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Biofilms Formed by Isolates from Recurrent Vulvovaginal Candidiasis Patients Are Heterogeneous and Insensitive to Fluconazole

Leighann Sherry,[a] Ryan Kean,[a,b] Emily McKloud,[a] Lindsay E. O'Donnell,[a] Rebecca Metcalfe,[c] Brian L. Jones,[d] Gordon Ramage[a]

Oral Sciences Research Group, Glasgow Dental School, School of Medicine, Dentistry and Nursing, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, United Kingdom[a]; Institute of Healthcare Policy and Practice, School of Health, Nursing and Midwifery, University of West of Scotland, Paisley, United Kingdom[b]; Sandyford Sexual Health Service, NHS Greater Glasgow & Clyde, United Kingdom[c]; Microbiology Department, Glasgow Royal Infirmary, United Kingdom[d]

ABSTRACT Vulvovaginal candidiasis (VVC) is a global health problem affecting ~75% of women at least once in their lifetime. Here we examined the epidemiology of VVC in a patient cohort to identify the causative organisms associated with VVC. Biofilm-forming capacity and antifungal sensitivity profiles were also assessed. We report a shifting prevalence of *Candida* species with heterogeneous biofilm-forming capacity, which is associated with altered antifungal drug sensitivity.

KEYWORDS *Candida*, biofilm, vulvovaginal candidiasis, fluconazole

Fungal infections play a surprisingly substantial, yet unrecognized, health burden on the global population (1). Vulvovaginal candidiasis (VVC) is one example of such infections; it is estimated to be the most common fungal infection in a number of countries worldwide (2–4). Approximately 138 million women worldwide complain of >4 episodes of VVC per year due to treatment failure, clinically defined as recurrent VVC (RVVC) (5–7). These unresolved infections not only have a high impact on the quality of life of these women, but can also lead to further health complications (8). *Candida albicans* is historically reported as the predominant organism isolated from VVC, accounting for over 90% of infections (9, 10). However, evidence of a dynamic shift in yeast epidemiology has been demonstrated through an increasing prevalence of non-*C. albicans* species (NCAS), which accounts for 11 to 80% of infections, depending on geographical location (8). Nevertheless, *C. albicans*, a well-characterized biofilm-forming organism, remains a prominent pathogen in this disease. Resistance to antifungal therapy as a result of biofilm formation is a likely contributor to failed treatment. While it is widely accepted that biofilms contribute to the pathogenesis of bacterial vaginosis (BV) (11, 12), their role in VVC remains contested despite overwhelming evidence to suggest otherwise (13–15).

An anonymized series of high vaginal swabs (HVS) ($n = 300$) were obtained throughout April 2016 from women visiting their general practitioner (GP) and referral clinics in the NHS Greater Glasgow and Clyde area for at least the second time (16). These women were symptomatic at the time of sampling, with the causative organism identified using matrix-assisted laser desorption–ionization-time of flight mass spectrometry (MALDI-TOF MS), with *Escherichia coli* used pre- and post-yeast sampling to ensure accuracy of testing.

A total of 71% ($n = 212$) were identified as *C. albicans*, followed by 15% ($n = 47$) as *Candida glabrata*, 6% ($n = 17$) as *Candida dubliniensis*, and 3% ($n = 10$) as *Candida parapsilosis* (Fig. 1). The remaining 5% of isolates included *Candida tropicalis*, *Candida lusitaniae*, and *Candida guilliermondii*. These data are in line with recent epidemiological

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Address correspondence to Gordon Ramage, gordon.ramage@glasgow.ac.uk.

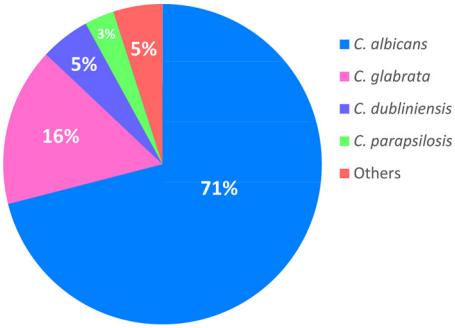

FIG 1 Distribution of organisms isolated from VVC patients. A total of 300 VVC isolates were identified using MALDI-TOF MS, with yeast species proportionally represented in the figure.

patterns showing a shift in NCAS prevalence within VVC (8). However, a caveat of our study is the limitation of a single geographical location, which may influence the species distribution. Future studies should include various institutes worldwide in order to fully assess the shift in VVC epidemiology.

To determine the biofilm-forming capability of these isolates, all VVC strains ($n = 300$) were standardized to 1×10^6 cells/ml in RPMI 1640 and grown as biofilms in 96-well plates for 24 h. Biofilms were washed with phosphate-buffered saline (PBS) and biomass was assessed using the crystal violet (CV) assay (17). Here we have shown that vaginal isolates were able to form differential biofilms, regardless of species (Fig. 2). *C. albicans* displayed the greatest heterogeneity with regard to biofilm biomass, with isolates ranging from OD_{570} (optical density at 570 nm) of 0.008 to 1.478, with a mean of 0.416. The second most prevalent species, *C. glabrata*, had significantly lower biomass than *C. albicans* ($P < 0.05$) and *C. dubliniensis* ($P < 0.01$), with a mean OD_{570} of 0.271. This apparent biofilm heterogeneity may affect the management of VVC infections, as these sessile communities are known to be notoriously recalcitrant to antifungal therapy and biofilm heterogeneity has been shown to correlate with success of *in vitro* antifungal therapy (17).

Planktonic and biofilm antifungal susceptibility testing was carried out as described previously to determine the MICs (18). Briefly, cells were standardized in RPMI 1640 before being treated with fluconazole (FLZ) (Sigma, Dorset, UK) for 24 h, at a range of concentrations (0.0625 to 32 mg/liter). Planktonic MIC (pMIC) was determined as the lowest concentration able to completely inhibit growth on visual inspection. Sessile MIC (sMIC) analysis were performed on 24 h preformed biofilms, with sMIC recorded at

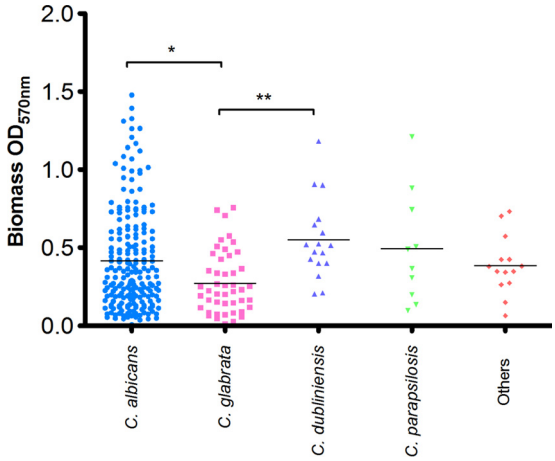

FIG 2 VVC isolates display varied biofilm formation. A total of 300 VVC isolates were screened for biofilm formation using a biomass stain, as described in Materials and Methods. Each isolate was tested in quadruplicate, with the mean represented by a horizontal black bar. Statistical analysis was carried out using a one-way ANOVA. *, $P < 0.05$; **, $P < 0.01$.

TABLE 1 Susceptibility profile of vaginal *Candida* isolates to fluconazole

Profile parameter	Fluconazole MIC (mg/liter) for[a]:									
	C. albicans (*n* = 212)		*C. glabrata* (*n* = 47)		*C. dubliniensis* (*n* = 17)		*C. parapsilosis* (*n* = 10)		Other *Candida* spp. (*n* = 14)	
	pMIC	sMIC	pMIC	sMIC	pMIC	sMIC	pMIC	sMIC	pMIC	sMIC
Range	0.0625–>32	0.125–>32	<0.0625–>32	0.5–>32	0.125–>32	0.125–>32	1–>32	1–>32	0.0625–>32	1–>32
MIC_{50}	4	>32	4	>32	4	>32	1	4	1	>32
MIC_{90}	>32	>32	>32	>32	>32	>32	16	>32	>32	>32

[a]*n* = 300. Abbreviations: pMIC, planktonic MIC; sMIC, sessile MIC.

50% inhibition using an XTT (2,3-bis[2-methoxy-4-nitro-5-sulfo-phenyl]-2H-tetrazolium-5-caboxanilide) metabolic reduction assay (19). Here we have shown that FLZ, the first line antifungal used to treat VVC, was ineffective against most isolates, with planktonic MICs ranging from < 0.0625 to >32 mg/liter (Table 1). Specifically, the $pMIC_{50}$ for FLZ was 4 mg/liter for *C. albicans*, *C. glabrata*, and *C. dubliniensis*, although for biofilms $pMIC_{50}$ was >32 mg/liter. When planktonic cells were stratified based on identification as *C. albicans* and NCAS it was shown that 41% and 26% of the isolates, respectively, were insensitive to FLZ at >32 mg/liter, whereas for sessile cells this rose to 51% and 56% of the isolates, respectively. Interestingly, similar susceptibility profiles were observed for *C. albicans* and *C. glabrata*, despite *C. glabrata* being known to be a low biofilm former (20). This reduced sensitivity in *C. glabrata* can be associated with its intrinsic resistance to fluconazole due to the overexpression of multidrug transporters (21).

VVC is not a reportable disease, making epidemiological studies difficult. However, this study provides a snapshot of the species identified within a VVC population, demonstrating that NCAS are responsible for an increasing number of these infections. This corresponds with previous studies reporting an ongoing dynamic shift in yeast epidemiology (22, 23), which is potentially driven by inappropriate use of over-the-counter azoles (10). Irrespectively, *C. albicans* remained the most dominant species in this study, which raises the question of why a high number of isolates displayed reduced susceptibility to FLZ. We demonstrated the ability of these clinical isolates to form heterogeneous biofilms. The presence of these communities in VVC may explain why *C. albicans* infections remain unresponsive to FLZ therapy, an antifungal highly ineffective against *C. albicans* biofilms (24). We cannot discount the potential for heteroresistance phenotypes within these populations (25). The contribution of biofilms to VVC pathogenesis remains poorly understood, though many researchers are beginning to consider them to be important determinants of disease (13, 14), further emphasizing the need for research in this field. Collectively, the data from this investigation highlight the necessity for careful consideration of the causative organism in VVC, the biofilm phenotype, and its accentuated antifungal sensitivity profiles, all of which may improve antifungal treatment in this area.

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