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In vitro susceptibility of oral *Candida* to seven antifungal agents

T. Kuriyama[1], D. W. Williams[1], J. Bagg[2], W. A. Coulter[3], D. Ready[4], M. A. O. Lewis[1]

[1]Department of Oral Surgery, Medicine and Pathology, School of Dentistry, Cardiff University, Cardiff, UK, [2]Infection Research Group, Glasgow Dental Hospital and School, University of Glasgow, Glasgow, UK, [3]Oral Research Centre, School of Dentistry, Queen's University of Belfast, Belfast, UK, [4]Department of Microbiology, Eastman Dental Hospital, University College London Hospitals, London, UK

Kuriyama T, Williams DW, Bagg J, Coulter WA, Ready D, Lewis MAO. In vitro *susceptibility of oral* Candida *to seven antifungal agents.*
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The *in vitro* susceptibility of 618 *Candida* isolates to fluconazole, itraconazole, voriconazole, ketoconazole, miconazole, amphotericin B, and nystatin was determined. The isolates were obtained from 559 patients who had attended the UK dental hospital departments in Cardiff, Belfast, Glasgow or London. Antifungal susceptibility was assessed using a broth microdilution method following the National Committee for Clinical Laboratory Standards (NCCLS) M27-A guidelines. The majority of the test strains were *C. albicans* ($n = 521$) with few of these being resistant to fluconazole (0.3%). A low incidence of fluconazole resistance (0–6.8%) was similarly evident with all non *albicans* species (*Candida glabrata*, 5 of 59 resistant; *Candida krusei*, 0 of 7 resistant; *Candida tropicalis*, 0 of 13 resistant; *Candida parapsilosis*, 0 of 12 resistant; other *Candida* species, 0 of 6 resistant). Voriconazole, ketoconazole, and miconazole also revealed high activity against both *C. albicans* and non *albicans* isolates, and 23.7% of *C. glabrata* isolates were found to be resistant to itraconazole. There was little difference in the antifungal susceptibilities of *Candida* isolated from patients who had a history of previous antifungal therapy compared with those who had not received antifungal treatment. In summary, this surveillance study of antifungal susceptibility of oral candidal isolates in the UK, through the collaboration of four dental hospitals, demonstrates that oral *Candida* species have a high level of susceptibilities to a range of antifungal agents.

Key words: antifungal agent; antifungal susceptibility; *Candida*; *in vitro* activity; oral disease

Dr. David W. Williams, School of Dentistry, Cardiff University, Heath Park, Cardiff, CF14 4XY, UK
Tel.: + 44 29 20742548;
fax: + 44 29 20742442;
e-mail: williamsdd@cardiff.ac.uk
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Up to 75% of healthy individuals carry the yeast *Candida* as part of their normal commensal oral microflora (3). However, *Candida* is an opportunistic pathogen which can cause acute or chronic infection in certain individuals. Predisposing factors to oral candidosis include the wearing of a denture (prosthesis), smoking, immunosuppression, xerostomia and the receipt of broad-spectrum antibiotic therapy (3). *Candida albicans* is generally considered the most pathogenic *Candida* species and has been identified as the most prevalent yeast encountered in oral candidosis. However, it is becoming increasingly evident that other non-*albicans* species can contribute to the development of oral candidosis (3). *Candida* species have also been implicated in other forms of oral disease such as epithelial dysplasia, squamous cell carcinoma, angular cheilitis, burning mouth syndrome, lichen planus, endodontic infections, and periodontitis (9, 11, 12, 21).

Antifungal agents are often prescribed to manage oral candidosis (5). The polyenes (amphotericin B or nystatin) and the azole, miconazole, all of which are applied topically, are most frequently used to treat superficial candidosis. However, systemic azole antifungal therapy (fluconazole or itraconazole) can also be used to treat superficial candidosis and chronic forms of the infection. Prophylactic use of antifungals is also frequently employed in the management of oral candidosis in immunocompromised individuals, such as those suffering with AIDS or leukaemia.

Information concerning the antifungal susceptibility of *Candida* is important in the prediction of the likely efficacy of subsequent treatment. *C. albicans* is generally assumed to be susceptible to most antifungal agents, although some non-*albicans* species frequently exhibit resistance (2, 16). The recent increased use of antifungal therapy has raised concerns over the potential for the emergence of resistance of *Candida* to antifungals (14, 21). Indeed, the continued exposure of *Candida* to antifungals in certain patient groups has already been shown to alter the susceptibility of strains (7, 8, 17).

The aim of the present study was to assess the *in vitro* susceptibility of oral *Candida* isolates from UK patients to seven frequently used antifungal agents. The study also determined whether previous exposure to antifungal therapy affected the subsequent susceptibility of candidal isolates.

Material and methods

Candida isolates

Test strains of *Candida* were obtained from routine specimens taken from patients attending the Dental Hospitals in Cardiff, Glasgow, Belfast and London (Eastman), between January 2000 and June 2003. A total of 618 clinical *Candida* isolates were obtained from 553 patients with oral disease (oral candidosis, 362; burning mouth syndrome, 48; squamous cell carcinoma, 28; lichen planus, 24; xerostomia, 24; surgical wound infection, 12; angular cheilitis, 11; aphthous stomatitis, 9; leukoplakia, 9; endodontic infection, 6; geographic tongue, 2; squamous cell papilloma, 3; pemphigoid, 3; fissured tongue, 2; others, 10). In addition, isolates were recovered from six individuals who had a previous history of oral candidosis. Specimens were obtained by imprint culture, swab or the concentrated oral rinse method (19). Isolates were cultured on Sabouraud's dextrose agar (Oxoid, Hampshire, UK) under aerobic conditions at 37°C for 48 h. Candidal colonies were identified to species level using either the API 32C (bioMérieux, Basingstoke, UK) or the Auxacolor® 2 (Bio-Rad, Marnes-la-Coquette, France) system.

In vitro activity of antifungals

The *in vitro* activities of fluconazole, voriconazole (Pfizer, Surrey, UK), itraconazole, ketoconazole, miconazole (Janssen, Beerse, Belgium), amphotericin B and nystatin (Bristol-Myers Squibb, Middlesex, UK) were assessed. All antifungal agents were generously provided by the respective manufacturers. The minimum inhibitory concentration (MIC) for each drug to the test isolates of *Candida* was determined using a broth microdilution method following the National Committee for Clinical Laboratory Standards (NCCLS) M27-A guidelines (15).

Each isolate was subcultured on Sabouraud's dextrose agar for 24 h at 35°C. Resulting candidal colonies were suspended in 0.85% NaCl buffer and then inoculated in RPMI 1640 medium (Sigma, Poole, UK) containing 0.165 M MOPS (Sigma) and 2% glucose, which was incubated aerobically at 35°C for 48 h. *C. albicans* ATCC 90028, *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 were used as control strains for each test. The range of fluconazole concentrations tested was 0.06–64 μg/ml and for itraconazole, voriconazole, ketoconazole, miconazole, amphotericin B and nystatin the range was 0.015–16 μg/ml. Tests were conducted in flat-bottomed microtiter plates and the reading was performed after shaking the plates. In addition to visual end point readings, the optical density of each strain culture was measured with a microplate spectrophotometer set at 405 nm to determine the MICs (1). In the case of the spectrophotometer readings, the azole cut-off value was 50% of the reading of the growth control wells. For polyene antifungals a cut-off value of 100% was used.

Antifungal activity was expressed as the MIC of each isolate to the drug. The following resistance breakpoints were used according to NCCLS guidelines (15) or based on previous investigations (2, 4, 6, 10):

- fluconazole: resistant, ≥64 μg/ml; susceptible dose dependent, 16–32 μg/ml; susceptible, ≤8 μg/ml;
- itraconazole: resistant, ≥1 μg/ml; susceptible dose dependent; 0.25–0.5 μg/ml; susceptible ≤0.125 μg/ml;
- voriconazole: resistant, ≥8 μg/ml; susceptible dose dependent, 2–4 μg/ml; susceptible, ≤1 μg/ml;
- ketoconazole: resistant, ≥4 μg/ml;
- miconazole: resistant, ≥8 μg/ml;
- amphotericin B: resistant, ≥2 μg/ml;
- nystatin: resistant, ≥16 μg/ml.

Information regarding previous dental and medical histories, including receipt of any antifungal agent in the preceding 6 months was obtained by interview and/or from the patient records. The antifungal susceptibility of isolates from patients who had previously received an antifungal agent was compared with those of isolates from patients who had not received antifungals. Comparison of resistance rates was performed using a Mann–Whitney *U*-test.

Results

In vitro susceptibility of *Candida* isolates

The identity of candidal isolates is presented in Table 1. The majority of isolates were *C. albicans* ($n = 521$) and *Candida glabrata* ($n = 59$).

Only two of the 521 isolates (0.3%) of *C. albicans* were found to be resistant, with all remaining strains (99.7%) susceptible to fluconazole (MIC, ≤8 μg/ml). Moreover, 54 of the 59 *C. glabrata* isolates and all isolates of *C. krusei*, *C. parapsilosis,* and *Candida tropicalis* were susceptible to fluconazole. Only five *C. albicans* strains were resistant to itraconazole, with 402 out of 521 strains being fully susceptible to this antifungal. In the case of *C. glabrata* and *C. krusei*, resistance occurred in 23.7% and 14.3% of isolates, respectively. Voriconazole exhibited the lowest MIC to *C. albicans* with only two resistant *C. albicans* isolates being detected. Overall, 610 of 618 candidal strains tested were susceptible to this agent. Most of the candidal isolates were found to be very susceptible to ketoconazole and miconazole, although 14.3% of *C. krusei* isolates were resistant to miconazole. In this study, 5% of *C. albicans* isolates were

Table 1. In vitro susceptibility of 618 *Candida* isolates to seven antifungal agents

Antifungals (breakpoint, μg/ml)	*C. albicans* ($n = 521$)	*C. glabrata* ($n = 59$)	*C. krusei* ($n = 7$)	*C. parapsilosis* ($n = 12$)	*C. tropicalis* ($n = 13$)	*Candida* spp.[a] ($n = 6$)
Fluconazole (64)	0.12/2 (0.3)	2/8 (6.8)	0.5/2 (0)	0.25/0.5 (0)	0.5/2 (0)	0.25/4 (0)
Itraconazole (1)	0.03/0.5 (1.0)	0.25/2 (23.7),	0.25/1 (3.14)	0.12/0.5 (0)	0.25/0.5 (7. 7)	0.5/2 (33.3),
Voriconazole (8)	≤ 0.015/0.12 (0.3)	0.06/0.5 (0)	0.5/1 (0)	≤ 0.015/0.03 (0)	0.03/0.12 (0)	0.06/0.12 (0)
Ketoconazole (4)	0.03/0.5 (3.6)	0.12/0.5 (3.4)	0.25/1 (0)	0.03/0.12 (0)	0.06/0.12 (0)	0.06/0.12 (0)
Miconazole (8)	0.03/0.5 (0)	0.06/0.25 (0)	0.5/8 (3.14)	0.25/0.5 (0)	0.12/2 (0)	0.03/0.25 (0)
Amphotericin B (2)	0.5/1 (5.0)	0.5/1 (3.4)	1/4 (3.14)	0.5/1 (0)	0.5/1 (0)	0.5/1 (0)
Nystatin (16)	1/1 (0)	1/2 (0)	1/2 (0)	1/2 (0)	1/1 (0)	0.5/1 (0)

Data are expressed as MIC_{50} μg/ml/MIC_{90} μg/ml (resistance rate, %).
Resistance rate is calculated as 'number of resistant isolates to a drug'/'number of total isolates'.
[a]*Candida rugosa* ($n = 2$), *Candida dubliniensis* ($n = 3$) and *Candida lusitaniae* ($n = 1$).

Table 2. In vitro susceptibility of 473 candidal isolates from 432 patients as related to previous antifungal therapy

Antifungals	No previous antifungal ($n = 422$ [a])	Previous fluconazole ($n = 37$[b])	Previous itraconazole ($n = 14$[c])
Fluconazole	0.25/2 (0.5)	0.12/4 (0)	0.5/4 (0)
Itraconazole	0.06/0.5 (2. 8)	0.06/0.5 (4.5)	0.12/0.5 (0)
Voriconazole	= 0.015/0.25 (0)	≤ 0.015/1 (0)	≤ 0.015/0.5 (0)
Ketoconazole	0.03/0.5 (2. 8)	0.03/0.5 (0)	≤ 0.015/0.25 (0)
Miconazole	0.03/0.5 (0)	0.03/1 (2.7)	0.06/0.5 (0)
Amphotericin B	0.5/1 (5.0)	0.5/1 (4.5)	0.5/1 (0)
Nystatin	1/2 (0)	1/2 (0)	1/2 (0)

Data are expressed as MIC_{50} μg/ml/MIC_{90} μg/ml (Resistance rate, %).
[a]*C. albicans*, 360 isolates; non-*albicans* species, 62 isolates.
[b]*C. albicans*, 30 isolates; non-*albicans* species, 7 isolates.
[c]*C. albicans*, 11 isolates; non-*albicans* species, 3 isolates.

resistant to amphotericin B based on the selected resistant breakpoint, and the highest resistance rates to this agent were evident with *C. krusei* isolates (14.3%). However, it was worth noting that the MICs did not exceed 4 μg/ml for any of the tested strains. No candidal strain was found to be resistant to nystatin based on the breakpoint values used, although nystatin exhibited slightly higher MICs compared with amphotericin B.

There was no noticeable difference in the antifungal MICs for isolates from England, Wales, Scotland and Northern Ireland (data not shown).

Cross-resistance

In the present study, a total of seven *Candida* isolates were found to be resistant to fluconazole. Five of the fluconazole-resistant isolates (71.4%) and 18 of 611 (2.9%) of fluconazole-susceptible isolates were found to be resistant to itraconazole. There was a significant difference in incidences of resistance to itraconazole between the fluconazole-resistant and fluconazole-susceptible *Candida* isolates ($P < 0.001$). In addition, the prevalence of resistance to ketoconazole in fluconazole-resistant *Candida* isolates (three of seven) was also significantly higher than that recorded for fluconazole-susceptible isolates (18 of 611, $P < 0.002$). In contrast, only one fluconazole-resistant strain was resistant to voriconazole and none was resistant to miconazole. All fluconazole resistant isolates were susceptible to amphotericin B and nystatin, and the MICs for these polyene antifungals were similar when compared with the fluconazole-susceptible isolates (data not shown).

Relation between *in vitro* susceptibility and history of antifungal therapy

In this study, information regarding past antifungal therapy was obtained from 432 patients. Patients who received topical treatment with amphotericin B ($n = 3$) or miconazole ($n = 2$) were excluded. A total of 51 individuals had been treated with antifungal systemic therapy prior to collection of the specimen; fluconazole (50 mg daily) had been taken by 34 patients and itraconazole (100 mg daily) by 12 patients. The mean duration of fluconazole therapy was 16 days (range, 7–49 days) and for itraconazole therapy, 14 days (range, 10–15 days). The MICs for all the antifungal agents tested against candidal isolates from patients who had received fluconazole were not notably different from those values for isolates obtained from individuals who had not previously taken antifungal therapy (Table 2). This feature was similarly evident with itraconazole.

Discussion

It has been suggested that due to the trailing growth phenomenon in the test medium, visual determination of MIC endpoints for some azoles can be complicated and unreliable (1). This was indeed evident in the present study for fluconazole, itraconazole, voriconazole, and ketoconazole (data not shown). It has been reported that spectrophotometric readings of broth microdilution tests provide a more objective assessment of MIC endpoints (1) and this proved to be the case in the present study.

Fluconazole is a triazole agent that is established as a first-line antifungal for the treatment of oral candidosis. Although isolates of *C. albicans* have been found to be susceptible to fluconazole (16), recently an increase in the isolation of azole-resistant *C. albicans* strains has been reported (21). In the present study, almost all of the candidal isolates were found to be susceptible to fluconazole (MICs ≤8 μg/ml). There was no difference in the antifungal susceptibility with respect to the geographic origin of isolates. The results of this study would indicate that fluconazole remains an effective agent for the treatment of oral candidosis with a low incidence of *in vitro* resistance.

Itraconazole is prescribed as an alternative to fluconazole for treating oral candidosis. In this study, although a higher prevalence of itraconazole resistance was evident for *C. glabrata* and *C. krusei* strains compared with fluconazole, the majority of *C. albicans* strains were found to highly susceptible to this antifungal. *C. albicans* is the predominant candidal species encountered in oral candidosis and is regarded as the most pathogenic species. Therefore, itraconazole is likely to have a role in the treatment of oral candidosis.

Voriconazole is a new triazole drug (18), and there were very few strains that were resistant to this agent. Moreover, voriconazole revealed the lowest MIC to *C. albicans*. These results suggest that voriconazole may have a role in the treatment of oral candidal infections, although voriconazole currently is not licensed in the UK for the treatment of oral candidosis.

While hepatotoxicity limits systemic use of ketoconazole, a topical role for this antifungal remains, as highlighted by its effectiveness against both *C. albicans* and non-*albicans* species. Miconazole is employed topically to treat oral candidal infections. The results of this study would support continued clinical use of these agents.

Amphotericin B is traditionally used in topical formats, although it may be administrated systemically for the treatment of systemic infections in hospitalized patients. In the present study, most of the *C. albicans* isolates were susceptible to amphotericin B, and there was no strain that exhibited MICs greater than 4 μg/ml for this agent. Nystatin is a polyene antifungal not dissimilar in structure to amphotericin B. Although MICs for nystatin were comparatively slightly higher than amphotericin B, resistant strains were not detected. These results would support the effectiveness of topical amphotericin B

and nystatin therapy for superficial candidosis.

It has been suggested that *Candida* species demonstrate azole cross-resistance (13, 20). In this study, the incidence of resistance to itraconazole and ketoconazole in fluconazole-resistant isolates was significantly higher than recorded for fluconazole-susceptible isolates ($P < 0.002$). The results would appear to confirm the presence of cross-resistance among oral *Candida* species to certain azole antifungals. Itraconazole and ketoconazole would not therefore be recommendable agents for treatment of oral candidoses that involve fluconazole-resistant *Candida* strains. In contrast, low incidences of resistance to voriconazole and miconazole in fluconazole-resistant isolates were evident. Despite being azole antifungals, these two agents may be used to treat candidal infections where fluconazole resistance is evident. *Candida* isolates that were resistant to fluconazole revealed similar MICs and resistance rates to amphotericin B and nystatin compared with the fluconazole-susceptible isolates. This finding supports the belief that there is no cross-resistance between the polyenes and azoles.

It has been reported that exposure to antifungal agents, especially fluconazole, can result in the emergence of resistance in *Candida* strains (7, 8, 17). In this study, the susceptibility of *Candida* from patients who had received fluconazole for all tested antifungals did not differ from isolates obtained from individuals who had not previously taken antifungal therapy. This feature was similarly evident with itraconazole. Those studies that have reported a correlation between administration of antifungals and increased incidence of resistance have obtained samples from patients who had taken repeated and/or long-term antifungal therapy, or who had been given prophylaxis for fungal infections due to underlying immunosuppression such as AIDS (7, 8, 17). In contrast, the subjects in the present study were patients who were not immunocompromised and had received antifungal therapy for approximately 2 weeks only. The differences in susceptibility of candidal strains in the mouth reported here and other studies could in part be due to the different population studied; larger studies would be required to confirm this.

The present study provides valuable surveillance data on the antifungal susceptibility of a large number of oral *Candida* isolates collected from four geographically diverse areas of the UK. The results suggest that despite an increasingly widespread use of triazole antifungal drugs, such as fluconazole, resistance to these agents among immunocompetent outpatient populations remains rare. While this is reassuring, attention to local factors, in particular denture hygiene and correction of any systemic predisposing factor, must remain the first principle of treatment of oral candidosis. As with the prescribing of any antimicrobial agent, the use of a systemic antifungal drug must be justified on a case-by-case basis. Furthermore, to ensure that the high incidences of susceptibility identified in this study are retained, efforts must be maintained to avoid inappropriate or unnecessary prescribing of these antifungals.

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