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Fluorescent Capillary Electrophoresis Is Superior to Culture in Detecting *Candida* Species from Samples of Urinary Catheters and Ureteral Stents with Mono- or Polyfungal Biofilm Growth

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ABSTRACT Molecular techniques in fungal detection and identification represent an efficient complementary diagnostic tool which is increasingly used to overcome limitations of routinely used culture techniques. The aim of this study was to characterize *Candida* sp. representation in samples from urine, urinary catheter, and ureteral stent biofilm using *ITS2* ribosomal DNA (rDNA) amplification followed by fluorescent capillary electrophoresis (f-*ITS2*-PCR-CE) and to compare the results with those obtained by culture. A total of 419 samples were analyzed, and 106 (25.2%) were found positive, out of which 17 (16%) were polyfungal. The positivity rate did not differ between samples from catheters and stents (23.6% versus 20.9%) or between catheter and stent corresponding urine samples (40.2% versus 30.2%). Ten different *Candida* species were detected, with *Candida parapsilosis* (31.4%), *Candida albicans* (26.5%), and *Candida tropicalis* (12.4%) predominating. f-*ITS2*-PCR-CE was evaluated as substantially less time-consuming and 8.3 times more sensitive than the routinely applied culture technique with 1 μl of urine/sonicated fluid inoculated, detecting 67 (19.9%) versus 8 (2.4%) positive samples out of 337 initially analyzed samples. The culture sensitivity considerably improved to 1.7 times lower than that of f-*ITS2*-PCR-CE after the inoculation volume was increased to 100 μl in the additional 82 samples. Moreover, the molecular technique, unlike routine cultivation, enabled precise pathogen composition determination in polymicrobial samples. In conclusion, the f-*ITS2*-PCR-CE method was shown to be a quick and efficient tool for culture-independent detection and identification of fungi in urinary tract-related samples, demonstrating a higher sensitivity than culture.

KEYWORDS *Candida*, biofilms, capillary electrophoresis, fungi, panfungal PCR, polyfungal sample, ureteral stents, urinary catheters

Ureteral stents and urinary catheters are valuable tools in urological practice although they offering, like all synthetic medical intracavity devices, an ideal surface for microbial colonization (1). They can be colonized by a wide range of microorganisms, including both bacteria and fungi, usually forming a community or multispecies biofilm. Colonization usually does not affect the patient's clinical status in any significant way. On the other hand, it can be an important source of infection, and biofilms may be critical for clinical infection development (2). Colonization has gained importance mainly due to an increasing number of immunocompromised patients who are particularly susceptible to infections. *Candida* spp. are the most frequently detected fungal agents in urinary tract samples. However, in many instances, a clinical laboratory

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report indicates that candiduria represents colonization or procurement specimen contamination rather than an invasive infection (3).

For many years, *Candida albicans* has been a predominant fungal species isolated from the urinary tract. With the increasing access and use of fluconazole, non-*albicans Candida* (NAC) species have emerged, and they can even dominate *C. albicans* in some patients (3). However, the most frequently occurring fungus is still *C. albicans*, followed by *Candida glabrata*, *Candida tropicalis*, and *Candida krusei* (4).

Traditionally, diagnosing *Candida* urinary tract infections has relied on clinical signs and symptoms and on microbiological urine culture, which is still the recommended method of choice (5). Species identification is based mostly on phenotypic features and is usually time-consuming as classical diagnostic work flow takes up to several days (6). Cultivation-independent approaches based on DNA isolation are superior to cultures in rapidity and in identifying species which cannot be easily grown and/or distinguished by a routine phenotypic approach. In order to characterize polyfungal samples, several molecular techniques, employed mainly for environmental material testing in the field of molecular ecology, can be applied. These community profiling techniques include, for instance, denaturing/temperature gradient gel electrophoresis (DGGE/TGGE), single-strand conformation polymorphism (SSCP), terminal restriction fragment length polymorphism (T-RFLP), cloning (7), or fluorescent capillary electrophoresis (CE) (8). The last method, based on utilizing the variability of the *ITS2* ribosomal DNA (rDNA), has been proven to be superior to classical electrophoresis for analyzing DNA due to its higher separation efficiency, speed, and higher sensitivity and the fact that there is no need for sequencing (8, 9).

Therefore, the aim of this study was to characterize *Candida* occurrence and species prevalence in the urinary catheter and ureteral stent biofilm and in urine samples using fluorescent *ITS2* rDNA amplicon detection by capillary electrophoresis (f-*ITS2*-PCR-CE) and to validate this approach using a comprehensive set of clinical samples.

MATERIALS AND METHODS

Determining reference polymicrobial samples. A combination of different concentrations (as the number of CFU/milliliter) of seven clinical *Candida* species, *C. albicans*, *C. glabrata*, *C. tropicalis*, *Candida parapsilosis*, *C. krusei*, *Candida kefyr*, and *Candida lusitaniae*, were used to determine the ability of capillary electrophoresis to evaluate more than one fungal strain in a sample. Therefore, two fungal species were mixed in equal ratios or in 1:10, 1:100, and 1:1,000 ratios in triplicates; DNA was extracted as described below, and samples were evaluated by f-*ITS2*-PCR-CE. Accurate cell concentration was determined by assessing the CFU count on Sabouraud agar plates (Himedia, India) supplemented with vancomycin (5 mg) and amikacine (20 mg/liter) after 48 h of cultivation at 37°C.

Sample collection and clinical material preparation. Urinary catheters (C; $n = 127$), ureteral stents (S; $n = 67$), and corresponding urine samples (U-C, $n = 101$; U-S; $n = 64$), were collected from 161 patients (125 males and 36 females; median ages, 76.0 and 65.5 years, respectively) hospitalized in the Department of Urology, St. Anne's University Hospital, Brno, Czech Republic, during the years 2012 to 2014, irrespective of patient diagnosis or underlying disease, as performed previously by others (10), except for the consecutive sampling approach in our study ($n = 196$). Thus, both infectious and noninfectious samples were included. In total, 419 samples were analyzed (Table 1). Catheter removal was based on a urologist's decision. The study was approved by the Ethics Committee of the St. Anne's University Hospital in Brno; no informed consent was required because neither human cells nor human tissues were processed, and no procedure in addition to standard care was performed.

Stents and catheters were aseptically removed from the body; 5-cm-long tips (both the proximal and distal parts of the stents and the distal part of the catheters) were snipped off and placed into sterile tubes with 5 ml of brain heart infusion (BHI) medium for sonication. The sonication procedure was based on a previously described protocol (11) and consisted of two 5-min sonications interspaced by 2 min of vortexing. In parallel, urine samples obtained through the catheter or stent before their removal were also treated aseptically. Urine collection was impossible in 29 and 3 sampling cases of catheters and stents, respectively, because of patients' oligo-anuria at the time of collection. In 3 U-C cases, urine without a catheter was collected. Sonicates and urine samples were subsequently cultured and, in parallel, used for direct DNA extraction.

Phenotypic identification. The suspension (1 μl in 337 samples and 100 μl in an additional 82 samples) was inoculated to blood agar (with 7% of sterile sheep blood; Columbia Blood Agar Base, Oxoid, United Kingdom), UriSelect 4 (Bio-Rad, France), and Sabouraud agar (Himedia, India) supplemented with vancomycin (5 mg) and amikacine (20 mg/liter) and cultured for 48 h and with Sabouraud agar for 1 week at 37°C to isolate individual strains. All isolated strains were identified at the species/genus level using ChromAgar *Candida* (CHROMagar, France), conventional biochemical tests (Micro-LA-tests; Lachema,

TABLE 1 Detection of fungi using f-*ITS2*-PCR-CE[a]

Material[b]	No. (%) of samples				
	Total analyzed	Negative	Positive	Monofungal[c]	Polyfungal[c]
C	127	97 (76.4)	30 (23.6)	26 (86.7)	4 (13.3)
U-C	101	60 (59.4)	41 (40.6)	30 (73.2)	11 (26.8)
S[d]	127	111 (87.4)	16 (12.6)	16 (100)	0 (0)
U-S	64	45 (70.3)	19 (29.7)	17 (89.5)	2 (10.5)
Total	419	313 (74.8)	106 (25.2)	89 (84)	17 (16)

[a]The table shows an overview of molecular detection in clinical samples. *C. robusta* detected in sonication fluids (material C and S) was evaluated as a contaminant and was excluded from the results. These results also include three non-*Candida* fungal species, identified by sequencing as *Geotrichum candidum*, *Rhodotorula rubra*, and *Cryptococcus neoformans*, in 2 U-C, 1 U-C, and 1 polyfungal U-C sample, respectively. *Candida* was detected in 8 samples (3 *C. parapsilosis*, 3 *C. albicans*, 1 *C. tropicalis*, and 1 *C. famata* isolate) out of 29 and 2 (*C. albicans*) out of 3 catheter and stent samples without a corresponding urine sample, respectively. In 3 U-C cases without a corresponding catheter sample, no *Candida* was detected.

[b]C, urinary catheter; U-C, urine from patients with catheter; S, ureteral stent; U-S, urine from patients with stent.

[c]Percentage of positive samples.

[d]Data for the stent category are based on the results of 127 unique samples. In the disjunction of results of proximal and distal tip samples, there were 14 (20.9%) *Candida* sp. positive stents; no stent was polyfungal.

Czech Republic; API bioMérieux, France). Culture results were provided in a quantified manner as a number of CFU per urinary catheter or urinary stent and urine sample.

To verify ambiguous results, matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS) analysis, not easily available for routine use at the time of sample collection, was performed. A MALDI Biotyper with FlexControl, version 3.4, software (Bruker Daltonik) was used according to the manufacturer's instructions. The manufacturer-recommended cutoff scores were used for identification, with scores of $\geq$2.000 indicating identification to the species level, scores between 1.700 and 1.999 indicating identification to the genus level, and scores of $<$1.700 indicating no identification.

DNA extraction. The genomic DNA from clinical specimens was extracted by a QIAamp DNA Blood Mini kit (Qiagen, Germany), according to the manufacturer's instructions with the following modification. Briefly, 2 ml of the urine sample and 1 ml of the catheter or stent sonicate were centrifuged at 23,000 rpm for 20 min and at 14,000 rpm for 10 min, respectively. The supernatant was removed, and the sediment was incubated with 50 μl of lysis buffer (500 mM EDTA, pH 8.0, 1 M Tris, pH 8.0, 43.2 μl/ml Triton X-100), 20 μl of lysozyme (180 mg/ml; Sigma-Aldrich, USA), and 20 μl of lyticase (5 U/μl; Sigma-Aldrich, USA) for 60 min at 37°C and treated according to the manufacturer's instructions. DNA samples were stored at −20°C. Sterile DNA-free water (Qiagen, Germany) was used as a specimen to check for possible contamination during the extraction step.

PCR amplification by using a fluorescently labeled primer (f-*ITS2*-PCR). The internal transcribed fungal genomic rDNA spacer region *ITS2* was amplified using the previously described panfungal primers UNF1 (5′-GCATCGATGAAGAACGTAGC-3′) and UNF2 (5′-AACTATACGAATTCAAGTCGCC-3′). Due to the final amplicon detection with capillary electrophoresis, a UNF1 primer 5′ fluorescently labeled with a 6-carboxyfluorescein (6-FAM) dye was used. PCR was performed in a total volume of 20 μl of HotStarTaq Master Mix (Qiagen, Germany) with 0.5 μM of each primer and 2 mM final Mg^{2+} concentration, using cycling conditions as described previously (8). A negative control of both DNA extraction (see above) and amplification (sterile DNA-free-water as a template) was used in each PCR run.

Capillary electrophoresis (f-*ITS2*-PCR-CE). FAM-labeled *ITS2*-PCR amplicons were separated and detected in terms of their size using capillary electrophoresis on an ABI Prism 3130 Avant Genetic Analyser (Life Technologies, USA). The instrument was adjusted according to the manufacturer's instructions.

One microliter of PCR amplicon was mixed with 0.25 μl of a GeneScan 500 6-carboxy-X-rhodamine (ROX) dye size standard (Life Technologies, USA) and 9.75 μl of highly deionized (HiDi) formamide (Life Technologies, USA). After 2 min of denaturation at 95°C and quick freezing at −80°C, the samples were injected into a 36-cm capillary column containing the POP-7 (Life Technologies, USA) high-performance polymer. Electrophoretic parameters were set at 16-s injection times, 1.2-kV injection voltages, 15-kV electrophoresis voltages, and 60°C oven temperature. Finally, PCR product lengths were analyzed using GeneMapper, version 4.1, software. A successful analysis was derived from the red peaks being assigned to the ROX-labeled size standard GeneScan 500 (Life Technologies, USA).

The fungi present in each sample were evaluated by comparing *ITS2* lengths with our previously prepared internal laboratory database of the reference strain *ITS2* lengths. Identification was considered successful if the analyzed sample length appeared within a 0.3-nucleotide (nt) interval of the respective reference strain's length (8).

Data interpretation and statistical analysis. For positivity rate evaluation and data set characteristics (Table 1), proximal and distal stent tip samples were handled as independent ones. For *Candida* sp. prevalence evaluation and statistics addressing clinical material, a disjunction of proximal and distal stent tip results was applied. A statistical analysis employing a two-tailed Fischer's exact test was performed

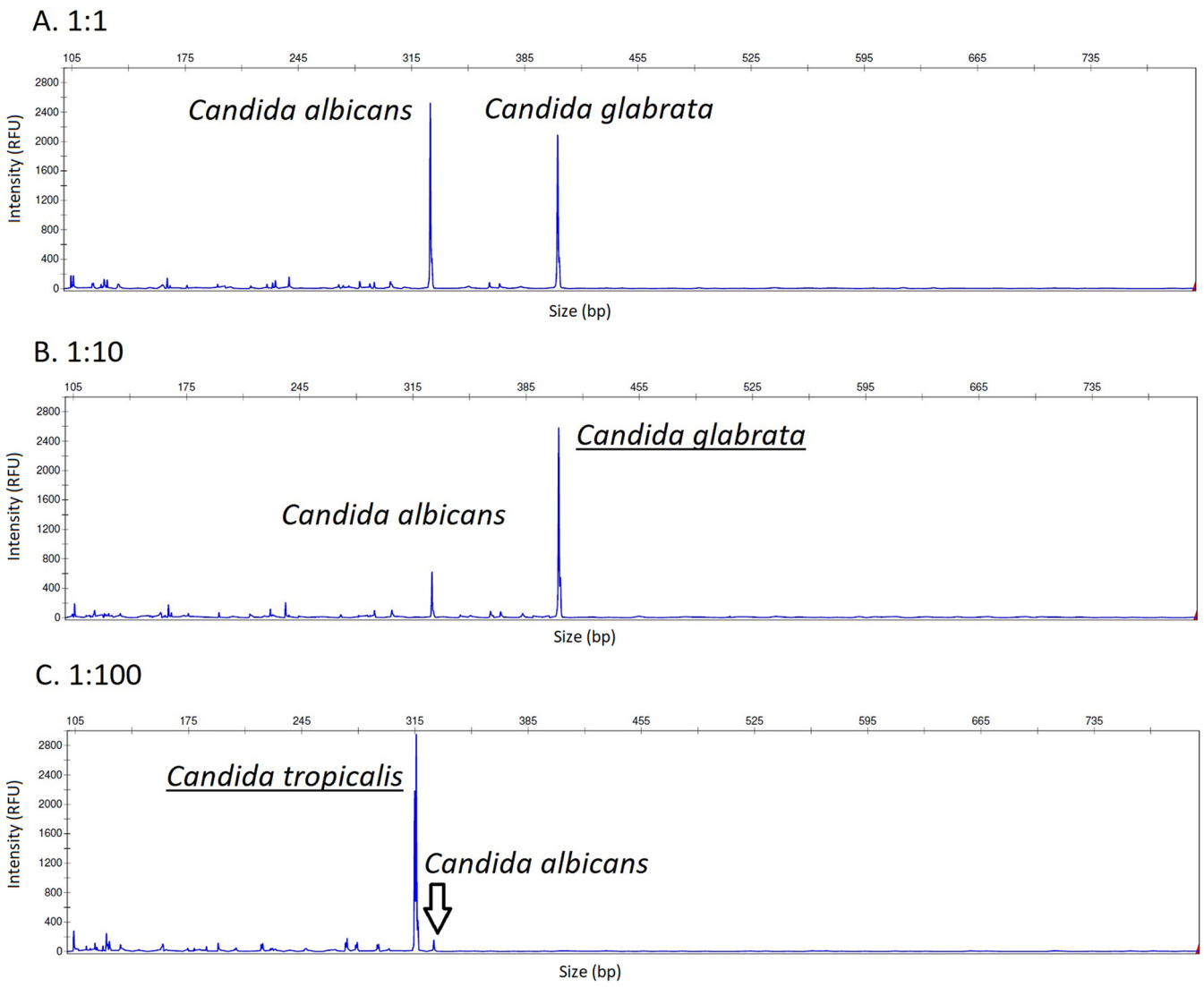

FIG 1 f-*ITS2*-PCR-CE analysis of mixed *Candida* sp. populations with different CFU ratios at the beginning of DNA extraction. It was possible to differentiate two *Candida* spp. if present in equal concentrations (A) or 10-fold-different concentrations (B). Only the major species was reliably identified if its concentration was 100-fold higher (C). Major species are underlined in panels B and C. RFU, relative fluorescence units. The range of the *x* axis is set to 100 to 800 bp.

to test categorical data. Nonparametric analysis of variance (ANOVA) was used to test mutually species prevalence in catheter- and stent-related urine samples and in catheters and stents separately.

***ITS2* sequencing.** The representative *ITS2* rDNA amplicons were separated on a 2% agarose gel, excised, purified using a QIAquick Gel Extraction kit (Qiagen, Germany) according to the manufacturer's instructions, and sequenced as described previously (12).

RESULTS

Evaluation of artificial polyfungal samples. At first, we examined cultivation-independent f-*ITS2*-PCR-CE performance in detecting individual *Candida* sp. in cell suspensions containing various mixed populations of two different species. Each sample was assessed three times. The results showed that both fungal species were detected in all samples if present in equal or 10-fold different concentrations, while only the major species was identified if the concentration difference was 100-fold or higher (Fig. 1).

Analysis of clinical specimens. In total, 106 out of 419 (25.3%) of the C, U-C, S, and U-S samples were positive using cultivation-independent f-*ITS2*-PCR-CE (Table 1 provides a detailed description). Among the positive samples, 89 (84.0%) were monofungal; the remaining samples contained more than one fungal pathogen (Tables 1 to 3).

TABLE 2 Summary of *Candida* species prevalence[a]

Identified species	No. (%) of positive samples[b]				
	C	U-C	S[c]	U-S	Total
C. parapsilosis	14 (40)	17 (34.7)	1 (7.1)	6 (28.6)	38 (31.4)
C. albicans	10 (28.6)	8 (16.3)	7 (50.0)[d]	6 (28.6)	32 (26.5)
C. tropicalis	6 (17.1)	7 (14.3)	1 (7.1)	1 (4.8)	15 (12.4)
C. robusta	0[e]	8 (16.3)	0[e]	3 (14.3)	11 (9.1)
C. krusei	1 (2.9)	1 (2.0)	4 (28.6)[d]	2 (9.5)	9 (7.4)
C. famata	2 (5.7)	4 (8.2)	1 (7.1)	1 (4.8)	8 (6.7)
C. lusitaniae	1 (2.9)	2 (4.1)	0	0	3 (2.5)
C. glabrata	1 (2.9)	1 (2.0)	0	0	2 (1.7)
C. lipolytica	0	1 (2.0)	0	1 (4.8)	2 (1.7)
C. dubliniensis	0	0	0	1 (4.8)	1 (0.8)
Total	35	49	14	21	121

[a]The table shows the prevalence of *Candida* spp. detected by f-*ITS2*-PCR-CE in clinical samples, regardless of mono- or polyfungal infection. *C. robusta* detected in sonication fluids (materials C and S) was evaluated as a contaminant and was excluded from results. *Candida* was detected in 8 samples (3 *C. parapsilosis*, 3 *C. albicans*, 1 *C. tropicalis*, and 1 *C. famata* isolate) out of 29 and 2 samples (*C. albicans*) out of 3 catheter and stent samples without a corresponding urine sample, respectively. In 3 U-C cases without a corresponding catheter sample, no *Candida* was detected.

[b]C, urinary catheter; U-C, urine from patients with catheter; S, ureteral stent; U-S, urine from patients with stent.

[c]Results for stents represent a disjunction of results from stent proximal and distal tip samples.

[d]In one case of *C. albicans* and *C. krusei*, respectively, representatives were detected in the proximal and distal tip samples.

[e]*C. robusta* was excluded as a contaminant if detected in catheter or stent sonicate.

With respect to different clinical materials, surprisingly, the positivity rate did not differ between samples from either the catheters and stents (23.6% versus 20.9%; $P = 0.72$) or between catheter- and stent-corresponding urine samples (40.2% versus 30.2%; $P = 0.24$), as has been reported in bacteria previously (13). No stent was found to be polyfungal. We detected 10 different fungal species, with 38 *C. parapsilosis* (31.4%), 32 *C. albicans* (26.5%), and 15 *C. tropicalis* (12.4%) strains predominating (Table 2). Correct species identification was confirmed by *ITS2* sequencing in all representative *Candida* species (Table 2). Moreover, due to the absence of non-*Candida* species in our

TABLE 3 Characterization of polyfungal samples identified by f-*ITS2*-PCR-CE[a]

Identified species[b]	No. (%) of positive samples[c]		
	C	U-C	U-S
C. albicans > *C. parapsilosis*		1	
C. albicans > *C. robusta*		1	
C. famata > *C. lusitaniae*		1	
C. famata > *C. robusta*		1	
C. krusei > ***C. albicans***			1
C. lipolytica > *C. tropicalis*		1	
C. parapsilosis > ***C. albicans***	1		1
C. parapsilosis > *C. tropicalis*		2	
C. tropicalis > *C. glabrata*	1	1	
C. robusta > ***C. albicans***		1	
C. robusta > *C. famata*		1	
C. parapsilosis > ***C. albicans***	1		
C. albicans > *C. parapsilosis*		1	
C. famata > *C. parapsilosis* > *C. lusitaniae*	1		
Total	4 (23)	11 (65)	2 (12)

[a]The table shows the characterization of polyfungal samples identified by f-*ITS2*-PCR-CE. *C. robusta* detected in sonication fluids (materials C and S) was evaluated as a contaminant and was excluded from the results. *C. albicans* is in boldface, and polyfungal-tending NAC species (species with two or more times higher prevalence in polyfungal than mono-fungal samples) are underlined. No cooccurrence of *C. albicans* with polyfungal-tending NAC species is depicted.

[b]Species in the same line are in order of decreasing signal.

[c]C, urinary catheter; U-C, urine from patients with catheter; U-S, urine from patients with stent. No stent was found to be polyfungal.

library, sequencing was also used to identify three detected non-*Candida* species: *Geotrichum candidum* was identified in 2 U-C samples, and *Rhodotorula rubra* and *Cryptococcus neoformans* were each identified in one U-C sample. No statistical difference was observed in species prevalences mutually between catheters and stents or between catheter- and stent-corresponding urine samples.

We identified 17 polyfungal samples with 35 representatives (Tables 1 and 3). *C. glabrata* was identified exclusively in polyfungal samples. *Candida famata*, *Candida lipolytica*, and *C. lusitaniae* showed more than 2-fold-higher prevalence in poly- than monofungal samples. Interestingly, these four polyfungal-tending NAC species were detected exclusively together with other NAC species in six polyfungal samples. In seven polyfungal samples containing *C. albicans*, apart from the previously mentioned NAC species, such as *C. parapsilosis*, *Candida robusta*, or *C. krusei*, were detected. Conversely, *C. krusei* prevalence was more than three times higher in mono- than polyfungal samples, and *Candida dubliniensis* was detected exclusively in one monofungal sample. Unsurprisingly, the highest detection rate of more than one species in a sample was observed in 11 out of 41 positive catheter urine samples (26.8%).

To be comprehensive, the most frequently identified species from urinary catheters and ureteral stents was *Candida robusta* (*Saccharomyces cerevisiae*) (data not shown). However, when samples with BHI medium as a negative control were analyzed, we observed that the species' DNA had been present in the medium. Therefore, this species was evaluated as contamination coming from the BHI medium used to sonicate solid material samples. Thus, sonicates containing only *S. cerevisiae* were evaluated as negative, and *S. cerevisiae* was not considered an additional agent in any positive catheter or stent sample.

f-*ITS2*-PCR-CE comparison with phenotypic identification. The same clinical samples were also analyzed by culture-dependent phenotypic identification, considered a gold standard. However, using this technique in a routine setting with just 1 μl of sonicate fluid/urine inoculation volume, we identified only eight strains (2.4% of the total number of 337 analyzed samples) belonging to four different species. Thus, an 8.3-fold-lower sensitivity than that with f-*ITS2*-PCR-CE (67 out of 337 samples positive, 19.9%) was achieved. *C. albicans* was found in four samples (50%), two catheters and two stents; *C. krusei* was identified in two stent samples (25%); and *C. parapsilosis* and *C. tropicalis* were each identified only once (12.5%), with both found in catheters. The fungal population reached 10^3, 10^4, and 10^5 CFU/ml, in 4, 3, and 1 positive sample, respectively.

In order to increase the sensitivity of classical culture techniques, since the culture positivity rate was lower than expected, 100 μl of sonication fluids and urine instead of the routinely used 1 μl was inoculated in an additional 82 clinical samples. Then, 23 samples (28.0%) were culture positive, while 39 (47.6%) were positive using f-*ITS2*-PCR-CE analysis (Table 4), indicating a much improved sensitivity, but culture sensitivity was still 1.7-fold lower than that of the molecular approach. An overall higher f-*ITS2*-PCR-CE positivity rate, regardless of the material type, in this additional sample set (compare 39.5%, 64.5%, 22.3%, and 50% of positive samples of additionally tested samples to 16.9%, 31.3%, 11.9%, and 28.3% positive samples of those initially tested in C, U-C, S, and U-S groups, respectively) is probably related to the different nature of samples because no difference was registered in the collection strategy or f-*ITS2*-PCR-CE analysis.

Correspondingly, more polyfungal samples (17.4% of culture-positive samples) were found when a 100-μl inoculation volume was applied than with a 1-μl inoculation volume (0.0%), while 23.1% of positive samples were polyfungal using f-*ITS2*-PCR-CE. However, in only 1 of 4 polyfungal samples was the second pathogen identified to species level by culture (Table 4).

When the consistency of identification between both methods was considered, 56 out of 82 samples (68.3%) were in full concordance (43 samples were concordantly negative), 10 samples (12.2%) were concordantly positive but discrepant or partially

TABLE 4 Comparison of culture and f-*ITS2*-PCR-CE results by using a higher inoculation volume for culture (100 μl)

	Culture method result		f-*ITS2*-PCR-CE result		No. (%) of positive samples[b]			
Result type	Identification[a]	No. (%) of samples tested	Identification[a]	No. (%) of samples tested	C	U-C	S	U-S
Concordant	Negative	43	Negative	43	23	11	7	2
	C. tropicalis	5	*C. tropicalis*	5	3	2		
	C. albicans	6	*C. albicans*	6	2	1	2	1
	C. parapsilosis	2	*C. parapsilosis*	2	1	1		
Partially concordant	*C. albicans* > *Candida* sp.	2	*C. albicans*	2	1	1		
	C. albicans > *Candida* sp.	1	*C. albicans* > *C. parapsilosis*	1		1		
	Candida sp.	2	*C. parapsilosis*	2	1	1		
	Candida sp.	1	*C. parapsilosis* > *C. albicans*	1	1			
	C. tropicalis	2	*C. tropicalis* > *C. glabrata*	2	1	1		
	C. albicans > *C. glabrata*	1	*C. parapsilosis* > *C. albicans*	1	1			
	Rhodotorula rubra	1	Non-*Candida* sp.[c]	1		1		
Discrepant	Negative	16	*C. albicans*	3	2			1
			C. famata	2	1	1		
			C. lusitaniae	1		1		
			C. parapsilosis	5	1	4		
			C. robusta	1		1		
			C. famata > *C. lusitaniae*	1		1		
			C. parapsilosis > *C. tropicalis*	1		1		
			C. robusta > *C. albicans*	1		1		
			C. robusta > *C. famata*	1		1		
Total		82		82	38	31	9	4
Negative		59 (72)		43 (52)	23	11	7	2
Positive		23 (28.0)		39 (47.6)	15 (39.5)	20 (64.5)	2 (22.3)	2 (50)
Polyfungal[d]		4 (17)		9 (23)				

[a]Species in the same line are in order of decreasing signal.
[b]C, urinary catheter; U-C, urine from patient with catheter; S, stent; U-S, urine from patient with stent.
[c]Identified as *Rhodotorula rubra* by sequencing.
[d]Number (percentage) of polyfungal samples out of positive samples.

discrepant at species level identification, and the remaining 16 samples (19.5%) were culture negative but PCR positive (Table 4). Importantly, there was no sample that was culture positive and f-*ITS2*-PCR-CE negative.

DISCUSSION

We optimized the method for *ITS2* rDNA length polymorphism analysis using fluorescent capillary electrophoresis (f-*ITS2*-PCR-CE) and evaluated the technique's efficiency in our previous study (8), and we now proceeded to evaluate its applicability for urinary catheter, ureteral stent, and urine clinical samples. This technique enabled us to distinguish 26 out of 29 tested medically important *Candida* species. This method is usable for closely related species which are difficult to phenotypically distinguish, such as *C. dubliniensis* and *C. albicans* or *Candida fabianii* and *Candida pelliculosa*. *Candida guilliermondii, Candida fermentati*, and *Candida carpophila* were indistinguishable because of identical amplicon lengths. Amplicon length varied from 229 nucleotides (*C. lipolytica*) to 420 nucleotides (*C. kefyr*) (8).

Catheters and stents are valuable tools in urologic practice, but colonizing microorganisms might concurrently be a source of serious infection (1). To the best of our knowledge, here we provide the most comprehensive study focused on *Candida* identification in urinary catheters and ureteral stents, alongside corresponding urine samples, both by culture-dependent and culture-independent techniques. In addition, we compared the contribution of molecular culture-independent f-*ITS2*-PCR-CE for fungal detection and *Candida* sp. identification in clinical samples with culture-dependent phenotypic identification.

Candida species identification is mostly based on phenotypic features. Although

urine culture is more time-consuming than the molecular-based methods and is not a very sensitive approach, it is considered a standard, widely used method to detect stent colonization (14, 15). In our previous study we reported f-*ITS2*-PCR-CE as a sensitive and efficient tool for culture-independent identification of a substantial number of clinically important *Candida* species that was also applicable to polyfungal specimens. Moreover, it was shown to be fast, making a difference of more than 3 h compared with the time required for panfungal PCR followed by sequencing, the method currently used in the clinical setting in our laboratory (8). This study confirmed the higher sensitivity of f-*ITS2*-PCR-CE than that of a culture approach even though the sensitivity of cultivation was substantially improved by using 100-fold-higher volume of sonicate or urine than was routinely applied. Using f-*ITS2*-PCR-CE, we detected considerably more fungal species than by cultivation followed by phenotypic identification (19.9% versus 2.4% and 47.6% versus 28.0%, when 1 μl and 100 μl of urine/sonicated fluid, respectively, were inoculated). The detection limit of a cultivation-dependent approach in this setting was roughly 10^3 CFU/ml. Thus, f-*ITS2*-PCR-CE had high sensitivity and could therefore be applied to rapidly detect fungi in clinical urological specimens.

Of note, the BHI medium used to sonicate different clinical materials was largely contaminated by *C. robusta* DNA (*S. cerevisiae*), identified by f-*ITS2*-PCR-CE not by cultivation. A possible explanation is the presence of yeast extract DNA in BHI broth after inactivation of the living cells. For this purpose, we suggest using another medium for sonication, such as Ringer solution or phosphate-buffered saline (PBS)/phosphate buffer, as reported previously by (10, 16).

Higher f-*ITS2*-PCR-CE sensitivity was probably also caused by detection of DNA fragments of floating or dead fungal cells and by the multicopy nature of *ITS2* rDNA in the genome. Both of these probable causes raise the question of the clinical relevance of *Candida* sp. detection and identification.

Candida spp. are often considered contaminants, and verifying their presence does not prove clinical impact (4). On the other hand, they might be a source of serious infection, particularly in immunocompromised patients (17). Kauffman et al. (18) stated that *Candida* growth in urine represents a spectrum of states, including external perineal colonization, catheter infection, cystitis, or even secondary seeding from an undetected bloodstream infection. A debate exists about the threshold of microorganism concentration of 104 versus 105 CFU/ml in urine that may potentially be used as a criterion for treating candiduria (18). One of the limitations of culture-independent f-*ITS2*-PCR-CE analysis is that it is impossible to determine the cell concentration in the sample because of the difficulty of standardization (calibration) caused by different genomic *ITS2* rDNA copy numbers in various fungal species. However, quantitation may be achieved by combining the f-*ITS2*-PCR-CE and cultivation-based approaches. Detecting nonliving cells still may be of diagnostic value, particularly in culture-negative samples analyzed from treated patients.

Numerous authors (19–22) reported that the most frequently occurring fungal microorganism in clinical materials is *C. albicans*, followed by *C. glabrata*, *C. tropicalis*, and *C. krusei*. We detected mostly *C. parapsilosis* (31.4%), *C. albicans* (26.5%), and *C. tropicalis* (12.4%), followed by *C. robusta* (9.1%) and other NAC species. The high prevalence of saprophytic urinary tract commensals (*C. parapsilosis* and *C. robusta*) is not surprising because our sampling strategy did not consider each patient´s diagnosis. f-*ITS2*-PCR-CE showed the capability to distinguish even between species of *C. parapsilosis* complex (8); therefore, we do not assume any misidentification. Biofilms are critical for developing clinical infection. *C. albicans* and *C. parapsilosis*, which were often identified in analyzed materials, have lower incidences of fluconazole, voriconazole, amphotericin B, and echinocandins resistance than other NAC species (23–26). As NAC specie resistance together with prevalence in infections is increasingly being reported (22), NAC species detection and identification must not be neglected. Of note, fungi identified in this study (including *R. rubra* and *G. candidum*) are common urinary tract pathogens or commensals.

We did not detect *C. glabrata* on stents and catheters or in urine samples, except in two polyfungal samples (Tables 2 and 3), although its higher adherence to epithelial

cells and silicone than other NAC species and *C. parapsilosis* or *C. tropicalis*, respectively, was reported (22, 23). In contrast to these findings, Shin et al. (27) reported lower *C. glabrata* biofilm-forming capabilities than other NAC species after culture in nutritionally rich medium.

In addition, unlike cultivation, f-*ITS2*-PCR-CE enabled more efficient polyfungal sample composition analysis. Although the culture-based polyfungal sample detection rate improved by increasing the inoculation volume, the capacity to distinguish individual pathogens in complex samples remained low; both strains were identified to the species level in just 1 out of 4 samples. Wolcott et al. (28) stated that it is important to identify all of the species present in biofilm because minor microbial constituents can provide a multitude of different advantages to their neighbors, including increased virulence. However, it should be pointed out that f-*ITS2*-PCR-CE does not allow minor species identification in polymicrobial samples present in a 100-fold-lower concentration than major species (Fig. 1).

We recognized groups of *Candida* spp. according to poly- or monofungal sample inclinations. *C. glabrata*, *C. famata*, *C. lipolytica*, and *C. lusitaniae* can be suspected as polyfungal-tending species because they were present in polyfungal samples more often or even exclusively. Of note, these four NAC species were not detected together with *C. albicans* in any case. Mutually opposed higher *C. krusei* prevalence in stents and higher *C. parapsilosis* prevalence in catheters (Table 2) could imply that *C. krusei* has an inhibitory effect on *C. parapsilosis*, a phenomenon well described in cases of *C. krusei* and *C. albicans* (29, 30). The possible inhibitory effect of *C. krusei* on other NAC species is supported by a 3-fold-higher prevalence in monofungal than in polyfungal samples. This hypothesis needs to be supported by further investigations.

In this study, we provided an example of the previously reported f-*ITS2*-PCR-CE method used in urinary tract-associated samples. This technique was found to be more sensitive and more specific than routine culture both in mono- and polyfungal samples. We suggest further improvement of f-*ITS2*-PCR-CE by prospectively broadening the in-house database of non-*Candida* fungi distinguishable by the unique *ITS2* length and including an internal amplification control.

Further studies are desired to clarify and identify the *Candida* sp. colonizers, their mutual relationships, and involvement in infection.

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