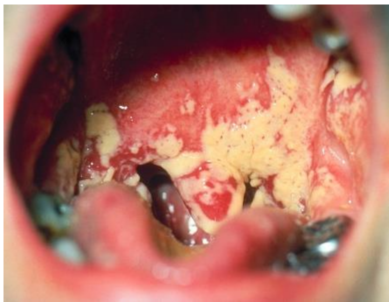
UNIVERSITY OF OSLO
FACULTY OF DENTISTRY

Oral candidiasis

and molecular epidemiology of *Candida glabrata*



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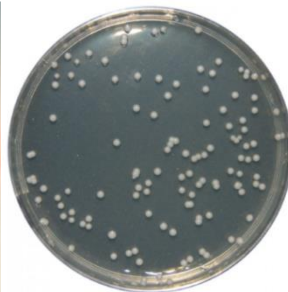

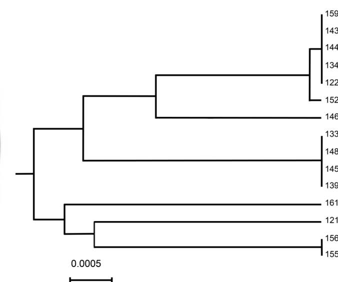

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Preface

In the summer of 2012 and 2013, I got to be a part of a PhD project on the characterization of the yeast fungus *Candida glabrata*. The project focused on genetic characterization, using a method called multilocus sequence typing (MLST) to detect variations in the genome of different *C. glabrata* strains. *C. glabrata* was until recently considered a commensal in humans, one of the many harmless microorganisms that inhabit the human body. However, with the increasing number of patients with reduced host response in medicine, *C. glabrata* is becoming an increasingly important human pathogen.

In the PhD project, we examined over 250 strains of *C. glabrata*, all sampled from human sources, mainly from systemic infections. While this is not strictly the dentist's area of expertise, it is still possible to draw parallels to oral yeast infections, also known as oral candidiasis. Dentists and dental hygienists may not know of *C. glabrata*, but will know a different yeast, more common in the oral cavity, namely *Candida albicans*. *C. albicans* is prevalent in both systemic and oral infections.

While our work with *C. glabrata* was very interesting, it was not my intention to use it in my master thesis. The idea came later, as I saw that connections could be drawn between systemic infections and oral candidiasis, and *C. glabrata* and *C. albicans*. I decided to write a two-part thesis. Part 1 is a literary review of oral candidiasis, where I focus on clinically relevant information, such as diagnosis and treatment of the disease. Part 2 is a discussion of *C. glabrata*, and molecular methods used in the PhD project. Lastly, I got to work with a small selection of *C. glabrata* strains, specifically for this thesis, and I present my results at the end of part 2.

Part 1: Oral candidiasis

Introduction

The oral cavity is home to many different microorganisms, including bacteria, viruses, fungi, and sometimes protozoa (1). The different tissues and fluids in the oral cavity, as well as the range of foods that pass through, provide a unique environment that allows various microorganisms to thrive (1). Sometimes the organisms live in complete harmony with their host, other times they can cause harm in the form of dental caries, periodontitis, and other infections. The shift from harmless to harmful was described by P.D. Marsh (2) in the ecological plaque hypothesis.

Candida species[1], commonly *Candida albicans* (3, 4), can be present in the mouth of healthy individuals without causing disease to its host (3-5). Like bacteria, fungi can be part of the natural oral microflora[2], and like with bacteria, a shift in the balanced microflora can lead to infection and disease. Oral candidiasis is one term used to describe such an infection, and other names are oral candidosis, oral thrush (specifically pseudomembranous candidiasis, see below), and candidal stomatitis. It is also referred to as a biofilm disease (6).

Candidiasis is defined as an infection caused by a fungi of the genus *Candida*, and the term oral candidiasis is only used when describing a clinically visible lesion in the oral cavity (4). The lesion can vary in size, shape and colour, largely dependant on the predisposing factors behind the disease (4). The patient's complaints can vary from none, to extremely painful and completely disabling (7). With this wide array of clinical presentations, it is important for the dental practitioner to have the knowledge for diagnosis and treatment of oral candidiasis.

Pathogenesis and predisposing factors

The formation of an oral candidiasis lesion is usually caused by the establishment of a complex biofilm[3] containing *C. albicans* as well as other microorganisms (4). The biofilm adheres to oral surfaces, such as teeth, mucosa and dentures, and triggers an immune response heavy in neutrophils (4). The biofilm is the perfect environment for the fungal cells to thrive,

[1] Genus of yeast that exists in humans, both harmless and a cause of infection.
[2] The collection of microorganisms living in the oral cavity, of any given individual at any given time.
[3] Organized group of microorganisms that adhere to each other and any surface, particularly teeth and dentures in the mouth.

as the neutrophils cannot reach outside the body's own borders (4). *C. albicans* pathogenicity[4] is linked to it phenotypic switching, between the commensal yeast form, and the invasive hyphal form (8). Hyphae are elongations of the fungal cells, a tube without constrictions that can aid the pathogen in invading its host (8). *C. albicans* can invade the superficial layers of the oral epithelium, and cause proteolytic breakdown of E-cadherin (4). E-cadherin is an important structural protein in the oral cavity, responsible for keeping the epithelium continuous, and a barrier against harmful substances (9). When E-cadherin is broken down, the tissue weakens and the protective barrier is compromised (9). *C. albicans* uses this to migrate deeper into the tissue (9). Less is known about the pathogenicity of *Candida glabrata*, which will be discussed further in part 2.

Hyposalivation

Saliva has antibacterial and antifungal properties that help protect the healthy patient from infection. Proteins such as secretory IgA, lysozyme, mucin and lactoferrin have inhibitory effects on *Candida* that stop adhesion and multiplication on the mucosal surface (10). When a patient suffers from hyposalivation, and the quantity or quality of saliva is affected, the risk of infection increases (10, 11). Causes of hyposalivation include taking medicine, especially polypharmacy[5], cancer treatment, nutritional deficiency, and Sjögren's syndrome[6] (3, 6, 10, 11).

Biofilm

With good oral hygiene, the biofilm is constantly being disturbed. Though oral hygiene alone will not prevent dental caries and periodontitis, it is an important prerequisite for reducing the prevalence of disease. When the biofilm is left alone for some time, it becomes a highly structured, highly protected, community of microorganisms, which is capable of doing harm, also in the form of oral candidiasis (6). The candidal hyphae and extrapolymeric material increases biofilm growth and structural integrity (6). The non-shedding surfaces of the oral cavity comprise the teeth, and the surface increases with the addiction of restorations, orthodontic appliances, and removable dentures (6). When the patient is unable or unwilling to maintain proper oral hygiene, the risk of oral candidiasis will increase (6).

[4] An organism's ability to cause disease.
[5] Regular use of many different drugs, causing their adverse effects to pile up.
[6] Chronic, autoimmune disease that causes destruction of the salivary glands.

Antibiotics

The use of broad-spectrum antibiotics alters the oral microflora (5). Destruction of the normal bacterial population favours yeast growth, as it decreases the competition for nutrition and cell adhesion (5). Therefore the use of broad-spectrum antibiotics is a well known predisposing factor for oral candidiasis (5, 6).

General predisposing factors

A functioning immune system is essential to fight all threats of infection. Topical corticoids, such as steroid inhalers for treating asthma, can give characteristic circumscribed lesions that will be discussed later. Systemic immunosuppressive medication, used to treat autoimmune, inflammatory, and neoplastic disorders, will increase susceptibility to infection (5). For this reason, hospitalized cancer patients are especially at risk for developing severe oral candidiasis, as well as dangerous invasive candidiasis (12). HIV-infected patients and patients with diagnosed AIDS are at risk for the same reason (5). Other high-risk groups are discussed below.

Prevalence and high-risk groups

Reports have shown that 20-75 % of the general population are carriers of one or more species of *Candida*, without showing any symptoms of infection (13). *C. albicans* is considered a commensal[7] in healthy individuals, so it will often exist in the oral cavity without any corresponding candidiasis. *C. albicans* is the most frequent cause of oral candidiasis (70-80 % (3, 11, 14)). Isolating *C. albicans* from the oral cavity has shown a higher incidence in immunocompromised[8] patients than in healthy individuals (see table 1).

[7] Organism living in a host without causing harm to it.
[8] Having an immune system that is somehow impaired or damaged.

Population group	Incidence of *C. albicans* in the oral cavity (%)					
	Lynch (1994) (15)	Cannon et al. (1999) (16)	Akpan et al. (2002) (13)	Grimoud et al. (2003) (17)	Samaranayake et al. (2009) (7)	Giannini, et al. (2011) (11)
Healthy adults	20	18	30-45	-	-	-
Healthy children	9	-	45-65	-	-	-
Neonates, infants	16	13	45	-	-	-
People who wear removable dentures	60	-	50-65	-	-	-
Patients in hospitals or care facilities	-	41	65-88	67	-	-
Patients with leukaemia, undergoing chemotherapy	-	-	90	-	-	-
Patients with HIV	-	-	95	-	84-100	90

Table 1: Incidence of *C. albicans* in the oral cavity of different population groups. The numbers are pulled from different review articles, from 1994 to 2011. The early studies have listed the incidence in healthy population groups, while the later studies focus on patients with HIV. With the growing amount of immunocompromised patients, widespread use of antibiotics and chemotherapy, as well as increased life expectancy of HIV-infected individuals, the incidence of opportunistic fungal infections also increase, but in different population groups than were previously examined and with several other species than *C. albicans* involved (7).

Though *C. albicans* is the most common cause of yeast infections, the non-*albicans Candida species*[9] are increasing in prevalence. *C. glabrata* is the second most common species in candidiasis or candidemia[10], and the number one non-*albicans Candida spp*. in blood stream infections (18). In patients younger than 13 years, *Candida parapsilosis* is almost as common as *C. albicans*, causing 34 % of all candidemias (18). *C. glabrata* is less common in infants and children (19). Unlike most *Candida spp.*, *C. glabrata* does not produce hyphal structures. *C. glabrata* is common in blood stream infections, and resistance to azole antifungals[11] is common (6). *C. glabrata* was not seen as an important oral pathogen, but due to its opportunistic nature, and resistance to azoles, it is becoming increasingly involved in oral candidiasis, especially in HIV and cancer patients (20). *Candida tropicalis* is important in

[9] *Candida species* other than *C. albicans*.
[10] A type of fungemia, or blood infection.
[11] Group of commonly used antifungal agents.

patients with neutropenia[12] and leukaemia, and is potentially very aggressive (18, 19). *Candida krusei* is also associated with haematological malignancies and neutropenia (19). Azole resistance is common in *C. krusei,* which is a concern, especially to high-risk patients (6).

Classification

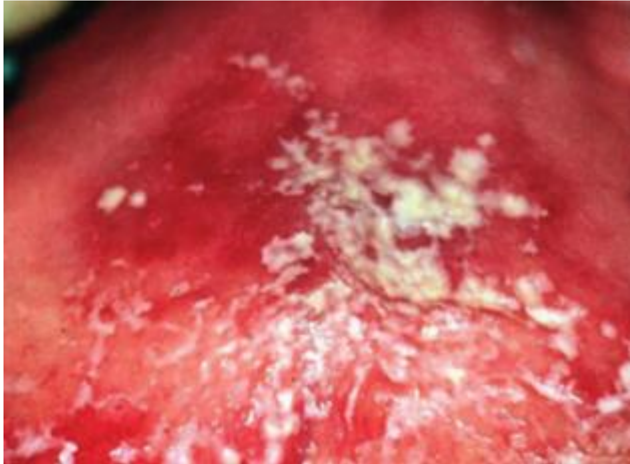
Oral candidiasis can be divided into many different types, often by its clinical appearance in the mouth, and sometimes by its predisposing factors. The following are the most commonly described types of oral candidiasis.

Pseudomembranous candidiasis

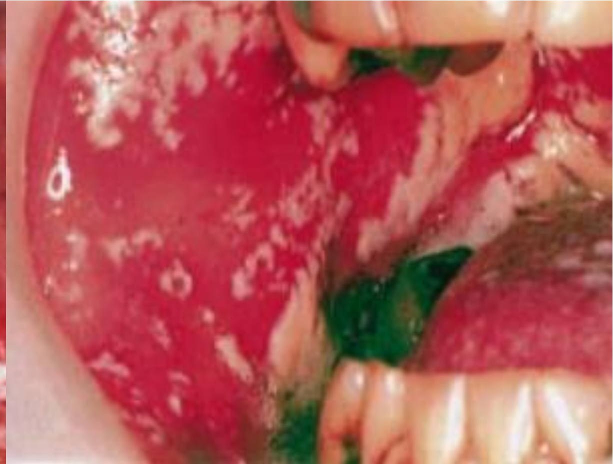
Pseudomembranous candidiasis, or *oral thrush*, is the most commonly diagnosed and most easily recognizable form of oral candidiasis (11). In this type of infection, the mucosa is covered in a white or yellow pseudomembrane, consisting of fibrin, desquamated epithelial cells, inflammatory cells, and sometimes bacteria or food debris (3, 13). The plaque is also heavily infiltrated by fungal hyphae (13, 21). With some pressure, the membrane can be removed, and underneath the mucosa is erythematous and inflamed (4, 11, 14, 15). If removal of the membrane reveals bleeding mucosa, the patient is most likely suffering from additional conditions such as erosive lichen planus or pemphigus, which are often associated with oral candidosis in the affected areas (7).

The infection can often be asymptomatic, and other times the patient will describe discomfort, burning, tenderness or changes in taste when large parts of the mucosa are involved (4, 7). Most commonly affected is the buccal mucosa, tongue, soft palate and oropharynx (3, 4, 11, 13).

[12] Immune disorder affecting the production of neutrophil granulocytes, causing an abnormally low number of these cells.



Pseudomembranous candidiasis
From Farah et al. 2010 (5)



Acute pseudomembranous candidiasis
From Akpan et al. 2002 (13)

Erythematous candidiasis

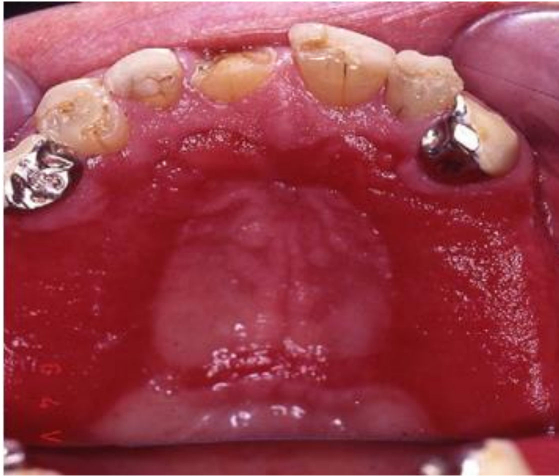
Erythematous candidiasis is probably the most common form of oral candidiasis. Due to its less pathognomonic appearance[13], however, it is not as easily diagnosed as its pseudomembranous counterpart (7). Erythematous candidiasis appears as a red, more or less circumscribed lesion in the hard palate or dorsum linguae (3, 4). It can persist chronically and is usually asymptomatic (3, 4). A burning or itching sensation can occur (11). Some distinguish between symptomatic and asymptomatic erythematous candidiasis (5, 14). A bright red tongue can occur, and the differential diagnosis in these cases is low serum B12, folate and iron (14).

Denture stomatitis, or *chronic atrophic candidiasis* (11, 22), is a type of erythematous candidiasis. It appears as lesions in the palate, and is caused by removable dentures, when the wearer does not remove it at night or fails to clean it properly (3, 4). An ill-fitted denture is also a contributing factor, as repeated trauma against the mucosa can cause an increase in penetration of *Candida*-antigens and –toxins (1). The lesion is restricted to the mucosa covered by the denture, and is typically asymptomatic (14, 15).

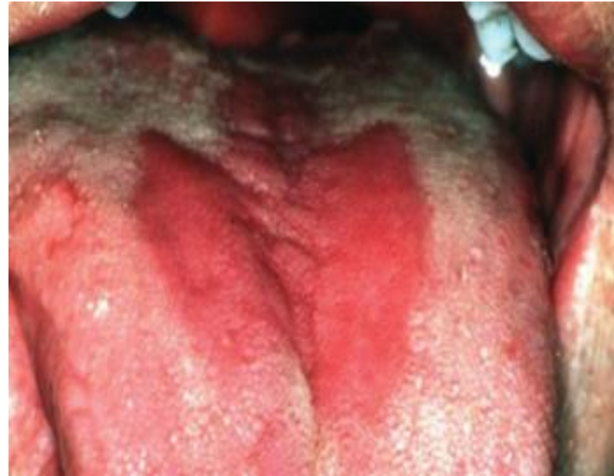
When erythematous candidiasis affects the tongue, a smooth red patch appears where the filiform papillae atrophy. If this patch is round or oval, and is located in the middle of the tongue, it is called *median rhomboid glossitis* (3, 4, 7), or *central papillary atrophy* (14). This

[13] Typical or characteristic for a certain disease.

type of lesion may be caused by bacteria as well as *Candida* and other fungi, so the etiology is not completely clear (7). Predisposing factors for this particular type of lesion is smoking and the use of steroid inhalers (14).



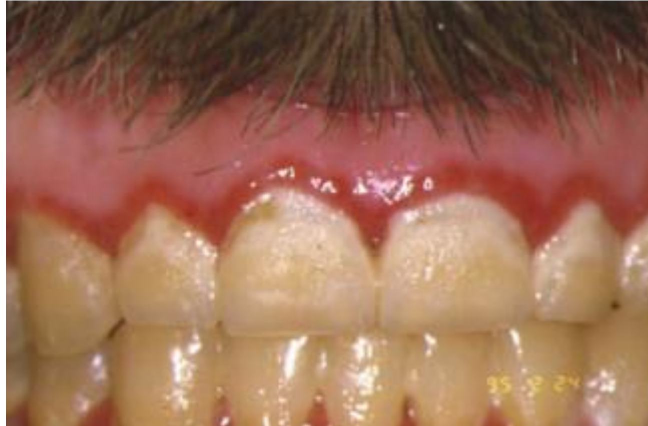
Erythematous candidiasis in a patient wearing a removable prosthesis
From Giannini et al. 2011 (11)



Median rhomboid glossitis
From Farah et al. 2010 (5)

Linear gingival erythema

Linear gingival erythema is a specific type of oral fungal infection that most frequently affects patients with HIV (7, 11). The lesion is a red band stretching along the gingival margin, and can be mistaken for gingivitis (7). Though the diagnostic criteria are not entirely clear, linear gingival erythema is defined as *"a nonplaque-induced gingivitis presenting a distinct erythematous band of at least 2 mm along the margin of gingivae, with either diffuse or punctuate erythema of the attached gingivae"* (7). Improved oral hygiene, even with regular professional cleaning, is not an efficient treatment (7, 11). The fungus *Saccharomyces cerevisiae*, *Candida dubliniensis,* and opportunistic bacteria are thought to be the pathogens associated with this type of lesion (7).



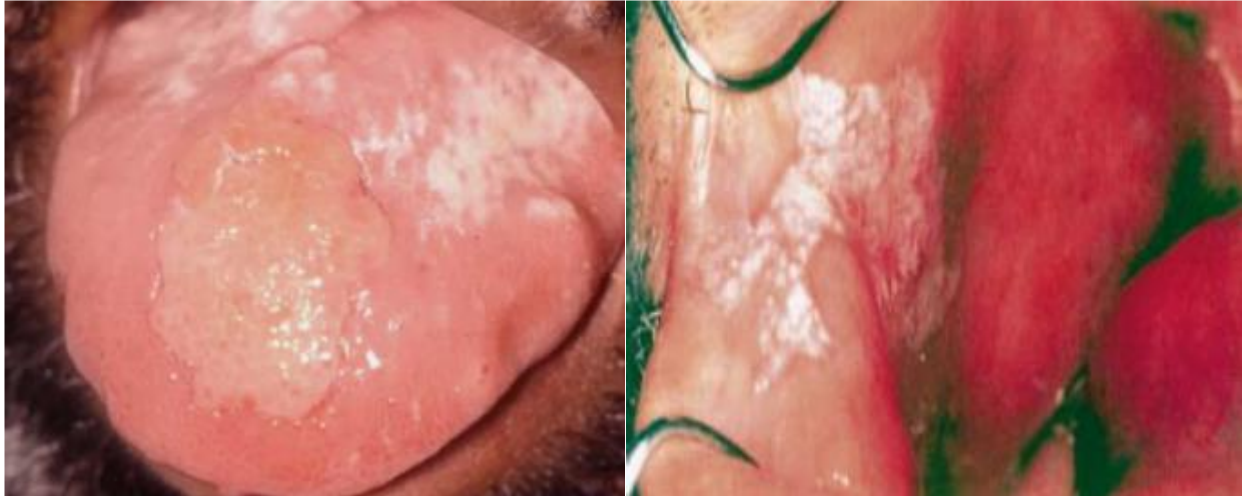
Linear gingival erythema

From Samaranayake et al. 2009 (7)

Hyperplastic candidiasis

Hyperplastic candidiasis is the least common form of oral candidiasis, but its malignant potential[14] makes it an important one (11, 13, 14). It is also called *candidal leukoplakia*, and like ordinary leukoplakia[15], it appears as white lesions that cannot be rubbed off (3, 7, 14). Its appearance varies greatly, from small, translucent, slightly raised lesions, to large, plaque-like areas that feel hard and rough on palpation (7). It is most commonly found on the buccal mucosa and is associated with smoking (13, 15). Though it cannot be rubbed off, it can be separated from leukoplakia by microbiological tests, attempting treatment with anti-fungal medicine, or by taking a biopsy for histological examination (7). The fungi's hyphae often invade the oral epithelium, which is hyperplastic (13). As previously mentioned, the lesions of hyperplastic candidiasis can sometimes turn malignant, but there is controversy regarding the importance of *Candida spp.* as a contributing risk factor (11, 13).

[14] Potential for developing into a cancerous lesion.
[15] A white mucosal lesion that can not be diagnosed as anything else.



Hyperplastic candidiasis in an immunosuppressed patient
From Muzyka 2005 (22)

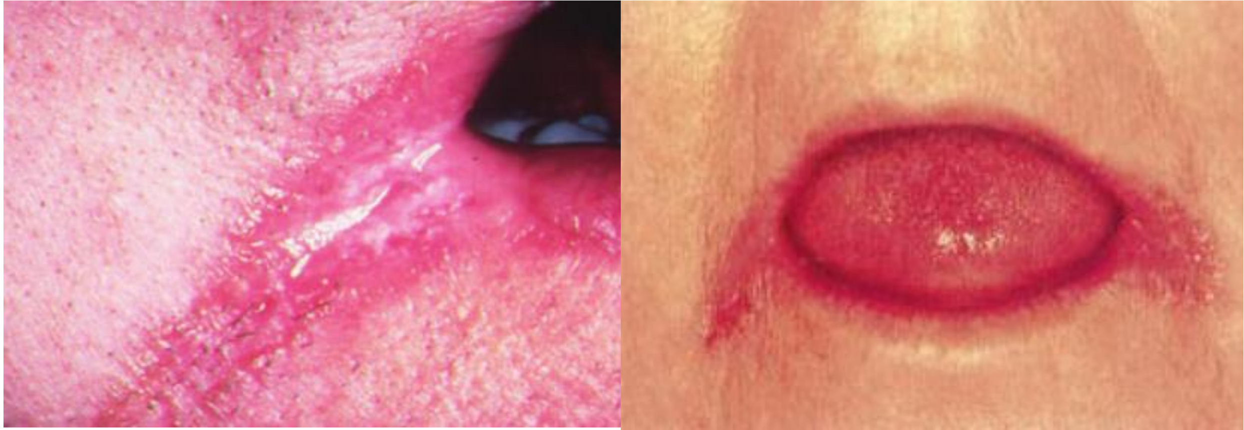
Chronic hyperplastic candidiasis
From Akpan et al. 2002 (13)

Angular cheilitis

Angular cheilitis is a multifactorial condition that can be caused by bacteria, especially *Staphylococcus aureus*[16] (3, 7, 11), fungi, or a combination of both. It is also affected by the loss of vertical dimension[17], vitamin B12 deficiency and iron deficiency anaemia (7, 13-15). The connection between folic-acid deficiency and angular cheilitis was made in 1971 by J.A. Rose (23), who found a significantly higher occurrence of folic-acid deficiency in patients with angular cheilitis. Folic-acid therapy was also found to heal the lesions, though this occurred in only two of the patients, and thus was not conclusive (23).

Angular cheilitis affects the corners of the mouth and the surrounding skin and mucosa. Folds in the skin create a constantly moist environment, with perfect growth conditions for both bacteria and fungi (3, 13, 14). The result is a red, sensitive lesion, with fragile skin that can rupture when stretched, such as when opening the mouth wide during dental treatment (4, 11). Treatment of the fungal infection will often cure the lesion, but if the vertical dimension is not improved (denture relining, see below), or the nutrient deficiencies are not treated, the lesions will most likely reoccur (15).

[16] Bacteria commonly involved in skin and lung infections.
[17] Facial height, affected by the size of the alveolar ridges and the presence of teeth.



Angular cheilitis in an elderly denture wearer
From Samaranayake et al. 2009 (7)

Angular cheilitis
From Akpan et al. 2002 (13)

Diagnostic tests

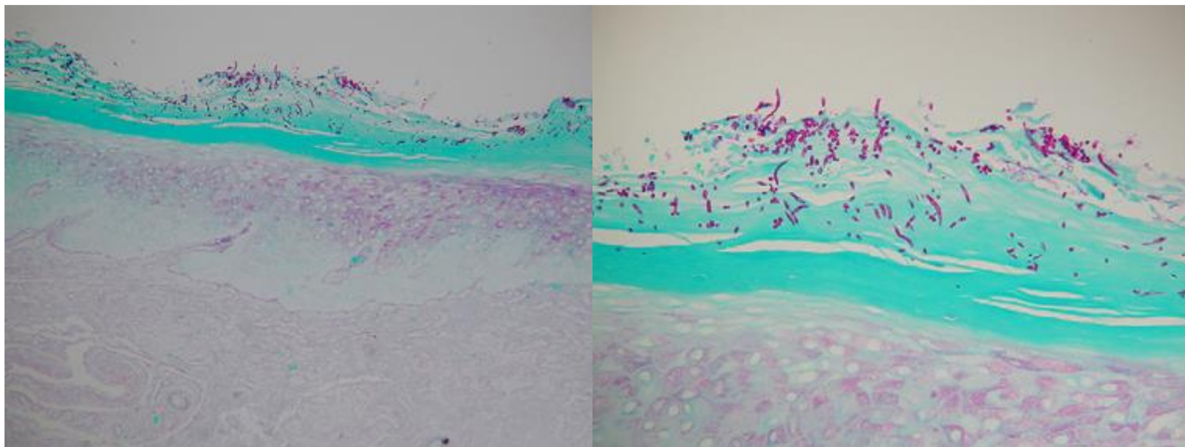
Diagnosis of oral candidiasis relies largely on recognition of its physical appearance. However, tests should be made to confirm the diagnosis and determine susceptibility to antifungals. This can be done using a microscope to see the fungal cells in a sample, or macroscopically with a fungal culture (3, 4).

Smear staining

For a microscopic test, a smear of the lesion is made. The smear can be stained in a number of different ways, and fixed to glass microscope slide with alcohol, or by simply air drying, before viewing under a microscope (3-5). Periodic acid-Schiff staining (PAS) can confirm the presence of hyphae in the case of pseudomembranous candidiasis, but usually not with other types (5). A potassium hydroxide (KOH) solution can also be used, with gentle heating, which causes the epithelial cells from the mucosa to dissolve, leaving a clearer view of the fungal cells (3, 4). Care should be taken when making a microbiological sample. The sampled area should be representative for the actual infection, but this might be difficult as the mouth looks quite different when open and closed. Areas that appear distant might actually be in constant contact when the patient closes their mouth (6). While the mucosa should be swabbed, an adjacent non-renewing surface will often carry a lot of extra organisms (6). A moistened wooden tongue blade can be used with good results (11).

Biopsy

In cases of hyperplastic candidiasis, or rather, in cases where it is suspected, a biopsy should be made along with the smear stain (3, 4). The mucosal tissue sample is placed in 10% formalin, before further processing, embedding in paraffin, and cutting (11). A thin section is placed on a glass microscope slide and stained (for example PAS), and can be examined under a microscope (11). In the case of a superficial fungal infection, the fungi can be found in the parakeratin layer, the outmost layer for the epithelium (11).

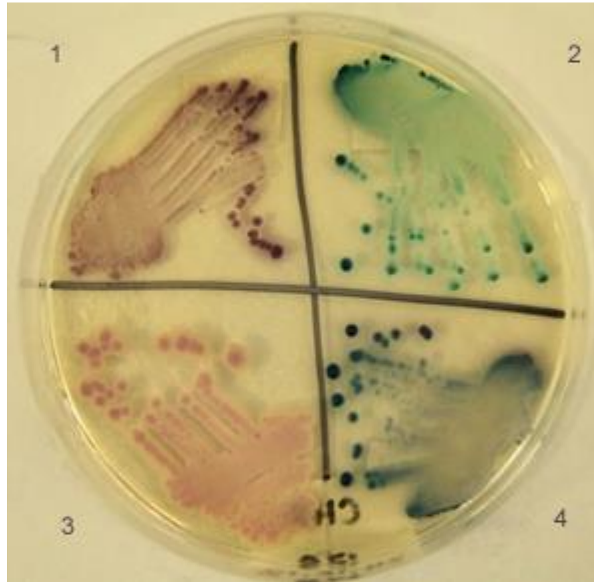


***C. albicans* within the parakeratin layer (PAS stain, hyphae are magenta in colour)**

From Giannini et al. 2011 (11)

Fungal cultures

Fungal cultures can be cultured on Sabouraud dextrose agar (SDA) representing a common selective fungal growth medium (3-5). White colonies appear and indicate a positive culture result, but it does not necessarily indicate infection (11). The cultures can be analyzed further for identification of the species and/or for testing of antifungal susceptibility (5). To determine sensitivity, or resistance, to an antifungal medication, fungal cultures with an Epsilometer test, or E-test, can be used (24). An agar with chromogenic substrate (chromagar) can be used with mixed-species samples, as the different species and their enzymes will react differently, their colonies will have various colours (3). For further identification of species, a method called *matrix assisted laser desorption ionization time-of-flight*, or MALDI-TOF, can be used. Quick diagnostic methods are increasingly important, and the MALDI-TOF method has gradually become both reliable and fast when used for diagnostic purposes. The method analyses proteins and other large molecules, and has been found to effectively discriminate between species of fungi (25).



Chromagar showing different *Candida spp*.[18]

1=*C. glabrata*; 2=*C. albicans*; 3=*C. krusei*; 4=*C. tropicalis*.

Differential diagnosis

As described above, hyperplastic candidiasis has the same appearance as leukoplakia, which has the potential to turn cancerous (26). A number of white and red lesions in the oral mucosa can have other causes than oral candidiasis (6). A common cold, or an allergy to oral care products can cause erythema, sore mouth, and nonspecific inflammation which appears similar to oral candidiasis (6). Mucocutaneus disease, such as oral lichen planus (5, 6), lichenoid reactions, discoid lupus erythematosus, erythema multiforme, pernicious anaemia, and epithelial dysplasia (5), can clinically resemble erythematous candidiasis. Oral lichen planus lesions can in addition be infected by *Candida*, and a study by Kragelund et al. found that non-*albicans Candida* were overrepresented in these cases (27). Gingivitis, periodontitis, and angular cheilitis can be caused by bacteria, *Candida*, or a combination of both. Several medications, such as methotrexate[19], can also cause redness in the oral mucosa (6). Chemical burns, traumatic lesions, and syphilis can cause a pseudomembrane similar to the one of pseudomembranous candidiasis (5).

[18] Photo from Leading International Fungal Education, http://www.life-worldwide.org/
[19] Cytostatic drug.

Treatment of oral candidiasis

Identifying the cause of infection for each individual patient is the first step in treating oral candidiasis. Correcting the risks can be simple or challenging, depending on their severity. In many cases it becomes necessary to involve the patient's physician, if the underlying condition is not so severe that they are involved from the start. The dentist's role can vary from curing the infection completely, to providing palliative care of the oral conditions.

Oral hygiene

In cases such as denture stomatitis, instructing the patient in proper oral hygiene is important (6, 13). If the patient is incapable of managing their own oral hygiene, their care takers must be instructed to do it for them. The initial treatment should include treating both the mouth and the denture with antifungal creams or ointments, and later the denture can be cleaned using soap, bleach or chlorhexidine[20] (4). Oral hygiene is important for immunocompromised patients, but complete recovery is not expected without some other form of treatment.

Ill-fitted dentures

As mentioned above, ill-fitted dentures can be a contributing cause of denture stomatitis (1). In addition to improving oral hygiene, regular relining of the denture is recommended for prophylaxis and treatment of denture stomatitis (28). Tissue conditioning is a temporary form of relining dentures, which provides the denture with a soft, cushioning base, allowing the mucosa to heal and normalize in shape (28). After about two weeks, sometimes in combination with topical antifungal therapy, the soft material can be replaced by a permanent relining, often preformed by the dental technician. Alternatively, an autopolymerising, hard reline material can be used (28). Relining dentures to improve the vertical dimension, after resorption of the alveolar bone, will also be helpful for treating angular cheilitis (13).

Hyposalivation

Treating hyposalivation is a part of treating fungal infection, and strategies include frequent hydration and saliva substitutes (4). *Pilocarpine* and *cevimeline* can be prescribed as they increase salivary flow (4), but they are not registered for use in Norway. If hyposalivation is caused by medication, the prescriber should be consulted in regards to finding an alternative (4).

[20] Chemical with antibacterial and antifungal properties. Suitable for disinfection of the mouth.

Steroid inhalers

If the use of steroid inhalers is a contributing cause of infection, the patient should rinse the mouth after use, or alternatively use a spacer device that causes less of the medication to end up in their mouth (4).

Immunosuppression

The most difficult patient to treat is the immunosuppressed patient. This could be a patient undergoing treatment for an autoimmune disease, receiving cancer treatment, suffering from HIV infection, living with a genetic disorder or any number of rare conditions that affect the immune system. Immunosuppression during cancer treatment is usually temporary, the greatest challenge being the resulting hyposalivation (4, 11). Patients with recurring oral candidiasis due to HIV infection should always be treated in collaboration with their physician. The prophylactic use of antifungal medicine in these patients is sometimes necessary, but can be problematic as it can lead to resistant organisms (4, 29).

Topical antifungal therapy

After the predisposing factors have been identified, and as they are being managed, the infection can be treated with antifungal medicine. The approved first line of treatment in *simple cases* of oral candidiasis is topical agents (4, 13, 14). They have few adverse effects in comparison to systemic treatment, and can be used in combination with systemic treatment as it lessens the dose and duration (13). The topical agents exist as oral suspensions, pastilles, creams, and tablets. In order for the medicine to have optimal effect, the patient should not rinse their mouth, eat or drink for at least one hour after use (4). There is large variety of these medications world-wide. In Norway, nystatin (Mycostatin), clotrimazole (Canesten, Klotrimazol), and miconazole (Daktar) are among the available choices.

Nystatin

Nystatin is widely used and comes in many different formulations, usually liquid suspensions, creams and pastilles, and its efficacy is largely dependant on contact with the oral mucosa or fungal cells (4, 22). It is poorly absorbed from the gastrointestinal tract, so it can be swallowed after oral use, but sometimes with the adverse effect of nausea, leading to vomiting and diarrhoea (10). It has a bitter taste, despite a high sugar content, and patient compliance can be difficult (7, 22). Nystatin is a polyene antifungal agent, and it works by binding to

ergosterol[21] in fungal cell membranes (10). The amphipathic molecule inserts itself into the cell membrane where it aggregates and forms pores, causing efflux of cations, and resulting in cell death (30).

Clotrimazole

Clotrimazole is an alternative to nystatin that is especially useful in angular cheilitis, due to its effect on both *Candida* and *Staphylococci* (7, 22). In Norway it is available as a cream. It rarely causes adverse effects when applied topically, but nausea, vomiting and local skin irritation may occur (7). Clotrimazole belongs to the imidazoles, a subgroup of azole antifungals (7). The molecule binds to the fungal cell wall, causing alterations to the permeability that result in cell death (10).

Miconazole

Miconazole is a broad spectrum antifungal agent that can be used both topically and systemically, though its systemic use has been stopped as there are less toxic alternatives (7). It is an azole drug, and it has antifungal and antibacterial properties. It has several mechanisms, one of which is inhibition of ergosterol synthesis (10). Miconazole should not be used in combination with the anticoagulant warfarin, as it can increase anticoagulant effect to a potentially dangerous level (4, 7).

Systemic antifungal therapy

Systemic antifungal therapy is preferred in severely immunocompromised patients, and the treatment is usually led by a physician. *Fluconazole* is the preferred agent (4). Fluconazole is absorbed well in the gastrointestinal tract and can be taken orally with good effect (13). Because of its long half-life, fluconazole tablets need only be administered once a day (4). It has some side effects, like nausea, headache, gastrointestinal discomfort and abdominal discomfort, but these are usually mild (7). Like miconazole, fluconazole disrupts the ergosterol synthesis in the fungal cell, causing the leakage in the cell membrane and cell death (13). Fluconazole is usually effective against *C. albicans*, but *C. glabrata* is frequently resistant.

[21] Molecule found in fungal cell membranes, with the same function as cholesterol in animal cells, namely maintaining proper membrane permeability and fluidity.

Invasive candidiasis – systemic infections

Like the oral cavity, *Candida spp*. can infect the skin, respiratory system, genitalia, and the rest of the gastrointestinal tract. These are all categorized as superficial infections. Invasive candidiasis is a term that excludes the superficial infections, and includes more severe conditions such as candidemia, endocarditis (affecting the heart), and meningitis (affecting the brain) (12). Patients in intensive care with complex medical and surgical disorders are especially at risk, with high prevalence, morbidity, and mortality (31, 32). These are, for example, patients with burns, neutropenia, HIV infection and pancreatitis (33). While there are many different antifungals available for treatment of invasive candidiasis, there is no early reliable diagnostic test. This is why targeted treatment might be difficult, and is increasingly accompanied by early treatment strategies, such as prophylactic antifungal treatment (29). However, the rise of non-*albicans Candida* involved in candidiasis might be attributed to wide use of azoles, to which *C. glabrata*, amongst others, have shown resistance (29). Selection of azole-resistant *Candida spp*. should always be a concern when prescribing antifungals.

Part 2: Molecular epidemiology of *Candida glabrata*

Molecular epidemiology (ME)

Epidemiology is the study of health and disease in a population, as well as the cause, patterns and effects of disease and death. Included in the term are all forms of health related topics and diseases. Epidemiological studies can determine the risk factors of various diseases, and is therefore of high importance to public health issues (34).

"I propose the following definition of ME, which clearly states its double goal:

> *(i) definition, identification and tracking of pathogen species, subspecies, strains, clones and genes of interest by means of molecular technology and evolutionary biology;*
>
> *(ii) evaluation of the impact of the genetic diversity of a pathogen on its relevant medical properties, such as pathogenicity or drug resistance (downstream studies)."*

– Michel Tibayrenc (35).

ME has the same aim as other branches of epidemiology, but the methods in use are on a molecular scale, looking at proteins, DNA, RNA, and other cell structures that can determine the cause and development of disease. Why is molecular epidemiology in particular important? When dealing with microorganisms, there are few phenotypic traits to look at, and an individual organism's pathogenicity can rarely be determined by appearance only. With methods in ME, we can now discriminate between strains based on their genetic variations, which will enable us to separate phenotypically identical strains.

Multilocus sequence typing

Multilocus sequence typing (MLST) was developed by Maiden et al. (36) for the pathogen *Neisserria meningitidis*[22] in 1998, but it has since then been applied to many different bacteria and fungi. MLST is a tool in ME used to genetically discriminate between strains on a sub-species level[23]. Previously used methods (1980´s and 1990´s) with the same purpose include pulse-field gel electrophoresis (PFGE) and multilocus enzyme electrophoresis (MLEE), which used electrophoretic gels to separate different strains.

[22] Bacteria that can cause meningitis.

[23] Separating the individual strains within a given species.

MLEE analyses the core metabolic, or 'housekeeping', genes, by looking at the differing electrophoretic mobilities of the gene products[24] (37). These genes are suitable for such studies because they typically evolve at a slow rate. They are essential for the cells survival, and thus, frequent mutations in these genes can cause the entire population to die. Genes associated with virulence typically evolve at an accelerated rate, as these are highly influenced by the cells environment (37).

MLST, like MLEE, uses housekeeping genes to distinguish between strains. While MLEE examines the cell's enzymes in order to study the genome, MLST looks at the DNA sequence of the corresponding genes directly. In MLST, 4-8 gene fragments are used, variations within the genes are assigned alleles, and variation in these alleles give each strain an allelic profile, or sequence type (ST) (37). The housekeeping genes utilized must not overlap, and instead be spread around the chromosome. It is important to have an ideal amount of diversity within the gene fragments, but whether it is a definite housekeeping gene or not, is sometimes a difficult distinction (37).

The DNA sequences themselves, their alleles, and sequence types can all be stored in an MLST database. The disadvantage of MLEE was that the results were difficult to compare between laboratories. MLST, however, does not face that problem, as the results are indexed directly by the nucleotide sequence, readily available for download from the Internet. While MLST is great at discovering single base mutation, it is insensitive to homologous recombination[25], in which large sections of DNA is rearranged (37).

In 2002, MLST was found to be very useful in subspecies characterization of *C. albicans* (38). The challenge of using MLST with *C. albicans* is that it is a diploid organism[26], and the presence of heterozygosity[27] can complicate the process. Despite this, many satisfactory results have been achieved, and an online global database was established.

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[24] The gene products are placed on a gel, and a current is run through it. This causes them separate by size and/or charge. The result can be visualized with colouring agents and a UV light.

[25] Large sections of DNA are moved, replaced, or switched, which happens during DNA repair, meiosis and horizontal gene transfer.

[26] Cells that have two homologous copies of each chromosome. Haploid is the counterpart, only having one set of chromosomes.

[27] The two chromosome copies in a diploid organism have different alleles.

MLST and *C. glabrata*

As described in Part 1, *C. glabrata*, formerly classified as *Toruplopsis glabrata* (39), has emerged as an important opportunistic pathogen, and is increasingly implicated in human infections. *C. glabrata* was thought to be commensal and a part of the normal flora in humans, but has been found to be prevalent in systemic infections and is associated with high mortality (20). *C. glabrata* is the second or third most commonly isolated *Candida spp.* from reported cases of candidiasis, depending on the site of infection (20). It is the most prevalent non-*albicans Candida* in humans (40).

In 2003, Dodgson et. al. developed an MLST scheme for *C. glabrata* (41). They found 6 loci (*FKS, LEU2, NMT1, TRP1, UGP1* and *URA3*, see table 2) that were variable enough to produce 30 STs among 109 strains. They tested 9 additional loci, but it did not increase discrimination. The amount of variable sites was similar to what had been previously found in *C. albicans*. The system for *C. albicans* seems to be more discriminating than the one for *C. glabrata*, which can be owed the fact that *C. albicans* is diploid, while *C. glabrata* is haploid (41).

In addition to creating the MLST scheme for *C. glabrata*, they identified 5 major clades among the isolates, 3 of which exhibited significant geographical bias (41). They also looked for an association between fluconazole resistance and ST, but found no correlation. In a later study (42), they used MLST and found some occurrences of recombination in *C. glabrata*, which has a predominantly clonal population structure[28]. This was later supported by Lott et. al. (43), who used MLST to find evidence for both recombination and clonality in the species.

[28] No horizontal gene transfer, see below.

Locus	Gene product	GeneBank accession no.	Primer	Primer sequence (5'-3')	Annealing temp. (°C)
FKS	1,3-Beta-glucan synthase	AF229171	FKS F1[a] FKS R1[b]	GTCAAATGCCACAACAACAACCT AGCACTTCAGCAGCGTCTTCAG	55.0
LEU2	3-Isopropylmalate dehydrogenase	U90626	LEU2F1 LEU2R1	TTTCTTGTATCCTCCCATTGTTCA ATAGGTAAAGGTGGGTTGTGTTGC	54.0
NMT1	Myristoyl-CoA, protein N-myristoyltransferase[c]	AF073886	NMT1F1 NMT1R1	GCCGGTGTGGTGTTGCCTGCTC CGTTACTGCGGTGCTCGGTGTCG	59.0
TRP1	Phosphoribosyl-anthranilate isomerase	U31471	TRP1F1 TRP1R1	AATTGTTCCAGCGTTTTTGT GACCAGTCCAGCTCTTTCAC	50.0
UGP1	UTP-glucose-1-phosphate uridylyltransferase	AB037186	UGP1F1 UGP1R1	TTTCAACACCGACAAGGACACAGA TCGGACTTCACTAGCAGCAAATCA	57.0
URA1	Orotidine-5'-phosphate decarboxylase	L13661	URA3F1 URA3R1	AGCGAATTGTTGAAGTTGGTTGA AATTCGGTTGTAAGATGATGTTGC	53.0

Table 2: Oligonucleotide primers for amplification and sequencing of loci used in MLST scheme. Table 2 from Dogdson et. al.(41)
[a]Forward primer
[b]Reverse primer
[c]CoA, coenzyme A.

Material and study design

As mentioned in the preface, my thesis is part of a much bigger project, using more than 250 strains of *C. glabrata*. My part consists of 15 strains, and was chosen randomly after the majority of the 250 strains had their DNA extracted, amplified and sequenced. All the samples were gathered from human hosts, but sites and indications vary (see table 3). The strains are assigned numbers according to their place in the original project.

Strain	Source	Strain	Source
122	Venous catheter needle	**121**	Intestinal anastomosis
133	Blood	**143**	Intestinal aspirate
134	Tracheal secretion	**144**	Oesophagus
139	Blood	**155**	Oral
145	Expectorate		
146	Blood		
148	Blood		
152	Blood		
156	Pharyngeal secretion		
159	Pleura		
161	Thorax		

Table 3: The 15 strains and their source. On the right are strains gathered from systemic infections, blood and respiratory organs. On the left are strains gathered from the oral cavity, as well as other locations in the gastrointestinal tract.

Methods: PCR, DNA sequencing and MLST

Polymerase chain reaction (PCR)

PCR is a highly efficient method of amplifying specific DNA segments. In a short amount of time, millions of copies can be synthesized. The process is done in vitro, excluding bacteria from the method. The desired DNA segment is denatured and annealed[29] with tailored primers (see table 2), and the reaction cycles through different temperatures, allowing the specific reactions to occur. Every round each strand of DNA doubles, causing exponential accumulation of the defined segment. PCR has simplified how molecular studies are done, and many additional uses have been discovered since its introduction in 1985 (44).

In this study, we used the primers defined by Dodgson et al. (41) to amplify the specific loci we needed for the MLST. If the sequence is known, primers can be synthesized exactly to fit the segments we are looking for. Commercial laboratories can provide researchers with custom primers, and usually they can be ordered and received in a few days.

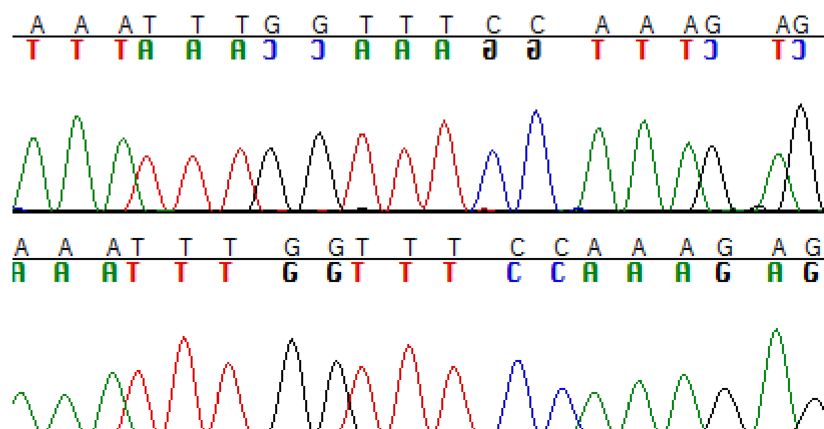
[29] Breaking the bonds between the two strands of the DNA molecule, and then recreating the bonds with a primer.

Once the DNA was extracted from the different strains of *C. glabrata*, we could begin amplifying the six different segments. Some care had to be taken in establishing the best protocols[30] for the PCR, and we found that the different primers needed different conditions for optimal reactions. The quality of the amplified sequences was examined using gel electrophoresis, which was also a useful tool in finding the best protocols.

DNA sequencing

There are many different methods of sequencing DNA, but the purpose is to determine the order of nucleotides within a selected DNA strand. This process has countless uses, and in this study our aim was to determine the specific sequence at each loci of each *C. glabrata* strain, so we could use that information for the MLST.

Using Sequencher[31], DNA sequence analysis software, the forward and reverse sequence for each strain and each locus were assembled and corrected.



Example of a Sequencher electropherogram[32]

MLST

The finished sequences were then typed, using the MLST database to match the sequence to its allele. The alleles from each locus were then matched up to the established STs.

[30] The temperature of each step in the process, as well as the time it remains at each temperature.
[31] Sequencher by Gene Codes Cooperation. See http://www.genecodes.com/ for more information.
[32] Visual representation of a DNA sequence in sequencing software after electrophoresis automatic sequencing.

Results and discussion

Strain	FKS	LEU2	NMT1	TRP1	UGP1	URA3	ST
121	7	7	11	10	New allele - A	9	New ST - A
122	3	9	26	4	New allele - C	4	New ST - C
133	5	7	8	7	3	6	3
134	3	9	26	4	New allele - C	4	New ST - C
139	5	7	8	7	3	6	3
143	3	9	26	4	New allele - C	4	New ST - C
144	3	9	26	4	New allele - C	4	New ST - C
145	5	7	8	7	3	6	3
146	3	4	4	3	3	4	7
148	5	7	8	7	3	6	3
152	3	9	26	4	New allele - B	4	New ST - B
155	1	2	2	1	2	1	8
156	1	2	2	1	2	1	8
159	3	9	26	4	New allele - C	4	New ST - C
161	12	4	16	5	1	8	25

Table 4: The strains with their alleles and STs. New alleles were assigned a letter (A, B and C), as were the resulting new STs. The ST can only be determined once the alleles for each locus are found.

In the larger project, the MLST results can be used to analyse the genetic diversity of *C. glabrata*, but the 15 strains I worked with are not sufficient to draw any conclusions. I will, however, discuss some of the possible results from these types of studies in molecular epidemiology.

Locus	Fragment length (bp)	No. of alleles	No. of alleles (previously registered)*	No. of polymorphic sites	% variable nucleotide sites
FKS	589	5	25	8	0,01
LEU2	512	4	18	5	0,01
NMT1	607	6	34	15	0,03
TRP1	419	6	23	9	0,02
UGP1	616	6	13	9	0,02
URA3	602	5	20	8	0,01

Table 5: The characteristics of each locus. The fragment length shows the amount of basepairs (bp) in each locus. No. of alleles is the amount of different alleles found in this project, and this is compared to the amount of alleles in the MLST database. No. of polymorphic sites shows the amount of polymorphic sites within each locus, and this is used to calculate the percentage of variable nucleotide sites[33].

*from http://cglabrata.mlst.net/, cited 22.3.14

New alleles and sequence types

In studies with a higher number of strains (>1000), one can expect to find new variations in the genes that have not previously been registered in the MLST database. Table 5 shows the amount of alleles for each locus previously registered.

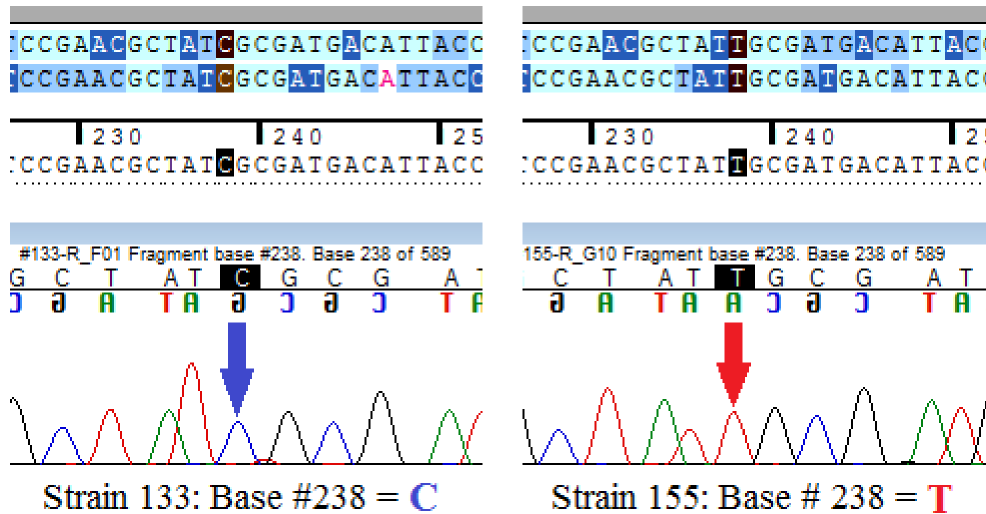
Out of the 15 strains, there were *3 new alleles* not yet registered, and they were all in the *UGP1* gene (see table 4). They were temporarily named A, B and C. A only differed from a previous allele (UGP1-5 allele) in one polymorphic site. B also differed from a previous allele (UGP1-3 allele) in one site. C's closest match was also UGP1-3 allele, but the one differing site was not the same as B.

C. glabrata has 70 STs previously registered. In these 15 strains, 7 STs were found, 3 were new (see table 4). The new alleles in UGP1 made up 3 new STs, also named A, B and C. A was closest to ST 16. B and C were close matches with each other, but not to any previous ST.

The percentage of variable nucleotide sites is quite low for all the loci (0,01-0,03 %, see table 5). Dodgson et al. (41) found 1,3-3,5 % variable nucleotide sites in their study with 109 isolates of *C. glabrata*.

[33] The location of a base that varies from strain to strain.

FKS



Example of a polymorphic site: In the FKS locus, base 238 of 589 is a polymorphic site. In strain 133 this is a C (blue) and in strain 155 this is a T (red).

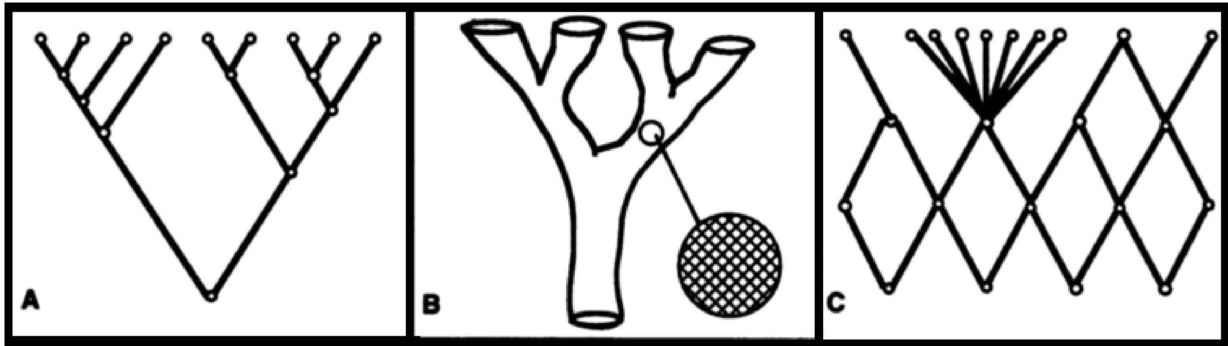
Geographical distribution

When large amounts of strains are gathered from different continents, it is possible to see if the species' genetic diversity is linked to their physical location in the world. MLST is particularly useful for this purpose, as data from all over the world is comparable. Dodgson et al. found that *C. glabrata* has distinct genetic clades[34] that are more common in different geographical regions (41).

Population structure

A population of microbes can have different sets of genetic population structures. It was previously believed that bacteria adhere to a *clonal* population structure (45). This means that one individual is the source of all genetic material in each descendant, and that any variation is the result of mutations within each individual. See Fig.1 A below. Alternatively, a *panmitic* population structure allows horizontal gene transfer between individuals, see Fig. 1 B. This represents a complete meshwork of exchange of genetic information, and the ancestors will be much more difficult to track than in clonal populations (45). The third population structure is the *epidemic* where genetic exchange is swapped freely in an underlying pandemic situation with the rise of branches of purely clonal complexes on top, which can be responsible for epidemic disease outbreaks.(see Fig. 1 C) (45).

[34] A group of individual organisms within a species, the ancestor and all its descendants.



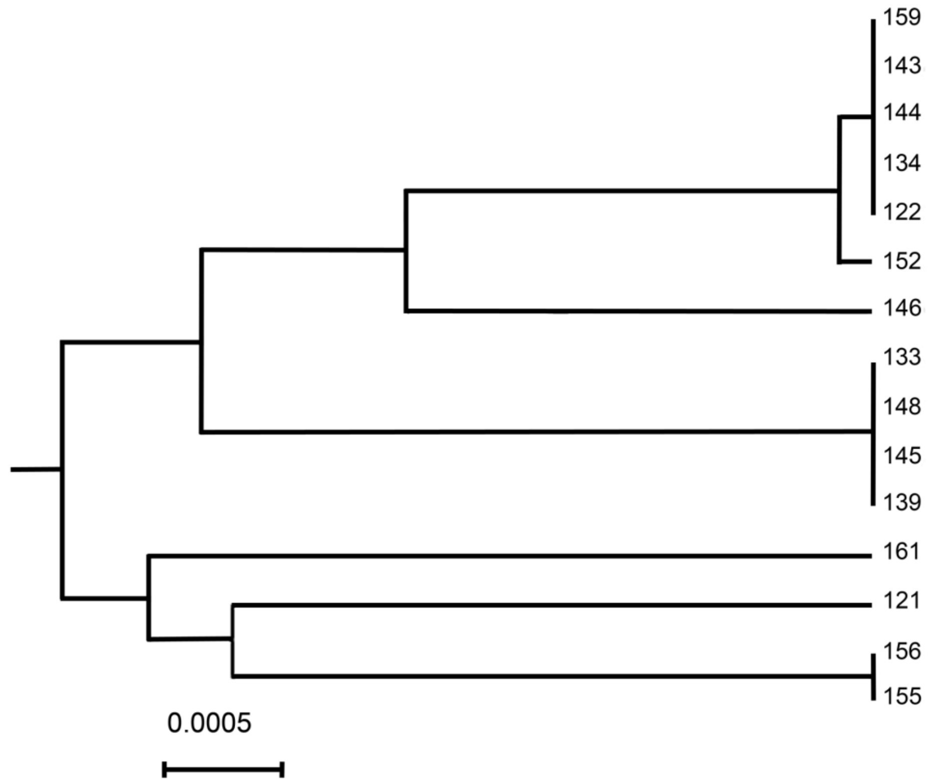
Models of different population structures

Fig. 1 from Smith et al. 1993 (45)

Calculations can be done to determine the likely population structure of a species. The index of association (I_A) is one parameter, developed by Brown et al. (46) in 1980 for the study of wheat. With a larger number of strains, it is possible to determine if *C. glabrata* has a clonal population structure. Dodgson et al. (42) and Lott et all. (42) both concluded that, although *C. glabrata* has a mainly clonal population structure, recombination most likely also plays a role.

Phylogenetics

A *phylogenetic tree*, or *dendrogram*, is a diagram showing the relationship between different species, or different strains within a species, based on their genetic determinants. In my project, the STs decide how the strains are related to each other based on concatenated sequences of 6 genes (MLST) of the *C. glabrata* strains investigated.



Phylogenetic tree shows the relationship between different strains of *C. glabrata*

In the coming years, more information will be available as methods in molecular epidemiology are under constant development. This will provide us with further understanding and clarity when studying the world of microorganisms.

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