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ARTICLE

Multidrug-resistant endemic clonal strain of *Candida auris* in India

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Abstract *Candida auris* is a recently described rare agent of fungemia. It is notable for its antifungal resistance. A total of 15 *C. auris* isolates, originating from seven cases of fungemia, three cases of diabetic gangrenous foot, and one case of bronchopneumonia from a tertiary care hospital in south India, were investigated. All of the 15 isolates were identified by sequencing and 14 of these along with 12 *C. auris* isolates previously reported from two hospitals in Delhi, north India, two each from Japan and Korea were genotyped by amplified fragment length polymorphism (AFLP). In vitro antifungal susceptibility testing (AFST) was done by the Clinical and Laboratory Standards Institute (CLSI) broth microdilution method. *Candida auris* isolates were misidentified as *Candida haemulonii* by VITEK. All were resistant to fluconazole [geometric mean minimum inhibitory concentration (MIC) 64 μg/ml] and 11 isolates were resistant to voriconazole (MIC ≥1 μg/ml). Forty-seven percent of the *C. auris* isolates were resistant to flucytosine (MIC ≥64 μg/ml) and 40 % had high MIC (≥1 μg/ml) of caspofungin. Breakthrough fungemia developed in 28.6 % of patients and therapeutic failure in 4 (66.7 %) patients. Interestingly, the 26 Indian *C. auris* isolates from north and south India were clonal and phenotypically and genotypically distinct from Korean and Japanese isolates. The present study demonstrates that *C. auris* is a potential emerging pathogen that can cause a wide spectrum of human mycotic infections. The prevalence of a *C. auris* endemic clonal strain resistant to azoles and other antifungals in Indian hospitals with high rates of therapeutic failure in cases of fungemia is worrisome.

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Introduction

Candida auris was identified as a new species in 2009 from the external ear canal of a Japanese patient [1]. This pathogen cannot be identified by commercial methods used for yeast identification. It has recently been described as an agent of fungemia, so far reported only from Korea and India [2, 3]. Sequence analysis of the D1/D2 domain of 26S rDNA and internal transcribed spacer (ITS) regions of nuclear rRNA genes of *C. auris* demonstrates a close phylogenetic relationship to *Candida haemulonii*, with which it is usually misidentified by commercial identification systems such as VITEK 2 and API 20C [2–4]. Moreover, *C. auris* is reported to be resistant to fluconazole and exhibits reduced susceptibility to voriconazole and itraconazole [2–4]. Further, persistent fungemia occurs in patients despite fluconazole and amphotericin B therapy, resulting in fatal outcome [3]. This pathogen has recently assumed a greater clinical significance due to the local prevalence and transmission of drug-resistant clonal strains in healthcare units [2, 4, 5]. Herein, we report *C. auris* as an etiologic agent of fungemia and its isolation from deep sterile sites in patients from a tertiary care hospital

in south India. Our results indicate that the strains of *C. auris* exhibiting resistance to azole antifungal agents, from both north and south India, were clonal.

Materials and methods

Fungal isolates and their morphological and physiological characterization

The study analyzed 245 yeast isolates from cases of fungemia and other invasive mycosis cultured during the period November 2011 to June 2013 at a university hospital in Kochi in southern India. Of the 245 yeast isolates, 15 (6.1 %) isolates, initially identified as *C. haemulonii* by VITEK® 2 Compact (bioMérieux, Marcy I'Etoile, France), were confirmed as *C. auris* by ITS and D1/D2 sequencing, and were included in the present study. The phenotypic identification was based on the colony color of the isolates on CHROMagar™ Candida medium (Difco, Becton, Dickinson and Company, Baltimore, MD, USA), germ tube test, and morphology on rice Tween 80 agar. Growth of the isolates at 37, 42, and 45 °C was also tested.

Sequencing of ITS and D1/D2 regions

Amplification of the ITS region of the ribosomal subunit and D1/D2 region of the large ribosomal subunit was done, followed by sequencing and alignment of both the strands using the Sequencing Analysis 5.3.1 software (Applied Biosystems, Foster City, CA, USA), as described previously [6–8]. GenBank Basic Local Alignment Search Tool (BLAST) searches (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi) were performed for species identification. For phylogenetic analysis, we included ITS and D1/D2 sequences of the recently reported 12 new clonal strains of *C. auris* prevalent in two hospitals in northern India (CBS 12766–12777), two Korean isolates, and a solitary Japanese isolate [1–3]. Sequences were aligned using the ClustalW program (v1.82) and the final alignments were edited manually.

Amplified fragment length polymorphism (AFLP)

Of the 15 south Indian *C. auris* isolates, the genotypic diversity of 14 isolates was determined together with the previously described clonal north Indian ($n=12$), Japanese ($n=2$), and Korean ($n=2$) isolates using amplified fragment length polymorphism (AFLP) fingerprint analysis [9]. *Candida auris* reference isolates (KCTC 17809, KCTC 17810, JCM 15448, and DSMZ 21092), *C. haemulonii* (CBS 7801, CBS 7802, CBS 5149, and CBS 5140), *C. duobushaemulonii* (CBS 7798–7800 and CBS 9754), and *C. pseudohaemulonii* (KCTC 1787, CBS 10004, and JCM 12453) were used as controls to assign the AFLP genotypes. Briefly, approximately 50 ng of genomic DNA was subjected to a combined restriction–ligation procedure containing EcoRI and MseI restriction enzymes (New England Biolabs, Beverly, MA, USA) and complementary adaptors. Prior to further usage, the restriction–ligation reaction was diluted by adding 80 μl Tris/HCl (pH 8.3) buffer. One microliter of the diluted product was used for amplification in a final volume of 25 μl, for which the selective primers EcoRI (5′-FLU-GACTGCGTACCAATTC AC-3′) and MseI (5′-GATGAGTCCTGACTAAC-3′) were used. One microliter of the 10× diluted amplicon was added to a mixture of 8.9 μl water and 0.1 μl LIZ600 internal size marker (Applied Biosystems), followed by heating the diluted sample for 1 min to 95 °C and subsequent fragment analysis on an ABI 3500xL Genetic Analyzer (Applied Biosystems). Raw data were analyzed using Bionumerics v6.0 (Applied Maths, Sint-Martens-Latem, Belgium) and a dendrogram was generated using standard Pearson and unweighted pair group method with arithmetic mean (UPGMA) settings.

In vitro antifungal susceptibility testing (AFST)

Antifungal susceptibility testing (AFST) was performed using the Clinical and Laboratory Standards Institute (CLSI) broth microdilution method, following the M27-A3 guidelines [10]. The antifungals tested were amphotericin B (Sigma, St. Louis, MO, USA), fluconazole (Pfizer, Groton, CT, USA), itraconazole (Lee Pharma, Hyderabad, India), voriconazole (Pfizer), posaconazole (Merck, Whitehouse Station, NJ, USA), isavuconazole (Basilea Pharmaceutica, Basel, Switzerland), flucytosine (Sigma), caspofungin (Merck), micafungin (Astellas, Toyama, Japan), and anidulafungin (Pfizer). CLSI-recommended *Candida krusei* ATCC6258 and *Candida parapsilosis* ATCC22019 were used as quality control strains with each test. The minimum inhibitory concentration (MIC) end points for azoles and echinocandins were defined as the lowest drug concentration that caused a prominent decrease in growth (50 %) vis-à-vis the controls and read visually after 24 h, as validated recently by Pfaller et al. [11, 12]. For amphotericin B, the MIC was defined as the lowest concentration at which there was 100 % inhibition of growth compared with the drug-free control wells. The geometric mean (GM) and MICs for each drug at which 50 % (MIC_{50}) and 90 % (MIC_{90}) of the tested isolates were inhibited were also determined. Newly revised clinical breakpoints (CBPs) defined for *C. albicans* were used for the interpretation of MIC data. These are described as susceptible (S) ≤2 μg/ml, susceptible dose dependent (SDD) 4 μg/ml, resistant (R) ≥8 μg/ml for fluconazole; S ≤0.12 μg/ml, SDD 0.25–0.5 μg/ml, R ≥1 μg/ml for itraconazole; S ≤0.12 μg/ml, intermediate (I) 0.25–0.5 μg/ml, R ≥1 μg/ml for voriconazole; and S ≤0.25 μg/ml, I 0.5 μg/ml, R ≥1 μg/ml for echinocandins [13]. Epidemiological cutoff values (ECVs) were used for amphotericin B (≤2 μg/ml),

Table 1 Clinical summary of 12 patients with *Candida auris*-associated infections from University Hospital Kochi, India, 2011–2013

Case no.	1	2	3	4	5	6	7
Age/sex	87/F	80/F	79/M	30/M	20/M	55/M	66/M
Diagnosis	Disseminated papillary thyroid carcinoma	POVD[a], CKD[b], DM[c]	POVD, CKD, COPD[d], DM	NHL[e], b/l[f] hydronephrosis	IgA nephropathy, CKD, bronchopneumonia	Pituicytoma, sepsis, ARF[g], hypocortisolism, DM	Aneurysm of arch of aorta
Sample	Blood	Excised tissue	Excised tissue	Blood	BAL[m]	Blood, CVC[n] tip	Blood
Risk factors							
Immunosuppression	Yes	Yes	Yes	Yes	Yes	Yes	No
Neutropenia ($<10^9$ cells/L)	No	No	No	Yes	No	Yes	No
CVC	Yes	Yes	No	Yes	Yes	Yes	Yes
Broad-spectrum antibiotics	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Parenteral nutrition	Yes	No	No	Yes	Yes	Yes	Yes
Surgery within 30 days	Yes	Yes	No	No	No	Yes	Yes
Intensive care	Yes	Yes	No	Yes	Yes	Yes	Yes
Antifungals within 30 days	No	No	No	No	Yes	No	Yes
Concomitant bacteremia	No	Not done	Not done	Yes	No	Yes	Yes
Indwelling urinary catheter	Yes	Yes	No	Yes	Yes	Yes	Yes
Day of isolation	Day 13	Day 6	Day 7	Day 18	Day 25	Day 19	Day 49
Therapy (days)	NA	Amputation	Amputation	FLU[o] (3 days)	FLU (10 days), CAS[p] (14 days)	FLU (8 days)	FLU (11 days) followed by CAS (24 days)
CVC removal	NA	No	No	Yes	No	Yes	Yes
Breakthrough fungemia	No	No	No	No	No	No	Yes
Outcome	NA[q]	Survived	Survived	Expired	Survived	Expired	Survived

Case no.	8	9	10	11	12
Age/sex	79/M	48/F	69/M	2/M	60/M
Diagnosis	Aortic stenosis, sepsis, ARF	RHD[h]	POVD, NHU[i], DM	Short bowel syndrome, intestinal perforation, pneumonia, sepsis	IE[j], HF[k], CVA[l], bacterial meningoencephalitis, DM
Sample	Blood, pus	Blood	Excised tissue	Blood, CVC tip	CVC tip
Risk factors					
Immunosuppression	No	No	Yes	Yes	Yes

Table 1 (continued)

Case no.	8	9	10	11	12
Neutropenia ($<10^9$ cells/L)	No	No	No	No	No
CVC	Yes	Yes	No	Yes	Yes
Broad-spectrum antibiotics	Yes	Yes	Yes	Yes	Yes
Parenteral nutrition	Yes	Yes	No	Yes	No
Surgery within 30 days	Yes	No	Yes	Yes	No
Intensive care	Yes	Yes	Yes	Yes	Yes
Antifungals within 30 days	Yes	Yes	Yes	No	No
Concomitant bacteremia	Yes	Yes	No	Yes	Yes
Indwelling urinary catheter	Yes	Yes	Yes	Yes	Yes
Day of isolation	Day 42	Day 5	Day 21	Day 39	Day 48
Therapy (days)	FLU (15 days) followed by AMB[p] (1 day)	FLU (7 days)	FLU (14 days), amputation	FLU (10 days) followed by AMB (21 days)	FLU (8 days)
CVC removal	No	No	No	Yes	Yes
Breakthrough fungemia	Yes	No	No	No	No
Outcome	Expired	Expired	Survived	Survived	Survived

[a] Peripheral occlusive vascular disease

[b] Chronic kidney disease

[c] Diabetes mellitus

[d] Chronic obstructive pulmonary disease

[e] Non-Hodgkin's lymphoma

[f] Bilateral

[g] Acute renal failure

[h] Rheumatic heart disease

[i] Non-healing ulcer

[j] Infective endocarditis

[k] Heart failure

[l] Cerebrovascular accident

[m] Bronchoalveolar lavage fluid

[n] Central venous catheter

[o] Fluconazole

[p] Caspofungin

[q] Not available

Dosage: FLU 400 mg once daily, CAS loading dose of 70 mg followed by 50 mg daily, AMB 0.5–1 mg/kg body weight

flucytosine (≤0.5 μg/ml), and posaconazole (≤0.06 μg/ml) [13].

Patient information

The clinical details of the patients, such as demographic characteristics, clinical diagnosis, risk factors for candidemia, antifungal therapy, and outcome, were collected retrospectively. In cases of candidemia, therapeutic failure was defined either as the persistence of *Candida* despite the administration of 3 days of antifungal therapy or as the development of breakthrough candidemia while receiving antifungal agents for 3 days.

Results

Microbiology

A total of 15 *C. auris* isolates originated from 12 patients. Of these, seven isolates were cultured from blood, three each from central venous catheter (CVC) tips and surgically excised tissues, and one each from bronchoalveolar lavage (BAL) fluid and pus (Table 1). All isolates developed a pink color on CHROMagar™ Candida medium and showed ovoid to elongated budding yeast cells occurring singly or in pairs. The isolates grew well at 37 °C and 42 °C, whereas no growth was seen at 45 °C. They were inhibited on 0.01 % cycloheximide-containing medium. In addition, all *C. auris* isolates assimilated *N*-acetylglucosamine (NAG), unlike the reference Korean and Japanese *C. auris* isolates.

Molecular characterization

ITS sequences of 15 test isolates (GenBank accession nos. KF689009–KF689023) showed 99 % homology (query coverage ranging from 98 to 100 %) with Korean and the previously described north Indian *C. auris* isolates (accession nos. EU884184, EU884180, and KC692050) in GenBank. The D1/D2 sequence similarity of our isolates (GenBank accession nos. KF689024–KF689038) was 99 % with the Korean isolates (GenBank accession numbers EU881965, EU881967, and EU881968). Of the 15 south Indian *C. auris* isolates, 14 have been deposited at the CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands, and the accession numbers assigned are CBS 12874–12887. *Candida auris* isolates from the present study clustered together with the previously published north Indian *C. auris* isolates in the ITS phylogenetic tree and were distinct from the Japanese and Korean isolates. AFLP analysis showed separate clades of *C. haemulonii*, *C. duobushaemulonii*, and *C. pseudohaemulonii* distinct from *C. auris*. The 26 *C. auris* isolates, including 12 from north and 14 from south India, were clonal and clustered together, irrespective of their geographical origin, with an overall similarity of 80.2 % (Fig. 1). They were genotypically distinct from the Japanese and the Korean isolates, which had a similarity of 63.4 % with the Indian *C. auris* isolates (Fig. 1). In addition, AFLP clearly showed that the most genetically related sibling species of *C. auris*, i.e., *C. haemulonii*, *C. duobushaemulonii*, and *C. pseudohaemulonii*, had a similarity of 9.9 % among these three species versus *C. auris* (Fig. 1).

Antifungal susceptibility testing

All 15 *C. auris* isolates were resistant to fluconazole with GM MICs of 64 μg/ml and 11 (73 %) were resistant to voriconazole (MIC ≥1 μg/ml). Furthermore, 47 % of *C. auris* isolates were resistant to flucytosine ($n=7$; MIC ≥64 μg/ml). In addition, 40 % of *C. auris* isolates revealed high MICs (≥1 μg/ml) of caspofungin. Posaconazole exhibited the most potent activity, with a GM MIC of 0.03 μg/ml, followed by itraconazole (GM MIC, 0.14 μg/ml) and isavuconazole (GM MIC, 0.23 μg/ml). Amphotericin B showed good activity, with a GM MIC of 0.64 μg/ml. Also, micafungin (GM MIC, 0.10 μg/ml) and anidulafungin (GM MIC, 0.14 μg/ml) were highly active (Table 2).

Clinical evaluation of the patients

The clinical features, risk factors, and response to antifungal therapy of the 12 patients with *C. auris*-associated infections are summarized in Table 1. Seven patients were diagnosed to have candidemia and three had an infection of a gangrenous diabetic foot. Of the fungemic patients, two patients also had CVC tip cultures positive for *C. auris*, while another patient had post-operative wound sepsis due to *C. auris*. In one patient, only a CVC tip culture was positive and in a solitary patient, BAL fluid cultures yielded *C. auris*. The age group of patients ranged from 2 to 87 years (mean age 56.2 years). A mean of 7 of the 10 risk factors for candidiasis were present in each patient. The most common risk factor, present in all the patients, was the concomitant use of broad-spectrum antibiotics and the least was neutropenia, present in two (16.7 %) patients. Nine (75 %) patients had immunosuppressive conditions, such as diabetes mellitus in 5 patients (55.5 %), chronic kidney disease in 4 (44.4 %), and hematologic malignancies or history of cancer chemotherapy in 3 (33.3 %) patients. Among the other important risk factors, admission to the intensive care unit and the presence of an indwelling urinary catheter were noted in 11 (91.6 %) patients each, followed by CVC placement in 10 (83.3 %). Other risk factors included parenteral nutrition in 8 (66.6 %), concomitant bacteremia and a recent history of surgery in 7 (58.3 %) each, and the use of antifungals in 5 (41.6 %) patients. Breakthrough fungemia

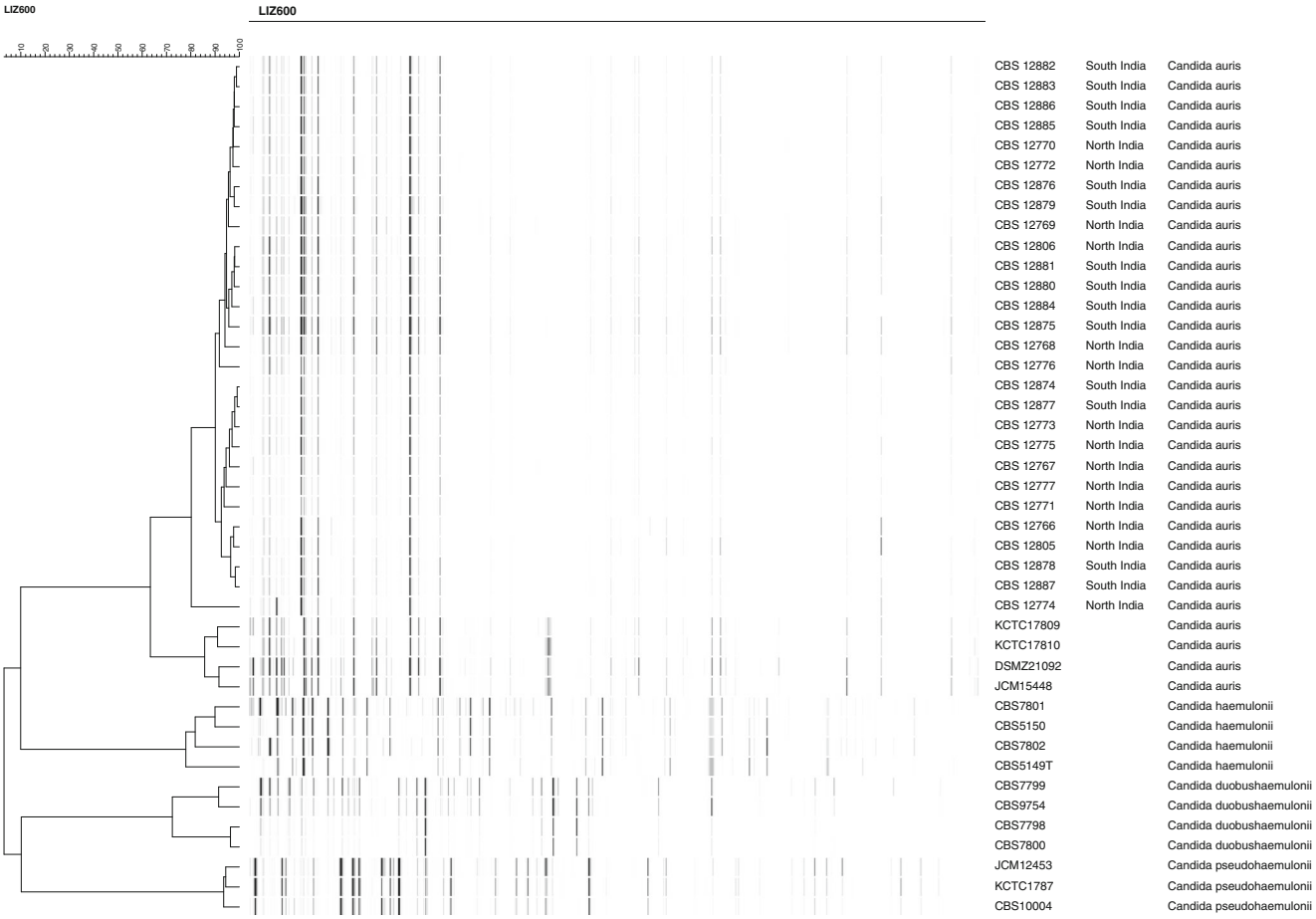

Fig. 1 Amplified fragment length polymorphism (AFLP) analysis showing fingerprinting of *Candida auris* isolates from India (north and south), Japan, Korea, and members of the *C. haemulonii* complex. The dendrogram was constructed using unweighted pair group method with arithmetic mean (UPGMA) analysis in combination with the Pearson correlation coefficient and was restricted to fragments in the range 60–400 bp. The *scale bar* indicates the percentage similarity

was observed in two (28.6 %) patients. Details of therapy were available for 11 patients. Of these, 9 (81.8 %) received antifungal therapy, the details of which are given in Table 1. Persistent fungemia was noted in 4 of 6 patients whose records were available. Of these six patients, cultures of CVC could be done in only two patients. Overall, 4 (36.4 %) patients expired, all of whom had fungemia. Of the remaining seven cases that survived, three underwent surgical excision of the gangrenous tissue; the solitary case of bronchopneumonia was treated with fluconazole and caspofungin, while another case (case 12) was managed by removal of the central line.

Discussion

Although *C. auris* has been previously reported as a rare agent of fungemia, this report highlights the isolation of *C. auris* from diverse clinical samples not reported so far [2, 3]. The isolation of *C. auris* from pus, surgically resected tissues, and BAL fluid suggests its potential role in varied diseases. In addition, the study documents seven well-characterized cases of fungemia due to this rare pathogen. In the present study, *C. auris* represented 8.6 % of annual candidemia cases in a tertiary care hospital. Previously, one-third (30 %) cases of candidemia have been attributed to this pathogen in a single tertiary care hospital of north India [2]. To date, there is a paucity of information regarding the clinical characteristics and antifungal susceptibility profiles of *C. auris*. The major risk factors associated with *C. auris* infections in the present study were similar to those of invasive candidiasis, which were broad-spectrum antibiotics, indwelling catheters, and immunosuppressive conditions [14]. Breakthrough fungemia developed in 28.6 % patients and therapeutic failure was observed in 66.7 % patients with fungemia. Also, previously high rates of therapeutic failures in patients with fungemia have been reported with this pathogen [2, 3]. In the present study, therapeutic failure was observed in patients with fungemia who received fluconazole, to which the isolate was highly resistant. The successful treatment outcome in the remaining two patients could be attributed to the timely institution of caspofungin and amphotericin B. It is noteworthy that low MICs of amphotericin B have been reported

Table 2 In vitro antifungal susceptibility profile of *Candida auris* isolates ($n=15$)

MIC (μg/ml)	Drugs									
	AMB[a]	FLU[b]	ITC[c]	VRC[d]	ISA[e]	POS[f]	FC[g]	CAS[h]	MFG[i]	AFG[j]
MIC_{50}[k]	1	64	0.125	1	0.25	0.06	0.5	0.5	0.125	0.125
MIC_{90}[l]	1	64	0.25	2	0.425	0.06	64	1	0.125	0.25
GM[m]	0.64	64	0.14	1.16	0.23	0.03	3.62	0.64	0.10	0.14
Range	0.25–1	64	0.06–0.25	0.5–4	0.06–0.5	0.015–0.125	0.25–64	0.25–1	0.06–0.125	0.125–0.25

[a] Amphotericin B
[b] Fluconazole
[c] Itraconazole
[d] Voriconazole
[e] Isavuconazole
[f] Posaconazole
[g] Flucytosine
[h] Caspofungin
[i] Micafungin
[j] Anidulafungin
[k] MIC at which 50 % of test isolates were inhibited
[l] MIC at which 90 % of test isolates were inhibited
[m] Geometric mean of MICs

previously [2–4], as well as in the present study. In contrast, *C. haemulonii*, with which *C. auris* is commonly misidentified by routine identification systems, is notably resistant to amphotericin B and azoles [4, 15]. Therefore, the correct identification of *C. auris* by sequencing and the need for undertaking antifungal susceptibility testing for this pathogen can hardly be overemphasized. It is difficult to ascertain the clinical significance of *C. auris* isolates recovered from patients with diabetic foot and BAL. Although the histopathologic evidence was lacking in aforementioned cases, the favorable response to surgical excision of the gangrenous tissue supports a causative role of this agent. Also, the case with bronchopneumonia from which no obvious etiological agent except *C. auris* was isolated had underlying risk factors and responded well to caspofungin. The patient had indwelling catheters, which could be the source of infection. However, blood and catheter tips were not cultured. The south Indian *C. auris* isolates also assimilated NAG, just like the previous isolates from north India [2]. Thus, the emerging Indian *C. auris* isolates were phenotypically and genotypically distinct from the Japanese and Korean strains. Additionally, AFLP analysis of 26 Indian *C. auris* strains from three geographically distant hospitals located at a distance of 2,081 km, one in Kochi, south India, and two in Delhi, north India, revealed the presence of an endemic clonal strain, suggesting widespread transmission. The fact that not only geographical clusters of *C. auris* could be segregated, as well as that the sibling species of *C. auris*, i.e., *C. haemulonii*, *C. duobushaemulonii*, and *C. pseudohaemulonii*, were revealed to be only distantly related by AFLP analysis, indicated that this genotyping method is a good discriminatory tool to study the epidemiology of this emerging yeast pathogen. To conclude, *C. auris* is an emerging pathogen which shows reduced susceptibility to antifungal agents and demonstrates a potential to cause a wide spectrum of human mycotic infections.

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Conflict of interest JFM received grants from Astellas, Merck, and Schering-Plough. He has been a consultant to Astellas, Basilea, and Merck, and received speaker's fees from Merck. All other authors: no potential conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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