# Clinical-oriented polyphasic identification, typing and susceptibility methodology for *Candida rugosa* isolates to establish potential virulence markers and processing guidelines.

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## Abstract

*Candida rugosa* is a rare as yet, though emerging pathogen, attracting increasing international interest. A collection of 10 clinical strains from the southern Balkan peninsula was subjected to polyphasic detection, identification and typing techniques, such as cultures in differential media, microscopy, assimilation tests, immunefluorescence, *in vitro* drug susceptibility (microdilution CLSI methodology and E-test) to multiple agents and both simple and amplification-based molecular techniques, such as electrophoretic karyotyping, RFLP-PFGE, PCR fingerprinting with mini- and microsatellite DNA, ribotyping and multiple PCR-RFLPs. Similarities and differences to other members of the genus were noted to determine appropriate identification, typing and treatment techniques and regimens for use in epidemiological studies and clinical applications, as the resulting *C. rugosa* profiles were in different degrees distinct but generally not dissimilar to other *Candida* species. TTC assimilation and Dalmau cultures allow identification, but typing is better achieved with electrophoretic karyotyping and specific PCR-RFLP, though the respective results are similar but not matching, indicating discontinuous intraspecific microevolutionary events.

**Keywords:** *Candida rugosa, TTC culture, bloodstream fungal infections, electrophoretic karyotyping, PCR-RFLP*

## Introduction

In the past two decades, an increased incidence in nosocomial bloodsteam fungal infections has occurred, witch are by now the fourth cause (8-15%) of nosocomial bloodstream infections (BSI) in USA and Europe. Among these infections, candidemia represent >50% with an overall attributable mortality of 38% (25, 26, 48).
Prior use of corticosteroides and broad-spectrum antibiotics, invasive procedures, and patient's status are major predisposing factors for development of candidemia in both, immunocompromised and immunocompetent patients.
Although *C. albicans* remains the most common cause of candidemia, infections due to non-*C. albicans* species have been increased dramatically in recent years worldwide (35-65%) , especially in patients with haematological malignancies, bone marrow recipients, intensive care unit (ICU) and surgical patients (16, 25, 43).
Among non-*C. albicans Candida* species, *C. rugosa* (a common animal pathogen) constitutes a rare but emerging cause of candidemia in humans, documented since 1985

only in a few case reports and an outbreak in ICU patients (4, 5, 10, 36, 42). Sugar et al (42), reported that the common risk factors for most of 18 fungaemia cases were burn wound and surgical nystatin prophylaxis. The majority of the strains tested were resistant to both nystatin and amphotericin B. Also, Dube et al, (10) reported 15 episodes of candidemia due to *C. rugosa* in a burn unit where topical nystatin use was associated with the increased fungaemia caused by nystatin-resistant *C. rugosa* isolates. A cluster of *Candida rugosa* candidemia involving six patients hospitalized in ICU was reported in Brazil with four of them dying, despite treatment with amphotericin B (13).
Other authors have reported that isolates of *Candida* species collected from 6082 bloodstream infections and from 145 candidemic patients included 5 and 8 strains of *C. rugosa* respectively (6**,** 28**).** Additionally, Pfaller et al (32) reported that 13 strains of *C. rugosa* (2.02%) were isolated from the blood specimens of 643 patients **,** while Ng KP et al (22), isolated 7 strains of *C. rugosa* from 1114 various clinical specimens: 3 from blood (4.1%), 2 from urine (3.8%) and 2 from skin (5.4%). Eventually, the common risk factors for most episodes of proved *C. rugosa* BSI were burn wounds, surgical nystatin prophylaxis and invasive medical procedures such as indwelling catheters, surgery and haemodialysis (4, 5, 10, 16).
Apart from a higher appearance (5%) in a recent report from Brazil (4), the incidence of *C. rugosa* candidemia worldwide is low (<1%) (16), concerning hospital- acquired infections not only in immunocompromised (36,42), but in immunocompetent patients as well (1, 4, 5, 10, 20, 24, 29, 46 ).

Identification of microorganism species and subspecies, as well as susceptibility to antifungal agents, are essential to understanding the epidemiology of candidemia, and have been well documented for the most common species (*C. albicans, C. parapsilosis, C. glabrata, C. tropicalis*), but there is a lack of information for the rare pathogens such as *C. lusitaniae, C. guilliermondii, C. rugosa, C. kefyr, C. famata* (30, 31).
There is strong evidence that *C. rugosa* strains show various degrees of *in vitro* resistance to amphotericin B and –less- to fluconazole (23%) and 5-flucytosine (15%), while new extended-spectrum triazoles (voriconazole, posaconazole, ravuconazole) demonstrate excellent *in vitro* activity (10, 16, 27, 32).
Although decreased *in vitro* susceptibility to amphotericin B has been related to poor clinical outcome in *Candida rugosa* BSI (17), there is a discrepancy in clinical response for susceptible *C. rugosa* isolates (MIC values ≤1 mg/L) (5), which may not be surprising since amphotericin B MIC values >0,5 mg/L tend to be associated with clinical failure in humans (39).
Given that *C. rugosa* is one of the emerging etiologic agents of candidemia, these results indicate the urgent need to understand the mechanisms of amphotericin B and other antifungal drugs' resistance in *C. rugosa* clinical isolates. To our knowledge, there is no published data so far, for possible mechanisms of drug resistance in *C. rugosa*. We also used six different molecular methods, in order to identify DNA subtypes of *C. rugosa* isolates.

## Materials and methods

*Organisms*

Ten *C. rugosa* clinical isolates were studied, 8 derived from hospital infections in Bulgaria and 2 in Greece. The majority (6 strains) were isolated from haematocultures of candidaemic ICU patients, 1 strain from prosthetic valve (ICU patient suffering from endocarditis), 1 from bronchoalveolar lavage fluid (ICU patient suffering from bronchopneumonia), 1 from urine culture and 1 from contact lens-induced corneal ulcer.

All isolates were identified as *C. rugosa* by microscopy, carbohydrate assimilation profile by API ID 32C (BioMérieux, France), pseudohyphae formation in Dalmau plate cultures (17), and by general morphology both on CHROMagar Candida™ (CHROMagar, Paris, France) and on solid medium containing 2,3,5-triphenyl-tetrazolium chloride (TTC) prepared as described previously (38), including proper standards where needed.
*C. rugosa* strains were examined by indirect immunofluorescence (IFA) with the anti-*C. dubliniensis* serum, courtesy of the developers' (2).

*Susceptibility testing*
Susceptibilities to amphotericin B, fluconazole, itraconazole, ketoconazole, 5-flucytosine and voriconazole were determined by E-test on RPMI 1640 agar and by the broth microdilution method according to the M27-A document of the Clinical Laboratory Standards Institute (CLSI- previously NCCLS) (21) and using the interpretive criteria for susceptibility to fluconazole, itraconazole and 5-flucytosine by Rex et al. (37) and a susceptibility breakpoint of ≤1 μg/mL for amphotericin B and voriconazole (30, 41).
The reference strains *C. albicans* 90028 and NCPF 3153A, *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258, obtained from the Public Health laboratory Service (Bristol, UK) were used as quality controls in broth microdilution assays.

***Molecular typing***
*I. Electrophoretic karyotype*
Agarose plugs containing intact chromosomes were prepared as previously described (9, 14) and run at (i) 120 sec pulse time for 10h at 4,5 V and at a reorientation angle 120$^{o}$, (ii) 240 sec pulse time for 16h at 4 V and at a reorientation angle 120$^{o}$ and (iii) 900 sec pulse time for 10h at 2,5 V and at a reorientation angle 106$^{o}$.
Identical plugs, treated with either *Sfi* I or *Not* I restriction endonucleases were prepared and run as described earlier (7, 9). All pulse field gel electrophoresis (PFGE) assays were performed five times, starting from fresh cultures each time, to ensure the stability of both karyotype and *Sfi* I/*Not* I restriction fingerprints (PFGE-RFLPs).

*II. PCR-based typing*
*C. rugosa* DNA was extracted with the Cheelex-100 method as described earlier (44) and used for (i) PCR ribotyping of the IGS region (34), (ii) PCR fringerprinting of mini- and microsatellite DNA with M13 and RY primers respectively (8, 18), (iii) PCR-RFLP of ITS-1 and ITS-2 rDNA regions with ITS-1/-2 and ITS-3/-4 primers respectively and digestion with *Msp* I restriction endonuclease (3) and (iv) PCR-RFLP of fungus-specific *L1A1* gene with P-450-1/-2 primers (19) and digestion with *Msp* I restriction endonuclease.
The generated profiles of PCR fingerprinting and ribotyping, were separated in 1,8% standard agarose gels (Sigma) in x 0,5 TBE stained with 1 mg/ml ethidium bromide, after electrophoresis for 2 h and viewed on a UV transillumination (Herolab, E.A.S.Y., Weisloch, Germany), while 2,5% gels were used for PCR-RFLP analyses.

## Results

Compared with the reference *C. tropicalis* and *C. dubliniensis* strains producing maroon and pink colonies respectively (17, 38), all *C. rugosa* reducing TTC presented variable

colony color ranging from white to pink (Fig 1a), a fact partly explained by the discovery of the *C. pararugosa* species.
On CHROMagar Candida™ (CHROMagar, Paris, France) all *C. rugosa* isolates were light purple, with slight differences of shade between them (Fig 1b), much like *C. lusitaniae* isolates (11).

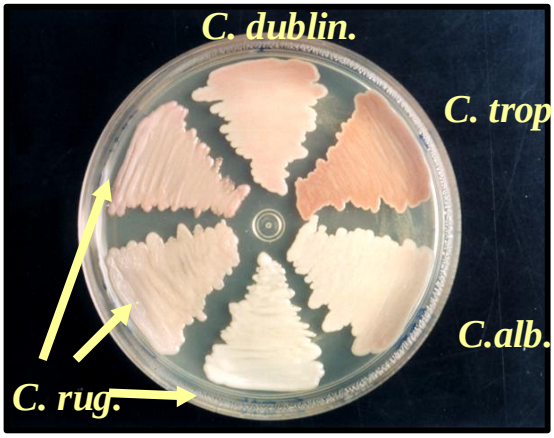


**Figure 1a.** TTC culture of *C. albicans*, *C. tropicalis*, *C. dubliniensis* and *C. rugosa* isolates.

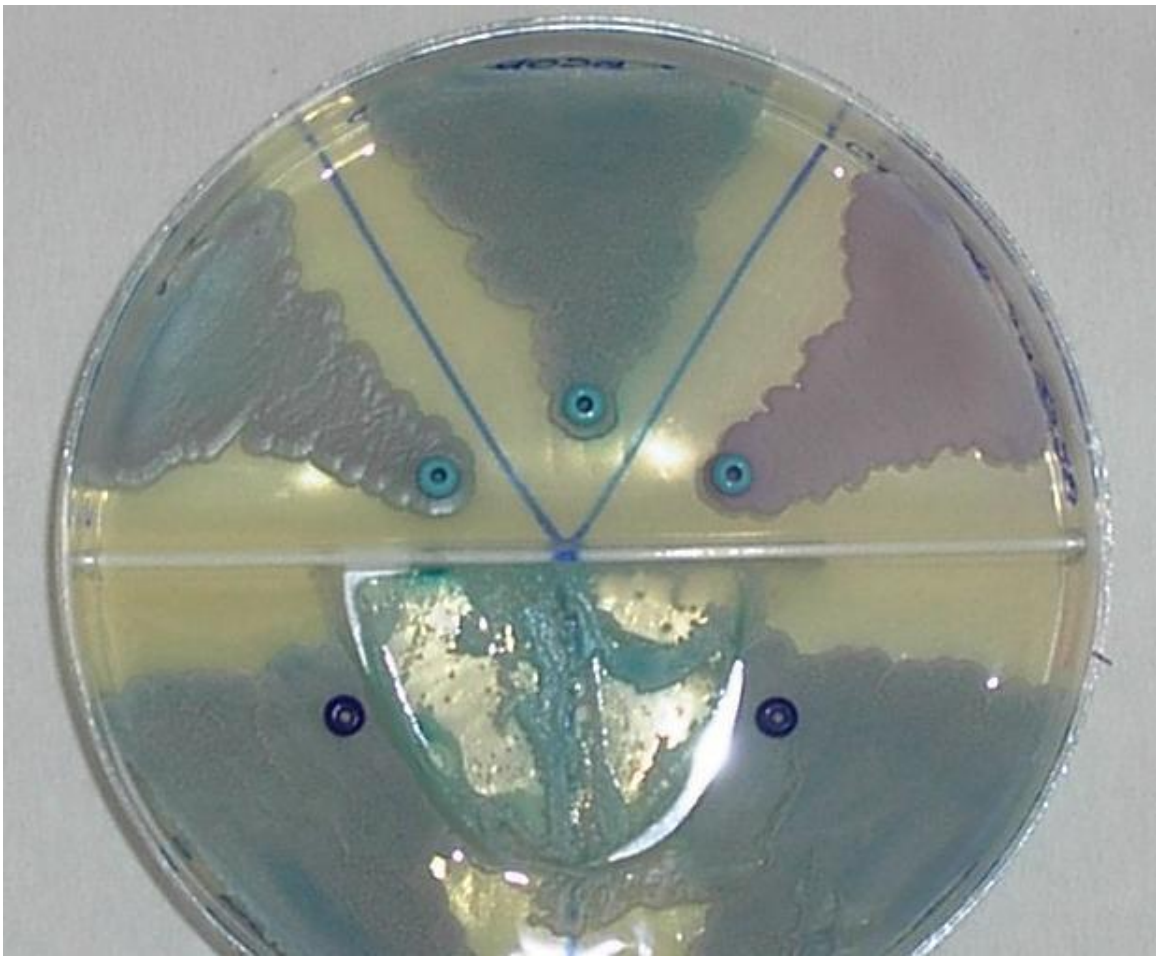

**Figure 1b.** *C. rugosa* isolates on CHROMagar Candida™ (CHROMagar, Paris, France)

Indirect immunofluorescence (IFA) with a rabbit polyclonal anti-*C. dubliniensis* antiserum proved all *C. rugosa* positive, with 80-90 % of the cells (including

pseudohyphae) being reactive with the antiserum, with high membrane and cytoplasmic adsorption (Figure 2). The expression of antigens reacting with the anti-*C. dubliniensis* serum is homogeneous in most cells of *C. dubliniensis* and *R. rubra* strains. Contrarily, it is very heterogeneous in the reacting cells (only 10% of the total) of the *C. krusei* strains studied (2). No other *Candida* species (*C. albicans, C. stellatoidea* types I&II, *C. glabrata, C. guilliermondii, C. parapsilosis, C. tropicalis*) show any reactivity.

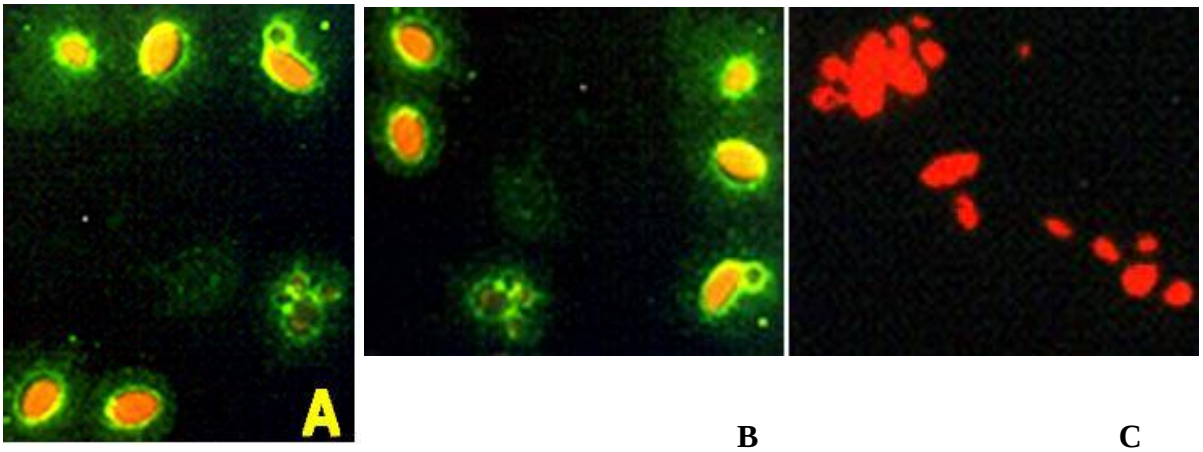


**Figure 2.** Indirect immunofluorescence (IFA) with a rabbit polyclonal anti-*C. dubliniensis* antiserum. A: *C. dubliniensis* (positive control) B: *C. rugosa* (positive reaction) C: *C. albicans* (negative control)

The microdilution method (Table 1) showed all *C. rugosa* strains susceptible to amphotericin B (MIC range 0,25-1 μg/ml), fluconazole (MIC range 2-8 μg/ml), itraconazole (MIC range 0,03-0,25 μg/ml), ketoconazole (MIC range 0,016-0,125 μg/ml) and voriconazole (MIC range 0,03-0,125 μg/ml.), while 7 of the 10 isolates were resistant to 5-fluorocytocine (30, 41).

**Table 1.** MIC values of *C. rugosa* strains

| Strain | Amphotericin B | Fluconazole | Itraconazole | Ketoconazole | 5-Fluocytocine | Voriconazole |
|---|---|---|---|---|---|---|
| 1 | 0,5 | 2 | 0,03 | 0,016 | >64 | 0,03 |
| 2 | 0,5 | 2 | 0,06 | 0,016 | >64 | 0,03 |
| 3 | 0,5 | 2 | 0,06 | 0,016 | >64 | 0,03 |
| 4 | 0,5 | 2 | 0,03 | 0,016 | >64 | 0,06 |
| 5 | 0,5 | 2 | 0,06 | 0,016 | >64 | 0,03 |
| 6 | 0,5 | 2 | 0,06 | 0,016 | >64 | 0,03 |
| 7 | 0,5 | 4 | 0,06 | 0,060 | 0,5 | 0,03 |
| 8 | 0,5 | 2 | 0,06 | 0,016 | >64 | 0,03 |
| 9 | 1 | 4 | 0,125 | 0,125 | 0,5 | 0,25 |
| 10 | 0,25 | 8 | 0,25 | 0,125 | 0,5 | 0,125 |

The molecular typing methods showed different suitability for *C. rugosa* strains. Best results were obtained with PFGE karyotyping, (Fig 3) were 3 subtypes of 7, 2 and 1 strains were discriminated, containing 6, 3 and 7 chromosomes respectively sized 950 kbp <>2.500 kbp. The greek strains were autonomously clustered in the second subtype with 3 chromosomes, whereas the majority of the Bulgarian strains (7/8) had 6 chromosomes and consisted subtype A, the other one being typed alone, with 7

chromosomes. The numbers are more or less in accordance with literature, both regarding the chromosome numbers (9) and the average type size/content [<4 isolates (35)].

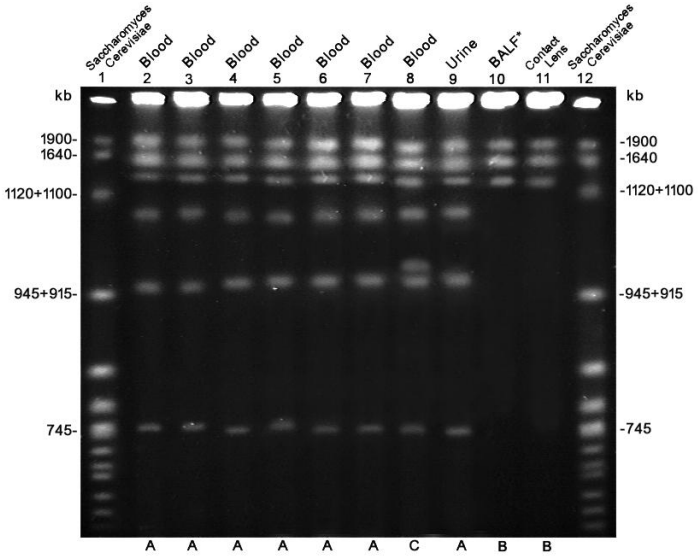


**Figure 3.** *C. rugosa* isolates' PFGE karyotyping. Subtypes (3) marked at the bottom end.

PFGE after restriction digestion with *Sfi* I (Fig 4) revealed only two types: one for the greek and the other for the Bulgarian strains, with 10-14 bands of 45<>450 kbp.

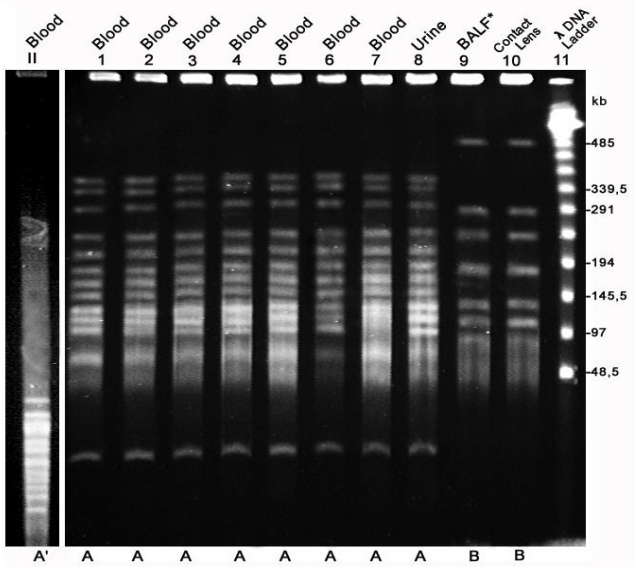


**Figure 4.** *C. rugosa* isolates' PFGE-RFLP with *Sfi* I. Subtypes (2) marked at the bottom end.

The PCR methods were also ambiguous, but stable. Both MLP typing and PCR-RFLP targeted on the ITS-1/-2 regions clustered all strains together. On the other hand, PCR-RFLP with P450-1/-2 primers and *Msp* I restriction endonuclease (Fig 6) and ribotyping with IGS L/R primers (Fig 5) produced 3 types each, matching in discriminatory power the electrophoretic karyotype. It must be noted that the clustering was different in all three cases.

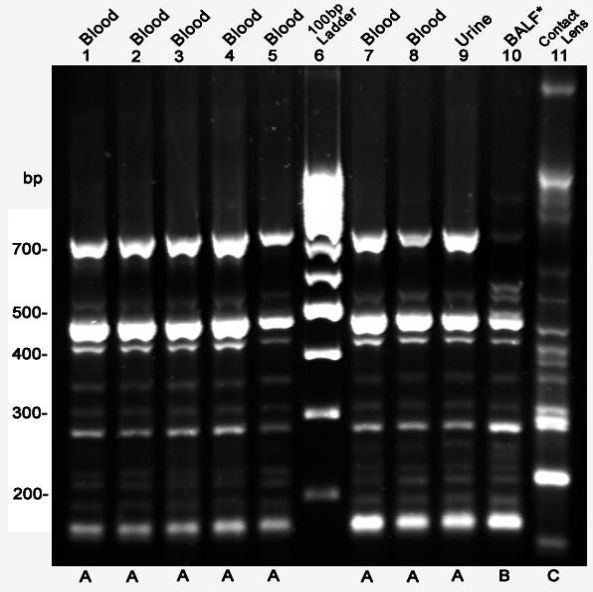


**Figure 5.** Ribotyping using SL-SR primer pair targeted on the IGS sequence. Subtypes (3) marked at the bottom end.

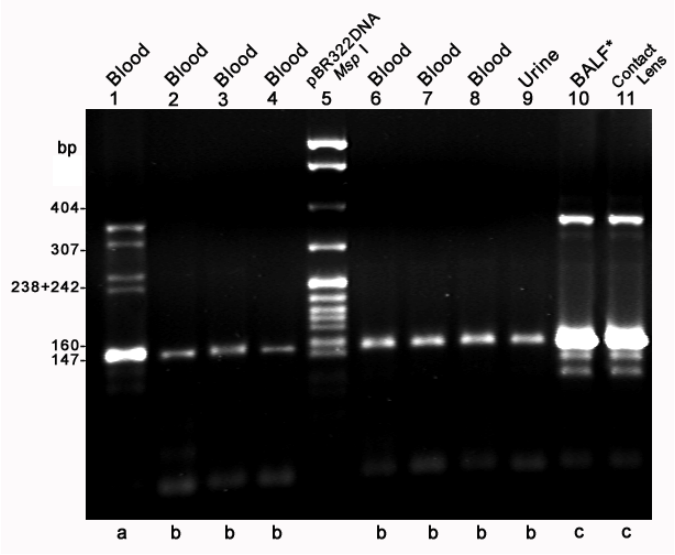


**Figure 6.** Typing with PCR-RFLP using $P450_{1/2}$ primer pair targeted on the *L1A1* gene and *Msp* I restriction endonuclease. Subtypes (3) marked at the bottom end.

## Discussion

*C. rugosa* isolates are still a rarity, as no study includes more than 26, whereas the identification is usually performed by microscopic morphology and by biochemical assays such as API ID 32C. We studied phenotypic characters up to now underappreciated in the literature, hoping to establish a correlation between clinical characteristics and simple and fast phenotypic assays such as the reduction of TTC (Fig 1), up to now encountered exclusively in *C. tropicalis* and *C. dubliniensis* among *Candida* species.

*C. rugosa* demonstrate fluorescence distinctive from that of *C. dubliniensis*. Strains with increased MICs to amphotericin B showed low voriconazole MIC values (Table 1). Susceptibilities of *C. rugosa* strains to ketokonazole were studied to test the *in vitro*

activity of an imidazole-derived agent, as possible alternative antifungal therapy in future. No differences were identified in the ITS region or in the minisatellite sequences, while one Bulgarian and one Greek strain showed polymorphism in the *L1A1* gene sequences. Ribotyping proved to be a rapid typing method giving comparable results to those obtained by PFGE of *Sfi* I and *Not* I digested chromosomes.
Electrophoretic karyotyping revealed differences in the number of chromosomes between Bulgarian and Greek strains, but the sample is too small to substantiate any geographical grouping. One Greek strain, exhibiting high frequency switching, was not of a stable ribotype or *Sfi* I / *Not* I PFGE types after testing consecutive subcultures of distinct phenotypes. The difference in grouping of PCR and electrophoretic karyotyping, which both showed identical discriminatory power and outperformed the other molecular typing methods (as noted by Dib *et al* -9- previously), imply a rather complex, disrupted and possibly multifocal mechanism of microevolution within the species, allowing no predictions about the genetic predisposition of the species.

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# Πολυφασική μελέτη ταυτοποίησης, τυποποίησης και προσδιορισμού ευαισθησίας στελεχών για προσδιορισμό δεικτών λοιμογονικότητας και ανάπτυξη εργαστηριακών διαδικασιών κλινικής εφαρμογής.

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## Περίληψη

Η *Candida rugosa* είναι ακόμη σπάνιος παθογόνος παράγων αλλά η επίπτωσή της βαίνει αύξουσα, με αποτέλεσμα σημαντικό διεθνές ενδιαφέρον. Δέκα κλινικά στελέχη της νοτίου βαλκανικής εξετάστηκαν πολυφασικά, με τεχνικές συμβατικής και μοριακής μυκητολογίας. Έγιναν καλλιέργειες σε διαφοροποιητικά υλικά, έλεγχοι αφομοίωσης, μικροσκοπική εξέταση, ανοσοφθορισμός, εν δοκιμίω εξετάσεις αντοχής σε αντιμυκητιακά (τόσο με τη μέθοδο των μικροαραιώσεων όσο και με E-test) και ηλεκτροφορητικός καρυότυπος, ανάλυση περιοριστικών πέψεων σε ηλεκτροφόρηση παλλομένου πεδίου, περιοριστικές πέψεις σε προϊόντα PCR, αποτύπωση ίχνους μικροδορυφορικού και μινιδορυφορικού DNA και ριβοτυπία. Οι ομοιότητες και διαφορές με τα αποτελέσματα άλλων ειδών του γένους σημειώθηκαν για να επιλεγούν οι μέθοδοι με την μεγαλύτερη δυναμική για επιδημιολογικές μελέτες και κλινικές εφαρμογές. Η *Candida rugosa* επέδειξε γενικά διακριτά αλλά όχι ασύμβατα αποτελέσματα σε3 σχέση με άλλα είδη του γένους. Οι καλλιέργειες σε υλικά Dalmau και TTC επιτυγχάνουν ταυτοποίηση, ενώ για τυποποίηση βέλτιστες λύσεις είναι ο ηλεκτροφορητικός καρυότυπος και οι περιοριστικές πέψεις σε συγκεκριμένα προϊόντα PCR, με παρόμοια αλλά όχι συμβατά αποτελέσματα, γεγονός που υποδεικνύει ασυνεχή μικροεξελικτικά γεγονότα εντός του είδους.

**Λέξεις ευρετηρίου:** *Candida rugosa, καλλιέργεια σε TTC, μυκηταιμία, ηλεκτροφορητικός καρυότυπος, PCR-RFLP*