Molecular epidemiology and treatment outcomes of vulvovaginal candidiasis in Namibian women

Cara Mia Dunaiski

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by

CARA MIA DUNAISKI

Student number: 28200846

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DECLARATION

I, the undersigned, declare that the thesis, which I hereby submit for the degree PhD (Medical Microbiology) at the University of Pretoria, is my own original work and has not been previously submitted by me for a degree at this or any other tertiary institution. I further declare that all sources cited or quoted are specified and recognised by means of an inclusive list of references.

Signature

2 November 2023

Date

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LIST OF ABBREVIATIONS

AMR:	Antimicrobial resistance
AST:	Antimicrobial susceptibility testing
ATCC:	American Type Culture Collection
AV:	Aerobic vaginitis
BMD:	Broth microdilution
BV:	Bacterial vaginosis
BVAB:	Bacterial vaginosis associated bacteria
CI:	Confidence interval
CT:	*Chlamydia trachomatis*
DNA:	Deoxyribonucleic acid
EUCAST:	European Committee on Antimicrobial Susceptibility Testing
HIV:	Human immunodeficiency virus
IL:	Interleukin
IM:	Intramuscular
INF-γ:	Interferon-gamma
IQR:	Interquartile range
LMICs:	Low- to middle-income countries
MBL:	Mannose binding lectin
MDR:	Multidrug resistant
MIC:	Minimum inhibitory concentration
MLST:	Multilocus sequence typing
NIP:	Namibia Institute of Pathology

NG:	*Neisseria gonorrhoeae*
NLRP:	Nod-like receptor protein
NO:	Nitric oxide
OR:	Odds ratio
PCR:	Polymerase chain reaction
PMN:	Polymorphonuclear
PRR:	Pattern recognition receptor
RVVC:	Recurrent vulvovaginal candidiasis
SDA:	Sabouraud dextrose agar
SNPS:	Single nucleotide polymorphisms
ST:	Sequence types
STI:	Sexually transmitted infection
TLR:	Toll-like receptor
UPGMA:	Unweighted pair group method with arithmetic mean
VDS:	Vaginal discharge syndrome
VVC:	Vulvovaginal candidiasis
WHO:	World Health Organization

LIST OF PUBLICATIONS, CONFERENCE ATTENDANCE AND AWARDS

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by Cara Mia Dunaiski

Supervisor: Prof Remco P.H. Peters (University of Pretoria, University of Cape Town, Foundation for Professional Development)

Co-supervisor: Prof Marleen M. Kock (University of Pretoria/ National Health Laboratory Service)

Department: Medical Microbiology, Faculty of Health Sciences, University of Pretoria

Degree: PhD Medical Microbiology

SUMMARY

Vulvovaginal candidiasis (VVC) is a common condition in women of childbearing age worldwide and usually presents as vaginal discharge syndrome (VDS). Other important causes of VDS are bacterial vaginosis (BV) and sexually transmitted infections (STIs). The most common STI causing VDS include *Trichomonas vaginalis*. Vaginal dischare syndrome (VDS) can also be caused by *Neisseria gonorrhoeae*, *Chlamydia trachomatis* and *Mycoplasma genitalium*. However, these are more commonly associated with endocervical infections, and may not present with VDS. Syndromic treatment is the standard of care for VDS, i.e. empirical antibiotics are provided without diagnostic testing. Performance of the syndromic approach depends on the aetiology of VDS and presence of antimicrobial resistance but there is little information from sub-Saharan Africa.

The aim of this PhD study was to describe the aetiology of VDS and determine outcome of syndromic treatment of VDS in Namibian women. This PhD study had three components: first, a cross-sectional evaluation was completed to determine the aetiology of VDS. De-identified vaginal swabs (*n*=253) sent to the routine laboratory at the Namibia Institute of Pathology in Windhoek were tested for STIs using real-time PCR, BV by smear microscopy and VVC by culture and drug susceptibility testing. Second, a prospective cohort study of women (*n*=109) with VDS at Katutura Intermediate Hospital in Windhoek was conducted to determine incidence, risk factors and microbial aetiology of treatment failure. Last, comprehensive molecular microbiological analysis of phenotypic and genotypic resistance including multi-

locus sequence typing was conducted through whole genome sequencing analysis of all *Candida glabrata* isolates (*n*=21) obtained in the two studies.

Vulvovaginal candidiasis was the main aetiology (43%) of VDS in the cross-sectional study followed by BV (39%) and STIs (36%); multiple infections were present in 34% of women. *Candida albicans* was the most common fungal species (79%); most isolates (98%) were susceptible to fluconazole. In contrast, no non-albicans *Candida* species were susceptible to fluconazole.

Vulvovaginal candidiasis aetiology at baseline was similarly high in the prospective cohort study with 41% with VVC, 43% of women diagnosed with BV and 40% with STI. At 30 days follow-up, treatment failure was reported by 31% of women, with 18% reporting recurrent and 13% persistent VDS after syndromic treatment. Incidence of treatment failure was 3.6 per 100 person-days at 7 days follow-up and 1.0 per 100 person-days at 30 days follow-up. Vulvovaginal candidiasis was the main risk factor for treatment failure (OR 4.3, 95% CI 1.7–11, *p*=0.002). Microbial evaluation of treatment failure attributed most cases to untreated (31%) and azole-resistant (23%) VVC.

Twenty-one fluconazole-resistant *C. glabrata* isolates were obtained from the two studies. Whole genome sequencing analysis showed non-synonymous single nucleotide polymorphisms in antifungal resistance genes in 95% of isolates. Single nucleotide polymorphisms were also detected in genes associated with polyene, echinocandin and multiple class antifungal resistance despite full phenotypic susceptibility to these drug classes. Multilocus sequence typing classified isolates in eight sequence types.

The results show that VVC is an important cause of VDS in Namibian women, with *C. albicans* as the main species followed by *C. glabrata*. Untreated and fluconazole resistant VVC constitute an important risk factor for VDS treatment failure. Therefore, clinical consideration of VVC in women with VDS and access to fungal culture and susceptibility testing is warranted, especially among women with treatment failure.

Word count: 499

Keywords: Antifungal resistance; *Candida albicans*; *Candida glabrata*; Namibia; sexually transmitted infections; sub-Saharan Africa; vaginal discharge syndrome; vulvovaginal candidiasis; treatment outcomes.

CHAPTER 1

1.1 Introduction

Vulvovaginal candidiasis (VVC) is a common condition in women of childbearing age, with 70-75% of healthy women facing symptomatic VVC at least once in their childbearing years. [1, 2] Recurrent or persistent VVC may occur in 10-20% of women with symptomatic VVC. [3] The only study in Namibia exploring VVC and published in 2016, reported that among a sample population of 335 symptomatic and asymptomatic women among newly diagnosed adult people living with human immunodeficiency virus (HIV), 18.2% had symptomatic VVC. [4] In two recent studies, recurrent vulvovaginal candidiasis (RVVC) was estimated to occur in 37,390 females per year in Namibia and in over a million females per year in South Africa. [5, 6]

The aetiology of VVC in more than 90% of cases is *Candida albicans.* [7] *Candida* non-albicans species have recently emerged globally and the circulating species may differ by geographical location. [7] The most significant of these non-albicans *Candida* species is *Candida glabrata* owing to its high prevalence of resistance to azoles. [7] *Candida krusei, Candida tropicalis, Candida parapsilosis* and, very rarely, *Saccharomyces cerevisiae*, are other potential pathogens that may cause VVC. [8] In addition, *Candida africana*, a *Candida albicans* related species, has been reported as a cause of VVC. [9]

The main cause of VVC is an overgrowth of the commensal *Candida* spp. in the vagina. [10] Vulvovaginal candidiasis is usually acute and symptomatic. Abnormal vaginal discharge is a frequent genital tract symptom amid women and is a major sign of VVC. [8, 11] Abnormal vaginal discharge can also be observed due to sexually transmitted infections (STIs) caused by *Chlamydia trachomatis, Neisseria gonorrhoeae, Mycoplasma genitalium* and *Trichomonas vaginalis* and in bacterial vaginosis (BV). [11] In addition to abnormal vaginal discharge, appearing white and curd-like, other signs and symptoms of VVC include painful and/or burning, stinging, or itching sensation associated with urination, difficult or painful sexual intercourse, and enflamed and swollen vaginal wall. [3, 12, 13] Nonetheless, a lack of abnormal vaginal discharge upon clinical examination is quite common and does not exclude vaginal infections. Women with a genital infection that do not present with abnormal discharge may therefore not obtain suitable treatment, as it is the main clinical indicator of vaginal infection. [14, 15] Although uncomplicated VVC is a manageable disease, complicated VVC including RVVC has a severe effect on women and their partners; bodily, mentally, sexually and economically. [16, 17] It is generally recommended that VVC be diagnosed with an approach

including both clinical features and diagnostic methods, as asymptomatic colonization is not treated. [8] Undetected or not treated VVC can lead to complications such adverse pregnancy and birth outcomes, and the acquisition and transmission of human HIV, herpes simplex virus-2 (HSV-2) and other STIs, when exposed. [17, 18]

The classes of drugs available for the treatment of *Candida* infections include azoles, polyenes, echinocandins and the pyrimidine analogue flucytosine. [19] Treatment of elementary vulvovaginal *Candida albicans* infection is simple and a single-dose intravaginal treatment, for example clotrimazole vaginal ovules, are efficacious in 80-90% of the primary infection or episode, [18, 20] However, this type of treatment does not prevent recurrences and azole resistance rates may accelerate to over 15% in women with VVC and repeated exposure to intravaginal treatment. [20] In both Namibia and South Africa the first line drug used for VVC is clotrimazole. [14, 21] The Namibian Standard Treatments Guideline (2011), as well as the South African Sexually Transmitted Infections Management Guidelines (2018) recommends the insertion of a clotrimazole vaginal pessary (available in different dosages), as well as the local application of clotrimazole topical cream to the vulva for 7 days after symptoms resolve. [14, 21, 22]

Antifungal resistance of *Candida* spp. is a mounting problem globally. [23] Non-*albicans* *Candida* infections and the rates of azole resistance, including to clotrimazole, differ topographically, probably due to prescribing patterns of clinicians for both the treatment of and prophylaxis against invasive candidiasis. [24]

Dysbiosis may also be caused by multiple and mixed vaginal and cervical infections, in which more than one pathogen is responsible for the manifestation of symptoms. [2] Mixed infections usually occur with BV, VVC and trichomoniasis, in combinations of two or all three. [2] Additionally, co-infections can also occur with *C. trachomatis*, *M. genitalium* and *N. gonorrhoeae.* [25, 26] Globally the burden of STIs remains high, with an approximated 376 million new infections of the four treatable STIs- chlamydia, gonorrhoea, syphilis and trichomoniasis in 2016. [27] In Africa, *C. trachomatis* has an estimated annual incidence rate of 12 million infections, *N. gonorrhoeae* has an estimated annual incidence rate of 11.4 million and *T. vaginalis* has an estimated annual incidence rate of 37.4 million. [28] In addition, a 10% prevalence of *M. genitalium* has been reported in studies from various African countries. [29, 30] These STIs may all present as VDS or remain asymptomatic. Antimicrobial resistance (AMR) is emerging in *N. gonorrhoeae* and *M. genitalium* infection while *C. trachomatis* and *T. vaginalis* resistance is rare. Bacterial vaginosis (BV) affects women of reproductive age and

can cause symptoms in about 50% of women globally with the highest prevalence in sub-Saharan Africa. [31] It is the most frequent cause of vaginal discharge, and is instigated when *Lactobacillus* spp., the principal species in healthy vaginal flora, is substituted by anaerobes, mainly *Gardnerella vaginalis*. [32-35] The recommended first-line therapy for BV is metronidazole or clindamycin. [36-38] These antibiotics show equivalent short-term efficiency, with cure rates of 80-90% at one month. [36-39] It is, however, associated with significant morbidity and high recurrence rates. [38] Bacterial vaginosis recurrence rates are about 80% three months subsequent to efficacious treatment. Moreover, there are some cases in which treatment is unsuccessful leading to persistent BV. [31]

The interactions between bacteria and fungi in the vagina evidently play a significant role in the pathogenesis of genital infections. [2] Bacterial and fungal biofilms in vaginal infections, particularly VVC and BV, have been implicated in treatment failure and recurrence. [40, 41]

In Namibia, like other low- to middle-income countries, syndromic management of VDS is the status quo of care, where clinicians treat patients by means of a demarcated set of symptoms, generally disregarding an etiological diagnosis. [21] This may lead to over-prescribing antimicrobial drugs and the risk of AMR development and selection. [42] Many genital infections are asymptomatic and consequently syndromic management may result in a noteworthy number of infections being undiagnosed and untreated. [43-46] This poses a great public health risk, as untreated genital infections are associated with long-term genital and reproductive tract morbidity and proliferate the risk of HIV acquisition and transmission. [42] Another well documented limitation of the syndromic management approach is resource constraints for surveillance. [47]

Candida AMR surveillance and the molecular characterisation of the causal mechanisms of resistant phenotypes are imperative to establish appropriate empirical treatment regimens, and furthermore to describe the emergence of novel mechanisms in local yeast populations. [48] Whole genome sequencing (WGS) imparts comprehensive proof of the present AMR burden and vital surveillance data where deficient. [49] Insights in the antifungal resistant *Candida* in Namibia can be obtained by investigating predisposed groups such as women with multiple VVC episodes. Many studies have shown that women with repeat VVC episodes are at higher risk of acquiring antifungal resistant VVC due to repeat or prolonged exposure to first-line antifungal drugs. [50]

The emergence of *Candida* strains resistant to azole antifungals (e.g. clotrimazole) poses a significant public health threat globally. The possibility of untreatable *Candida* is real, as there are species including *Candida auris* that are multidrug resistant. Additionally, there are no vaccines in development and few alternative antifungal treatments are available to treat VVC [51]. The reasons for the emergence of AMR *Candida* are multifactorial in Namibia and include inadequate VVC burden surveillance leading to inadequate laboratory diagnoses and incorrect treatment interventions. Additionally, inadequate AMR stewardship may lead to selection pressure enacted by extensive use of ineffective antifungals.

Therefore, this study will describe the molecular epidemiology and treatment outcomes of vulvovaginal candidiasis in Namibian women by evaluating microbial aetiology, AMR patterns and treatment outcomes.

1.2 Aim

To study the molecular epidemiology and treatment outcomes of vulvovaginal candidiasis in Namibian women.

1.3 Objectives

- To determine the frequency of *Candida* spp. and concurrent STIs in symptomatic women by utilizing microscopy, culture and molecular identification techniques.
- To determine the phenotypic and genotypic antifungal susceptibility patterns of *Candida* spp. in specimens obtained from symptomatic women with primary and recurrent vulvovaginal candidiasis.
- To perform molecular epidemiological analysis of antifungal resistant *Candida* species to determine genotype distribution in relation to demographic and bio-behavioural characteristics, and co-infections using multilocus sequence typing.
- To assess whether concurrent presence of *Chlamydia trachomatis*, *Trichomonas vaginalis*, *Neisseria gonorrhoeae*, *Mycoplasma genitalium* and BV- and AV-related microorganisms has an impact on spontaneous clearance and/or treatment outcomes of vulvovaginal candidiasis by using molecular identification techniques.

1.4 Outline of thesis

Chapter 1 provides the introduction of challenges faced in Namibia with understanding the burden of VVC and potential threat posed by antifungal resistant infection. **Chapter 2** provides further background and contextualises the study through up-to-date review of the relevant literature. **Chapter 3** describes the aetiology of vaginal discharge syndrome in Namibian women with VDS, including the prevalence of *Candida* infection, distribution of *Candida* spp. and the phenotypic antifungal drug resistance profile. **Chapter 4** provides unique data on the the incidence, risk factors, and microbial aetiology of VDS treatment failure in Namibia. **Chapter 5** provides a detailed analysis of genotypic antifungal resistance and molecular epidemiology of *Candida glabrata* isolates from women with VVC in Namibia. This information is beneficial to policy makers in the re-evaluation of the syndromic management approach used in resource-limited settings. Finally, **Chapter 6** presents a synopsis and discussion of the core outcomes of this thesis and its impact on public health.

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CHAPTER 2

Literature Review

This literature review contextualizes the present PhD study in the relevant literature. The focus of this literature review is on the epidemiology of vulvovaginal candidiasis (VVC) and its clinical manifestations, on the history of *Candida* antimicrobial regimens and development of resistance to them, on the treatment outcomes associated with VVC and other vaginal infections, such as bacterial vaginosis (BV), aerobic vaginitis (AV) and sexually transmitted infections (STIs), on the genetic resistance mutations of *Candida* to previously and currently recommended antimicrobials; on the detection and genotyping of resistant *Candida* strains and on future treatment regimens for VVC.

2.1 Epidemiology of vulvovaginal– and recurrent vulvovaginal candidiasis

Vulvovaginal candidiasis is a common global problem and is the second most common cause of genital infection in women of childbearing age, with 70-75% of healthy women facing symptomatic VVC at least once in their childbearing years, with some experiencing intermittent and often obdurate forms of the disease. [1, 2]

Complicated VVC may occur in 10-20% of women with symptomatic VVC. [3] A study by Djomand *et al* published in 2016, the only study in Namibia exploring VVC, reported that among a sample population of 335 women 18.2% had symptomatic VVC. [4] In two recent studies, recurrent VVC (RVVC) was estimated to occur in 37,390 females per year in Namibia and in over a million females per year in South Africa. [5, 6] A projected 8-10% of women are susceptible to RVVC, experiencing four or more symptomatic episodes per year, with the majority of episodes taking place between 19 to 35 years of age. [7, 8]

Data of prevalence rate of VVC, and particularly RVVC, have historically been limited. Vulvovaginal candidiasis is mainly diagnosed via a syndromic approach without laboratory testing. This means that many asymptomatic cases may be missed in these epidemiological studies. The only global prevalence study reported that the worldwide prevalence of RVVC was estimated around 138 million women annually in 2013 (Figure 2.1). [7]

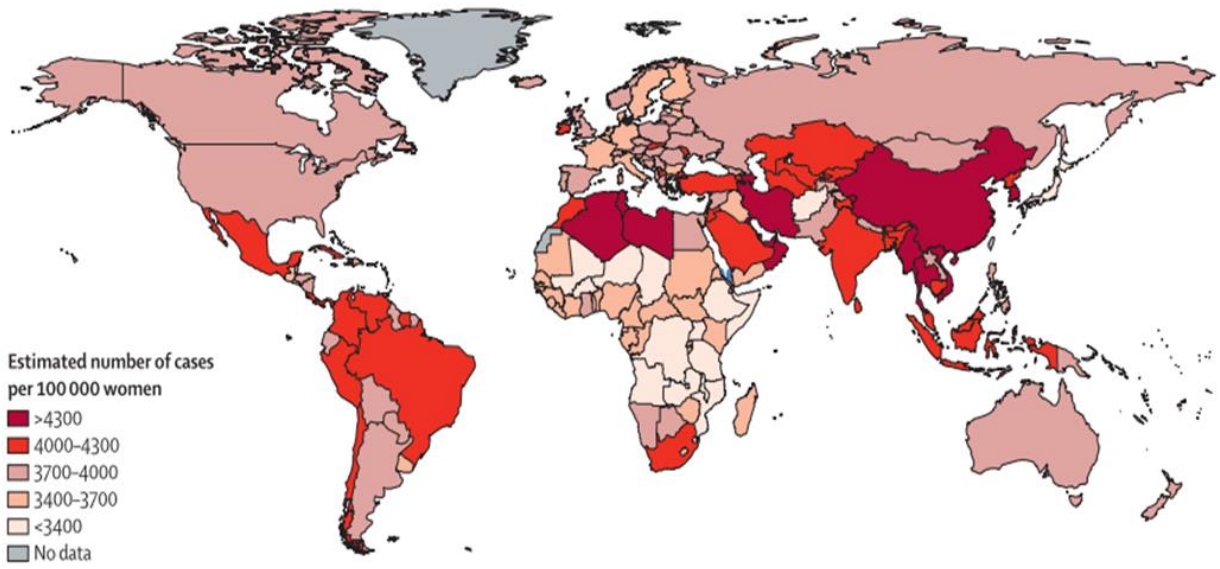

Figure 2.1 Estimated global prevalence of recurrent vulvovaginal candidiasis per 100 000 women[1] (Denning et al., 2018) 10.1016/S1473-3099(18)30103-8

Typically, *Candida* species, from the phylum *Ascomycota*, are harmless eukaryotic commensal yeasts that can be isolated from various sources, including the environment, humans and other mammals. [9] The mucosal surfaces of the gastrointestinal and genitourinary tracts of humans are inhabited by *Candida* species as part of the normal commensal flora in healthy individuals. The aetiology of VVC stems from an overgrowth of *Candida* spp. in the vagina VVC and generally only manifests when the host's immunity becomes compromised. [9, 10]

The cause of VVC in more than 90% of cases is *Candida albicans.* [11] Recently, it has been reported that non-*albicans Candida* species have emerged and that the circulating species differ by geographical location. [11] The most significant of these non-*albicans Candida* species is *C. glabrata* owing to its significant resistance to azoles. [11] *Candida krusei, C. tropicalis, C. parapsilosis* and, very rarely, *Saccharomyces cerevisiae*, are other potential pathogens that may lead to VVC. [12] In addition, *C. africana*, a *C. albicans* related species, has also been reported as a cause of VVC. [13]

[1] Figure 2.1 Global prevalence of recurrent vulvovaginal candidiasis per 100 000 women (in 2013). Estimated using the 6% flat rate for women 15-54 years of age. Prevalence primarily reflects the variable demographics of women in their reproductive years globally.

2.2 Clinical manifestation of vulvovaginal candidiasis

Vulvovaginal candidiasis is defined as an acute inflammatory condition of the vulva and vagina. [11] It is a multifactorial disorder, where a combination of dysbiosis in vaginal microbiota composition, host associated factors and genetics, immunological response as well as *Candida* strains are projected to support disease onset (Figure 2.2). [14]

Bradford and Ravel described that an increase in fungal load may be associated to the severity of vaginal discharge [15]. This supports the idea that inflammatory cells are recruited and triggers vaginal symptoms when a fungal burden threshold has been reached. [15]

The presence of *Candida* species and its ability of dimorphic transition in the vaginal tract can play two roles. In its yeast form, it can be beneficial to the host by retaining balanced microbiota, and is usually seen in asymptomatic women. However, in its hyphal form, it can be detrimental by causing vaginal dysbiosis leading to VVC. [1] Tissue damage ensues due to the breach of mucosal barriers and the secretion of degradative enzymes by hyphae. [1]

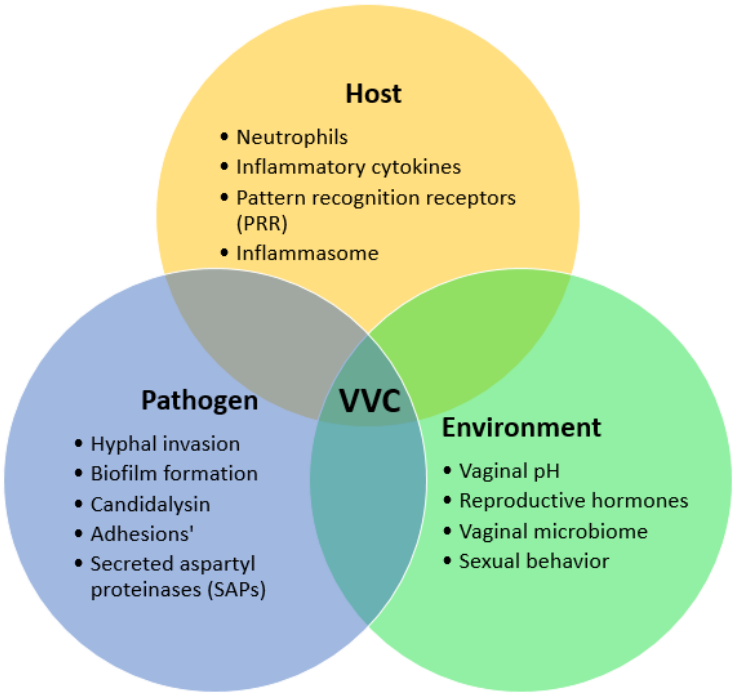

Figure 2.2 Various contributions from host, pathogen, and environment that are necessary to drive the manifestation of VVC[2] (Willems *et al.*, 2020) 10.3390/jof6010027

[2] Figure 2.2 Vulvovaginal candidiasis (VVC) is a multifactorial disease. Multiple inputs from host (yellow), pathogen (blue), and environment (green) are required to drive disease onset and symptomatic infection. Each circle represents major contributing factors to the immunopathogenesis of VVC.

Vulvovaginal candidiasis can be classified into uncomplicated and complicated VVC, as summarised in Table 2.1.

Uncomplicated VVC is classified in sporadic or infrequent VVC, mild-to-moderate VVC, likely to be *Candida albicans* and non-immunocompromised women. [16] The diagnosis of uncomplicated VVC is initially suggested by a clinical examination characterised by vaginal discharge, dysuria or itching, occasionally supplemented by burning sensation on micturition and dyspareunia. [7]

Table 2.1 Classification of vulvovaginal candidiasis (Source: CDC Sexually Transmitted Diseases Treatment Guidelines, 2015).

	Severity and/ or frequency of symptoms	**Predisposing factors**	**Microbial aetiology**	**Treatment strategy**
Un-complicated VVC	Mild-to-moderate Sporadic/ infrequent	None (non-immunocompromised women)	Usually *C. albicans*	Usually topically applied azole drugs
Recurrent VVC	More severe than uncomplicated VVC ≥ four episodes/ year	No apparent predisposing/ underlying conditions Pregnancy, Immunocompromised Immunosuppression Uncontrolled diabetes	*C. albicans*, *C. glabrata* and other non-*albicans Candida* species	Longer duration oral or topical azole therapy; oral fluconazole for first-line maintenance regimen
Severe VVC	Severe vulvovaginitis (i.e., extensive vulvar erythema, oedema, excoriation, and fissure formation)	Pregnancy, Immunocompromised Immunosuppression Uncontrolled diabetes	*C. albicans*, *C. glabrata* and other non-*albicans Candida* species	Longer duration of topical azole or higher dose of fluconazole in two sequential oral doses
Non-*albicans* VVC	Minimally symptomatic/ no symptoms	Pregnancy, Immunocompromised Immunosuppression Uncontrolled diabetes	Non-*albicans Candida* species	Longer duration of therapy with a non-fluconazole azole regimen (oral or topical) as first-line therapy.

The discharge is typically white, curd-like and mainly odourless, and the vaginal wall is enflamed and swollen (Figure 2.3). [7, 17]

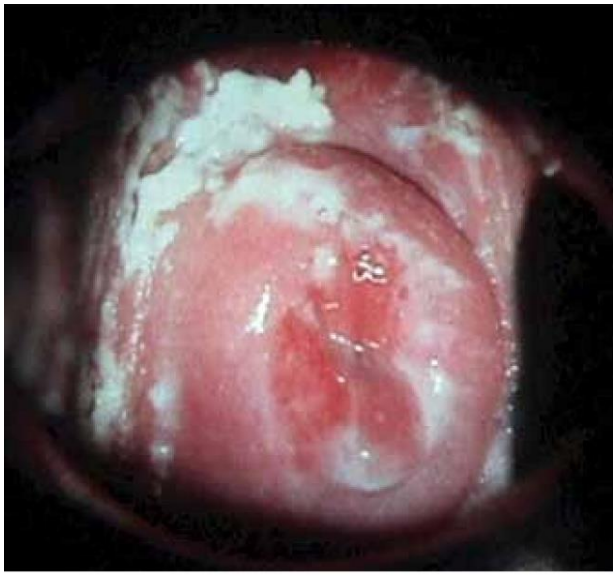

Figure 2.3 White curd-like vaginal discharge associated with vulvovaginal candidiasis[3] (Powell, 2010) 10.12968/BJON.2010.19.17.78559

Vaginal discharge is a frequent genital tract symptom amid women. [18] Vaginal discharge might be caused by either a typical physiologic manifestation or a pathological expression, often making it difficult to differentiate abnormal from normal discharge. [18] Normal physiologic deviations transpire usually due to biological or hormonal changes and pathological vaginal discharge may originate from the vagina or the cervix. [19] Infection with *Candida* spp., usually presents with discharge of vaginal origin. In addition to *Candida*, discharge is also found in other genital infections including aerobic vaginitis (AV), bacterial vaginosis (BV), and to STIs with *Trichomonas vaginalis*, *Neisseria gonorrhoea*e, *Chlamydia trachomatis*, and *Mycoplasma genitalium.* [18] It is however important to note that a lack of vaginal discharge upon clinical examination does not exclude vaginal infections that may cause vaginal discharge and is therefore, one of the chief causes for infected women to not obtain suitable treatment. [20] Because the signs and symptoms of the disease are generic, it is difficult to diagnose the disease centred merely on the patient's medical history and clinical manifestation. Therefore, laboratory diagnosis, by means of microscopy, culture and antimicrobial susceptibility is essential. [21, 22]

[3] Figure 2.3 Candidiasis. White curd-like vaginal discharge associated with vulvovaginal candidiasis.

Complicated VVC may occur in 10-20% of women with symptomatic VVC. [16] It has been reported that *Candida albicans* as species causes 80-95% cases of symptomatic VVC. [23] With regards to RVVC, the severity of symptoms differs from moderate to severe, but perpetually upsets the quality of life of women and is concomitant with substantial stress. Adding together pruritus, pain, and discomfort, women with RVVC frequently registers disparagement, humiliation, debilitation, and complications with their sexual life and intimate relationships, universally noting qualms about STIs attained from their partner. [8]

Vulvovaginal candidiasis, both incident and recurrent infection, and other urogenital infections including BV, AV and STIs, are associated with a wide variety of financial, psychosocial, reproductive and genital health implications. [8, 24] This is especially evident in cases of untreated, persistent and recurrent infections, which may possibly lead to pelvic inflammatory disease (PID). [8, 25] Implications of birth outcomes may include ectopic pregnancy, reproductive dysfunction and adverse pregnancy outcomes, such as pre-term birth, foetal infections and post-delivery infections. [26] Although many healthy women experience at least one episode of symptomatic VVC during their lifetime, some women may be more susceptible to severe VVC. [27, 28] Severe VVC may lead to serious reproductive problems in pregnant women including chorioamnionitis which may lead to preterm birth and in rare cases, systemic candidiasis in neonates after delivery. [26]

Therefore, complicated VVC always requires special diagnostic and therapeutic deliberation. [16] Vaginal cultures should be attained from women with complicated VVC to ratify clinical diagnosis and ascertain uncommon species, together with non-*albicans* species. *Candida glabrata* is not simply identified via microscopy as it does not produce pseudohyphae or hyphae. [16]

2.3 Laboratory criteria for diagnosis

The diagnosis of vaginitis encompasses several critical steps to establish the cause and therefore, the appropriate treatment strategy. The first step includes taking the history and performing a clinical examination. [67, 68] Vaginitis usually presents with abnormal discharge, dysuria, odour or itching. [67, 68] The subsequent steps in the diagnostic workflow should typically include wet mount microscopy, the measurement of pH and the Whiff test. [29] Thereafter, specific laboratory tests, such as the Amsel criteria, Nugent score, culture and molecular identification techniques are used to identify the specific cause of vaginitis. [30, 31]

Vulvovaginal candidiasis diagnostics include wet mount and Gram stain microscopy for the detection of yeast cells (Figure 2.4). A culture on a dextrose and chloramphenicol agar plate is also performed for the isolation of yeast colonies, and a germ tube test may be used to presumptively identify *C. albicans.* [32] A germ tube test can be used to distinguish between *Candida albicans* and non-albicans *Candida*, where a short hyphal (filamentous) extension arises laterally from a yeast cell, with no constriction at the point of origin is when incubated in a proteinaceous media seen in *Candida albicans* and *Candida dubliniensis.* A negative germ tube tests is characterized by no hyphal extension arising from a yeast cell or a short hyphal extension constricted at the point of origin, and include *C. tropicalis, C. glabrata* and other yeasts. [32]

If available, the use of chromogenic agar or the Vitek 2 automated system (bioMérieux, France) may be used to identify non-*albicans Candida* spp. [32] Antifungal susceptibility test protocols, which provide minimal inhibitory concentration (MIC) values and clinical breakpoints to recommended treatment strategies, include those established by the Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST). [33]

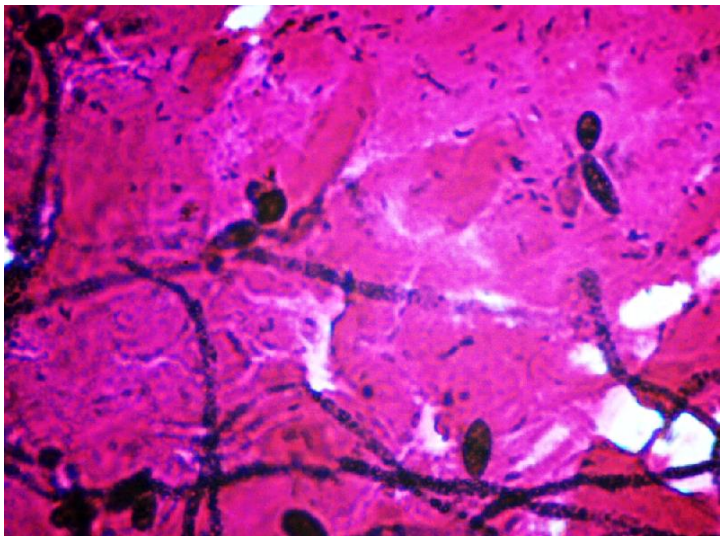

Figure 2.4 Gram stain reaction of vaginal fluid smear indicating Gram positive yeasts with purple colour appearance using 100x objective (Dunaiski, C.M., 2022).

However, the uncommon species may have to be confirmed using other techniques. [34] The matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) can be used to identify unidentified *Candida* spp. by analyzing their protein profiles. [33, 35] The

mass spectra obtained from these yeast isolates can then be compared to the existing spectra in databases by finding the closest match. [33, 35]

Several molecular techniques, from polymerase chain reaction (PCR) to next generation sequencing (NGS) have been developed to identify medically important yeasts in recent years, especially considering the limitations of commercial systems like API 20C (bioMérieux, United States of America) and Vitek 2 bioMérieux, United States of America). [32]

2.4 Pathogenesis of VVC

The ability of *Candida* spp. to persist within the host and cause infection has been attributed to its diverse virulence features (Figure 2.5), which include adhesion capacity, biofilm production, dimorphism, interference with the immune system, synergism, the secretion of extracellular enzymes and several other predisposing factors. [36]

2.4.1 Changes in vaginal microbiota

In addition to the role of virulence factors in the pathogenesis of VVC, changes in the vaginal microbiota also play a significant role in the progression of the disease. The health of the vagina is pre-empted by the microorganisms that inhabit and defend it against infections. [36] A healthy vaginal microbiota is dominated by the *Lactobacillus* spp., which provide a number of protective functions: it inhibits the growth of potential pathogens by competing for nutrients; it releases antimicrobial compounds that activate an immune response and finally, it maintains the low vaginal pH by producing lactic acid. [37]

Vaginal dysbiosis is the shift in the distribution and abundance of commensal microorganisms, such as *Lactobacillus* spp., enteric bacteria, *Gardnerella vaginalis* and *Candida albicans*, or an acquired infection, such as a STI can lead to a variety of vulvovaginal diseases termed vaginitis. [38] It should however be noted that the composition of the female genital tract microbiota is specific to each woman and is most likely to have originated early on in life via exposure to important birthing-associated maternal microbes. [39]

2.4.2 Adhesion capacity

In addition to changes in the vaginal microbiota, the attachment of *Candida* to the host cells also plays a role in the virulence of the organism. [36] Regardless of the existence of several pattern recognition receptors (PRR) on the epithelial surface, yeast forms of *Candida*

asymptomatically settle in the vaginal epithelium. [37] Thereafter, *C. albicans* begins to transition from its harmless yeast form to its pathogenic hyphal form. [37]

2.4.3 Dimorphism

Candida albicans is a dimorphic species that can propagate as yeast or filamentous forms. [40] The yeast-to-hypha switch occurs under morphogenesis-inducing conditions such as increases in temperature, oestrogen, elevated vaginal pH, and microbiota disruption, followed by its recognition by the PRRs. [37] In addition to *C. dubliniensis*, *C. albicans* is the only other C*andida* species adept of establishing true hyphae. Hyphae are deemed to account for essential roles in disease progressions including adhesion and tissue invasion. Irrespective of both species having the ability of producing hyphae, *C. albicans* is a more effective pathogen. [40] The production of hyphae ultimately leads to an increase in the hyphal biomass. [37] Hyphae-associated virulence factors including *Candida* lysin, secreted aspartyl proteinases (SAPs) are expressed and in turn activates nod-like receptor protein 3 (NLRP3) inflammasome signalling, eliciting inflammatory cytokines and chemokines in the vaginal epithelium. [37] The initial migration of polymorphonuclear leukocytes (PMNs) to the vaginal lumen is the result. [37]

2.4.4 Biofilm production

The ability of *Candida* to form biofilms, consisting of yeast, hyphae, and commensal bacteria, is a chief virulence factor of *Candida* that plays a role in the adherence to the tissue and colonisation of *Candida*. Additionally, biofilms are able to shield *Candida* from external factors including the host immune system defences and antifungal drugs. [41, 42]

Candida biofilms budding on the vaginal epithelial lining exhibits the archetypal biofilm infrastructure with the adherence cells of embedded in an extracellular matrix. [42] Biofilm features are governed by the ability of each species to produce extracellular polymeric substances (EPS), as well as dimorphic growth, the biofilm substratum, carbon source obtainability and additional reasons. [41] Differences in these features are therefore important in presenting the pronounced complexity and diversity within pathogenic yeasts of the *Candida* genus, the consequences in the persistence of colonisation and infections, and also the antifungal resistance usually linked with *Candida* biofilms. [41]

2.4.5 Production of extracellular enzymes

Candida, like any other microorganism, produce innate hydrolytic enzymes that stimulates the penetration of *Candida* into the host tissues. [36] The role of these enzymes, which include extracellular phospholipases and proteases, is essential in destroying cell membranes made up of lipids and proteins. [36] The destruction of the epithelium cell membranes via the action of phospholipase allows the hyphal tip of *Candida* spp. to enter the cytoplasm, thereby, playing a significant role in the invasion of host tissues in lesions of candidiasis. [36] Proteinase production is produced to amplify colonisation and penetration of microorganisms into host tissues. [36] In addition, these proteases allows *Candida* to evade the host's immune system by degrading several proteins vital in host defences. [36] These proteins include immunoglobulins, complements, as well as cytokines. [36]

2.4.6 Interference with host immune system

The immunopathogenesis of VVC (Figure 2.5) starts with the asymptomatic colonization of yeast *Candida*. Symptomtic VVC occurs when morphogenesis-inducing conditions, such as a change in vaginal pH, causes the yeast-to-hyphae switch. This switch furthmore leads to innate immune activation and the recruitment of polymorphonuclear cells (PMNs). [36, 37]

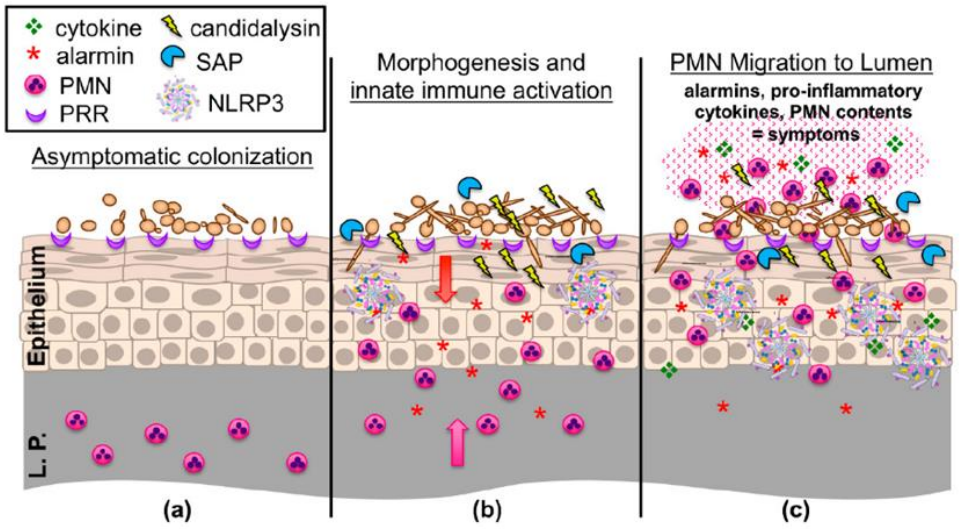

Figure 2.5 The immunopathogenesis of *C. albicans* vaginitis[5] (Willems *et al.*, 2020). 10.3390/jof6010027

[5]Figure 2.5 An updated working model of the immunopathogenesis of *C. albicans* vaginitis. (a) The asymptomatic colonization of yeast forms. (b) Yeast-to-hypha switch under morphogenesis-inducing conditions leading to increased recognition by PRRs and the expression of hypha-associated virulence factors, including candidalysin, secreted aspartyl proteinases (SAPs). Inflammatory cytokines and chemokines released results in migration of PMNs. (c) Continued expression of innate immune effectors leading to symptomatic infection and characteristic immunopathology.

Biofilm growth has been shown to afford *Candida* protection from the host immune response, as both neutrophils and mononuclear cells are less effective in killing *Candida* biofilm cells. [36] This is because mononuclear cells become ensnared in biofilms and are not able to efficiently activate or phagocytise fungal cells; and because neutrophils have impaired function against both *C. albicans* and *C. parapsilosis* biofilms. [36]

It has been shown that gene polymorphisms in innate immune effectors including PRRs, toll-like receptor 2 (TLR2), mannose-binding lectin (MBL), the NLRP3 inflammasome, and cytokine interleukin-4 (IL-4), play a role in the multifactorial susceptibility to RVVC by causing a cascade of immunological events. [37, 43] A polymorphism in TLR2 reduces the production of IL-17 and interferferon-gamma (IFN- γ); a polymorphism in codon 54 in MBL2 causes a defect of the normal function of MBL2, which is to promote complement activation and *Candida* killing; a polymorphism in the NLRP3 inflammasome causes hyperinflammation by overproduction of IL-1β; and the −589C/T polymorphism in the IL-4 gene results in a raised concentration of IL-4 and reduced concentration of MBL and nitric oxide (NO) in vaginal fluid. [37, 43] Interleukin-4 has been recognised to inhibit macrophage activation and NO production. [37, 43]

2.4.7 Synergisms

The interactions between bacteria and fungi in the vagina unmistakably contributes a significant role in the pathogenesis of genital infections. [1] There are bacterial species that have been shown to inhibit the growth of *Candida* hyphal/ virulent forms, and vice versa. The co-culturing of vaginal yeast and bacteria in some studies have reported that bacteria, especially lactobacilli, may curb the dimorphic transition from yeast-to-hyphae in *Candida* by maintaining a low pH and producing bactericidal compounds. [1] Other species that have been found to inhibit *Candida* from transforming into its virulent hyphal form include *Pseudomonas aeruginosa*, *Escherichia coli* and *Acinetobacter baumannii*. [44, 45] Moreover, examples of other bacteria-fungi interactions include: interactions of *Streptococcus* species, including Group B *Streptococcus*, with *C. albicans* have been found to promote augmented biofilm production and adhesion to host epithelia; *C. albicans* might have an inhibitory effect on the growth of *Neisseria gonorrhoeae*; and the fungal-bacterial co-infections between *E. coli* and *C. albicans* is possibly associated with disease severity. [45]

2.5 Risk factors of VVC and RVVC

The inception of most VVC cases are triggered via an eclectic array of predisposing factors. These include the use of antibiotics, increased oestrogen levels, contraceptives, uncontrolled diabetes mellitus, socio-demographic factors, behavioural factors, immune suppression following disease and psycho-emotional stress. [25, 37]

Recurrent vulvovaginal candidiasis is an enervating, continuing condition in women and its pathogenesis is inadequately surmised. Regardless, the triggering events of this disease in some patients have been reported. The predisposing factors have been observed in women with diabetes, women needing repeated antibiotics, women having cystic fibrosis, and women with a history of frequent episodes of VVC. [8]

Other clinical conditions can also facilitate RVVC occurrence, including exposure to chronic stress and deficient antioxidant micronutrients that may cause a defect in the immune system. In addition, intrinsic factors counting glycaemia, insulin resistance, chronic stress, antioxidant capacity, general immune status, vaginal inflammation and microbiota, are involved in predisposing women to developing RVVC. [14] Figure 2.6 demonstrates the various risk factors involved in the pathogenesis of RVVC. [14]

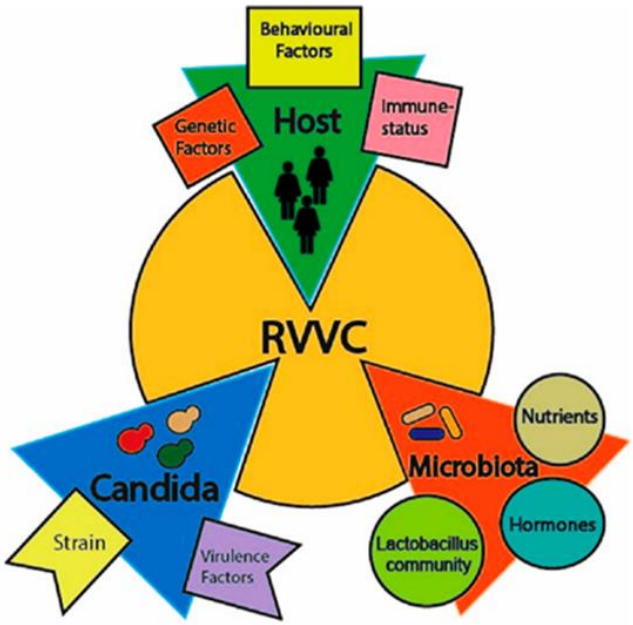

Figure 2.6 The risk factors associated with recurrent vulvovaginal candidiasis (RVVC) susceptibility[6] (Rosati *et al.* 2020) 10.3390/microorganisms8020144

[6] Figure 2.6 The factors contributing to recurrent vulvovaginal candidiasis (RVVC) onset.

2.5.1 Socioeconomic-demographics

The incidence of VVC has been reported to increase from the age of 20, due to the onset of sexual activity. The peak of VVC cases are in the age range from 30-40, and declines in female older than 40 years old. [25] Amid the RVVC population, the peak proportion of cases were in the age range of 26-40, which echoes the described hormonal and behavioural risk factors to infection. [7] The incidence of RVVC among women older than 40 years, differs from that of VVC in that the RVVC rates in the premenopausal and postmenopausal populations are substantially high. This may be reflected from the occurrence of prospective age-linked health conditions, for example the use of exogenous oestrogen to remedy atrophy. [7] Low levels of education in women also show an increased risk for vaginal infections. [46]

Vulvovaginal candidiasis has been found to be considerably higher among women with polygamous husbands. [47] Even though the role of sexual transmission of *Candida* is still contentious, penile colonisation with *Candida* has been described. [46, 48] In a review by Arfiputri *et al.*, the majority of patients with VVC were married, supporting the notion that marital status may play a role in the colonisation and pathogenesis of *Candida* in the vagina. [48]

2.5.2 Increased oestrogen levels

A well-studied contributor to the pathogenesis of VVC is that of increased oestrogen levels. These can be observed from natural hormonal fluctuations, such as during puberty and menopause, high oestrogen oral contraceptives, hormone replacement therapies, and pregnancy. [7] Pregnancy, in particular, is a major predisposing factor of VVC in women of reproductive age, as increased levels of reproductive hormones which provides glycogen, which is a carbon source for *Candida.* [48] In addition, hormone replacement therapy has been shown to not only increase the risk of VVC, but also RVVC. [7]

2.5.3 Non-hormonal contraception

Non-hormonal contraceptives such as intrauterine device (IUD), spermicides, condoms and diaphragms may also trigger *Candida* infection as they can alter the vaginal ecosystem and lead to vaginal dysbiosis. [7] In particular, IUDs may trigger *Candida* infections due to various factors involved in the virulence of the organism, including proteinase production and antifungal resistance. [49]

2.5.4 Uncontrolled diabetes

Hyperglycaemia, usually seen in patients with diabetes mellitus, may contribute to risk of VVC. [50] The increased glucose concentrations stimulates changes that augment yeast growth and adhesion, as well as interfering with the host's immune response. In particular, hyperglycaemia reduces the killing capacity of *Candida* by neutrophils by causing a lessening in the indiscriminate motion of neutrophils, chemotaxis, phagocytosis and microbial death, ultimately triggering increased colonisation of *Candida.* [50] In addition, the higher prevalence of VVC among diabetic patients has also been found to relate to increased aspartyl proteinases production, which are important virulence factors of *Candida.* [50]. Diabetes mellitus is also a risk factor to RVVC. [51] Impaired glucose tolerance and glucosuria in type 2 diabetes mellitus demonstrates an increased risk in the pathogenesis of RVVC in women. [51]

2.5.5 Antioxidant capacity

Chronic stress and reduced antioxidant capacity have also been related in particular to RVVC. A study by Akimoto-Gunther and colleagues, measured lower levels of early-morning cortisol levels and overall antioxidant capacity in women with RVVC. [51] A decline in antioxidant capacity plays a role in an impaired immune status. Immunodeficiency in turn plays a role in the pathogenesis of RVVC via reduced modulation of signal transduction factors, immune-mediated cytokine production and cell-mediated elimination of *Candida*, as observed with a declining antioxidant capacity. [37]

2.5.6 Sexual behaviour and hygiene

There is a variety of behavioural aspects that may trigger the growth of *Candida.* Behavioural patterns related to sex, including the menarche, sexual debut, the number of partners, semen, lubricants and oral sex, have all been associated with VVC due to its impact on the vaginal microbiota. [48] It is however important to note that there are various sociodemographic aspects that play a role in the sexual behaviour of women. [52] One of the most important of these aspects include age. [52] Dubbink and his team described that women less than 25 years frequent bars more often, practice oral sex and have concurrent sexual partners .[52] In addition to age, higher risk sexual behaviour has also been linked to the place of residence and age, economic status and education, as well as ethnicity and (human immunodeficiency virus) HIV status. [52]

Personal hygiene can influence the composition of the vaginal microbiota. [39] Therefore, practices such as continuously using panty liners and using tampons during menstruation, are

factors that increases the risk of triggering VVC. [53] Panty liners, which are non-breathable feminine hygiene products, can transfer intestinal flora, like *Escherichia coli*, to the vaginal area, and induce a humid area for the propagation of microorganisms. [54, 48] In addition to *E. coli*, *Candida* from the intestinal flora can also be transferred from the rectum to the vagina via inappropriate wiping techniques .[45] The type and fit of clothing and underwear also play a role in potentially triggering VVC. [48] Both the use of panty liners and tight fitting clothing and underwear have been associated with RVVC, as it induces a hyperactive immune response. [45, 54]

Vaginal douching for hygiene is a common practice among women worldwide. Vaginal pH, guarding acidic mucus, vaginal mucosal immune response and adaptive immunity are the most vital mechanisms of protection against the invasion and proliferation of pathogenic microorganisms. [55, 56] However, these components come under threat as vaginal douching can alter the vaginal microbiota and thereby increasing the risk for VVC. [55, 56] Several VVC-linked adverse health outcomes are associated with vaginal douching. These include pelvic inflammatory disease, preterm delivery, and sexually transmitted infections (STIs). [55]

2.5.7 Co-infections with other genital tract infections

Predisposition for severe VVC may be augmented by immune function changes seen in disorders such as HIV. [39] In addition, vaginal infections may contribute to the acquisition and transmission of HIV, herpes simplex virus-2 (HSV-2) and other STIs, when exposed. [47, 57, 95] Studies have reported that abundant diverse genital bacteria may escalate HIV risk. [59, 60] In particular, BV shows an exacerbated association with these outcomes, and has a significant relationship with STIs. [61] In certain high risk populations it had been reported that women with BV have a 60% chance to acquire HIV and that there's a three-time likelihood for HIV to be transmitted an uninfected partner. [62] This may be due to disturbances in the vaginal microbiota observed in women with BV, augmented genital inflammation and HIV target cell activation via inflammatory cytokine induction. [62]

In addition to BV, VVC and other vaginal pathogens are related to STIs. [63] These associations may be due to various factors such sexual behaviour and mucosal barrier disruption which exacerbates the risk of HIV acquisition. [63] Several studies describe that symptomatic VVC is observed in HIV-infected women at a greater frequency than that of HIV-uninfected women. [60, 61] These dire clinical implications are also seen in varying degrees in aerobic vaginitis (AV), *C. trachomatis*, *T. vaginalis* and *M. genitalium* vaginal infections to mention but a few. [59, 64]

2.5.8 Use of antimicrobials

Another extraneous factor that contributes to changes in the composition of the vaginal microbiota is the use of antibiotics. [1] Antibiotics play a role in impairing the normal vaginal microbiota that may cause overgrowth of *Candida* in the vagina, thereby increasing the risk of developing VVC. [65, 66] The use of antimycotic therapy may also be a contributing factor to RVVC as some strains of yeast may illustrate inducible resistance and synergism. [67]

2.5.9 Availability and quality of health services

The availability and quality of reproductive health remain a challenge in low-income countries. [68] Due to the absence of diagnostics capacity, syndromic management is the status quo for low-income countries, including countries in sub-Saharan Africa. [18, 69] The standard treatment of vaginal discharge in women include broad spectrum antibiotics used to treat the symptoms of either STIs or other genital tract infection. [20, 22] The exacerbation of VVC and RVVC may however be augmented by asymptomatic cases that are left untreated. [7, 70, 71]

2.6 Vulvovaginal candidiasis antimicrobial treatment

The classes of drugs available for the treatment of *Candida* infections includes azoles, polyenes, echinocandins and the pyrimidine analogue flucytosine in the form of topical, oral, prescription and over-the-counter treatments. [7] Treatment of elementary vulvovaginal *C. albicans* infection is simple prescription or over-the-counter single-dose intravaginal treatment, for example vaginal ovules containing antifungals, are efficacious in 80% -90% of the primary infection or episode. [7, 37] However, this type of treatment does not prevent recurrences and azole resistance rates may accelerate to over 15% in women with VVC and repeated exposure to intravaginal treatment. [28]

In many low- to middle-income countries (LMICs), including Namibia and South Africa, the first line drug used for VVC is clotrimazole. [72, 73] The Namibian Standard Treatments Guideline (Figure 2.7) (2011), as well as the South African Sexually Transmitted Infections Management Guidelines (2015) recommends the insertion of a clotrimazole vaginal pessary (available in different dosages), as well as the local application of clotrimazole topical cream to the vulva for 7 days after symptoms resolve. [72, 74]

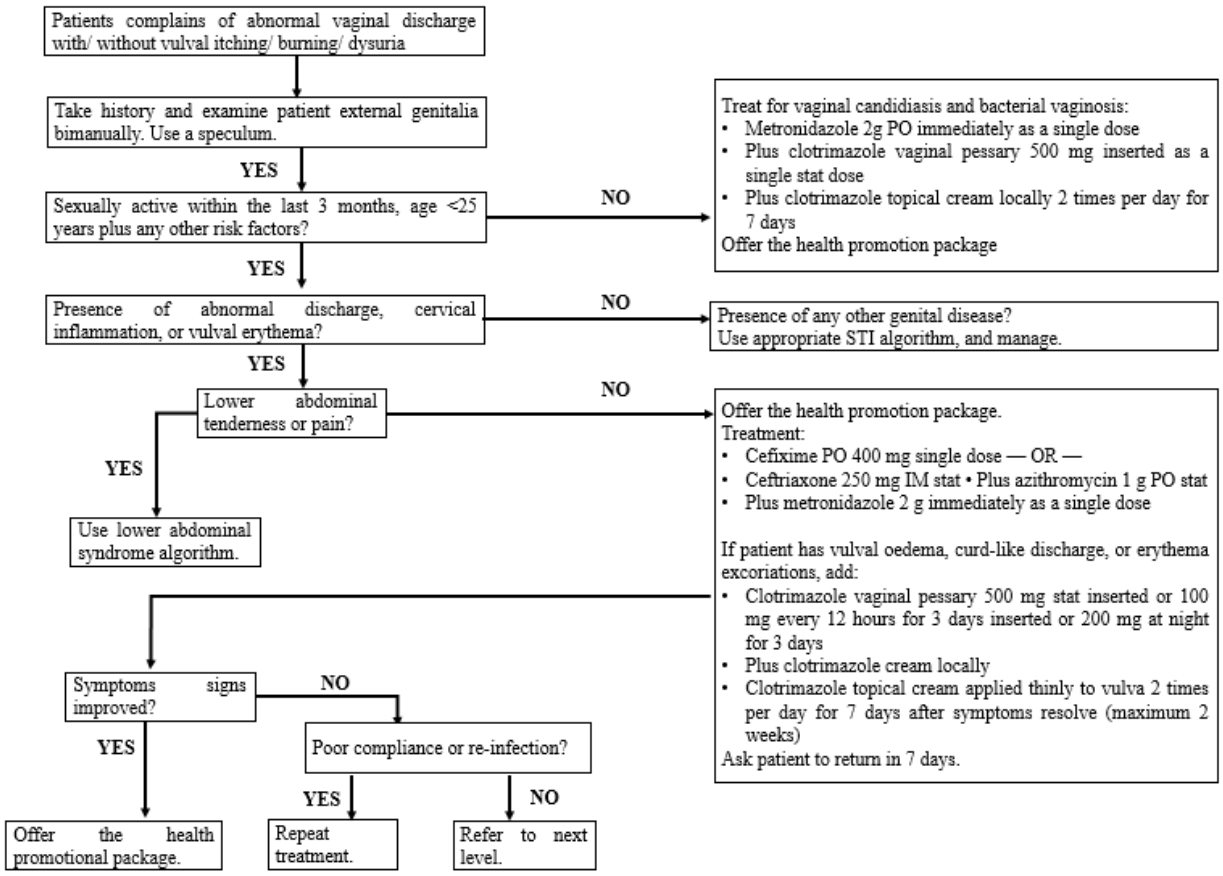

Figure 2.7. The treatment algorithm for vaginal discharge syndrome in Namibia[7] (Ministry of Health and Social services of Namibia, 2011).

Complicated VVC, including RVVC and non-*albicans Candida*, are treated differently. Suppressive therapy is used in patients suffering from RVVC, which usually alleviates symptoms fully during treatment. [8] However, suppressive therapy might have to be repeated or prolonged as some patients might experience symptomatic episodes. [8] Non-*albicans* VVC have been reported to produce milder symptoms. [37]

Nevertheless, inherent azole resistance and acquired resistance mechanisms may complicate treatment of the non-albicans *Candida* species. As with RVVC, extended antifungal regimens or unconventional treatment methods are necessary for disease clearance, but may also contribute to acquisition of antifungal resistance. [37]

[7] Figure 2.7 Algorithm for vaginal discharge syndrome

Generally azoles are used to treat VVC by means of fungistatic activity against *Candida.* [75] Azoles act by binding to and inhibiting the intracellular target enzyme lanosterol 14α-demethylase (ERG11p) that is involved in the biosynthesis of ergosterol. [76] Ergosterol is a vital constituent of the fungal plasma membrane, which retains the integrity of the fungal cell wall. [76] In addition, azoles are preferred due to their cogent bioavailability, antifungal efficacy, and suitable safety. [77] Fluconazole is an oral antimycotic drug that can be used as a single-dose treatment at 150 mg to treat uncomplicated VVC. [78] The use of fluconazole is, however, not included in most national standard treatment guidelines of LMICs for the treatment of VVC. Other azoles used as an antimycotic against VVC includes itraconazole, voriconazole, ketoconazole and posaconazole. [79] It is also very uncommon for polyenes, echinocandins and flucytosine to be prescribed for the treatment of VVC.

Echinocandins have however been demonstrated to be effectual against some *Candida* biofilms, which are typically recalcitrant to triazole therapy. [79] Nonetheless, with the dawn of azole drug resistance and recurrent infections the need for alternative therapies have become imperative for the clearance of *Candida.* [79]

Probiotic therapy to the mucosal surface is one of the alternative therapies that have been suggested for the treatment or prevention of VVC. [79] *Sacchoromyces cerevisiae* and *Lactobacillus* spp. have been found to help protect against *C. albicans* via adherence inhibition, accelerated clearance, blockade of the yeast-to-hypha transition, and reduced expression of established virulence effectors. [79, 80] Probiotic use has shown to decrease the risk of recurrence, and if used in conjunction with antifungal treatments may provide a long term cure against RVVC. [79, 80, 81] Other alternate treatments consist of vaginal vinegar shower, povidone iodine, boric acid, tea tree oil, and garlic. [79]

2.7 Treatment Outcomes

Although readily available, antibiotic and antifungal treatments are not always effective due to many reasons. [82, 83] Complications with therapy may arise due to the treatment algorithm, patient compliance, antimicrobial usage, treatment failure, interactions between drugs and microbial resistance, leading to persistent and recurrent infections. [7, 79]

2.7.1 Persistent infections

Persistent infections are defined as detection of the same pathogen at follow-up that do not desist regardless of the administration of treatment. The causes for persistent positivity may

include antimicrobial/antifungal resistance, treatment failure, slow clearance of detectable bacterial nucleic acids, or reinfection. [7] Certain sexual behaviours, such being newly diagnosed with HIV and having numerous sexual partners, have also been independently associated with persistent positivity. [84] The formation and persistence of biofilms in BV and VVC have also been linked to recurrent and persistent infections due to treatment failure. [85] Though *C. trachomatis, N. gonorrhoeae*, *M. genitalium* and *T. vaginalis* infections are remediable, some studies have described persistent positivity after applicable therapeutic management. [59, 86]

2.7.2 Recurrent infections

Recurrent infections are defined as more than one symptomatic episode despite therapeutic interventions. Recurrent infections of common vaginal infections are incipient and are a significant cause for concern. [1, 7] Recurrent BV has been characterised by repetition rates as high as 30–50% within three months, RVVC is characterized by ≥ four repetitive episodes a year and recurrence rates of recurring T. *vaginalis* have been described as high as 5–8% within two months of primary diagnosis. [1, 85] High rates of relief of symptoms have been shown to correlate with patients that had their episodes diagnosed and treated by a physician with prescription antifungal drugs. [7] The opposite is true among women who self-diagnose and self-medicate with over-the-counter drugs. [7, 37] Failure to initiate and maintain a prolonged suppressive therapy regimen has been a common cause for relapse in women suffering from RVVC. . In addition, different vaginal bacterial pathogens such as *Streptococcus agalactiae* and *Gardnerella vaginalis* have been isolated in samples from women with RVVC, indicating the presence of mixed infections. [87]

2.7.3 Antifungal resistance

Antifungal resistance of *Candida* spp. is a mounting problem universally. [88] Figure 2.8 illustrates the various mechanisms responsible for antifungal drug resistance. [89] Even though antifungal resistance has been reported less often in *C. albicans*, it shows an increase with long-term antifungal use and with recurrent infections. [2] Antifungal resistance can be either intrinsic or extrinsic, and numerous of the non-*albicans Candida* species, for example *Candida krusei*, are intrinsically resistant or less susceptible to several classes of antifungals, where others, counting *Candida glabrata*, develop acquired resistance upon exposure to antifungal agents. [2].

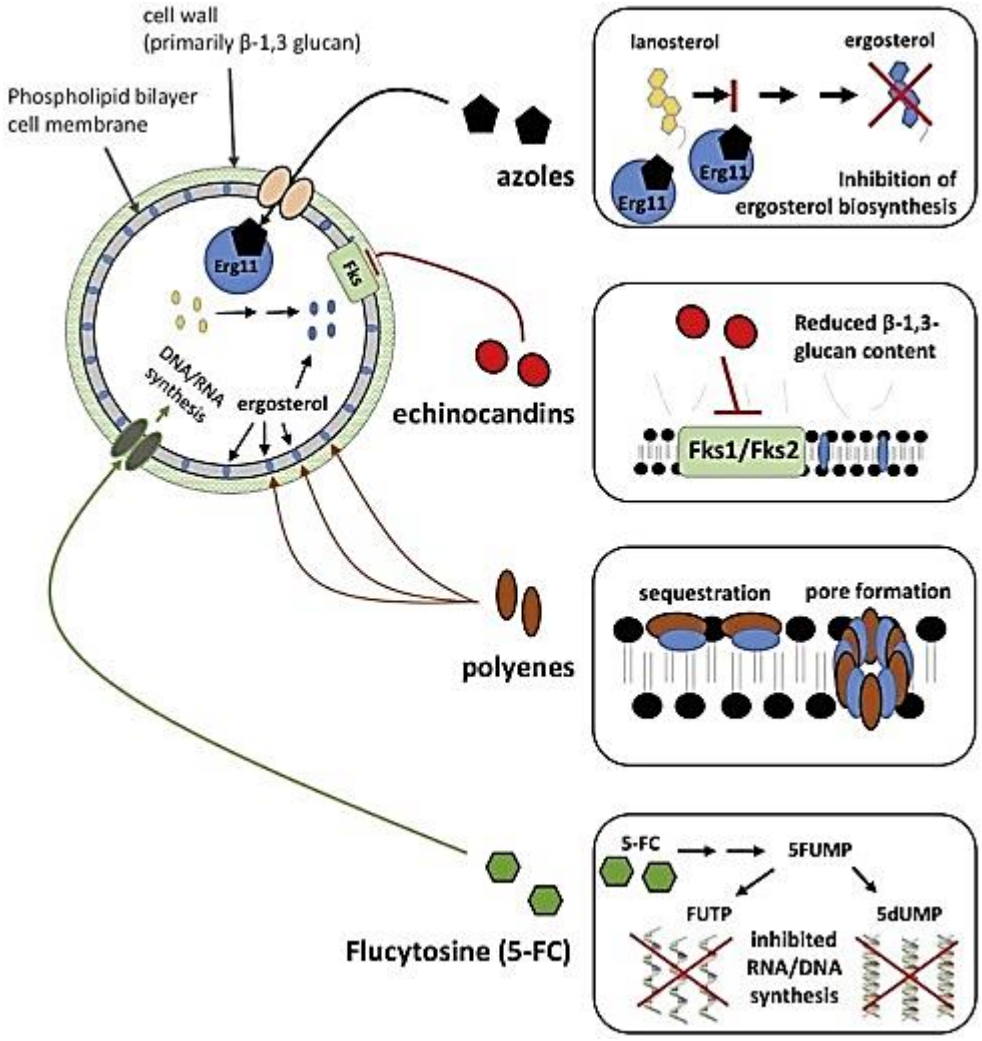

Figure 2.8 Mechanism of antifungal drug resistance in *Candida* (Morio *et al.*, 2017) 10.1016/j.ijantimicag.2017.05.012

Azole resistance can be seen in *Candida* isolates in both VVC and RVVC. [90] *Candida albicans* isolates that demonstrate resistance to fluconazole, itraconazole, and voriconazole from VVC patients have been reported worldwide. [91] With regard to RVVC, there is a variability in studies. Regardless, azole resistance in *Candida* isolates has also been reported in patients with RVVC. [28]

[8] Figure 2.8 Mode of action for allantifungal drug classes. Azoles inhibit lanosterol14-α-demethylase and thus the formation of ergosterol, a key membrane sterol. Echinocandins inhibit the formation of β-1,3-glucans, which are essential components of the cell wall. Polyenes induce membrane instability and form destabilizing ion-channels. Flucytosine is converted to compounds, which inhibit both RNA and DNA synthesis.

Non-*C. albicans* infections and the rates of azole resistance differ topographically, probably due to prescribing patterns of clinicians for both the treatment of and prophylaxis against invasive candidiasis. [90] *Candida tropicalis* usually shows a moderate level of fluconazole resistance and is intrinsically resistant to 5-fluorocytosine. [2] However, acquired resistance to azole antifungals may ensue subsequent to exposure to these antifungals. [2] The causes of azole resistance among *Candida* spp. includes mutations, which lead to the overexpression of the drug target and the up-regulation of transporters. [90, 92]

Figure 2.9 illustrates the various mechanisms involved in fluconazole resistance in different *Candida* species. [93]

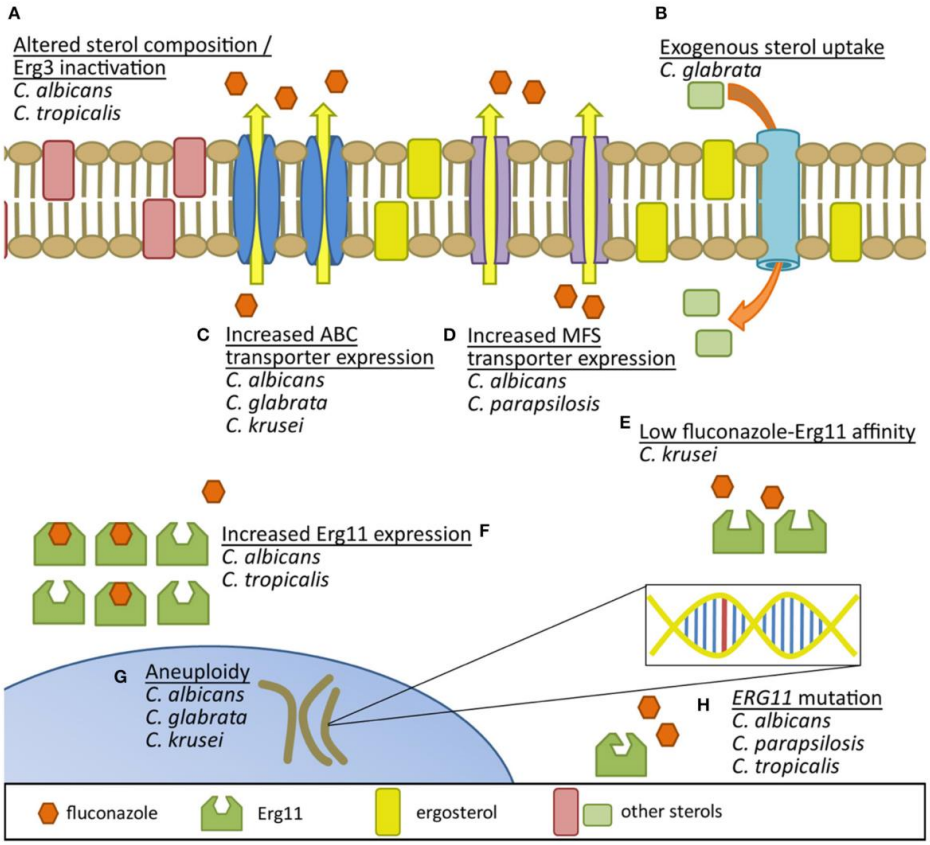

Figure 2.9 Fluconazole resistance mechanisms in *Candida* species[9] (Whaley *et al.*, 2016) 10.3389/fmicb.2016.02173

[9] Figure 2.9 comparison of documented fluconazole resistance mechanisms in *Candida* species. (A) Erg3 inactivation results in utilization of alternative sterols in the yeast membrane. (B) Uptake of exogenous sterols helps circumvent endogenous sterol production inhibition by fluconazole. Increased production of both (C) ATP-binding cassette efflux pumps and (D) major facilitator superfamily transporters reduces intracellular accumulation of azoles. (E) Inherently low affinity of fluconazole binding to species-specific Erg11 may decrease fluconazole's potential to inhibit the protein. (F) Increased expression of Erg11 protein can help overcome azole activity and (G) aneuploidy may promote genetic adaptation to azole exposure. (H) Mutations in ERG11 can also result in proteins with reduced affinity for fluconazole binding.

The primary target for azole antifungals, cytochrome P450 lanosterol 14a-demethylase, is encoded by the *ERG11* gene. [2] Therefore, overexpression and mutation of this gene have been associated with azole antifungal resistance. [2]

Additionally, the up-regulation of *CDR1* and *MDR1* genes, encoding the efflux proteins of the ATP-binding cassette (ABC) transporter family and the major facilitator superfamily (MFS), has been described to confer to the active efflux of azole drugs in a number of *Candida* spp. [2] Respiration deficiency, which predominantly reduces the production of ATP and reactive oxygen species in mitochondria, has also been reported to cause azole resistance in both *C. albicans* and *C. glabrata.* [2]

Echinocandins, including micafungin, anidulafungin and caspofungin, show a relatively low resistance rate with *C. albicans* and most *Candida* spp., except *C. glabrata.* [108] In *C. glabrata* echinocandins resistance is escalating and there is reason for apprehension as many isolates display cross-resistance to azole antifungal agents. [76] In addition, *C. parapsilosis*, a species with recognised reduced susceptibility to echinocandins, has been recovered from patients treated with caspofungin suggesting acquired resistance. [2] Echinocandin resistance is facilitated by point mutations in two hot spots of *FKS* genes (*FKS1, FKS2, FKS3*), which encode the target enzyme β-1,3-D-glucan synthase that constitutes the echinocandin drug target enzyme (Figure 2.8). [2]

Amphotericin B, from the polyene class of antifungal drugs, utilises its fungicidal activity by means of binding to ergosterol in the fungal cell membrane. [2] This leads to the disruption of cellular permeability. [2] Amphotericin B resistance in *Candida* is quite rare and may be caused by combined mutations in *ERG11* and in *ERG3* or *ERG5* and single mutations in *ERG6* or in *ERG2.* [2] Flucytosine, another uncommonly used antifungal for the treatment of VVC, is actively transported into the fungal cell by permease, which is encoded by *FCY2.* [2] It is consequently converted via the enzymes cytosine deaminase or uracil phosphoribosyl transferase encoded by the *FCY1* and *FUR1* genes, respectively and exert flucytosine resistance by inhibiting transcription, DNA replication, and protein synthesis (Figure 2.8). [11]

2.7.4 Drug interactions

The treatment for BV with clindamycin or metronidazole, for example, have been found to lead to antimicrobial resistance (AMR) among vaginal aerobic bacteria. [81] Antibiotic drugs have also been found to contribute to antifungal resistance by reducing the amount of good bacteria,

i.e. lactobacilli, thereby providing favourable conditions for *Candida* growth. [65] In addition, drug susceptibility studies have demonstrated that fungal cells may modulate the action of antibiotics and that, conversely, bacteria can affect antifungal activity. [65, 94] Additionally, antifungal resistance can be attributed to prior inappropriate fluconazole dosing and recent exposure to caspofungin or fluconazole. [90]

2.7.5 The role of bacterial vaginosis and aerobic vaginitis

Bacterial vaginosis is symptomatic in about 50% of women of reproductive age globally with the highest prevalence in Sub-Saharan Africa (Figure 2.10). [61, 95]

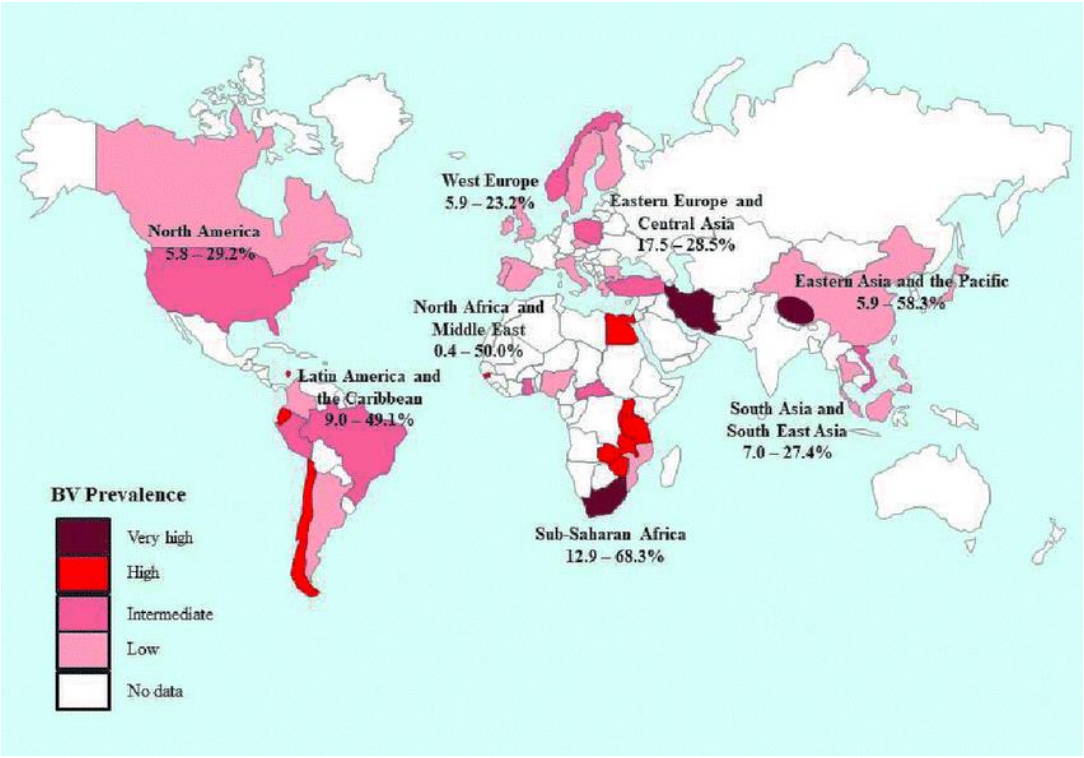

Figure 2.10 Global burden of bacterial vaginosis in 2013[10] (Wong *et al.*, 2018) PMID: 30580358

It is the most frequent cause of vaginal discharge, and is instigated when *Lactobacillus* spp., the principal species in healthy vaginal flora, is substituted by anaerobes, including *Gardnerella vaginalis*, *Atopobium vaginae*, *Prevotella* spp. *Mycoplasma hominus* and *Ureasplasma* spp. [96, 39] However, *G. vaginalis* is the most common and is associated with biofilm production that plays a pivotal role in the initiation and persistence of BV. [97]

[10] Figure 2.10 Global burden of bacterial vaginosis (2013). Data extracted from a systematic review report.

Clinically, a grey profuse homogenous malodourous vaginal discharge may be observed in women. [31] However, these symptoms may be missed in asymptomatic women. [61] The clinical criteria for diagnosing BV is performed via the Amsel criteria. [29] The Amsel criteria are founded on the fulfilment of a maximum of three out of the four specified criteria. [29] The criteria includes a vaginal pH of more than 4.5, an rise in distinctive thin, homogenous vaginal discharge, the release of a fishy amine odour after vaginal secretions are blended with 10% potassium hydroxide (KOH), i.e. a positive Whiff test, and lastly, the appearance of clue cells in wet smears. [67] In addition to the Amsel criteria, a vaginal swab Gram stain is examined to determine the abundance of microbial flora using Nugent scoring. [31] In addition to the unpleasant symptoms, BV may be accompanied with antagonistic sequelae of the reproductive system. Women with BV are in danger of developing PID and it could increase the risk for the acquisition and transmission of STIs and HIV. [95]

Numerous studies have recognised several sexual risk behaviours and other risk factors related with BV. [98] Women with a higher number of sexual partners in their lifespan, who are unmarried, and an earlier sexual debut, are commercial sex workers and carry out regular douching, were found more likely to suffer from BV. [98] Additional epidemiological risk factors consist of a high number of vaginal sexual intercourse per week, a history of pregnancy and cigarette smoking. [98] There is further suggestion that oral and pre-vaginal anal sex may escalate the risk of BV. [99] Independent relationship of BV prevalence may also include ethnicity, education level, and use of hormonal contraceptive pills. [100]

Aerobic vaginitis contributes to approximately 7-12% of cases of vaginitis. It is far less common than BV, and therefore its clinical significance is underestimated. [101] Aerobic vaginitis (AV) is characterised by vaginal dysbiosis, aerobic and enteric bacteria, vaginal inflammation and deficient vaginal epithelial maturation. [101] The diagnosis of AV is determined via a set of criteria established by Donders. The criteria for diagnosis includes elevated yellow secretion, a pH value greater than or equal to 5, a negative Whiff test, the presence of more than 10 leukocytes, the absence of lactobacilli and microbiologically isolated microorganisms:, such as *Escherichia coli, Staphylococcus aureus* and Group B Streptococci (Table 2.2). [102, 103]

Characteristics that AV shares with BV include the diminished or absence of lactobacilli, increased vaginal discharge and an increase in vaginal pH. There are however several noteworthy differences between AV and BV as elucidated in Table 2.2 (adopted from Kaambo *et al*., 2018). [104]

Table 2.2 Comparison of clinical, microbiological and diagnostic characteristics of aerobic vaginitis (AV) and bacterial vaginosis (BV) (Kaambo *et al.*, 2018) 10.3389/fpubh.2018.00078

Characteristic	Aerobic vaginitis (AV)	Bacterial vaginosis (BV)
Clinical		
Discharge	Yellow to green and mucoid; rotten smelling	White and watery; fishy smelling
pH	> 4.5; often >6.0	≥ 4.5
Whiff test*	Negative	Positive
Epithelial inflammation	Present	Absent
Erosions and ulcerations	Present	Absent
Dyspareunia	Severe	Absent/ mild
Microbiological		
Common pathogens	Commensal aerobic and enteric bacteria; including *Escherichia coli*, *Staphylococcus aureus*, coagulase-negative staphylococci, group B *Streptococcus* and *Enterococcus faecalis*	Commensal anaerobic bacteria; including *Gardnerella vaginalis* *Atopobium vaginae* and *Megasphaera* species
Wet mount microscopy		
Shed epithelial cells	Parabasal cells	Clue cells
***Lactobacillus* species**	Displaced	Displaced
Cocci and coarse bacilli	Present	Absent
Vaginal leukocytes	Present	Absent

* The Whiff test produces a fishy smell when a solution of potassium hydroxide is added to the discharge sample

Aerobic vaginitis is generally diagnosed by means of phase contrast wet mount microscopy in which the AV score is calculated. [104] The AV score is dependent on the lactobacillary grade, the presence of inflammation, the proportion of toxic leukocytes, the characteristics of the microflora and the presence of immature epithelial cells. Much like the Nugent score, the AV score indicates normal, intermediate and severe AV with scores ranging from 0-3 respectively. [104] The Nugent score has three categories, including normal vaginal flora, which has a score of 0-3, intermediate vaginal flora with a score of 4-6 and BV flora, which has a score of 6-10. [105]

The recommended first-line therapy for BV is metronidazole or clindamycin. [98, 99] These antibiotics have been found to be show equivalent short-term efficacy, with cure rates of 80–

90% at one month. [98, 99] It is, however, associated with significant morbidity and high recurrence rates. [112] Bacterial vaginosis recurrence rates are about 80% three months subsequent to efficacious treatment. [61] Moreover, there are some cases in which treatment is unsuccessful leading to persistent BV. [61] Aerobic vaginitis may be treated with antibacterial, hormonal and non-steroidal anti-inflammatory drugs. [106]

2.7.6 The role of sexually transmitted infections

Globally the burden of STIs remains high, with an approximated 376 million new infections of the four treatable STIs- chlamydia, gonorrhoea, syphilis and trichomoniasis in 2016. [3] In Africa, *Chlamydia trachomatis* has an estimated annual incidence rate of 12 million infections, *Neisseria gonorrhoeae* has an estimated annual incidence rate of 11.4 million and *Trichomonas vaginalis* has an estimated annual incidence rate of 37.4 million. [64] In addition, a 10% prevalence of *Mycoplasma genitalium* has been reported in studies from various African countries. [108] These STIs may all present as VDS or remain asymptomatic. Antimicrobial resistance is emerging in *N. gonorrhoeae* and *M. genitalium* infection, while *Chlamydia trachomatis* and *T. vaginalis* resistance is rare.

Chlamydia trachomatis is a Gram-negative obligate intracellular bacterium and is the main cause of bacterial STIs. [109] It poses a threat to global public health due to the impact asymptomatic infections have on long-term reproductive outcomes. [110] Exposure to *C. trachomatis* can cause chlamydial conjunctivitis during vaginal childbirth in about 60−70% of infants. [111] Common chlamydia symptoms in women comprise abdominal pain, abnormal vaginal discharge, intermenstrual bleeding, painful intercourse, burning while urinating, vaginal bleeding after intercourse and yellow discharge with a strong odour. [108] Chronic infections can be established as *C. trachomatis* is able to evade the host's immune systems and migrate to the upper genital tract .[109]

Neisseria gonorrhoeae is an obligate human pathogen and is the second most commonly diagnosed and curable bacterial STI worldwide, with an estimated 87 million new cases in 2016. [3, 112] Gonorrhoea is a very infectious bacterial infection transmitted through genital, oral and anal sex. In women, gonococcal cervicitis manifests in the endocervical canal. Signs and symptoms in women include a green/yellow vaginal discharge with an unpleasant odour, postcoital bleeding, dysuria and/ or vaginal itching. Antibiotic treatment is usually effective in most local infections. [112] It should, however, be noted that *N. gonorrhoeae* is asymptomatic in up to 70-90% of women and repeat *N. gonorrhoeae* infections are common. [112] Positivity can

usually be observed in up to 40% of patients within one year subsequent to diagnosis. [112] In addition, antimicrobial resistance in *N. gonorrhoeae* strains has led to a lack of effective treatment that has resulted in in a major public health problem. [113]

Trichomonas vaginalis is the most ubiquitous non-viral sexually transmitted pathogen worldwide and accounts for an estimated 180 million infections per year. [114] *Trichomonas vaginalis* is a flagellate protozoan, facultative anaerobic, extracellular human parasite transmitted by sexual intercourse, which typically settles in the vagina, cervix and periurethral gland. [115] A significant number of women with *T. vaginalis* infection are asymptomatic [115]; however, symptomatic patients presents with the characteristic symptoms of trichomoniasis including a greenish yellow malodorous vaginal discharge, coital bleeding, supplemented by local irritation signified by vulvovaginal erythema, dysuria, pruritus and oedema. [115] The vaginal pH of women with trichomoniasis is often above 4.5, and in some cases a pH >6 is observed. [114] Other clinical manifestations may include upper reproductive syndromes, such as PID, tubular pathology and an increased risk in preterm birth. [115] The risk factors for *T. vaginalis* infections include a higher number of lifetime sexual partners and irregular use of condoms. [115] *Trichomonas vaginalis* infection can persist for months in women and, if left untreated, can increase the risk for HIV acquisition. [115]

Mycoplasma genitalium causes cervicitis and VDS in women and may be accompanied by PID, infertility and preterm birth. These impediments, principally, PID, may be linked with recurrent *M. genitalium* infections. [108] The regularity of *M. genitalium* infection has been linked to specific high risk populations for STIs, such as men who have sex with men, commercial sex workers and pregnant women, in whom transmission of infection to a foetus might have adverse consequences. [108] The prevalence of *M. genitalium* has been reported in studies from various sub-Saharan countries of up to 10%. [116] Azithromycin is the first-line treatment for *M. genitalium* infections. However, the management of *M. genitalium* has been complicated by multidrug resistance .[116]

2.7.7 Interactions between fungi and bacteria

Bacterial and fungal biofilms in vaginal infections, particularly VVC and BV, have been implicated in treatment failure and recurrence. [44, 87] The expeditiousness of genital infections may also be caused by the overgrowth of *Candida* spp. and the depletion of *Lactobacillus* spp. in vaginal dysbiosis. Biofilms related to *Candida* and *Gardnerella* genital infections may act as a reservoir for *C. trachomatis*, revealing the links between VVC and BV, and STI acquisition

and transmission. [30] A study by Kruppa and colleagues demonstrated a novel interaction between *C. trachomatis* and *C. albicans* via the binding of elementary bodies of *C. a trachomatis* to *C. albicans* yeast and hyphal forms. [45] This binding was shown to considerably decrease the capacity of *C. trachomatis* to infect human cervical epithelial cells, thereby decreasing its disease progression. [117]

2.7.8 Concurrent and mixed vaginal infections

Dysbiosis may also lead to co- and mixed vaginal infections, in which more than one pathogen are responsible for the manifestation of symptoms. [1] Concurrent or mixed infections usually occur with BV, VVC and *T. vaginalis*, in combinations of two or all three. [1] Several studies have elucidated that *T. vaginalis* is a common denominator for co-infections with other STIs, such as *C. trachomatis*, *M. genitalium* and *N. gonorrhoeae.* [117] Additionally, bacterial and viral STIs, have on several occasions been associated with BV and/or VVC. [1] The bacteria associated with BV play an important role in the pathogenesis of *M. genitalium* in addition to other STIs such as *C. trachomatis* and *N. gonorrhoeae* infections. [118] However, it is usually uncommon for *M. genitalium* to have a co-infection with *C. trachomatis.* [118] Nonetheless, concurrent genital infections may increase the susceptibility to long-term complications and transmission for individual STIs. [110] A study by Dubbink and colleagues observed that nearly half of the women with genital *C. trachomatis* infection also had a concurrent *N. gonorrhoeae* infection in certain sub-Saharan African populations. [119] This in turn may increase susceptibility to long-term complications and transmissibility for individual STI. [119]

2.8 Detection of antimicrobial resistance in *Candida* spp.

Antifungal susceptibility test protocols, which provide MIC values and clinical breakpoints to recommended treatment strategies, include those established by the Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST). [33, 120]

The CLSI and EUCAST have established reference methods to carry out antifungal susceptibility tests founded on broth microdilution (BMD) of yeast cultures. Both of these protocols provide MIC values and clinical breakpoints, which are used to recommended treatment strategies. [33, 120] The fully automated Vitek 2 system (bioMérieux, United States of America) is one of the commercial antifungal susceptibility tests that is widely used; however, results are not always consistent with reference methods and only include antimicrobial

susceptibility (AST) for caspofungin, fluconazole, flucytosine, micafungin and voriconazole. [33] In addition, there are several nucleic acid-based methods used to detect mutations conferring resistance. [33]

Matrix Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS can also been used for antibiotic resistance testing. [34] The MALDI-TOF method is used to identify microorganisms by analyzing their protein profiles .[34] The mass spectra obtained from isolates is then compared to the existing spectra in databases by finding the closest match. [34] The susceptibility of yeasts can therefore be analysed by the detection of the mass spectrometric profile subject to antifungal concentration changes. It has been used to detect *C. albicans, C. glabrata* and *C. tropicalis* strains resistant to a number of azoles, as well as echinocandin susceptibility in *C. albicans* and *C. glabrata.* [34]

Any polymerase chian reaction (PCR) or hybridization technique capable to distinguish single-point mutations can theoretically be useful to investigate the presence of an established set of resistance conferring mutations. [121, 122, 123] The study of genes involved in the mechanism of drug resistance can therefore be studied to support the therapeutic options to VVC. [122]

2.9 Molecular epidemiology

Molecular epidemiology applies molecular typing techniques to identify determinants, to study dynamics and distribution of diseases in human populations. [33, 123]

2.9.1 Importance and relevance of conducting molecular epidemiological analyses

Molecular typing techniques, including multilocus sequence typing (MLST), have demonstrated its usefulness in the epidemiology of microbial pathogens, by expediting the considering of the changing aspects of infectious organisms in human populations, the multifaceted interactions between commensal and infectious organisms, the origins of infection, the development of drug resistance in populations and the genetic similarity of isolates of the equivalent species. [33, 123] By identifying these factors, molecular typing allows for improved treatment and the prompt and true identification of the pathogenic strains. [123]

2.9.2 Genotyping of *Candida* spp.

Candida spp., in particular *C. albicans* displays a raised level of genetic diversity and strains may vary significantly in virulence, antifungal susceptibility, and additional clinically pertinent

factors. [123] By utilising genotyping methods, small genetic variations signifying adaptation to prolonged host response may be detected. [123]

Preceding to the arrival of molecular typing techniques, epidemiological studies of the *Candida* were dependent on phenotypic approaches including bio typing and antimicrobial susceptibility testing. [123] However, these methods possess poor inter-laboratory reproducibility, as well as poor discriminatory ability. [123] A selection of techniques for typing strains of *Candida* spp. have been designated, which have illustrated the connotation of genotypes with infection varieties, the likely connection between certain strain types and the host microbiota, and the connection between azole susceptibilities and particular *Candida* spp. genotypes. [123]

2.9.3 Genotyping methods

Multilocus enzyme electrophoresis (MLEE) uses the principle of electrophoresis to directly reflect allelic differences at defined loci of enzymes and can therefore discriminate among the gene products of different alleles for a number of loci. [123]

Methods that target DNA more directly include electrophoretic karyotyping (EK), restriction fragment length polymorphism (RFLP) analysis and random amplified polymorphic DNA (RAPD) analysis. [123] These approaches can identify variations in the DNA sequences of restriction endonuclease cleavage sites that may be instigated by single nucleotide polymorphisms (SNPs), insertions or deletions, translocations and recombination occurrences. [123]

Electrophoretic karyotyping (EK) of *C. albicans* isolated from women with RVVC demonstrated the genetic microevolution of *Candida* by determining that *Candida* in the vagina comes from of a single strain. [123]

Restriction fragment length polymorphism (RFLP) techniques have been broadly employed to fingerprint *Candida* spp. to study population structure, the method of reproduction and microevolution. [147]

Five clades of *C. albicans* with geographic specificity, named clades I, II, II, SA and E, were determined using DNA fingerprinting with the use of a probe called Ca3. In addition, clade-specific drug resistance was also identified using this method of molecular typing. [123]

Recently, short tandem repeats or microsatellites have been gradually used, due to the hypervariability of these DNA regions, as molecular indicators for genotyping of a variety

organisms. [147] This method is called multilocus microsatellite typing (MLMT). The microsatellite loci that have been used for the strain typing of *C. albicans* are called CAI, CAIII, CAV, CAVI and CAVII, and may be found in coding or noncoding regions. [123] A study by Wang *et al*., used ABC genotyping of *C. albicans* strains using CAI microsatellite typing to compare polymorphism from different sources. [121] In addition, the study of Wang *et al.* also distinguished genotypes (A, B and C) via the amplification of an intron-containing region of the 25S rDNA gene and found that the 25S rDNA genotype A was foremost in VVC isolates and that *C. albicans* strains from VVC were less susceptible to itraconazole than the strains from non-VVC isolates. [123]

2.9.4 Multilocus sequence typing

One of the most commonly used molecular typing techniques today is that of MLST. The MLST of *C. albicans* consists of the amplification and DNA sequencing of seven housekeeping genes. [123] The sequence variations caused by SNPs for each locus are identified as separate alleles and are assigned a corresponding integer, which corresponds to a sequence type (ST). [33, 123, 124]

An array of important epidemiological findings have been determined using MLST. [123] Some of these findings includes that that *Candida* infections frequently emerges from an endogenous source, minor genetic variations may occur with persistent *Candida* strains that are sustained by hosts over extended periods of time, that microvariations of persistent isolates may occur in the same individual between recurrent infections and that drug susceptibility may be geographically linked. [123]

The MLST database of *Candida* species is questionably the most beneficial system for the epidemiological and population analysis of this yeast species. Figure 2.11 illustrates the number of isolate profiles in the C. *albicans* MLST database in 2013. The MLST database reveals the clades that have been identified for these *C. albicans* and other *Candida* spp. [124] Regardless, isolates from many parts of the world are still absent or are understated in the database. The addition of isolates from these parts may disclose further clades and permit a more exact global assessment of the population structure of *Candida* to be revealed. [123]

Figure 2.11 The worldwide distribution of *C. albicans* isolates incorporated in the MLST database in 2013[11] (McManus *et al.*, 2014).

2.10 Whole genome sequencing

The advent and advances of nucleic acid sequencing technologies have transformed the manner in which biological research is approached. [33] There are many NGS techniques that are currently available for a variety of applications, including clinical diagnostics, the discovery of biomarkers, the control of outbreaks and the evaluation of drug resistance. [33]

Whole genome sequencing (WGS), which is one of the techniques of NGS, allows for the study of the evolutionary pathway and phylogeny of the pathogenic yeasts, including extensive assessments of genetic variations from *Candida* isolates from different clades related to commensalism and pathogenicity connected with VVC and RVVC. [123, 125] In addition, the genes involved in intrinsic and endogenous drug resistance, can undergo amplification, targeted sequencing, as well as WGS to decipher drug resistance in *Candida* spp. [33, 123]

[11] Figure 2.11 Global distribution of C. albicans isolates currently included in the MLST database. Numbers beside each pin indicate the number of isolates currently included in the MLST database from each country (date accessed 20.08.2013). Geographical location information is available for 2083 of the 2244 isolates currently in the MLST database, but is not available for the remaining isolates. Further epidemiological and DST information for these isolates can be retrieved from the *C. albicans* MLST database online (http://calbicans.mlst.net/). This figure highlights the geographical locations that are currently underrepresented in the MLST database; examination of isolates recovered from these locations may reveal the presence of additional MLST clades.

2.11 Summary

Vulvovaginal candidiasis is a common cause of genital infection in women of childbearing age, with some experiencing uncomplicated forms of the disease and others experiencing complicated forms of the disease, including recurrent VVC. [1, 2, 7] Vulvovaginal candidiasis is caused by an overgrowth of *Candida* spp. in the vagina, which is usually a commensal, and generally manifests due to a disruption of vaginal microbiota. [9, 10] *Candida albicans* is the predominant causative agent of VVC, followed by *Candida glabrata*. [11] *Candida glabrata* has beome increasingly significant due resistance to azoles. [11]

Azoles are the most commonly used class of antifungals to treat VVC. [72, 74] Many resource-limited countries use the syndromic approach to manage genital tract infections, including VVC. [72, 73] However, complications with this approach may lead to persistent and recurrent infections due to the lack of laboratory testing, poor patient compliance and follow-ups, inappropriate antimicrobial usage and microbial resistance. [7, 79] Exposure to antifungal drugs and long-term antifungal use and with recurrent infections may lead to the development of acquired resistance to several *Candida* spp. in particular *C. glabrata*. [2, 28] Multilocus sequence typing can elaborate on the genetic similarity of *Candida* spp. in relation to the antifungal drug resistance in populations [33, 123]. Additionally, WGS can be utilised to detect genes involved with antifungal resistance. [33]

The emergence of multidrug resistant *Candida* strains, including *C. glabrata* and *C. auris* poses a major universal public health threat. Data of prevalence rate of VVC and AMR in *Candida* spp. have precedingly been limited in resource-constrained settings, including Namibia. Ineffectual VVC burden surveillance attributing to deficient laboratory diagnoses and inappropriate treatment protocols may be some of the causes of AMR *Candida* in Namibia.

There are no previous studies that have investigated AMR patterns, nor the associated genes, of *Candida* species in Namibia. Investigating the burden of VVC, the presence of AMR *Candida* and the factors that may contribute to treatment failure in women with VVC will provide much needed comprehensive epidemiological and microbiological data to improve the management of VVC in Namibia by highlighting the need for diagnostic testing. Therefore, the aim of this study is to study the molecular epidemiology and treatment outcomes of vulvovaginal candidiasis in Namibian women.

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Chapter 3: Importance of *Candida* species and fluconazole resistance in women with vaginal discharge syndrome in Namibia

Objective(s) addressed:	• To determine the frequency of *Candida* spp. and concurrent STIs in symptomatic women by utilising microscopy, culture and molecular identification techniques. • To determine the phenotypic and genotypic antifungal susceptibility patterns of *Candida* spp. in specimens obtained from symptomatic women with primary and recurrent vulvovaginal candidiasis.
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1. Overview of the chapter

According to the literature review in Chapter 2, vulvovaginal candidiasis (VVC) is a globally prevalent cause of genital tract infection in women of childbearing age. Syndromic algorithms are used in low-to-middle-income countries to manage vaginal discharge syndrome (VDS). Abnormal vaginal discharge is a clinical presentation of vulvovaginal candidiasis, sexually transmitted infections including those caused by *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Trichomonas vaginalis* and *Mycoplasma genitalium*, as well as bacterial vaginosis. Because of the dynamic nature VDS, factors such antimicrobial resistance and co-infections, may have an affect on treatment outcomes. Due to resource limitations, laboratory diagnosis of the microbial aetiologies of VDS, in particular antimicrobial susceptibility testing, are limited. In-depth knowldeg of the burden of VVC and associated co-infections, as well AMR patterns are necessary for the appropriate management of VDS. Therefore, chapter 3 addressed objective 1 and objective 2 of this study. It provided the prevalence of *Candida* infection, antifungal resistance, and coinfections in Namibian women with VDS. In this chapter, a high prevalence of VVC (43%) in women with VDS in Namibia, as well as a high frequency of non-*albicans Candida* species that are resistant to fluconazole (100%) was noted.

2. Relevance of the chapter

Investigating the prevalence of *Candida* infection, antifungal resistance, and coinfections in Namibian women with VDS can provide data to motivate for the improvement of treatment strategies and the development and implementation of diagnostic testing for the management of VVC and other microbial aetiologies of VDS.

3. Author contributions

Cara M. Dunaiski:	Conceptualisation, Methodology, Formal Analysis and Investigation, Writing – Original Draft, Review & Editing, Software and Data Curation
Marleen M. Kock:	Conceptualisation, Methodology, Formal Analysis and Investigation, Writing – Review & Editing, Resource, Supervision
Hyunsul Jung:	Formal Analysis and Investigation, Writing –Review & Editing, Resource, Supervision
Remco P. H. Peters:	Conceptualisation, Methodology, Writing – Review & Editing, Resource, Supervision

CHAPTER 3

Importance of *Candida* species and fluconazole resistance in women with vaginal discharge syndrome in Namibia

The editorial style of Antimicrobial Resistance and Infection Control has been followed as the chapter was published in this journal.

Abstract

Background. Vaginal discharge syndrome (VDS) is a common condition in primary healthcare. Clinical management targets sexually transmitted infections (STIs) and bacterial vaginosis (BV); there is a limited focus on *Candida* infection as cause of VDS. Lack of *Candida* treatment and antifungal resistance, if present, may result in VDS treatment failure. This study aimed to determine the prevalence of *Candida* infection, antifungal resistance, and coinfections in Namibian women with VDS.

Methods. A cross-sectional study was performed using 253 vaginal swabs from women with VDS from the Namibian central laboratory. Demographic data was collected, and phenotypic and molecular detection of *Candida* species was performed followed by fluconazole susceptibility testing of *Candida* isolates. BV was diagnosed using Nugent score microscopy; and molecular detection of *Chlamydia trachomatis*, *Neisseria gonorrhoeae* and *Trichomonas vaginalis* was performed. Data were analyzed using appropriate statistical tests.

Results. *Candida* species was detected in 110/253 women (43%). Ninety women (36%) had *Candida albicans* and 24 (9.5%) had non-albicans *Candida* species isolated. The non-albicans species detected were 19 (17%) *Candida glabrata*, 4 (3.5%) *Candida krusei*, and 1 (0.9%) *Candida parapsilosis*. *Candida albicans* were more frequently isolated in younger and pregnant women as opposed to non-albicans *Candida*. Almost all (98%) *Candida albicans* isolates were susceptible to fluconazole while all non-albicans *Candida* species were fluconazole resistant. STIs were diagnosed in 111 women (44%): 30 (12%) *C. trachomatis*, 11 (4.3%) *N. gonorrhoeae*, and 70 (28%) *T. vaginalis*; 98 (39%) women had BV. There was a statistically significant association between *C. albicans* and age (p = 0.004) and pregnancy (p=0.04). *Candida* infection alone was diagnosed in 30 women (12%), combined with STIs in 42 women (17%) and was concurrent with BV in 38 women (15%). *Candida* infection was more often detected in swabs from women without *C. trachomatis* detected (6.4% vs. 16%; OR 0.30; 95% CI 0.10–0.77, p = 0.006).

Conclusions. The high prevalence of *Candida* infection, especially those due to non-albicans *Candida* species that are resistant to fluconazole, is a great concern in our setting and may lead to poor treatment outcomes. Access to microbiological testing for *Candida* species in the context of syndromic management is warranted.

Keywords

Candida albicans, non-albicans *Candida* species, vaginal discharge syndrome, antifungal susceptibility testing, sexually transmitted infections, Namibia, sub-Saharan Africa.

3.1 Background

Vaginal discharge syndrome (VDS) is the most common gynaecological condition among women of reproductive age [1]. Vulvovaginal candidiasis (VVC) is the most common aetiology of VDS, accounting for about 90% of symptomatic vaginal infections [2, 3]. Up to 75% of healthy women face symptomatic VVC at least once in their childbearing years, with some experiencing intermittent and often obdurate forms of the disease [4, 5]. A previous study from Namibia reported that symptomatic VVC was present in 61/335 (18.2%) of women newly diagnosed with HIV (human immunodeficiency virus) that were enrolled for antiretroviral therapy (ARV) treatment [6]. Additionally, recurrent VVC (RVVC) may occur in 10%-20% of women with symptomatic VVC [7]. In two recent studies, RVVC, defined as four or more symptomatic episodes per year, was estimated to occur in 37,390 females per year in Namibia and in over a million females per year in South Africa [8, 9]. There are several factors contributing to RVVC, which include treatment failure, co-infections and antifungal resistance [10].

The aetiology in more than 90% of the VVC cases is *Candida albicans* [11]. Non-*albicans Candida* species have emerged as an important aetiology of VVC as its prevalence and antifungal resistance is a mounting problem globally [10, 11]. The most significant of these non-*albicans Candida* species is *Candida glabrata* owing to its intrinsic resistance or low susceptibility to azoles [12]. Other than C. *glabrata*, C. *krusei*, C. *tropicalis*, C. *parapsilosis* and, very rarely, *Saccharomyces cerevisiae*, are other potential pathogens that may lead to VVC [13].

In addition to VVC, VDS may also be caused by sexually transmitted infections (STIs), such as *Chlamydia trachomatis*, *Trichomonas vaginalis*, *Neisseria gonorrhoeae*, and bacterial vaginosis (BV) [2, 3]. In Namibia, like other low- to middle-income countries (LMICs),

syndromic management of VDS is the standard of care, where clinicians treat patients empirically, without aetiological diagnosis, based on a set of symptoms [14]. This approach is associated with under-treatment as STIs frequently remain asymptomatic, but over-treatment with unnecessary use of antibiotics also occurs [15, 16]. Determination of the microbiological aetiology of VDS is essential to guide empirical treatment algorithms and to guide effective prescription of antifungal drugs for presumed candidiasis. Most of such studies focus on STIs and do not include *Candida* species, or antifungal resistance. Comprehensive evaluation of microbial aetiology of VDS is essential to better understand the occurrence of *Candida* species, as well as the intersection with STIs and BV. The aim of this study was to determine the prevalence of *Candida* species, fluconazole resistance and co-infections in swabs collected from women with VDS in Namibia.

3.2 Methods

3.2.1 Study design, setting and population

This cross-sectional study was conducted using 253 vaginal swabs collected from women with VDS between February and July 2021 at primary healthcare facilities across Namibia. The vaginal swabs were collected by healthcare workers at primary healthcare facilities, which were sent to the diagnostic laboratory for routine diagnostic testing.

The laboratory requisition forms were assessed for inclusion and exclusion in the study. Vaginal swabs from women aged 18 to 49 years of age with ‘vaginal discharge’ as diagnostic indication recorded by the clinician were included, while swabs were excluded if more than two weeks old.

3.2.2 Detection and identification of *Candida* isolates

Vaginal swabs were inoculated on the chromogenic *Candida* agar plates (CHROMagar™, France), which were incubated (Thermo Scientific, USA) at 37°C for 24 to 36 hours. Colonies isolated on chromogenic *Candida* agar was used to identify *Candida* spp., according to colony colour as per manufacturer’s instructions. In each test, the reference strain C. albicans American Type Culture Collection (ATCC) 14053 was used for quality control. If there were no visible colonies within 3 days, the sample was considered negative for *Candida*. Colonies were inoculated on the Sabouraud Dextrose Agar (SDA) plates (Oxoid, United Kingdom) in order to be stored with 50% glycerol (Merck, Germany) at -20°C prior to DNA extraction.

3.2.4 Molecular confirmation of Candida species

Deoxyribonucleic acid (DNA) extraction and purification from vaginal swabs were performed using the Quick-DNA™ Fungal/ Bacterial Miniprep Kit (Zymo Research, USA) according to the manufacturer's guidelines. Multiplex PCR was performed using the One Taq® Quick-Load 2× Master Mix (New England BioLabs, USA), a universal *Candida* primer pair targeting ITS1 and ITS2 and C. albicans specific primers according to Rad *et al*. [17]. The PCR products were then analysed with the 50 bp DNA ladder (New England BioLabs, United States of America) by gel electrophoresis through a 2% agarose gel and ultraviolet visualisation [17]. *Candida albicans* ATCC 14053, *C. glabrata* CBS2175, *C. parapsilosis* CBS2195, *C. tropicalis* CBS94, and *C. krusei* CBS473 were included in each PCR reaction as positive controls; nuclease-free water (BioConcept, Switzerland) was used as negative control.

3.2.5 Fluconazole antifungal susceptibility patterns of *Candida* isolates

The fluconazole susceptibility of *Candida* isolates was determined using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) microbroth dilution (MBD) method to determine the minimum inhibitory concentration (MIC) [18]. The EUCAST breakpoints were used to assign the *Candida* species into the clinical categories "susceptible", "intermediate" and "resistant" [18]. Quality control isolates will include *C. parapsilosis* ATCC 22019 (susceptible) and *C. krusei* ATCC 6258 (resistant) [19].

3.2.6 Detection of bacterial vaginosis

A Gram-stained vaginal smear prepared from vaginal swab was examined under a microscope and evaluated for BV by Nugent scoring [20, 21]. Nugent scores from 0 to 3 are considered as "Normal"; 4 to 6 as "Intermediate"; and 7 to 10 as "BV" [22].

3.2.7 Detection of sexually transmitted infections

Molecular detection of *Chlamydia trachomatis* and *N. gonorrhoeae* was performed using the LightMix 480 HT CT/NG assay (TIB MOLBIOL, Berlin, Germany), while a validated in-house real time-PCR assay as described elsewhere was performed for detection of *T. vaginalis* [23].

3.2.8 Statistical analyses

Data were captured into a study-specific Epi Info™ database version 7.2.4.0 (Centres for Disease Control and Prevention (CDC), USA) and exported into RStudio version 2021.09.1 (RStudio, USA) for analysis. Data are presented as absolute value with proportion, and median with range. The chi-squared test, with Fisher's Exact if appropriate, was used to compare dichotomous variables between groups, while the Mann-Whitney test was used for continuous variables between groups. Logistic regression was used to calculate associations of age and pregnancy between *Candida albicans* and non-albicans *Candida* species isolated from women with yeast infections. A p-value < 0.05 was considered statistically significant.

3.3 Results

3.3.1 Study population

A total of 253 vaginal swabs from women with VDS were included. The median age of these women was 29 years (interquartile range (IQR) 24 - 34), 58 (23%) were HIV-infected and 60 (24%) were pregnant.

Candida isolates were detected in vaginal swabs from 110 (43%; 110/253) women; there was no association with any of the demographic variables (Table 3.1). In addition, *Chlamydia trachomatis* was detected from 30 women (12%), *N. gonorrhoeae* from 11 (4.3%) and *T. vaginalis* from 70 (28%). Bacterial vaginosis was present in 98 women (39%) while 69 (27%) and 98 (39%) belonged to an intermediate and normal Nugent score category.

Any *Candida* infection was less likely detected in swabs from women with *Chlamydia trachomatis* (6.4% vs. 16%; OR 0.30; 95% CI 0.10–0.77, $p = 0.006$). There was no relationship between *Candida* infection and age, HIV, *N. gonorrhoeae, T. vaginalis*, and BV.

Table 3.1 Demographic factors and coinfections in women with and without *Candida* infection in Namibia

Characteristics	**Total (*n*=253)**	***Candida* species isolated**		**OR**	**95% CI**	***p*-value**
		Yes (*n*=110)	**No (*n*=143)**			
Median age in years (IQR)	29 (24–34)	28 (23–32)	30 (24–36)			0.11
Pregnancy status						
Pregnancy	60 (24)	31 (28)	29 (20)	1.54	0.83–2.9	0.18
Not pregnant	193 (76)	89 (78)	114 (80)			
HIV status						
HIV-positive	58 (23)	23 (21)	35 (24)	0.82	0.43–1.5	0.55
HIV-negative	195 (77)	87 (79)	108 (76)			
Chlamydia trachomatis	30 (12)	7.0 (6.4)	23 (16)	0.30	0.10–0.77	0.006
Neisseria gonorrhoea	11 (4.0)	5.0 (4.5)	6.0 (4.2)	1.1	0.26–4.4	1.0
Trichomonas vaginalis	70 (28)	30 (27)	40 (28)	0.97	0.53–1.7	1.0
Bacterial vaginosis	98 (39)	38 (34)	60 (42)	0.73	0.42–1.3	0.24

Data are presented as number (n) with proportion (%) unless indicated otherwise.

Abbreviations: OR, odds ratio; CI, confidence interval; IQR, interquartile range; HIV, human immunodeficiency virus.

3.3.2 Distribution of *Candida* species recovered from vaginal swabs

Among the 110 women who tested positive for *Candida*, 114 *Candida* isolates were detected using culture methods, i.e. both *C. albicans* and *C. glabrata* were detected in two vaginal swabs. *C. albicans* was the most common isolate (*n* = 90, 79%), followed by the following non-albicans *Candida* species: *C. glabrata* (*n* = 19, 17%), *C. krusei* (*n* = 4, 3.5%) and *C. parapsilosis* (*n* = 1, 0.9%) (Table 3.2). Molecular methods confirmed phenotypic identification in all isolates (100% concordance).

Table 3.2 Distribution of 114 *Candida* species isolated from 110 swabs from women with vaginal discharge syndrome in Namibia

***Candida* species**	**No. of isolates**	**Prevalence (%)**	**95% CI**
C. albicans	90	79	71–86
C. glabrata	19	17	11–25
C. krusei	4	3.5	1.4–8.7
C. parapsilosis	1	0.9	0.04–4.8
TOTAL	**114**	**100**	

Data are presented as number (n) with proportion (%) unless indicated otherwise.

Abbreviations: CI, confidence interval.

There were significant differences between women with *C. albicans* and non-*albicans Candida* spp. with regards to age (*p* = 0.01) and pregnancy (*p* = 0.002) (Table 3.3).

Table 3.3 Demographic factors and coinfections in women with *Candida albicans* versus non-albicans species isolated from vaginal swabs in Namibia (*n*=106)

Syndrome/ Infection	***Candida albicans* (n=86)**	**Non-albicans *Candida* (n=20)**	**COR**	**95% CI**	***p*-value**
Age (years), median (IQR) [a]	27.5 (23–32)	29.5 (23–37)	0.94	0.90–0.98	0.01
Pregnancy[a]				1.0–3.5	
Pregnant	27 (31)	0 (0)	1.9		0.002
Not pregnant	59 (69)	20 (100)			
HIV status					
HIV positive	17 (20)	2 (10)	2.2	0.45–21	0.52
HIV negative					
Chlamydia trachomatis	7 (8.1)	0 (0)	-	-	0.34

Table 3.3 Demographic factors and coinfections in women with *Candida albicans* versus non-albicans species isolated from vaginal swabs in Namibia (*n*=106) *(continue)*

Syndrome/ Infection	*Candida albicans* (n=86)	Non-albicans *Candida* (n=20)	COR	95% CI	*p*-value
Neisseria gonorrhoea	4 (4.7)	0 (0)	-	-	1.00
Trichomonas vaginalis	21 (24)	2 (10)	2.9	0.61–28	0.23
Bacterial vaginosis	32 (37)	5 (25)	1.8	0.54–6.8	0.44

Data are presented as number (n) with proportion (%) unless indicated otherwise.
Four women with concurrent *Candida albicans* and non-*albicans* species are not included in this analysis.
Abbreviations: COR, crude odds ratio; CI, confidence interval; IQR, interquartile range; HIV, human immunodeficiency virus.
[a] Multivariate analysis: adjusted odds ratio for age is 0.94 (95% CI 0.90 –0.98, *p*=0.01) and for pregnancy 1.9 (95% CI 1.0–3.5, *p*=0.002).

Similar results were observed with the multivariate analysis, where both age (p = 0.004) and pregnancy (p = 0.04) were found to be independently associated with *C. albicans* isolated from vaginal swabs. *Candida albicans* are more likely to occur in pregnant women (adjusted odds ratio 1.9; 95% CI 1.0–3.5, p=0.002) and in younger women, as *C. albicans* is less likely to occur with increasing age (adjusted odds ratio 0.94; 95% CI 0.90–0.98, p=0.01).

3.3.2 Fluconazole susceptibility pattern

The overall drug susceptibility pattern of *Candida* isolates against fluconazole is shown in Table 3.4. Fluconazole resistance was low in *C. albicans* isolates, but high in all non-albicans *Candida* isolates: *C. glabrata* (74%), *C. krusei* (100%) and *C. parapsilosis* (100%). There was no significant association between fluconazole susceptibility of *C. albicans* with demographic factors or coinfections.

Table 3.4 *In vitro* fluconazole susceptibility of *Candida* isolates (*n*=114) collected from women with vaginal discharge in Namibia

Candida species	No. of isolates (%)	MIC range (mg/L)	MIC breakpoints[a]		
			Susceptible	Intermediate	Resistant
C. albicans	90 (79)	2–4	88 (98)	0 (0)	2 (2.0)
C. glabrata	19 (16)	0.001–16	0	5 (26)	14 (74)
C. krusei	4 (3.5)	–[b]	0	0	4 (100)
C. parapsilosis	1 (0.9)	>4	0	0	1 (100)

[a] EUCAST (version 7.3.2) breakpoints were used for interpretation.

[b] No breakpoints: *C. krusei* intrinsically resistant to azole antifungals.

3.3.3 Relationship between *Candida* species, STIs and BV

Multiple infections were common: two or more concurrent infections were detected in 86 (34%) women (Figure 3.1). Vaginal infections caused by *Candida* alone occurred in 30/110 (27%) women. Concurrent infections occurred in 80/110 (73%) of women with *Candida* and included those with STIs (*n* = 42, 38%) including *T.* vaginalis (*n* = 30, 27%), *Chlamydia trachomatis* (*n* = 7, 6.4%) and *N. gonorrhoeae* (*n* = 5, 4.5%). In addition, BV co-occurred with *Candida* in 15% of the study population. Concurrent infections with BV (*n* = 86/98, 88%) included *T. vaginalis* (*n* = 24, 24%), *Chlamydia trachomatis* (*n* = 19, 19%) and *N. gonorrhoea* (*n* = 5, 5.0%). However, neither *Candida* spp., *C. trachomatis*, *N. gonorrhoeae* nor *T. vaginalis* was detected in 52 women (21%).

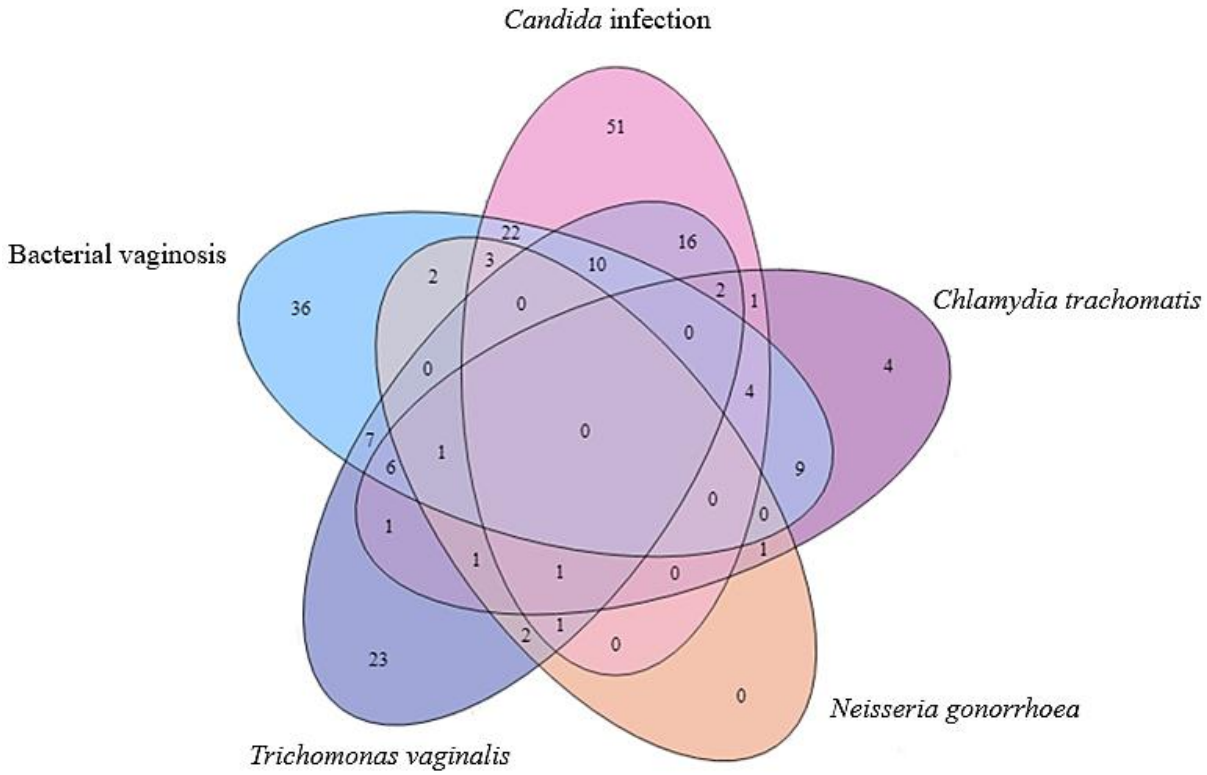

Figure 3.1 Interactions between various aetiologies of VDS.

3.4 Discussion

Vaginal discharge syndrome (VDS) is a common cause of gynaecological visits among women globally [1, 24]. Most studies report only on specific microbiological aetiology of this condition, usually STIs, but only a few have studied microbial aetiology comprehensively.

Consequently, VVC and antifungal resistance is often not reported [14, 25, 26]. This study provides a comprehensive analysis of microbial aetiology of VDS in Namibian women; it highlights a high prevalence of *Candida* species, including fluconazole-resistant non-albicans *Candida* species, and its concurrence with STIs and BV.

Candida species were detected in 110 (43%) of swabs collected from women with VDS, mostly from primary health care facilities, in our study making it the most common microbial aetiology. Studies from sub-Saharan Africa reported a wide range of *Candida* prevalence in women with VDS: 21% in Rwanda [27], 25% in Ethiopia [28], 26% in Mauritania [29], 29% in Senegal and Gabon [15, 30], 38% in Cameroon [31], 39% in Benin [32], , 45% in South Africa [33], 49% Burkina Faso [34], 55% in Nigeria [35] and 66% in Tanzania [36]. The geographic differences in the reported prevalence described in different settings might be owing to environmental, behavioural, socioeconomic factors, as well differences in study methodologies [28].

In this cross-sectional study, no significant association with older age, pregnancy or HIV seropositive status was observed although these are known risk factors for VVC [37-41]. However, we did observe a significant association between the presence of *Candida* infection and detection of *Chlamydia trachomatis*, but not with the other STIs, where *C. trachomatis* was less likely to be detected in women with *Candida* COR, 0.30, p=0.006). A study by Kruppa and colleagues demonstrated a novel interaction between *Chlamydia trachomatis* and *C. albicans* via the binding of elementary bodies of *Chlamydia trachomatis* to *C. albicans* yeast and hyphal forms. This binding was shown to considerably decrease the capacity of *Chlamydia trachomatis* to infect human cervical epithelial cells, thereby decreasing its disease progression [42]. In contrast, another study illustrated that biofilms related to VVC that may act as a reservoir for *Chlamydia trachomatis* [3]. STIs have been suggested in other studies as risk factor for VVC; however, this relationship should be further confirmed [43, 44].

In our study, like most other studies looking at the species distribution of *Candida*, the most prevalent *Candida* species isolated was *C. albicans*, followed by *C. glabrata* [45, 46]. *Candida glabrata* is the most relevant non-*albicans Candida* species, owing to its ability to develop acquired resistance subsequent to exposure to azole antifungals [11, 12, 47]. In our study, low rate of fluconazole resistance was found in *C. albicans* isolates (<5%), but most non-*albicans Candida* isolates (n = 19, 17%) were fluconazole resistant. These findings are similar to reports from Africa and other parts of the world [31, 46]. Antifungal resistance of *Candida* spp. is a

mounting problem universally [47-49]. High rates of fluconazole resistance has been demonstrated in several countries including China [50], Iran [51], Ethiopia [37], Pakistan [52], Brazil [53], Cameroon [31] and Uganda [46], to mention but a few. The use of azole antifungals may stimulate the selection of resistant subpopulations of *Candida* by shifting colonisation to more intrinsically resistant species, especially *C. krusei* or *C. glabrata* [54]. In our study, non-albicans species that were not susceptible to fluconazole were detected in 24/253 (9.5%) women with VDS, which is 22% of all women with *Candida* infection, highlighting the challenge in management of *Candida* species in the syndromic management context. Since pregnancy predisposes women to VVC, which in turn could increase the risk for poor pregnancy outcomes, it is reassuring that the pregnant women in this study did have non-albicans *Candida*, and could therefore be appropriately treated [55]. We observed an association between older age and the isolation of non-albicans species vs. *C. albicans* [4]. Similarly, some studies show that non-albicans species, such *C. glabrata*, are associated with older age when compared to *C. albicans*, which may be due to the exposure of several risk factors such as the use of hormonal contraceptives and broad spectrum antifungals [56, 57].

Our study demonstrates the complex microbial aetiology of VDS. Several women in this study experienced more than one infection, and some up to three infections at once, which may lead to overlapping diagnosis and conditions. Hence, these findings questions whether empirical approach for the management of VDS based on symptoms is appropriate or not [2].

This study has several limitations. First, limited demographic and clinical information was available from study participants due to collecting specimens submitted to the laboratory. Reliance on the information provided by the requesting clinician might have resulted in some misclassification. Second, the culture and molecular methods used target *Candida* species that cause vaginal infections; other *Candida* species might have been missed by these assays.

3.5 Conclusion

This study highlights a high prevalence of VVC in women with VDS in Namibia. The high frequency of non-albicans *Candida* species that are resistant to fluconazole is a great concern and may contribute to poor treatment outcomes. Access to microbiological testing for *Candida* species in the context of syndromic management is warranted.

3.6 Acknowledgements

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3.7 Abbreviations

AMR:	Antimicrobial resistance
AST:	Antimicrobial susceptibility testing
ATCC :	American Type Culture Collection
BV:	Bacterial vaginosis
CI:	Confidence interval
DNA:	Deoxyribonucleic acid
EUCAST:	European Committee on Antimicrobial Susceptibility Testing
HIV:	Human immunodeficiency virus
LMICs:	Low- to middle-income countries
MIC:	Minimum inhibitory concentration
OR:	Odds ratio
PCR:	Polymerase chain reaction
RVVC :	Recurrent vulvovaginal candidiasis
SDA:	Sabouraud dextrose agar
STI:	Sexually transmitted infection
VDS:	Vaginal discharge syndrome

VVC: Vulvovaginal candidiasis

WHO: World Health Organization

3.8 Author contributions

CD was responsible for the design of the study, acquisition of data, conducting the experimental work, analysis of data, writing the article and final approval of manuscript. RP, MK and HJ were responsible for supervising the design and implementation of the study, substantively revising the article and approving the submitted version. All authors read and approved the final manuscript.

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3.10 Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

3.11 Ethics approval and consent to participate

Ethical clearance was obtained from the Human Research Ethics Committee of the University of the Pretoria (Ref: 518/2020), the Namibia University of Science and Technology Faculty of Health and Applied Sciences Research Ethics Board (Ref: FHAS 11/2020) and regulatory approval from the Namibian Ministry of Health and Social Services (Ref: 17/3/3/CMD). As approved by the Ethics committees, anonymised swabs were used and no individual informed consent was obtained.

3.12 Consent for publication

Not applicable.

3.13 Competing interests

The authors declare that they have no competing interests.

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Chapter 4: Prospective cohort study of treatment outcome of vaginal discharge syndrome in women in Windhoek, Namibia

Objective(s) addressed:	• To determine the frequency of *Candida* spp. and concurrent STIs in symptomatic women by utilising microscopy, culture and molecular identification techniques. • To determine the phenotypic and genotypic antifungal susceptibility patterns of *Candida* spp. in specimens obtained from symptomatic women with primary and recurrent vulvovaginal candidiasis. • To assess whether concurrent presence of *Chlamydia trachomatis*, *Trichomonas vaginalis*, *Neisseria gonorrhoeae*, *Mycoplasma genitalium* and bacterial vaginosis– and aerobic vaginitis–related microorganisms has an impact on spontaneous clearance and/or treatment outcomes of vulvovaginal candidiasis by using molecular identification techniques.
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1. Overview of the chapter

Chapter 3 focussed on the prevalence of VVC, antifungal resistance, and coinfections in Namibian women with VDS. Poor treatment outcomes have been linked to persistent and recurrent infections related to several risk factors including patient socio-economic demographics, patient sexual behaviour, antimicrobial resistance, co-infections and suboptimal treatment strategies. As the syndromic management approach is used in resource-constrained settings, the management of women with repeat or persistent VDS is problematic without access to diagnostic testing. However, there is limited data on VDS treatment outcomes in sub-Saharan Africa. This chapter (chapter 4) addresses objectives 1, 2 and 4 of this study, which described the incidence, risk factors and microbial aetiology of treatment failure in women with VDS. Prevalence of microbial aetiologies and antifungal resistance in chapter 3 is similar to

that at baseline in chapter 4. Untreated or fluconazole-resistant vulvovaginal candidiasis (VVC) and bacterial vaginosis (BV) were the main causes of treatment failure, which was common in women with VDS in Namibia following syndromic management.

2. Relevance of the chapter

Data derived from this chapter highlights the importance of access to laboratory diagnostic testing to optimise the treatment outcomes of persistent or recurrent VDS. It can therefore be used to develop laboratory diagnostics and alternative treatment strategies, including fungal culture and antimicrobial susceptibility tesing, in settings that utilise syndromic management.

3. Author contributions

Cara M. Dunaiski:	Conceptualisation, Methodology, Formal Analysis and Investigation, Writing – Original Draft, Review & Editing, Software and Data Curation
Marleen M. Kock:	Conceptualisation, Methodology, Formal Analysis and Investigation, Writing – Review & Editing, Resource, Supervision
Hyunsul Jung:	Formal Analysis and Investigation, Writing –Review & Editing
Remco P. H. Peters:	Conceptualisation, Methodology, Formal Analysis and Investigation, Writing – Review & Editing, Resource, Supervision

CHAPTER 4

Prospective cohort study of treatment outcome of vaginal discharge syndrome in women in Windhoek, Namibia

The editorial style of Sexually Transmitted Diseases has been followed as the chapter has been accepted for publication in this journal.

Short summary

This prospective cohort study shows that vulvovaginal candidiasis is associated with treatment failure in women presenting with vaginal discharge syndrome in Windhoek, Namibia.

Abstract

Background. Syndromic treatment is the standard of care for vaginal discharge syndrome (VDS) in resource-constrained settings. However, the outcomes of VDS treatment have not been well documented. This study aimed to determine the incidence, risk factors, and microbial etiology of treatment failure in women with VDS.

Methods. This prospective cohort study of women with VDS was conducted between September 2021 and March 2022 at Katutura Intermediate Hospital in Windhoek, Namibia. Microbiological analyses of sexually transmitted infections (STI) (*Chlamydia trachomatis*; *Neisseria gonorrhoeae*; *Trichomonas vaginalis*; *Mycoplasma genitalium*), bacterial vaginosis (BV), and vulvovaginal candidiasis (VVC) were performed. Treatment outcomes were assessed at 7 and 30 days after treatment, followed by microbial investigation in case of treatment failure.

Results. One hundred and nine women were enrolled and 94 (86%) completed the follow-up. At baseline, 37/109 (40%) women were diagnosed with STI, 47/109 (43%) with BV and 45/109 (41%) with VVC. *Candida albicans* (33/45; 73%) was the main pathogen in VVC, with fluconazole resistance detected in 8/33 (24%) isolates; 10/12 (80%) of non-*albicans Candida*

species showed resistance. The incidence of treatment failure was 3.6 per 100 person-years at 7 days and 1.0 per 100 person-years at 30 days follow-up; 17/94 (18%) women had recurrent VDS and 12/94 (13%) women had persistent VDS. VVC (OR 4.3; 95% CI 1.7-11, *p*= 0.002) at baseline was associated with treatment failure.

Conclusions. Treatment failure after syndromic management of VDS is common in resource-constrained settings. Access to diagnostic testing, including fungal culture and susceptibility testing, is recommended to improve outcomes.

Keywords

Vaginal discharge syndrome, treatment outcomes, sexually transmitted infections, Namibia, Sub-Saharan Africa.

4.1 Introduction

Vaginal discharge syndrome (VDS) is a common gynecological condition in women of reproductive age [1,2]. Normal vaginal discharge varies in women and presents with a clear, white, or clear, thick, mucous-like, and typically odorless vaginal discharge that may change in quantity and consistency depending on various periods in the menstrual cycle, pregnancy, and the use of hormonal contraceptives [3]. VDS signifies shifts in the quantity, thickness, smell, and/or color of vaginal discharge, and often has diverse infectious etiologies [4]. Important causes are sexually transmitted infections (STIs) such as *Chlamydia trachomatis*, *Neisseria gonorrhoeae, Trichomonas vaginalis*, *Mycoplasma genitalium,* bacterial vaginosis (BV), and vulvovaginal candidiasis (VVC) [1,5]. For example, etiological surveillance studies from South Africa and Zimbabwe reported 41-45% of cases due to STI, but also BV (26-56%) and VVC (22-28%) [1,6,7]. Mixed etiology of VDS is common; approximately 30% of individuals with an STI have a mixed infection, and 44% with BV and 29% with VVC have a concurrent STI [6,7].

Syndromic management is the standard of care for VDS in most resource-constrained settings in sub-Saharan Africa, including Namibia [1,4]. The syndromic algorithm (Supplementary File 1) guides empirical treatment with a combination of antibiotics for VDS, based on clinical history and physical examination. This strategy is relatively cheap and easy to implement, but is disadvantageous as antibiotics are used excessively for those presenting with VDS, contributing to the development and emergence of antimicrobial resistance, and asymptomatic infections remain untreated. Another important but often neglected limitation of the syndromic management approach is that the clinical management of individuals with repeat or persistent VDS is challenging without access to diagnostic testing [1,4]. The etiology of treatment failure is diverse and includes repeated infection from the same or a new sex partner (e.g., *C. trachomatis* or *N. gonorrhoeae*), suboptimal treatment efficacy (e.g., single dose vs. course of metronidazole for *T. vaginalis* infection), antimicrobial resistance (e.g., macrolide resistance in *M. genitalium*), and etiology not covered by the syndromic management regimen (e.g., non-*albicans Candida* species resistant to azole drugs) [8].

Although VDS is a common presentation in primary healthcare, treatment outcomes of syndromic management are poorly documented in resource-constrained settings. A study conducted in rural South Africa demonstrated persistent and recurrent STI-associated symptoms after previous treatment [9]. In addition, there is little evidence to guide the clinical management of patients with VDS treatment failure, especially those with limited access to diagnostic tests. Disappointment with healthcare services due to resistance and recurrence of symptoms following treatment is an important reason reported by women to stop seeking healthcare despite their symptoms [9].

We conducted a prospective cohort study to determine the treatment outcomes in women with VDS in a resource-constrained setting in Windhoek, Namibia. The incidence of persistent and recurrent VDS was determined, and the microbiological etiology was investigated.

4.2 Methods

4.2.1 Study Design and Setting

This prospective cohort study was conducted between September 2021 and March 2022 at Katutura Intermediate Hospital, where gynecological and obstetric health services for women are provided in Windhoek. Katutura Intermediate Hospital is situated in the socioeconomically disadvantaged area of Windhoek's largest township, Katutura.

Women (18–49 years) presenting with VDS as confirmed by healthcare workers were recruited and treated in line with the Namibian syndromic algorithm (Supplementary File 1). After the study questionnaire was completed, a physical examination was conducted and two vaginal swabs for microbiological investigations were collected by the healthcare worker; all women received treatment in line with the syndromic algorithm. Women who were menstruating at baseline or reported antibiotic use for less than one week preceding presentation were not eligible to participate in the study.

Follow-up visits were scheduled 7 and 30 days after the baseline visit to determine treatment outcomes. For participants who lived far from the healthcare facility and had limited financial resources and time to travel, the treatment outcome was tracked telephonically. Participants reporting persistent or recurrent discharge either at the 7-day or 30-day follow-up visit were asked to return to the facility for further evaluation and treatment.

Written informed consent was obtained from all the participants. This study was approved by the Human Research Ethics Committee of the University of Pretoria, Pretoria, South Africa (ref: 518/2020), the Namibia University of Science and Technology Faculty of Health and Applied Sciences Research Ethics Board (Ref: FHAS 11/2020) and regulatory approval from the Namibian Ministry of Health and Social Services (Ref: 17/3/3/CMD).

4.2.2 Laboratory investigations

Specimen collection and processing was the same at the baseline and follow-up visit. Two vaginal swabs for microbiological investigations were collected at each time point by a nurse using Amies transport gel swabs (Copan Diagnostics, United Kingdom). The first vaginal swab specimen was used to prepare vaginal smears for Nugent scoring and fungal culture with drug-susceptibility testing. Vaginal smears were Gram stained, and Nugent scoring was performed for BV diagnosis [10]. Nugent scores from 0 to 3 were considered as "Normal"; 4 to 6 as "Intermediate"; and 7 to 10 as "BV" [10]. In addition, smears were assessed for the presence of budding yeast or pseudohyphae.

Chromogenic *Candida* agar plates (CHROMagar™, France) were inoculated and incubated for 18—24 h (Thermo Fischer Scientific, USA) at 37°C, and yeast growth was assessed daily. Presumptive *Candida* spp. colonies on chromogenic *Candida* agar plates were identified according to colony color as per the manufacturer's instructions. The reference strain, *C. albicans* American Type Culture Collection (ATCC) 14053, was used for quality control. Presumptive *Candida* spp. colonies were subcultured on Sabouraud dextrose agar plates (Oxoid, United Kingdom) for further antifungal susceptibility testing and DNA extraction for species identification using PCR.

Antifungal susceptibility testing to fluconazole was performed on isolated *Candida* spp. using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) microbroth dilution (MBD) method to determine the minimum inhibitory concentration (MIC) [11]. Minimum inhibitory concentrations were interpreted according to EUCAST breakpoints version 7.3.1 (2017) [11]. *C. parapsilosis* ATCC 22019 (fluconazole-susceptible) and *C. krusei* ATCC 6258 (fluconazole-resistant) were used as quality control strains [11].

Yeast DNA was extracted from colonies isolated on Sabouraud dextrose agar plates (Oxoid, United Kingdom) that were inoculated and incubated for 18–24 h at 37°C in Sabouraud

dextrose broth (Oxoid, United Kingdom) using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, USA) according to the manufacturer's guidelines. The ITS1 region and a specific DNA fragment within the ITS2 region were amplified (BIORAD T100, France) using multiplex PCR [12]; yeast species were assigned based on the amplified product size, with *Candida albicans* ATCC 14053, *C. glabrata* CBS2175, *C. parapsilosis* CBS2195, *C. tropicalis* CBS94, and *C. krusei* CBS473 as positive controls.

The second swab was obtained for the detection of STIs and BV by initially placing it in phosphate buffer saline (New England Biolabs, United Kingdom) until well suspended, and subsequently performing DNA extraction using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, USA). *Chlamydia trachomatis* and *Neisseria gonorrhoeae* were detected using a LightMix 480 HT CT/NG assay (TIB MOLBIOL, Berlin, Germany) on a LightCycler® 480 System (Roche Diagnostics GmbH, Mannheim, Germany). Validated in-house real-time PCR assays were used to detect *T. vaginalis* and *M. genitalium* [13,14].

The Allplex™ Bacterial Vaginosis Plus Assay (Seegene, Republic of Korea) was used according to the manufacturer's instructions to detect and quantify *Lactobacillus* spp., *L. crispatus*, *L. gasseri*, *L. jensenii*, and BV-related bacteria (*Gardnerella vaginalis*, *Atopobium vaginae*, *Bacteroides fragilis*, bacterial vaginosis–associated bacteria 2, *Megasphaera* Type 1, and *Mobiluncu*s spp., including *Mobiluncus mulieris* and *Mobiluncus curtisii*) in the samples of participants with a Nugent score of 7–10. The Seegene viewer software analysis algorithm (Allplex™ BV plus algorithm), based on quantification of the bacterial targets, was used to interpret, and analyze the results.

4.2.3 Clinical definitions and statistical analysis

Treatment failure was defined as any VDS symptom reported at the follow-up visit, whereas the absence of such symptoms was defined as treatment success. Treatment failure at 30 days

was further classified as recurrent VDS (no symptoms at the 7-day follow-up visit) or persistent VDS (symptoms at the 7-day follow-up visit). Persistent infection was defined as the detection of the same pathogen at both follow-up visits, while incident infection was defined as the detection of a pathogen at follow-up that was not detected at baseline. Incidence rates were determined at the 7-day and 30-day follow-up visits.

Data were captured into a study-specific Epi Info™ database version 7.2.4.0 (Centers for Disease Control and Prevention, USA) and exported into Statistical Package for the Social Sciences Statistics version 28.0.1.0 (IBM SPSS Statistics, USA) for statistical analysis.

Data are presented as absolute number with proportions, median with ranges and mean with standard deviation. The chi-square test or Fisher's exact test, if appropriate, was used to compare categorical variables between groups; odds ratios (OR) were provided with 95% confidence intervals (CI). The Mann-Whitney U test was used to compare continuous variables between the groups. Forward logistic regression analysis was performed to calculate adjusted odds ratios from variables with $p<0.1$ in the crude analysis. A p-value of equal to or less than 0.05 was defined statistically significant.

4.3 Results

4.3.1 Baseline characteristics of study population

During the study period, 120 women with VDS were assessed for eligibility; 109 (91%) were eligible, and 109 (100%) consented to participate. The reasons for non-eligibility were recent antimicrobial drug use (*n*=9) and age <18 or >50 years (*n*=2).

The mean age of the study participants was 28 years (standard deviation 6.6 years), eight (7.0%) were living with HIV, 26 (24%) were pregnant, and 83 (76%) reported their relationship status as single (**Table 4.1**). Seventy-seven participants (71%) had a history of VDS in the past year.

VDS had an infectious etiology in 96 (88%) of the women; a cause was not identified in 13 (17%).

STI were detected in 59 (54%) women (**Table S4.1**): *C. trachomatis* in 24 (22%), *N. gonorrhoeae* in 16 (15%), and *T. vaginalis* in 23 (21%). Seventeen (16%) women tested positive for *M. genitalium*. Forty-seven women (43%) had BV as defined by the Nugent score; 29 (26%) were classified as having intermediate vaginal dysbiosis and 33 (30%) as having normal flora. Among the 43 women with a positive BV PCR, the most common BV-related bacteria were *Gardnerella vaginalis* (100%), *Atopobium vaginae* (94%), and BVAB-2 (92%); only one woman had *Lactobacillus* species detected (**Table S4.2**).

Vulvovaginal candidiasis (VVC) was diagnosed in 45 (41%) women, with *C. albicans* being the most common species (*n* = 33, 73%), followed by *C. glabrata* (*n* = 5, 12%), *C. tropicalis* (*n* = 4, 10%), and *C. krusei* (*n* = 3, 7.0%) (**Table 4.2**). Antifungal susceptibility testing for fluconazole was performed (**Table 4.2**). Fluconazole resistance was low in *C. albicans* isolates (24%) but high in all non-*albicans Candida* isolates: *C. glabrata* (80%), *C. krusei* (67%), and *C. tropicalis* (100%).

Mixed etiologies were detected in 57 (52%) women with infectious causes of VDS (**Figure 4.1**):19 (17%) had two or more concurrent STIs, 26 (23%) had a combination of STI and BV, 22 (20%) had STI and VVC, and concurrent BV and VVC were present in 16 (15%) women.

4.3.2 VDS treatment outcome

Follow-up data were available for 94 (86%) of the 109 enrolled women. The median time for participants to return for the 7-day follow-up visit was 7 days (range, 7–14 days) and 31 days (range, 28–49 days) for the 30-day follow-up visit. An STI at baseline was not associated with VDS treatment failure; however, women with VVC at baseline (69%) were more likely to

experience treatment failure than women without VVC (34%) (OR 4.3, 95% CI 1.7–11, *p*=0.002) (**Table 4.3**).

Treatment success was achieved in 65 women (69%) women (**Figure 4.2**). At the 7-day follow-up visit, the incidence of treatment failure was 3.6 cases per 100 person-days (95% CI 2.2–5.1), with 24/94 (26%) women reporting persistent or recurrent VDS. At the 30-day follow-up visit, the incidence of VDS treatment failure was 1.0/100 person-days (95% CI 0.65–1.4); 17/94 (18%) women had recurrent VDS and 12/94 (13%) women had persistent VDS. Women living with HIV were more likely to experience recurrent VDS than those without HIV infection (adjusted OR=5.9, 95% CI 1.06–33, *p*=0.042). Pregnancy, vaginal douching, and a history of VDS were not associated with the risk of treatment failure in multivariate analysis (**Table S4.3**). Women with BV had treatment failure more frequently than those without BV (55% vs. 37%; OR 2.1, 95% CI 0.87–5.1).

4.3.3 Microbial aetiology of VDS treatment failure

Of the 29 women reporting VDS at the 30-day follow-up visit, 13 (45%) returned to the facility for evaluation (eight women with recurrent and five with persistent VDS). Based on microbiological data (**Table S4.4**), four cases were attributed to untreated VVC at baseline, three to fluconazole-resistant *Candida* infections (one *C. albicans* and two *C. glabrata*), and four to BV. There was one incident STI, and the cause could not be identified in one case.

4.4 Discussion

Syndromic management without diagnostic testing is the standard of care for VDS in sub-Saharan Africa[1]. This prospective study shows that treatment failure following syndromic management is common in women with VDS in Namibia and that this may present as recurrent

or persistent symptoms. We confirmed that the microbial etiology of VDS is diverse and showed that VVC and BV are important conditions, especially in cases of treatment failure.

The microbial etiology of VDS in our study was representative of women with VDS in our region. At baseline, STI were diagnosed in 40% of women with VDS, while 43% had BV and 41% had VVC. This is in line with etiologic surveillance and research reports from South Africa, Zimbabwe, and Namibia, where STI was diagnosed in 40%-50% of VDS cases [1,6]. These studies also showed that mixed etiology is common, which is, multiple STIs and various combinations of STI, BV, and VVC. The diverse aetiology highlights the clinical challenges and unnecessary use of antibiotics associated with VDS treatment in syndromic management settings.

Unlike other reports on VDS etiology, we conducted a microbial analysis of VVC in this study. As expected, *Candida albicans* was the most common species, but 21% of women with VVC had non-*albicans Candida* species, especially *C. glabrata*, *C. tropicalis* and *C. krusei* [15-19]. Interestingly, 24% of *C. albicans* and 80% of non-*albicans Candida* strains showed *in vitro* resistance to the first-line drug, fluconazole. These rates of antifungal resistance are in line with reports from other parts of the world, such as Cameroon and Uganda, as well as a previous report that included a different cohort from Namibia [20-22]. The observed occurrence of fluconazole-resistant strains generates a serious concern about *Candida* treatment in our syndromic management setting, given the lack of access to antifungal susceptibility testing and limited access to alternative, non-azole, and therapeutic options.

To our knowledge, no other studies have reported VDS treatment outcomes in sub-Saharan Africa. The present study revealed that VDS treatment failure was common in our cohort, with one-third of women reporting either persistence (13%) or recurrence (18%) of symptoms one month after treatment, with an incidence of 1.0 per 100 person-days of follow-up. There are various reasons for persistent or recurrent symptoms [8]. First, VVC may persist if it is not

covered by an empirical antibiotic regimen. Vulvovaginal candidiasis (VVC) was associated with a four-fold increased risk of treatment failure in our study, and microbial evaluation of cases showed that some women did not receive *Candida* treatment at baseline due to a lack of indicative signs during physical examination. Second, the efficacy of single-dose metronidazole may be suboptimal for the empirical treatment of *T. vaginalis* infection and BV [23,24]. Indeed, we observed a trend towards treatment failure in women with BV; although not statistically significant, this is in line with clinical practice and clinically relevant. Third, antimicrobial resistance may occur. Although unlikely to occur for STIs (ceftriaxone resistance in *N. gonorrhoeae* is rare in Africa and macrolide resistance in *M. genitalium* is uncommon) [25,26], we observed fluconazole resistance in a substantial proportion of women with *Candida* infection [20,27]. Other studies have demonstrated that azole resistance is an important cause of VVC treatment failure [27,28]. Finally, a repeat STI within one month from the same or a new sex partner may occur, but this likely only contributes to a small proportion of cases [29,30]. We did not observe an association between a new sexual partner and treatment failure, and only one incident of STI was detected among the small group of women with microbiological evaluation of their treatment failure.

This study had several limitations. Our sample size was relatively small and recruited at a single healthcare facility; a larger study population is required to confirm and extrapolate our findings. Furthermore, selection bias may have occurred as a large proportion of women reported a history of VDS in the preceding year, although the frequency is largely in line with other studies from our region. Also, we used a relatively short period of 7 days of antibiotic use preceding presentation as exclusion criterion which might have resulted in the occasional detection of remnant DNA of a recently treated infection. Second, we verified the VDS treatment outcomes telephonically for practical reasons. This may have resulted in reporting and desirability biases; therefore, the true proportion of women with treatment failure may have been larger. In

addition, microbiological follow-up data were only available for a limited number of women. Furthermore, we used a broad definition of VVC based on clinical presentation, microscopy and culture. Possibly, low level commensal yeast presence instead of overgrowth may have been present; however, interactions between VVC, BV and STIs are dynamic and complex. Finally, despite extensive efforts, several women were lost to follow up. However, it is unclear how this may have affected our results.

In conclusion, our study showed that treatment failure of syndromic management is common in the Namibian setting and that it is mainly caused by untreated or fluconazole-resistant *Candida* infection and BV. As recommended by the World Health Organization, access to laboratory diagnostic testing is essential to optimize the treatment outcomes of persistent or recurrent VDS and should certainly be extended beyond STI testing. Investing in diagnostic and therapeutic options for VVC in settings that implement syndromic management is required.

4.5 Abbreviations

ATCC	American Type Culture Collection
BV	Bacterial vaginosis
BVAB	Bacterial vaginosis associated bacteria
CI	Confidence interval
CT	*Chlamydia trachomatis*
DNA	Deoxyribonucleic acid
EUCAST	European Committee on Antimicrobial Susceptibility Testing
IM	Intramuscular
MIC	Minimum inhibitory concentration
NG	*Neisseria gonorrhoeae*
PCR	Polymerase chain reaction

STI	Sexually transmitted infections
VDS	Vaginal discharge syndrome
VVC	Vulvovaginal candidiasis

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Table 4.1. Baseline characteristics of study population of 109 women with vaginal discharge syndrome, Windhoek, Namibia, 2021-2022

Characteristic	Number (%)
Age (years, mean ± SD)	28 ±6.6
Marital status	
Single	83 (76)
Co-habiting	15 (14)
Married	11 (10%)
New sexual partner(s) in the past month	
Yes	9 (8.0)
No	100 (92)
Time since last sexual intercourse*	
≤ a week ago	58 (53)
≥ a week ago	50 (46)
Condom use during last sex act*	
Yes	45 (42)
No	63 (63)
Vaginal douching	
Yes	23 (21)
No	86 (79)
Pregnancy	
Yes	26 (24)
No	83 (76)
Use of hormonal contraception	
Yes	23 (21)
No	86 (79)
HIV status*	
Unknown	14 (13)
Positive	8 (7.0)
Negative	87 (80)

Data are presented as number (*n*) with proportion (%) unless indicated otherwise.

Note. SD, standard deviation; HIV, human immunodeficiency virus.

*N=108 due to one participant not ever engaging in sexual intercourse.

Table 4.2. . *In vitro* fluconazole susceptibility of *Candida* isolates (*n*=45) collected from women with vaginal discharge in Namibia.

***Candida* species**	**Number of isolates**	**MIC range (mg/L)**	**MIC breakpoints[a]**		
			Susceptible	**Intermediate**	**Resistant**
Candida albicans	33 (73)	2–4	24 (72)	1 (3.0)	8 (24)
Candida glabrata	5 (11)	0.001–16	1 (20)	0 (0)	4 (80)
Candida krusei	3 (7.0)	–[b]	1 (33)	0 (0)	2 (67)
Candida tropicalis	4 (9.0)	>4	0 (0)	0 (0)	4 (100)

Data are presented as number (*n*) with proportion (%) unless indicated otherwise.

Note. MIC, minimum inhibitory concentration.

[a] EUCAST (version 7.3.2) breakpoints were used for interpretation.

[b] No breakpoints: *C. krusei* intrinsically resistant to azole antifungals.

Table 4.3. Microbiological predictors of treatment failure in Namibian women with vaginal discharge syndrome (*n*=94).

Infection	Total (*n*, %)	Treatment failure (*n*=29)	Treatment success (*n*=65)	Crude OR (95% CI)	*p*-value
Chlamydia trachomatis				0.76 (0.25-2.4)	0.63
Yes	19 (20)	5 (17)	14 (22)		
No	75 (80)	24 (83)	51 (78)		
Neisseria gonorrhoea				0.88 (0.25-3.1)	1.00
Yes	14 (15)	4 (14)	10 (15)		
No	80 (85)	25 (86)	55 (85)		
Trichomonas vaginalis				1.3 (0.44-3.6)	0.65
Yes	20 (21)	7 (24)	13 (20)		
No	74 (79)	22 (76)	52 (80)		
Mycoplasma genitalium				1.4 (0.47-4.4)	0.56
Yes	16 (17)	6 (21)	10 (15)		
No	78 (83)	23 (79)	55 (85)		
Vulvovaginal candidiasis				4.3 (1.7-11)	**0.002**
Yes	42 (45)	20 (69)	22 (34)		
No	52 (55)	9 (31)	43 (66)		
Bacterial vaginosis				2.1 (0.87-5.1)	0.098
Yes	40 (42)	16 (55)	24 (37)		
No	54 (58)	13 (45)	41 (63)		

Data are presented as number (*n*) with proportion (%) unless indicated otherwise.

Note. OR, odds ratio; CI, confidence interval.

Figure 4.1. Infectious aetiology identified in women with vaginal discharge syndrome in Windhoek, Namibia, 2021-2022

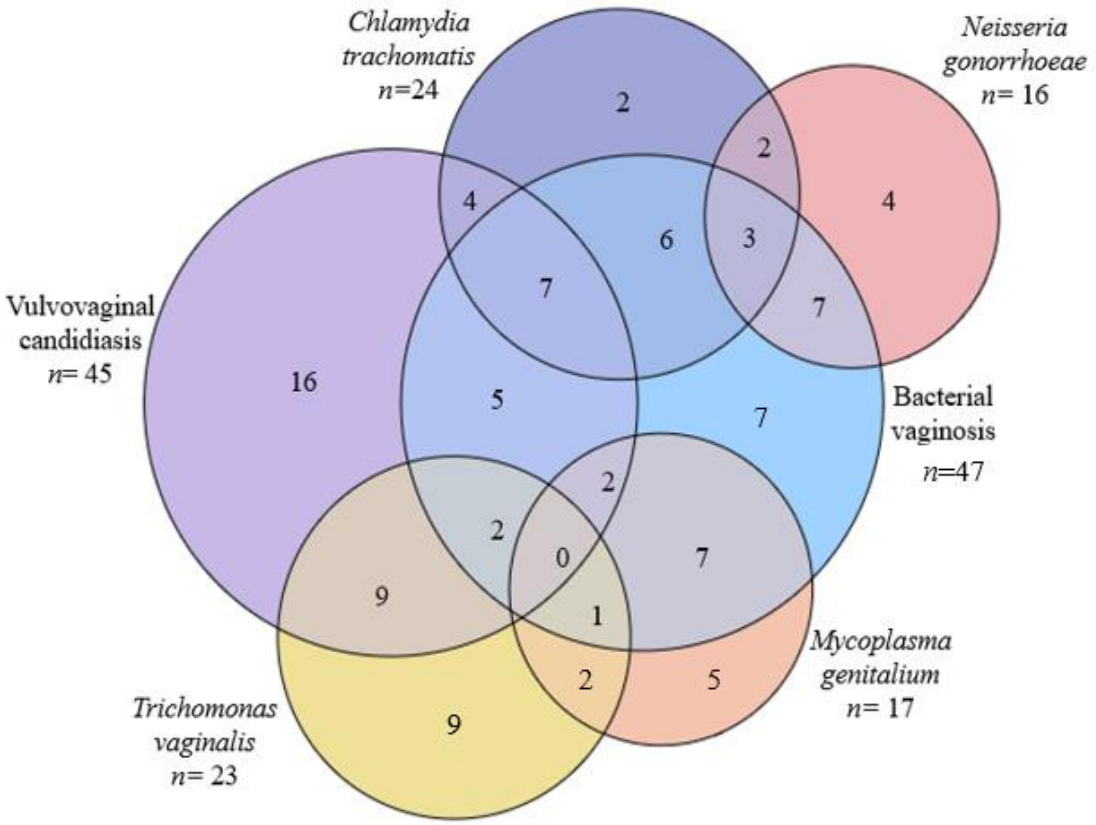
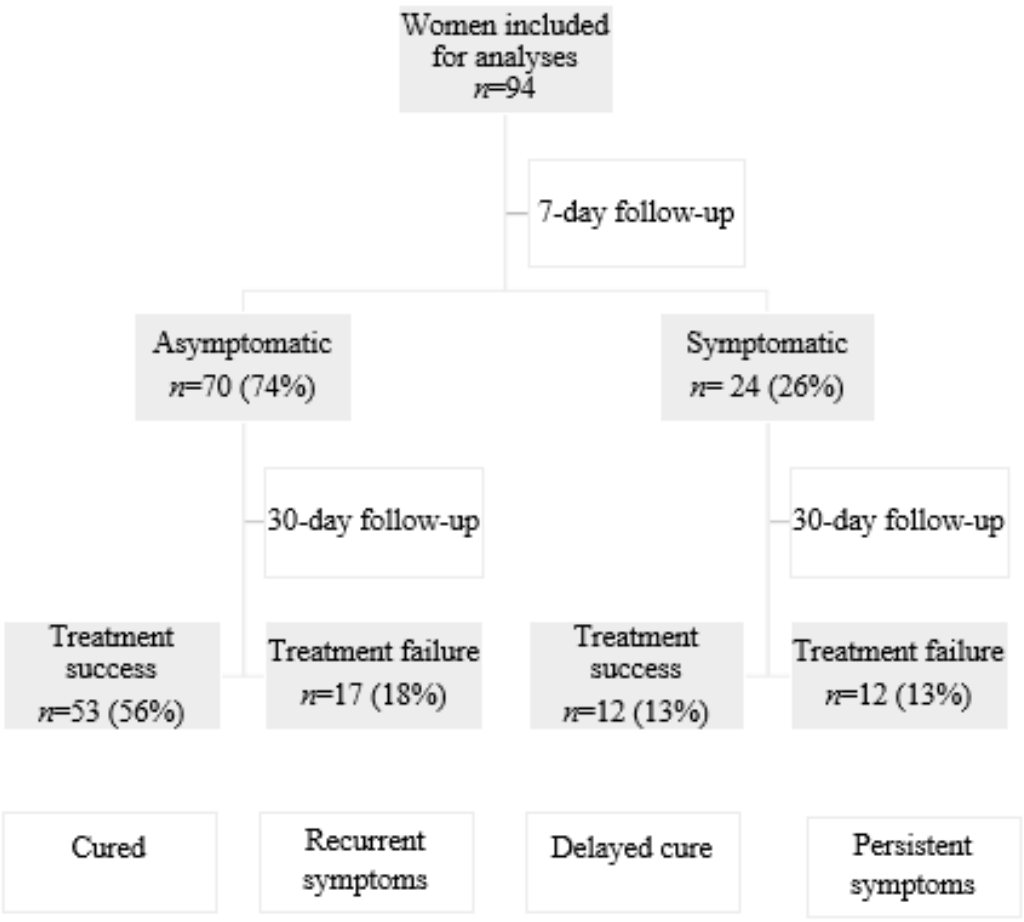


Figure 4.2. Treatment outcomes of vaginal discharge syndrome in women in Windhoek, Namibia, 2021 - 2022.



Chapter 5: Molecular epidemiology and antimicrobial resistance of vaginal *Candida glabrata* isolates in Namibia

Objective(s) addressed:	• To determine the phenotypic and genotypic antifungal susceptibility patterns of *Candida* spp. in specimens obtained from symptomatic women with primary and recurrent vulvovaginal candidiasis. • To perform molecular epidemiological analysis of antifungal resistant *Candida* species to determine genotype distribution in relation to demographic and bio-behavioural characteristics, and co-infections using multilocus sequence typing.
Publication status:	In Press
Authors:	Cara M. Dunaiski, Marleen M. Kock, Wai Yin Chan, Arshad Ismail and Remco P. H. Peters
Journal:	Medical Mycology

1. Overview of the chapter

Selected isolates, i.e. *Candida glabrata* isolates, from the previous chpaters (chapter 3 and 4) were subjected antifungal susceptibility testing (AST), as well as whole genome sequencing (WGS) to address objectives 2 and 3 in this chapter (chapter 5). Among the *C. glabrata* isolates, all exhibited phenotypic resistance to azole class antifungal drugs, (fluconazole and voriconazole), and phenotypic susceptibility to the polyene class (amphotericin B), and the echinocandins (anidulafungin). The whole genome sequence analysis revealed several single nucleotide polymorphisms in antifungal resistance genes, eight distinct sequence types (STs) (ST55, ST114, ST22, ST15, ST283, ST284, ST285, ST286) and four new STs not previously reported (ST283, ST284, ST285, ST286) in the fluconazole-resistant *C. glabrata*. Of the known STs, the commonest ST amongst the Namibian isolates was ST15 (38%).

2. Relevance of the chapter

This chapter provides the first genomic characterisation of *Candida glabrata* in Namibia. This study provides a detailed report on the STs associated with antimicrobial resistance, and higlights the importance of including *C. glabrata* strains from resource-constraint settings. Information obtained from this data can be used to explore ways to improve the syndromic management guidelines by the introduction of laboratory diagnoses for *Candida* species identification, AST and the investigation of new and alternative thaerpaeutic strategies or antifungals.

3. Author contributions

Cara M. Dunaiski:	Conceptualisation, Methodology, Formal Analysis and Investigation, Writing – Original Draft, Review & Editing, Software and Data Curation
Marleen M. Kock:	Conceptualisation, Methodology, Formal Analysis and Investigation, Writing – Review & Editing, Resource, Supervision
Wai Yin Chan:	Formal Analysis and Investigation, Writing – Review & Editing, Resource
Arshad Ismail:	Formal Analysis and Investigation, Writing – Review & Editing, Resource
Remco P. H. Peters:	Conceptualisation, Methodology, Formal Analysis and Investigation, Writing – Review & Editing, Resource, Supervision

CHAPTER 5

Molecular epidemiology and antimicrobial resistance of vaginal *Candida glabrata* isolates in Namibia

The editorial style of Medical Mycology has been followed as the chapter was published in this journal.

Abstract

Candida glabrata is the most common non-albicans *Candida* species that causes vulvovaginal candidiasis (VVC). Given the intrinsically low susceptibility of *C. glabrata* to azole drugs, investigations into *C. glabrata* prevalence, fungal susceptibility profile, and molecular epidemiology are necessary to optimise the treatment of VVC. This molecular epidemiological study was conducted to determine antifungal drug profile, single nucleotide polymorphisms (SNPs) associated with phenotypic antifungal resistance and epidemic diversity of *C. glabrata* isolates from women with VVC in Namibia. *Candida glabrata* isolates were identified using phenotypic and molecular methods. Antifungal susceptibility of strains was determined for fluconazole, itraconazole, amphotericin B and anidulafungin. Whole genome sequencing was used to determine SNPs in antifungal resistance genes and sequence type (ST) allocation. Among *C. glabrata* isolates, all (20/20; 100%) exhibited phenotypic resistance to the azole class antifungal drug, (fluconazole), and phenotypic susceptibility to the polyene class (amphotericin B), and the echinocandins (anidulafungin). Non-synonymous SNPs were identified in antifungal resistance genes of all fluconazole-resistant *C. glabrata* isolates including *ERG6* (15%), *ERG7* (15%), *CgCDR1* (25%), *CgPDR1* (60%), *SNQ2* (10%), *FKS1* (5.0%), *FKS2* (5.0%), *CgFPS1* (5.0%) and *MSH2* (15%). ST15 (*n*=8/20, 40%) was predominant. This study provides important insight into phenotypic and genotypic antifungal resistance across *C. glabrata* isolates from women with VVC in Namibia. In this study, azole resistance is determined by an extensive range of SNPs, while the observed polyene and echinocandin resistance-associated SNPs despite phenotypic susceptibility require further investigation.

Lay summary

Candida glabrata is inherently resistant to azole drugs. In this study, we identified a clone that was predominant in women with VVC in Namibia, and that harboured various mutations in

resistance-associated genes. This study provides important insight into antifungal resistance across *C. glabrata* isolates in a sub-Sahara African setting.

5.1 Introduction

Candida glabrata, recently reclassified as *Nakaseomyces glabrata*, is a non-hyphae-producing haploid yeast commensal and an important causal-agent of vulvovaginal candidiasis (VVC).[1] The SENTRY Antifungal Surveillance Program reported a decrease in invasive *Candida albicans* isolates but an increase in fluconazole-resistant *Candida glabrata* isolates from 8.6% in 1997 to 10.1% in 2014 and in echinocandin-resistant isolates from 1.7% to 3.5% over the period 2006-2014 globally.[2] In Africa, a 2020 study in Ghana reported fluconazole resistance (39%), voriconazole resistance (36%) and nystatin resistance (13%) in *C. glabrata* isolates from pregnant women.[3] More recently, a study conducted in Namibia demonstrated a high rate of fluconazole resistance in *C. glabrata* isolates (74%) obtained from women with VVC.[4] Vulvovaginal candidiasis, usually caused by *Candida albicans*, responds generally well to first-line empirical treatment with topical or systemic azole drugs. However, this is not the case for *C. glabrata* infection that is often resistant to first-line treatment, and where pathogen-direct treatment is generally challenging given the limited access to diagnostics and appropriate therapeutic options in Africa. Complicated cases such as recurrent VVC caused by azole-resistant *C. glabrata* are associated with considerable morbidity to women and constitute a financial burden for patients due to repeated healthcare attendance.[5-7]

Drugs of the azole class are considered the first-line antifungal therapy for VVC.[8,9] Treatment outcome is generally good for *C. albicans* infection, but *Candida glabrata* exhibits innately low susceptibility to azole drugs, especially fluconazole, which may result in treatment failure.[10,11] Alternative treatment options are the echinocandin class drugs (anidulafungin, caspofungin and micafungin), the polyenes (nystatin and amphotericin B) and the pyrimidine analogue flucytosine which are all active against *C. glabrata.*[12] Amphotericin B and flucytosine resistance in *C. glabrata* is relatively uncommon.[13] However, development of drug resistance is an important concern and better understanding of its underlying mechanisms is required to optimize treatment regimens and outcomes [14].

Due to its haploid nature, resistance in *C. glabrata* can emerge by single amino acid alterations and the subsequent accumulation of polymorphisms, particularly during antifungal therapy.[13,15] A gain-of-function mutation in the transcription factor pleiotropic drug resistance CgPDR1,

and the overexpression of the drug-efflux transporter genes *CgCDR1*, *CgCDR2*, and *CgSNQ2* are the most common causes for azole resistance in *C. glabrata* [16]. However, other mechanisms may also contribute to azole resistance, including single nucleotide polymorphisms (SNPs) in genes encoding the target enzyme *ERG11*p, which is a vital enzyme in ergosterol biosynthesis.[16] Echinocandin resistance is most commonly caused by SNPs in hotspot regions of the drug target protein encoded by *FKS1* and *FKS2*, which encode 1,3-β-D-glucan synthase, the target of echinocandins in *C. glabrata.*[17] Low rates of susceptibility in pyrimidine analogues such as flucytosine, have been linked with SNPs in *FUR1*, *FCY1* (encoding enzymes involved in the pyrimidine pathway) and *FCY2* (encoding a cytosine permease) genes in *C. glabrata.*[15] Furthermore, the deletion of *FPS1* or *FPS2*, encoding aquaglyceroporins, may lead to an increased accumulation of flucytosine within *C. glabrata*, by interfering with transmembrane transport systems.[15,18] Multidrug phenotypic resistance in fluconazole, amphotericin B and echinocandins has been observed in certain *C. glabrata* genotypes that harbour particular polymorphisms in the *MSH2* gene.[19,20]

Detection of genetic polymorphisms in the genomes of clinical isolates is an important tool to elucidate the emergence of antifungal drug resistance.[21] Multilocus sequence typing (MLST) can be utilised to perform fungal population structure studies.[22,23] In this study, we determined antifungal susceptibility and used whole genome sequencing to study genetic determinants of antifungal resistance and determine the MLST sequence types (STs) for 20 *C. glabrata* isolates collected from women with VVC in Namibia.

5.2 Materials and methods

5.2.1 Study design

Twenty fluconazole-resistant *C. glabrata* isolates were obtained from vulvovaginal specimens from women presenting with vaginal discharge syndrome. Vulvovaginal swabs from two studies were included: a cross-sectional study conducted at the Namibia Institute of Pathology, Namibia's reference laboratory, to determine the prevalence of *Candida* infection, antifungal resistance, and coinfections in specimens from Namibian women with vaginal discharge syndrome;[4] and a prospective cohort study conducted at the Katutura Intermediate Hospital in Windhoek, Namibia that had the aim to measure the incidence and microbial aetiology of vaginal discharge syndrome treatment failure in a resource-constrained setting in Namibia. Clotrimazole pessary and topical cream are standard of care for VVC in Namibia. Clinical

guidelines for the management of vaginal discharge treatment failure and for azole-resistant candidiasis are currently not in place.

5.2.2 Antifungal susceptibility testing

Candida glabrata isolates were identified with chromogenic *Candida* agar plates (CHROMagar™, France), and were confirmed using multiplex PCR to amplify the ITS1 region between the 18S and 5.8S rRNA genes and a specific DNA fragment within the ITS2 region (BIORAD, France).[24] Broth microdilution method was performed for antifungal susceptibility testing for all strains and *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 as control strains as described by the European Committee of Antimicrobial Susceptibility Testing (EUCAST).[25] Minimal inhibitory concentrations (MICs) were determined for fluconazole, itraconazole, polyene amphotericin B, and anidulafungin. Multidrug resistance (MDR) was also evaluated and is defined as resistance to at least one antifungal in three or more drug classes.

5.2.3 DNA preparation and whole genome sequencing

Genomic extraction and purification from *Candida glabrata* isolates were performed using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, USA) according to the manufacturer's guidelines. The Qubit® 2.0 fluorometer (Invitrogen, Oregon, USA) and Qubit® dsDNA HS assay kit was used for quantification of the extracted genomic DNA to a concentration of >10ng/µl. Multiplexed, paired-end libraries (2 x 150bp) were prepared using the Illumina DNA Prep kit (Illumina, San Diego, USA), followed by sequencing on the Illumina NextSeq 2000 platform (Illumina, San Diego, USA) with 100x coverage.

5.2.4 Whole genome sequencing analysis

The raw reads were assessed for quality using FastQC,[26] and low-quality bases and adaptor sequences were trimmed with Trim Galore v.0.6.5.[27] Filtered reads were de novo assembled using the UGAP pipeline (https://github.com/jasonsahl/UGAP), which uses the SPAdes v.3.15.5 assembler,[28] genome completeness was score using BUSCO v.5.2.2 against the saccharomycetes_odb10 database and the genome assembly quality was assessed using Quast v.5.2.0.[29] The genome sequences data are being submitted to the National Centre for Biotechnology Information under BioProject submission number: SUB12333157.

Single nucleotide polymorphisms in genes associated with antifungal resistance in *Candida glabrata* were identified by mapping sequenced reads to *C. glabrata* CBS138 (GenBank Accession No. GCA_0002545.2) as the reference genome using Geneious Prime® 2023.1.1 (Biomatters, Auckland, New Zealand). Low-quality variants were filtered for mapping quality (> 30), read depth (>20), read base quality (>25) and variant frequency (> 0.25). Only non-synonymous SNPs were reported. The genes inspected included *ERG6, ERG7, ERG9, ERG11, CgCDR1, CgPDR1*, *SNQ2* (for azole resistance), *FKS1 and FKS2* (for echinocandin resistance), *ERG2* and *ERG6* (for polyene resistance), *FCY1, FCY2, CgFPS1, CgFPS2* (for 5-fluorocytosine resistance), and *MSH2* (for multidrug resistance).

Multilocus sequence typing profiles, for the six loci (*FKS, LEU2, NMT1, TRP1, UGP1* and *URA3*) of each isolate were assigned from whole genome sequence data, using the *C. glabrata* database at the MLST website [30]. Novel *C. glabrata* STs were allocated and included to the *C. glabrata* MLST database.

5.2.5 Statistical analyses

Data are presented as an absolute value with proportions, and as median with interquartile range in case of continuous data. Comparison of data between groups was not performed due to the nature of the study and the relatively small sample size.

The genes and frequency of SNPs in *C. glabrata* isolates were illustrated via a heat map generated on Microsoft Excel version 365 (Microsoft Corporation, USA). A UPGMA dendrogram showing the similarities was constructed based on MLST of six housekeeping gene loci using Geneious Prime® 2023.1.1 (Biomatters, Auckland, New Zealand) showing the sequence type based phylogenetic relationship of all *C. glabrata* isolates contained in the study.[31]

5.2.6 Ethical clearances

Ethical clearance was obtained from the Human Research Ethics Committee of the University of the Pretoria (Ref: 518/2020), the Namibia University of Science and Technology Faculty of Health and Applied Sciences Research Ethics Board (Ref: FHAS 11/2020) and regulatory approval from the Namibian Ministry of Health and Social Services (Ref: 17/3/3/CMD).

5.3 Results

A total of 20 fluconazole-resistant *Candida glabrata* isolates from women with vulvovaginal candidiasis in Namibia were included in this study: 14 from the cross-sectional evaluation of 253 swabs at the Namibia national reference laboratory and six from 109 participants in the prospective cohort study of women with vaginal discharge syndrome at Katutura hospital in Windhoek. Median age was 28 years (interquartile range (IQR) 18 – 58), 4/20 women were living with HIV, and 2/20 were pregnant.

5.3.1 Antifungal susceptibility profile

All *C. glabrata* (*n*=21, 100%) isolates were resistant to the azole antifungals and all were susceptible to amphotericin B and anidulafungin (Table 5.1).

5.3.2 Single nucleotide polymorphisms associated with resistance

Whole genome sequencing assembly of the 20 *C. glabrata* isolates resulted in genomes with an average of 12 164 285 bp in size (range from 12 136 421 to 12 234 316), an average GC content of 38% for each isolate and an overall BUSCO completeness score above 97% When aligned against the reference genome CBS138, an average of 99% coverage query was observed. Overall, an average of 95% of sequencing reads were mapped to the *C. glabrata* reference genome with a median read depth coverage of 75-fold.

High-quality non-synonymous SNPs in antifungal resistance genes such as *CgCDR1*, *CgPDR1*, *SNQ2*, *FKS1*, *FKS2*, *CgFPS1* and *MSH2* were identified in 12/20 (60%) fluconazole-resistant *C. glabrata* isolates (Table 5.2).

Single nucleotide polymorphisms (SNPs) in *CgPDR1* (60%) and *CgCDR1* (25%) were detected in 20 azole-resistant isolates. Isolates exhibiting fluconazole resistance, also had SNPs in *CgCDR1*; His58Tyr (2/20, 10%), Gly384Val (1/20, 5.0%), Ala74Pro (1/20, 50%), Lys979Arg (1/20, 5.0%), and Ile1478Met (1/20, 5.0%). Single nucleotide polymorphisms in the *CgSNQ2* gene resulted in amino acid substitutions observed in 2/20 isolates (10%), Lys1156Asn in one and one other isolate harbouring two SNPs, Ser1506Pro and Phe1282Leu.

Three isolates harboured a SNP in *ERG6* (15%), resulting in amino acid substitution, Arg48Lys; however, this nonsynonymous mutation is a polymorphism that is not connected with resistance to amphotericin B. There were also SNPs in the *ERG7* (15%); Gln683Leu

(1/20,5.0%), Glu668Lys (1/20, 5.0%), Ile497Thr (1/20, 5.0%), Thr732Ala (2/20, 10%), Val438Phe (2/20, 10%).

Single nucleotide polymorphisms (SNPs) in the *MSH2* gene were observed in 3/20 (15%) isolates with three locations of SNPs – Glu456Asp, Gln310His and one isolate harbouring two SNPs at Ser934Leu and Ile110Met (Table 5.2).

Although phenotypic resistance to the echinocandin anidulafungin was not observed, SNPs resulting in amino acid substitutions in the *FKS1* and *FKS2* genes were present. One isolate contained an *FKS1* mutation (5.0%) leading to the amino acid substitution Ile1174Val, and another *FKS2* (5.0%) nonsynonymous mutation leading to the amino acid substitution Thr926Pro (Table 5.2).

5.3.3 Molecular epidemiology of *Candida glabrata*

A dendrogram was constructed of the 20 *C. glabrata* isolates (Figure 5.1). In total, 34 point-SNPs were detected, and only twenty-two were formerly reported (Supplementary Table S5.1).

Eight distinct STs were defined including four new STs not previously reported in the *C. glabrata* MLST database.[32] Of the known STs, the commonest ST amongst the Namibian isolates was ST15 (8/20, 40%) followed by ST55 (3/20, 15%), ST114 (2/20, 10%) and ST22 (1/20, 5.0%).

5.4 Discussion

Vulvovaginal candidiasis is a common condition in primary healthcare and affects 75% of women of reproductive age.[12] A rise of VVC has been reported due to azole-resistant *C. glabrata.*[33] This study is among a few from sub-Saharan Africa to provide an in-depth analysis of *C. glabrata* antifungal resistance and molecular epidemiology of isolates from Namibian women with VVC.

All *C. glabrata* strains analysed in this study were resistant to the azole class of antifungals. Several countries have also reported high rates of azole resistance in *C. glabrata* including isolates from Uganda, Cameroon and Nigeria.[34-36] Since azole antifungals, in particular clotrimazole, are commonly used to treat VVC, *C. glabrata* may acquire resistance mutations from its over-use leading to high prevalence of resistant strains.[3] Similar to our study, low rates of phenotypic resistance to polyene and echinocandin drugs have been reported.[13,20,37,38]

Whole genome sequencing revealed SNPs of several multidrug transporter genes including *CgCDR1*, *CgPDR1* and *SNQ2* that are associated with the overexpression of drug efflux pumps.[39,40] The transcription factor, CgPDR1, which induces the gene expression of the *CgCDR1/2* and *CgSNQ2* efflux pumps, has been reported as a significant cause of azole resistance in *C. glabrata*.[39,41] Previously, 67 gain-of-function mutations in *CgPDR1* have been described that are associated with intrinsically high expression of the efflux pumps.[39,42] In this study, 10 possible contributory SNPs were detected in 12 of the 20 azole-resistant strains. Five of these SNPs were not reported before. Multi-azole resistance has previously been found resulting from gain-of-function mutations in *CgPDR1*,[39,43,44] however, no gain-of-function mutations were observed in this study. Single nucleotide polymorphisms were detected in genes expressing other efflux pumps; however, the importance of these remains ambiguous as these SNPs may also be present in azole-susceptible isolates.[44] Mutations in *CgPDR1* were mostly observed in ST55. This is in line with reports of fluconazole-resistant ST55 isolates from bloodstream infections in South Korea.[45]

This study did not observe any *ERG9* and/or *ERG11* SNPs in genes encoding the target of azole agents in our azole-resistant *C. glabrata* isolates. The absence of these SNPs in azole-resistant isolates has previously been reported.[46-48] A study by Li *et al.* demonstrated that gene expression involved in ergosterol biosynthesis may mediate antifungal resistance in *C. glabrata* in response to azole stress.[49]

This study did not show phenotypic resistance to amphotericin B but harboured a non-synonymous mutation in *ERG6* (Arg48Lys) in four isolates; this polymorphism has been detected among drug-susceptible and drug-resistant strains and is not associated with loss of ergosterol and alteration in the total cell sterol profiles.[50] Although phenotypic susceptibility was demonstrated against the echinocandin anidulafungin, in this study, *FKS* polymorphisms were detected but these nonsynonymous mutations are not connected with resistance.[42] SNPs associated with high-level resistance phenotypes, such as *FKS1* S629P and *FKS2* S663P, were not detected.[8,44, 51,52] Echinocandins are not used for the treatment of VVC in Namibia, which may explain the absence of phenotypic resistance as echinocandin exposure is the main driver of acquired resistance.[2,44]

Antifungal susceptibility was not tested for 5-fluorocytosine; however, a SNP was observed in *CgFPS1*, known to confer 5-fluorocytosine resistance, in two *C. glabrata* isolates. As with the

azole class, resistance to 5-fluorocytosine usually develops post-treatment.[41,44] The clinical relevance of the observed SNP is unknown.

Single nucleotide polymorphisms in the DNA mismatch repair gene *MSH2* have been described in numerous fungal species, including *C. glabrata*, as genetic drivers of MDR involving fluconazole, amphotericin B and echinocandins.[53] In contrast to other studies reporting approximately 50% of *C. glabrata* isolates to contain *MSH2* gene SNPs, only 15% of our isolates harboured a mutation in *MSH2* and this was not reflected in the drug resistance profile as MDR.[41, 51] Interestingly, the known mutation, Val239Leu, which is associated with hypermutability, was absent in our set of isolates harbouring SNPs in the *MSH2* gene.[1] This explains the lack of MDR in our study despite the *MSH2* gene mutation.[1,19]

We observed a variety of known and novel sequence types; ST15, ST55 and ST22 that were detected in our study and have been reported globally.[15,19,32,41,51,54-72]Sequence type 15 and ST114 were reported from Tanzania; the only other African country that reported *C. glabrata* STs.[32] Other important sequence types like ST3, ST5, and ST7 that have been reported in Australia, Europe, United States of America, Japan, Korea, China and South America, were not identified in this study.[41,60] We identified eight clusters with strains from multiple genes associated with antifungal resistance allocated to each cluster. This confirms the widespread nature of individual STs of azole-resistant *C. glabrata* strains circulating in Namibia.

This study has several limitations. First, the sample size is relatively small. Nevertheless, this study provides an important and microbiologically comprehensive overview of the *C. glabrata* strains circulating in this population with the paucity of epidemiological data and showing the concerning high prevalence of drug resistance. Second, only fluconazole-resistant isolates from vaginal swabs in women with VVC were included, therefore differences and associations between SNPs, STs, anatomical sites and phenotypic antifungal resistance could not be assessed. Third, we used 6-loci-MLST to get an impression of genetic relatedness. Multigene phylogeny would have provided higher discriminatory power but was not done due to the small sample size. Last, we were not able to collect data about treatment history with fluconazole or other azoles to provide better insight observed antifungal resistance.

Antimicrobial resistance in *C. glabrata* is a global health concern and there is a paucity of data from sub-Saharan Africa. This study shows a high rate of azole resistance against the azole antifungal drugs, but not to the other classes, in the *C. glabrata* isolates from Namibia. This

highlights the importance of access to diagnostic and antifungal susceptibility testing, and the need to develop evidence-based clinical guidelines for management of treatment failure and azole-resistant candidiasis in our syndromic management setting.

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Tables and Figures

Table 5.1. Minimum inhibitory concentrations of *Candida glabrata* isolates collected from women with vaginal discharge syndrome in Namibia, 2021-2022 (*n*=20).

Isolate	MIC (mg/L)			
	FLC	ITR	AMP	ANI
N0021	128	8	0.03	0.016
N0082	128	8	0.13	0.016
N0083	128	8	0.25	0.016
N0094	128	8	0.06	0.016
N0095	64	8	0.5	0.016
N0096	128	8	0.03	0.016
N0099	128	8	0.25	0.016
N0132	64	8	0.5	0.016
N0160	32	8	0.25	0.03
N0163	32	4	0.5	0.016
N0165	32	4	0.25	0.016
N0172	128	8	0.5	0.016
N0209	64	8	0.25	0.016
N0227	128	8	0.5	0.016
K0010	64	8	0.5	0.016
K0054	128	8	0.13	0.016
K0012	128	8	0.03	0.016
K0085	64	8	0.13	0.016
K0118	64	8	0.13	0.016
K0120	128	8	0.03	0.016

Abbrreviations: ID, identification; MIC, minimum inhibitory concentration; ST, sequence type, (refers to MLST sequence type); FLU, fluconazole, ITR, itraconazole, AMB, amphotericin B, ANI, anidulafungin

EUCAST (version 7.3.2) breakpoints were used to classify strains as susceptible or resistant.

Fluconazole resistance MIC>16 mg/L; Itraconazole susceptibility cannot be categorized with breakpoints as there is insufficient evidence that the species in question is a good target for therapy with the drug; Amphotericin B resistance: MIC> 1.0 mg/L; Anidulafungin resistance: MIC>0.06 mg/L.

Table 5.2. Antifungal resistance-associated SNPs identified in *Candida glabrata* isolates (*n*=21).

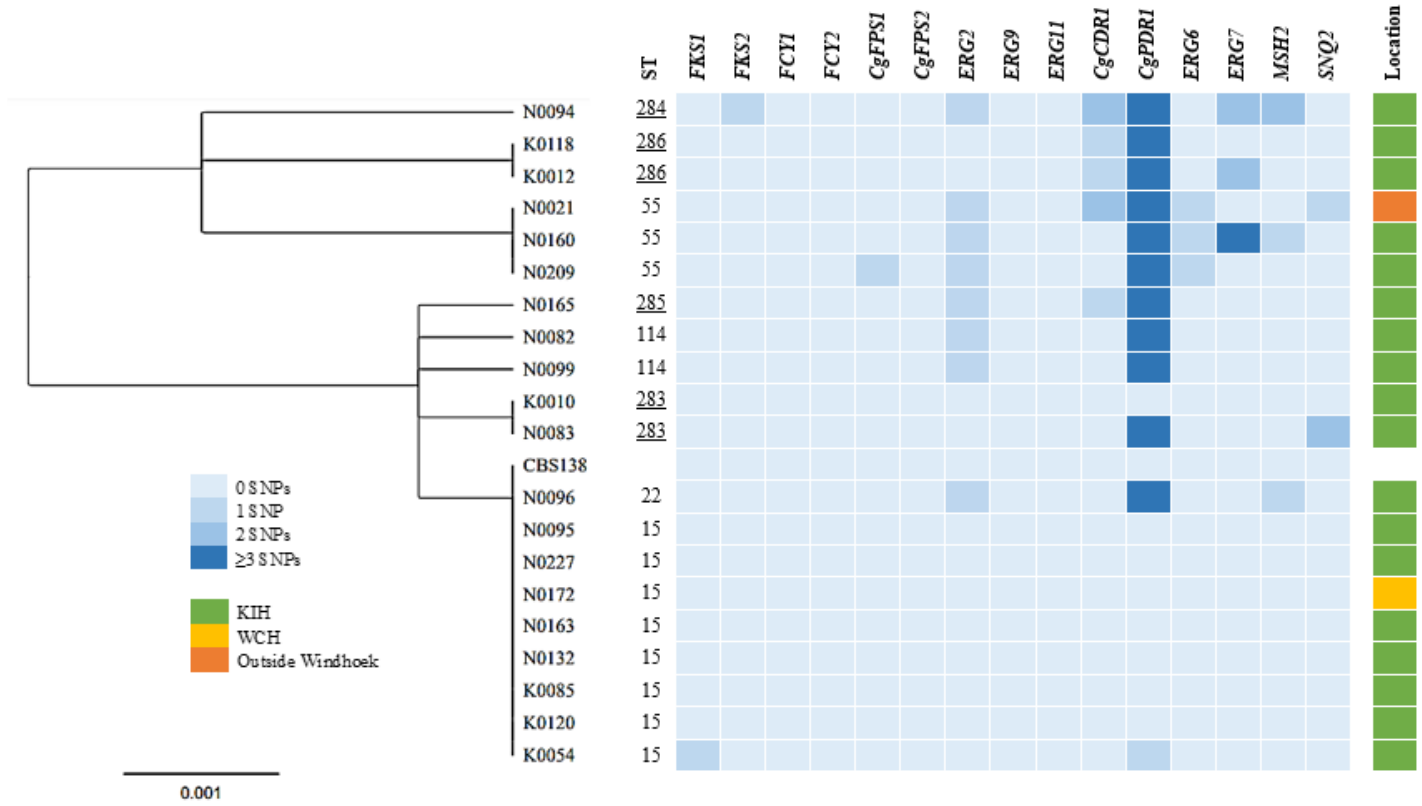
Drug Class	Phenotypic resistant isolates (*n*, %)	Isolates with resistance associated SNPs (*n*, %)	Gene	Isolates harbouring SNPs in genes (*n*, %)	Mutation	Isolates with this SNP (*n*, %)
Azole	20 (100)	12 (60)	*ERG6* [1]	3 (15)	Arg48Lys	3 (15)
			ERG7 [1]	3 (15)	Gln683Leu	1 (5.0)
					Glu668Lys	1 (5.0)
					Ile497Thr	1 (5.0)
					Thr732Ala	2 (10)
					Val438Phe	2 (10)
			CgCDR1 [1,42]	5 (25)	His58Tyr	2 (10)
					Ile1478Met	1 (5.0)
					Ala74Pro	1 (5.0)
					Lys979Arg	1 (5.0)
					Gly384Val	2 (10)
			CgPDR1 [1,42, 40, 46]	12 (60)	Ser76Pro	8 (40)
					Val91Ile	11 (55)
					Leu98Ser	11 (55)
					Thr143Pro	11 (55)
					Glu259Gly	3 (15)
					Gln15Lys	1 (5.0)
					Gln18Lys	1 (5.0)
					Thr51Ala	1 (5.0)
					Gly189Val	2 (10)
					Asp963Tyr	1 (5.0)
			SNQ2	2 (10)	Lys1156Asn	1 (5.0)
					Ser1506Pro	1 (5.0)
					Phe1282Leu	1 (5.0)

Table 5.2. Antifungal resistance-associated SNPs identified in *Candida glabrata* isolates (*n*=21) (*continued*).

Drug Class	Phenotypic resistant isolates (*n*, %)	Isolates with resistance associated SNPs (*n*, %)	Gene	Isolates harbouring SNPs in genes (*n*, %)	Mutation	Isolates with this SNP (*n*, %)
Amphotericin B	0 (0)	8 (40)	*ERG2*	8 (40)	Ile207Val	8 (40)
	0 (0)		*ERG6*	3 (15)	Arg48Lys	3 (15)
Echinocandin	0 (0)	2 (10)	*FKS1*	1 (5.0)	Ile1174Val	1 (5.0)
			FKS2 [42]	1 (5.0)	Thr926Pro	1 (5.0)
5-fluorocytosine	NP	1 (5.0)	*CgFPS1* [42]	1 (5.0)	Asn27His	1 (5.0)
Multidrug	0 (0)	3 (15)	*MSH2* [42,19]	3 (15)	Ser934Leu	1 (5.0)
					Ile110Met	1 (5.0)
					Glu456Asp	1 (5.0)
					Gln310His	1 (5.0)

Note. No resistance was conferring mutations were observed in this study, and all SNPs were non-synonymous.

Figure 5.1. UPGMA dendrogram of Namibian *Candida glabrata* isolates based on MLST of six housekeeping gene loci.



Note. Sequence types (STs) for genes and the frequency of single nucleotide polymorphisms (SNPs) is indicated to the right of the tree. Location where isolates were obtained are indicated and novel STs are underlined.

Abbreviations. KIH, Katutura Intermediate Hospital; WCH, Windhoek Central Hospital.

CHAPTER 6

Concluding discussion

Vulvovaginal candidiasis (VVC) is a mucosal infection caused by *Candida* species that affects millions of women annually and is considered an imperative public health problem. [1] There are only four main classes of antifungals in clinical use to treat *Candida* infections, namely azoles, polyenes, echinocandins and pyrimidine analogues. [2] In recent years, the extensive use of antifungal drugs, especially from the azole class, has stimulated a shift in candidiasis epidemiology, wherein the incidence of *C. albicans* has declined, while other species that are innately less susceptible to this drug, including *C. glabrata*, are increasing. [2] The emergence of resistant *Candida* strains, has been progressively described with the fluconazole resistance prevalence of the four most frequently isolated species of *Candida* ranging from 0.3%, in *C. albicans*, to 8.1% in *C. glabrata.* [3] A twenty-year old antifungal susceptibility surveillance program reported an increase of 8.6% to 10.1% in fluconazole resistance from 1997 to 2014 for *C. glabrata*, thereby demonstrating a slow decline in the likelihoods of successful treatment. [3, 4]

In response to the increasing threat of fungal infections, existing and emerging resistance, and concerns regarding treatment options, the World Health Organization (WHO) recently published its first ever list of fungal "priority pathogens" of which the most important criteria for ranking priorities was antifungal resistance. [5] *Candida albicans* was ranked as a critical priority pathogen, followed by C. *glabrata*, *C. tropicalis* and *C. parapsilosis* as high priority pathogens. [5] *Candida* antifungal resistance surveillance programs are critical to recognise the causal drivers of the transmission of antimicrobial resistant infections and to offer substantiated evidence for reconsiderations of national standard treatment and public health policy guidelines. [6]

Vulvovaginal candidiasis frequently occurs as co-infections with sexually transmitted infecions (STIs), including *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Trichomonas vaginalis* and *Mycoplasma genitalium*, as well as bacterial vaginosis (BV). [7] Globally the burden of STIs remains high, with an approximated 376 million new infections of the four treatable STIs; chlamydia, gonorrhoea, syphilis and trichomoniasis in 2016. [8] In Namibia, like other low- to middle-income countries (LMICs), syndromic management of genital tract infections is the standard of care, i.e. clinicians treat patients with suggestive symptoms with an empirical combination of antibiotics guided by an algorithm and without diagnostic testing. [9] This may lead to over-prescribing antimicrobial drugs and the risk of antimicrobial resistance (AMR). [10] Many

genital infections are asymptomatic and consequently syndromic management may result in a noteworthy number of infections being undiagnosed and untreated. [11] This poses a great public health risk, as untreated genital infections are associated with long-term genital and reproductive tract morbidity and proliferate the risk of human immunodeficiency virus (HIV) acquisition and transmission. [10] Genital tract infections should be viewed as dynamic infections that can occur alone or in combination with other infections. [12] The various causes of genital tract infections, including VVC and STIs have health implications and therefore, characterising pathogenic bacteria and yeasts in genital tract infections is key in providing appropriate patient/ disease management.

6.1 General summary of findings

The aim of this PhD was to describe the molecular epidemiology and treatment outcomes of vulvovaginal candidiasis in Namibian women by evaluating microbial aetiology, AMR patterns and treatment outcomes. The results from this PhD study (Chapter 3 and 4) were determined from a population presenting with vaginal discharge syndrome (VDS). The results from the cross-sectional study (Chapter 3) and the prospective cohort study (Chapter 4) confirmed a high rate of VVC (43% and 41% of women with VDS, respectively) in women, with *C. albicans* as the most common species (36% and 73% of all *Candida* infections, respectively), followed by *C. glabrata* (17% and 11%, respectively), a high rate of BV (39% and 43%, respectively), as well as a high rate of women with multiple infectious aetiologies of VDS (34% and 84%, respectively). Studies from neighbouring countries report a similar aetiology distribution of VDS with high rates of VVC, BV and large proportions of women with multiple aetiologies. [13] Although syndromic management is useful for multiple aetiologies, the addition of routine diagnostics, in particular antimicrobial susceptibility testing (AST), may reduce poor treatment outcomes in this population with a high burden of VVC and mixed genital infections. With regards to mixed infections, we found a significant association between the concurrent presence of VVC and *C. trachomatis*, where *C. trachomatis* was found to be less likely detected in women with VVC (Chapter 3). The importance between *C. albicans* and *C. trachomatis* infection is supported by studies that revealed interactions between these two organisms via the binding of elementary bodies of *C. trachomatis* to *C. albicans* yeast and hyphal forms. [14, 15]

High rates of azole resistance was detected among non-*albicans Candida* isolates, particularly *C. glabrata*, collected from both the national reference laboratory (14/19, 74%) (Chapter 3) and the Katutura Intermediate Hospital (4/5, 80%) (Chapter 4). The high prevalence of azole-resistant

Candida species observed in this study follows the trends reported in other studies in Africa. [16-19] Clotrimazole is the only antifungal recommended in the syndromic management guideline for VVC in Namibia, and is also recommended for persistent and recurrent infections. [9, 20] In the absence of availability and access to other antifungal drugs to treat VVC and currently lack of recommendation for AST, antifungal resistance can develop due to over-use of clotrimazole via selection pressure. [21] These findings highlight the importance of including diagnostic testing, in particular antifungal culture and susceptibility testing, to the national treatment guideline to prevent poor treatment outcomes.

Treatment failure among women with VDS was high, with one-third of women reporting either persistence (13%) or recurrence (18%) of symptoms one month after treatment. This cohort study is the first to describe VDS treatment outcomes in sub-Saharan Africa. Vulvovaginal candidiasis caused by untreated and by azole-resistant *Candida* strains was the main cause of treatment failure in our cohort (23%) (Chapter 4). Azole resistance is a cause for treatment failure of VVC, [22] Another cause for treatment failure was persistent or recurrent BV (31%), which has been observed in several studies. [7] The provision of information to women with VDS concerning possible persistence or recurrence of their symptoms, as well as vaginal smear microscopy for VVC and BV may help improve outcomes. Our data also suggest that an in-depth microbiological work-up for *Candida* species should be incorporated in management of VDS treatment failure in our setting.

A significant association was found between treatment failure and HIV positivity, where women living with HIV were prone to VDS treatment failure (OR=5.9, 95% CI 1.1–33, p=0.025) (Chapter 4). This could be attributed to altered susceptibility to VVC due to changes in the vaginal immune response brought about by highly active antiretroviral therapy. [8]

In previous studies, correlations have been described between *Candida glabrata* phenotypic antifungal resistance and genetic resistance mutations. [23] The present study is one of the first studies in Africa to offer a comprehensive genomic analysis of *C. glabrata* among women experiencing VVC. Whole genome sequencing analysis revealed several mutations in genes associated with azole resistance (Chapter 5), especially, the n(2)-Cys(6) transcription factor *(CgPDR1)* gene, in which multi-azole resistance has been found in gain-of-function mutations. [24, 25] No gain-of-function mutations were detected despite presence of phenotypic resistance for fluconazole and itraconazole. Phenotypic resistance to amphotericin B and anidulafungin was not observed (Chapter 5). However, a non-synonymous mutation in the *ERG6* gene, which led to the

amino acid substitution, Arg48Lys, was associated with amphotericin B, was detected and was previously reported. [26] Furthermore, *FKS*, *ERG6* and *CgFPS1* mutations, albeit not associated with high-level resistance phenotypes, were detected in the present study. The syndromic algorithm does not include echinocandins, polyenes, nor 5-fluorocytosine for the treatment of VVC, which may explain the absence of phenotypic resistance as exposure to these drugs is the main driver of acquired resistance. [3, 27] The emergence of multidrug resistance in *C. glabrata* has been reported in several studies. [27, 28] In this study, the known mutation, Val239Leu, which is associated with hypermutability, was absent in isolates harbouring mutations in the *MSH2* gene. [26] This explains the lack of multidrug-resistant isolates despite the *MSH2* gene mutation. [29] The observed phenotypic azole resistance of *C. glabrata*, as well as mutations in genes associated with antifungal resistance, warrants further exploration on AMR in Namibia. A systematic review reported a high clotrimazole efficacy rate for VVC in the general population. However, the same review reported that standard treatment strategies, including clotrimazole, are not effective against non-albicans *Candida* infections, including *C. glabrata* .[30] Therefore, high rates of azole resistance, particular in *C. glabrata* VVC, may implore improvements to the syndromic management guidelines by the introduction of laboratory diagnoses for species identification and AST.

The present study is one of two studies in Africa to report distinct *C. glabrata* strains circulating in Namibia based on the novel sequence types (STs) that were identified (Chapter 5). Eight distinct STs, including four novel STs, were identified with ST15 and ST55 as the most common (38% and 19% of twenty-one isolates, respectively). Numerous studies conducted globally also reported these STs. [21, 28, 31, 32] This study and another in Tanzania reported the only ST114 among *C. glabrata* in sub-Saharan Africa. [33] However, further studies are recommended. Mutations in *CgPDR1* were mostly observed in ST55. This is in line with reports of ST55 recovered as fluconazole-resistant isolates from bloodstream *C. glabrata* infections in South Korea. [34] The identification of novel *C. glabrata* STs accentuates the significance of including *C. glabrata* strains from resource-constraint settings when exploring novel and alternative treatment programs or antifungals as *C. glabrata* strains could have dissimilar genetic resistance mutations from *C. glabrata* strains from well-resourced settings.

There were a few limitations noted in the present PhD study. These limitations include a small sample size for the prospective cohort (Chapter 4) and for WGS analysis (Chapter 5). Deployment and recruitment of eligible participants was challenging resulting of the Coronavirus disease

(COVID-19) epidemic and associated movement and health restrictions, reflecting the low number of participants that were recruited and returned for a follow-up in the Namibian public health setting. Furthermore, limited demographic and clinical information was obtainable from study participants in the cross-sectional study (Chapter 3). Regardless of the low number of participants, the current study provided a thorough overview of VVC in Namibian women, with an alarming message of high prevalence of VVC, and high rates of VDS treatment failure due to untreated and drug resistant VVC. However, it should be noted that due to low numbers of participants that returned for a follow-up visit, the actual contribution of untreated and drug resistanct VVC to treatment should be explored further,

6.2 Perspectives on improving syndromic management of VVC in Namibia

Vulvovaginal candidiasis is a common global problem and one of the most common causes of genital infection in women of reproductive age. [35] Despite this burden, gaps in disease surveillance, the absence of routine diagnostics, and a lack of comprehensive epidemiological and microbiological research of genital infections have led to limited data on epidemiology *Candida* strains circulating in Africa and their mechanisms of resistance. [36]

The arrival of WGS has provided a powerful tool in determining the current burden and underlying molecular mechanisms of AMR in VVC. [21] However, WGS studies for *Candida* antimicrobial resistance surveillance prove to be challenging in resource-limited settings due to lack of financial resources and the absence of routine laboratory diagnostic infrastructure.

In the present study, a comprehensive WGS analysis of *C. glabrata* among women with azole-resistant VVC was conducted. Vulvovaginal candidiasis and azole resistance were highly prevalent in this particular population. There is an urgent need to improve health services targeting female genital and sexual health, including VVC, STIs and other genital tract infections.

This can be achieved via cost-effective strategies including reinforcing national service delivery of female reproductive health care; strengthening adherence to national standard treatment guidelines by healthcare professionals; strengthening aetiological and antimicrobial surveillance systems; initiating of routine diagnostics for VVC and STI causal management; and introducing phenotypic resistance guided treatment in populations prone to treatment failure. This can be achieved by developing standardized diagnostic protocols for detection and phenotypic resistance of VVC and common STIs, implementing routine screening programs for women at higher risk, improving guidelines for the management of VDS, educating healthcare providers on these

guidelines and establishing a system for continuous monitoring and surveillance of resistance pattern of *Candida* and STIs.

In Namibia, the syndromic management approach for infections causing VDS has been implemented for over two decades. [9] However, its efficacy on improving treatment outcomes has never been assessed. A South African study reported neither an improvement nor decline in *C. trachomatis* and *N. gonorrhoeae* infections over the past 30 years. [37] However, a decline in syphilis was reported. [37] The benefit of the syndromic approach is the delivery of same-day treatment for patients using treatment algorithms that are cheap and user-friendly for healthcare workers to appreciate and implement. [38] However, there are some challenges to the implementation of this approach including excessive use of antimicrobials, poor antimicrobial stewardship and sustained transmission of infections causing VDS due to untreated asymptomatic infections. [10, 39, 40] Additionally, noncompliance to treatment guidelines by healthcare workers may also lead to poor treatment outcomes. [41] A study conducted in South Africa reported on the poor implementation and adherence of STI guidelines. [42] In this study the treatment failure was noteworthy and therefore, the continual monitoring of the syndromic management of infections causing VDS should be prioritised as part of national healthcare agendas to improve treatment outcomes. Key areas for monitoring include follow-up examinations to track treatment progress and address any concerns or new symptoms that may arise; laboratory testing to determine the cause and antimicrobial susceptibility of the infection; and providing patients with information regarding the expected duration of treatment, potential side effects, the importance of completing the full course of prescribed medications, safe sexual practices to prevent reinfection and transmission of STIs. Laboratory diagnostics are fundamental in detecting the *Candida* species, STI pathogens, BV pathobionts and describing AMR strains in patients experiencing treatment failure. Repeat testing, including antimicrobial resistance testing, should be considered after treatment completion to confirm the eradication of the causative organism, especially for persistant or recurrent cases. Hence, implementation of routine laboratory diagnostics in our settings to guide treatment and monitor treatment outcomes in patients experiencing VDS is necessary and would necessitate a concerted effort among stakeholders involved policy-making and resource allocation. These may be achieved by identifying the key stakeholders involved in women's reproductive health; organizing stakeholder consultation sessions to gather input on current challenges, opportunities, and priorities in managing infections; establishing a multidiscsiplinary task force to collaborate on developing and implementing strategies to improve policies, infrastructure and procedures; presenting evidence-based practices and research findings to stakeholders, highlighting the impact

of improved infrastructures, policies and procedures on patient outcomes; and demonstrating cost-effectiveness and long-term cost savings of investing in preventative measures, early detection and appropriate treatment strategies. A study comparing targeted point-of-care testing with syndromic management has demonstrated a substantial reduction in the prevalence of curable symptomatic STIs by introducing diagnostic testing to support the syndromic management for STIs. [43] Additionally, the deployment of routine molecular laboratory diagnostics in both public and private healthcare divisions would also afford much sought after data for improving surveillance programmes. An abundance of molecular diagnostics assays are available for detection of VVC, curable STIs and BV. Some include the Cepheid GeneXpert® CT/NG, the Xpert® TV, the AllplexTM STI/BV and the AllplexTM Candidiasis Panel Assays, [44] With the prevailing molecular diagnostics infrastructure already established for rapid detection of *Mycobacterium tuberculosis* and COVID-19 in Namibia, these can be utilised to deploy further routine molecular diagnostics for VVC, STIs and BV. [45] Regardless of the countless options available for molecular based testing to detect the pathogens causing VDS, microscopy facilities for VVC and BV are readily available and are cost-effective. [46] The implementation of routine laboratory diagnostics will further assist antimicrobial stewardship in a clinical setting via targeted therapeutic management and therefore the reduction of the over prescription of antimicrobials for unsubstantiated aetiologies. [47] Monitoring the local AMR trends via AMR surveillance systems to detect emerging resistant strains can offer evidence for amendment of the national standard management guidelines for urogenital infections, including VVC and STIs. This is necessary to address the burden of AMR among VVC and STIs.

In Namibia, AMR surveillance is spearheaded by the Namibian Antimicrobial Resistance National Action Plan in the context of *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp. (ESKAPE) pathogens [48] and the WHO Global Antimicrobial Surveillance Program (GLASS) for AMR surveillance in the context of *Candida* spp. bloodstream infections and Enhanced Gonococcal Antimicrobial Surveillance Programme (EGASP) [47], thereby excluding organisms or infections, such as VVC, that may be underestimated. Regardless, Namibia's surveillance capacity for resistant pathogens needs reinforcement as reports on AMR from Namibia are also limited and the resistance trends of many pathogenic isolates from different specimen types, including urogenital specimens, have not been described. [48]

Candida AMR has developed to clotrimazole and fluconazole, the current first line therapeutic drugs. [49] In Namibia, no second-line therapy is included in the national standard treatment guideline for VVC. Therefore, one of the crucial approaches in the conservation of effective use of this antifungal is the implementation of suitable antimicrobial stewardship programmes, including the identification of aetiological *Candida* species and AST to ensure the appropriate treatment and reduction of the over-use of azole antifungals. [50] The development of bedside molecular diagnostics assays to provide resistance-guided therapy for *Candida* may be useful in reducing the consumption of azole antifungals. Local boric acid and topical nystatin have been shown highly efficacious for non- *albicans Candida* strains and RVVC, is generally well tolerated by patients and is economical. [51, 52] Therefore, including local boric acid and topical nystatin in Namibia is feasible.

6.3 Future perspectives

Vulvovaginal candidiasis causes substantial morbidity worldwide if treatment outcomes are poor. [51] Because of the growing incidence of drug resistance in *Candida* infection, in particular persistent and recurrent infections, there is great interest in establishing alternative treatment strategies. [53] The recent emergence and increased prevalence of multidrug resistant fungal species has propelled research into novel treatments. [54] Increased knowledge about immune responses and genetic testing has opened the doors to recognise those at greatest risk and in need to alternative therapeutic management schemes. [55, 56] However, VVC has largely been neglected as a public health priority in light of the emergence of HIV, STIs and other fungal infections with high mortality rates including cryptococcal meningitis and hospital-acquired *Candida auris* infection. [55] Global and local resources for VVC management needs to be mobilised to improve treatment outcomes.

To adequately manage VVC, new approaches, including prevention, laboratory diagnostics and therapeutic management, are necessary. In the interim, the introduction of routine laboratory diagnostics in designated populations, such as those predisposed to complicated VVC, is warranted in Namibia to manage the burden of VVC. There are numerous impediments, however, that need to be addressed to effectually deploy routine diagnostics in VVC management, including the availability of funding, the financial feasibility, infrastructural considerations, laboratory operations and the enhanced training of laboratory staff. A concerted effort from policy makers and healthcare professionals are therefore warranted. Prospectively, it is essential to spearhead the development and implementation of accurate point-of-care tests (POCTs), that include pathogen

detection AST at the primary healthcare centres to direct the appropriate treatment and management of VVC. By this means, the use of inappropriate antifungals and the onward transmission of antifungal resistant strains will decrease.

To combat the emergence of resistant *Candida* strains non-antimicrobial therapeutic interventions and vaccines are necessary; the development pipeline currently has several promising options.

In a study by Izbu and colleagues, the novel therapy, liposomal lyophilised powder formulation of panomycocin, was developed and found to be effective against VVC. [57] It showed stability at vaginal pH and is secreted by the yeast *Wickerhamomyces anomalus* and hydrolyses the exo-β-1, 3-glucans, which are essential polymers for fungal cell wall integrity, causing cell wall disruption and targeted cell death. [57] Some studies have evaluated *Candida* virulence factors, such as phospholipase and protease production to develop alternative treatment intervention. [56] Furthermore, numerous studies propose that targeting the nod-like receptor protein 3 (NLRP3) inflammasome during VVC may be a viable therapeutic alternative for disease management as the NLRP3 inflammasome contributes to the clinical immunopathology of VVC. [58] Immunotherapy with recombinant cytokine interleukin-17 (IL-17) or IL-22 have also been suggested for the development of new drugs due to their importance in antifungal immune response. [57] The use of medicinal plant formulations has also increased in traction because of its potential health benefits. [59] An example includes liquorice extract that has demonstrated promising anti-*Candida* activity by inducing cytoplasmic damage. [59]

Because of the nature of vaginal infections, it is also important to also investigate drugs against mixed vaginal infections including BV and trichomonal vaginitis. A nifuratel-nystatin combination for the treatment of mixed vaginal infections has been found to be active against these infections as recorded by Obiero *et al.* [12] Boric acid has historically been used in several regimes for non-*albicans* VVC. [60] A study by Marrazzo *et al.* (2019) investigated the effectiveness of new boric acid anti-VVC treatments with improved anti-biofilm activity, called TOL-463, and found it to be effective to treating VVC, especially in vaginal insert form. [60] In addition, this drug has also shown effectively against recurrent BV. [61]

Historically, numerous anti-*Candida* vaccines with different modalities have been proposed but only a few of them have been tested in clinical trials, due to the genomic variation and phenotypic plasticity across *Candida* strains and species, and its difficulty in mounting protective immunity

in immunocompromised or immunosuppressed individuals. [61] Therefore, further research and development efforts are required to develop an effective vaccine for VVC.

In conclusion, the findings highlighted in this project include a high prevalence of VVC in women with VDS; a high frequency of non-*albicans Candida*, particularly *C. glabrata*, that are resistant to azole antifungals; a noteworthy occurrence of treatment failure after syndromic management of VDS, particularly in those with VVC; and a broad spectrum of SNPs in genes associated with resistance to antifungal drugs. The findings of this project proposes the inclusion of diagnostics to the case management of VVC to improve treatment outcomes.

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APPENDIX A Ethical approval certificates

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

Institution: The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IORG #: IORG0001762 OMB No. 0990-0279 Approved for use through February 28, 2022 and Expires: 03/04/2023.

13 August 2020

Approval Certificate
New Application

Ethics Reference No.: 518/2020
Title: Molecular epidemiology and treatment outcomes of vulvovaginal candidiasis in Namibian women

Dear Mrs CM Dunaiski

The **New Application** as supported by documents received between 2020-07-27 and 2020-08-12 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2020-08-12 as resolved by its quorate meeting.

Please note the following about your ethics approval:

- Ethics Approval is valid for 1 year and needs to be renewed annually by 2021-08-13.
- Please remember to use your protocol number (518/2020) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers
MBChB MMed (Int) MPharmMed PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health)

Research Ethics Committee
Room 4-60, Level 4, Tswelopele Building
University of Pretoria, Private Bag x323
Gezina 0031, South Africa
Tel +27 (0)12 356 3084
Email: deepeka.behari@up.ac.za
www.up.ac.za

Fakulteit Gesondheidswetenskappe
Lefapha la Disaense tša Maphelo

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

Institution: The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IORG #: IORG0001762 OMB No. 0990-0279 Approved for use through February 28, 2022 and Expires: 03/04/2023.

Faculty of Health Sciences Research Ethics Committee

30 July 2021

Approval Certificate
Annual Renewal

Dear Mrs CM Dunaiski

Ethics Reference No.: 518/2020
Title: Molecular epidemiology and treatment outcomes of vulvovaginal candidiasis in Namibian women

The **Annual Renewal** as supported by documents received between 2021-06-29 and 2021-07-28 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2021-07-28 as resolved by its quorate meeting.

Please note the following about your ethics approval:

- Renewal of ethics approval is valid for 1 year, subsequent annual renewal will become due on 2022-07-30.
- Please remember to use your protocol number (518/2020) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

On behalf of the FHS REC, Dr R Sommers
MBChB, MMed (Int), MPharmMed, PhD
Deputy Chairperson *of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria*

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health)

Research Ethics Committee
Room 4-60, Level 4, Tswelopele Building
University of Pretoria, Private Bag x323
Gezina 0031, South Africa
Tel +27 (0)12 356 3084
Email: deepeka.behari@up.ac.za
www.up.ac.za

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UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

Institution: The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 18 March 2022 and Expires 18 March 2027.
- IORG #: IORG0001762 OMB No. 0990-0278 Approved for use through August 31, 2023.

Faculty of Health Sciences **Research Ethics Committee**

14 July 2022

Approval Certificate
Annual Renewal

Dear Mrs CM Dunaiski,

Ethics Reference No.: 518/2020 – Line 2
Title: Molecular epidemiology and treatment outcomes of vulvovaginal candidiasis in Namibian women

The **Annual Renewal** as supported by documents received between 2022-06-14 and 2022-07-13 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2022-07-13 as resolved by its quorate meeting.

Please note the following about your ethics approval:

- Renewal of ethics approval is valid for 1 year, subsequent annual renewal will become due on 2023-07-14.
- Please remember to use your protocol number (518/2020) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

On behalf of the FHS REC, Dr R Sommers
MBChB, MMed (Int), MPharmMed, PhD
Deputy Chairperson *of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria*

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health)

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Faculty of Health Sciences

Faculty of Health Sciences **Research Ethics Committee**

20 June 2023

Approval Certificate
Annual Renewal

Dear Mrs CM Dunaiski,

Ethics Reference No.: 518/2020 – Line 3
Title: Molecular epidemiology and treatment outcomes of vulvovaginal candidiasis in Namibian women

The **Annual Renewal** as supported by documents received between 2023-05-23 and 2023-06-14 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2023-06-14 as resolved by its quorate meeting.

Please note the following about your ethics approval:

- Renewal of ethics approval is valid for 1 year, subsequent annual renewal will become due on 2024-06-20.
- Please remember to use your protocol number (518/2020) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

On behalf of the FHS REC, Professor C Kotzé
MBChB, DMH, MMed(Psych), FCPsych, Phd
***Acting Chairperson**: Faculty of Health Sciences Research Ethics Committee*

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health)

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APPENDIX B Data collection forms

The Molecular Epidemiology and Treatment Outcomes of Vulvovaginal Candidiasis in Namibian Women

Participant code _____

Date _____

Instructions

Thank you for participating in this study. We would like to ask you to complete this questionnaire. Please answer the questions by ticking the appropriate option and writing where indicated. Please answer the questions as accurately and honestly as possible. Feel free to skip any questions you are not comfortable with—it is better for us to have no information than incorrect information. Please do not hesitate to ask the researcher if you need clarification of specific question(s).

Part 1: Demographical Information

***Your age today (write in print) _____**

To which ethnic group do you belong?

Oshiwambo		Damara		Coloured	
Oshiherero		Kavango		Baster	
Nama		Caprivian		San	
White		Other (write in print):			

How do you consider your marital status?

Single		Co-habiting		Married		Divorced		Widow/er	

In which city/ town do you live? (write in print)

City/town	
Region	

Which level of school did you complete?

None		Primary		Secondary		Tertiary	

Are you currently employed?

Unemployed		Student		Employed		Self-employed	

Part 2: Behavioural Information

Are you currently pregnant?

*Yes		No		Unsure	

Which of the following methods of contraception are you currently using to prevent pregnancy? (tick multiple if applicable)

Oral contraceptives		IUCD		Injections		Implant	

Do you do any vaginal douching? If so, when last?

Yes < week ago		Yes < month ago		No recent douching	

Which products did you use to douche? (Write in print)

How many sexual partner(s) do you currently have? (Write number in print) _____

Have you have any new sexual partner (s) in the past week/ month?

Yes		No	

When was your last sexual intercourse?

< 1 day ago		< 1 week ago		<1 month ago		< 1 year ago	

Did you use a condom the last time that you had sex?

None		Male condom		Female condom		Both male & female condoms	

In the past 6 months, have you engaged in any of the following (tick multiple if applicable):

Vaginal sex		Oral sex		Anal sex	

Part 3: Clinical History

When last did you use antibiotics?

Present		< 1 week ago		< 1 month ago		> 1 month		Never	

Do you recall which antibiotics you used? (Write in print)

Why did the doctor prescribe the antibiotics? (Write in print)

When last did you use of over-the-counter antifungals?

Present		< 1 week ago		< 1 month ago		> 1 month		Never	

Do you know your HIV status?

Yes, it's Positive		Yes, it's Negative		Unknown	
Known, but unwilling to disclose					

Are you currently taking antiretrovirals?

Yes		No	

When was your last Pap smear?

< 1 day ago		< 1 week ago		<1 month ago		< 1 year ago		Never	

Do you have any of the following diseases?

Diabetes mellitus		Rheumatoid arthritis		Cancer		High blood pressure	
Tuberculosis							

Other (write in print):

+Part 4 : Gynaecological History , Clinical Examination and Treatment Interventions

What is the reason for visit today? (Write in print)

Have you experienced vaginal discharge, itching, burning and painful sexual intercourse before?

Yes		No	

How many times this year? (Write number in print): _____

Did you receive treatment for those symptoms?

Yes		No	

Which treatment did you receive? (Write in print)

Have you been treated for STI this past year?

Yes		No	

If so, how many times? (Write number in print): _____

Symptoms as reported by participant:

Do you have a vaginal discharge? If so, how long has this been?

Yes		No	

If so, how long has this been?

1 week		2weeks		3 weeks		1 month		> a month	

What is the colour of the vaginal discharge?

Clear		White		Yellow		Yellow-green	

What is the consistency of vaginal discharge?

Watery		Mucoid		Diffuse		Curd-like	

Does your vaginal discharge smell like any of the following?

Normal		Fishy		Rotten	

Other (write in print):_____

Does your vagina itch?

Yes		No	

If so, how long has this been going on?

1 week		2weeks		3 weeks		1 month		> a month	

Does your vagina burn when you urinate?

Yes		No	

If so, for how long has it been?

1 week		2weeks		3 weeks		1 month		> a month	

Do you experience pain during sexual intercourse?

Yes		No	

If so, for how long has it been?

1 week		2weeks		3 weeks		1 month		> a month	

Do you experience vaginal bleeding during or after sexual intercourse? If so, for how long has it been?

Yes		No	

If so, for how long has it been?

1 week		2weeks		3 weeks		1 month		> a month	

Do you experience lower abdominal pain related to sex?

Yes		No	

If so, for how long has it been?

1 week		2weeks		3 weeks		1 month		> a month	

Have any of the symptoms happened again (recurred)?

Yes		No	

Number of recurrences/ year (write number in print): _____

Clinical symptoms and signs as per healthcare worker's observations:

Vaginal discharge

Yes		No	

Colour of vaginal discharge

Clear		White		Yellow		Yellow-green	

Consistency of vaginal discharge

Watery		Mucoid		Diffuse		Curd-like	

Odour of vaginal discharge

Normal		Fishy		Rotten	

Other (write in print): _____

Inflammation

No inflammation		Redness		Swelling		Erosions	

<u>Additional observations (additional investigation):</u>

Vaginal fluid pH

≤ 4.5		4.5-7.0		7.0-10	

Therapeutic Intervention

Treatment prescribed

*Exclusion criteria: women below the age of 18 years; women that are pregnant, menstruating, on any previous antimicrobial treatment for any indication less than one week prior to recruitment in the study.

+ Extracted from clinical records

$ To be recorded on return visits and telephonic communication

APPENDIX C Laboratory procedures

Protocol for DNA extraction from vaginal swabs

1. Place swab tip in up to 1000 µl of water or isotonic buffer (e.g., PBS) until well suspended.
2. Remove the tip and 200 µl of the suspension to a ZR BashingBead™ Lysis Tube.
3. Add 750 µl BashingBead™ Buffer to the tube.
   NOTE: Cap tube tightly to prevent leakage.
4. Secure in a bead beater fitted with a 2 ml tube holder assembly and process at maximum speed for ≥ 5 minutes.
   Note: Required processing time will vary depending on the device and application and therefore should be evaluated on a case by case basis.
5. Centrifuge the ZR BashingBead™ Lysis Tube (0.1 & 0.5 mm) in a microcentrifuge at 10 000 RCF (7750 RPM) for 1 minute.
6. Transfer up to 400 µl supernatant to a Zymo-Spin™ III-F Filter in a Collection Tube and centrifuge at 8000 RCF (6000 RPM) for 1 minute. Discard the filter.
7. Add 1,200 µl of Genomic Lysis Buffer to the filtrate in the Collection Tube from Step 6.
8. Transfer 800 µl of the mixture from Step 5 to a Zymo-Spin™ IICR Column in a Collection Tube and centrifuge at 10 000 RCF for 1 minute.
9. Discard the flow through from the Collection Tube and repeat Step 8.
10. Place the Zymo-Spin™ IICR Column in a new Collection Tube.
11. Add 200 µl DNA Pre-Wash Buffer to the Zymo-Spin™ IICR Column and centrifuge at 10 000 RCF for 1 minute.
12. Add 500 µl g-DNA Wash Buffer to the Zymo-Spin™ IICR Column and centrifuge at 10 000 RCF for 1 minute.
13. Transfer the Zymo-Spin™ IICR Column to a clean 1.5 ml microcentrifuge tube and add 100 µl (35 µl minimum) DNA Elution Buffer directly to the column matrix.
14. Centrifuge at 10 000 RCF for 30 seconds to elute the DNA.
15. Duplicate the DNA extract, by aliquoting 50 µl of the eluted DNA into another clean 0.5 mL tube. **Ultra-pure DNA is now ready for use in your experiments**

Protocol for DNA extraction from yeast culture

1. Suspend approximately 1 yeast colony (≈10^8 fungal cells) that have been resuspended in up to 200 µl of water or isotonic buffer (e.g., PBS).
2. Add the suspension to a ZR BashingBead™ Lysis Tube.
3. Add 750 µl BashingBead™ Buffer to the tube.
   NOTE: Cap tube tightly to prevent leakage.
4. Secure in a bead beater fitted with a 2 ml tube holder assembly and process at maximum speed for ≥ 5 minutes.
   Note: Required processing time will vary depending on the device and application and therefore should be evaluated on a case by case basis.
5. Centrifuge the ZR BashingBead™ Lysis Tube (0.1 & 0.5 mm) in a microcentrifuge at 10 000 RCF (7750 RPM) for 1 minute.
6. Transfer up to 400 µl supernatant to a Zymo-Spin™ III-F Filter in a Collection Tube and centrifuge at 8000 RCF (6000 RPM) for 1 minute. Discard the filter.
7. Add 1,200 µl of Genomic Lysis Buffer to the filtrate in the Collection Tube from Step 6.
8. Transfer 800 µl of the mixture from Step 5 to a Zymo-Spin™ IICR Column in a Collection Tube and centrifuge at 10 000 RCF for 1 minute.
9. Discard the flow through from the Collection Tube and repeat Step 8.
10. Place the Zymo-Spin™ IICR Column in a new Collection Tube.
11. Add 200 µl DNA Pre-Wash Buffer to the Zymo-Spin™ IICR Column and centrifuge at 10 000 RCF for 1 minute.
12. Add 500 µl g-DNA Wash Buffer to the Zymo-Spin™ IICR Column and centrifuge at 10 000 RCF for 1 minute.
13. Transfer the Zymo-Spin™ IICR Column to a clean 1.5 ml microcentrifuge tube and add 100 µl (35 µl minimum) DNA Elution Buffer directly to the column matrix.
14. Centrifuge at 10 000 RCF for 30 seconds to elute the DNA.
15. Duplicate the DNA extract, by aliquoting 50 µl of the eluted DNA into another clean 0.5 mL tube. **Ultra-pure DNA is now ready for use in your experiments**

Procedure for Nugent score

Perform Nugent score according to Nugent, R. P. (1991) [1].

1. Prepare a vaginal smear by rolling a vaginal swab onto a glass slide.
2. Heat fix the smear by passing it through a flame.
3. Perform a Gram stain [2]:
    - Flood the slide for 1 minute with 0.5% crystal violet,
    - Tilt the slide, and rinse slide gently with water,
    - Flood the slide with (1%) Lugol's iodine for 1 minute,
    - Tilt the slide and wash off the iodine with water,
    - Decolourise with acetone until colour ceases to run out of the smear,
    - Rinse with water,
    - Flood the slide with 0.1% counterstain carbol fuchsin/ safranin for 30 seconds to 1 minute,
    - Rinse with water,
    - Remove excess water and leave to air dry.
4. Evaluate each Gram-stained smear for the following morphotypes under oil immersion (x1, 000 magnification):
    - Large gram-positive rods (*Lactobacillus* morphotypes),
    - Small gram-variable rods (*G. vaginalis* morphotypes),
    - Small gram negative rods (*Bacteroides* spp. morphotypes),
    - Curved gram variable rods (*Mobiluncus* spp. morphotypes),
    - Gram positive cocci.
5. Quantitate each morphotype according to the table below:

Table 1. Nugent scoring system for Gram stained vaginal smears

Organism morphotype	**Description**	**Number/ oil immersion field**	**Score**
Lactobacillus morphotypes	Parallel sided, gram positive bacilli	>30	0
		5-30	1
		1-4	2
		<1	3
		0	4
Mobiluncus morphotypes	Curved, gram negative rods	>5	2
		<1-4	1
		0	0
Gardnerella/ Bacteroides morphotypes	Tiny, gram variable coccobacilli and pleomorphic rods with vacuoles	>30	4
		5-30	3
		1-4	2
		<1	1
		0	0

Total score: 0-3= Normal; 4-6= Intermediate; 7-10= Bacterial vaginosis

Protocol for *Candida* PCR

Protocol for Multiplex PCR for *Candida* species identification was adopted from Rad and colleagues (2012) [3].

Primers

Primers were ordered at Inqaba Biotechnology (Table 1).

Table 1. Primers for PCR amplification and detection *Candida* species

Primers	**Sequence**
ITS1	5-TCCGTAGGTGAACCTGCGG-3
ITS2	5-GCTGCGTTCTTCATCGATGC-3
CA3	5-GGTTTGCTTGAAAGACGGTAG-3
CA4	5-AGTTTGAAGATATACGTGGTAG-3

Lyophilised primers were dissolved and diluted in PCR grade water (Inqaba Biotechnology). Primers were diluted to 100 µM stock solution according to manufacturer's instructions (Table 2). Thereafter, prepared stock solutions were stored until use at −20°.

Table 2. Quantity of PCR grade water to make 100 µM stock solution

Primers	**Quantity of PCR grade water to make 100 µM stock solution (µL)**
ITS1	364.21 µL
ITS2	267.58 µL
CA3	263.84 µL
CA4	289.63 µL

The primers should be diluted to 0.16 µM using PCR grade water prior to PCR amplification.

Preparation of reaction mixture

The Mastermix, primers and purified DNA were removed from the −20°C freezer and allowed to thaw at room temperature. After all the reagents were completely thawed, each tube was gently vortexed to mix and thereafter briefly centrifuged to allow all precipitants to be resuspended.

Thereafter, the reaction mixture was assembled on ice in PCR tubes as per Table 4.3. Between samples the tips were changed to prevent contamination.

Table 3. Reaction mixture

Reagent	Volume (µL)	Concentration
Water	15 µL	
Primer	2µL each	0.16 µM each
PCR Mastermix	25 µL	
Purified DNA	2 µL	
	50 µL	

PCR amplification process

PCR amplification process was carried out with a BIORAD T100 thermal cycler under the following conditions:

- Initial denaturation (94°C, 3 min);
- 35 cycles of denaturation (94°C, 1 min),
- Annealing (60°C, 1 min),
- Extension (72°C, 1 min);
- Final extension (72°C, 5 min).

PCR product analysis

Five µL PCR products were analyzed by electrophoresis through a 2% agarose gel containing ethidium bromide (0.5µg/mL), and UV visualization were performed according to the protocols provided (UVdoc, GAS9000, England). Positive controls were included in each PCR experiment and consisted of one strain of each *C. albicans* ATCC14053 (218 or 219, and 110 bp), *C. glabrata* CBS2175 (482 or 483 bp), *C. parapsilosis* CBS2195 (229 bp), *C. tropicalis* CBS94 (218 bp), and *C. krusei* CBS573 (182 bp). Pyrogen-free water was used as negative control. Species identification was possible by comparison with a 50-bp DNA ladder.

Protocol for Real Time–Polymerase chain reaction for *T. vaginalis*

Protocol for real-time PCR for *T. vaginalis* detection was adopted from Pillay and colleagues [4] and the LightCycler® 480 Probes Master Instructions for Use (version 11).

General Required Materials

Standard Laboratory Equipment

- LightCycler® 480 Instrument II
- Microwell Plate Centrifuge
- Microcentrifuge
- Micropipettes (P10 or P20, P200, P1000 or equivalent)

Disposables

- Nuclease-free, aerosol-resistant pipette tips
- 1.5 mL microcentrifuge tubes
- LightCycler® 480 Multi-well Plate 96-well – White
- LightCycler® 480 Sealing Film

Reagents

- FastStart TaqMan® Probe Master or LightCycler®480 Probes Master,2x concentration (red cap)
- LightCycler®480 Probes Master, Water, PCR Grade (colourless cap)
- Primers and probes (see below)

Sample Materials

- Template DNA, up to 500 ng complex genomic DNA

Preparation of stock solution for primers and probes (Clean room)

Primers and probe sequences are obtained from Pillay and colleagues (2007) (Table 1). Oligonucleotides were synthesised by Inqaba Biotechnology.

Table 1. Primers for real-time PCR amplification for detection of *Trichomonas vaginalis*

Primer/ probe	Primer/ probe name	Sequence
Primers	F-TV001	5′-A AAG ATG GGT GTT TTA AGC TAG ATA AGG-3′
	R-TV002	5′-T CTG TGC CGT CTT CAA GTA TGC-3’
Probe	TV003P	5′-Cy5-AG TTC ATG TCC TCT CCA AGC GTA AGT-BMN-Q620/BMN-Q650-3′

1. Dissolve lyophilised primers and probes in PCR grade water (Promega) to prepare a 100 µM stock solution according to manufacturer’s instructions.
2. Store prepared stock solutions until use at −20°C.

Preparation of the 10x primer-probe mix (Clean room)

Prepare the 10x primer-probe mix prior to PCR amplification as per Table 2:

Table 2. Preparation of 10x primer-probe mix

Reagent	Volume (µL)	Final concentration
Forward primer	25 µL	5 µM
Reverse primer	25 µL	5 µM
Probe	7.5 µL	1.5 µM
Water, PCR grade	442.5 µL	-
Total volume	**500 µL**	-

Calculation: $V_1 = \frac{C_2 V_2}{C_1}$

Primer: $V_1 = \frac{(5)(500)}{100} = 25\ \mu L$ Probe: $V_1 = \frac{(1.5)(500)}{100} = 7.5\ \mu L$

Preparation of PCR reaction mixture (Clean room)

Prepare the PCR reaction mixture according to manufacturer’s instructions (LightCycler® 480 Probes Master Instructions for Use, version 11).

1. Remove the 2x LightCycler® 480 Probes Master mix (red cap), the 10x primer-probe mix and PCR-grade water (colourless cap) from the −20°C freezer and were allowed to thaw on ice. After all the reagents were completely thawed, each tube was briefly spun in a microcentrifuge.

2. Prepare the PCR reaction mixture in a 1.5 mL reaction tube as per Table 3. Before transferring the reagents into a tube, gently pipette up and down to allow all precipitants to be resuspended. When mixing each reagent, mix carefully by pipetting up and down.

Table 3. Preparation of the PCR reaction mixture

Reagent	**Final concentration**	**Volume per reaction (µL)**	**Volume for z[a] reactions (µL)**
LightCycler®480 Probes Master (2x concentration, red cap)	1x	10	10 x z
10x primer-probe mix	Primers: 0.5 µM each Probe: 0.15 µM	2	2 x z
Water, PCR grade (colourless cap)	-	3	3 x z
Total volume	-	15	15 x z

[a] z = the number of reactions to be run + two additional reactions (extra amount for preventing shortage of reagents)

3. Pipette 15 µl of the PCR mixture into each well of the LightCycler® 480 Multiwell Plate.
4. Add 5 µl PCR-grade water instead of a template DNA in a negative control well. Mix by pipetting up and down.

※When loading PCR mixture/mixing water in the wells, make sure not to cause air bubbles. Air bubbles may interrupt with PCR reactions and cause inaccurate results.

Addition of template DNA (DNA workstation-PFGE lab)

1. Add 5 µl of the DNA template into each well of the LightCycler® 480 Multiwell Plate. Mix by pipetting up and down.

※When mixing the DNA in the wells, make sure not to cause air bubbles. Air bubbles may interrupt with PCR reactions and cause inaccurate results.

2. Seal the plate with the LightCycler® 480 Sealing Film.

PCR amplification (PCR/Gel electrophoresis lab)

PCR amplification is carried out on the LightCycler 480 II instrument (Roche Diagnostics). It is recommended to program the LightCycler® 480 Instrument before preparing the PCR reaction mixture.

Programming of the instrument

1. Switch on the instrument (main switch is at the back of the instrument) and wait until the instrument has initialised and is ready (left LED becomes green).
2. Switch on the computer and sign in (ID: operator; PW: LC480).
3. Double-click on the icon of the LightCycler 480 software.
4. Sign in with the user login details.
5. Click “New Experiment” from the Overview window.
6. Choose the detection format from a pull-down menu.
7. Select a reaction volume.
8. Program the PCR cycling conditions as per Table 4.

Table 4. Setup of the LightCycler® 480 System PCR experiment

Setup					
Block type				Reaction volume (µL)	
96				20	
Detection format	Excitation filter			Emission filter	
Monocolour hydrolysis probe: Cy5	618			660	
Programs					
Program name	Cycles			Analysis Mode	
Pre-incubation	1			None	
Amplification	50			Quantification	
Cooling	1			None	
Temperature targets					
Pre-incubation					
Target (°C)	Acquisition Mode	Hold [hh:mm:ss]	Ramp Rate [°C/s]	Acquisition [per °C]	
95	None	00:10:00	4.4	–	

Table 4. Setup of the LightCycler® 480 System PCR experiment *(continue)*

Amplification				
Target (°C)	Acquisition Mode	Hold [hh:mm:ss]	Ramp Rate [°C/s]	Acquisition [per °C]
95	None	00:00:20	4.4	–
60	Single	00:01:00	2.2 (Target °C ≥50°C)	–
Cooling				
Target (°C)	Acquisition Mode	Hold [hh:mm:ss]	Ramp Rate [°C/s]	Acquisition [per °C]
40	None	00:00:10	1.5	–

9. Go to "Subset Editor" tab in the left column of the software window. Create a subset by pressing + button and rename the subset as desired.
10. Click the newly created subset and click the wells containing the PCR reaction mixture with the Ctrl key. Click "Apply" to save the selection.
11. Go to "Sample Editor" tab. Select the created subset from the subset selector drop-down menu.
12. Enter sample information (Sample Name, Sample Type etc.) and select the desired colour for each sample.
13. Go back to "Experiment" tab – "Run Protocol" section. Check that the cycling conditions are correct.

Plate loading and starting the experiment

1. Load the prepared LightCycler® 480 Multiwell Plate in the instrument (click grey button next to the 2nd LED).

※Make sure the plates are correctly loaded as you have entered in the subset editor.

2. Click "Start Run" and save experiment in the desired folder to continue.
3. The window will automatically switch to the "Data" section to show progress of the experiment. When the experiment is finished, the status message will display as "Run Complete".

Real-time PCR analysis

1. Click “Analysis” tab in the left column of the software window.
2. Double-click “Abs Quant/2nd Derivative Max” or “Abs Quant/Fit Points” to create new analysis.
3. Select the subset from the drop-down menu, “Amplification” and if desired, insert the name for the analysis. Click √ button to proceed.
4. Click “Calculate”
5. To export the curve image, right-click the image and click “Export Chart”.

Generation of test report

1. To generate the report, click “Report” tab in the left column of the software window.
2. Select the items in the menu you want to display in the report.
3. Click “Save” button (small disket icon in the right column) to save experiment.
4. Click “Generate” button” to display the preview.
5. Click “PDF” to export the report to PDF file in the desired folder.

Procedure real-time PCR assay for detection of *M. genitalium*

Protocol for real-time PCR for *Mycoplasma genitalium* detection was adopted from Edberg and colleagues [5] and the LightCycler® 480 Probes Master Instructions for Use (version 11).

General Required Materials

Standard Laboratory Equipment

- LightCycler® 480 Instrument II
- Microwell Plate Centrifuge
- Microcentrifuge
- Micropipettes (P10 or P20, P200, P1000 or equivalent)

Disposables

- Nuclease-free, aerosol-resistant pipette tips
- 1.5 mL microcentrifuge tubes
- LightCycler® 480 Multi-well Plate 96-well – White
- LightCycler® 480 Sealing Film

Reagents

- FastStart TaqMan® Probe Master or LightCycler®480 Probes Master,2x concentration (red cap)
- LightCycler®480 Probes Master, Water, PCR Grade (Colourless cap)
- Primers and probes (see below)

Sample Materials

- Template DNA, up to 500 ng complex genomic DNA

Preparation of stock solution for primers and probes (Clean room)

Primers and probe sequences are obtained from Edberg and colleagues (2008) (Table 1). Primer oligonucleotides were synthesised by Inqaba Biotechnology, and probes were synthesized by Integrated DNA Technologies.

Table 1. Primers for real-time PCR amplification and detection of *Mycoplasma genitalium*

Primer or probe	Primer or probe name	Sequence	Nucleotide position
Forward primer	MgPa-355F	5'-GAGAAATACCTTGATGGTCAGCAA-3'	1420 in MgPa operon sequence
Reverse primer	MgPa-432R	5'-GTTAATATCATATAAAGCTCTACCGTTGTTATC-3'	1497 in MgPa operon sequence
Probe	MgPa-380	5'-6'FAM-ACTTTGCAATCAGAAGGT-BHQ-2™-3'	1445 in MgPa operon sequence

3. Dissolve lyophilised primers and probes in PCR grade water (Promega) to prepare a 100 µM stock solution according to manufacturer's instructions.
4. Store prepared stock solutions until use at −20°C.

Preparation of the 10x primer-probe mix (Clean room)

Prepare the 10x primer-probe mix prior to PCR amplification as per Table 2:

Table 2. Preparation of 10x primer-probe mix

Reagent	**Final concentration**	**10x Concentration**	**Volume (µL)**
Forward primer	0.9 µM	9.0 µM	45 µL
Reverse primer	0.9 µM	9.0 µM	45 µL
Probe	0.25 µM	2.5 µM	12.5 µL
Water, PCR grade		-	397.5 µL
Total volume		-	**500 µL**

Calculation: $V_1 = \frac{C_2 V_2}{C_1}$

Primer: $V_1 = \frac{(9)(500)}{100} = 45\ \mu L$ Probe: $V_1 = \frac{(2.5)(500)}{100} = 12.5\ \mu L$

Preparation of PCR reaction mixture (Clean room)

Prepare the PCR reaction mixture according to manufacturer's instructions (LightCycler® 480 Probes Master Instructions for Use, version 11).

5. Remove the 2x LightCycler® 480 Probes Master mix (red cap), the 10x primer-probe mix and PCR-grade water (colourless cap) from the −20°C freezer and were allowed to thaw on ice. After all the reagents were completely thawed, each tube was briefly spun in a microcentrifuge.
6. Prepare the PCR reaction mixture in a 1.5 mL reaction tube as per Table 3. Before transferring the reagents into a tube, gently pipette up and down to allow all precipitants to be resuspended. When mixing each reagent, mix carefully by pipetting up and down.

Table 3. Preparation of the PCR reaction mixture

Reagent	**Final concentration**	**Volume per reaction (µL)**	**Volume for z[a] reactions (µL)**
LightCycler®480 Probes Master (2x concentration, red cap)	1x	10	10 x z
10x primer-probe mix	Primers: 0.9 µM each Probe: 0.25 µM	2	2 x z
Water, PCR grade (colourless cap)	-	3	3 x z
Total volume	-	15	15 x z

[a] z = the number of reactions to be run + two additional reactions (extra amount for preventing shortage of reagents)

7. Pipette 15 µl of the PCR mixture into each well of the LightCycler® 480 Multiwell Plate.
8. Add 5 µl PCR-grade water instead of a template DNA in a negative control well. Mix by pipetting up and down.

※When loading PCR mixture/mixing water in the wells, make sure not to cause air bubbles.

Air bubbles may interrupt with PCR reactions and cause inaccurate results.

<u>Addition of template DNA (DNA workstation-PFGE lab)</u>

9. Add 5 µl of the DNA template into each well of the LightCycler® 480 Multiwell Plate. Mix by pipetting up and down.

※When mixing the DNA in the wells, make sure not to cause air bubbles. Air bubbles may interrupt with PCR reactions and cause inaccurate results.

10. Seal the plate with the LightCycler® 480 Sealing Film.

PCR amplification (PCR/Gel electrophoresis lab)

PCR amplification is carried out on the LightCycler 480 II instrument (Roche Diagnostics). It is recommended to program the LightCycler® 480 Instrument before preparing the PCR reaction mixture.

Programming of the instrument

11. Switch on the instrument (main switch is at the back of the instrument) and wait until the instrument has initialised and is ready (left LED becomes green).
12. Switch on the computer and sign in (ID: operator; PW: LC480).
13. Double-click on the icon of the LightCycler 480 software.
14. Sign in with the user login details.
15. Click “New Experiment” from the Overview window.
16. Choose the detection format from a pull-down menu.
17. Select a reaction volume.
18. Program the PCR cycling conditions as per Table 4.

Table 4. Setup of the LightCycler® 480 System PCR experiment

Setup		
Block type		Reaction volume (µL)
96		20
Detection format	Excitation filter	Emission filter
Monocolour hydrolysis probe: FAM	465	510

Table 4. Setup of the LightCycler® 480 System PCR experiment (*continued*).

Programs					
Program name	Cycles		Analysis Mode		
Pre-incubation	1		None		
Amplification	45		Quantification		
Temperature targets					
Pre-incubation					
Target (°C)	Acquisition Mode	Hold [hh:mm:ss]	Ramp Rate [°C/s]	Acquisition [per °C]	
95	None	00:10:00	4.4	–	
Amplification					
Target (°C)	Acquisition Mode	Hold [hh:mm:ss]	Ramp Rate [°C/s]	Acquisition [per °C]	
95	None	00:00:10	4.4	–	
60	Single	00:00:30	2.2	–	

19. Go to “Subset Editor” tab in the left column of the software window. Create a subset by pressing + button and rename the subset as desired.
20. Click the newly created subset and click the wells containing the PCR reaction mixture with the Ctrl key. Click “Apply” to save the selection.
21. Go to “Sample Editor” tab. Select the created subset from the subset selector drop-down menu.
22. Enter sample information (Sample Name, Sample Type etc.) and select the desired colour for each sample.
23. Go back to “Experiment” tab – “Run Protocol” section. Check that the cycling conditions are correct.

Plate loading and starting the experiment

24. Load the prepared LightCycler® 480 Multiwell Plate in the instrument (click grey button next to the 2nd LED).

※Make sure the plates are correctly loaded as you have entered in the subset editor.

25. Click “Start Run” and save experiment in the desired folder to continue.
26. The window will automatically switch to the “Data” section to show progress of the experiment. When the experiment is finished, the status message will display as “Run Complete”.

Real-time PCR analysis

27. Click “Analysis” tab in the left column of the software window.
28. Double-click “Abs Quant/2nd Derivative Max” or “Abs Quant/Fit Points” to create new analysis.
29. Select the subset from the drop-down menu, “Amplification” and if desired, insert the name for the analysis. Click √ button to proceed.
30. Click “Calculate”
31. To export the curve image, right-click the image and click “Export Chart”.

Generation of test report

32. To generate the report, click “Report” tab in the left column of the software window.
33. Select the items in the menu you want to display in the report.
34. Click “Save” button (small disket icon in the right column) to save experiment.
35. Click “Generate” button” to display the preview.
36. Click “PDF” to export the report to PDF file in the desired folder.

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5. Edberg A, Jurstrand M, Johansson E, et al. A comparative study of three different PCR assays for detection of Mycoplasma genitalium in urogenital specimens from men and women. J Med Microbiol 2008; 57: 304-309.

APPENDIX D Gel electrophoresis of *Candida* species PCR

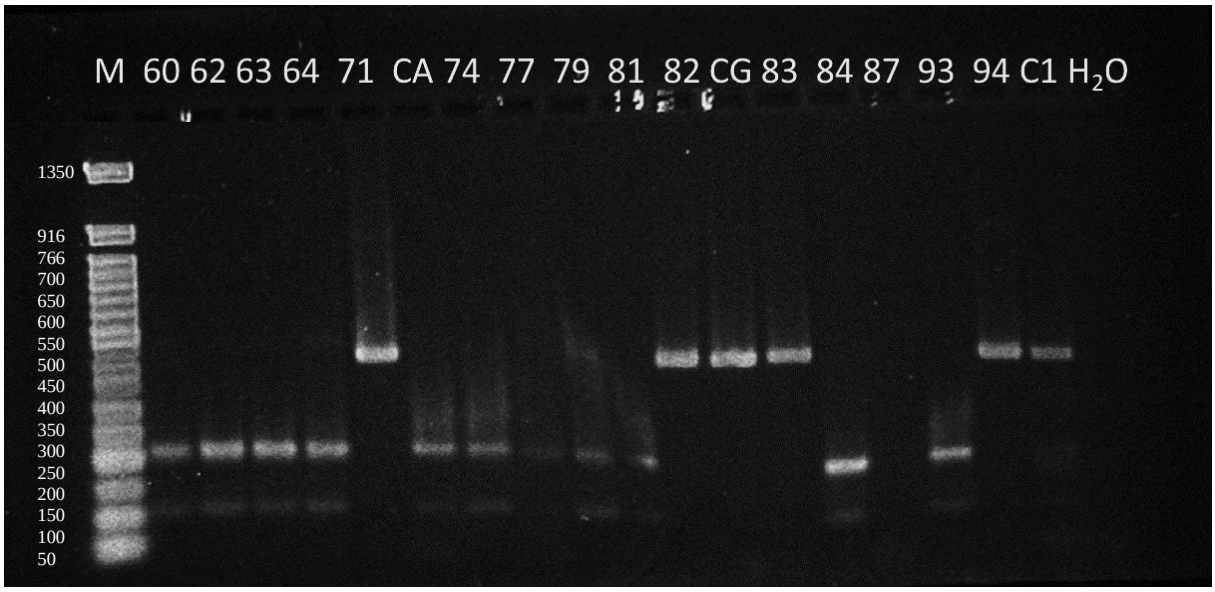

Figure 1. Electrophoresis of the PCRs resolved in a 2% agarose gel. M, molecular size marker. NC, negative control. (A) Lanes: 60-94, Patient DNA; (B) Lanes: CA, *C. albicans*; CG, *C. glabrata* (482/483 bp); C1, *C. albicans* (218 bp and 110 bp), *C. krusei* (183 bp), *C. glabrata* (482/483 bp); C2, *C. tropicalis* (218 bp); C3, C. *parapsilosis* (229 bp).

APPENDIX E Supplementary tables

Table S3.1. Demographic factors and coinfections in women with fluconazole sensitive and resistance Candida albicans species in Namibia (*n*=90).

Characteristics	**Sensitive (n=88)**	**Resistant (n=2)**	**OR**	**95% CI**	***p*-value**
Age (years), median (IQR)	28 (23–32)	[a]28.5			0.88
Pregnancy	27 (31)	0 (0)	Inf.	0.08–Inf.	1.00
HIV	18 (20)	1 (1.1)	0.26	0.0032–21	0.38
Chlamydia trachomatis	7 (8.0)	0	Inf.	0.015–inf.	1.00
Neisseria gonorrhoeae	4 (4.5)	0	Inf.	0.0078–inf.	1.00
Trichomonas vaginalis	23 (26)	0	Inf.	0.015–inf.	1.00
Bacterial vaginosis	33 (38)	0	Inf.	0.11–inf.	0.58

Data are presented as number (n) with proportion (%) unless indicated otherwise.

Abbreviations: OR, odds ratio; CI, confidence interval; IQR, interquartile range; HIV, human immunodeficiency virus.

Inf. = If any of the cell frequencies in 0, then fisher test does not compute a confidence interval.

a Only 2 isolates that are resistant to fluconazole, i.e. unable to calculate an IQR

Figure S4.1. Syndromic algorithm for management of vaginal discharge syndrome in women in Namibia.

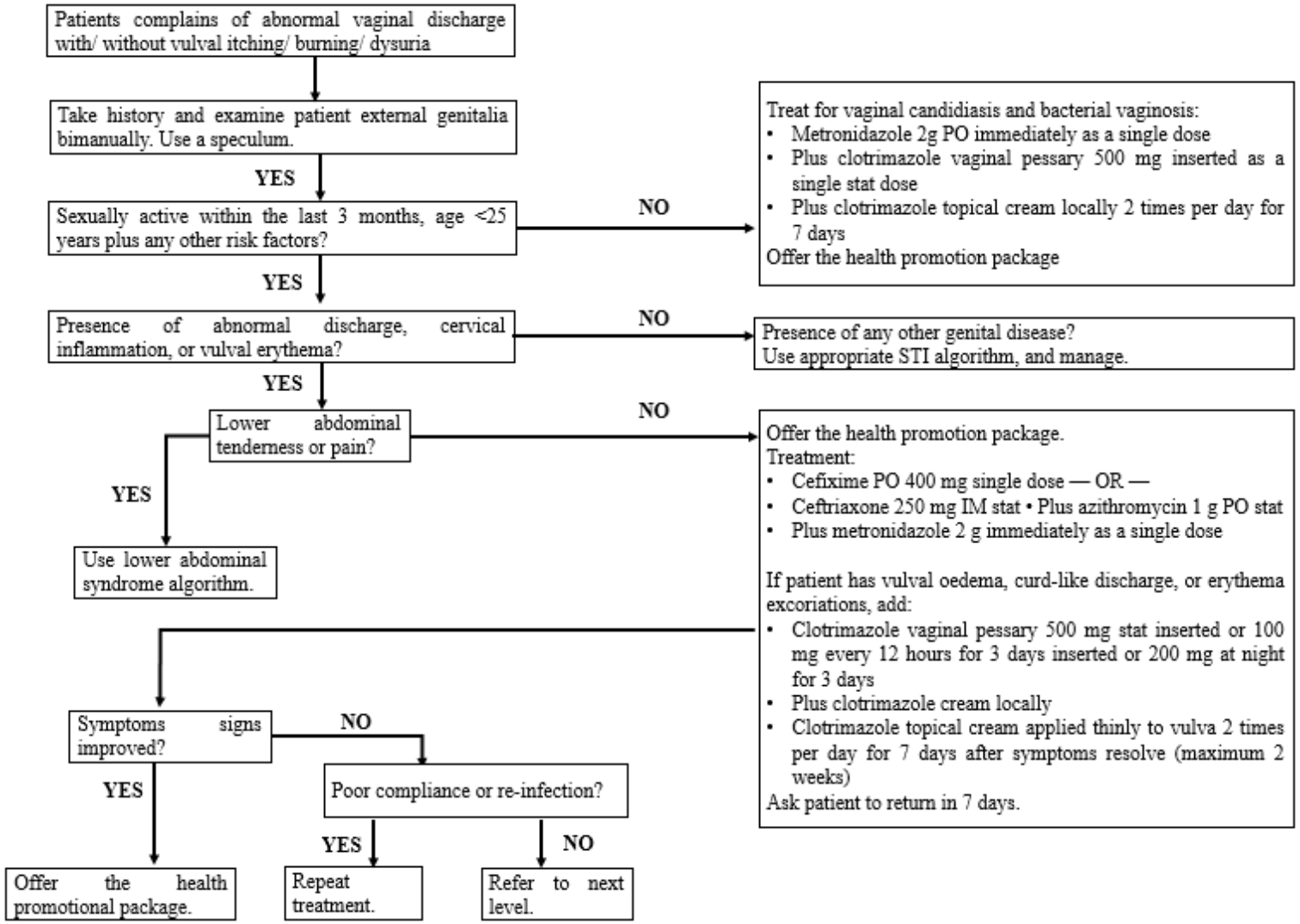

Table S4.1. Microbial aetiology of vaginal discharge syndrome in 109 women in Windhoek, Namibia, 2021-2022.

Infection	**Total (*n*, %)**
Chlamydia trachomatis	
Yes	24 (22)
No	85 (78)
Neisseria gonorrhoea	
Yes	16 (15)
No	93 (85)
Trichomonas vaginalis	
Yes	23 (21)
No	86 (79)
Mycoplasma genitalium	
Yes	17 (16)
No	92 (84)
Bacterial vaginosis	
Yes	47 (43)
No	62 (57)
Vulvovaginal candidiasis	
Yes	45 (41)
No	64 (59)

Data are presented as numbers (*n*) with proportions (%) unless indicated otherwise.

Table S4.2. Bacterial species in Namibian women with bacterial vaginosis (*n*=43).

Organisms	**Total (*n*, %)**
Atobium vaginae	
Yes	39 (91)
No	4 (9.0)
BVAB-2	
Yes	40 (93)
No	3 (7.0)
Gardnerella vaginalis	
Yes	43 (100)
No	0 (0)
Lactobacillus spp.	
Yes	1 (3.0)
No	42 (97)
Mobiluncus spp.	
Yes	23 (53)
No	20 (47)
Megasphaera Type1	
Yes	31 (72)
No	12 (28)

Data are presented as number (*n*) with proportion (%) unless indicated otherwise.

Table S4.3. Predictors of treatment failure in women presenting with VDS (*n*=94).

Characteristics	Total (%)	Treatment failure (*n*=29)	Treatment success (*n*=65)	Unadjusted			Adjusted		
				OR	95% CI	*p*-value	*OR*	*95%CI*	*p-value*
Age (years, mean ± SD)	28 ± 6.6	29 ± 6.6	28 ± 6.7			0.54			–
Marital status				0.892	0.49–1.6	0.71	–	–	–
Single	72 (77)	21 (72)	51 (78)						
Co-habiting	13 (14)	4 (14)	9 (14)						
Married	9 (9.0)	4 (14)	5 (8.0)						
New sexual partner(s) in the past week/ month				3.2	0.79–13	0.098	–	–	–
Yes	9 (10)	5 (17)	4 (6.0)						
No	85 (90)	24 (83)	61 (94)						
Time of last sexual intercourse				2.4	0.95–5.9	0.061	2.4	0.95–5.9	0.064
≤ a week ago	48 (51)	19 (66)	29 (45)						
≥ a week ago	46 (48)	10 (34)	36 (55)						
Condoms use at last sex act				2.1	0.87–5.1	0.12	–	–	–
Yes	40 (43)	16 (55)	24 (37)						
No	54 (57)	13 (45)	41 (63)						
Vaginal douching				0.95	0.32–2.8	0.93	–	–	–
Yes	19 (11)	5 (7.0)	14 (12)						
No	75 (79)	24 (83)	51 (78)						
Pregnancy				0.638	0.21–1.9	0.59	–	–	–
Yes	21 (22)	5 (17)	16 (25)						
No	73 (78)	24 (83)	49 (75)						

Table S4.3. Predictors of treatment failure in women presenting with VDS (*n*=94) (*continued*).

Characteristics	Total (%)	Treatment failure (*n*=29)	Treatment success (*n*=65)	Unadjusted			Adjusted		
				OR	95% CI	*p*-value	*OR*	*95%CI*	*p-value*
Use of hormonal contraception				0.247	0.053–1.2	0.060	0.247	0.053–1.2	0.077
Yes	17 (18)	2 (7.0)	15 (23)						
No	77 (82)	27 (93)	50 (77)						
HIV status*				5.9	1.1–33	0.032	5.9	1.1–33	0.042
Unknown	13 (14)	2 (7.0)	11 (17)						
Positive	7 (7.0)	5 (17)	2 (3.0)						
Negative	74 (79)	22 (76)	52 (80)						
Time of last Pap smear*				0.21	0.078–0.56	0.003	0.21	0.078–0.56	0.002
< a month ago	55 (64)	10 (39)	45 (75)						
> a month ago	31 (36)	16 (61)	15 (25)						
Co-morbidities‡				2.8	0.33–25	0.43	–	–	–
Yes	7 (7.0)	1 (3.0)	6 (9.0)						
No	87 (93)	28 (97)	59 (91)						
Self-reported History of VDS				2.1	0.79–5.6	0.19	–	–	–
Yes	71 (76)	19 (66)	52 (80)						
No	23 (24)	10 (34)	13 (20)						
Number of self-reported VDS episodes this past year (*n*=73)				0.89	0.24–3.3	1.0	–	–	–
≤ 2 episodes	58 (79)	15 (71)	43 (83)						
≥ 3episodes	15 (21)	6 (29)	9 (17)						

Table S4.3. Predictors of treatment failure in women presenting with VDS (*n*=94) (*continued*).

Characteristics	Total (%)	Treatment failure (*n*=29)	Treatment success (*n*=65)	Unadjusted			Adjusted		
				OR	95% CI	*p*-value	*OR*	*95%CI*	*p-value*
Documented treatment for VDS this past year				1.2	0.51–3.0	0.66	–	–	–
Yes	52 (55)	15 (52)	37 (57)						
No	42 (45)	14 (48)	28 (43)						

Data are presented as number (*n*) with proportion (%) unless indicated otherwise.

Note. OR, odds ratio; CI, confidence interval; SD, standard deviation; HIV, human immunodeficiency virus; VDS, vaginal discharge syndrome.

*N=86 due to incomplete responses from participants on time of last Pap smear.

⁋N=86 due to participants that couldn't recall time of last antibiotic use.

⁑N=81 due to unknown HIV status.

‡Co-morbidities include *n*=2 Hypertension, *n*=1 Arthritis, *n*=1 Glaucoma, *n*=3 Asthma.

Table S4.4. Aetiology of VDS treatment failure in Namibian women (*n*=13)

Causes	**Total (*n*, %)**	**Persistent (*n*=5)**	**Recurrent (*n*= 8)**
VVC not covered by syndromic treatment	4 (31)	3 (75)	1 (25)
Drug-resistant VVC	3 (23)	0 (0)	3 (100)
Recurrent, persistent or drug-induced BV	4 (31)	2 (50)	2 (50)
Incident STI infection	1 (8.0)	0 (0)	1 (100)
Unclear aetiology	1 (8.0)	0 (0)	1 (100)

Data are presented as number (*n*) with proportion (%) unless indicated otherwise.

Table S5.1. Antifungal resistance mechanisms of *Candida glabrata* species (adopted from María *et al*. 2021).

Antifungal drug class	**Target pathway**	**Antifungal drug target**	**Mechanisms of action**	**Genes involved in resistance**	**Mechanism of resistance**
Azoles	Ergosterol in cell membrane	Lanosterol 14-α-demethylase	Inhibition of ergosterol synthesis thus depleting membranes of ergosterol causing accumulation of toxic sterol precursors	*ERG6* *ERG7* *ERG9* *ERG11*	Increased drug efflux and incorporation of non-ergosterol sterols into cell membrane
	ATP-binding cassette (ABC) transporters in cell membrane	ATP-binding cassette (ABC) transporters	Activation of transporters leading to drug influx	*CgPDR1* *CgCDR1* *SNQ2*	Overexpression of ATP-binding cassettes leading to drug efflux
Echinocandins	β-1,3-glucan in cell wall	β-1,3-glucan synthase	Inhibition of β-1,3-glucan synthesis thus disrupting cell wall stability	*FKS1* *FKS2* *FKS3*	Altered conformation of β-1,3-glucan synthase subunits leading to reduced drug affinity
Polyenes	Ergosterol in cell membrane	Ergosterol	Sequesters ergosterol out of membranes and induces pore formation causing ion leakage	*ERG6* *ERG11*	Increased drug efflux and incorporation of non-ergosterol sterols into cell membrane
Pyrimidine analogues	DNA and protein synthesis	Cytosine permease and deaminase	Inhibits pyrimidine metabolism	*FCY1* *FUR1*	Reduced drug uptake
Multidrug	Multidrug transporters	Thymidylate-synthetase	Inhibits the thymidylate-synthetase enzyme interfering with DNA	*MDR1*	Mutations in *MSH2* involved in mismatch repair that causes increased mutation rate that leads to a higher rate of antifungal resistance