. The enzyme PBP2a is responsible for MRSA strains being resistant to penicillin and similar antibiotics. The carbapenemases have spread broadly among Gram negative bacteria and include KPC, NDM, VIM, IMP, and OXA-48-like families. Increased dissemination has resulted in the emergence of several variants from each of these families, leading to varying levels of resistance; the difference in resistance between similar variants can be substantial. Therefore, the ability to identify not only the type of resistance, but also the specific variant(s) present from a bacterial sample is vitally important and can provide critical information to clinicians to aid in determining appropriate therapy.

MATERIALS AND METHODS

Bacterial isolates were acquired from the CDC and FDA Antibiotic Resistance Isolate Bank and American Type Culture Collection. Each isolate was grown on agar (tryptic soy or sheep's blood) or in broth (tryptic soy) for 16-24 hours. Those grown on agar were harvested with a 10 µL loop tool and mechanically lysed via bead-beating or sonication. Isolates grown in broth were centrifuged at 4,000 g for 25 minutes with supernatant decanted, followed by resuspension with 0.9% NaCl. The solutions were centrifuged a second time with supernatant decanted. Pellets were resuspended in either 8M urea 50mM Tris for bottom-up analysis or 6M guanidinium chloride 50mM Tris (250mM for MRSA strains). Lysates were mechanically lysed via bead-beating, then centrifuged with the protein-containing supernatant extracted.

Lysates for bottom-up analysis were digested with trypsin following standard protocols and separated on C18 spin columns. Separation of protein digests were performed using a binary gradient of water and a 10:10:80 mixture of water, isopropanol and acetonitrile in 0.2% formic acid.

Intact proteins were first separated with either an online solid phase extraction or a 25 cm Thermo Scientific™ ProSwift™ RP4H reverse-phase column. This has been performed on multiple LC platforms, such as Thermo Scientific™ Ultimate™ 3000, Thermo Scientific™ Vanquish™ Horizon, Thermo Scientific™ EASY-nLC™ and an in-house-built prototype similar to the EASY-nLC. Proteins were eluted with a binary gradient of water and acetonitrile, both in 0.2% formic acid. LC-MS analysis of intact lysates in 6M guanidinium chloride involved a 10 minute valve switch delay, such that excess salts could be washed from the column and transferred to waste. After the delay, the valve switch delivered the flow to the ionization source. In some cases, lysates in guanidinium chloride were buffer-exchanged into 5% acetonitrile, 0.2% formic acid in water to avoid the need to divert to waste.

For PBP2a sample preparation, proteins were extracted by gently lysing cells over ice with 50 mM HEPES, 100 mM NaCl, 1mM EDTA, 0.5% SDS, 1 mM EDTA, and 1 mM PMSF, pH 7.0. Following lysis, extracts were centrifuged and filtered prior to strong cation exchange fractionation and subsequent dialysis[5].

Mass analysis of intact proteins was performed to help detect and identify resistance markers directly from lysates, using Thermo Scientific™ Q-Exactive™ HF mass spectrometers. Detection of intact proteins was initially performed using LC-MS to identify potential resistance markers. Further characterization of intact proteins was performed via LC-MS/MS and was validated on multiple strains for each type of resistance marker.

DATA ANALYSIS

Bottom-up data analysis was performed using Thermo Scientific™ Proteome Discoverer™ 2.5 with Sequest HT with databases produced from several Uniprot entries (www.uniprot.org), as well as databases of common resistance markers from ResFinder (cge.cbs.dtu.dk/services/ResFinder/). With the aid of Protein Prospector (prospector.ucsf.edu/prospector/mshome.htm), protein mass spectra were manually interpreted.

RESULTS

Bacterial lysates have substantial amounts of proteins in them, making the identification of one or more specific proteins particularly challenging. To identify and characterize specific resistance markers, each element of LC, MS and MS/MS is essential. To date, we have identified and characterized PBP2a and 5 types of carbapenemases using this approach, with multiple variants of each resistance marker and across multiple species of bacteria.

The process of developing methods for detection of resistance markers tends to follow general steps, including chromatographic separation, detection of candidate intact proteins, and fragmentation of those candidates for confirmation of protein identity. In some cases the mature protein mass differs from the predicted mass due to a discrepant mature protein sequence, post-translational modifications, or both.

Figure 1. Chromatographic separation for bacterial cell lysis using an analytical column. . Peaks represent extracted ion chromatograms of KPC-2 fragment b_{269}. Top panel illustrates separations reasonable for protein detection, while the middle and lower panel illustrate the ability to significantly reduce elution timescale for an overall shorter analysis time. The dashed line illustrates a valve switch from waste to the MS, avoiding high salt-content being transferred to the source. Buffer exchange eliminates the need for a valve change, and can reduce the overall elution time

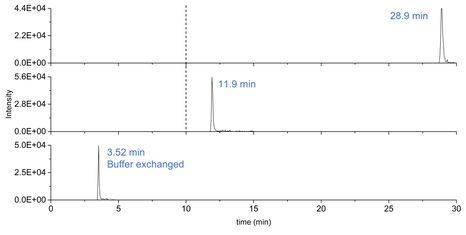
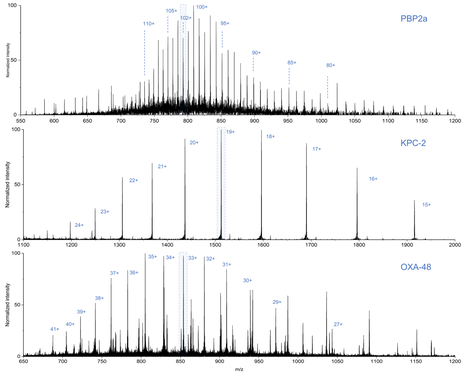


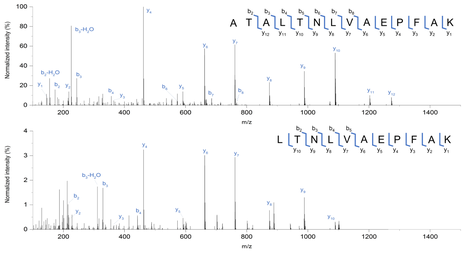
Figure 2. MS detection of intact proteins under chromatographic separations. Mass spectra of the intact proteins PBP2a of MRSA (top panel), KPC-2 (middle panel) and OXA-48 (lower panel) are illustrated below, using various chromatographic techniques. Shaded areas represent precursor ions for MS/MS.



MS/MS for Exploit Differences and to Identify

The specific function of bacterial proteins can affect where they tend to be located within the cells. For the two classes of proteins discussed herein, PBP2a and carbapenemases, they will tend to be localized toward the periphery of the cells. Gram negative proteins can be generated as pro-proteins, with a signal peptide guiding them to their final location, often in the periplasm, before being cleaved. Proteins from both Gram positive and Gram negative can have additional post-translational modifications as well.

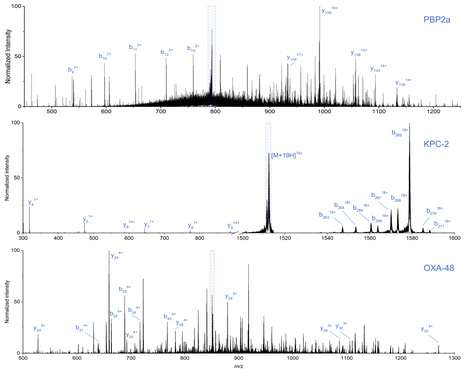
Figure 3. Detection of N-terminal tryptic peptides of KPC-2 formed from competing signal peptide cleavage sites, with intensity normalized to the top panel. Most predictions of mature protein sequence previously suggested cleavage N-terminal of "LTN" sequence yet the much more dominant cleavage site occurs N-terminal to "ATA." Consequently, the dominant form of the mature protein begins with the "ATA" sequence.



General differences between the predicted and the observed mature protein may include:

- **Unexpected cleavage site for signal peptide**
- **Post-translational modifications**

Figure 4. MS/MS of intact resistance markers. Fragmentation of PBP2a (top), KPC-2 (middle) and OXA-48 (lower) provides extremely high protein identification confidence. Annotated fragments are assigned based on ± 10 ppm *m/z* tolerance, correct charge state, and S/N ≥ 3 (as shown in Table 1). Shaded areas show precursor *m/z* windows.



Resistance Marker Detection

Isolated intact protein ions, as observed in MS, can be combined with sequence-informative fragment ions from MS/MS data to confidently identify the protein. However, in some cases, MS/SM fragment ions on their own can identify the protein even though the precursor is not observed and a fixed isolation window is used. In this study, a set of criteria have been established to confidently identify resistance markers, even if they are not directly observed in MS alone.

Table 1. Criteria for peak assignment as diagnostic fragment. For any protein to be positively detected, it must have at least 3 diagnostic fragments.

#	Criteria
1	Fragment must be generated from correct precursor ion
2	Fragment *m/z* must be within ± 10 ppm of theoretical value
3	Fragment must have S/N ≥ 3
4	Fragment must have correct z

Resistance Marker Detection vs Susceptibility Testing

Resistance detection and susceptibility testing follow two separate approaches, yet compliment each other well. Some differences and practical considerations are listed below.

Resistance Detection:

- **Identifies specific resistance markers and, consequently, the mechanism of resistance**
- **Can allow for specific variant of a particular resistance type (i.e. KPC-3 vs KPC)**
- **Can determine the presence of multiple resistance markers simultaneously**
- **Only focuses on specified targets, will not detect resistances beyond its scope**

Susceptibility Testing:

- **Offers evaluation of broad antibiotic resistance, such as carbapenemase activity**
- **Generally struggles to provide insight into specific mechanisms of resistance**

Diversity of Resistance Markers and Species

We have observed the same resistance markers expressed across multiple species with no or minimal variation in the mature protein. For Gram positive bacteria, *Staphylococcus aureus* was the main species investigated. For Gram negative bacteria, three main groups represent the vast majority of species harboring carbapenemase genes.

Table 2. Variants of different resistance types that have been evaluated thus far.

Resistance Marker	Variants Detected Based on Available Strains
PBP2a	SCC*mec*-I, -II, -III, -IV, -V, -VI
KPC	-2, -3, -4, -5
OXA-48-like	-48, -181, -232
NDM	-1, -4, -5, -6, -7
VIM	-1, -2, -4, 27
IMP	-1, -4, -14

Table 3. Groups of bacteria investigated and the corresponding resistance markers detected

Type	Types of Resistance Markers Detected
Staphylococcus aureus	PBP2a
Enterobacterales	KPC, OXA-48-like, NDM, VIM, IMP
Pseudomonas spp.	KPC, NDM, VIM, IMP
Acinetobacter spp.	VIM, IMP, NDM

CONCLUSIONS

- **LC-MS/MS with high resolution and accurate mass analysis can be used to rapidly detect and identify enzymes conferring antibiotic resistance in a straight-forward manner and with relatively simple experimental setup.**
- **This approach offers the ability, in most cases, to distinguish between specific variants of a particular resistance marker (e.g. KPC-2 and KPC-3) across multiple species**
- **The use of LC-MS/MS allows for the detection of multiple resistance markers in a single sample through chromatographic separation**
- **MS/MS can be used to distinguish separate resistance markers even when co-isolated and co-fragmented**
- **MS/MS tends to show higher sensitivity towards detection of resistance markers than MS alone**

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ACKNOWLEDGEMENTS

The authors wish to thank collaborators, clinicians, colleagues, and subject matter experts that have provided helpful discussions, especially to Dr. Nate Ledeboer and Mary Ann Silvius. We also wish to thank the CDC and FDA Antibiotic-Resistant Isolate Bank for providing bacterial isolates for research purposes.

TRADEMARKS/LICENSING

PO66118 EN0921S