Analysis of Intact Resistance Markers for Metallo-β-Lactamases in Bacterial Pathogens

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ABSTRACT

Purpose: The complete characterization of mature metallo-β-lactamases (NDM, VIM,IMP) for the development of rapid detection assays of common clinical variants.

Methods: Top-down and bottom-up mass spectrometry methods were used to characterize metallo-β-lactamase targets and to develop a five minute assay for detection in Gram negative pathogens.

Results: Metallo-β-lactamase characterization revealed a variety of mature forms of VIM and IMP in different bacterial orders depending on the nature of signal peptidase I. Lipidation of NDM was found to vary as well across different bacterial orders. All variants for any metallo-β-lactamase could be analyzed in a 5 minute time frame.

INTRODUCTION

Metallo-β-lactamases have broad activity against carbapenem, penicillin, and cephalosporin antibiotics. The most common types observed are the zinc ion-based enzymes VIM (Verona Integron-encoded metallo-β-lactamase), IMP (imipenem metallo-β-lactamase) and NDM (New Delhi metallo-β-lactamase) found in Gram negative bacterial pathogens. With a global mortality rate over 60%, rapid detection of these disease markers is critical in determining timely and appropriate therapy. Although a variety of protein-based diagnostic approaches exist to detect this class of resistances, no single approach can determine the enzyme type, exact variant, and variations in the mature expressed form of the disease marker. Here we present an intact protein tandem mass spectrometry-based method based on previous characterization studies of other carbapenemases[1,2] for the characterization and detection of this important class of resistances in bacterial pathogens.

MATERIALS AND METHODS

Sample Preparation

Bacterial isolates were obtained from American Type Culture Collection (ATCC), and the Centers for Disease Control and Prevention and Food and Drug Administration Antibiotic Resistance Isolate Bank. Isolates were grown on either tryptone soya agar or broth for 20-24 hours at 37C. Colonies were harvested, suspended in a water/formic acid solution and mechanically lysed via bead-beating. Extracts were then centrifuged and the supernatant filtered prior to LC-MS/MS analysis. Samples were then divided for bottom-up and top-down analyses, using C_{18} reverse phase and RP4H monolithic columns.

Test Method(s)

Tryptic peptides were separated using a Thermo Scientific™ Vanquish™ Horizon UHPLC system equipped with a Thermo Scientific™ Hypersil GOLD™ C_{18} column, sample line preheater (60C), and connected to an Ion Max API source. A binary gradient of solvent A (0.1% formic acid in water) and solvent B (10% water, 10% isopropanol, 0.1% formic acid in acetonitrile) was used, increasing from 10% to 50% solvent B over 60 minutes at a flow rate of 60 µL/min.

Intact protein samples were separated using a Thermo Scientific™ Ultimate™ 3000 HPLC system coupled with a Thermo Scientific™ ProSwift™ RP-4H column or MAbPAC RP column, maintained at 60C, and connected to a Thermo Scientific™ Ion Max API source. A binary gradient of solvent A (0.2% formic acid in water) and solvent B (0.2% formic acid in acetonitrile), increasing from 2% to 50% solvent B, at a flow rate of 200 µL/min for 60-120 minutes was used for protein characterization.

Tryptic peptide data was collected in data-dependent mode on a Thermo Scientific™ Q Exactive™ HF mass spectrometer. Peptide fragmentation was performed using HCD at a normalized collision energy of 30eV. Fragment spectra were collected at 30,000 resolving power, 3 µscans/spectrum, with mass isolation at a width of 2 m/z, and a maximum injection time of 100 ms.

Top-down protein data acquisition was performed on a Q Exactive HF mass spectrometer with implementation of instrument protein mode settings. Single stage MS data acquisition was performed over a range of 750 – 1500 m/z during protein elution, with data collected at 120,000 resolving power. Fragmentation spectra were acquired with a 2 - 3 m/z isolation window, at a resolving power of 120,000 (at m/z 400), an AGC target value of 2x105, with 5-10 µscans/spectrum, and a maximum injection time of 200 ms.

Data Analysis

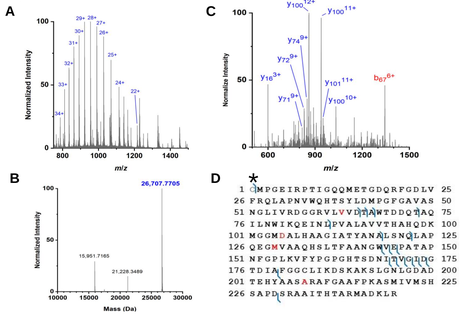
Peptide spectra were identified using Proteome Discoverer 2.5 software with SEQUEST HT and searched against a reviewed database of manually annotated pathogen protein sequences and a database of common contaminants proteins. Data were deconvoluted using the Xtract algorithm and 'averagine' model to determine NDM protein molecular weight. Interpretation of fragment spectra was performed with the MS-Product and MS-Isotope utility from Protein Prospector as well as the ProSight Lite software tool for top-down proteomics. Average precursor mass and a ±10 ppm fragmentation tolerance was used for ProSight Lite data processing.

RESULTS AND DISCUSSION

NDM

Detection of endogenous NDM-1 lipoprotein by top-down LC-MS/MS analyses was performed with intact protein extracts from representative *K. pneumoniae* strains. Two different strategies for collisional-activation were employed for characterization via tandem mass spectrometry. First, in-source dissociation (isCID) was used to generate peptide fragments within the ion optics of the MS system. Secondly, traditional tandem mass spectrometry using mass isolation and direct detection of product ions was employed. The isCID process has previously been used to enable sequencing of N-terminal and C-terminal fragments from intact ionized proteins[3]. The full scan mass spectrum was acquired from m/z 700 to1500 and produced a charge state distribution between +18 to +35 (**Figure 1A**). The molecular weight of the intact protein determined experimentally was 26,707.7705 Da (**Figure 1B**). Isolation and high-energy collisional dissociation (HCD) of the most intense precursor ion charge state (+28 at m/z = 955.46) produced multiple sequence-specific fragment ions enabling NDM-1 protein identification (**Figure 1C**). The majority of MS/MS product ions generated were C-terminal y-ions with only two abundant fragments that could be potentially assigned as N-terminal b-ions. Confirmation of the N-terminal lipid structure and dependent b-ion fragment ion assignment is presented in the next panel. Dissociation of the +28 NDM-1 precursor ion yielded 39 fragments that could be assigned (**Figure 1D**), representing approximately 16% of all cleavable bonds in the protein[4].

Figure 1. A) ESI charge state distribution of the NDM-1 lipoprotein from LC-MS analysis of a protein extract from the *K.pneumoniae* CDC106 strain. (B) Molecular weight determination of the intact NDM-1 protein. (C) HCD fragmentation of the most intense charge state of NDM-1 with corresponding sequence-specific b- and y-ion fragments. (D) HCD fragmentation map of intact NDM-1 corresponding to the b- and y-type fragments observed. The amino acids labeled in red are associated with the point mutations in common variants including NDM-5, -6, and -7. The asterisk at the N-terminus indicates the location of the lipidation of NDM. (Adapted from reference 4).



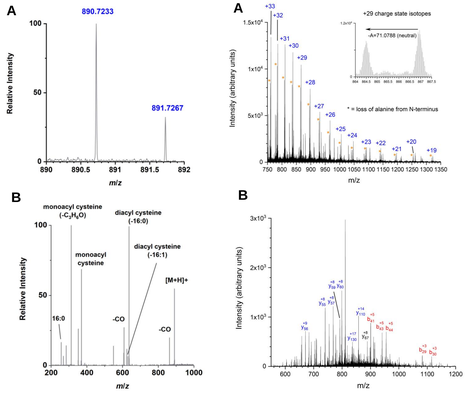
From these initial tandem mass spectrometry experiments, a singly charged fragment ion at m/z = 890.72 and an abundant fragment ion at m/z = 1344.39, z = +6 were observed on a routine basis. The deconvoluted neutral mass difference between the two fragments was found to be equivalent to the theoretical difference between the cysteine in the lipobox (located at amino acid 26) and the alanine residue at the amino acid found at position 67. Isolation and fragmentation of the singly charged product ion at m/z = 890.7233 was performed with the MSn function on a Fusion Lumos Tribrid MS system (**Figure 2A**). Dissociation of this species generated fragments at m/z = 636.50 and m/z = 370.27, which are consistent with the initial neutral loss of an unsaturated fatty acid (16:1, MW = 254.22 Da) and the subsequent loss of another saturated fatty acid and the loss of CO (16:0 + CO, MW = 266.22 Da) to generate a monoacylated cysteine with a single saturated fatty acid (16:0), respectively (**Figure 2B**). The neutral loss of CO (-27.99 Da) was also observed from the triacylated precursor ions and the diacylated product ion. Lower abundant fragment ions were observed for the diacylated and monoacylated species with an observed mass difference of -2.01 Da. This difference corresponds to the neutral loss of the saturated fatty acid, rather than the unsaturated chain. The loss of the unsaturated chain therefore may be the more favorable dissociation process. The fragment ion at m/z = 312.23 is postulated as the rearrangement of the monoacylated cysteine and the loss of acetone (C_3H_6O, 58.04 Da). The proposed structure of this modified N-terminal cysteine in shown in **Figure 2C**. Using this chemical information for the modified cysteine ($C_{54}H_{99}O_6NS$), the calculated theoretical monoisotopic mass of the NDM-1 protein for *K.pneumoniae* is 26707.5673 Da, within 0.2 Da (7.6 ppm) of the observed deconvoluted nmass[4].

IMP

In the initial characterization experiments the extraction and lysis conditions are such that the IMP resistance marker protein unfolds and releases the ligand bound Zn^{2+} ion associated with its activity. Therefore, upon mass spectrometric detection only the single polypeptide chain is observed in the full scan mass spectrum. In **Figure 3A** is shown the electrospray mass spectrum of IMP-1 from *Pseudomonas aeruginosa*. Two different mature forms of the protein were observed differing by an N-terminal alanine residue (see inset **Figure 3A**).

Figure 2. (A) Isolation of the triacylated cysteine fragment ion of NDM-1 following HCD fragmentation of the +27 precursor ion charge state. (B) HCD fragmentation of the isolated triacylated cysteine fragment (MS^3) produced neutral loss product ions helping to enable deduction of lipid structure. (C) Proposed structure of triacylated cysteine of NDM-1. Lipid chains are labeled as R1, R2, and R3. (Adapted from reference 4).

Figure 3. (A) Full scan mass spectrum of IMP-1 derived from *Pseudomonas auriginosa* showing two forms of the mature IMP protein (minus one alanine residue, see inset). The asterisk indicates the truncated mature form of IMP-1 (B) HCD fragmentation of isolated m/z 811 ion (+31 charge state) produced diagnostic b- and y-type fragment ions facilitating identification of IMP-1.



This difference in the mature forms of the IMP resistance protein can be attributed to two factors. The first is the nature of the binding grove associated with the signal peptidase I enzyme which is responsible for cleaving the signal peptide of IMP-1 to generate the mature protein as it is transported into the periplasm of Gram negative microorganisms. Another factor responsible for differing mature forms of IMP is the actual signal peptide sequence itself which can also influence the cleavage process. The data observed to date is also a function of the variation in the sequence homology of signal peptidase I across different bacterial families (enterobacteriaceae, pseudomonadaceae and acetobacteraceae) and the origin of species for the original metallo-β-lactamse variant.

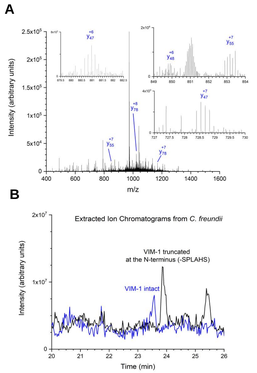
In order to confirm the presence of IMP-1 the +31 charge state of IMP-1 was subjected to the HCD fragmentation process. Figure 3B shows the diagnostic b- and y-type ions generated via MS/MS of the isolated charge state. Similar studies were conducted on IMP-4 and IMP-14 derived from *Klebsiella pneumoniae* and *Pseudomonas auriginosa* respectively (data not shown).

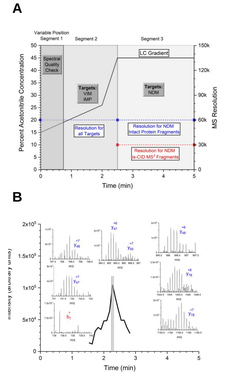
VIM

VIM is similar in nature to IMP in that the mature form of the protein releases the bound Zn^{2+} metal ions upon unfolding in the presence of organic solvent. The ESI mass spectrum of the intact species produces a charge state distribution similar to that of IMP-1 (data not shown). As with other metallo-β-lactamases tandem mass spectrometry of the intact species yields diagnostic fragments specific to VIM-1 as shown in **Figure 4A**. Interestingly in Enterobacteriaceae, two forms of the mature VIM-1 protein were observed that could be separated by LC-MS as shown in **Figure 4B**.

Figure 4. (A) Tandem mass spectrum of the +31 charge state of VIM-1 derived from *Citrobacter fruendii*. Inset shows diagnostic fragment ions specific to VIM-1 in the presence of overlapping contaminants from mass isolation (B) Extracted ion profile of the two mature forms of VIM-1 found in *Citrobacter fruendii* separated via LC-MS/MS

Figure 5. (A) LC-MS/MS gradient conditions, MS resolution settings, and target protein monitoring segments for detection of metallo-β-lactamases and other carbapenemases (B) Extracted ion profile of specific diagnostic ions of VIM-1 from a five minute LC-MS/MS run.





The truncated form of VIM-1 was found to be in a ratio of 3:1 in Enterobacteriaceae, while no truncated forms of the VIM variant were observed in Pseudomonadaceae and Acetobacteraceae. The rationale for this again can be explained by the differences in the binding site of signal peptidase I across these bacterial families and the differences in the signal peptide sequences of the VIM variants.

All the aforementioned metallo-β-lactamases can be analyzed in a five minute LC-MS/MS run as illustrated in **Figure 5A**. In addition to these resistance proteins, two other major classes of carbapenemases (KPC and OXA-40 Like) can be analyzed in this time frame as well. The method is typically divided into three segments including a full scan quality control check in segment 1 (which can be performed at any time position in the LC run provided no carbapenemases are eluting), the analysis of VIM and IMP in segment 2, and the detection of NDM in segment 3. In **Figure 5B** is shown the elution of the truncated form of VIM-1 at the two minute mark using a modified form of the method from **Figure 5A**. The insets represent some of the diagnostic fragments present for the confirmation of VIM-1 from any given microbial pathogen sample. For this dataset, the full scan spectral quality check was performed between the three and four minute mark.

CONCLUSIONS

- Individual protein variants of metallo-β-lactamases can be readily identified from any Gram-negative pathogen on a five minute timescale. This information can be readily utilized for epidemiological considerations.
- The lipidation of NDM varies between bacterial families, while the mature forms of IMP and VIM are dependent on the signal peptide sequence and nature of the signal peptidase I enzyme.
- This information on rapid intact protein resistance analysis can be used to enhance diagnostic methods and advance our ability to monitor and contain antibiotic-resistant bacteria.

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TRADEMARKS/LICENSING

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