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ORIGINAL ARTICLE

Diversity and antifungal susceptibility of Norwegian *Candida glabrata* clinical isolates

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Background: Increasing numbers of immunocompromised patients have resulted in greater incidence of invasive fungal infections with high mortality. *Candida albicans* infections dominate, but during the last decade, *Candida glabrata* has become the second highest cause of candidemia in the United States and Northern Europe. Reliable and early diagnosis, together with appropriate choice of antifungal treatment, is needed to combat these challenging infections.

Objectives: To confirm the identity of 183 *Candida glabrata* isolates from different human body sites using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) and VITEK®2, and to analyze isolate protein profiles and antifungal susceptibility. The minimum inhibitory concentration (MIC) of seven antifungal drugs was determined for the isolates to elucidate susceptibility.

Design: A total of 183 *C. glabrata* isolates obtained between 2002 and 2012 from Norwegian health-care units were analyzed. For species verification and differentiation, biochemical characterization (VITEK®2) and mass spectrometry (MALDI–TOF) were used. MIC determination for seven antifungal drugs was undertaken using E-tests®.

Results: Using VITEK®2, 92.9% of isolates were identified as *C. glabrata*, while all isolates (100%) were identified as *C. glabrata* using MALDI-TOF. Variation in protein spectra occurred for all identified *C. glabrata* isolates. The majority of isolates had low MICs to amphotericin B ($\leq$1 mg/L for 99.5%) and anidulafungin ($\leq$0.06 mg/L for 98.9%). For fluconazole, 18% of isolates had MICs $>$32 mg/L and 82% had MICs in the range $\geq$0.016 mg/L to $\leq$32 mg/L.

Conclusions: Protein profiles and antifungal susceptibility characteristics of the *C. glabrata* isolates were diverse. Clustering of protein profiles indicated that many azole resistant isolates were closely related. In most cases, isolates had highest susceptibility to amphotericin B and anidulafungin. The results confirmed previous observations of high MICs to fluconazole and flucytosine. MALDI-TOF was more definitive than VITEK®2 for *C. glabrata* identification.

Keywords: C. glabrata; *identification*; *biochemical*; *MALDI-TOF*; *MICs*

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In healthy humans, yeasts are part of the commensal microbiota. However, these yeast species can act as opportunistic pathogens at different parts of the body, including the oral cavity. Patients with a reduced immune response, experiencing surgical procedures, with a reduced blood supply, in receipt of broad-spectrum antibiotics or immune suppressing medication, are at risk of opportunistic fungal infections. Associated with the expanding elderly population there has been an increase in severe systemic fungal infections (1–3). Such infections result in increased morbidity and mortality as well as high costs to society. Hospital infections caused by different *Candida* species can result in up to 30 extra hospital days for those affected (4). Mortality due to invasive

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Candida infections is in the range 5–71% (5). Thus, it is highly important to prevent and treat these infections, including those of the oral cavity. *Candida albicans* is the predominant cause of fungal infection in humans. Based on the data from global epidemiological studies, significant geographical variation is evident (6–8), both in incidence and response of *C. glabrata* to antifungal drugs. Several reports indicate that *C. glabrata* infections have increased and are now the second most prevalent cause of candidosis (3, 8–10), although this can vary with age, gender (8), and geographical location. Data from the ARTEMIS Antifungal Surveillance Program between 2001 and 2007 showed that *C. glabrata* accounted for 11.6% of *Candida* isolated from all body sites, with a frequency ranging from 7.4% in Latin America to 21.1% in North America (7).

Infection with *C. glabrata* is also a concern because of the reduced antifungal susceptibility of this species, especially to azoles (1, 3, 7, 8). An increasing number of studies report that *C. glabrata* and other *Candida* species also develop resistance to echinocandins (11–14) and co-resistance to both azoles and echinocandins has been found in bloodstream infection isolates (11). Rapid and precise testing procedures for species identification with reliable prediction of antifungal resistance patterns are essential in making appropriate treatment decisions. The use of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) requires only 1 h of analysis and is increasingly being adopted as a time-saving diagnostic to supplement conventional identification methods such as morphological and biochemical approaches.

The aims of the present study were twofold and were to confirm identity of the test isolates using MALDI-TOF and the VITEK®2 (BioMérieux, Inc., Durham, NC, USA) system, and also compare isolate diversity in terms of protein profiles and antifungal susceptibility patterns. The *C. glabrata* isolates were from different human sites, namely the oral cavity and gastrointestinal tract (OGI tract), respiratory tract (R tract), and blood. Susceptibility testing and minimum inhibitory concentration (MIC) determination to seven different antifungal drugs was performed to assess associated resistance.

Materials and methods

Clinical isolates and reference strains

A total of 183 *C. glabrata* isolates from the Microbiological Diagnostic Service (Department of Oral Biology (DOB), Faculty of Dentistry, University of Oslo, Norway) and the Norwegian Mycological Reference Laboratory (Department of Microbiology, Oslo University Hospital, Rikshospitalet, University of Oslo (OUS), Norway) were included in the study. These isolates were collected between 2003 and 2011 from patients attending different Norwegian health-care units. All isolates had previously been definitively identified as *C. glabrata* using routine identification methods at the time of isolation. Fifteen isolates originated from the OGI tract, 17 from the respiratory tract, 150 from blood, and one was of unknown origin. The distribution of isolates in patients and their site of isolation are presented in Table 1. Four different *Candida* reference strains from the American Type Culture Collection (ATCC®) (*C. glabrata* ATCC-15545D-5™, *C. parapsilosis* ATCC-22019D-5™, *C. albicans* ATCC-90028D-5™ and *C. dubliniensis* ATCC-MYA-646™) were included as controls. The Norwegian Institute of Public Health provided a single reference strain of *C. glabrata*. Isolates were cultured from frozen stocks on to CHROMagar® Candida (Paris, France) and incubated aerobically for 48 h at 37°C. One single colony was re-cultivated from each plate for subsequent testing.

Biochemical identification

Biochemical identification was based on assessing carbon and nitrogen assimilation and enzymatic activities. The yeast identification card in the VITEK®2 (BioMérieux) system includes 46 biochemical tests and can identify up to 50 different yeast species within 18 h by comparison with an integrated database (BioMérieux Clinical Diagnostics website, April 2012, June 2014). Identification is categorized as: 'Excellent' (96–99%), 'Very Good' (93–95%), 'Good' (89–92%), 'Acceptable' (85–88%), 'Low Discrimination', and 'Inconclusive'. These categories are defined by numbers of possibilities. If two possible identities exist, the result is presented as below 'Acceptable', 'Low Discrimination' represents 2–3 options and 'Inconclusive' >3 options. When identity scores were below 'Acceptable', the sample was retested. Preparation and analyses of isolates were performed according to the manufacturer's instructions. All isolates had previously been definitively identified as *C. glabrata* according to routine identification methods used in clinical microbiology laboratories. The identity of isolates that led to a low-discrimination level result was subsequently confirmed by 18S rRNA sequencing.

Protein profiling

All isolates were subcultured from CHROMagar® Candida by inoculating one single colony on Sabouraud dextrose agar, which was incubated aerobically for 24 h at 37°C. Prior to MALDI-TOF MS analysis, an ethanol/formic acid extraction procedure was performed according to the manufacturer's instructions (Bruker Daltonics, GmbH, Bremen, Germany). All isolates were analyzed in duplicate using high-performance liquid chromatography (HPLC) quality reagents throughout. Briefly, using a 10-µL inoculation loop, a colony of 24-h yeast culture was transferred to 300 µL of sterile water (HPLC-quality) in a 1.5-ml Eppendorf Safe Lock Tube™, and suspended and mixed with 900 µL of absolute ethanol. After centrifugation (13,000 rpm for 2 min, Heraeus® Megafuge® 1.0), the

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Table 1. Origin of the 183 investigated *C. glabrata* isolates including reference strains in the 10 clusters in the protein profile cluster dendrogram

Cluster no.	Protein profile no.	Strain	Source	Azole resistance
	43	*C. albicans*		
	48	*C. albicans*		
	2	*C. albicans,* ATCC® 90028D-5™		
	4	*C. dubliniensis,* ATCC® MYA-646™		
	123	*C. tropicalis*		
	1	*C. parapsilosis*, ATCC® 22019D-5™		
	5	*E. coli*		
	11	*C. glabrata* clinical isolate	Blood	
	17	*C. glabrata* clinical isolate	R tract	VO
1	49	*C. glabrata* clinical isolate	R tract	VO
	51	*C. glabrata* clinical isolate	R tract	FL, VO
	58	*C. glabrata* clinical isolate	R tract	FL, VO
	171	*C. glabrata* clinical isolate	Blood	FL, VO
	54	*C. glabrata* clinical isolate	Bi	
	55	*C. glabrata* clinical isolate	OGI tract	
	191	*C. glabrata* clinical isolate	Blood	
	170	*C. glabrata* clinical isolate	OGI tract	VO
	50	*C. glabrata* clinical isolate	R tract	FL, VO
	56	*C. glabrata* clinical isolate	Blood	
	169	*C. glabrata* clinical isolate	Blood	VO
	179	*C. glabrata* clinical isolate	Blood	
	165	*C. glabrata* clinical isolate	Blood	VO
2	3	*C. glabrata*, ATCC® 15545D-5™		
		C. glabrata clinical isolates ($n = 16$)		FL (3), VO (12)
3		*C. glabrata* clinical isolates ($n = 48$)		FL (3), VO (7)
4		*C. glabrata* clinical isolates ($n = 13$)		FL (9), VO (29)
5		*C. glabrata* clinical isolates ($n = 2$)		FL (4), VO (9)
6		*C. glabrata* clinical isolates ($n = 14$)		
7		*C. glabrata* clinical isolates ($n = 40$)		FL (6), VO (30)
8		*C. glabrata* clinical isolates ($n = 5$)		FL (1), VO (5)
9		*C. glabrata* clinical isolates ($n = 15$)		FL (2), VO (5)
	13	*C. glabrata* clinical isolate	Blood	FL, VO
	24	*C. glabrata* clinical isolate	Blood	VO
	31	*C. glabrata* clinical isolate	Blood	FL, VO
	32	*C. glabrata* clinical isolate	Blood	FL, VO
	30	*C. glabrata* clinical isolate	R tract	
	35	*C. glabrata* clinical isolate	OGI tract	
	16	*C. glabrata* clinical isolate	Blood	FL, VO
10	29	*C. glabrata* clinical isolate	Blood	FL, VO
	36	*C. glabrata* clinical isolate	OGI tract	FL, VO
	37	*C. glabrata* clinical isolate	OGI tract	
	18	*C. glabrata* clinical isolate	R tract	VO
	20	*C. glabrata* clinical isolate	Blood	FL, VO
	25	*C. glabrata* clinical isolate	R tract	VO
	26	*C. glabrata* clinical isolate	Blood	FL, VO
	34	*C. glabrata* clinical isolate	OGI tract	FL, VO

Protein profile numbers refer to identification by the MALDI-TOF MS analysis, see results section. Blood isolates ($n = 150$). Isolates from other sources than blood ($n = 33$) includes: OGI tract (oral cavity and gastro intestinal tract; $n = 15$), R (respiratory) tract ($n = 17$), and Bi (biopsy) from an unknown site ($n = 1$). Azole resistance: MICs $>$32 mg/L toward fluconazole (FL) and MICs $>$0.125 toward voriconazole (VO). Number of resistant isolates in parentheses.

supernatant was removed. Centrifugation was repeated and the resulting pellet dried for 2 min at room temperature before re-suspension in 50 μL of 70% (v/v) formic acid and mixing with 50 μL acetonitrile. The sample was centrifuged (13,000 rpm for 2 min, Heraeus® Megafuge® 1.0) and 1 μL of supernatant placed on the target plate. The sample was dried at room temperature before being covered with 1 μL of matrix (α-cyano-4-hydroxy-cinnamic acid (HCCA), Bruker Daltonics) and air-dried again. The HCCA matrix, 10 mg, was dissolved in 250 μL of organic solution (trifluoroacetic acid (TFA) 2.5%, acetonitrile 50%, and water 47.5%) by vortex mixing at room temperature. Reference strains were included as positive controls in all analyses. In accordance with the manufacturer's protocol, an *Escherichia coli* standard (Bruker Bacterial Test Standard (BTS), Bruker Daltonics) was included. This bacterial standard is 'spiked' with two additional high molecular weight proteins, enabling calibration across the entire mass range (4–17 kDa) of proteins used for identification of microorganisms (www.bruker.com). Bioinformatic analysis was performed with the integrated MALDI Biotyper 3.0 software, which compared the mass spectra from the samples to profiles stored in the reference library. Results of pattern matching were presented as log score values in the range 0–3. According to the manufacturer's guidelines, a score of 2.3–3.0 gives a highly probable species identification, while scores between 2.0 and 2.299 provide confidence to genus level and probable species identification (Bruker Daltonics MALDI Biotyper 3.1). For *C. glabrata*, a log score of 2.0 was chosen for species identification at OUS. When scores were below 2.0, samples were retested, and the spectra from each isolate with the two best matches (2.0 or above) chosen.

Clustering and correlation analysis of protein spectra

The heterogeneity of the raw protein-spectrum set was analyzed by the MALDI Biotyper managed by an external MATLAB software tool, integrated in the equipment. Results were visualized in a clustering dendrogram (Fig. 1), representing the closeness of individual spectra (the x-axis as protein profile numbers) to one another. Composite correlation index (CCI) analysis is a statistical tool of the MALDI Biotyper to visualize the similarity between protein spectra in a heat map. A composition of correlations of all the intervals provides the CCI. In the heat map grid, the different colors of the squares at the intersection of two fungal colonies (in the diagram called 'groups') indicate their relationship. Dark red represents a close relation, whilst blue are more distantly related (Bruker Daltonics MALDI Biotyper 3.1 User Manual Revision 1). The clustering and correlation analysis of protein spectra was achieved using Maldi Biotyper Compass version 4.1.

MIC determination/testing

MIC determination or testing was done using E-tests® (BioMérieux) as recommended by the manufacturer.

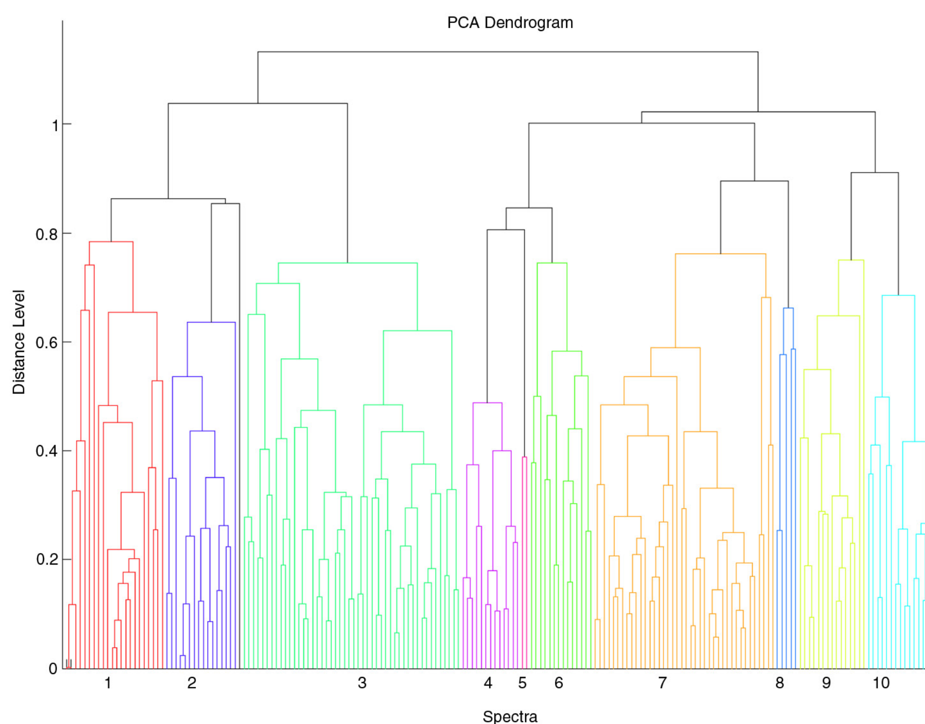

Fig. 1. Protein profile cluster (Principal Component Analysis (PCA)) dendrogram of the 183 *C. glabrata* and reference strains grouped into 10 clusters based on proteomic profiling. The clusters are marked by color and numbered 1–10 (Maldi Biotyper Compass version 4.1).

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The E-test® is a fast and cost-effective alternative to 'gold standard' procedures developed by the Clinical and Laboratory Standards Institute (CLSI) (15) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (16). The agreement between both methods is considered high (17).

Susceptibility of all 183 isolates to amphotericin B (polyene), fluconazole, and voriconazole was determined. The susceptibility of 179 isolates was established to anidulafungin, 146 isolates to caspofungin, 71 isolates to micafungin and 36 isolates to flucytosine (nucleoside analog). The E-test® was applied to inoculated agar and incubated at 35°C for 48 h. MICs for amphotericin B were determined as the lowest concentration of antifungal agent inhibiting any growth. For azoles and echinocandins, 80% reduction of growth represented the MIC. Flucytosine MICs were determined as the lowest concentrations that caused a 90% reduction in growth. Reading and interpretation of the results were in accordance with the manufacturer and procedures of the Norwegian Mycological Reference Laboratory (OUS), which is based on recommended breakpoints (BPs, mg/L) given by EUCAST (www.eucast.org. 2015).

Clinical BPs

The BPs for susceptibility testing are regularly revisited and re-established according to shifts in clinical relevance, epidemiological differences, and available antimicrobials. New recommended BPs were given for amphotericin B, fluconazole, anidulafungin, and micafungin in 2014 by EUCAST (Table 2). There are no established European BPs for caspofungin, voriconazole, or flucytosine. Isolates susceptible to anidulafungin as well as micafungin were considered susceptible to caspofungin (www.eucast.org. 2015).

Results

Culture and purification

Colonies of all isolates appeared white on CHROMagar® Candida corresponding to the *C. glabrata* control strains included in the study.

Table 2. Antimicrobial susceptibility of *C. glabrata* to five of the antifungals tested

	MIC (mg/L) Proportion of **all** isolates (%)			**MIC** (mg/L) Proportion of **blood** isolates (%)			**MIC** (mg/L) Proportion of **other** isolates (%)		
	S	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**
Amphotericin B	**≤1**		**>1**	**≤1**		**>1**	**≤1**		**>1**
Total: *n* = *183*									
Blood: *n* = *150*	99.5		0.5	100		0	97.0		3.0
Other isolates: *n* = *33*				**100**		**0**			
Fluconazole	**≤0.002**		**>32**	**≤0.002**		**>32**	**≤0.002**		**>32**
Total: *n* = *183*									
Blood: *n* = *150*		82.0	18.0		85.3	14.7		66.8	33.2
Other isolates: *n* = *33*					***82.6***	***17.4***			
Voriconazole*	**≤0.125**		**>0.125**	**≤0.125**		**>0.125**	**≤0.125**		**>0.125**
Total: *n* = *183*									
Blood: *n* = *150*	31.2		68.8	34.0		66.0	18.1		81.9
Other isolates: *n* = *33*				***34.8***		***65.2***			
Anidulafungin	**≤0.06**		**>0.06**	**≤0.06**		**>0.06**	**≤0.06**		**>0.06**
Total: *n* = *179*									
Blood: *n* = *147*	98.9		1.1	98.6		1.4	100		0
Other isolates: *n* = *32*				***95.7***		***4.3***			
Micafungin	**≤0.032**		**>0.032**	**≤0.032**		**>0.032**	**≤0.032**		**>0.032**
Total: *n* = *71*									
Blood: *n* = *64*	98.6		1.4	98.4		1.6	100		0
Other isolates: *n* = *7*									

NORM 2012 – results (*n* = 23) in italic/bold. Susceptible (S), intermediate susceptible (I), and resistant (R) according to the recommended breakpoints (BPs, mg/L) given by the European Committee on Antimicrobial Susceptibility Testing – EUCAST in 2014.

*Due to insufficient evidence that *C. glabrata* is a good target for therapy with voriconazole, no EUCAST breakpoints were available in 2014, and BPs made for *C. albicans*, *C. tropicalis*, and *C. parapsilosis* were used.

Biochemical identification

Identification using biochemical analysis (VITEK®2) showed that 170 of the 183 (92.9%) isolates had a discrimination confidence level compatible with *C. glabrata*. A 'Low discrimination' score was obtained for 13 strains, with two (15.4%) originating from the gut and 11 (84.6%) from blood. Isolates with a low identity score were verified by 18S rRNA sequencing. The five most common positive biochemical tests were: D-glucose assimilation (dGLUa) and Leucine-Arylamidase (LeuA) (positive for 100% isolates), D-mannose assimilation (dMNEa) (positive for 99.5% isolates), D-trehalose assimilation (dTREa) (positive in 93.4%), and acetate assimilation (ACEa) (positive for 92.9% isolates). All isolates with a 'Low discrimination' score were negative for the ACEa test, and one of these isolates was positive in the dTREa test. The identification messages, confidence levels, and source distribution are listed in Supplementary file.

Protein profiling

All isolates were identified as *C. glabrata* using the MALDI-TOF MS, with a log score 2.0 or above. In the clustering and correlation analysis, all protein spectra were sorted by group numbers as protein profile numbers (different from isolate numbers) by the software program (Maldi Biotyper Compass version 4.1) (Fig. 1).

Clustering and correlation analysis of protein spectra

The protein profile dendrogram distributed the protein spectra into 10 clusters (Fig. 1 and Table 1). An enlarged view of cluster 1 is presented in Supplementary file. This cluster contained two subclusters: the first covered all seven reference/quality controls from other species, and the second subcluster contained 15 clinical *C. glabrata* isolates (Supplementary Fig. 2). Cluster number 2–10 included clinical *C. glabrata* isolates. The *C. glabrata* reference strain (ATCC®15545D-5™) was placed in cluster 2.

Results from the CCI analysis were presented as a heat map grid in which all the non-*C. glabrata* controls and reference strains were indicated by dark blue lines in the diagram, demonstrating no relationship to the *C. glabrata* isolates. For the *C. glabrata* group, most were expressed by different shades of red, orange, and yellow, indicating close relationships. All intersections of identical groups were represented by a dark red color making a diagonal line in the map grid (Fig. 2).

MIC determination

MIC testing revealed that 99.5% of isolates had MIC values ≤ 1 mg/L for amphotericin B, and included 100% of blood isolates and 97% of other isolates. For fluconazole, 82% of isolates MICs in the range ≥ 0.02 mg/L to ≤ 32 mg/L, and 18% had MICs > 32 mg/L. There were fewer fluconazole resistant isolates from blood (14.7%) compared with other sources (33.2%). None of the isolates were totally susceptible to fluconazole. For voriconazole, 31.2% of isolates had MICs ≤ 0.125 mg/L (34% from blood, 18.1% of isolates from other sites), while 68.8% of isolates had MICs > 0.125 mg/L (66% from blood, 81.9% of isolates from other sites). For anidulafungin, MICs > 0.06 0.06 mg/L were recorded for 1.1% of isolates, leaving almost all (98.9%) isolates susceptible to this agent. Blood isolates were slightly less susceptible to this agent (98.6% versus 100%). The only two isolates resistant to anidulafungin in the test (no. 20 and no. 99) were from blood, and both were sensitive to amphotericin B (MIC, 0.125 mg/L). For the 71 isolates tested against micafungin, 98.6% had MICs ≤ 0.032 mg/L, and the only resistant isolate (no. 20) was also resistant to anidulafungin. Based on BPs made for *C. albicans*, *C. tropicalis*, and *C. parapsilosis,* isolate no. 20 was susceptible to amphotericin B (MIC $= 0.125$ mg/L) and voriconazole and had intermediate susceptibility against fluconazole (MIC $= 2$ mg/L).

The MIC results from the five currently most used antifungals are shown in Table 2, while results from all the antifungal gradient agar diffusion tests (E-test®) are presented in Supplementary file.

Discussion

C. glabrata infections have increased in recent decades and are now the second most prevalent cause of candidemia in the United States and Northern Europe (3, 8–10). A rapid and reliable diagnosis of these infections, combined with antifungal resistance determination, are needed, especially when patients are critically ill.

Screening patient samples on CHROMagar® Candida provides an initial presumptive identification of *Candida* species. Colonies of certain clinically significant *Candida* species exhibit different colors on this agar. However, *C. glabrata* colonies appear white on this medium, which is similar to some other species. After screening, further analyses are therefore necessary to definitively identify the *Candida* species.

The VITEK®2 provides a biochemical characterization of the species of interest and is based on 46 biochemical reactions and is capable of identifying up to 50 different yeasts. In this study, the VITEK®2 identified 92.9% of isolates as *C. glabrata*. All but one *C. glabrata* isolate tested positive in the following three tests: dGLUa, LeuA, and dMNEa. *C. glabrata* is claimed to ferment and assimilate two sugars only, glucose and trehalose, and this is atypical for the majority of *Candida* species, which normally demonstrate a wide repertoire for sugar utilization. Under certain conditions, *C. glabrata* hydrolyzes trehalose into glucose more rapidly than other species and on this basis, several commercial tests have been developed specifically for rapid and cost-effective presumptive identification of *C. glabrata* (18). In our study, a 'Low probability' score from the VITEK®2 system was obtained for 13 isolates, in which one isolate was positive for the trehalose-assimilation-test (dTREa). In contrast, whenever the ACEa test (ACEa)

was negative, the probability score was 'low'. Our results indicate that the specific ACEa test and not the trehalose-assimilation test was crucial for identification of *C. glabrata*. VITEK®2 is widely used in clinical microbiology, but it is not able to identify all species despite continuous update of the software database.

The second diagnostic tool used was MALDI-TOF MS. All isolates were identified as *C. glabrata* with the MALDI-TOF MS analysis. The score was ≥ 2.0 for two of the duplicate tests. Prior to analysis, a standard protein extraction of the yeast isolates was performed and the cutoff for species level was set to 2.0, according to the manufacturer's instructions and protocols at OUS. A more uncomplicated and time-saving alternative would be 'on-plate extraction' or 'on-target lysis' procedures and eventually lowering the interpretative cutoff from 2.0 to >1.7 (19, 20), although this would not give the most predictable results. Another way of reducing procedural time might be to grow the yeasts in liquid media (21).

The dendrogram (Principal Component Analysis (PCA) dendrogram) created from the acquired protein profiles distributed the isolates into 10 clusters (Fig. 1). Cluster no 1 contained two subclusters: the first of which covered all seven reference/quality controls from other species, and the second subcluster contained 15 clinical *C. glabrata* isolates (Maldi Biotyper Compass version 4.1). Cluster nos. 2–10 contained *C. glabrata* strains from different sources, and all included blood isolates. The two smaller clusters (nos. 5 and 8) included blood samples only, but no cluster had samples only from the other sources. Our results indicate little difference in protein profiles between isolates according to their origin (source). Due to a limited number of isolates of oral ($n = 7$) and gastrointestinal tract ($n = 8$) origin compared with blood, no conclusions can be drawn. All clinical *C. glabrata* isolates assembled in cluster no 1 had 'Excellent' or 'Very Good' identification in VITEK®2 analysis (Table 1). Eight out of the 13 isolates with a 'Low probability' score were in cluster no. 10 and had a different protein expression profile from the other *C. glabrata* isolates. This observation may associate with their antifungal resistance. All eight (seven originating from blood and one from the gastrointestinal tract) had high MICs for fluconazole and voriconazole.

CCI analysis illustrated diversity in protein expression. In the heat map diagram, variation in color indicative of variation in protein expression occurred with most isolates grown under standardized conditions. All reference strains were deemed to have distant relationships in terms of their protein spectra to *C. glabrata*. For *C. glabrata*, most isolates were represented as different shades of red, orange, and yellow indicating close relationship between protein spectra. However, some isolates also showed signs of greater differences in protein spectra with blue and green colors in some of the intersection squares (Fig. 2).

Correct and rapid species identification is crucial for appropriate antifungal treatment. Despite the diversity in protein spectra, MALDI-TOF MS seems, according to our investigation, to be the most reliable and rapid method (within 1 h), while the VITEK®2 results were not available until the next day. These conclusions are in accordance with those made by others (22).

MIC determination is of utmost importance in clinical microbiology for *in vitro* susceptibility testing of the drugs of choice. Almost all isolates in this study were susceptible to amphotericin B (MIC ≤ 1 mg/L in 99.5%), anidulafungin (MIC ≤ 0.06 mg/L in 98.9%), and micafungin (MIC $\leq$ 0.032 mg/L in 98.6%). In recent decades, an increase in echinocandin resistance among *C. glabrata* isolates has been reported and has the order of 4.3–18.9% (11, 12, 14, 23). A review by Arendrup and Perlin (13) described emerging resistance in America and resistance on a smaller scale in Europe. In Denmark, echinocandin resistance rates increased from zero in 2004–2007 to 0.9% in 2008–2009, 1.2% in 2010–2011 and 3.1% in 2012–2013 (13). Our findings are in agreement with these recent values and appear to follow the same resistance incidence as in Denmark. The Norwegian annual reports from 2008 to 2013 provide a slightly different picture, showing zero resistance in the first 3 years (24–26), increasing to 8% resistance in 2011, and declining to 4.3% in 2012 and to zero resistance in 2013 (12, 23, 27).

Based on EUCAST-BPs from 2014, in this study, total susceptibility to fluconazole was not evident for any isolates, with 18% being resistant (MIC >32 mg/L) and 82% intermediately susceptible (>0.002 mg/L MIC $\leq$ 32 mg/L). Our results were in accordance with those from the last two NORM reports (23, 27) as well as findings from other countries where fluconazole resistance in *C. glabrata* has been increasing from 5.6% in 1997–2007 (7) to 11.9% in 2008–2011 (28). Among isolates from blood, resistance to fluconazole was 14.7% while the proportion was 33.2% for other isolates.

Antifungal susceptibility and BPs as well as laboratory procedures have changed over the years. In our study, clinical blood isolates ($n = 150$) were collected between 2003 and 2011, while isolates in the different NORM reports ($n < 40$) were collected yearly (www.vetinst.no/nor/Publikasjoner/NORM-NORM-VET). These differences make comparison difficult.

For voriconazole, all isolates had MICs ≤ 32 mg/L, indicating an *in vitro* activity superior to fluconazole, a result in accordance with findings elsewhere (29). However, cross-resistance between fluconazole and voriconazole is frequently seen, especially with *C. glabrata* (11, 30). Due to insufficient evidence that *C. glabrata* is a good target for therapy with voriconazole, no European BPs are available, and clinical interpretations have not been made. Considering the BPs for *C. albicans*,

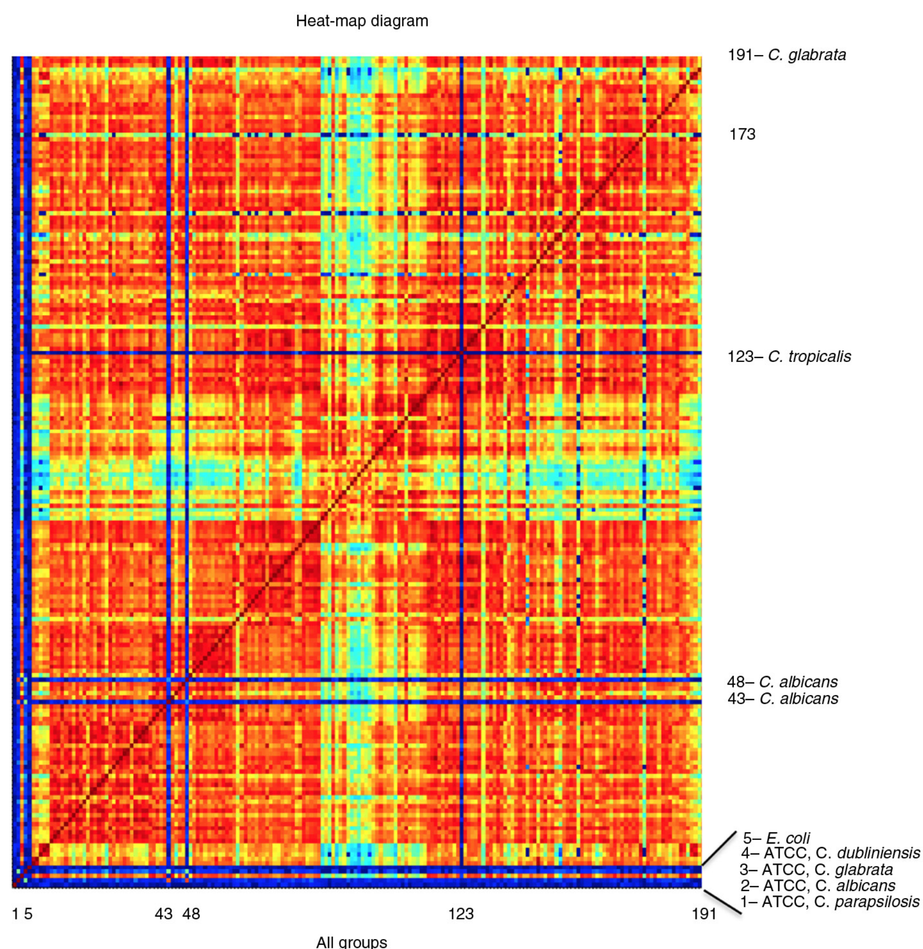

Fig. 2. CCI matrix of 183 *C. glabrata* isolates versus reference strains presented in a heat map diagram. Strains different from *C. glabrata*, expressed by dark blue lines, are marked by species names and protein profile numbers (different from sample numbers). The dark red diagonal line represents the intersections of identical groups. The diagram illustrates variation in protein spectra between *C. glabrata* and other species as well as among all isolates identified as *C. glabrata* in which blue and green shades represent distance between some of the protein spectra (Maldi Biotyper Compass version 4.1).

C. tropicalis, and *C. parapsilosis*, 68.8% of the isolates would be regarded as resistant to voriconazole.

C. glabrata isolates with high MICs toward the triazoles were represented in all protein profile clusters; however, cluster no. 10 had a higher representation. This cluster (no. 10) contained 15 isolates (eight from blood, four from OGI tract, three from R tract), 12 having high MICs to voriconazole and 9 to fluconazole. Eight of the 13 isolates, which had low identity scores in the VITEK®2 test, were in cluster 10, and all of them were simultaneously resistant to both triazoles tested (MIC $>$32 mg/L to fluconazole, MIC $>$0.125 mg/L to voriconazole). It would be interesting to further investigate the relationship between the resistance and protein cluster profiles.

Primary resistance to flucytosine (5-fluorocytosine) is reported as low (2–12%) for *Candida* (31), although higher for some species such as *C. krusei* (28% R) (32) and *C. glabrata* (18% R) (31). The frequent development of flucytosine resistance has led to limited use as a monotherapy and is mainly utilized in combination with amphotericin B. While was previously registered in Norway, flucytosine is now unregistered but can still be used on exemption from registration in patients with cryptococcal meningitis (33). In our study, a total of 36 isolates were tested against flucytosine and 55.6% had MICs $\geq$32 mg/L. There is no EUCAST BP for flucytosine, although in 90% of these isolates, macro-colonies were found, retested separately, and no inhibition of growth was registered.

In conclusion, MALDI-TOF MS, correctly identified in 100% of the isolates (cf. 92% by VITEK®2) and in less time (1 h versus 18 h) than the VITEK®2, thereby representing the most effective technique. Clustering and CCI analysis showed variation in protein spectra between all strains identified as *C. glabrata* by the MALDI-TOF MS, although they had a signature profile detected as *C. glabrata*. It is important to further understand the protein profile

clustering since we observed that eight of 13 strains with azole resistance clustered together, whereas the metabolic profile from VITEK®2 analysis resulted in a low ID score. If the clustering profile could indicate high-azole MICs among the samples, it could be a rapid supplement to the MIC determination/testing.

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Conflict of interest and funding

The authors declare that there are no conflicts of interest.

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