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Highlights

- Mass spectrometry analysis offers new diagnosis avenues for fungal diseases
- Fungal trehalose is a relevant target for the mass spectrometry-based diagnosis MS-DS
- High resolution mass spectrometry improves the diagnostic value of MS-DS

Abstract

The rapidity of the diagnosis of invasive candidiasis (IC) is crucial to allow the early introduction of antifungal therapy that dramatically increases the survival rate of patients. Early diagnosis is unfortunately often delayed because *Candida* blood culture, the gold standard diagnostic test, is positive in only 50% of cases of IC and takes several days to obtain this result. Complementary non-culture-based methods relying on the detection of *Candida* cell wall polysaccharides in the serum, β-glucans and mannans, by enzymatic and immunological reagents have been successfully developed to allow a more efficient patients care. We have previously demonstrated that detection of circulating glycans by mass spectrometry could provide a reliable and cost-effective early diagnosis method called MS-DS for Mass Spectrometry of Di-Saccharide. Here, by comparing patient's sera and *Candida albicans* strains deficient in carbohydrates synthesis, we demonstrate that trehalose derived from fungal metabolism can be specifically targeted by MS-DS to allow early diagnosis. In particular, the use of *C. albicans* strains deficient in the synthesis of trehalose synthesizing enzymes Tsp1 and Tsp2 show that MS-DS results were correlated to the metabolism of trehalose. Finally, we demonstrate that the performance of the IC diagnosis can be significantly improved by using high resolution mass spectrometry, which opens new perspectives in the management of the disease.

Keywords

Diagnosis, Mass Spectrometry, Candida albicans, Fungal infection, Trehalose, Serum

1. Introduction

Invasive candidiasis (IC) are major life-threatening nosocomial invasive fungal infections with a low survival rate [1,2]. Despite constant progress made in antifungal therapy, the high rates of morbidity and mortality associated with IC were only marginally reduced over the last few years mostly because of delayed diagnosis [3]. Indeed, whereas the rapidity and reliability of the diagnosis of IC is of paramount importance to allow the early introduction of antifungal therapy to improve patient survival rate, blood culture, the gold standard diagnostic test, requires several days to be positive, which occurs only in 50% of cases of IC [4]. Biomarkers based diagnostic methods rely on the detection of *Candida*-derived molecules in the sera of patients, including fragments of cell wall polysaccharides β-glucans (BDG) and mannans (Mnn). They are clinically recommended as useful adjuncts to blood cultures [5,6]. However, they also represent an increasing economic burden for hospitals considering the increasing prevalence of Invasive Fungal Infections (IFI) in intensive care units.

In order to provide improved methods for early detection of IC, we have previously demonstrated that application of mass spectrometry for detection of fungal molecules circulating in patients' sera could be a reliable alternative to biochemical and immunological detection methods currently used for IC diagnosis [7]. Application of such a diagnostic method takes advantage of the massive introduction in clinical mycology laboratories of matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (MS) equipment allowing rapid and accurate identification of hospital fungal isolates [8]. We have shown that mass spectrometry analysis of free oligosaccharides purified from the sera of IC patients reveals the presence of a prominent signal at *m/z* 365, tentatively attributed to $[M+Na]^+$ adduct of Hex_2. This disaccharide was shown to efficiently and early diagnose, among hospitalized at risk patients, those presenting life threatening IC [7,9,10]. The attribution of the diagnostic signal as a di-hexoside was further consolidated by the observation in mass spectrometry of a specific signal at *m/z* 477 in sera from IC patients, following permethylation of total purified oligosaccharide [7]. Quantification of the signal was performed by establishing the "MS-DS index," defined as the ratio of *m/z* 365 over *m/z* 361 matrix signal intensities (percent *m/z* 365 versus m/z 361). This new biomarker was designated as MS-DS (mass spectrometry-disaccharide) with reference to its nature and the technology leading to its identification. Mono- and multi-center clinical trials not only demonstrated that MS-DS favourably compared to BDG and Mnn detection in terms of specificity/sensitivity for the diagnosis of IC, but also that it could be used with even higher performances for the diagnosis of invasive aspergillosis and mucormycosis [9,10]. In the present report, using a combination of gas-chromatography coupled to mass spectrometry (GC/MS), mass spectrometry and *C. albicans* strains deleted for genes encoding carbohydrates synthesizing enzymes, we have demonstrated that MS-DS targets the trehalose. This molecule is generated by *C. albicans* metabolism during stress

conditions and infection process. Furthermore, we have shown that the performance of MS-DS could be improved by using high resolution mass spectrometry.

2. Materials and Methods

2.1. Culture of *C. albicans*

C. albicans wild-type (WT) strain SC5314, *tps1*Δ and *tps2*Δ strains were grown in 20 mL of Sabouraud liquid medium at 37°C overnight [11,12]. *C. albicans* WT cells were washed twice in PBS after centrifugation at 2200 x g for 5 min. and suspended at 10^8 cells/mL, either in 1 mL control serum or in 0,5 mL RPMI, with or without NaCl 1M, H_2O_2 0.5 mM, NO 7.5 mM or in Tris-HCl at pH2 depending on the applied stress conditions. To reveal the trehalose release from *C. albicans* in trehalose undetectable serum from healthy subject, *C. albicans* WT cells were incubated four hours in serum at 37°C. These *C. albicans* cells were centrifuged at 2200xg for 5 min and 200 μL of supernatant were sampled for MS-DS assay. In order to evaluate the influence of stress conditions on MS-DS index, *C. albicans* WT, *tps1*Δ and *tps2*Δ cells were incubated in RPMI and Tris-HCl either at 37°C or at 60°C for 1 hour, the fungal cells were then centrifuged at 2200 x g for 5 min and the supernatant was collected for MS-DS assay.

2.2. Measurement of MS-DS index

Before MS-DS assay, sera were subjected to EDTA treatment as previously described whereas *in vitro* culture supernatants in RPMI and Tris-HCl were directly subjected to MS-DS assay [13]. The same purification procedure was then applied for both serum and culture supernatants, as previously described [7]. Briefly, 100 µL of supernatant was applied to a mixed-bed chromatography column of activated charcoal-celite (50:50 by weight) and RP medium (Sepra ZT 30 µm, 85 A; Phenomenex, Torrance, CA, USA). The column was conditioned by washing with ethanol/ water (20:80, volume/volume) and equilibrated with water. After loading the sample, the column was washed with 4 mL of water and the oligosaccharide fraction was eluted with 600 µL of 5% ethanol. The solution was dried down under vacuum and resuspended in 40 µL water. One μL of solution was then spotted onto the MALDI plate followed by 1 µL of ionic liquid matrix (2,5-dihydrobenzoic acid-pyridine, DHB-Py). The sample spots were dried by heating at 50°C for 2 min [7]. Samples were analyzed using either a 4800 MALDI-TOF/TOF analyzer (Applied Biosystems/MDS Sciex) for low resolution acquisition or a MALDI-LTQ-Orbitrap XL (Thermo Fisher Scientific, Waltham, Massachusetts, USA) for high resolution at a fixed laser intensity and 1000 accumulated shots/spectrum within an *m/z* 300 to 800 range. Following acquisition of MS spectra for individual samples, the area for the signals corresponding to trehalose (*m/z* at 365.1 for MALDI TOF/TOF analyser and at 365.12 for MALDI-LTQ-Orbitrap XL) and to ubiquitous matrix signal trehalose (*m/z* at 361.1 for MALDI TOF/TOF analyser and at 361.17 for MALDI-LTQ-Orbitrap

XL) were established, then the MS-DS indexes were computed as a percentage of the first compared to the second.

2.3. Measurement of mannan and glucan polysaccharides/oligosaccharides in sera

The BDG levels were determined by using the Fungitell kit (Associates of Cape Cod Inc., Falmouth, MA, USA) according to the manufacturer's instructions. The recommended cut-off of 80 pg/ml was used to determine clinical relevance. Measurement of serum Mnn levels was performed by using the Platelia *Candida* Ag Plus test (Bio-Rad, Marnes la Coquette, France) according to the manufacturer's instructions. The recommended cut-off was used to determine clinical relevance. For both tests, serum samples with positive results of >500pg/ml were diluted and tested again.

2.4. GC/MS analysis

The samples were permethylated before their analysis in GC-MS, according to the method of Ciucanu and Kerek [14]. Briefly, the dried samples were taken up in a DMSO/NaOH/ICH_3 mixture and incubated for 2 h in an ultrasonic bath. The reaction was stopped by adding water and the permethylated compounds were extracted with $CHCl_3$ and washed seven times with water. The permethylated samples were finally dissolved in acetonitrile.

The purified permethylated compounds were injected in GC-MS (Thermo Fisher Scientific, Waltham, Massachusetts, USA) on a 30 m x 0.25 mm SOLGEL-1MS column (SGE Analytical Science, Melbourne, Australia) via a Ross injector. The column was coupled to a Finnigan Automass II mass spectrometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) with a mass limit of *m/z* 1000. The mass analyses were carried out in Electronic Impact (EI) mode with an ionization energy of 70 eV and a source temperature of 150° C.

2.5. Ethics statement.

All sera used in this study were obtained from patients monitored at Lille University Hospital. When no results were available from routine tests, BDG and Mnn levels were determined retrospectively from residual frozen samples. No additional sampling was necessary. As sera were taken from a registered biological collection, patient consent was not required according to the French law. Agreement for the establishment of a biological collection of IFI samples was obtained from the French Ministry of Education and Research under reference DC- 2008-642. Institutional review board approval was given by the the ethical committee (Comité de Protection des Personnes Nord-Ouest IV).

2.6. Statistical analysis

The Mann-Whitney two-tailed test was used to compare the distributions of biomarkers in the different groups, and the nonparametric correlation test (Spearman's rank test) was used to analyze the correlation between them. GraphPad Prism 6 was used to generate receiver operating characteristic (ROC) curves and derived cut-offs and graphs. A p value <0.05 was considered to be statistically significant.

3. Results and Discussion

3.1. Mass spectrometry detects trehalose released by *C. albicans* in patient's serum

To date, serum biomarkers used for the diagnosis of IC consist in oligosaccharides resulting from catabolism of fungal cell wall polysaccharides. These are detected either by antigenic or biochemical tests when derived from the mannans using Platelia assay (Bio-Rad, Marnes la Coquette, France) or the β-glucans using Fungitell assay (Associates of Cape Cod Inc., Falmouth, MA, USA)), respectively. Considering the diversity of cell wall polysaccharides (CWP) and their quantitative importance in fungal cells, one expects that MS-DS may as well detect these circulating metabolites. Among these, Man_2 derived from the tetramannanoside epitope of the Platelia test [15–17] or Glc_2 from the pentaglucoside triggering the Limulus biochemical pathway [18] would be appropriate candidates resulting from additional degradation by endogenous or host glycosidases. Initial analysis of monosaccharide composition by GC/MS of oligosaccharide fractions purified from IC patients only shows the presence of glucose, which suggests that Hex_2 is actually a di-glucoside (data not shown). However, the presence of incompletely removed blood glucose could also account for a high glucose content, so monosaccharide composition does not provide unambiguous results.

No less than nine natural isomeric diglucosides exist in nature, including eight reducing diglucosides, kojibiose (Glcα1-2Glc), nigerose (Glcα1-3Glc), maltose (Glcα1-4Glc), isomaltose (Glcα1-6Glc), sophorose (Glcβ1-2Glc), laminaribiose (Glcβ1-3Glc), cellobiose (Glcβ1-4Glc), gentibiose (Glcβ1-6Glc), and one none-reducing trehalose (Glcα1-1αGlc), precluding the identification of the potential disaccharide only based on hexose composition and mass spectrometry screening. Among those diglucosides, several ones may be derived from CWP such as β-glucans, considered previously or from α-glucans that has been recently described in *C. albicans* [19]. In addition, dozens of dihexosides containing other monosaccharides are very common in nature including lactose (Galβ1-4Glc), sucrose (Glcα1-2βFru) and di-mannosides that may also originate from the cell-wall mannans. In order to identify the disaccharide isomer observed in sera from IC patients, we first analyzed by GC/MS a library of common disaccharides to establish their chromatographic and spectroscopic parameters. Authentic standards were permethylated and their retention times established in standard GC/MS conditions. As shown in Figure 1A, reducing disaccharides exhibit two retention times due to the α–β mutarotation of their reducing monosaccharides whereas non-reducing disaccharides (trehalose and sucrose) show only one. Examples of GC-MS chromatograms and EI-MS spectra of trehalose and maltose are shown in Figures 1B and 1C, respectively. Sera from control individuals and IC patients were treated according to standard oligosaccharide procedure and permethylated before analysis by GC/MS. Prior to GC/MS

analysis, all sera were tested for BDG, Mnn and MS-DS index. These data confirmed that these five patients presented MS-DS discriminatory levels for IC when compared to control subjects (Table 1). The five IC patients showed positive results for BDG and had high MS-DS indexes between 1000 and 2500. Only two out of five were positive for Mnn, in accordance with the known low sensitivity of this antigenic test that is compensated by a very high specificity [20]. GC/MS chromatograms of all sera from IC patients showed a strong signal around 10.60 min (Fig. 1D) suggesting the presence of trehalose. These data were confirmed by co-injection of samples with trehalose authentic standard (data not shown). Electronic impact (EI) pattern of this signal (Fig. 1D) shows a set of hexose-containing fragments. In particular, A-series ions at *m/z* 155, 187 and 219 typify the presence of a released permethylated hexose generated by the cleavage of inter-glycosidic bond whereas ions at *m/z* 275 and 307 strongly suggests the presence of a permethylated Hex(1-1)Hex disaccharide. These chromatographic and mass-spectrometric behaviors are identical to those observed for an authentic permethylated trehalose standard (Fig. 1B), establishing the nature of the observed di-saccharide as trehalose. By comparison, other di-glucosides presented different patterns. In particular, EI-MS spectrum of permethylated maltose did showed the major fragment ions at m/z 219, 187 and 155 typifying the release of glucose but intense signals at J1 and F1 fragments at *m/z* 279 (pMeHex-O-CH=CH_2) and 305 (pMeHex-O=CH_2-CH_2=CH_2-O-CH_3) that specifically typify (1-4) linkages (Fig. 1C). It is noteworthy that some of the sera also contained minor proportions of maltose as characterized by the presence of two signals around 11.60 and 11.70 min. and EI-MS profile. Further screening of patient sera showed that maltose was seldom identified along trehalose. On the contrary, GC/MS analysis of control serum never showed the presence of permethylated disaccharides, in agreement of a negative MS-DS index.

A

Disaccharide standards	Structures	Retention times (min.)
Maltose	Glcα1-4Glc	11.58 – 11.78
Isomaltose	Glcα1-6Glc	11.56 – 11.79
Laminaribiose	Glcβ1-3Glc	11.12 – 11.35
Lactose	Galβ1-4Glc	11.35 – 11.52
Di-mannoside	Manα1-3Man	11.23 – 11.38
Trehalose	Glcα1-1αGlc	10.60
Sucrose	Glcα1-1βFru	10.03

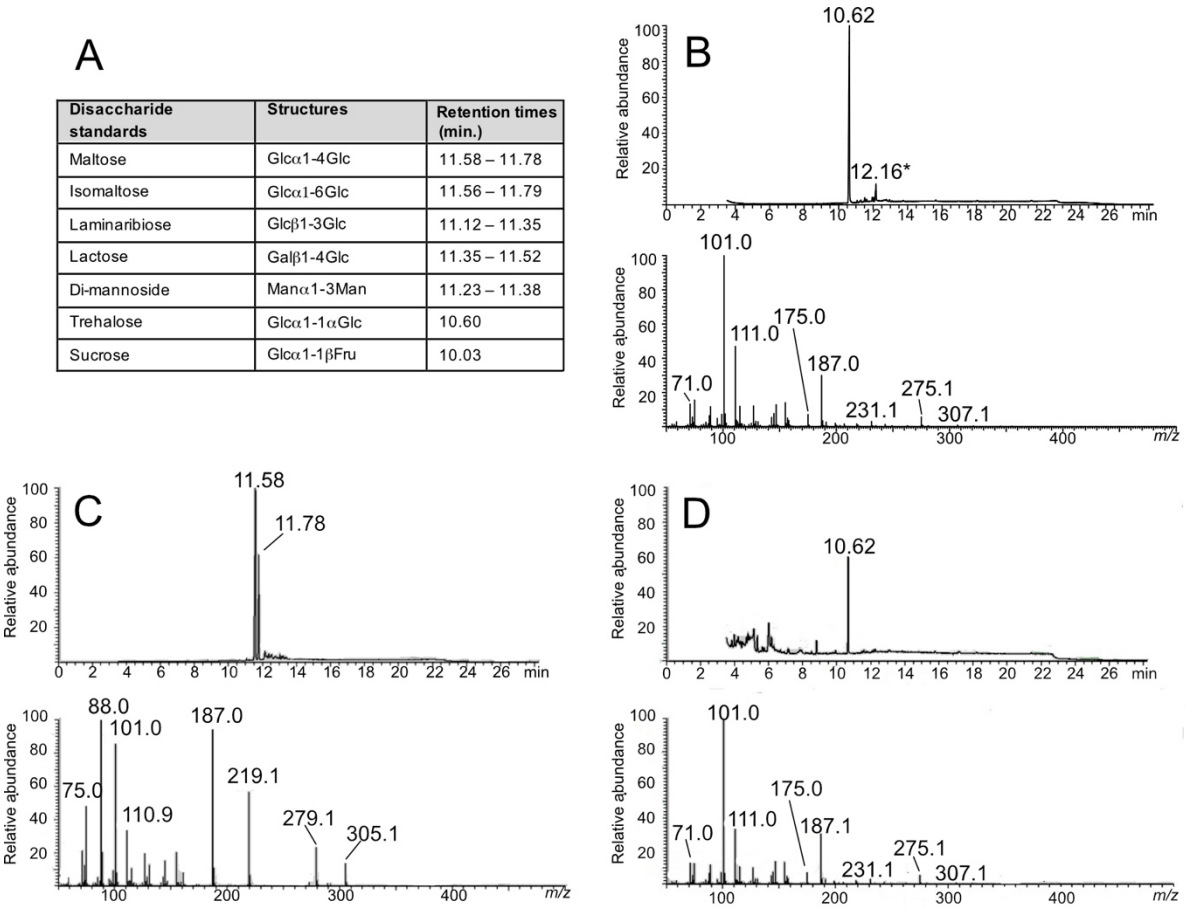


Fig. 1. Identification of circulating disaccharide. (**A**) Structure of disaccharide standards analyzed by GC-MS and retention times of their permethylated derivatives. GC/MS chromatograms (top panels) and EI-MS spectra of analysis (bottom panels) of (**B**) permethylated trehalose standard (**C**) permethylated maltose standard and (**D**) permethylated oligosaccharide fraction purified from the serum of IC patient 2 (Table 1). Similar results were obtained for the other four patients. * contaminant in trehalose standard.

Patient n°	Species identified	Delay vs BC (days)	Fungitell® (pg/mL)	Platelia plus® Ag (pg/ml)	MS-DS
IC patients from ICU					
Patient 1	*C. albicans*	0	81	0	2000
Patient 2	*C. albicans*	3	1328	1859	1000
Patient 3	*C. albicans*	4	323	0	1000
Patient 4	*C. glabrata*	0	1928	214	1000
Patient 5	*C. tropicalis*	7	446	0	1500
Healthy blood donors					
Control 1	/	/	/	/	15
Control 2	/	/	/	/	15
Control 3	/	/	/	/	18

Table 1. Summary data of the different purified sera from the cohort selected for the characterization of the disaccharide. BC, time of first positive blood culture. ICU, Intensive care unit. MS-DS indexes were acquired on MALDI-TOF MS.

Altogether, structural analysis established the presence of trehalose as a major disaccharide component in the serum of IC patients. These data disagree with the initial assumption that diagnostic disaccharide used in MS-DS methodology was originating from the degradation of CWP, either mannan or β-glucan. Trehalose is known to be synthesized by numerous yeast species including *C. albicans*. It serves not only as a storage carbon source but also as a protectant against oxidative and temperature stresses [21–23]. Of particular importance, oxidative stress and high temperature generate an important build-up of intracellular trehalose in *C. albicans* [23–25]. It could also be partially released to the outside by trehalose transporter(s) through a yet uncharacterized mechanism. Thus, it comes as no surprise that the release by *C. albicans* of trehalose in the blood stream during infection in stressful conditions may be used for diagnosis purpose.

3.2. MS-DS effectively follows trehalose release *in vitro*

In order to further analyze the release mechanisms of trehalose from *C. albicans*, we evaluated if the trehalose production *in vitro* could be detected by MS-DS. To do that, *C. albicans* was cultured in Sabouraud medium, washed in PBS and 10^8 *C. albicans* cells per mL were transferred to a β-glucan-free serum collected from a healthy subject. After incubation, *C. albicans* cells were pelleted down and the MS-DS index was established from the serum supernatant according to standard protocol. Prior to cell transfer the serum was showed to be negative for MS-DS (Table 1). MS-DS index of *C. albicans* suspension in three different sera were established immediately after transfer, then every hour. Transfer of *C. albicans* into serum immediately triggered a strong response, followed by a steady decline of MS-DS index (Fig. 2). Permethylation and analysis of corresponding supernatant by GC/MS demonstrated the presence of trehalose in all three serum samples at T0 (data not shown). No other disaccharide was identified, confirming that *C. albicans* induces trehalose release in serum *in vitro*. Furthermore, the steady reduction of trehalose level observed through MS-DS index is in agreement with literature as it is believed that the reduction of serum trehalose level displays the ability of *C. albicans* to respond to its microenvironment stress that contributes to its success as a pathogen and its resistance against stress conditions. This trehalose level is gradually decreased during 4 hours and is likely related to trehalose-hydrolyzing enzymes such as acid trehalase (Atc1) that promotes the use of *C. albicans* trehalose as a carbon source [26,27]. The acid trehalase is linked to the external cell wall in *C. albicans* and involved in the hydrolysis of trehalose. In line with these observations, Pedreño *et al.* showed that *C. albicans* atc1 mutant strain that lacks acid trehalase activity could not grow on trehalose as sole carbon source [27]. Altogether, these data demonstrate that MS-DS can be used to follow the release of trehalose *in vitro*, similarly to *in vivo* conditions.

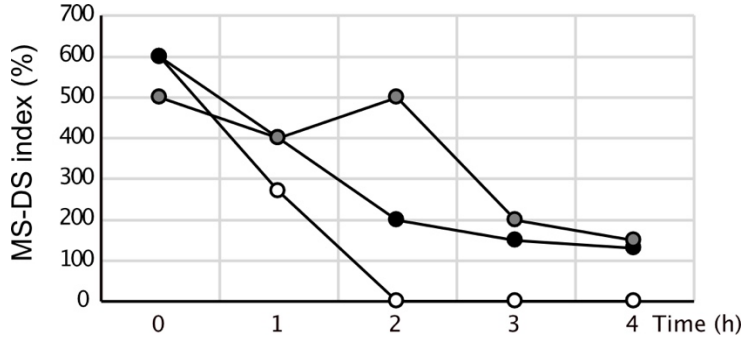


Fig. 2. Induction of MS-DS response in serum. *C. albicans* were washed in PBS, transferred in serum (10^8 cells/mL). After incubation at 37°C at different time points, cells were pelleted down and MS-DS index measured in supernatants. Black, gray and white circles show results from three independent experiments in three different control sera from healthy blood donors.

3.3. MS-DS index is associated to the trehalose metabolism in yeast

Trehalose is biosynthesized intracellularly in *C. albicans* cytoplasm. Trehalose biosynthetic pathway is directly connected to the glycolytic pathway under the dependence of two main enzymes: trehalose-6-phosphate synthase (Tps1) and trehalose-6-phosphate phosphatase (Tps2). First Tps1 converts uridine diphosphate (UDP)-glucose and glucose-6-phosphate into trehalose-6-phosphate (T6P) and UDP. Then Tps2 hydrolyzes T6P into trehalose and free phosphate. Trehalose may in turn be cleaved back into glucose by two differentially regulated cell-wall associated and cytoplasmic trehalases [28] (Fig. 3A). Of particular interest, trehalose cannot be synthesized by vertebrates, making it a very specific and relevant target for the diagnosis of human infections as exemplified by the robustness of MS-DS assay to diagnose IFIs [9,10].

Furthermore, the synthesis of trehalose is considered to be involved in the virulence and pathogenesis of yeast because of their functions in resistance to physical and biological stress conditions and biofilm formation [29–32]. As such it is known to be accumulated into cells through induction of its biosynthetic pathway that includes Tps1 and Tps2 under a number of external stress such as temperature and oxidative stresses [23,25,33,34].

In order to confirm that the observation of disaccharide signal in IC serum is related to trehalose biosynthetic pathway, we have evaluated the *in vitro* MS-DS index under different stress conditions using *C. albicans* strains deficient in the trehalose biosynthetic enzymes Tps1 or Tps2. Indeed, it was previously demonstrated that inactivation of such enzymes considerably reduced the capacity of *C. albicans* to synthetize trehalose [31,35]. In order to evaluate the impact of trehalose synthesis on MS-DS index, *C. albicans* cells were grown in Sabouraud culture medium, washed in PBS and transferred in RPMI (10^7 cells/mL) before being subjected to various stresses for one hour: temperature (60°C), osmotic shock (NaCl 1M), low pH (pH2), periodic oxidation (H_2O_2 0.5 mM) and nitric oxide stress (NO 7.5 mM). Then cells were pelleted

down and the MS-DS index was established from the culture medium supernatant according to standard protocol. It is noteworthy that we used lower cell density in order to be able to standardize the procedure with TPS deficient strains that grow slower than WT. As shown in Figure 3B, the transfer of cells from Sabouraud to RPMI (CTL) did not induce a background MS-DS signal in none of the strains studied, establishing that RPMI is an appropriate incubation medium to determine the trehalose release. In contrast, for *C. albicans* WT strain, temperature, oxidation and nitric oxide stresses triggered significant increase of MS-DS index, contrarily to osmotic shock and low pH that did not. Again, analysis by GC/MS of the positive supernatant confirmed that the increase of MS-DS index was directly correlated to the appearance of trehalose in the culture medium (data not shown).

*C. albicans tps1*Δ strain did not respond to any of the temperature, oxidation and nitric oxide stresses registered for WT strain (Fig. 3B), demonstrating that deficiency in trehalose-6-phosphate synthase abrogates the cellular excretion of trehalose under stress condition. By contrast, *C. albicans* deficient in TPS2 strain showed a similar pattern of response than *C. albicans* WT strain toward temperature, oxidation and nitric stresses, albeit lower MS-DS index values. This maintenance of the trehalose release in *C. albicans tps2*Δ strain under stressful conditions is consistent with the trehalose-6-phosphate phosphatase unspecific dephosphorylation pathway previously suggested in *C. albicans* [33]. These experiments establish both that MS-DS response is associated with trehalose metabolism and that trehalose is the target metabolite of MS-DS.

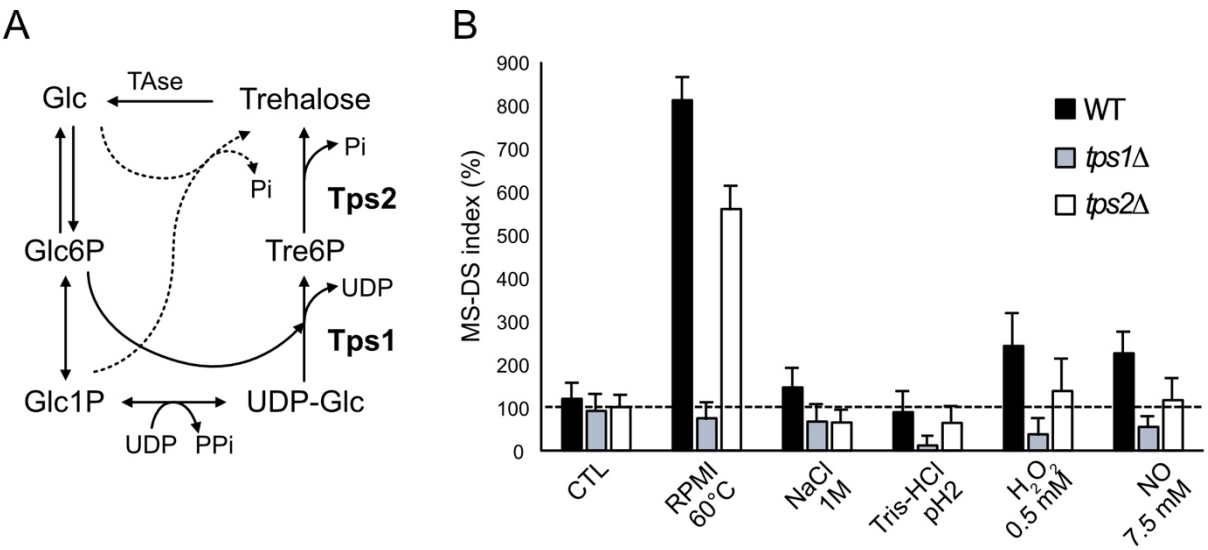


Fig 3. MS-DS response is associated to trehalose metabolism. (**A**) In plain line is represented the canonical biosynthetic pathway of trehalose in yeasts under the dependence of trehalose-6-phosphate synthase (Tps1) and trehalose-6-phosphate phosphatase (Tps2). In dotted line is shown an alternative

synthesis of trehalose involving a trehalose phosphorylase that has not been yet identified in *Candida species*.[34] Trehalose can be hydrolyzed back to Glc by trehalase (TAse) activity. (**B**) WT (in black), Tps1 deficient (in gray) and Tps2 deficient (in white) strains of *C. albicans* were incubated for 1 hour in RPMI at 37°C and subjected or not (CTL) to different stresses as indicated. MS-DS indexes were established from culture supernatants after one hour incubation in independent triplicate for each experiment.

3.4. MS-DS performance is improved by high resolution mass spectrometry

The identification of molecular mechanisms underlying MS-DS response in IC paves the way toward improving the robustness of the diagnostic method. One way of improvement is to evaluate to what extent trehalose contributes to the signal observed at *m/z* at 365 in MALDI-TOF MS that is used to calculate MS-DS and to exclude other potentially non-relevant contributing signals. Indeed, resolution of MALDI-TOF MS technology, especially expected with linear spectrometers routinely used for clinical applications, is not sufficient to discriminate signals within 1 mass unit. In contrast, MALDI-LTQ MS can generally provide a ten times higher resolution.

In order to evaluate if high resolution mass spectrometry may contribute to improve the robustness of the diagnosis method, we first identified trehalose signal in MALDI-LTQ MS. Based on its elemental composition $C_{12}H_{22}O_{11}Na$, trehalose adduct has a calculated average mass of 365.2868 m.u. and a monoisotopic mass of most abundant signal (85.35%) at 365.1060 m.u. The comparative analysis of trehalose standard shows a low resolution $[M+Na]^+$ signals at *m/z* 365.1 in MALDI-TOF MS and a high resolution signal at m/z 365.12 in MALDI-LTQ MS, well in line with the calculated theoretical *m/z* value of the most abundant monoisotopic signal (Fig. 4A). Then, the presence of these two signals where evaluated in three serums isolated from (1) an IC patient, (2) a colonized non-IC patient and (3) a blood donor. As shown in Figure 4B, and in agreement with previous reports, serum from IC patient shows a very robust low-resolution signal at *m/z* 365.1 for MALDI-TOF MS and high-resolution signal at *m/z* 365.12 for MALDI-LTQ MS indicative of the presence of trehalose. As expected, the analysis of the serum from colonized non-IC patient by MALDI-TOF MS shows also signals at *m/z* 365.1 and 365.12 for MALDI-TOF MS and MALDI-LTQ MS respectively, but at much lower relative intensity (Fig. 4C). Surprisingly, MALDI-LTQ MS spectrum showed in the same sample the presence of two very close signals that could not be discriminated by MALDI-TOF MS analysis: one at *m/z* 365.12 attributed to trehalose based on analyses of standard and IC serum and another one at *m/z* 365.29 (Fig. 4C). Finally, analysis of blood donor serum only shows residual diagnostic signals using both methods, with MS-DS index below 10%, again in agreement with the expected results (Fig. S1). However, non-specific signal at *m/z* 365.29 could still be observed on MALDI-LTQ MS spectrum, as well on every spectra at different

relative intensities. Altogether, analyses by MALDI-LTQ MS showed that the diagnostic target signal at *m/z* 365.1 in MALDI-TOF MS is made up at least two molecules, the trehalose released by *Candida* in IC patients and a ubiquitous and yet unidentified molecule.

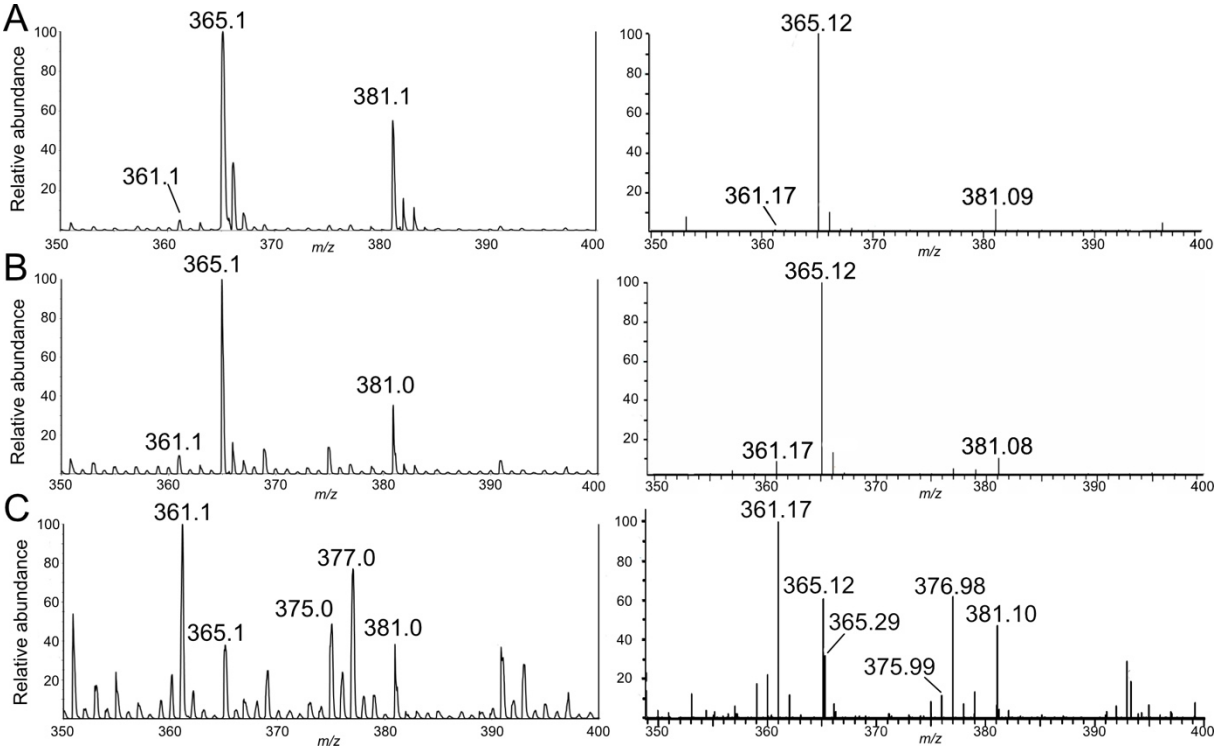
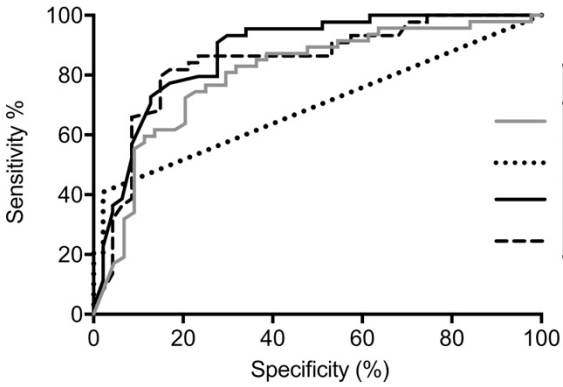


Fig. 4. High resolution MS distinguishes two compounds in the target signal at *m/z* 365. MALDI-TOF MS (left panels) and MALDI-LTQ MS (right panels) spectra of (**A**) trehalose standard, (**B**) purified serum of a IC patient, (**C**) purified serum of a non-IC patient colonized by *Candida*.

Considering that high resolution MS discriminates trehalose signal at *m/z* 365.12 from a non-specific signal at *m/z* 365.29, we have hypothesized that using only the former one may improve the overall diagnostic performance of MS-DS to identify IC patient by increasing the signal to noise ratio. To assess this assumption, we compared the performances of MS-DS index to discriminate IC patient on a cohort of 100 sera composed of 44 IC patients confirmed by blood culture (Table S1), 47 colonized non-IC patients (Table S2) and 9 healthy individuals (Table S3) using either low resolution signal at *m/z* 365.1 generated by MALDI-TOF MS, or high resolution trehalose signal at *m/z* 365.12 generated by MALDI-LTQ MS that excludes non-specific signal at *m/z* 365.29. The MS-DS index was established as the ratio of *m/z* 365.1 over *m/z* 361.1 matrix signal intensities for MALDI-TOF MS and as the ratio of m/z 365.12 over m/z 361.17 matrix signal intensities for MALDI-LTQ MS. The performances of the MS-DS index in the two instrumental configurations were also compared with those of BDG and Mnn detections.

Assessment of the diagnostic value of BDG detection, Mnn detection and MS-DS index was performed by comparing the IC and control groups. All the data were compiled and ROC curves were performed on each of the parameters taken into account for the 100 sera analyzed*, i.e.* BDG, Mnn and MS-DS index values of the two mass spectrometers MALDI-TOF MS and MALDI-LTQ MS. The cut-off of the MS-DS index MALDI-TOF MS was kept to 365%, but the one for MS-DS index using MALDI-LTQ MS was decreased from 325% to 255% to obtain a better sensibility/specificity trade-off (Fig. 5A). Data from Mnn and BDG were analyzed using the thresholds recommended by manufacturers. In terms of sensitivity/specificity ratios, BDG exhibited a high sensitivity, whereas Mnn showed an excellent specificity at the expense of a poor sensitivity, as previously observed (Fig. 5B). By quantifying the specific trehalose signal at *m/z* 365.12 on the MALDI-LTQ MS rather than the overall signal at *m/z* 365 on the MALDI-TOF MS, the sensitivity increased from 77% to 82% while the specificity remains the same at 83%. These data demonstrate that MS-DS performance can be significantly improved using higher resolution instruments when those will become widely available for clinical practices.

A



B

Test	Cutoff	Sensitivity (%)	Specificity (%)	AUC
BDG	80	80	62	0.8039
Mnn	62,5	38	100	0.6939
MS-DS TOF-MS	325	77	83	0.8767
MS-DS LTQ-MS	255	82	83	0.8436

Fig. 5. Comparison of BDG, Mnn and MS-DS index from 100 sera of the cohort. (**A**) ROC curves based on comparison between IC group and the corresponding control group: Mnn, dotted line; BDG, grey line; MALDI-TOF MS MS-DS index, black line; MALDI-LTQ MS MS-DS index, hatched line. (**B**) Cut-offs established by ROC curves and corresponding sensibility/specificity values. AUC, Area Under the Curve

4. Conclusion

The present study demonstrates that trehalose originated from fungal metabolism is a new target for the diagnosis of life-threatening invasive candidiasis. The test based on the calculation of the relative intensity of trehalose signal recorded by MALDI-TOF MS at *m/z* 365 *versus* the ubiquitous signal at *m/z* 361 (MS-DS) in any given serum sample exhibited very good performances with a sensitivity/specificity trade-off comparable, if not superior, to existing

enzymatic and immunological methods designed to quantify the presence of cell-wall polysaccharides originating from yeast. Over the last decade, MALDI-TOF MS using protein mass fingerprinting has emerged as an essential tool for microbial identification and diagnosis in clinical settings [36]. Compared with the traditional methods of morphological, physiological and biochemical identification, it has many advantages, including convenience, speed, accuracy and low cost. With regard to yeasts, MALDI-TOF MS was shown to reliably identify a large panel of *Candida* species involved in human infections [37,38]. However, such a MALDI-TOF MS application only concerns cultures having grown from human samples, and does not respond to the need for a rapid diagnosis crucial for IC patients care. To our knowledge, MS-DS is until now the only diagnostic approach that successfully uses MALDI-TOF MS to identify fungal metabolite released in the patient serum, without the need of fungal isolation. It appears as a complementary method to traditional MALDI-TOF MS applications that enables a rapid diagnosis of IFI, but without species identification, and thus participates in medical decision-making process and patient management. The identification of trehalose, a major fungal metabolite overexpressed during pathogenic processes, as the target of MS-DS further reinforce its indication for IFI. The data presented reinforced the conviction that the detection of pathogens metabolites in serum by mass spectrometry is the way to achieve a rapid and efficient diagnosis of numerous infectious diseases. Finally, we have demonstrated here that the continuous improvement of mass spectrometry performances, in particular in term of resolution, will further reinforce the performance of diagnosis based on the detection of circulating fungal metabolites.

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Supplementary data

Figure S1 – High resolution MS distinguishes two compounds in the target signal at *m/z* 365. MALDI-TOF MS (left panel) and MALDI-LTQ MS (right panel) spectra of purified control serum.

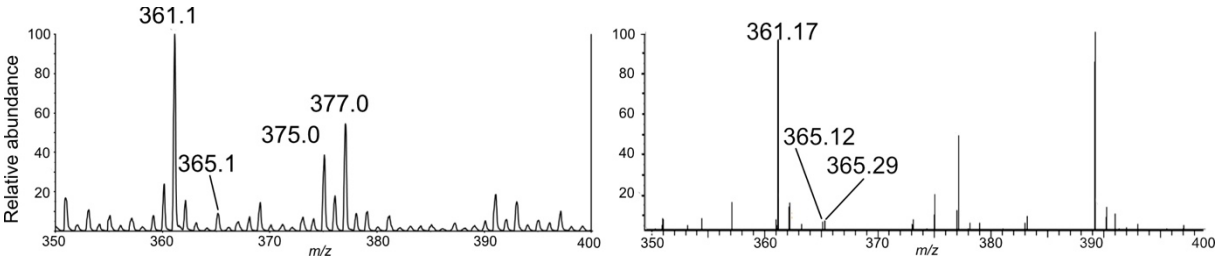

Table S1 – Characteristics of sera from patients with IC. Delay vs BC: number of days between serum sampling and first positive blood culture (BC). MS-DS indexes at low resolution were acquired on MALDI TOF MS, MS-DS indexes at high resolution were acquired on MALDI-LTQ MS.

Patient n°	Species isolated	Delay vs BC (days)	Fungitell® (pg/mL)	Platelia plus® Ag (pg/ml)	MS-DS low resolution	MS-DS high resolution
Ca23	*C albicans*	-5	315	236	800	1000
		1	912	>500	1000	2000
		8	896	2039	900	1000
Ca33	*C albicans*	-3	71	0	350	400
		7	113	0	500	300
Ca34	*C albicans*	-4	341	435	500	500
		3	277	363	500	700
		4	1704	1172	500	500
		10	264	401	650	500
		18	1088	373	700	700
Ca5	*C albicans*	-2	177	236	400	300
		0	170	84	230	500
		3	220	>2500	700	1000
		56	364	0	77	94
Ca8	*C albicans*	-2	816	0	220	60
		5	1056	0	700	700
		12	1712	0	600	900
Ca12	*C dubliniensis*	-3	1488	0	153	350
		-2	497	0	800	2000
		3	0	0	115	200
Ca29	*C albicans*	-7	1088	105	380	350
		-5	536	0	220	60
		0	2832	614	700	1000
Ca45	*C albicans*	-1	0	0	200	75
		4	323	0	1000	2000
		14	75	0	350	92
Ca18	*C glabrata*	J0	1928	214	1000	500
		6	324	73	700	2000
		12	3904	2210	230	220
Ca40	*C glabrata*	-25	101	77	400	300
		5	84	0	400	450
		12	63	0	800	1000
Ca52	*C parapsilosis*	0	968	0	450	700
		2	1464	0	500	1000
		9	760	0	1000	900
Ca44	*C tropicalis*	-6	108	0	300	260
		-1	36	0	400	1000
		6	11	0	450	1000
Ca37	*C tropicalis*	-4	133	0	230	51
		0	60	507	800	2000
		7	446	0	1500	4000
Ca53	*C krusei*	-4	113	0	400	500
		-1	203	0	400	700
		1	261	0	500	800

Table S2 – Characteristics of sera from colonized patients without positive blood culture test and *Candida* species isolated. MS-DS values at low resolution were acquired on MALDI-TOF MS, MS-DS values at high resolution were acquired on MALDI-LTQ MS.

Patient n°	Species identified	Fungitell® (pg/mL)	Platelia plus® Ag (pg/ml)	MS-DS low resolution	MS-DS high resolution
Tem1	C tropicalis	47	0	280	250
		0	0	50	20
		134	0	92	110
Tem 2	C albicans	12	0	150	142
		468	0	139	65
		1392	0	350	400
Tem 3	C glabrata	12	0	52	100
		0	0	65	62
Tem 4	C parapsilosis	84	0	110	50
		101	0	60	70
		56	0	164	140
Tem 5	C albicans	20	0	300	260
		38	0	300	118
		89	0	200	123
Tem 6	n.d.	120	0	400	230
		38	0	400	180
		42	0	40	65
Tem 8	C albicans	215	0	350	400
		109	0	48	10
		46	0	33	24
Tem 10	C albicans	120	0	137	50
		60	0	133	180
		49	0	122	50
Tem 14	C albicans	25	0	97	88
		36	0	280	800
		3584	0	700	400
Tem 16	C albicans	0	0	23	14
		87	0	22	27
		0	0	300	155
Tem 18	C parapsilosis	22	0	38	30
		0	0	55	60
		65	0	164	185
Tem 21	C albicans	100	0	120	40
		0	0	62	52
		41	0	500	2000
Tem 25	C kefyr	196	0,4	1000	900
		>500	0	600	2000
		314	0	39	110
Tem 26	C parapsilosis	0	0	53	37
		62	0	48	90
		42	0	34	61
Tem 27	C albicans	31	0	133	210
		336	0	150	125
		96	0	82	130
Tem 29	C albicans	42	0	96	143
		0	0	43	52
		11	0	52	40

Table S3 – Identification of sera from healthy individuals from l'Etablissement Français du Sang

Patient n°	MS-DS low resolution	MS-DS high resolution
EFS1	34	8
EFS6	45	8
EFS9	33	7
EFS10	8	12
EFS12	26	12
EFS14	13	18
EFS15	15	6
EFS16	18	6
EFS20	14	8