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MALDI MS profiling of serum extracts to identify Galactomannan Enzyme Immunoassay False Positivity via Immunoglobulin Products

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Introduction

- The clinical application of MALDI MS is growing as a result of it's ease of use, low cost per test and ability to identify biomarkers from body fluids which correlate with disease. We report a clinical scenario where MALDI MS profiling is used to investigate the origin of false positive Galactomannan enzyme immunoassay (EIA) results in a clinical laboratory.
- Therapeutic immunoglobulins (IVIG) are used as replacement or immunomodulatory therapy, but can transmit clinically important molecules (Ramsey *et al*., 2016). Indications for their use include replacement therapy in antibody-deficiency syndromes.
- Patients in these groups are at increased risk of invasive fungal disease (IFD) with *Aspergillus fumigatus*.
- Galactomannan antigen (GM) is a component of the cell wall of *Aspergillus fumigatus* and several other clinically important fungi. The Galactomannan enzyme immunoassay (GM-EIA) is used largely as a screening test for early detection of invasive aspergillosis in at-risk patients. Several immunodominant glycoproteins in *Aspergillus fumigatus* have been identified previously via immunoblot, but with the use of *Aspergillus fumigatus* anti-cell wall antibody (Haynes *et al*., 1990). These glycoprotein masses are described in Table 1.
- Some patients were found to become positive for the GM-EIA screening test following IVIG administration, although aspergillus culture, pan-fungal and aspergillus specific PCR were negative. The testing of the IVIG vials produced a similar pattern.
- A false-positive GM-EIA result can be interpreted as an early biomarker of invasive aspergillosis or other invasive fungal infection leading to further investigation or treatment.
- Here, the development of a MALDI-based assay to confirm a false-positive GM-EIA result was investigated.

Table 1: *Aspergillus fumigatus* immunodominant biomarkers MW recognised by anti-cell wall and EB-A antibodies

Anti-cell wall*	Anti-GM (EB-A)
11 KDa (major)	21 KDa (weak)
13 KDa (intermediate)	>46 KDa (diffuse)
14 KDa (intermediate)	
18 KDa (major)	
29 KDa (intermediate)	
38 KDa (minor)	
44 KDa (minor)	

*Major/intermediate/minor based on incidence in urine samples of patients with invasive aspergillosis

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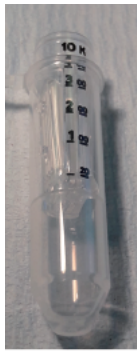
Methods and Materials

Simulated serum samples (26) were obtained which were positive for the GM-EIA assay (Platelia Aspergillus Ag, Bio-rad). The samples chosen had different strengths of GM scores (GMI) to represent a wide variation across a biological spectrum (GMI range 0.396-3.5). There were 3 negative (GMI range 0.089-0.214) and 3 positive (GMI range 2.688-4.278) patient serum samples. There was also the positive, cut-off and negative serum controls for the GM-EIA assay kit (GMI scores of 1.96, 0.923, 0.127 respectively). Commercial IVIG vials with known positive result were also obtained.

Sample preparation was optimised by comparing clean up on a novel MALDI target plate (Tethis SpA) designed to increase analyte retention with traditional approaches including C18, C4 ZipTip SPE, ultra-centrifugation (5/10 kDa MWCO), Dowex cation exchange, drop dialysis (with UHQ water), dilution into sinapinic acid MALDI matrix or 0.1% (aq.)TFA and carbon SPE extraction.

MALDI-MS profiling was performed using two linear MALDI-TOF mass spectrometers: AXIMA Performance™ (Shimadzu, Manchester, UK) and a new benchtop model (MALDI-8020™ (Shimadzu, Manchester, UK) to demonstrate robustness. The MALDI matrix used was sinapinic acid (20 mg/ml in 1:1 ACN/0.1% TFA). 1 uL of sample was spotted (in quadruplicate) with 1 uL matrix, using a modified pre-coat seed layer. BSA (5 pmol on target) was used as calibrant. Data was acquired over the range m/z 10,000-100,000 (up to 180,000 with IVIG samples) in positive ion linear mode, with pulsed extraction at 20,000Da. Further analysis was done using multivariate analysis software (Mass++DA, Shimadzu Japan).

1 elpmas Lu 0
+ 450 uL 0.1% TFA



Ultracentrifugation
10,000 rpm x 10 mins

Spot on target plate

MALDI-8020

Figure 1: Workflow for sample preparation

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Results

Sample preparation method development

- Sample preparation using the novel MALDI target resulted in significantly improved signal to noise and peak resolution compared with other typically used sample preparation methods.
- However, higher mass peaks (>28 KDa) were not observed – probably due to losses in the wash.
- Ultrafiltration using a 10 kDa MWCO device (Vivaspin™ or Amicon™), Figure 1, consistently produced signals for most *Aspergillus fumigatus* immunodominant biomarkers, particularly those for the cell wall.

Detection of *Aspergillus fumigatus* biomarkers

Despite the heterogeneous serum sample matrix, each of the GM-EIA assay-positive samples consistently resulted in detection of peaks for *Aspergillus fumigatus* cell wall glycoproteins (11, 13, 14, 18, 29, 38, 44 kDa).
Peaks for glycoproteins associated with *Aspergillus fumigatus* GM (21 kDa, diffuse peak >46 kDa) were less abundant and tentative. The 21 kDa peak is known to be weak (from previous from immunoblot studies) and the ambiguity of defining accurately a mass >46 kDa.
Despite this, the consistency of the cell wall antigen biomarkers demonstrate the diagnostic value for aspergillosis.
Regardless of the ranges in the GMI scores, there was no corresponding linear response on the signal to noise, which is unsurprising since MALDI is essentially qualitative rather than quantitative. The potential application of MALDI-MS would be in confirming the absence / presence of immunodominant biomarkers of *Aspergillus spp*. in the context of a suspected biological false positive GM-EIA result. Furthermore, MALDI-MS does not offer a cut-off score but rather a binary result indicating presence or absence of a series of immunodominant biomarkers of *Aspergillus spp*. Importantly, it is not subject to cross-reactivity errors which are inherent in immunoassays. A sample spectrum is shown in Figure 2.

Confirmation of false positive GM-EIA *Aspergillus fumigatus* result

Each of the IVIG drugs which were found to induce a positive GM-EIA result in patient sera and which themselves were positive for the immunoassay were negative for the MALDI-MS analysis – none of the *Aspergillus fumigatus* biomarkers (either for the cell wall or GM related antigens) were identified.
The peaks detected were related to the IVIG antibody, which, after removal (with a 100 kDa MWCO spin filter and analysis of the filtrate) produced a blank profile. This was done to assess if there were low abundance *Aspergillus fumigatus* biomarkers present. The MALDI-MS negative result for *Aspergillus fumigatus* indicates the positive immunoassay result is likely related to presence of an epitope on the IVIG antibody.

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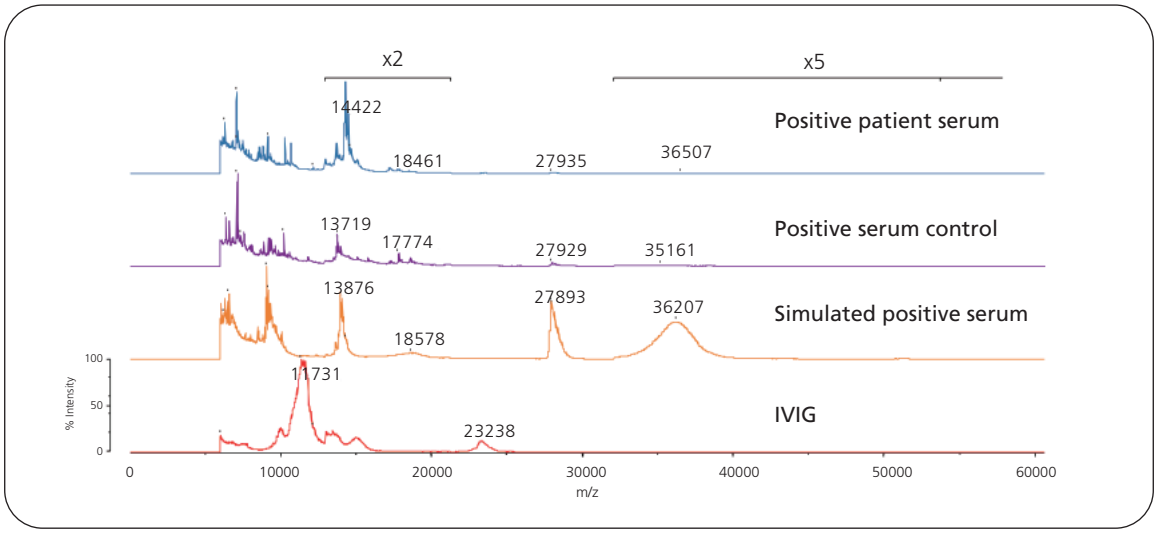

Figure 2: Example mass spectra of positive sera samples for *Aspergillus fumigatus* and false positive IVIG drug. Antibody peak in IVIG drug sample not shown

Statistical Analysis

The mass lists from the spectra were imported into a developmental statistical analysis software (Mass++DA™; Shimadzu, Japan). The most unique peaks for the positive *Aspergillus fumigatus* samples are shown in Table 2 and Figure 3. A comparison of the positive samples and positive controls with the IVIG drugs is shown in Figure 4.

Table 2: Peak matrix table for positive samples only

m/z	Positive AF (51)
13919	51
27836	50
10015	46
28028	43
14012	39
14075	36
10349	29
14263	25
36207	20
36000	15
10125	14
10214	14
10433	12
13606	12

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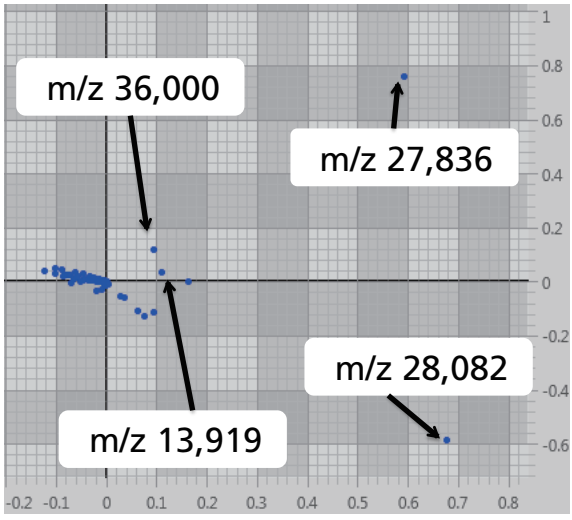

Figure 3: Loading plot (PCA) of positive sera and positive control samples

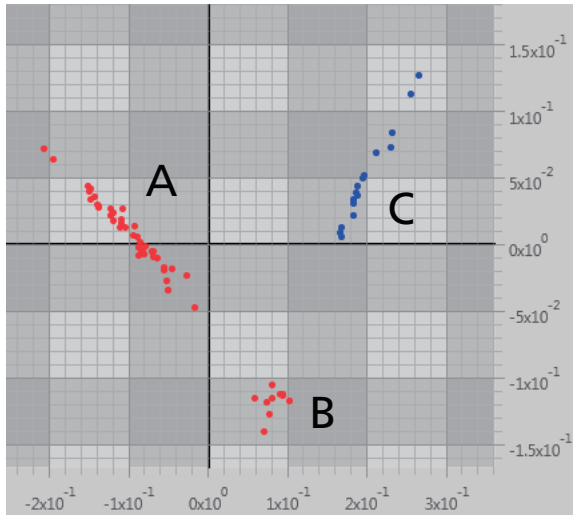

Figure 4: Score plot (PLS-DA) of IVIGs drugs (blue) and positive sera and positive control samples (red).
A: Simulated positive sera; B: Positive controls and positive patient sera; C: IVIGs

Conclusions

- A rapid sample preparation (10 min), MALDI-MS-based application for the interpretation of biomarkers of invasive fungal disease in immunocompromised patients receiving IVIG has been putatively demonstrated.
- This work has important clinical relevance and demonstrates how MALDI-MS may aid the clinical management of suspected false positive *Aspergillus fumigatus* EIA-GM results.

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