Discrimination of *Candida nivariensis* and *Candida bracarensis* among *Candida glabrata* sensu lato isolates from clinical isolates in Tunisia

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Abstract

Background

Candida nivariensis and *Candida bracarensis* are new species of *Candida that are* phenotypically similar to *Candida glabrata* sensu stricto, causing significant problems for their identification by traditional laboratory methods. This study used a singleplex PCR method for the rapid identification of members of the *Candida glabrata* species complex (*Candida glabrata* sensu stricto, *Candida nivariensis* and *Candida bracarensis*). Furthermore, we tried to choose an appropriate extraction method, which is an important factor for the success of the PCR approach.

Methods

A total of 163 clinical isolates cultured from urine samples, vaginal swabs, placenta, intrauterine device, and urinary catheter in patients from the Maternity and Neonatology Center of Monastir were screened. A singleplex PCR was used targeting the RPL31 gene for the discrimination between species of the *Candida glabrata* complex. Four different DNA extraction methods, two commercial kits (GF-1Tissue/Blood), the phenol−chloroform isoamylic method, and chelating resin, were applied to obtain and determine the most effective DNA extraction method. The DNA quantity and quality were determined using Nanodrop and PCR.

Results

The Singleplex PCR assay amplified a 1.061 bp amplicon from all 163 *Candida glabrata* sensu stricto isolates, thus identifying all clinical isolates in Tunisia as *Candida glabrata* sensu stricto. Low DNA concentrations were measured for all methods, and the results showed that with one method, PCR success was 100%. The results of DNA purity and quantity measurements show variant results.

Conclusion

Our results obtained from a collection of clinical *Candida glabrata* sensu lato isolates show that *Candida nivariensis* and *Candida bracarensis* are not clinically important or prevalent in Tunisia. For the extraction method, Chelex (chelating resin) turned out to be a rapid, low-cost method that can provide high-quality DNA.

Introduction

Candida spp. are fungal pathogens and remain the most common opportunistic fungi in humans (Tsega and Mekonnen 2019). The genus *Candida* (*C.*) belonging to the ascomycetous yeasts has over 350

heterogeneous species, but only 20 of them have been identified to cause human disease (Williams et al. 2011).

Guinea (Guinea 2014) reported that *Candida* infections are caused by only four species/species complexes comprising *C. albicans*, *C. glabrata*, *C. parapsilosis* and *C. tropicalis*. Although *C. albicans* is the most commonly isolated yeast species, an increasing number of *nonalbicans Candida* (N*AC*) species, such as *C. glabrata*, have been reported (Richter et al. 2005). However, the rapid emergence of *C. glabrata* as a main cause of invasive fungal infections leads him to rank second place after *C. albicans (Salma et al., 2015*). Reasons for this change in species distribution remain unclear but may be a consequence of the widespread use of azole derivates, particularly fluconazole, which has been widely used since the 1980s (Pfaller and Diekema 2007; Sanguinetti et al. 2015; Angoulvant et al. 2016; Kołaczkowska and Kołaczkowski 2016). Along with *C. glabrata* and belonging to the *Nakeseomyces* clade of *Saccharomycotina*, two new species, *C. nivariensis* and *C. bracarensis*, have been reported to be emerging pathogens (Enache-Angoulvant et al. 2011). *C. nivariensis* was first identified in 2005 from clinical samples (bloodstream, vaginal specimens and respiratory specimens). However, (Gabaldón et al. 2013) reported that *C. nivariensis* was isolated from plant surfaces, which suggests that it could be colonized from the environment. The two new species are phenotypically indistinguishable from *C. glabrata* based on conventional chromogenic media or biochemical panels such as the Yeast ID32C strips (BioMerieux Vitek Inc., Hazelwood, MO) (Alcoba-Flórez et al. 2005). These methods are not very efficient in new closely related *C. glabrata* complexes, and further identification techniques are recommended. Full differentiation within the complex is possible with molecular biology techniques, which allow the accurate species-specific identification of all clinical *C. glabrata sensu lato* isolates (Silva et al. 2009; Cornet et al. 2011). In fact, PCR techniques and DNA sequencing lead to full species identification. Discrimination between even closely related species may be of major importance on the one hand in the understanding of their clinical and epidemiological role in vulvovaginal candidiasis (VVC) and on the other hand from a therapeutic point of view, especially affecting resistance to antifungal agents.

In this way, our study aims to identify the presence of two species, *C. nivariensis* and *C. bracarensis*, from a collection of *C. glabrata* isolates from different sites in hospitalized and nonhospitalized patients.

Materials And Methods

Ethics statements

The study was carried out according to the Declaration of Helsinki Principles and all Tunisian pertinent regulations. The samples were obtained for routine diagnostic purposes from women who were managed by the Center of Maternity and Neonatology of Monastir at the request of the gynecologist. We confirmed that informed verbal consent was obtained from all subjects. During consultation, a clinical examination for signs of infection, such as vaginal discharge, was carried out by the gynaecologist. Consenting women were informed of the importance of biological analysis. Given the seriousness of the situation,

the women are convinced of the importance of this analysis of their health as well as that of the baby in case of pregnancy. After acceptance, the gynaecologist prescribes a request for analysis.

Clinical isolates

In this retrospective study, 163 *C. glabrata* isolates were analysed and collected from gynaecology departments from January 2015 to December 2016. Study materials included 163 *C. glabrata* clinical strains cultured from urine samples, vaginal swabs, placentas, intrauterine devices (IUDs) and urinary catheters in patients from the Maternity and Neonatology Center of Monastir (MNCM). Every strain came from one single patient and from routine mycological diagnostic examinations.

C. glabrata phenotypic identification

Strains were initially inoculated on Sabouraud medium with chloramphenicol at 30 °C for 48 h. Then, white creamy colonies were subjected to the germ tube test for the identification of *Candida* species. Hence, to differentiate *C. albicans* from *nonalbicans (NAC)*, a colony of yeast was added to a sterile test tube containing 0.5 ml human serum and incubated at 37 °C for 3 h. A drop of the serum mixture was placed on a clean microscope slide, covered with a cover glass, and microscopically examined. The appearance of germ tube formation indicated the positivity of the test. To induce chlamydospores and pseudohyphal production, yeasts were incubated on rice agar Tween 80 media for 24 to 48 h at 30 °C. The strains were subcultured on CHROMagar *Candida* medium (Becton Dickinson, Heidelberg, Germany), incubated at 37 °C and examined after 24 h for colony color and morphology. *Candida albicans* had a green colony on CHROMagar and was positive for germ tube formation, while *C. glabrata* colonies developed pink-colored colonies on CHROMagar *Candida* medium.

Biochemical identification was performed for all NAC isolates using different batches of Yeast ID32C. The ability of the isolates to assimilate carbohydrate source compounds was determined according to the manufacturer's instructions. Biochemically, only the fermentation of trehalose can distinguish *C. nivariensis* from *C. glabrata* sensu stricto.

Template DNA preparation and molecular identification

DNA extraction methods

DNA extraction was performed using two different methods, including two commercial DNA extraction kits (GF-1 Tissue DNA extraction and GF-1 Blood DNA extraction) and two manual DNA extractions (Phenol Chloroform Isoamylalcohol (PCI) and a chelating resin (Chelex® 100)) that are explained below. For each method, two strains of *Candida glabrata* were considered, and the examinations were repeated in quadruplicate. To compare these processes, the concentrations and purity of the acquired DNA were measured via a NanoDrop 2000/2000c spectrophotometer (Thermo Fisher Scientific, U.S.A.). The quality of the DNA (or PCR product) was evaluated by assessing the PCR and sequencing success.

DNA extraction with PCI

The isolates were lysed with 300 µl of TNNT (Tris HCl 1 M pH 7.2, Nonidet P40, NaOH 10 N, Tween 20) buffer. Proteinase K (Thermo Scientific, Massachusetts, USA, 20 mg/ml) was added to a final concentration of 200 µl/ml, and the samples were incubated at 65 °C for 3 hours. Consequently, equal volumes of equilibrated phenol were added to the samples (500 µl) and mixed gently for 5 min. After centrifugation at high speed (14000xg) for 4 min at room temperature, the upper phase was carefully removed and transferred to a new sterile 1.5 ml microtube. A mixture of phenol, chloroform, and isoamyl alcohol (25:24:1) was added in equal volumes to each sample. The samples were mixed gently and centrifuged for 4 min. The upper phase was again transferred to a new sterile 1.5 ml microtube. An equal volume of chloroform was added, and each sample was centrifuged for 4 min. The upper phase was once again transferred to a new sterile 1.5 ml microtube. The DNA samples in both groups were precipitated using 5 µl of 3 M sodium acetate (NaAc 300 mM, Ph) and at least two volumes of cold (-20 °C) ethanol. Subsequently, the samples were incubated at -80 °C for 30 min and centrifuged at 14000xg for 30 min at 4 °C. The ethanol was removed, and each DNA pellet was dried. The DNA samples were then resuspended in TE buffer (100 µl) and stored at 20 °C for subsequent analysis.

DNA extraction with two commercial kits: GF-1 Tissue DNA extraction/GF-1 Blood DNA extraction: Two methods were performed on each sample: one extraction using GF-1 Tissue DNA and one extraction using GF-1 Blood DNA. Both GF-1 extractions were carried out according to the manufacturer's protocol. The elution volume used in the final step was 100 µl of TE buffer for GF-1 tissue DNA extraction and GF-1 blood DNA extraction.

DNA extraction with Chelex® 100

Chelex® 100 resin (Bio–Rad Laboratories, CA, USA) is a chelating resin that uses ion exchange to bind transition metal ions. The resin is composed of styrene divinylbenzene copolymers containing paired iminodiacetate ions, which act as chelators for polyvalent metal ions (Phillips et al. 2012). During the extraction process, the alkalinity of the solution and the act of boiling the solution breaks down the cells and allows the chelating groups to bind to the cellular components, protecting the DNA from degradation.

DNA was extracted from phenotypically identified strains belonging to *C. glabrata* using a rapid method with Chelex® 100 resin (Bio–Rad Laboratories, CA, USA) performed using 5% Chelex in sterile H_2O according to the protocol outlined by (Walsh et al. 2013). The 100 µl DNA extract was removed from the Chelex resin resuspended in TE buffer (100 µl) and stored at 20 °C for subsequent analysis. for further analysis.

DNA quantity and quality

The DNA yield and DNA purity were determined using a Nanodrop 2000/2000c spectrophotometer (Thermo Fisher Scientific, USA). The absorbance ratios A260/280 nm and A260/230 nm were calculated to estimate the purity of the extracted DNA, whereby A260/280 nm was used for protein contamination and A260/230 nm was used for salt and phenol contamination. DNA is known to absorb light at 260 nm

and an A260/280 ratio of 1.8-2.0 and an A260/230 ratio of >1.8, indicating that the sample was of good purity with little or no contamination (Vesty et al. 2017). These two isolates were used for measurements.

PCR amplification

For rapid screening of *C. glabrata* sensu stricto, *C. nivariensis* and *C. bracarensis* isolates, a simple PCR amplification of the 60S ribosomal subunits was performed using primers previously described by (Enache-Angoulvant et al. 2011). The primers were used to amplify a fragment of 1.061 bp in *C. glabrata*, 902 bp in *C. bracarensis* and 665 bp in *C. nivariensis*. However, this method allows us to differentiate between these species with no need for sequencing. The reaction was performed in a final volume of 50 µl containing 1X PCR buffer, 1.5 mM MgCl2, 1.6 mM dNTP, 0.1 µM each primer and 2 U Taq polymerase. The amplification protocol was as follows: 3 min at 95 °C; 3 cycles of 30 s at 95 °C, 30 s at 62 °C, and 30 s at 72 °C; 3 cycles of 30 s at 95 °C, 30 s at 58 °C, and 30 s at 72 °C; 3 cycles of 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C; 3 cycles of 30 s at 95 °C, 30 s at 50 °C, and 30 s at 72 °C; 35 cycles of 30 s at 94 °C, 30 s at 50 °C, and 30 s at 72 °C; and a final elongation of 10 min at 72 °C. Negative controls and distilled H_2O were run simultaneously to detect possible contamination in both the extraction and amplification steps. Amplicons were separated by electrophoresis in a 1.5% agarose gel stained with ethidium bromide (0.5 µg/ml) at 100 V. The method allowed DNA amplification for all *C. glabrata*, *C. nivariensis* and *C. bracarensis* strains and the *Saccharomyces cerevisiae* strain but not DNA amplification for *C. albicans*, *C. parapsilosis, C. tropicalis*, and *C. krusei* (Enache-Angoulvant et al. 2011).

DNA sequencing and phylogenetic analysis

To confirm the results of the Singleplex PCR method, direct sequencing of the RPL31 gene amplicons was performed using the same set of primers that were used in the PCR assay (Eurofins MWG Operon, Munich, Germany). The obtained sequences were edited using Chromas software version 2.33 (http://ww.technelysium.com. au/chromas.html) and identified by comparison with the nucleotide-nucleotide Basic Local Alignment Search Tool (BLAST) (GenBank DNA sequence database, National Centre for Biotechnology Information) (www.ncbi.nlm.nih.gov/blast/) to confirm phenotypic identification. Species assignment was considered complete when a match of 98% or more between our sequences and those in GenBank was found. DNA sequence-based analyses were performed using the maximum parsimony method. The tree topology was supported by 1000 bootstrap replicates to determine node reliabilities with MEGA x software (Kumar et al. 2018). The nucleotide sequences of *C. nivariensis* and *C. bracarensis* were obtained from GenBank (JF690246 and JF690247).

Results

Phenotypic and biochemical identification

All 163 clinical isolates identified and conserved as *C. glabrata* sensu lato were streaked on CHROMagar *candida* medium and produced pink colonies. These isolates were not able to form germ tubes,

chlamydospores, pseudohyphae or ascospores. Biochimically, all isolates assimilated only trehalose and glucose. However, identification based on colony morphology and carbohydrate assimilation was unsuccessful. For biochemical identification, only the fermentation of trehalose can distinguish *C. nivariensis* from *C. glabrata*.

Molecular identification

DNA extraction methods

As explained in the Materials and Methods section, genomic DNA was extracted with different methods. Analysis of extracted genomic DNA showed that the shorter duration was associated with the commercial DNA extraction kit and the chelating resin. An advantage of these extraction methods is that the purification requires no toxic phenol/chloroform steps or isopropanol/alcohol precipitation. Thus, these methods do not generate hazardous waste or do not require a fume hood to operate. On the other hand, all silica membrane kits require an ethanol supply. The PCI extraction method is the most time consuming method, requiring many different steps. Moreover, toxic substances such as chloroform (carcinogen), which require a fume hood, have to be used. The differences between the protocols of extraction are described in *Table 1*.

DNA quantity and purity

In this section, 2 clinical *C. glabrata* isolates (Ce23 and Ce28) were used for determination of the quantity and purity of DNA. Variation in the yield and purity of DNA was observed among different methods (*Table 2*). The highest DNA quantity in ng/µl was obtained by applying the Chelex method. The lowest measurements were measured for the commercial extraction kit (GF-1 Blood) and PCI.

PCR amplification

To evaluate the effect of the DNA extraction method on the quality of DNA, the DNA extracted from all methods was used for PCR amplification. As shown in *Fig. 1*, a single and pure intense band on the agarose gel was observed for DNA extracted from the commercial kit and chelating resin, which was absent with the PCI method.

Identification of *Candida* species

PCR amplification of the RPL31 gene of 163 clinical *C. glabrata* sensu lato isolates was performed (extracted with the chelating resin method). The agarose gel electrophoresis of the PCR product demonstrated well separated and consistent bands with no impurity bands, yielding an expected size amplicon of 1.061 bp with genomic DNA from the reference strain of *C. glabrata* sensu stricto (ATCC64677) (*Fig. 2*). No amplification was obtained in PCR with DNA from reference strains of *C. albicans*. All 163 clinical *C. glabrata* sensu lato isolates used in this study were identified as *C. glabrata* sensu stricto strains. None of the clinical isolates yielded an amplicon of 902 bp (characteristic of *C. nivariensis*) or 665 bp (characteristic of *C. bracarensis*). Thus, PCR data showed a lack of detection of *C. nivariensis* and *C. bracarensis* among 163 clinical *C. glabrata* sensu lato isolates in Monastir, Tunisia.

DNA sequencing and phylogenetic analysis

The obtained DNA sequences were compared to those deposited from the GenBank database. Phylogenetic analysis was performed to confirm the genetic relationship between species. The

topology of the phylogenetic tree confirmed that Tunisian isolates (Cg36RPL31F and Cg18RPL31F) identified as *C. glabrata* were grouped in the same clade as *C. glabrata* (CP048121, CR380950 and CP048233). *Candida nivariensis* (CJ690246) and *C. bracarensis* (CJ690247) were grouped in the same clade. *Candida albicans* (AB105200) was used as an out group.

Discussion

To the best of our knowledge, this is the first study on the discrimination between Tunisian species of the *C. glabrata* complex by a singleplex PCR assay (Enache-Angoulvant et al. 2011). Distinction between species of the *C. glabrata* complex (*C. nivariensis*; *C. bracarensis* and *C. glabrata* sensu stricto) is important for appropriate treatment and may be important for the management of patients infected with various *C. glabrata* look-alike species (Aznar-Marin et al. 2016).

In Tunisia, the presence of *C. nivariensis* and *C. bracarensis* is unknown because the identification of *Candida* spp. is performed on a routine basis associated with phenotypic methods such as API ID32C and API 20C AUX (Esposto et al. 2013; Hou et al. 2018; Treviño-Rangel et al. 2018). The conventional methods for yeast identification, which depend on a combination of morphological and biochemical features, consistently fail to identify two less common species of this complex, tentatively identifying them as *C glabrata* (Mirhendi et al. 2011; Cai et al. 2020). Generally, *C. nivariensis* and *C. baracarensis* exhibit lower susceptibility to triazoles and amphotericin B than *C. glabrata* sensu stricto isolates warranting species-specific identification of all clinical *C. glabrata* sensu lato isolates (Correia et al. 2006; Lockhart et al. 2009; Li et al. 2014; Angoulvant et al. 2016; Asadzadeh et al. 2019). In addition, *C. nivariensis* and *C. bracarensis* produce creamy white colonies on CHROMagar Candida medium, which is not sufficient for the diagnosis or/and identification of these two species strains as *C. norvegensis* and *C. inconspicu*, and some strains of *C. glabrata* also produce the same colonies (Bishop et al. 2008; Lockhart et al. 2009). All 163 *C. glabrata* isolates tested on CHROMagar Candida medium developed only pink-colored colonies ascribed to *C. glabrata* sensu stricto. Thus, CHROMagar Candida medium seems to be useful for the discrimination between closely related species of the *C. glabrata* complex but does not permit its morphological differentiation from other related species, such as *C. bracarensis*, *C. norvergensis* and *C. inconspicua* (Bishop et al. 2008). However, a PCR-based technique is needed for rapid and specific discrimination. Several molecular approaches were applied: sequencing the ITS region and the D1−D2 region of the 26S rRNA gene (Alcoba-Flórez et al. 2005; Bishop et al. 2008), fingerprinting profiles using GTG5 and M13 primers (Wahyuningsih et al. 2008), species-specific peptide nucleic acid fluorescence in situ hybridization (PNA FISH) (Bishop et al. 2008), pyrosequencing of the ITS2 region, and multiplex PCR (Asadzadeh et al. 2019). Singleplex PCR assays have also been described (Alcoba-Flórez et al. 2005; Enache-Angoulvant et al. 2011) for the detection of the *C. glabrata* complex in a single test. In

our current study, before the achievement of singleplex PCR, different methods of extraction were tested (GF-1 Tissue/Blood, Chelating resin and PCI) to obtain a good quantity and quality of DNA from a clinical sample. In fact, in molecular diagnostic laboratories, the quality of DNA extraction is the key evaluation criterion (Javadi et al. 2014). Genomic DNA extraction is an important procedure for both clinical and experimental purposes, and DNA can be isolated from various fresh or frozen clinical specimens. The comparison of four different extraction methods showed that the Chelex method is the fastest and easiest to handle approach tested in this study. Moreover, in contrast to other methods, it is ecologically friendly with no toxic chemicals. The chelating resin method produced good quality genomic DNA compared to the quality and yield of the other DNA extraction methods. The extracted DNA should be free of contaminants, including the least amount of proteins, carbohydrates, lipids, other nucleic acids (RNAs), other cellular constituents that may interfere with restriction enzymes, ligases and thermostable DNA polymerases or any other PCR inhibitors. Removing contaminants is an important key factor for successful PCR since the quality and integrity of the isolated DNA will directly affect the results of all subsequent procedures. The two commercial extraction methods tested use a silica membrane with spin columns. All these spin procedures are based on the same principle and involve four steps: lysis, binding, washing and elution. All protocols are very easy to achieve using standard equipment commonly available in most laboratories (Lienhard and Schäffer 2019). The GF-1 Tissue/Blood produced good quality genomic DNA (gDNA) with an appreciably greater yield compared to the quality and yield of the other methods of extraction. The next method is PCI, which is one of the oldest DNA extraction protocols. This protocol is difficult to handle because of several supernatant pipetting steps, which demand pipetting experience and use a specific solution such as sodium dodecyl sulfate (SDS) and Proteinase K for digestion. In the next step, phenol and chloroform/isoamyl alcohol denature proteins. The spin down yields an upper aqueous layer containing DNA and an organic layer containing the precipitated proteins. To remove the precipitated proteins, extraction must be continued. The highest concentration of salt is used, and next, two washes of ethanol precipitate DNA. Then, the sample is resuspended in a suitable reagent containing EDTA (Santella 2006). Although this method mostly gives pure gDNA, the toxicity of phenol and labor intensity should be carefully considered. Moreover, the presence of phenol minimizes the quantitation of DNA detected by UV absorbance since phenol shows a high extinction coefficient at 260 nm. Additionally, excessive SDS above 0.01% could inhibit PCR by denaturing Taq polymerase (Yang et al. 2008). Therefore, extracted DNA had to be diluted 1:10 with PCR water before PCR amplification. Another major disadvantage of the phenol–chloroform protocol is the use of highly toxic reagents, and more time is required to extract gDNA from the samples in comparison with commercial methods. The total time for each DNA extraction protocol is also important, especially when a large number of samples need to be tested (Chacon-Cortes et al. 2012). The chelating resin-based procedure is a simple, one tube, minimal step extraction process that requires very little time. The resin prevents DNA degradation by binding metal ions ($Mg2+$) that catalyze the breakdown of DNA. The absorbance of DNA was measured at 260 nm to evaluate the quantity of the extracted DNA, and the ratio of A260/A280 nm was used to evaluate the DNA purity. This method was employed previously by other researchers to compare different DNA extraction methods (Yang et al. 2008). Small changes in the pH of the DNA solution may lead to a variation in the A260/A280 ratio (Wilfinger et al. 1997). In our study, the result of the DNA yield and purity

of each method were correlated. The Chelex method seemed to yield a greater quantity of DNA, and their A260/280 ratio indicated a high purity of DNA extracted. On the other hand, the concentrations of the DNA obtained using the commercial kits and PCI methods were lower, but the A260/280 ratio showed a high purity of the DNA obtained. When applied to clinical isolates, the chelating resin resulted in 100% successful DNA amplification and provided the best results when compared with the other two methods. Considering all criteria, the chelating resin method had the overall best performance. The use of chelex-100 has been recommended for DNA extraction in some studies, while other reports have not regarded it as being optimum because of its lowest efficiency for DNA amplification (Desloire et al. 2006). However, one common problem using Chelex is the hypothesis that Chelex extracts are not stable and not suitable for long-term conservation (Hajibabaei et al. 2005). Nevertheless, a modified Chelex extraction protocol (without a boiling step and yielding double-stranded DNA) was shown to be suitable for long-term storage (Lienhard and Schäffer 2019).

Although there are methodological differences between our study and others (Desloire et al. 2006; Yang et al. 2008; Lienhard and Schäffer 2019), the conclusion is the same that this method is simple, inexpensive, harmless, and quick, providing a good quantity of DNA and efficient for PCR.

In this study, we failed to identify the presence of *C. nivariensis* and *C. bracarensis* among the 163 clinical *C. glabrata* sensu lato isolates, implying that these two cryptic species are not clinically important or prevalent in Tunisia. The findings are consistent with two studies (Angoulvant et al. 2016; Asadzadeh et al. 2019) that reported a lack and a low prevalence of 0.12% for *C. nivariensis* and 0.01% for *C. bracarensis* among phenotypically identified *C. glabrata* strains. In fact, eight countries implicating 2560 *C. glabrata* sensu lato isolates failed to identify the presence of any *C. nivariensis* or *C. bracarensis*, meaning that these species are few or absent yeast pathogens in some geographical locations (Lockhart et al. 2009; Mirhendi et al. 2011; Esposto et al. 2013; Asadzadeh et al. 2019). Studies by (Alcoba-Flórez et al. 2005; Arastehfar et al. 2019) demonstrated that *C. nivariensis* was isolated from blood samples and absent from other clinical samples, such as urine, vaginal swabs, BAL, sputum and stool.

Conclusion

In Tunisia, this is the first systematic study regarding the epidemiology and identification of *C. nivariensis* and *C. bracarensis* isolates. We used simple and rapid singleplex PCR and compared three different methods of extraction to obtain a good quantity of DNA. Only the chelating resin method demonstrated the overall best performance. Furthermore, our study highlights the lack of detection of *C. nivariensis* and *C. bracarensis* in Tunisia. Other studies are needed by increasing the numbers of samples to reinforce the molecular identification (such as MALDI-TOF techniques) of these two rare species. Moreover, studies from multiple geographical locations and additional data are required to better characterize the frequency, geographical distribution, susceptibility profiles and clinical features of infections due to *C. nivariensis* and *C. bracarensis*.

Declarations

Competing Interests: The authors declare no competing interests.

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Tables

Table 1 Comparison of different extraction methods examined in this study

Extraction step	Commercial extraction kit		Manual extraction	
	GF-1 Tissue	GF-1 Blood	Chelating resin (Chelex® 100)	PCI
Lysis buffer	Tissue buffer and proteinase K	Blood buffer and proteinase K	Resin	TNNT and proteinase K
Cell lysis and homogenization	Incubation at 65°C and vortexing	Incubation at 65°C and vortexing	Boiling	Incubation at 65°C and vortexing
Extraction and DNA precipitation	Column and absolute ethanol	Column and absolute ethanol	Heat	Phenol chloroform and cold absolute ethanol
Store in	BE buffer	BE buffer	BE buffer	BE buffer
Approximate time for completion	17 min	25 min	25 min	7 ½ h

PCI : Phenol Chloroform Isoamylalcohol

TE: Elution Buffer

Table 2 Concentration measurements (ng/µl) of DNA extracts and DNA purity by UV spectrometry (NanoDrop), obtained from two *C. glabrata* species

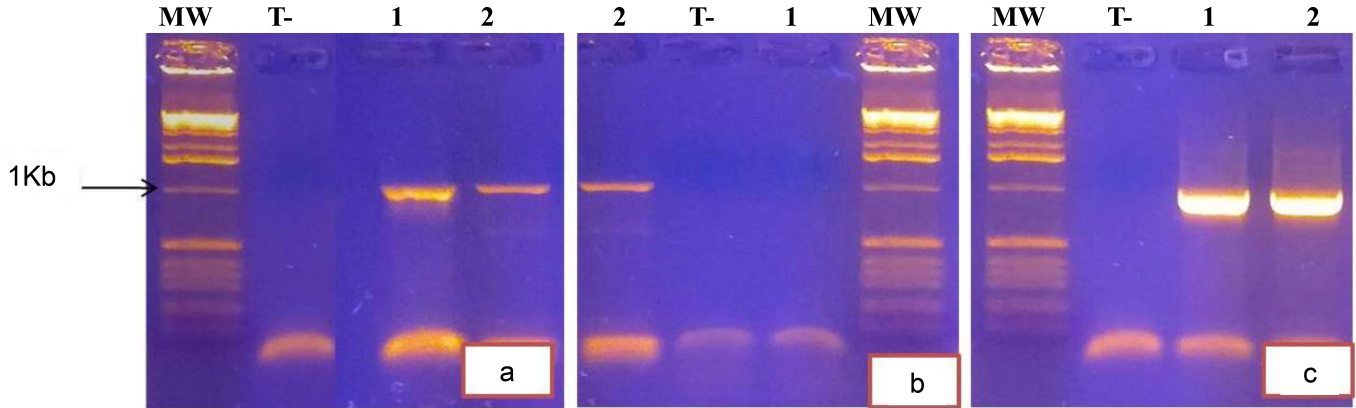| DNA extraction method	Isolat N°	DNA yield (ng/µl)	A260/280	A260/230	PCR product
GF-1 Blood	1	1.7	2.9	0.8	-
	2	3.9	1.8	2.2	+
GF-1 Tissu	1	44.8	1.95	1.87	+
	2	17.7	2.0	1.99	+
PCI	1	8	1.99	0.9	-
	2	3.7	1.96	1.0	-
Chelating resin	1	**58.1**	2.0	1.95	+
	2	**48.5**	1.98	1.49	+

Figures

Fig. 1 1.5% agarose electrophoresis gel of PCR products of DNA extraction methods. a: commercial extraction kit, 1 : GF-1Blood kit, 2 GF-1Tissue kit;
b: PCI; c: Chelex 100; T⁻ : Negative control ; MW : Molecular weight (1kb DNA Ladder, Promega®)

Figure 1

1.5% agarose electrophoresis gel of PCR products of DNA extraction methods. a: commercial extraction kit, 1 : GF-1Blood kit, 2 GF-1Tissue kit;

b: PCI; c: Chelex 100; T- : Negative control ; MW : Molecular weight (1kb DNA Ladder, Promega®)

Figure 2

Agarose gel electrophoresis of PCR amplified product of RPL31 gene from DNA samples which were extracted using Chelating resin method. T+: Straing of *C.glabrata* ; MW: Molecular weight 100 pb DNA ladder marker; N: Negative control

Figure 3

Maximum Parsimony analysis of *Candida glabrata* isolates and reference strains generated using RPL31 of rDNA sequences. Bootstrap values are shown above nodes. The *Candida albicans* isolate was used as the outgroup