1. INTRODUCTION

Candida is a genus of opportunistic yeasts, unicellular fungi that can cause oral, vaginal, lung, and sometimes systemic infections (Madigan and Martinko, 2006). *Candida* species that inhabit the oral cavity are usually harmless members of the microflora, but when they become pathogenic they manifest as Oropharyngeal Candidiasis (OPC). The most common form of OPC is pseudomembranous OPC, also known as *oral thrush*, in which white thick plaque forms in the oral cavity, causing mouth soreness, burning tongue sensation and taste changes. Sites affected include the buccal mucosa, tongue and palate. Erythematous or atrophic OPC is a less common form of OPC, which exhibits as a redness of the palate and tongue, and in addition may cause a feeling of metallic taste or burning (Samaranayake and Yaacob, 1990).

Correct and accurate identification of the candidal species infecting an oral candidiasis patient is highly important, as different antifungal agents are effective against different candidal species (Ellepola et al., 2003). Typical phenotype-based methods for distinguishing between candidal strains are sometimes not useful for diagnostic purposes because they differentiate some strains without any correlation to pathogenesis, or cannot make distinctions between epidemiologically dissimilar strains (Hunter and Fraser, 1989). In addition, candidal infections are typically treated with azole antifungal drugs, mainly fluconazole, however many infections are caused by non-*albicans Candida* (NAC) species that may have fluconazole resistance (Niimi et al., 1999). Some of the more common pathogenic NAC species include *Candida krusei*, which is resistant to fluconazole but sensitive to amphotericin B, ketoconazole and itraconazole, and *Candida tropicalis*, which has resistance to amphotericin B and fluconazole (Kremery and Barnes, 2002). Meanwhile *Candida dubliniensis* has many

phenotypic similarities to *Candida albicans* but has been reported to have fluconazole resistance (Sullivan et al., 2005). With the emergence of resistance to antifungal drugs (Mirhendi et al., 2006) it becomes of even greater importance to evaluate an antifungal drug's ability to combat various strains.

It is hoped that this research will shed more light on the microorganisms involved in oral disease pathogenesis, in addition to providing clinicians with more diagnostic tools for combating oral infections.

1.1 Research Objectives

1. To determine and compare the microbial loads of oral *Candida* species from periodontal patients, denture wearers with healthy oral cavity, and non-denture wearers with healthy oral cavity.
2. To differentiate oral *Candida* sp. based on genotype, in particular the characteristics of rDNA.
3. To assess the effectiveness of using candidal rDNA for identification of candidal species

2. LITERATURE REVIEW

2.1 Microbial Ecosystem in the Oral Cavity

The oral ecosystem is defined as being composed of oral microorganisms and the oral cavity (Theilade, 1990). The saliva that constantly bathes the oral cavity ensures that it is always moist, is stable at a warm temperature of about 34-36 °C, and is maintained at a mostly neutral pH of 6.75-7.25, which makes the oral cavity an ideal habitat for the growth of microorganisms (Marsh, 2003). Because of this, the oral ecosystem supports a wide range of microorganisms, comprising of numerous bacteria, yeasts, protozoa and mycoplasmas (Marcotte & Lavoie, 1998). In comparison to other microbial habitats in the human body such as the skin or the gastrointestinal tract, the oral cavity is one of the most densely populated with more than 500 microbial species isolated (Takahashi, 2005), and the microbial community is also considered relatively stable, with comparatively fewer differences between individuals (Costello *e*t al., 2009).

2.1.1 Factors Influencing the Microbial Ecosystem of the Oral Cavity

The growth of microorganisms in the oral cavity is influenced by many factors, including temperature, pH, oxidation-reduction potential, nutrient content, water availability, oral structures, salivary flow and the host immune system (Marcotte & Lavoie, 1998). Any alterations of these factors can upset the microbial homeostasis of the oral cavity, causing radical changes to the oral ecosystem, which in turn may have adverse effects on the oral health of the host. It has been suggested that changes in the oral environment can trigger a shift in the resident microflora, in which potentially pathogenic species that were previously not clinically significant become more

competitive under the new conditions. Hence, the selection of pathogenic microorganisms over non-pathogenic microorganisms in the oral microflora leads to the onset of periodontal disease (Marsh, 2003).

2.1.1.1 Temperature and pH

The oral cavity is maintained at a constant temperature of 35-36 °C and is bathed in saliva that regulates the pH at a mostly neutral level, all of which provides stable conditions for the growth of various microorganisms. However, changes in these environmental conditions can have drastic effects on the oral microflora. Small increases in temperature may have significant impacts on bacterial gene expression as well as the competitiveness of certain microorganisms. Meanwhile, exposure to low pH can inhibit or kill predominant plaque bacteria associated with healthy sites, resulting in increased colonization by acid-tolerant species such as *Lactobacillus* and mutans streptococci. Conversely, an increase in pH at the gingival crevice, which occurs during the host inflammatory response to periodontal disease, predisposes the site to the increased growth of pathogenic anaerobes such as *P. gingivilis* that grow optimally at pH 7.5 (Marsh and Martin, 2009).

2.1.1.2 Nutrients

The main source of nutrition for microorganisms in the oral cavity is saliva, which contains amino acids, peptides, proteins and vitamins. In addition, host diet can also influence the microbial ecology of the mouth. A high carbohydrate intake is associated with shifts in microbial populations of the dental plaque, predisposing a site to dental caries. Meanwhile, dairy products can have a protective effect against caries,

and nitrate from green vegetables can inhibit the growth of bacteria implicated in periodontal disease (Marsh and Martin, 2009).

2.1.1.3 Antimicrobial Factors

Saliva contains a variety of antimicrobial factors that act to suppress or remove harmful microorganisms from the oral cavity. Mucins are responsible for agglutinating oral bacteria, facilitating their removal from the mouth through swallowing. Lysozyme can aggregate Gram positive bacteria as well as Gram negative periodontal pathogens, in addition to hydrolyzing peptidoglycan, an important component in bacterial cell walls. Lactoferrin is a non-specific antimicrobial factor that is known to have bacteriostatic, bactericidal, fungicidal and anti-viral properties. Saliva also contains antimicrobial peptides such as histatins, defensins and cathelisidins. The major histatins found in saliva include histatin 3, which is effective at inhibiting yeast germination and histatin 5, which is comparatively more active at killing germinated yeast cells. There are two types of defensins found in the oral cavity: α-defensins, which are found primarily in neutrophils and β-defensins, which are found mainly in epithelial cells protecting mucosal surfaces. Other salivary antimicrobial agents include chitinase, which is implicated with breaking down yeast cell walls, cystatins, that control proteolytic activity, and chromogranin A, that have anti-fungal and anti-yeast properties (Marsh and Martin, 2009).

2.1.2 Oral Microbial Habitats

Different microorganisms have specific cell surface adhesins that bind to complementary specific receptors that can be found on different surfaces. Because of

this, oral microorganisms preferentially colonize different surfaces in the oral cavity, leading to each oral site having its own distinct population of microbial inhabitants (Gibbons, 1989).

2.1.2.1 Saliva

Saliva influences the oral microbial population in many ways. Saliva facilitates the colonization of certain microorganisms by adsorbing on to oral surfaces and forming an acquired pellicle layer that provides complemenary receptors for microbial attachment. Furthermore, saliva provides a rich nutrient source for microbial growth. On the other hand, saliva also plays a prominent role in removing and inhibiting microorganisms. Salivary factors aggregate bacteria in the mouth for removal by salivary flow and the physical act of chewing. Saliva also inhibits the growth of certain microorganisms through antimicrobial factors such as lysozyme, lactoferrin and secretory IgA. As such, the rate of salivary flow is an important factor for the overall microbial colonization of the oral cavity, as low flow rates reduce the protective function of saliva and increases the colonization and development of microorganisms in the oral cavity (Marsh and Martin, 2009).

Microorganisms found in the saliva are derived from microorganisms that have been dislodged from other oral sites (Marcotte & Lavoie, 1998). In an oral cavity colonized with *Candida* sp., the average concentration of oral yeasts in the saliva has been reported to be about 300-500 cells per ml (Cannon & Chaffin, 2001).

2.1.2.2 Gingival Crevice

The interface between a tooth and its surrounding gingival epithelium forms a crevice, also known as the gingival sulcus, that microorganisms can colonize. The microbial habitat of the gingival crevice is greatly influenced by gingival crevicular fluid (GCF), a serum-like fluid that flows into the mouth through the junctional epithelium of the gingivae. GCF removes non-adherent microbial cells and also introduces certain nutrients and host defence components such as IgG and leukocytes that regulate the microflora of the gingival crevice (Marsh and Martin, 2009).

2.1.2.3 Mucosal Surfaces

The mucosal surfaces of the palate and cheek are sparsely colonized by local microbes, with not more than 25 colony-forming units (CFUs) per epithelial cell (Theilade, 1990). The low microbial load is due to the frequent shedding, or desquamation, of the mucosal epithelial cells. However, certain specialized surfaces, such as the keratinized stratified squamous epithelium of the palate, can influence the microbial distribution of the oral cavity (Marsh and Martin, 2009). *Streptococcus mitis* and *Gemella hemolysans* have been found to be the predominant bacterial species of the buccal mucosa, while *S. mitis*, *S. infantis*, *Gemella hemolysans*, *Granullicatella elegans*, *Granulicatella adiacens* and *Neisseria subflava* are the predominant bacterial species of the palate surface (Aas et al., 2005).

2.1.2.4 Tongue

The surface of the tongue consists of saliva-coated desquamated epithilium for microbial adhesion, while the source of nutrition for microbes adhering to the papillary surface of the tongue is likewise derived from the saliva as well as the tongue epithilium (Takahashi, 2005). In comparison to other oral sites, the tongue surface has a high bacterial density, about 100 CFU per epithelial cell (Bowden et al., 1979). A wide range of bacteria can be found colonizing the surface of the tongue, including *Actinomyces* sp., *Streptococcus* sp., *Veillonella* sp., *Fusobacterium* sp. and *Prevotella* sp. (Takahashi, 2005), though several *Streptococcus* species such as *S. mitis*, *S. parasanguinis* and *S. salivarius* have been found to be the most predominant bacterial flora (Aas et al., 2005). A study comparing the bacterial flora of the tongue of healthy subjects and that of halitosis subjects revealed that *S. salivarius* was nearly absent amongst subjects suffering from halitosis, suggesting that there is a significant difference in microbial populations on the tongue surface between healthy and non-healthy individuals (Kazor et al., 2003). The presence of periodontal pathogens such as *P. gingivalis*, *E. corrodens* and *oral spirochetes* (Lee et al., 1999) suggests that the tongue may be a reservoir for microorganisms involved in periodontal diseases (Van der Valden et al., 1986).

2.1.2.5 Teeth Surfaces

Microorganisms can usually be found colonizing teeth surfaces in the form of dental plaque (Marcotte & Lavoie, 1998). Pyrosequencing of the dental plaque of healthy adults has revealed it to be comprised of about 1000 microbial phylotypes (Keijser et al., 2008).

2.1.2.6 Prosthodontic Applicances

The presence of dentures provides a low oxygen, low pH microenvironment that is conducive for the growth of *Candida* sp. Furthermore, acrylic dentures may provide an enhanced adherence surface in addition to reducing salivary flow. They have been found to predispose as many as 65% of elderly people wearing full upper dentures towards candidal infection (Akpan & Morgan, 2002).

2.2 Oral *Candida* Species

One member of the oral microflora is oral yeast, which is also known to sometimes become orally pathogenic due to its opportunistic characteristics. However, this does not mean *Candida* species are always pathogenic, in fact approximately 25-50% of healthy individuals have *Candida* species as part of their normal mouth flora, without suffering from any adverse effects (Odds, 1988).

2.2.1 Characteristics of *Candida* Species

Other than a few exceptions, *Candida* sp. have similar macroscopic and microscopic cultural characteristics. Candida is a yeast with a nuclear pore complex within the nuclear membrane, and a plasma membrane that contains large quantities of sterols. They are capable of both aerobic and anaerobic metabolisation of glucose, and require fixed carbon from environmental sources for growth (Lehmann, 1998).

2.2.2 Candidal Colonization of the Oral Cavity

Candida sp. are ubiquitous members of the oral microflora, and need to adhere to oral surfaces in order to be sustained in the oral cavity. The binding of candidal cells to oral surfaces is mediated by adhesin molecules, most of which are glycoproteins that are present in the fungal cell wall. Oral surfaces are covered by a salivary pellicle layer, consisting of salivary components that have been adsorbed by the oral surface from saliva, and oral yeasts adhere to oral surfaces by binding to these adsorbed salivary molecules (Cannon, 2001). Different conditions in the oral cavity may increase or decrease the prevalence of candidal colonization (Ryan, 1994).

2.2.2.1 Interaction with Bacterial Flora

In general, oral *Candida* species interact with local indigenous bacterial microflora in many ways, including competition for common nutrients, association with metabolic as well as toxic byproducts, and alterations of the microenvironment (Samaranayake, 1990).

2.2.2.1.1 Negative Interaction

Local bacterial microflora are known to negatively impact the prevalence of *Candida albicans* by competing for epithelial cell adherence sites (Samaranayake, 1990). Oral bacteria have also been reported to inhibit candidal hyphal phase transformation, which is associated with candidal invasion of the epithelium and pathogenesis (Nair, 2001). This inhibition could be caused by the production of butyric acid, an anti-inflammatory short-chain fatty acid (Bohmig, 1997) that can inhibit

candidal hyphal transformation (Hoberg, 1983) and is produced in large quantities as a by-product of lactic acid bacterial fermentation (Hove, 1994).

2.2.2.1.2 Positive Interaction

Positive relationships between *Candida* and pathogenic bacteria have also been observed, in particular the synergistic association between *Candida albicans* and *Staphylococcus aureus* (McFarlane, 1990).

2.2.2.2 Denture Wearers

Oral candidal colonization has been shown to be higher in denture wearers compared to non-denture wearers by 60-100% (Pires et al., 2002). An important factor in the pathogenesis and infection of *Candida* sp. in the oral cavities of denture wearers is the ability of the yeast to adhere to the acrylic resin and soft lining material surfaces of the denture (Waters et al., 1997). Studies have demonstrated that *Candida albicans* adheres more easily to soft lining materials compared to acrylic surfaces, and that retention is also higher on rougher surfaces compared to smooth surfaces (Radford et al., 1998).

2.2.2.3 Periodontal Disease

Periodontal diseases are inflammatory diseases that affect the periodontium, the tissue that supports the teeth (Slots & Rams, 1992). *Candida* sp. are an aerobic species that grow optimally at neutral to acidic pH (Odds, 1988), and require sufficient sugar supplies, primarily glucose, in order to live (Samaranayake et al., 1986). In contrast, the

organisms that are the most implicated with periodontal disease, gram-negative anaerobic microbes (Haffajee and Socransky, 1994), generally thrive in and create environments that have low oxygen tension (Loesche et al., 1983) and low pH (Eggert et al., 1991). In addition, the majority of anaerobic bacteria are dependent on nutrients such as proteins, glycoproteins and amino acids (Samaranayake et al., 1986), instead of sugars. Consequently, *Candida* sp. and periodontal anaerobic bacteria can be said to occupy different ecological niches and may thus thrive under different environmental conditions.

In further support of this, it has even been demonstrated that anaerobic oral microflora can inhibit the growth of yeasts (Kennedy, 1981). However, about 20% of patients suffering from adult-stage periodontitis are reported to be host to oral yeasts (Dahlen and Wilkstrom, 1996), the majority of which are *Candida albicans* species (Hannula et al., 1997). Thus far it is unknown whether any *Candida* species are involved in the development of periodontal disease.

2.2.2.4 Host Diet

Host diet can also be a factor in the growth of oral *Candida* species. Candidal growth in the saliva is enhanced by the presence of glucose, and a high carbohydrate diet can also enhance candidal adherence to oral epithelial cells (Ohman & Jontell, 1988).

2.2.2.5 Other Influencing Factors

A study has shown that *Candida albicans* prevalence is higher in the saliva of diabetic patients as well as in that of patients treated with antibiotics and corticosteroids (Knight and Fletcher, 1971). Thus, alterations in the microbial flora as a result of hormonal changes, illness, and medical treatment could have a significant effect on candidal colonization (Rogers and Balish, 1980).

2.2.3 Pathogenesis of Oral *Candida* Species

Oral *Candida* sp. are typically harmless, and only become pathogenic in certain situations, such as under conditions that allow them to increase their relative proportion to other members of the local flora (Ryan, 1994). Other predisposing factors to oral *Candida* infection include radiation therapy, iron deficiency, endocrine disorders and a compromised immune system (Scully et al., 1994).

2.2.3.1 Candidal Factors

The factors that are implicated during the initial stages of candidal infection are candidal adhesion to epithelial cell walls, which is promoted by several fungal cell wall components such as C3d receptors, mannoprotein, mannose and saccharins, the ability to bind to host fibronectin, and the degree of hydrophobicity. Other factors that influence candidal pathogenesis include endotoxins, proteinases, mycelia, germ tube formation, tumor necrosis factor induction and persistence within epithelial cells (Akpan & Morgan, 2002).

2.2.3.2 Salivary Secretion

Salivary flow rate is an important factor in oral candidal pathogenesis, as the secretion of saliva removes organisms from the mucosa, in addition to saliva containing antimicrobial proteins such as lysozyme, lactoferrin, sialoperoxidase and specific anticandida antibodies. Thus, impaired salivary gland function and any condition such as radiotherapy and Sjogren's syndrome that inhibits salivary secretion can result in higher risk of oral candidiasis (Peterson, 1992).

2.2.3.3 Denture Stomatitis

The high prevalence of *Candida* species in the oral cavities of denture wearers is often associated with denture-induced stomatitis, also known as denture sore mouth. Denture stomatitis is symptomized by red sores in the mucosal surface of denture-bearing tissue. (Budtz-Jorgensen et al., 1975). A study conducted in the United States concluded that one in three people who wear removable dentures have denture stomatitis (Shulman et al., 2005), while another study has shown that about one in two full upper denture users suffer from candida-associated denture stomatitis (Cannon, 1990).

2.2.3.4 Drugs

Drug therapy has also been shown to be a predisposing factor for oral candidiasis as drugs may suppress cellular immunity and phagocytosis. Broad spectrum antibiotics can have an impact on the local oral microflora, altering the environment so that it is more suitable for candidal proliferation. Furthermore, immunosuppressive

drugs can predispose to oral candidiasis by disrupting the mucosal surface and changing the character of the saliva (Akpan & Morgan, 2002).

2.2.4 Candidal Species of Importance

Among the candidal species of importance and growing emergence as prominent pathogens include *Candida albicans, Candida tropicalis, Candida krusei, Candida parapsilosis, Candida dubliniensis, Candida glabrata* and *Candida lusitaniae.*

2.2.4.1 *Candida albicans*

Candida albicans has long been established as the most common oral candidal species, constituting 34-85% of the yeast species isolated from the oral cavity (Odds, 1988), and can be isolated from the oropharynx of over 40% of normal individuals (Kleinegger, 1996). *Candida albicans* has also been considered the predominant species responsible for candidal infection (Pfaller et al., 2006), however recently there has been a global trend of decreasing rates of *Candida albicans* isolation (Pfaller et al., 2005).

2.2.4.2 *Candida tropicalis*

Candida tropicalis infections have been reported in 4% to 24% of candidemia patients (Pfaller and Diekema, 2007). *Candida tropicalis* is also considered a major cause of invasive candidiasis in cancer patients (Wingard, 1995).

2.2.4.3 *Candida krusei*

After *Candida albicans*, *Candida krusei* is the second most common oral *Candida* species, amounting to as much as 30% of oral yeast isolates (Odds, 1988). In addition, the occurrence of *Candida krusei* in infections has been seen to be increasing (Samaranayake and Samaranayake, 1994), and the latest findings also indicate that *C. krusei* has mutated and acquired echinocandin resistance, leading to a greater level of pathogenesis (Kahn et al., 2007).

2.2.4.4 *Candida parapsilosis*

The incidences of *Candida parapsilosis* have been on the rise, and *Candida parapsilosis* has been reported to be the second most common candidal species isolated from blood cultures (Trofa et al., 2008). It is also the most common species found on the hands of health care workers (Strausbaugh et al., 1994).

2.2.4.5 *Candidia dubliniensis*

As *Candida dubliniensis* shares many phenotypic characteristics with *Candida albicans*, distinguishing between these two closely-related species has been historically problematic (Pincus et al., 1999). However molecular analysis shows that *Candida dubliniensis* and *Candida albicans* are dissimilar by 13 to 15 nucleotides in the ribosomal RNA sequences (Sullivan et al., 1995).

2.2.4.6 *Candida glabrata*

Recently, *Candida glabrata* has emerged as one of the leading candidal pathogens. Reports indicate that *Candida glabrata* colonization and infection is rare in infants, but increases significantly with age (Pfaller et al., 2006). *Candida glabrata* is also known for having reduced susceptibility to fluconazole (Malani et al., 2005).

2.2.4.7 *Candida lusitaniae*

Candida lusitaniae is known for its amphotericin B resistence (Blinkhorn et al., 1989) and is now considered an emerging non-*albicans Candida* (NAC) pathogen (Krcmery and Barnes, 2002).

2.3 Methods for Differentiating *Candida* Species

2.3.1 Serotyping

Serotyping, in which whole cells of *Candida* sp. are agglutinated with rabbit antisera, produces only two distinct serotypes, which is not useful for diagnosis, and in addition is unreliable because different methods of serotyping can produce varying results (Brawner et al., 1992). As many as four resistogram methods of strain-differentiation of *Candida albicans* have been developed, but resistogram typing has been shown to have no correlation with pathogenesis, and there have been problems because of interpretation and reproducibility (Hunter & Fraser, 1989).

2.3.2 Biotyping

An established system for biotyping intraspecific candidal strains combines three biotyping tests, the API ZYM, API 20C and boric acid resistance tests. API ZYM revolves around biotyping candidal isolates based on the presence of five enzymes: valine arylamidase, phosphoamidase, alpha-glucosidase, beta-glucosidase and N-acetyl-beta-glucosaminidase. Meanwhile, the API 20C test differentiates isolates based on the yeast's ability to assimilate eleven carbohydrates: glycerol, L-arabinose, xylose, adonitol, xylitol, sorbitol, methyl-D-glucoside, N-acetyl-D-glucosamine, sucrose, trehalose and melezitose. This biotyping system was found to be simple to perform, reproducible and discriminatory (Williamson et al., 1987). However, for diagnosis it was problematic because many epidemiologically unrelated strains tended to have identical biotypes (Hunter & Fraser, 1989).

2.3.3 Selective Agar

The use of CHROMagar, a differential and selective medium, has been found to be most useful in identifying selected candidal species such as *Candida albicans*, *Candida krusei* and *Candida tropicalis*, although some confusion may arise because of ambiguities in colour, as identification is based on colony colouration when grown on the medium (Beighton et al., 1995).

2.3.4 Molecular Methods for Typing of Oral *Candida* Species

The diploid genome sequence of *Candida albicans* has been elucidated through whole-genome shotgun sequencing, and this has been a great help for molecular-based studies of *Candida* sp. (Jones et al., 2004).

2.3.4.1 Karyotyping

One of the earliest molecular methods for typing of *Candida* sp. is by karyotyping, in which yeast strains are identified based on the characteristics of their chromosomes. Because yeast chromosomes are too large to separate properly in normal gels, pulsed-field gel electrophoresis (PFGE) is employed instead. In PFGE, the orientation of the electric field and the gel is constantly changed, enabling separation of chromosome-sized fragments of DNA, producing distinct bands depending on the size of the fragments, which are then analysed for candidal typing (Schwartz & Cantor, 1984). PFGE has been used to distinguish phenotypically different strains of *Candida albicans* (Mahrous et al., 1990) and for karyotyping *Candida krusei* (Dassanayake et al., 2000).

2.3.4.2 Restriction Enzyme Analysis

Another way to differentiate candidal genotypes is through restriction enzyme analysis (REA), in which candidal DNA is extracted and then subjected to restriction enzymes that cut DNA at specific points, producing bands of variable lengths, which are visible through gel electrophoresis. This method was employed for genotyping candidal isolates by digestion with the *Eco*RI restriction enzyme, and was successful in

producing band patterns that sorted *Candida* species isolates into several mutually exclusive groups, and thus was informative for both epidemiological and taxonomic studies. In addition, three intense bands were identified to be present in each candidal isolate, and these were thought to be ribosomal RNA encoding genes, also known as rDNA (Scherer & Stevens, 1987).

2.3.4.3 Restriction Fragment LengthPolymorphisms in Ribosomal DNA

Another study investigated the presence of restriction fragment length polymorphisms (RFLPs) in *Candida albicans* rDNA. Digestion of extracted *Candida albicans* rDNA with *Eco*RI enzyme yielded six different classes based on restriction patterns (Magee et al., 1987). Another study used the *Hin*fI endonuclease to type 21 different *Candida* species, and was able to distinguish between *Candida albicans*, *Candida krusei*, *Candida tropicalis* and a few other candidal species, in addition to dividing them into mutually exclusive subgroups (Fujita and Hashimoto, 2000). The *Hinf*I restriction enzyme has also been succesfully used for genotyping *Candida krusei* strains (Sancak et al., 2004)

2.3.4.4 Random Amplified Polymorphic DNA

Random amplified polymorphic DNA (RAPD) is another molecular typing technique. RAPD involves the use of non-specific primers that anneal to random sites and amplify dispersed genomic sequences, producing distinct band patterns that are based on genetic polymorphisms (Welsh and McCleland, 1991). This method has been used for differentiating *Candida albicans*, *Candida parapsilosis* and *Candida glabrata* isolates (Valerio et al., 2006). In another study, four nonspecific primers, the 10-mer

oligonucleotide AP3, the microsatellite repeat sequences $(GTG)_5$ and $(AC)_{10,}$ and T3B, derived from tRNA intergenic spacers, were used separately to amplify the genomic DNA of 26 candidal species, and successfully generated distinct profiles for each species. The profiles also demonstrated distinctions between different strains of the same species (Thanos et al., 1996). A different study involving the use of a primer pair based on the sequence of a *Candida albicans* chitin synthase gene, *CHS1*, was successful in generating distinct bands for four medically important candidal species, *C. albicans, C. parapsilosis*, *C. tropicalis*, and *C. glabrata,* which is useful for the identification of these four species (Jordan, 1994).

2.3.4.5 Polymerase Chain Reaction (PCR) Amplification

Polymerase Chain Reaction (PCR) involves the application of primers that amplify specific sequences in the genome, generating bands that are visible through gel electrophoresis. This method has been used for identifying *Candida albicans* isolates through primer pairs that amplify a species-specific sequence in *C. albicans* mitochondrial DNA (Miyakawa et al., 1993).

2.3.4.6 Probe Hybridization

An alternative molecular approach for typing of candidal species based on genetic characteristics is probe hybridization. A DNA probe called 27A has been designed based on a DNA fragment that has about 10 copies dispersed among *Candida albicans* genomic DNA (Scherer & Stevens, 1988). In Africa, three genotype groups were identified in *Candida albicans* samples collected from the oral cavities of HIV-

positive patients through the use of the DNA fingerprinting probe Ca3 (Blignaut et al., 2002).

2.4 Analysis of the the Internal Transcribed Spacer Region of Candidal rDNA

In eukaryotic organisms, ribosomal RNA genes consist of repetitive sequences located in tandem clusters. Regions containing these tandem clusters are known as rDNA. In yeasts, the rDNA region contains multiple rRNA genes that are separated from each other by short nonstranscribed spacers (Lewin, 2004). The rDNA region is known to be the most conserved region in the genome, and thus is most suitable for studying phylogenetic differences (Iwen et al., 2002).

The fungal rRNA gene consists of sequences coding for the small subunit 18S rRNA, the 5.8S rRNA and the large subunit 28S rRNA. As can be seen in Figure 2.1, there are two Internal Transcribed Spacers (ITS) found in fungal rDNA, namely ITSI and ITSII. ITSI is situated between the 18S and 5.8S rDNA sequences, whereas ITSII is located between the 5.8S and 28S rDNA sequences. Thus, the rRNA gene can be imagined to be a series of sequences in the following order: 18S rDNA, ITSI, 5.8S rDNA, ITSII and finally 28S rDNA. In addition, two intergenic spacer (IGS) regions, IGSI and IGSII are located between the 28S rDNA end of an rRNA gene and the 18S rDNA end of the next rRNA gene. Of all these regions, ITSI and ITSII have been found to be most variable between different species of fungi, and thus useful for identification of fungal species (White et al., 1990).

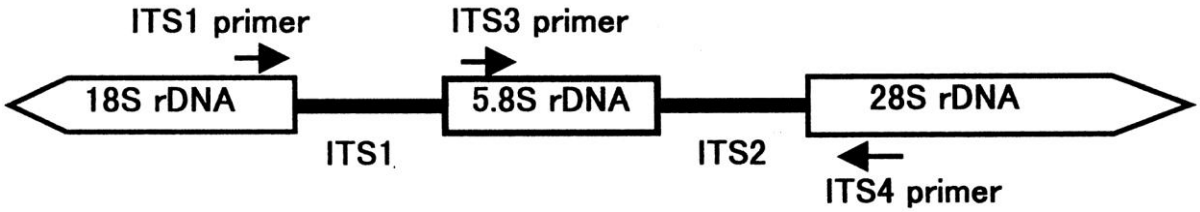

Figure 2.1: Candidal rDNA and the Locations of ITS1, ITS3 and ITS4 Primers

(Mirhendi et al., 2006)

2.4.1 ITSII Amplification

It has been reported that amplification of the ITSII region in the rDNA of various candidal isolates through the use of primers annealing to sequences in the fungal 5.8S (fluorescently labelled ITS86 primer) and 28S (ITS4 primer) ribosome coding genes, followed by analysis employing an automated capillary electrophoresis system, enables differentiation of eight *Candida* species, including *C. albicans, C. krusei* and *C. tropicalis*, with no intraspecific variety detected (Turenne et al., 1999).

2.4.2 Multiplex PCR

Multiplex PCR has been carried out, in which two different PCR reactions were conducted simultaneously, for the identification of 120 yeast isolates. The ITS1 and ITS4 primers amplified the ITSI-5.8S-ITSII region, while the ITS3 and ITS4 primers simultaneously amplified the ITSII region. This method was successful in differentiating 29 out of the 30 yeast species tested (Fujita et al., 2001).

2.4.3 Single-Strand Confirmation Polymorphism Analysis

A recent study compared the effectiveness of various fungal-specific primers for the identification of fungal species based on single-stranded confirmation polymorphism (SSCP) analysis. Four sets of PCR were compared, namely the ITS1 and ITS2 amplification of ITSI, the ITS3 and ITS4 amplification of ITSII, the ITS1 and ITS4 amplification of the ITSI-5.8S-ITSII region, and the invSR1R and LR12R amplification of the IGS region and 5S rRNA gene. The results concluded that the PCR products generated by the ITS1 and ITS2 primers were the most suitable for fungi identification (Kumar and Shukla, 2005).

2.4.4 Transposable Intron Amplification

A primer pair, CA-INT-L and CA-INT-R, which amplifies a transposable intron region in the 25S rRNA gene, has been used to classify *C. albicans* into four different genotypic strains (McCullough et al., 1999), while a different study using the same primers found five different genotypes (Tamura et al., 2001). Another study in China, again using the same primers, found only three different genotypes (Qi et al., 2005).

2.4.5 PCR Amplification and Enzyme Immunosorbent Assay Analysis

Another method combines both PCR amplification and enzyme immunosorbent assay (EIA) analysis for *Candida* species identification. This PCR-EIA technique involves the primer pair ITS3 and ITS4 for amplification of the 5.8S-ITSII region, followed by EIA species-specific probes to detect the amplified PCR product. This method has been used to correctly identify the *Candida* species in blood samples taken

from 31 patients with candidemia (Hee Shin et al., 1997). A similar method targeting the ITSII region was successful in accurately differentiating *Candida dubliniensis*, *Candida albicans*, *Candida glabrata*, *Candida krusei*, *Candida parapsilosis*, and *Candida tropicalis*. It was concluded that this method was more reliable than phenotypic methods for identifying *Candida dubliniensis* (Ellepola et al., 2003).

2.4.6 PCR Amplification and Restriction Fragment Length Polymorphism Analysis

Recently, carrying out both PCR and RFLP approaches upon candidal rDNA has proven useful for species identification. In one study, the primers ITS1 (targeting a sequence in the 18S rDNA) and ITS4 (targeting a sequence in the 28S rDNA) were used to amplify a region spanning ITSI, 5.8S and ITSII in the rDNA of various candidal isolates. After that, the amplified region was digested with the *Msp*I restriction enzyme, producing bands patterns that could distinguish between six medically significant *Candida* species, namely *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. glabarata*, *C. krusei*, and *C. guilliermondii* (Mirhendi et al., 2006). Another study employed *Msp*I and *BlnI* digestion of the amplified ITS1-5.8S-ITSII region to differentiate *C. albicans*, *C. tropicalis*, *C. krusei*, *C. glabrata*, *C. parapsilosis*, *C. guilliermondii* and *C. dubliniensis* (Shokohi et al., 2010).

METHODOLOGY

3.1 Research Materials

3.1.1 Chemicals

Agarose (Sisco Research Lab, Mumbai)

Boric Acid (BDH, England)

Chloroform (SystemChem AR)

Decon 90 (Decon, England)

Ethanol 95% (John Kollin Corporation, USA)

EDTA (Fluka, Germany)

EtBr Destroyer Sprayer (Favogen)

Germisep (Hovid, Malaysia)

Glycerol (Merck, Germany)

Isopropanol (R&M Chemicals, United Kingdom)

Lysozyme (Sigma, USA)

Lyticase (Fluka)

Proteinase K (Sigma, USA)

RNase A (Sigma, USA)

Sorbitol (R&M Chemicals, United Kingdom)

Sodium chloride (BDH, England)

Triton X-100 (R&M Chemicals, United Kingdom)

Tris-HCl (Sisco Research Lab, Mumbai)

3.1.2 Glasswares

Beaker (Bibby, UK)

Conical flask (Pyrex, England)

Glass beads, 3 mm diameter (Merck, Germany)

Glass bottle (Schott, UK)

3.1.3 Consumables

Aluminium foil (Diamond, USA)

Bunsen burner gas (Campingaz, France)

Latex Gloves (Unigloves, Malaysia)

Parafilm (Peching Plastic Packaging, Menasha)

Petri dish (Brandon, USA)

Pipette tips (Appendorf, Canada)

3.1.4 Media

Brain Heart Infusion Broth (Difco, France)

Brain Heart Infusion Agar (Difco, France)

Sabouraud Dextrose Agar (Difco, France)

3.1.5 Microbial Control Strains

Candida albicans (ATCC 14053), American Type Culture Collection, USA

Candida tropicalis (ATCC 13803), American Type Culture Collection, USA

Candida krusei (ATCC 14243), American Type Culture Collection, USA

Candida parapsilosis (ATCC 22019), American Type Culture Collection, USA

Candida dubliniensis (ATCC MYA-2975), American Type Culture Collection, USA

Candida glabrata (ATCC 90030), American Type Culture Collection, USA

Candida lusitaniae (ATCC 64125), American Type Culture Collection, USA

3.1.6 Equipments

Autoclave, HICLA VE HVE-50 (Hirayama, Japan)

Balancer (Denver Instrument, USA)

Chiller, 4 ºC (Mutiara, Malaysia)

Electric drying cabinet, Weifo KD-112 (Weifo, Singapore)

Freezer, -80 ºC, Hetofrig CL410 (Hetofrig, Denmark)

Fume cupboard, Ductless (Labcaire, England)

Gel-Pro Analyzer (Media Cybernetics, USA)

Gel Imaging System (Microlambda)

Icemaker (Nuove Tecnologie del Freldo, Italy)

Incubator (Memmert, Germany)

Laminar Air Flow Cabinet, ERLA CFM Series (Australia)

Micropipettors (Appendorf, Canada)

Microwave oven (Panasonic, UK)

Mastercycler, Gradient (Eppendorf, Germany)

pH Meter (Eutech Instuments)

Power Pack (BioRad, USA)

Spectrophotometer, Shimadzu UV160A (Shimadzu, Japan)

Thermal Printer (Mitsubishi Electric, Japan)

Vortex Mixer (Glas-Col, USA)

Water Bath (Grant, United Kingdom)

Water Distiller (J Bibby Merit, England)

Water Purifier System (ELGA, UK)

3.2 Research Methods

3.2.1 Research Outline

The overall research process consists of six stages, as detailed in Figure 3.1.

3.2.2 Sample Collection

Samples were collected from the oral cavity of a total of 45 individuals. Three target groups were identified for sample collection, consisting of 15 non-denture wearers with a healthy oral cavity (this acts as the control group), 15 upper full denture wearers with healthy oral cavity, and 15 non-denture wearers suffering from adult-stage periodontal disease. Samples were collected in accordance with ethical code DF 0B0702/2002(L) (refer to Appendix A).

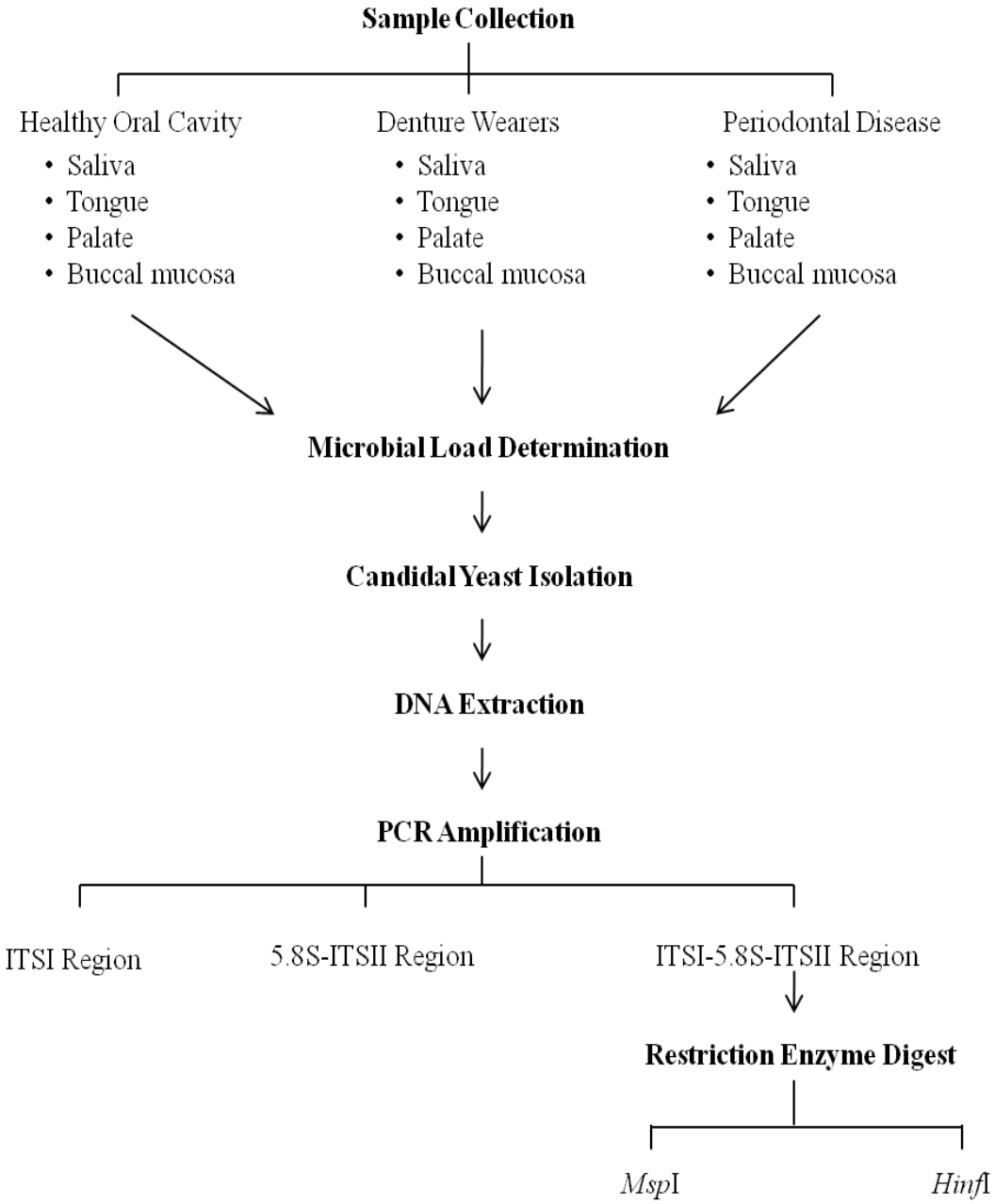

Figure 3.1: Six Stages of the Research Design

3.2.2.1 Preparation of Transport Medium

The transport medium consisted of phosphate buffer saline (PBS). Beforehand, 1 L of PBS was prepared by adding the ingredients as shown in Table 3.1 to 800 mL of sterile distilled water. The total pH of the mixture was then adjusted to 7.4 and the solution topped up to 1 L with sterile distilled water.

Table 3.1: Ingredients for 1 L of PBS

Materials	**Amount**
NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.24 g

Following preparation, 1.5 mL of the transport media was dispensed into sterile 2 mL microcentrifuge tubes before storing at 4 °C

3.2.2.2 Inclusion and Exclusion Criteria

The samples were taken from individuals of 35-65 years of age, who were non-smokers, not diabetic, and had not taken any antimicrobial treatment for the past 6 months prior to sampling. Each sampled individual was given an information sheet (refer to Appendix B) to ensure that they understood what they were participating in, and then asked to fill out a consent form (refer to Appendix C) before having their samples taken.

3.2.2.3 Sampling Sites and Collection Methods

In addition to collecting samples from saliva, samples were also collected from the surfaces of the tongue, palate and the buccal mucosa (cheek mucosal surface).

Samples from the tongue, palate and buccal mucosa surfaces were taken by brushing against the surface 10 times consecutively with a cytobrush. In order to collect saliva, a pea sized cotton ball was used to soak the saliva at the floor of the mouth. All of the samples were then transferred into the transport medium (refer to 3.2.2.1), which were stored in ice and brought to the laboratory.

3.2.3 Microbial Load Determination

3.2.3.1 Preparation of Agar Plates and Broth Mediums

Fresh agar plates were prepared prior to each sampling as well as prior to carrying out colony isolation. Fresh agar slants and broth media were also prepared prior to storage of the isolated colonies.

3.2.3.1.1 Sabouraud Dextrose Agar (SDA) Plates and Slants

SDA plates were prepared by first suspending 65 g of SDA powder in 1 L of distilled water. The suspension was boiled in a microwave oven and mixed through frequent agitation in order to ensure the powder was dissolved thoroughly. The solution was then autoclaved at 121 °C for 15 minutes at 15 psi. While still in liquid form, the

sterilized media was then poured into sterile petri dishes and left to solidify before storing at 4 °C in an inverse direction.

SDA slants were also prepared by dispensing 2.5 mL of the sterilized media, while still in its liquid form, into sterilized universal bottles that were then left to solidify at an inclined angle. The agar slants were stored at 4 °C.

3.2.3.1.2 Brain-Heart Infusion (BHI) Agar Plates

BHI agar plates were prepared by suspending 52 g of BHI agar powder in 1 L of distilled water, and then following the same steps of dissolving, autoclaving, dispensing and storage as outlined in 3.2.3.1.1 for SDA plates.

3.2.3.1.3 Yeast-Extract-Peptone-Dextrose (YEPD) Broth

The ingredients as shown in Table 3.2 were dissolved in 500 mL of distilled water and then autoclaved at 121 °C for 15 minutes at 15 psi.

Table 3.2: Chemical Ingredients Required for YEPD Broth

Materials	**Amount**
D (+) Glucose	10 g
Peptone	10 g
Yeast extract	5 g

3.2.3.2 Serial Dilution

Once the transport medium containing the samples were brought to the laboratory, serial dilution was carried out, in which the microcentrifuge tubes containing the samples were vortexed to ensure the microbes were evenly mixed in the broth, before pipetting 0.1 mL of the broth into 9.9 mL of sterile distilled water in a Falcon tube, which was also vortexed to produce a 10^2 dilution. From this first tube, 1 ml was transferred to a new tube containing 9 ml of sterile distilled water and vortexed, producing a 10^3 dilution. This step was serially repeated two more times to produce tubes containing 10^4 and 10^5 dilutions. The process of serial dilution is further illustrated in Figure 3.2.

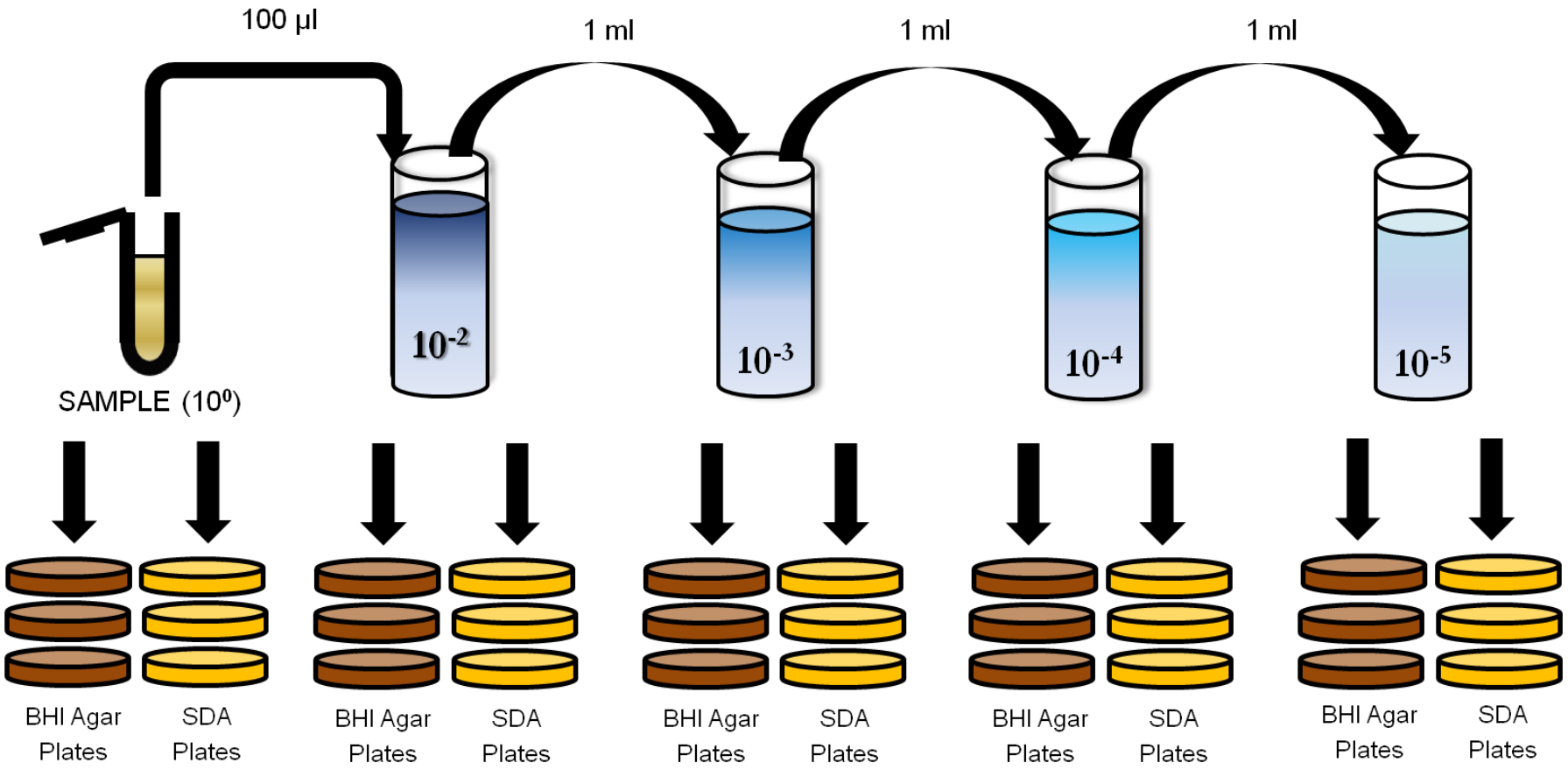

Figure 3.2: The Process of Serial Dilution and Plating

3.2.3.3 Microbial Plating

After the serial dilution was carried out, 100 µl was pipetted from the undiluted sample as well as from each serial dilution tube, all of which were vortexed prior to pipetting, and plated on triplicate SDA as well as BHI agar plates, producing plates that were inoculated with the following dilutions: 10^0, 10^2, 10^3, 10^4 and 10^5. The plates were incubated at 37 °C for 48 hours under aerobic conditions, to allow for the growth of candidal organisms on SDA plates, and the growth of both candidal and aerobic bacterial organisms on BHI plates, respectively. Following incubation, each plate was then scored for colonies, enabling the calculation of the total microbial, candidal and bacterial loads of each sample, expressed in colony-forming units (CFUs), as based on the following formulas:

$$\text{Microbial CFU/mL} = \frac{\text{Number of Formed Colonies}}{\text{Dilution Factor} \times \text{Volume (mL)}}$$

$$\text{Total Microbial Colony Forming Units} = \text{CFU/mL on BHI agar plates}$$

$$\text{Candidal Colony Forming Units} = \text{CFU/mL on SDA plates}$$

$$\text{Bacterial Colony Forming Units} = \text{Total Microbial Colony Forming Units} - \text{Candidal Colony Forming Units}$$

3.2.3.4 Isolation and Storage of Candidal Colonies

Fifteen unidentified yeast colonies were randomly selected for isolation. In addition, pure colonies of the following seven ATCC yeast species were also obtained as positive control samples: *Candida albicans, Candida tropicalis, Candida krusei, Candida parapsilopsis, Candida dubliniensis, Candida glabrata* and *Candida lusitaniae*. This was carried out by following the manufacturer's instruction: 0.5 mL of sterile distilled water was added to an ampoule containing lyophilised cells of the *Candida* species. Following rehydration of the cells in the ampoule, 100 µl of the suspension was then inoculated on an SDA plate and incubated at 37 °C for 24 hours.

The fifteen clinical colonies and seven ATCC colonies were isolated on SDA plates. This was conducted by streaking each sample on an agar plate with a sterile wire loop, and then incubating the plates at 37 °C for 48 hours, in order to obtain pure single colonies. Several pure colonies were then picked, subcultured on SDA slants (refer to 3.2.3.1.1) and incubated at 37 °C for 48 hours before placing in 4 °C for short term storage.

For long term storage, colonies from the agar slants were inoculated into 5 mL of YEPD broth (3.2.3.1.3) and incubated overnight at 37 °C. Then, 800 µl of the growth suspension was transferred to a sterile eppendorf tube, after which 200 µl of glycerol was added and mixed. The 20% glycerol stock was stored at -80 °C.

3.2.4 DNA Extraction

Genomic DNA was at first extracted from the ATCC control yeast colonies using several methods – a commercial yeast DNA extraction kit, a lyticase-based enzymatic extraction method, an extraction method based on rapid freeze-thawing and a glass bead disruption extraction method – before determining which method was best suited for candidal genomic DNA extraction. Prior to this, the necessary reagents, stock solutions and buffers were prepared accordingly.

3.2.4.1 Preparation of Yeast Cultures

Fresh yeast cultures were prepared before each DNA extraction procedure. Purified yeast colonies (see 3.2.3.4) were picked and inoculated into 2 mL microcentrifuge tubes containing 1.5 mL YEPD broth (see 3.2.3.1.3). The tubes were then incubated at 37 °C for 48 hours. The yeast cultures were standardized to 0.144 at a wavelength of 550 nm (10^6 cells per mL) using spectrophotometry.

3.2.4.2 Preparation of Reagents

An ethylenediamine tetraacetic acid (EDTA) stock solution (3.2.4.2.1) was required for preparation of Tris-EDTA (TE) buffer (3.2.4.2.3) as well as Tris-Borate-EDTA (TBE) buffer (3.2.4.2.4) stocks. A Tris-HCl (3.2.4.2.2) stock solution was also required for preparation of the TE buffer. It was necessary to prepare 10× TE buffer stock solution (3.2.4.2.3), as 1× TE buffer was required for storage of extracted DNA, while 3× TE buffer was needed for Lyticase-based enzymatic DNA extraction (3.2.4.4.4). Meanwhile, TBE buffer was prepared for gel electrophoresis of the DNA

extracts (3.2.4.5). Also, Lysis Buffer stocks (3.2.4.2.5) were prepared before carrying out the rapid freeze-thawing (3.2.4.4.2) and glass beads disruption (3.2.4.4.3) extraction methods. A sodium acetate solution (3.2.4.2.6) was also required for the glass beads disruption method.

3.2.4.2.1 Preparation of EDTA Solution

A 500 mL stock solution of 0.5M EDTA was prepared by weighing 93.05 g EDTA disodium salt and dissolving in 400 mL sterile deionized water and adjusting the pH to 8.0 with NaOH. The solution was then topped up to a final volume of 500 mL.

3.2.4.2.2 Preparation of Tris-HCl

A 500 mL stock solution of 1M Tris-HCl was prepared by dissolving 60.55 g Tris base in 400 mL deionized water and adjusting the pH to 7.5 with hydrochloric acid (HCl). The solution was then topped up to a final volume of 500 mL.

3.2.4.2.3 Preparation of Tris-EDTA (TE) Buffer

To prepare a 500 mL 10× TE buffer stock solution, 50 mL of the 1M Tris-HCl stock solution (3.2.4.1.2.2) was mixed with 10 mL of 0.5M EDTA (see 3.2.4.1.2) and 440 mL sterile deionized water. The 3× and 1× TE buffer solutions were later obtained by diluting the 10× TE buffer stock solution.

3.2.4.2.4 Preparation of Tris-Borate-EDTA (TBE) Buffer

Stock solutions of 5× TBE buffer were prepared by weighing 54 g Tris base and 27.5 g boric acid and dissolving both in 900 mL sterile deionized water. Then, 20 mL of 0.5M EDTA (see 3.2.4.2.1) was added and the solution was topped up to a final volume of 1 L. Later, 1× TBE buffer solutions were obtained by diluting the 5× stock solution.

3.2.4.2.5 Preparation of Lysis Buffer

The ingredients as shown in in Table 3.3 were dissolved in 500 mL of sterile distilled water in order to prepare Lysis Buffer stock.

Table 3.3: Chemical Ingredients Required for Lysis Buffer

Materials	**Amount**
Triton X-100	2%
SDS	1%
NaCl	100 mM
Tris-HCl (pH 8.0)	10 mM
EDTA (pH 8.0)	1 mM

3.2.4.2.6 Preparation of Sodium Acetate Solution

A 500 mL stock solution of 3M sodium acetate was prepared by dissolving 204 g of sodium acetate in 400 mL of deionized water and adjusting the pH to 5.2 with acetic acid. The solution was then topped up to 500 mL.

3.2.4.3 Preparation of 1% Agarose Gel

A fresh 1% agarose gel was prepared each time before running gel electrophoresis of DNA extracts (3.2.4.6). In order to prepare 50 mL of 1% agarose gel, 0.5 g of agarose powder was suspended in 50 mL TBE buffer (see 3.2.4.2.4) inside a conical flask. The flask was microwaved with frequent agitation in order to ensure the powder was properly dissolved in the solution. The process of heating was stopped at the boiling point. After 15 minutes in which the solution had cooled to body warmth temperature, 0.1 µl of ethidium bromide was pippeted into the solution and mixed well. The solution was then poured into a gel caster, and a well comb was inserted to form wells. After the gel had cooled and hardened, the well comb was removed.

3.2.4.4 Genomic DNA Extraction Methods

Four different genomic DNA extractions were employed in this study: the use of a commercial yeast genomic DNA extraction kit (3.2.4.4.1), an extraction method that uses a Triton X-100 lysis buffer and rapid freeze-thwaing for yeast cell disruption (3.2.4.4.2), an extraction method also employing the Triton X-100 lysis buffer but instead based on glass beads disruption (3.2.4.4.3), and an enzymatic extraction method based on Lyticase (3.2.4.4.4).

3.2.4.4.1 Commercial Yeast Genomic DNA Extraction Kit

DNA was extracted by following the procedure as given by the supplier of the MasterPure Yeast DNA Purification Kit. Fresh yeast cultures (3.2.4.1) were centrifuged at 4,000 rpm for 5 minutes at room temperature before discarding the supernatant and suspending the pellet in 300 µl Yeast Cell Lysis Solution (provided in the kit). The suspension was then incubated at 65 °C for 15 minutes, followed by placing on ice for 5 minutes. Afterwards, 150 µl MPC Protein Precipitation Reagent (provided in the kit) was added and the mixture was vortexed for 10 seconds before being centrifuged at 10,000 rpm for 10 minutes. The resulting upper aqueous layer was transferred to a new a tube, and then 500 µl isopropanol was added and gently mixed. The mixture was then centrifuged at 10,000 rpm for 10 minutes, and the supernatant was discarded by pipetting. Next, 500 µl 70% ice-cold ethanol was added and gently mixed before centrifuging at 12,000 rpm for 5 minutes. The supernatant was discarded and the pellet was air dried for about an hour before resuspending in 50 µl TE buffer (provided in the kit) and storing at -20 °C

3.2.4.4.2 Genomic DNA Extraction Based on Rapid Freeze-Thawing

This extraction method was based on the "Bust and grab" extraction protocol (Harju et al., 2004), with a few minor alterations. Tubes containing fresh yeast cultures (see 3.2.4.1) were centrifuged at 4,000 rpm for 5 minutes at room temperature. The supernatants were discarded and the remaining pellets were suspended in 200 µl Lysis Buffer (see 3.2.4.1.2.5). The suspended pellets were then placed in a -80 °C freezer for 2 minutes, and then immediately transferred to a 95 °C water bath for 1 minute. This

step was then repeated a second time before vortexing the suspension for 30 seconds and adding 200 µl chloroform. The resulting mixture was then vortexed for 2 minutes before centrifugation at 4,000 rpm for 3 minutes at room temperature. The resulting upper aqueous layer was transferred to a new tube containing 400 µl ice-cold 70% ethanol, gently mixed and then incubated at room temperature for 5 minutes. Following incubation, the mixture was centrifuged at 12,000 rpm for 5 minutes at room temperature and the supernatant was discarded. The pellet containing the DNA extract was washed in ethanol by adding 500 µl 70% ethanol, centrifuging at 4,000 rpm for 5 minutes at room temperature, and then discarding the supernatant. The tube was then air dried before suspending the DNA extract in 50 µl TE buffer (see 3.2.4.1.4) and storing it at -20 °C.

3.2.4.4.3 Genomic DNA Extraction Based on Glass Beads Disruption

Genomic DNA extraction based on glass bead disruption was carried out using a slightly modified protocol employed by Mirhendi (2006). Yeast cells from the yeast cultures (3.2.4.1.1) were pelleted by centrifugation at 4,000 rpm for 5 minutes and then suspended in 300 µl Lysis Buffer (3.2.4.2.5). Next, 300 µl of PCI (phenol-chloroform-isopropanol, 25:24:1) and 300 mg glass beads were added, and the mixture was vortexed for 5 minutes in order to disrupt the yeast cells.

Following centrifugation at 10,000 rpm for 5 minutes, the cell lysate, which had been collected in the supernatant, was then transferred to a new tube. In order to remove traces of phenol from the lysate, 500 µl of chloroform was added, vortexed for two minutes, and then centrifuged at 12,000 rpm for 3 minutes. The resulting upper aqueous layer was then transfered to a new tube. DNA precipitation was carried out by adding 20

µl of sodium acetate (3.2.4.2.6) and 220 µl of isopropanol. The mixture was gently mixed and incubated on ice for 15 minutes.

After incubation, the mixture was centrifuged at 12,000 rpm for 10 minutes and the supernatant was discarded. The resulting DNA precipitate was then washed in 500 µl of 70% ice-cold ethanol. Centrifugation was at 12,000 rpm for 5 minutes, followed by discarding of the supernatant. The washed DNA precipitate was finally air dried for an hour before being suspended in 50 µl of TE buffer (see 3.2.4.2.3). DNA extracts were stored at -20 °C.

3.2.4.4.4 Genomic DNA Lyticase-Based Enzymatic Extraction

The DNA extraction protocol that was employed was based on a slightly modified version of the Yeast DNA Mini-preparation Protocol (Lee, 1992). Fresh yeast cultures (3.2.4.1) were centrifuged at 4,000 rpm for 5 minutes at room temperature. The supernatant was discarded and the pellet was resuspended in 500 µl 1M sorbitol and vortexed. Then, 250 µl of Lyticase was added and the mixture was incubated at 37 °C for 30 minutes. Afterwards, the suspension was centrifuged at 12,000 rpm for 1 minute at room temperature. The supernatant was discarded, and the pellet was resuspended in 500 µl of 3x TE buffer.

Next, 25 µl of 20% sodium dodecyl sulphate (SDS) was added, and the solution was incubated in a water bath at 65 °C for 20 minutes. This was followed by adding 5 µl of Proteinase K, concentration 20 mg/ml, and incubation at 55 °C for 15 minutes. The next step was the addition of 400 µl 5M potassium acetate, pH 5.2, followed by incubation for 30 minutes on ice. The resulting solution was centrifuged at 12,000 rpm

for 5 minutes. Then, 750 µl of the supernatant from the centrifugation was transferred into a new microcentrifuge tube, and the pellet was discarded. To the new tube, 750 µl of isopropanol was added, and the resulting solution was mixed for 30 minutes at room temperature.

After mixing, the solution was centrifuged at 12,000 rpm for 5 minutes. The supernatant was discarded, and 300 µl of TE buffer (see 3.2.4.2.3) was added to the tube, being careful not to disturb the pellet. The tube was then treated with 1.5 µl of 10 mg/ml RNAse A for 1 hour at 37 °C, during which the pellet was observed to dissolve. DNA precipitation was then carried out by adding 30 µl of 3M sodium acetate, pH 5.2, followed by 300 µl of isopropanol. This was followed by centrifugation at 12,000 rpm for 10 minutes.

The supernatant was then discarded, and the pellet was washed in 500 µl of 70% ice-cold ethanol. Centrifugation was at 12,000 rpm for 5 minutes, followed by discarding of the supernatant. The resulting DNA precipitates were then air dried for an hour before being resuspended in 50 µl TE buffer (3.2.4.2.3) and stored at -20 °C.

3.2.4.5 Qualitative Confirmation of DNA Presence

The presence of DNA was detected by carrying out gel electrophoresis. After 1% agarose gel was prepared (3.2.4.3) and submerged in TBE buffer (3.2.4.2.4) in a gel tank, 5 µl of each DNA extract was mixed with 1 µl of DNA Loading Dye and pipetted into the wells of the gel. The gel was ran at 80 V for 80 minutes, after which the resulting bands of genomic DNA were visualized by viewing the gel under ultraviolet light.

3.2.4.6 Quantitative Determination of DNA Yield

After the presence of DNA had been confirmed, the optical density (OD) of the extracts was assessed using spectrophotometry. This was carried out by pipetting 5 µl of the DNA extract to a 2 mL microcentrifuge tube containing 495 µl sterile distilled water and vortexing. The 500 µl diluted DNA extract was then transferred to a clean cuvette, which was placed in the spectrophotometer. The total DNA yield of the extract was calculated based on the OD reading at 260 nm wavelength, using the following calculation:

Concentration of DNA = OD_{260} × 50 µg/mL × Dilution Factor

(1 OD reading at 260 nm wavelength corresponds to 50 µg/mL double-stranded DNA)

3.2.4.7 Determination of DNA Extract Purity

In addition to DNA yield, possible protein and phenol contamination of the extracts were also determined by taking the ratios of OD_{260}:OD_{280} and OD_{260}:OD_{270} wavelength, respectively. The DNA extract was considered to be free of protein contamination when the OD_{260}:OD_{280} ratio was 1.8 to 2.0, whereas it was considered free of phenol contamination when the OD_{260}:OD_{270} ratio was between 1.0 to 1.2.

3.2.4.8 Selection of Extraction Method for Clinical Samples

After extracting genomic DNA from the ATCC colonies using each of the different extraction methods and confirming the presence of DNA with gel

electrophoresis, the yield and purity of the DNA extracts was determined with spectrophotometry. Based on this, it was then determined which of the methods was most suitable for extracting DNA in this study, and the selected method was then used for all the selected clinical samples.

3.2.5 PCR Amplification

The extracted DNA samples were subjected to three different PCR amplifications using four different primers: ITS1 forward primer (5'-TCCGTAGGTGAACCTGCGG-3'), ITS2 reverse primer (5'-GCTGCGTTCTTCATCGATGC-3'), ITS3 forward primer (5'-GCATCGATGAAGAACGCAGC-3') and ITS4 reverse primer (5'-TCCTCCGCTTATTGATATGC-3'). In general the PCR protocols of the reactions were based on a slightly modified version of the Kumar & Shukla (2005) protocol, and all the reactions were carried out using an Eppendorf Mastercycler Gradient thermocycler. During each batch of reactions, a negative control, in which the DNA template was substituted with sterile distilled water, was simultaneously included.

3.2.5.1 Amplification of ITSI Region

The ITS1 and ITS2 primers were used to amplify the ITS1 region of the candidal rDNA, as illustrated in Figure 3.3.

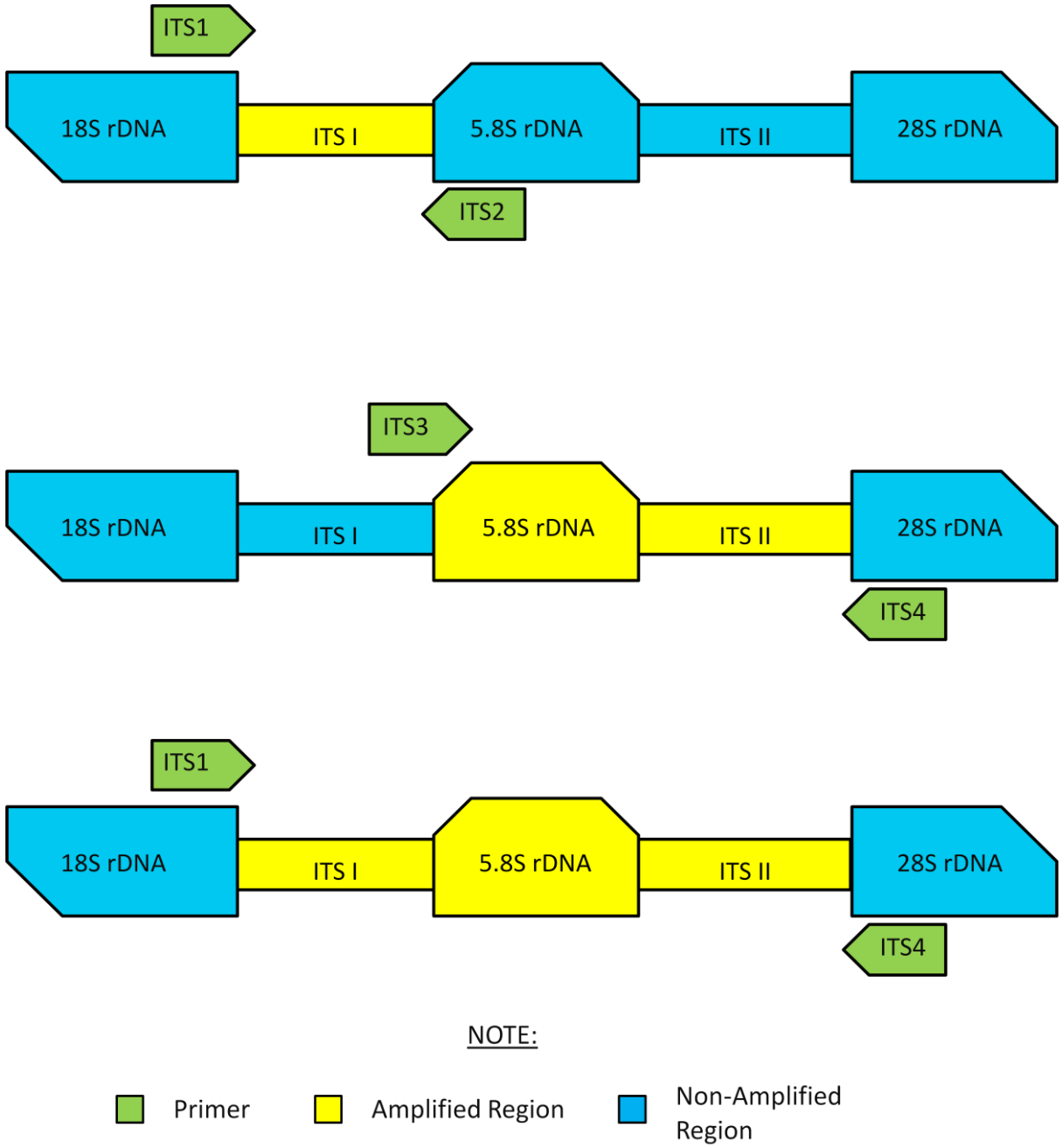

Figure 3.3: The regions amplified by each of the three primer pairs ITS1 and ITS4; ITS1 and ITS2; and ITS3 and ITS4

Prior to carrying out the reaction, all the tubes containing the reagents, buffers and DNA templates necessary for the reaction, with the exception of *Taq* Polymerase, were left to thaw at room temperature for about 15 minutes before vortexing. Each PCR reaction tube consisted of a mixture of 4 µl of the DNA template, 5 µl of 10× PCR buffer, 0.1µM of the ITS1 primer, 0.1µM of the ITS2 primer, 100µM of dNTP mixture and 1 U of *Taq* Polymerase. Sterile distilled water was used to top up the mixture to a total of 50 µl. The *Taq* Polymerase was added to the mixture last, after briefly thawing the tube containing *Taq* Polymerase. Each reaction mixture tube was vortexed before being placed in the thermocycler.

The amplification process consisted of an initial denaturation step at 96 °C for 10 minutes; 30 cycles of denaturation at 95 °C for 1 minute, annealing at 60 °C for 1 minute and extension at 72 °C for 1 minute; and a final extension of 72 °C for 10 minutes. After the reaction was completed, the tubes containing the PCR products were stored at -20 °C.

3.2.5.2 Amplification of 5.8S-ITSII Region

The ITS3 and ITS4 primers were used to amplify the 5.8S-ITSII region of the candidal rDNA, as illustrated in Figure 3.3.

Prior to carrying out the reaction, all the tubes containing the reagents, buffers and DNA templates necessary for the reaction, with the exception of *Taq* Polymerase, were left to thaw at room temperature for about 15 minutes before vortexing. Each PCR reaction tube consisted of a mixture of 4 µl of the DNA template, 5 µl of 10× PCR buffer, 0.1µM of the ITS1 primer, 0.1µM of the ITS2 primer, 100µM of dNTP mixture

and 1 U of *Taq* Polymerase. Sterile distilled water was used to top up the mixture to a total of 50 µl. The *Taq* Polymerase was added to the mixture last, after briefly thawing the tube containing *Taq* Polymerase. Each reaction mixture tube was vortexed before being placed in the thermocycler.

The amplification process consisted of an initial denaturation step at 96 °C for 10 minutes; 30 cycles of denaturation at 95 °C for 1 minute, annealing at 56 °C for 1 minute and extension at 72 °C for 90 seconds; and a final extension of 72 °C for 10 minutes. After the reaction was completed, the tubes containing the PCR products were stored at -20 °C.

3.2.5.3 Amplification of ITSI-5.8S-ITSII Region

The ITS1 and ITS4 primers were used to amplify the ITS1-5.8S-ITSII region of the candidal rDNA, as illustrated in Figure 3.3.

Prior to carrying out the reaction, all the tubes containing the reagents, buffers and DNA templates necessary for the reaction, with the exception of *Taq* Polymerase, were left to thaw at room temperature for about 15 minutes before vortexing. Each PCR reaction tube consisted of a mixture of 4 µl of the DNA template, 5 µl of 10× PCR buffer, 0.1µM of the ITS1 primer, 0.1µM of the ITS4 primer, 100µM of dNTP mixture and 1 U of *Taq* Polymerase. Sterile distilled water was used to top up the mixture to a total of 50 µl. The *Taq* Polymerase was added to the mixture last, after briefly thawing the tube containing *Taq* Polymerase. Each reaction mixture tube was vortexed before being placed in the thermocycler.

The amplification process consisted of an initial denaturation step at 95 °C for 10 minutes; 30 cycles of denaturation at 95 °C for 1 minute, annealing at 55 °C for 1 minute and extension at 72 °C for 90 seconds; and a final extension of 72 °C for 10 minutes. After the reaction was completed, the tubes containing the PCR products were stored at -20 °C.

3.2.5.4 Visualization of PCR Products

Following amplification, the PCR products were then visualized through gel electrophoresis, using 1.5% agarose gels containing ethidium bromide. The gels were prepared following the same procedure as detailed in 3.2.4.3, except that 0.75 g of agarose was used instead of 0.5 g. Once the gel had been prepared and submerged in TBE buffer inside a gel tank, 5 µl each of the extracted DNA samples were mixed with 1 µl of DNA loading dye and then pipetted into the wells. Gel electrophoresis was conducted at 90 V for 100 minutes, and the resulting bands were compared to a 100 bp DNA marker using the Gel Imaging System software to determine band size.

3.2.6 Restriction Endonuclease Enzyme Digestion

Following PCR amplification, the restriction enzymes *Msp*I and *Hinf*I were used to digest the ITS1-5.8S-ITSII PCR products, resulting in fragments of different sizes.

3.2.6.1 *Msp*I Digestion

The *Msp*I restriction enzyme cuts at the CCGG recognition site. Prior to carrying out the digestion, all the tubes containing the enzymes, buffers and PCR products

necessary for the digestion reaction were left to thaw at room temperature for about 15 minutes before vortexing. The *Msp*I digestion was carried out by mixing 10 µl of the PCR product together with 2 µl of 10× Buffer Tango, 18 µl of sterile distilled water and 1 µl of *Msp*I. The tubes containing the digestion mixture were then vortexed and then spun down before incubating at 37 °C for 1 hour.

3.2.6.2 *Hinf*I Digestion

The *Hinf*I restriction enzyme cuts at the GANTC recognition site. Prior to carrying out the digestion, all the tubes containing the enzymes, buffers and PCR products necessary for the digestion reaction were left to thaw at room temperature for about 15 minutes before vortexing. The *HinfI* digestion was carried out by mixing 10 µl of the PCR product together with 2 µl of 10× Buffer R, 18 µl of sterile distilled water and 1 µl of *Hinf*I. The tubes containing the digestion mixture were then vortexed and then spun down before incubating at 37 °C for 1 hour.

3.2.6.3 Restriction Fragment Visualization

The resulting restriction fragments were visualized through gel electrophoresis, using 2% agarose gels. The gels were prepared following the same procedure as detailed in 3.2.4.3, except 1 g of agarose was used instead of 0.5 g. The gels were ran at 70 V for 120 minutes.

4. RESULTS

4.1 Microbial Loads

The candidal and total microbial loads of the oral sampling sites were determined based on candidal and total microbial CFU counts, respectively. Bacterial loads were determined by calculating the difference between candidal and total microbial loads.

4.1.1 Comparison of Microbial Loads Between Groups

Statistical analysis was carried out to compare between the microbial loads for each site between the three different groups. The Shapiro-Wilk test for normality was used on SPSS analysis software to determine that the collected sample data was not normally distributed. Therefore, a non-parametric independent variable test, the Kruskal-Wallis test, was employed to determine if there were any significant differences between the three different groups.

According to the Kruskal-Wallis test, the average microbial loads between all three groups were significantly different (p value < 0.05) for candidal loads of the saliva ($H=12.84$, $p=0.006$), candidal loads ($H=12.601$, $p=0.006$) and bacterial loads ($H=14.28$, $p=0.006$) of the tongue, as well as the candidal loads ($H=15.973$, $p=0.003$) and bacterial loads ($H=14.605$, $p=0.003$) of the palate. There were no significant differences between all three groups for the microbial loads of the buccal mucosa site.

Furthermore, the control group was used as a baseline to determine whether the use of dentures or the onset of periodontal disease could have any significant difference on microbial loads in the oral cavity. Thus, a two-independent variable test, also known as the Mann-Whitney test, was used to compare between the average microbial loads at each of the oral sites of the sampled groups (the periodontal disease and denture wearer groups) and the control group. Table 4.1 shows the average CFU counts, represented in medians, of the candidal and bacterial loads, as well as the results of the Mann-Whitney non-parametric two-independent variable test, for each of the sampled oral sites of each of the three groups. All significance tests considered significance to be at p value < 0.05.

As can be seen, in the denture wearers group, the average bacterial loads of the saliva and the buccal mucosa have no significant difference with the control group, while all the other average microbial loads are significantly higher in comparison to the control group. Meanwhile, in the periodontal disease group, the average microbial loads of the tongue are significantly higher than in the control group, while the average microbial loads of the other three sites have no significant differences with the control group

Table 4.1: Median Microbial Loads of the Saliva, Tongue, Palate and Buccal Mucosa Sites of the Healthy Control Group, Periodontal Disease Group and Denture Wearers Group

Site	Healthy Control Group		Denture Wearers Group		Periodontal Disease Group	
	Median Candidal CFU/mL	*Median Bacterial CFU/mL*	*Median Candidal CFU/mL*	*Median Bacterial CFU/mL*	*Median Candidal CFU/mL*	*Median Bacterial CFU/mL*
Saliva	0.02×10^3	54.93×10^3	0.80×10^3 [b]	163.07×10^3 [a]	0.14×10^3 [a]	32.98×10^3 [a]
Tongue	2.61×10^3	281.57×10^3	244.77×10^3 [c]	$1{,}711.46 \times 10^3$ [d]	128.02×10^3 [h]	$1{,}049.435 \times 10^3$ [i]
Palate	2.52×10^3	44.06×10^3	40.07×10^3 [e]	165.90×10^3 [f]	3.68×10^3 [a]	85.160×10^3 [a]
Buccal mucosa	2.68×10^3	296.53×10^3	12.04×10^3 [g]	287.00×10^3 [a]	13.175×10^3 [a]	108.24×10^3 [a]

[a] No significant difference with the control group

[b] **Significantly higher** (U = 34, p = 0.001) than the control group

[c] **Significantly higher** (U = 47, p = 0.002) than the control group

[d] **Significantly higher** (U = 37, p = 0.003) than the control group

[e] **Significantly higher** (U = 29, p = 0.001) than the control group

[f] **Significantly higher** (U = 39, p = 0.012) than the control group

[g] **Significantly higher** (U = 36, p = 0.005) than the control group

[h] **Significantly higher** (U = 46, p = 0.006) than the control group

[i] **Significantly higher** (U = 45, p = 0.009) than the control group

4.1.2 Mean Percentages of Candidal Loads

The percentages of candidal loads out of total microbial loads were calculated for each sample. Table 4.2 shows the mean percentages of candidal loads out of total microbial loads for each sampled oral site of each of the three groups. Standard deviations are shown for each mean.

For the control group, it can be seen that bacteria clearly dominates at each of the oral sites, as the candidal load at each oral site constitutes only 0.6% to 17% of the total microbial load. Out of the five sampled oral sites in the control group, the surface of the tongue has the highest percentage of candidal load, while saliva contains the lowest. The ranking of mean candidal load percentage for the control group, in order from lowest to highest, is as follows: Saliva < Palate < Buccal mucosa < Tongue.

In the denture wearers group, the palate surface is the oral site which has the highest percentage of candidal load, followed by the surface of the tongue. Meanwhile saliva once again has the lowest candidal percentage. The saliva, tongue and palate surfaces have higher percentages of candidal load compared to the control group, while the cheek mucosal surface and the gingival sulcus have lower percentages. The ranking of mean candidal load percentage for the denture-wearers group, in order from lowest to highest, is as follows: Saliva < Buccal mucosa < Tongue < Palate.

Table 4.2: Mean Percentages and Standard Deviations of Candidal Loads out of Total Microbial Loads

Group	**Oral Site**	**Mean Percentage of Candidal Loads out of Total Microbial Loads**	
		Mean Percentage	*Standard Deviation*
Healthy Control	Saliva	0.61%	1.09
	Tongue	16.82%	30.78
	Palate	7.75%	9.65
	Buccal mucosa	11.81%	18.84
Denture Wearers	Saliva	1.41%	1.23
	Tongue	19.11%	24.95
	Palate	22.27%	25.85
	Buccal mucosa	8.71%	12.61
Periodontal Disease	Saliva	1.67%	3.77
	Tongue	11.42%	14.31
	Palate	13.41%	17.54
	Buccal mucosa	12.44%	23.87

Meanwhile for the periodontal disease group, the percentages of candidal load range between 1.6% and 14%. Saliva once again contains the lowest percentage of candidal load while the palate now has the highest. The percentages of candidal load at each site are higher than in the control group, with the exception of the tongue surface, which has a comparatively lower percentage. Apart from the saliva, the percentages of candidal load at all of the sampled oral sites are between 11% to 14%. The ranking of mean candidal load percentage for the periodontal disease group, in order from lowest to highest, is as follows: Saliva < Tongue < Buccal mucosa < Palate.

4.2 Assessment of Candidal DNA Extractions

Four different extraction methods were assessed for their effectiveness at extracting candidal genomic DNA for this study.

4.2.1 Estimation of Time and Materials Required for Extractions

Table 4.6 compares the estimated time and materials required for each of the four DNA extraction methods used in this study. As can be seen, the lyticase-based method appears to require the most materials in addition to being the most labor-intensive. The commercial kit method, the freeze-thaw method and the glass beads disruption method take around the same amount of time, however the glass beads disruption method becomes less practical and requires more time with large batch numbers. The freeze-thaw method is both the fastest and most convenient method while also being the cheapest as it requires the fewest amount of materials.

Table 4.3: Comparison of Required Time and Materials of Four DNA Extraction Methods

Method	**Estimated Extraction Time (minutes)**	**Required Materials (per sample)**													
		1.5 mL Centrifuge tubes	TE Buffer (µl)	70% EtOH (µl)	Sodium acetate (µl)	Iso-propanol (µl)	Lysis Buffer (µl)	Chlo-roform (µl)	PCI (µl)	Sor-bitol (µl)	Ly-ticase (µl)	SDS (µl)	Pro-teinase (µl)	Rnase A (µl)	3× TE Buffer (µl)
Commer-cial Kit	120	2	-	500	-	500	-	-	-	-	-	-	-	-	-
Rapid Freeze-Thawing	120	2	50	500	-	-	200	200	-	-	-	-	-	-	-
Glass Beads Disruption	150 (extra 10 mins. for each extra 2 samples)	3	50	-	20	220	300	500	300	-	-	-	-	-	-
Lyticase-Based	360 minutes	2	300	-	30	1050	-	-	-	500	250	25	5	1.5	500

4.2.2 Pilot Study of Candidal DNA Extraction Methods

The four DNA extraction methods were first carried out on the *Candida albicans* (ATCC 14053) control sample. The yeast cultures for each DNA extraction were standardized to 10^6 cells per mL by spectrophotometry (refer to 3.2.4.1). After confirming the presence of DNA in the extracts using gel electrophoresis, it was determined that only two of the four extraction methods were successful in extracting genomic candidal DNA. DNA extraction using those two methods was then carried out on four of the ATCC control species as samples: *Candida tropicalis* (ATCC 13803), *Candida parapsilosis* (ATCC 22019), *Candida glabrata* (ATCC 90030) and *Candida lusitaniae* (ATCC 64125). The yield and the purity of these DNA extracts were determined with spectrophotometry.

4.3.2.1 Qualitative Confirmation of Candidal DNA Extracts

Figures 4.1 and 4.2 show the presence of DNA in the extracts from four different extraction methods: Method 1 – Commercial Kit (see 3.2.4.2), Method 2 – Rapid Freeze-Thawing (see 3.2.4.3), Method 3 – Glass Beads Disruption (see 3.2.4.4) and Method 4 – Lyticase-Based (see 3.2.4.5), as visualized under ultraviolet light in 1% agarose gels that underwent electrophoresis at 70 V for 60 minutes. Based on visual confirmation of the DNA under ultraviolet light, it was determined that the commercial kit and glass bead disruption methods were succesful in extracting genomic DNA from the ATCC colonies.

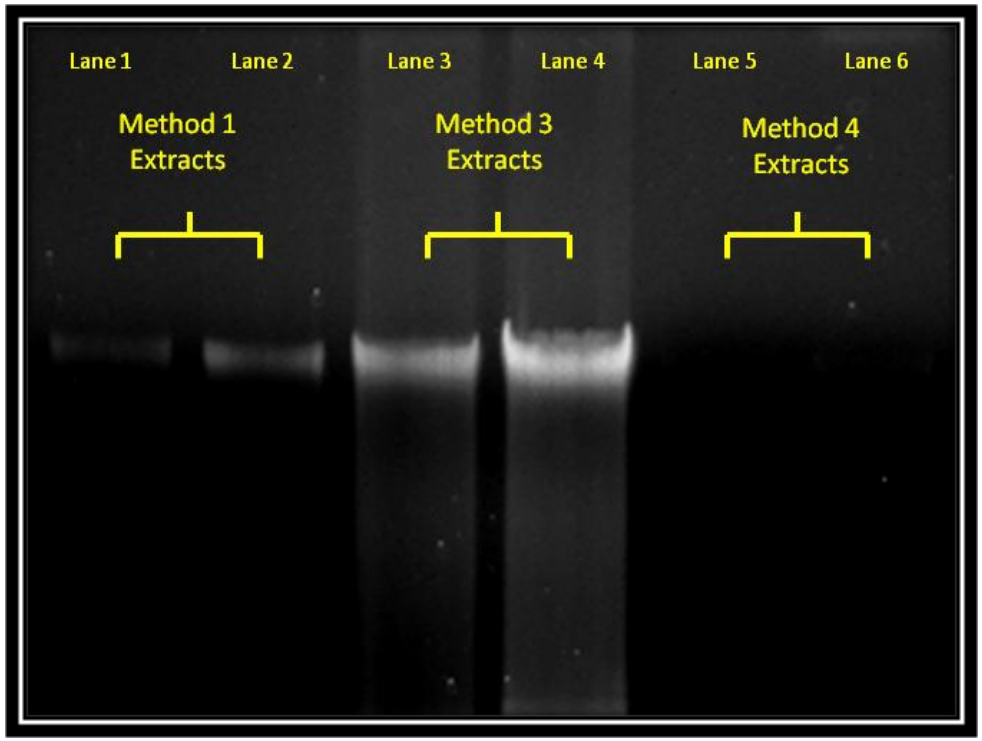

Figure 4.1: Gel Picture Comparing the Presence of Candidal DNA from the Extracts of Method 1 (Commercial Kit) in Lanes 1-3, Method 2 (Rapid Freeze-Thawing) in Lanes 4-5 and Method 3 (Glass Beads Disruption) in Lanes 6-7

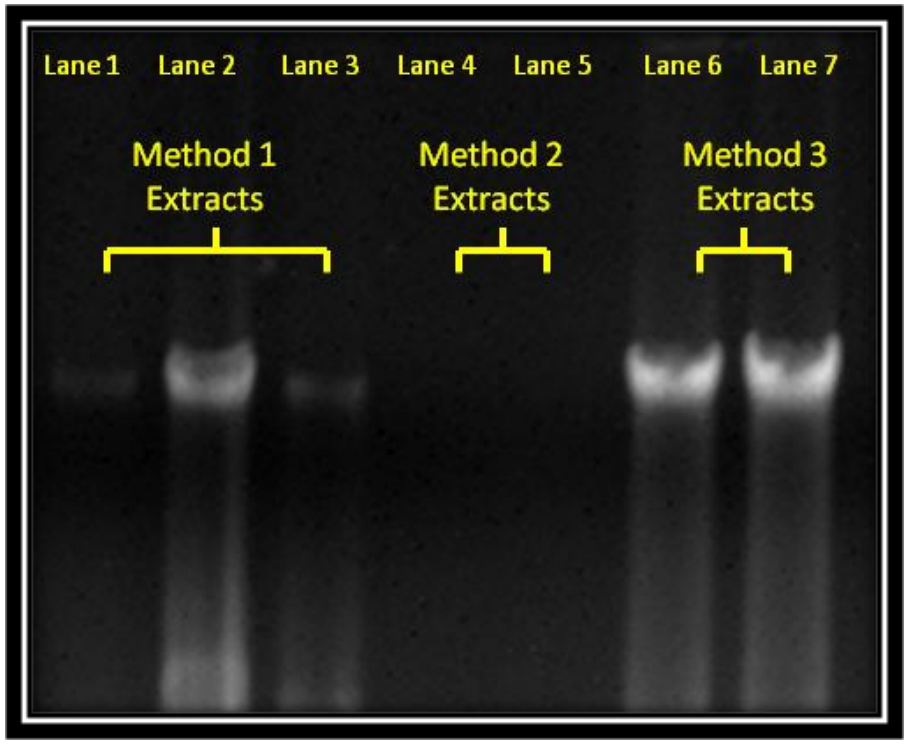

Figure 4.2: Gel picture comparing the presence of DNA from the extracts of Method 1 (Commercial Kit) in Lanes 1-2, Method 3 (Glass Beads Disruption) in Lanes 3-4 and Method 4 (Lyticase-Based) in Lanes 5-6

4.3.2.2 Quantitative Assessment of Candidal DNA Extracts

The yield and purity of the DNA extracts of four ATCC control samples using the two different methods was compared in order to determine which method was the best method to be used in this study.

4.3.2.2.1 DNA Yield

Table 4.7 compares the average concentration of the extracted DNA of four ATCC control species using two different methods, the glass beads disruption method and the commercial kit method. As can be seen, the glass beads disruption extraction method produced higher yields for the *Candida tropicalis* and *Candida lusitaniae* samples, whereas the commercial kit produced higher yields for the *Candida parapsilosis* and *Candida glabrata* samples.

4.3.2.2.2 DNA Purity

Table 4.8 compares the number of DNA extracts, from a total of 3 each, that were free of protein and phenol contamination between the commercial kit and glass beads disruption extraction method. As the table shows, with the exception of *Candida glabrata,* the glass beads disruption extraction method resulted in more extracts that were free of protein or phenol contamination.

Table 4.4: Mean Concentration of Extracted DNA of Four ATCC Control Species

Species	Glass Beads Disruption Method		Commercial Kit	
	Mean Concentration (µg/µL)	Standard Deviation	Mean Concentration (µg/mL)	Standard Deviation
Candida tropicalis (ATCC 13803)	1.528	1.099	1.318	0.647
Candida parapsilosis (ATCC 22019)	0.122	0.059	0.388	0.039
Candida glabrata (ATCC 90030)	1.481	1.383	5.61	1.732
Candida lusitaniae (ATCC 64125)	0.825	0.389	0.555	0.007

Table 4.5: Number of DNA Extracts Free From Protein and Phenol Contamination of Four ATCC Control Species

Species	Glass Beads Disruption Method		Commercial Kit	
	Number of Extracts Free From Protein Contamination	Number of Extracts Free From Phenol Contamination	Number of Extracts Free From Protein Contamination	Number of Extracts Free From Phenol Contamination
Candida tropicalis (ATCC 13803)	1 out of 3	3 out of 3	0 out of 3	2 out of 3
Candida parapsilosis (ATCC 22019)	2 out of 3	2 out of 3	0 out of 3	2 out of 3
Candida glabrata (ATCC 90030)	0 out of 3	3 out of 3	2 out of 3	3 out of 3
Candida lusitaniae (ATCC 64125)	3 out of 3	3 out of 3	1 out of 3	3 out of 3

4.3.3 Selection of Optimal DNA Extraction Method

Comparing between the commercial kit and glass beads disruption as methods for DNA extraction, it was noted that the glass beads disruption method is more labor-intensive and more costly. In terms of DNA yield, the commercial kit produced better DNA yields for two of the four tested ATCC species, whereas glass beads disruption resulted in better yields for the other two species. However, almost all of the glass beads disruption DNA extracts had better purity, in terms of being free of protein contamination, compared to the commercial kit DNA extracts. As DNA purity is highly important to ensure the success of PCR amplification, it was decided to use the glass beads disruption method for the extraction of DNA from the rest of the ATCC and clinical samples.

4.4 PCR Amplification of rDNA Regions

Three primer pairs were used to amplify three different rDNA regions of candidal genomic DNA: the ITSI region (4.4.1), the 5.8S-ITSII region (4.4.2) and the entire ITSI-5.8S-ITSII region (4.4.3). Following analysis of the sizes of the generated PCR productss and restriction fragments (see 4.5), the fifteen unidentified clinical samples were grouped into six different genotype profiles, designated in this study as A, B, C, D, E and F (see 4.6). For convenience, the clinical samples have been labeled throughout this chapter according to their genotype profile group.

4.4.1 Amplification of ITSI Region

Figures 4.3 and 4.4 show the ITSI PCR products of the seven ATCC control species, and the 15 clinical samples, respectively, as visualized on 1.5% agarose gels. Table 4.9 summarizes the sizes of all the ITSI PCR products.

With the exception of *C. glabrata* ATCC 90030 and the C1-C4 group which have a distinctively large ITS1 band size, all the tested samples have ITSI sizes ranging between 120 and 220. Three of the ATCC control species, namely *C. albicans* ATCC 14053, *C. tropicalis* ATCC 13803 and *C. parapsilosis* ATCC 22019 have identical band sizes, and *C. dubliniensis* ATCC MYA-2975 has a band size that is very close to the aforementioned three, implicating difficulties if ITS1 were to be used for species identification. Meanwhile, *C. krusei* ATCC 14243 and *C. lusitaniae* ATCC 64125 have distinctively smaller band sizes compared to the other samples. The F1 genotype is the only unidentified sample that does not share the same size with any of the ATCC species.

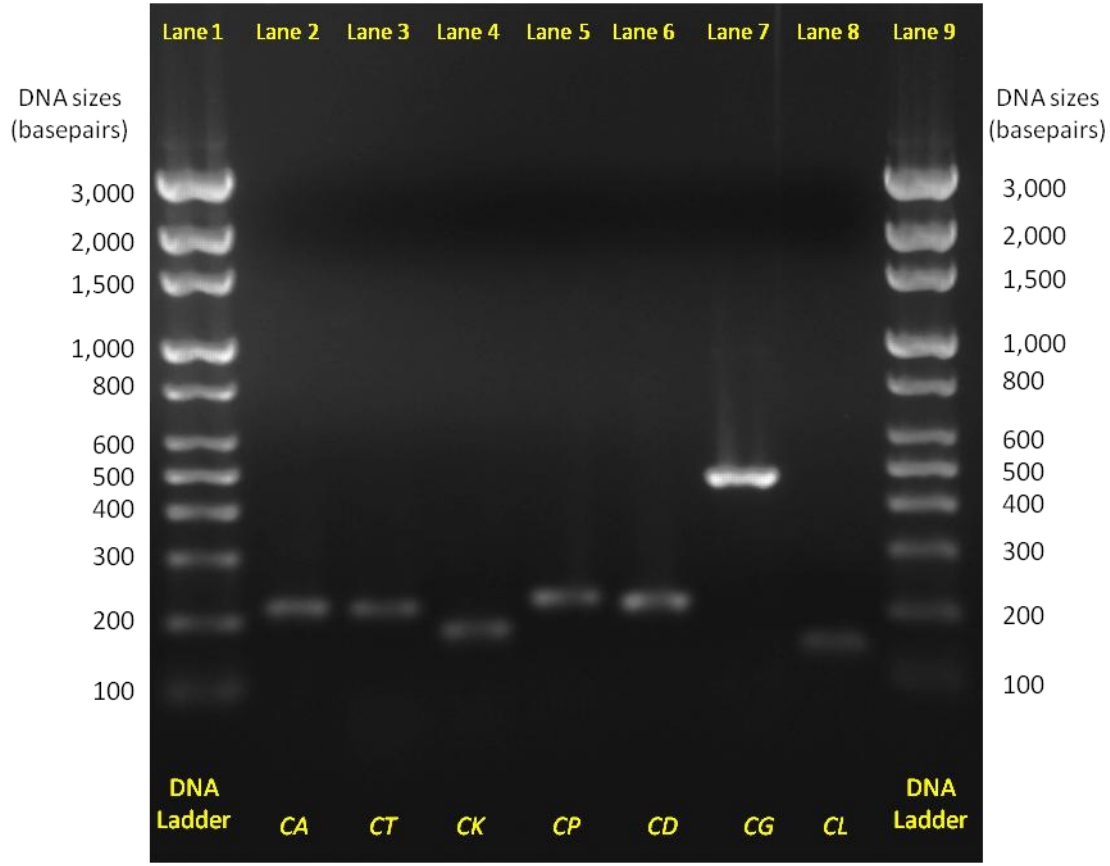

Figure 4.3: Gel Picture Showing the ITS1 PCR Products of *C. albicans* ATCC 14053 (CA) – Lane 2, *C. tropicalis* ATCC 13803 (CT) – Lane 3, *C. krusei* ATCC 14243 (CK) – Lane 4, *C. parapsilosis* ATCC 22019 (CP) – Lane 5, *C. dubliniensis* ATCC MYA-2975 (CD) – Lane 6, *C. glabrata* ATCC 90030 (CG) – Lane 7 and *C. lusitaniae* ATCC 64125 (CL) – Lane 8

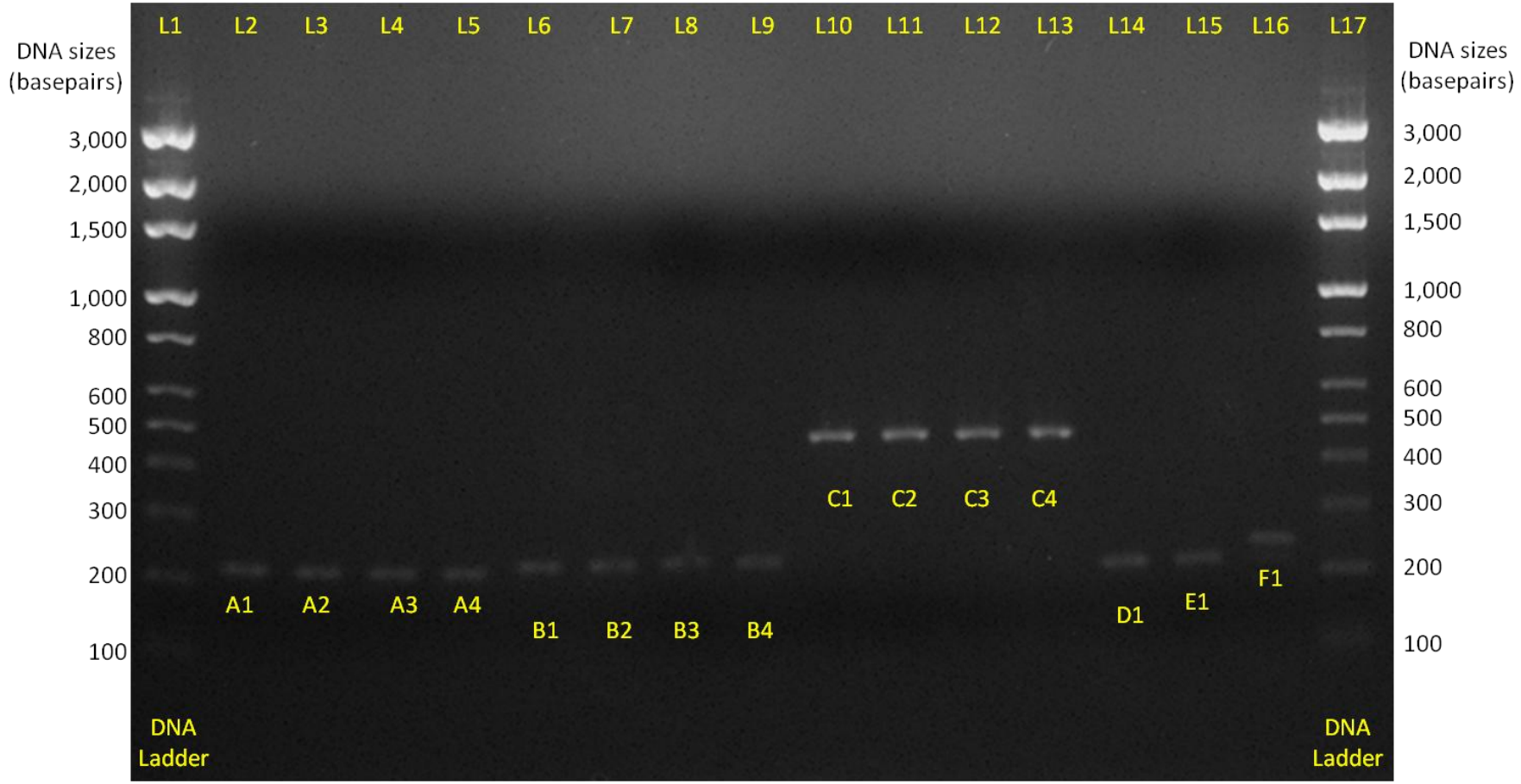

Figure 4.4: Gel Picture Showing the ITS1 PCR Products of the Following Clinical Samples: A1-4 (Lanes 2-5), B1-4 (Lanes 6-9), C1-4 (Lanes 10-13), D1 (Lane 14), E1 (Lane 15) and F1 (Lane 16)

Table 4.6: PCR Product Sizes of the Amplified ITSI Region

Samples	**ITSI Size** (basepairs)
C. albicans ATCC 14053 *C. tropicalis* ATCC 13803 *C. parapsilosis* ATCC 22019 A1, A2, A3, A4, B1, B2, B3, B4, D1	220
C. dubliniensis ATCC MYA-2975 E1	210
C. krusei ATCC 14243	170
C. glabrata ATCC 90030 C1, C2, C3, C4	480
C. lusitaniae ATCC 64125	120
F1	230

4.4.2 Amplification of 5.8S-ITSII Region

Figures 4.5 and 4.6 show the ITSI PCR products of the seven ATCC control species, and the 15 clinical samples, respectively, as visualized on 1.5% agarose gels. Table 4.10 summarizes the sizes of all the 5.8S-ITSII PCR products.

The band sizes of the 5.8S-ITSII region range between 250 and 420 basepairs. Once again *C. glabrata* ATCC 90030 has the largest band size and *C. lusitaniae* ATCC 64125 has the smallest, making both easily distinguishible. Unlike ITS1, *C. albicans* ATCC 14053, *C. krusei* ATCC 14243, *C. dubliniensis* ATCC MYA-2975 have the same band size, while *C. tropicalis* ATCC 13803 has a band size that is close to them and difficult to visually distinguish. Once again, the F1 genotype band size does not match that of any of the ATCC control species.

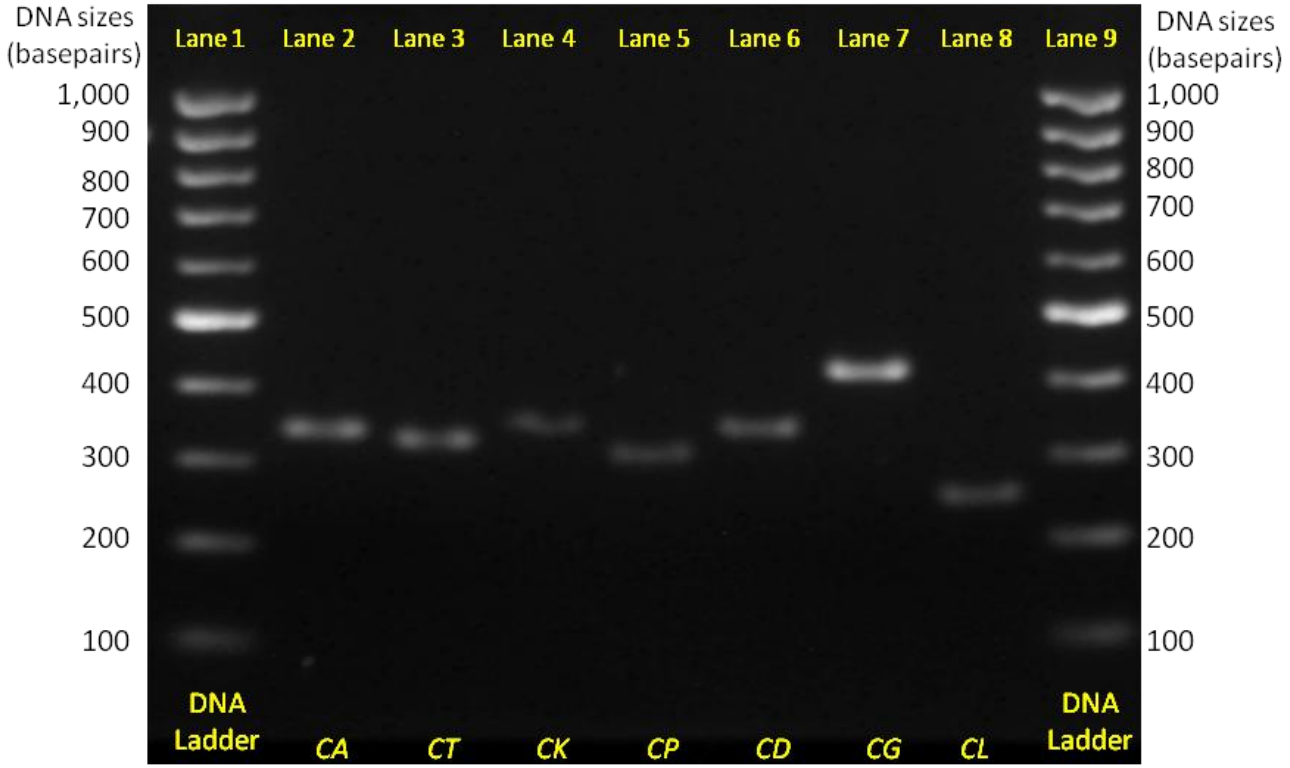

Figure 4.5: Gel Picture Showing the 5.8S-ITSII PCR Products of *C. albicans* ATCC 14053 (CA) – Lane 2, *C. tropicalis* ATCC 13803 (CT) – Lane 3, *C. krusei* ATCC 14243 (CK) – Lane 4, *C. parapsilosis* ATCC 22019 (CP) – Lane 5, *C. dubliniensis* ATCC MYA-2975 (CD) – Lane 6, *C. glabrata* ATCC 90030 (CG) – Lane 7 and *C. lusitaniae* ATCC 64125 (CL) – Lane 8

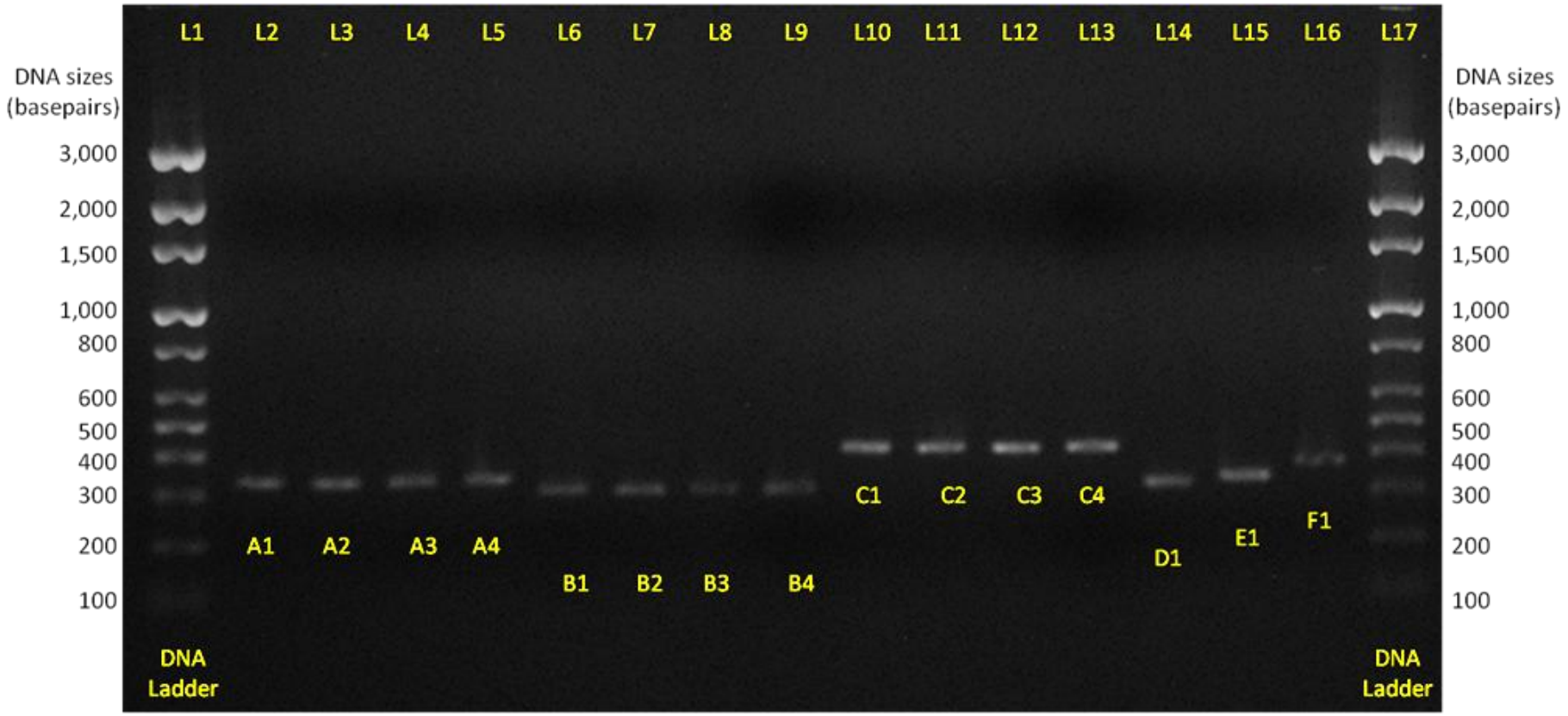

Figure 4.6: Gel Picture Showing the 5.8S-ITSI1 PCR products of the Following Clinical samples: A1-4 (Lanes 2-5), B1-4 (Lanes 6-9), C1-4 (Lanes 10-13), D1 (Lane 14), E1 (Lane 15) and F1 (Lane 16)

Table 4.7: PCR Product Sizes of the Amplified 5.8S-ITSII Region

Samples	**5.8S-ITSII Size** (basepairs)
C. albicans ATCC 14053 *C. krusei* ATCC 14243 *C. dubliniensis* ATCC MYA-2975 A1, A2, A3, A4, E1	340
C. tropicalis ATCC 13803 D1	320
C. parapsilosis ATCC 22019	300
C. glabrata ATCC 90030 C1, C2, C3, C4	420
C. lusitaniae ATCC 64125	250
F1	370

4.4.3 Amplification of ITSI-5.8S-ITSII Region

Figures 4.7 and 4.8 show the ITSI-5.8S-ITSII PCR products of the seven ATCC control ATCC species, and the 15 clinical samples, respectively, as visualized on 1.5% agarose gels. Table 4.11 summarizes the sizes of all the ITSI-5.8S-ITSII PCR products.

PCR amplification of the ITSI-5.8S-ITSII region generates band sizes of around 500 basepairs for almost all of the tested samples, with *C. glabrata* ATCC 90030 and *C. lusitaniae* ATCC 64125 once again distinctively having the largest and smallest band sizes, respectively. Two pairs of ATCC control species, *C. albicans* ATCC 14053 and *C. dubliniensis* MYA-2975, as well as *C. tropicalis* ATCC 13803 and *C. parapsilopsis* ATCC 22019, have similar band sizes. Unsurprisingly, the F1 genotype cannot be matched with any of the ATCC control species.

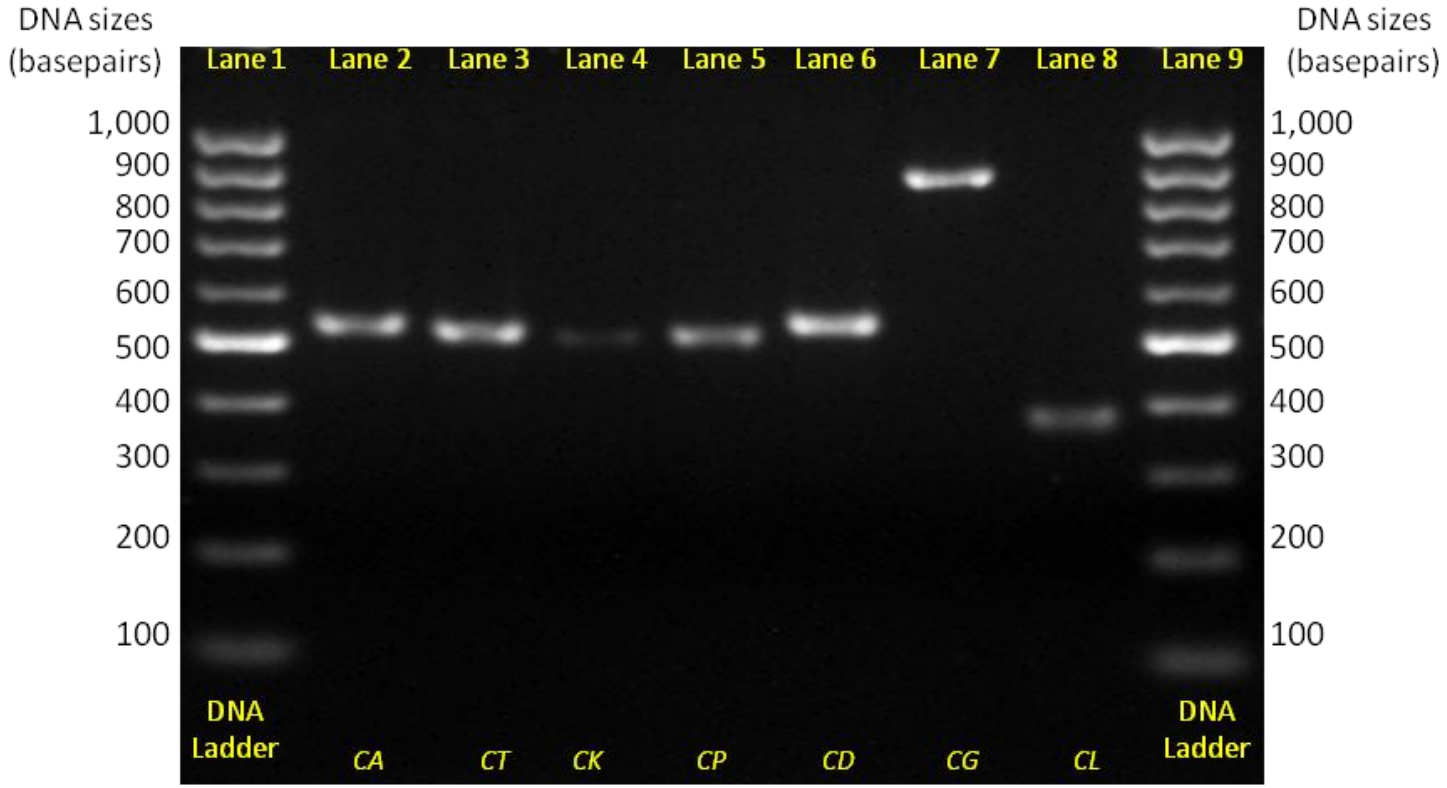

Figure 4.7: Gel Picture Showing the ITSI-5.8S-ITSII PCR Products of *C. albicans* ATCC 14053 (CA) – Lane 2, *C. tropicalis* ATCC 13803 (CT) – Lane 3, *C. krusei* ATCC 14243 (CK) – Lane 4, *C. parapsilosis* ATCC 22019 (CP) – Lane 5, *C. dubliniensis* ATCC MYA-2975 (CD) – Lane 6, *C. glabrata* ATCC 90030 (CG) – Lane 7 and *C. lusitaniae* ATCC 64125 (CL) – Lane 8

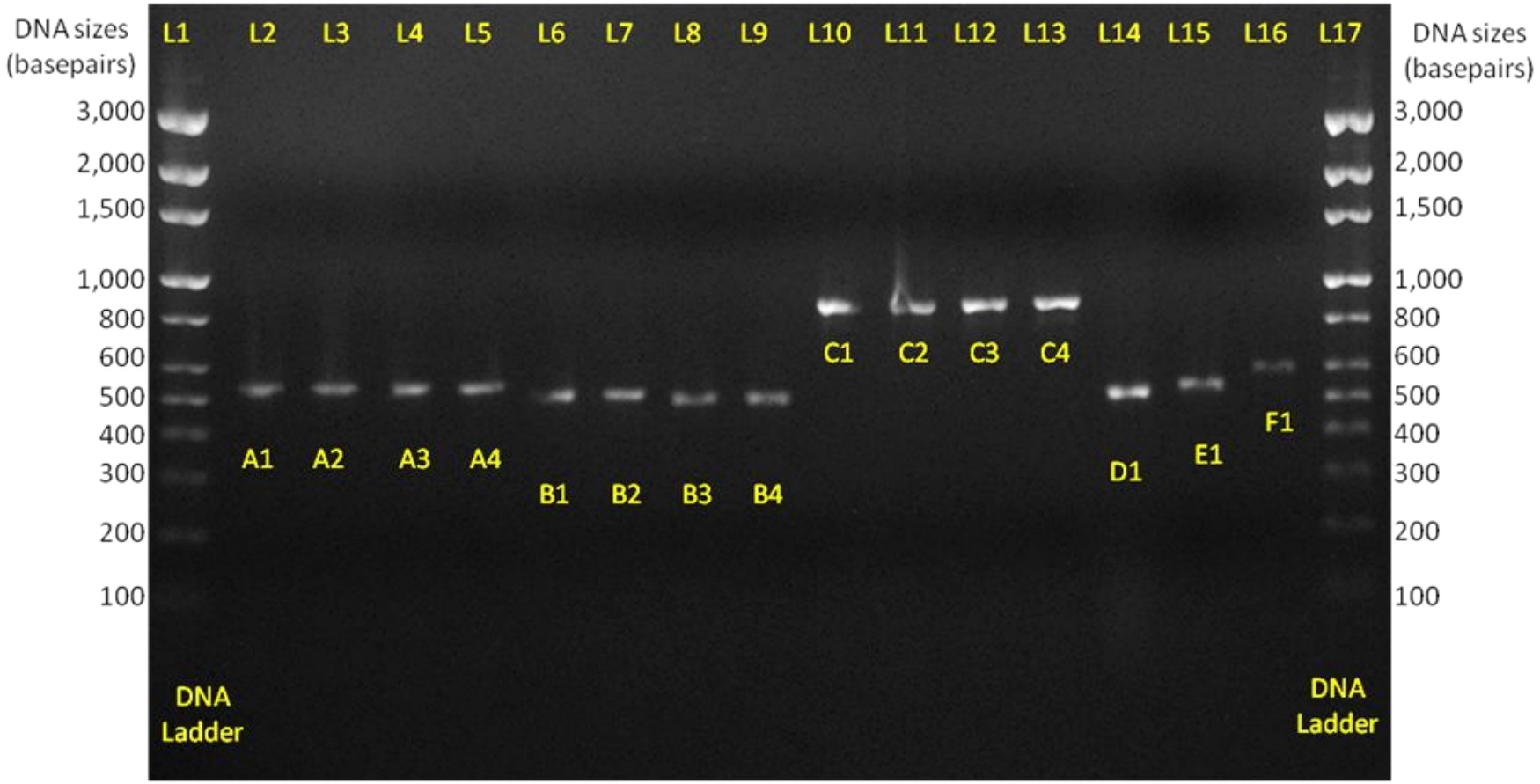

Figure 4.8: Gel Picture Showing the ITS1-5.8S-ITSII PCR Products of the Following Clinical Samples: A1-4 (Lanes 2-5), B1-4 (Lanes 6-9), C1-4 (Lanes 10-13), D1 (Lane 14), E1 (Lane 15) and F1 (Lane 16)

Table 4.8: PCR Product Sizes of the Amplified ITSI-5.8S-ITSII Region

Samples	**ITSI-5.8S-ITSII Size** (basepairs)
C. albicans ATCC 14053 *C. dubliniensis* MYA-2975 A1, A2, A3, A4, E1	540
C. tropicalis ATCC 13803 *C. parapsilopsis* ATCC 22019 B1, B2, B3, B4, D1	520
C. krusei ATCC 14243	510
C. glabrata ATCC 90030 C1, C2, C3, C4	900
C. lusitaniae	380
F1	630

4.5 Restriction Fragment Length Polymorphisms

The restriction enzymes *Msp*I and *Hinf*I were each used seperately to digest the ITSI-5.8S-ITSII PCR product.

4.5.1 *Msp*I Digestion

Figures 4.9 and 4.10 show the *Msp*I restriction fragments of the ITSI-5.8S-ITSII PCR products of the seven ATCC control ATCC species, and the 15 clinical samples, respectively, as visualized on 2% agarose gels. Table 4.12 summarizes the sizes of all the restriction fragments.

*Msp*I digestion of the ITSI-5.8S-ITSII region produced unique restriction fragment patterns for all of the ATCC species, with the exception of *C. albicans* and *C. dubliniensis*, which have identical patterns. The PCR product of *C. parapsilosis* was not cut at all, retaining its original size, indicating that it lacks the *Msp*I recognition site. The other patterns are very distinctive and easy to distinguish. For the clinical samples, it generated five different restriction fragment patterns, four of which could be matched to the restriction fragment pattern of at least one ATCC species. The F1 clinical sample once again has a very different genotype compared to the rest.

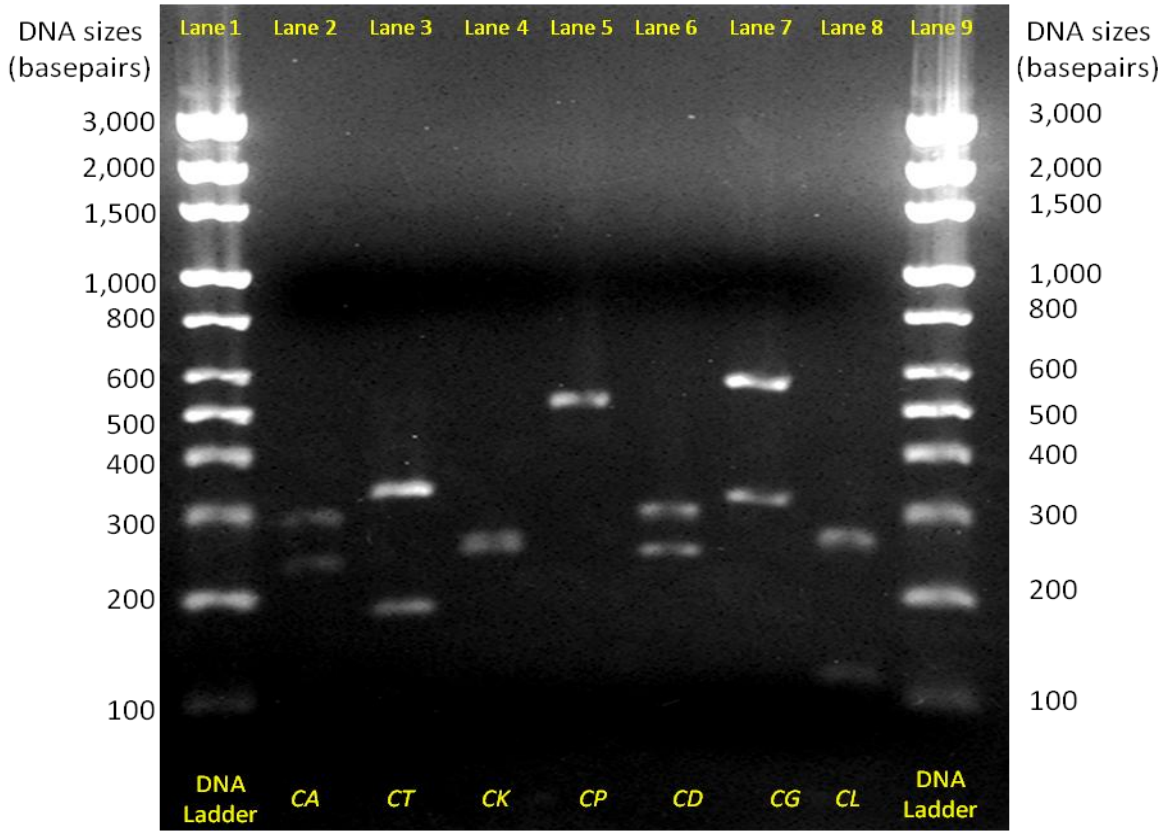

Figure 4.9: Gel Picture Showing the *Msp*I Restriction Fragments of the ITSI-5.8S-ITSII PCR Products of *C. albicans* ATCC 14053 (CA) – Lane 2, *C. tropicalis* ATCC 13803 (CT) – Lane 3, *C. krusei* ATCC 14243 (CK) – Lane 4, *C. parapsilosis* ATCC 22019 (CP) – Lane 5, *C. dubliniensis* ATCC MYA-2975 (CD) – Lane 6, *C. glabrata* ATCC 90030 (CG) – Lane 7 and *C. lusitaniae* ATCC 64125 (CL) – Lane 8

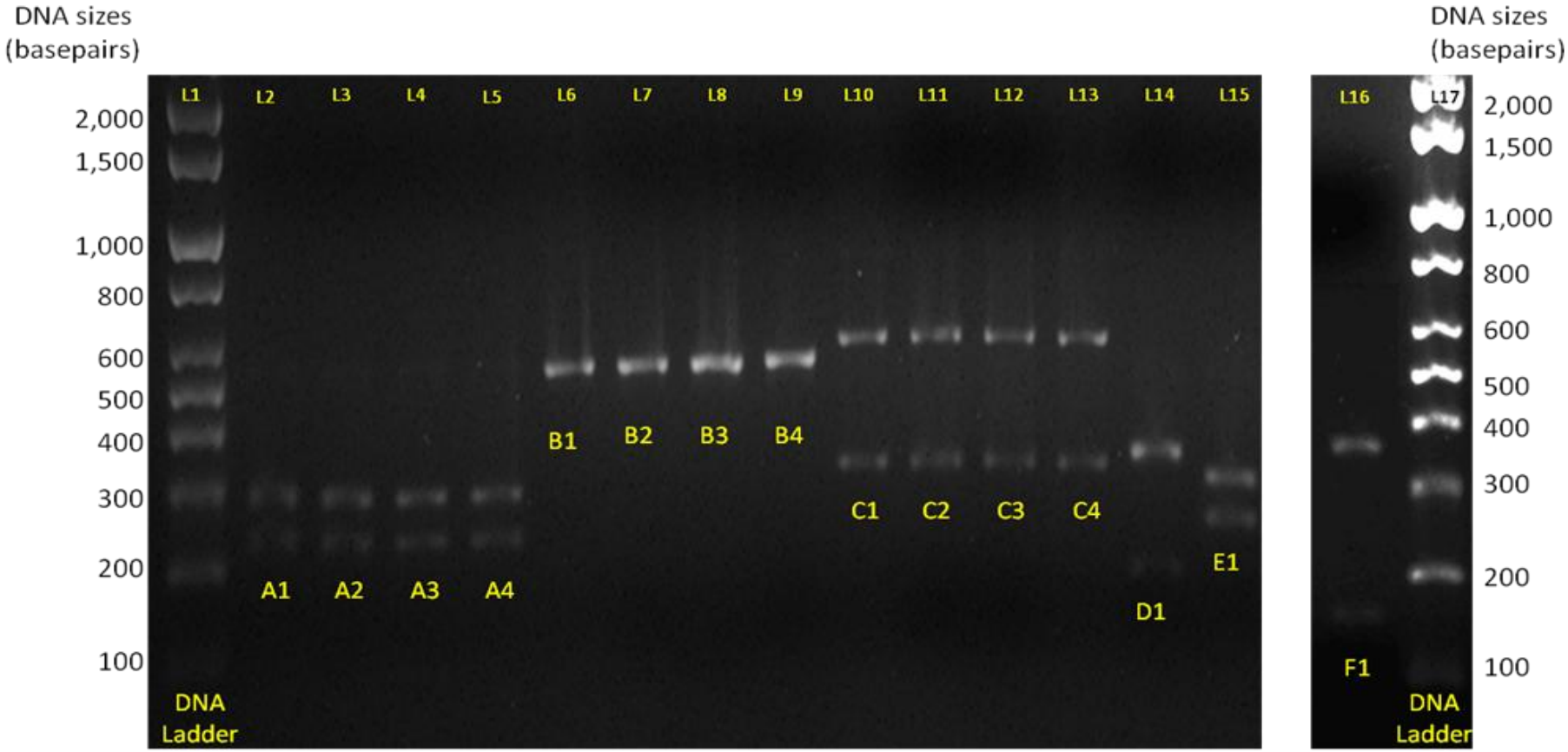

Figure 4.10: Gel Pictures Showing the *Msp*I Restriction Fragments of the ITSI-5.8S-ITSI1 PCR Products of the Following Clinical Samples: A1-4 (Lanes 2-5), B1-4 (Lanes 6-9), C1-4 (Lanes 10-13), D1 (Lane 14), E1 (Lane 15) and F1 (Lane 16)

Table 4.9: *Msp*I Restriction Fragment Sizes of the ITSI-5.8S-ITSII PCR Product

Samples	**Total ITSI-5.8S-ITSII Size** (basepairs)	***Msp*I Fragments Sizes** (basepairs)
C. albicans ATCC 14053 *C. dubliniensis* ATCC MYA-2975 A1, A2, A3, A4, E1	540	300, 240
C. tropicalis ATCC 13803 D1	520	340, 180
C. krusei ATCC 14243	510	260, 250
C. parapsilosis ATCC 22019 B1, B2, B3, B4	520	520
C. glabrata ATCC 90030 C1, C2, C3, C4	900	570, 330
C. lusitaniae ATCC 64125	380	260, 120
F1	630	370, 160

4.5.2 *Hinf*I Digestion

Figures 4.11 and 4.12 show the *Hinf*I restriction fragments of the ITSI-5.8S-ITSII PCR products of the seven ATCC control ATCC species, and the 15 clinical samples, respectively, as visualized on 2% agarose gels. In many cases, the sizes of the restriction fragments were too similar to the point that the fragments did not seperate from each other within the gel; in such cases the presence of the fragment was extrapolated based on the total size of the ITSI-5.8S-ITSII PCR product. The bands that were assumed to represent multiple fragments of identical sizes are represented in the table in brackets. Table 4.13 summarizes the sizes of all the restriction fragments.

*Hinf*I digestion of the ITSI-5.8S-ITSII region produced unique restriction fragment patterns for all of the ATCC species, with the exception of *C. tropicalis* and *C. parapsilosis*, which have identical patterns. Furthermore the fragment sizes of these two species are very similar to that of *C. albicans*, making distinguishing between them prone to error. Apart from that, *Hinf*I digestion generated easily distinguishible restriction fragment patterns for the other species. The F1 genotype has a unique *Hinf*I restriction fragment pattern.

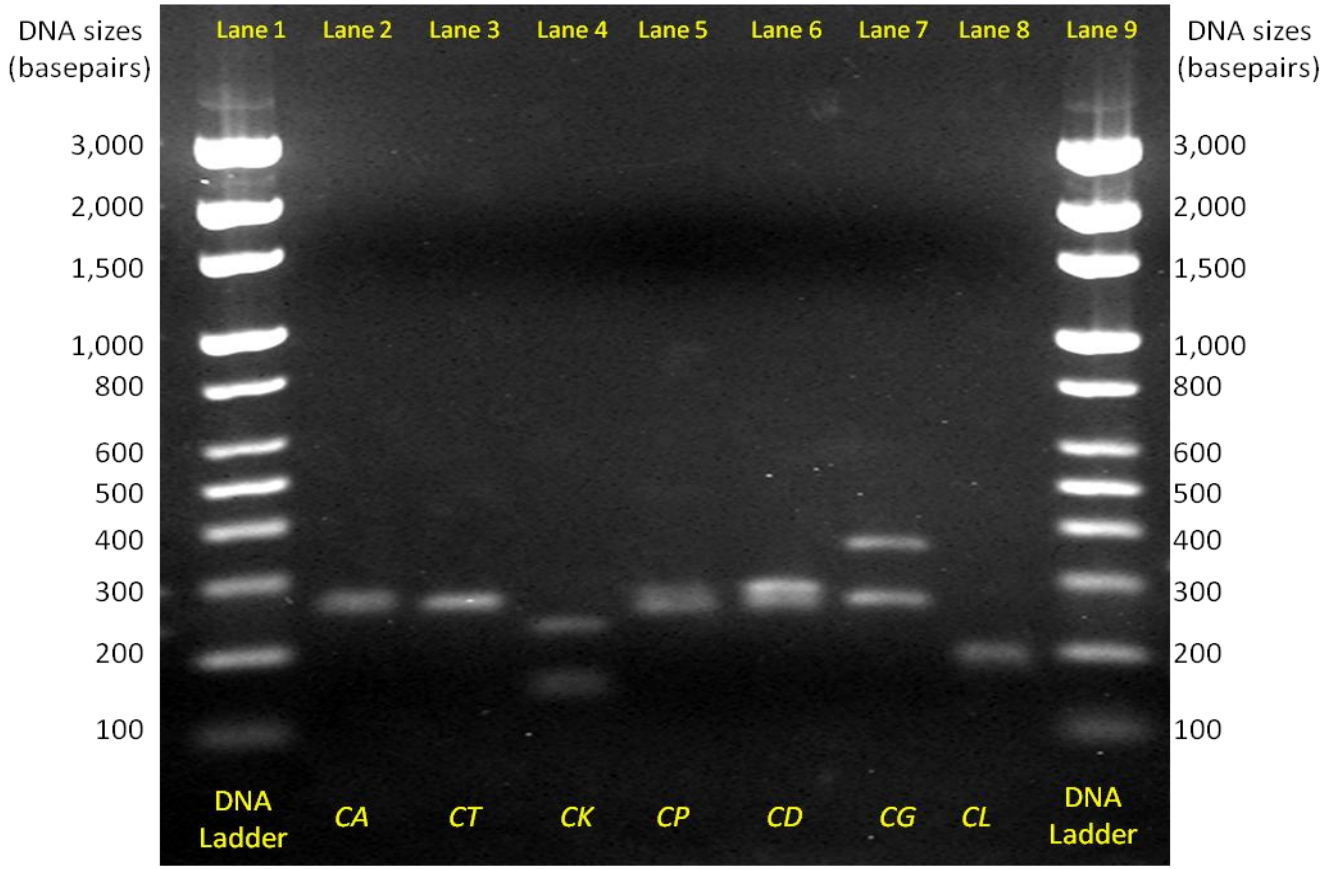

Figure 4.11: : Gel Picture Showing the *Hinf*I Restriction Fragments of the ITSI-5.8S-ITSII PCR Products of *C. albicans* ATCC 14053 (CA) – Lane 2, *C. tropicalis* ATCC 13803 (CT) – Lane 3, *C. krusei* ATCC 14243 (CK) – Lane 4, *C. parapsilosis* ATCC 22019 (CP) – Lane 5, *C. dubliniensis* ATCC MYA-2975 (CD) – Lane 6, *C. glabrata* ATCC 90030 (CG) – Lane 7 and *C. lusitaniae* ATCC 64125 (CL) – Lane 8

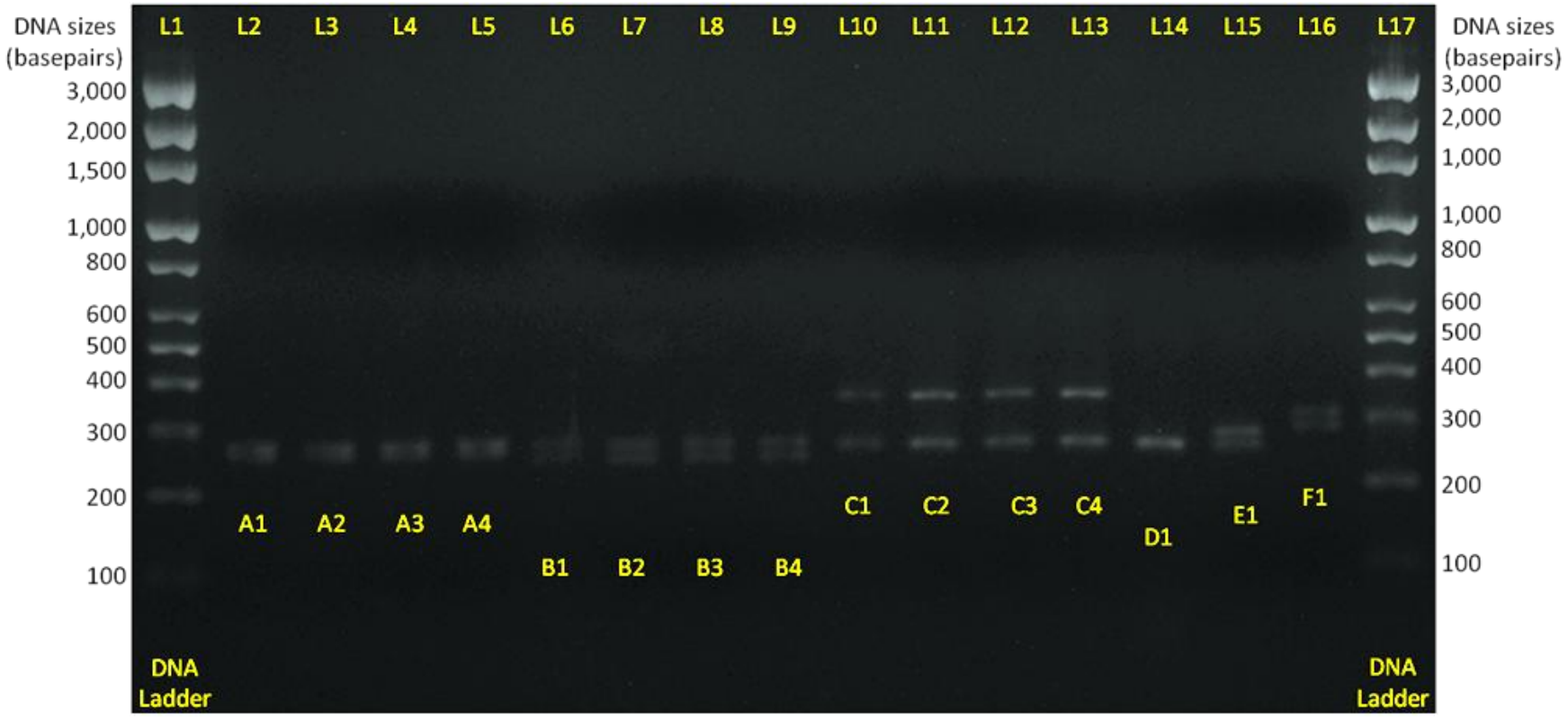

Figure 4.12: Gel Picture Showing the *Hinf*I Restriction Fragments of the 5.8S-ITSI1 PCR Products of the Following Clinical Samples: A1-4 (Lanes 2-5), B1-4 (Lanes 6-9), C1-4 (Lanes 10-13), D1 (Lane 14), E1 (Lane 15) and F1 (Lane 16)

Table 4.10: *Hinf*I Restriction Fragment Sizes of the ITSI-5.8S-ITSII PCR Product

Samples	**Total ITSI-5.8S-ITSII Size** (basepairs)	***Hinf*I Fragments Sizes** (basepairs)
C. albicans ATCC 14053 A1, A2, A3, A4	540	(270, 270)
C. tropicalis ATCC 13803 *C. parapsilosis* ATCC 22019 B1, B2, B3, B4, D1	520	(260, 260)
C. krusei ATCC 14243	510	240, (150, 150)
C. dubliniensis ATCC MYA-2975 E1	540	290, 260
C. glabrata ATCC 90030 C1, C2, C3, C4	900	570, 330
C. lusitaniae ATCC 64125	380	360, (270, 270)
F1	630	320, 290

4.6 Genotype Profiles

Based on the sizes of the PCR products (see 4.3) and restriction fragments (see 4.4), it was possible to construct a genotype profile for each of the seven ATCC samples and fifteen clinical samples. By matching the profiles of the clinical samples to the ATCC samples, it was possible to determine the species of fourteen out of fifteen of the clinical samples. This is summarized in Tables 4.14 and 4.15.

However, the F1 clinical species is unknown, as its genotype profile does not match any of the seven ATCC species genotype profiles. However, based on the sizes of the ITSI-5.8S-ITSII and 5.8S-ITSII PCR products, it can be surmised that the F1 species is *Candida famata* (Fujita et al., 2001).

It should be noted that the area amplified by the ITS1 and ITS2 primer pair, although refered to as "ITSI" in this thesis, also includes a small part of the 18S rDNA and the 5.8S rDNAs, whereas the ITS3 and ITS3 primer pair that amplifies an area designated as "5.8S-ITSII", similarly includes a small part of the 5.8S and 28S rDNAs. As such, the ITSI and 5.8S-ITSII regions may overlap with each other and the sum of their sizes do not necessarily equal to the size of the entire ITS-5.8S-ITSII region.

Table 4.11: Genotype Profiles of the Seven ATCC Candidal Control Species

Control Species	**ITSI**	**5.8S-ITSII**	**ITSI-5.8S-ITSII**	***MspI* Restriction Fragments**	***HinfI* Restriction Fragments**
C. albicans ATCC 14053	220	340	540	300, 240	(270, 270)
C. tropicalis ATCC 13803	220	320	520	340, 180	(260, 260)
C. krusei ATCC 14243	170	340	510	260, 250	240, (150, 150)
C. parapsilopsis ATCC 22019	220	300	520	520	(260, 260)
C. dubliniensis ATCC MYA-2975	210	340	540	300, 240	290, 260
C. glabrata ATCC 90030	480	420	900	570, 330	360, (270, 270)
C. lusitaniae ATCC 64125	120	250	380	260, 120	(190, 190)

Table 4.12: Genotype Profiles and Species Match of the Fifteen Clinical Samples

	A1-A4	**B1-B4**	**C1-C4**	**D1**	**E1**	**F1**
ITSI	220	220	480	220	210	230
5.8S-ITSII	340	300	420	320	340	370
ITSI-5.8S-ITSII	540	520	900	520	540	630
MspI Restriction Fragments	300, 240	520	570, 330	340, 180	300, 240	370, 160
HinfI Restriction Fragments	(270, 270)	(260, 260)	360, (270, 270)	(260, 260)	290, 260	320, 290
Species Match	*C. albicans*	*C. parapsilosis*	*C. glabrata*	*C. tropicalis*	*C. dubliniensis*	Possibly *C. famata*

5. DISCUSSION

5.1 Microbial Loads of *Candida* Species in the Oral Cavity

5.1.1 Intra-Oral Distribution of *Candida* Species in the Control Group

In the control group as well as in the other two tested groups, saliva is the oral site that has the lowest percentage of candida, with more than 99% of the microorganisms found in the saliva consisting of bacteria. As microorganisms in the saliva are derived from microbes dislodged from other oral surfaces (Marsh and Martin, 1992), salivary microbiota can be considered to reflect changes in the microbiota of the rest of the oral cavity (Li et al., 2005). Hence, the very low percentage of candida in saliva could be a reflection of the low yeast carriage in comparison to bacterial carriage in the oral cavity. In this study, the percentage of candidal load at all oral sites in all three groups is less than 24%. Furthermore, the presence of antimicrobial factors such as histatin, secretory IgA and lysozyme in the saliva (Marsh and Martin, 2009) could be another reason why candidal loads in the saliva are so low.

Meanwhile, the tongue surface was found to have the highest mean percentage of candidal load in the control group. The surface of the tongue is considered to be an ideal environment for candidal colonization because of the humidity, temperature and existence of hidden niches between the papillae of the tongue (Zadik et al., 2010). This is also supported by a study which concluded that the tongue is the primary oral reservoir for candidal species (Arendorf and Walker, 1980). As the most common oral candidiasis typically affects the surface of the tongue (Samaranayake and Yaacob,

1990), it can be surmised that the tongue may harbor many of the potentially pathogenic candidal species responsible for oral candidal infection.

Next to the tongue, the cheek mucosal surface has the highest percentage of candidal occurrence, indicating that the squamous stratified epithelium of the buccal mucosa is less ideal for candidal colonization compared to the papillary surface of the tongue. A possible reason for this is the continuous shedding by exfoliation of the buccal mucosa, as buccal epithelial cells have an estimated turnover rate of 5-6 days (Harris and Robinson, 1992).

Meanwhile, the palate has a lower percentage of candidal occurrences compared to the tongue and the cheek mucosal surfaces, indicating that *Candida* sp. does not compete as well against bacteria for adhesion sites on the surface of the palate. This may be because of the unique qualities of the squamous epithelium of the palate which is keratinized and is also less permeable compared to buccal mucosa (Harris and Robinson, 1992). Furthermore, as different oral sites have different cell surface receptors (Gibbons, 1989), it is also a possibility that the cell surface receptors of the palate favour the cell surface adhesins of bacterial species over candidal species.

5.1.2 Intra-Oral Distribution of *Candida* Species in the Denture Wearers Group

One of the most important roles of saliva in the oral microenvironment is the removal and clearance of microorganisms from the oral cavity (Marsh and Martin, 2009). As the presence of dentures in the oral cavity has the effect of slowing down salivary flow rate (Akpan & Morgan, 2002), this of course encourages the growth of oral microorganisms. Furthermore, denture use encourages the colonisation of oral

Candida (Shulman et al., 2005) in addition to predisposing towards candidal infection (Pires et al., 2002). This is in agreement with the results which show that there is a significantly higher candidal load in the saliva of denture-wearers compared to the control group, while there is no significant difference for the bacterial load. All of this indicates that candidal species in the oral cavity become increasingly competitive in the presence of dentures.

The results show that both the candidal and bacterial loads of the palate are significantly higher than in the control group. As dentures protect the palate from the washing action of saliva by trapping saliva in the palatal-denture space, it is unsurprising that the presence of dentures can cause an increase in microbial growth on the palate. However, something that differentiates the denture wearer group from the control group is that in the denture wearer group, the palate surface has the highest mean percentage of candidal load, being more than three times higher than in the control group. It has been found that immobilized salivary mucins can promote candidal adhesion to surfaces (Edgerton et al., 1993), thus it can be surmised that the arrested salivary flow in the palatal-denture space is responsible for greatly enhancing candidal growth, leading to a higher proportion of candidal species on the palate surface.

Elsewhere, the candidal loads on the cheek mucosal surface are significantly higher in comparison to the control group, while there is no significant difference in bacterial load. From this we can conclude that the presence of dentures also encourages candidal colonization on the cheek mucosal surface, which is in agreement with previous studies (Shulman *et al*., 2005), but has little to no effect on bacterial colonization of the buccal mucosa.

Meanwhile, like the palate surface, the tongue surface shows signficantly higher microbial loads for both candidal and bacterial populations in comparison to the control group. However, unlike the palate surface, there appears to be no drastic increase in candidal load percentage, as the candidal load percentage differs from the control group by about only 2%. Thus, it can be concluded that the slowed salivary flow rate in the oral cavity, which encourages oral microbial growth in denture wearers, also affects microbial growth on the tongue surface, however unlike at the other sites, it does not appear to allow candidal species to compete any better than bacteria. This is perhaps unsurprising as the tongue surface is known to have dense (Bowden et al., 1979) and diverse (Takahashi, 2005) bacterial populations, and thus it would be very tough for candidal populations to compete with them.

5.1.3 Intra-Oral Distribution of *Candida* Species in the Periodontal Disease Group

In periodontal patients, the tongue is the only sampled oral site which has microbial loads that are significantly different from the control group; at all the other sites there are no significant differences from the control group.

With the onset of periodontal disease, among the responses of the host immune system is the release of antibacterial factors, the production of antibodies stimulated by bacterial antigens (Marsh and Martin, 2009), as well as the elevation of non-specific antimicrobial factors in the saliva (Lamster et al., 2003). All of these salivary antimicrobial factors could be the reason why there are no significant differences between the microbial loads of periodontal patient saliva and the saliva of the control group, as the elevation of antimicrobial factors may be inhibiting any increased growth

of oral microorganisms that could occur as a result of periodontal disease, maintaining microbial growth at the same levels as in the control.

As saliva continuously bathes the oral cavity, the microbial growth inhibitory effect would also extend to the palate and cheek mucosal surfaces as well, which explains why the microbial loads of the palate and buccal mucosa surfaces of periodontal patients are not significantly different from the control group. However, on the tongue surface, both candidal and bacterial loads are found to be significantly higher, indicating that the tongue of periodontal patients provides a better environment for bacterial species to compete against oral *Candida* species. This is consistent with the suggestion that the tongue is a reservoir for bacterial species that cause periodontal disease (Van der Velden et al., 1986). It can thus be surmised that as periodontal disease-linked bacterial species become more competitive on the tongue surface during the onset of periodontal disease, the candidal colonization of the tongue is adversely affected, which is why the tongue of periodontal patients has a lower mean percentage of candidal load than the cheek and palate mucosal surfaces, demonstrating that in periodontal patients, the tongue is a less suitable environment for candidal colonization compared to those other sites.

There is another possible reason why the mucosal surfaces of the oral cavity are better candidal colonization sites than the tongue in periodontal patients. In addition to the washing action and antimicrobial factors of saliva that would also affect the microbial growth on the mucosal surfaces of the oral cavity, the oral epithelial cells of both the palate and cheek mucosal surfaces can also be stimulated by bacterial infection to produce interleukins such as interleukin-6 that inhibit microbial growth on the mucosal surface (Hedges et al., 1992), in addition to being actively involved with

neutrophils in the immune response to bacterial infection (Dale, 2000). It is possible that both of these oral epithelium-mediated immune responses are better at suppressing bacterial species rather than candidal species, allowing candidal populations on oral mucosal surfaces to compete better against oral bacterial populations. This is supported by studies which show that disrupting bacterial populations in the oral cavity promotes the growth of oral *Candida* (Scully et al., 1994).

Another difference from the control group is that in periodontal patients, the palate surface becomes the oral site which has the highest mean percentage of candidal load, overtaking both the tongue and cheek mucosal surfaces, indicating that in periodontal patients, the palate is the best environment for candidal species to compete against bacterial speciess. Something that differentiates the palate and cheek mucosal surfaces is the flow of gingival crevicular fluid, containing antibodies, neutrophils, leukocytes and other antimicrobial factors that have been stimulated by periodontitis (Marsh and Martin, 2009). It is possible that during the onset of periodontal disease, the flow of gingival crevicular fluid on the cheek mucosal surface also acts to suppress candidal colonization on the buccal mucosa, which is why the mean percentage of candidal load is higher on the surface of the palate.

5.2 Evaluation of DNA Extraction Methods

Comparing the four extraction methods based only on cost and time to carry out, the lyticase-based method appears to require the most materials in addition to being the most labor-intensive. The commercial kit method, the freeze-thaw method and the glass beads disruption method take around the same amount of time, however the glass beads disruption method becomes less practical and requires more time with large batch

numbers. The freeze-thaw method is both the fastest and most convenient method while also being the cheapest as it requires the fewest amount of materials.

Qualitative assessment of the DNA extracts, as visualized through gel electrophoresis, revealed that the lyticase-based and freeze-thaw extraction methods were unsuccesful in extracting candidal DNA. The complexity, inconvenience and the long duration of the lyticase-based method could be the reasons for its failure, as genomic DNA may have degraded when carrying out the DNA purification steps. In addition, many temperature-sensitive enzymes were involved in the lyticase-based method and deactivation of any of these enzymes could have been a cause. Meanwhile, the freeze-thaw extraction method is based on the premise that rapid freezing and thawing causes cells to lyse. In this procedure, the samples underwent only two cycles of rapid freezing and thawing, and this may have been insufficient to completely lyse the cells. It is also possible that the extremities of temperature required for rapid freeze-thawing were not achieved during the procedure.

The DNA yield and purity of the commercial kit and glass beads disruption extracts were compared to determine which was the better extraction method. The commercial kit produced higher yields for *Candida parapsilosis* and *Candida glabrata*, while the glass beads disruption extraction method had higher *Candida tropicalis* and *Candida lusitaniae* DNA yields. It is possible that minor differences in the structures of the yeast cell walls mean that enzymatic disruption is more effective against *C. parapsilosis* and *C. glabrata*, whereas mechanical disruption is better suited against *C. tropicalis* and *C. lusitaniae*.

Considering the heavy use of PCI (phenol-chloroform-isopropanol, 25:24:1), it is surprising that the glass beads disruption method produced only a few DNA extracts that were contaminated by phenol. This indicates that mixing the lysate with isopropanol and centrifuging is sufficient at removing traces of phenol from the lysate. Meanwhile, comparatively more of the commercial kit DNA extracts have protein contamination. After the centrifugation step of the lysate mixture with the "MPC Protein Precipitation Reagent" provided by the kit, it was observed that traces of protein precipitates had not completely pelleted and were suspended in the supernatant. Despite careful pipetting to ensure that none of these protein precipitates were also pipetted, it is highly likely that traces of the protein precipitate were also taken up in addition to the lysate and this is the cause of the protein contamination.

5.3 Differentiation of Oral *Candida* Species Based on ITS Region of rDNA

The PCR product sizes of the ITSI region for *C. albicans, C. tropicalis* and *C. krusei* are approximately in agreement with those reported by Kumar and Shukla (2005). The 5.8S-ITSII amplification for *C. albicans, C. krusei* and *C. lusitaniae* are consistent with the reports of Fujita et al. (2001), but the sizes for *C. tropicalis* and *C. parapsilosis* differ by about 10 basepairs. Meanwhile, the band sizes of the ITSI-5.8S-ITSII region for *C. albicans, C. tropicalis, C. krusei* and *C. parapsilosis* are in agreement with Mirhendi et al. (2006), but the reported product size for *C. glabrata* differs by 20 basepairs. Elsewhere, Fujita et al. (2001) reported similar product sizes for the species *C. tropicalis, C. parapsilosis* and *C. lusitaniae*, but the reported product sizes for *C. albicans* and *C. krusei* both differ with those observed in this study by about 10 basepairs. Lastly, the *Msp*I restriction fragment sizes of the ITSI-5.8S-ITSII amplicons for *C. albicans, C. tropicalis, C. krusei* and *C. parapsilosis* are consistent

with those reported by Mirhendi et al. (2006). However, the fragment sizes for *C. glabrata* differ by 13-16 basepairs.

As the most highly conserved region in the candidal genome, the candidal rDNA region is very suitable for comparing the genotypic differences between species (Iwen et al., 2002). Unfortunately, the band size of the ITSI amplification is incapable of distinguishing between *C. albicans, C. tropicalis* and *C. parapsilosis*, while 5.8S-ITSII cannot distinguish between *C. albicans, C. krusei* and *C. dubliniensis*. Meanwhile the ITSI-5.8S-ITSII band cannot distinguish between *C. albicans* and *C. dubliniensis*, as well as *C. tropicalis* and *C. parapsilosis.* Thus, clinical species identification would be problematic unless multiple PCR reactions are employed, as no single amplified region by itself can distinguish between all of the ATCC control species.

Comparing the ITSI, 5.8S-ITSII and the ITSI-5.8S-ITSII band sizes, there is very little difference between *C. albicans, C. dubliniensis*, *C. tropicalis*, *C. krusei* and *C. parapsilosis*.. Meanwhile, the sizes of *C. glabrata* and *C. lusitaniae* are markedly different, indicating that they are, by comparison, genetically more dissimilar from the other four species.

The sizes of the *C. albicans* and *C. dubliniensis* 5.8S-ITSII as well as ITSI-5.8S-ITSII bands are identical, which is unsurprising as these two species have always been closely linked and difficult to distinguish morphologically (Pincus et al., 1999). Meanwhile, *C. tropicalis* and *C. parapsilosis* have identical ITSI and ITSI-5.8S-ITSII bands, despite their differences in morphology. On the other hand, their 5.8S-ITSII bands differ by as much as 20 base pairs. Interestingly, both *C. tropicalis* and *C.*

parapsilosis have shorter 5.8S-ITSII regions compared to *C. albicans*, *C. dubliniensis* and *C. krusei*.

C. krusei distinguishes itself by having a smaller ITSI region compared to the other four candidal species it appears to be genetically closer related to. Elsewhere, *C. glabrata* and *C. lusitaniae*, by virtue of having significantly different ITSI-5.8S-ITSII region sizes from the others and from each other, also have substantially different ITSI and 5.8S-ITSII region sizes as well.

In addition to using PCR amplification, the *Msp*I and *Hinf*I restriction enzymes were assessed to determine if restriction enzyme analysis could be combined with PCR amplification of candidal rDNA as an effective means for species identification.

*Msp*I digestion of the ITSI-5.8S-ITSII PCR product resulted in restriction fragment patterns capable of distinguishing between *C. tropicalis*, *C. krusei*, *C. parapsilosis*, *C. glabrata* and *C. lusitaniae*. However, *C. albicans* and *C. krusei* produced identical banding patterns, indicating that they have identical recognition sites for *Msp*I. Each species has one *Msp*I recognition site, with the exception of *C. parapsilosis*, which has none. Meanwhile *Hinf*I digestion resulted in unique restriction fragment banding patterns for all the species with the exception of *C. tropicalis* and *C. parapsilosis*, which have identical restriction fragments. Furthermore, the sizes of the resulting restriction fragments are very close to the sizes of the restriction fragments of *C. albicans*, potentially making clinical identification problematic. The identical *Hinf*I recognition sites of *C. tropicalis* and *C. parapsilosis* further indicates that they are genetically related, despite their morphological differences. Each of the candidal species

tested has 2-3 *Hinf*I recognition sites, and for some species, they produced bands of similar sizes that overlapped each other when visualized in the gel.

While the F1 clinical sample genotype could not be matched with the genotype of any of the control samples, there is a possibility, based on the sizes of the ITSI-5.8S-ITSII and 5.8S-ITSII PCR products, that it is *Candida famata. Candida famata* is a hemiacomycetous yeast that is difficult to phenotypically distinguish from *Candida guilliermondii* (Desnos-Ollivier et al., 2008). It is known to be a common environmental isolate, but rarely recovered clinically (Rippon, 1988). However, recently it has been recognized as a potential emerging pathogen, and a recent study has shown that it is able to adhere to gingival epithelial cells and trigger an immune response from the epithelial cells (Bahri et al., 2010)

6. LIMITATIONS

This study is limited by its small sample size (n = 15). Thus, the results and conclusions that can be derived from this study are limited in scope. The comparison of candidal microbial loads in the oral cavity between different groups would be better supported if more individuals were sampled for each of the three groups. Meanwhile, the effectiveness of using candidal rDNA for the purposes of species identification as utilised in this study would be better demonstrated if more clinical samples were obtained in order to further investigate the usefulness of this candidal species identification method. It is recommended that future studies employ larger sample sizes in order to improve the veracity of the data presented in this study.

7. CLINICAL SIGNIFICANCE

A better understanding of the prevalence and intra-oral distribution of candida in the oral cavity under different conditions of periodontitis and denture use should be an invaluable guide for future studies concerning factors that are involved in candidal growth, and hopefully this will lead to clinical applications such as better management of oral candidal growth and ecological balance of the oral microbiota, as well as better treatment and prevention of candidal pathogenesis.

This study also illuminates the usefulness of the ITS regions of fungal rDNA, and their potential use as a means for identifying candidal species based on genotype. As the differing sizes of the restriction fragments of the ITSI-5.8S-ITSII region has been shown to be able to distinguish between different candidal species, it may be possible to construct genetic probes that target those specific fragments, enabling swift and precise identification of candidal species.

8. CONCLUSION

In fulfilment of the first objective of this study, the candidal loads of the saliva, tongue, palate and buccal mucosa of denture-wearers were found to be significantly higher compared to the control group, while only the candidal loads of the tongue of periodontal patients were found to be significantly higher, whereas at all the other sites there were no significant differences. Meanwhile, the mean percentage of candidal loads were the lowest in the saliva for all three groups, while it was the highest on the tongue surface of the control group, and highest on the palate surface of denture-wearers and periodontal patients.

For the second objective, fifteen clinical samples were randomly isolated and were then differentiated into six different genotypes based on the sizes of their ITS regions and restriction fragments. Out of these six different genotypes, five were succesfully matched to the genotype profile of a control species, enabling identification of their species.

In terms of fulfilling the third and final objective, out of the seven control species of *C. albicans, C. tropicalis, C. krusei, C. parapsilosis, C. dubliniensis, C. glabrata* and *C. lusitaniae*, *Msp*I restriction digest of the amplified ITSI-5.8S-ITSII region was successful in differentiating between all those species, with the exception of not being able to distinguish between *C. albicans* and *C. dubliniensis*, whereas the *Hinf*I digest could differentiate all of the aforementioned species with the exception of not being able to distinguish between *C. tropicalis* and *C. parapsilosis*. Combining the results of both digestions would enable candidal species identification of these seven species.

Future studies could concern further investigation of these and other restriction fragments as well as the possibility of developing hybridization probes for rapid genotype-based species identification.

APPENDIX A

UNIVERSITI MALAYA
KUALA LUMPUR

UM.D/PD/211/07

23 Februari 2007

Dr. Wan Himratul Aznita Wan Harun
Jabatan Biologi Mulut

Melalui:

Ketua
Jabatan Biologi Mulut
Fakulti Pergigian

Puan,

KELULUSAN ETIKA

Dengan segala hormatnya perkara tersebut di atas dirujuk.

Sukacita dimaklumkan bahawa Jawatankuasa Etika Fakulti Pergigian telah meluluskan projek puan yang bertajuk 'The Virulence Determination of Candida Species In The Oral Cavity'. Nombor kelulusan MEC projek tersebut ialah DF OB0702/0002(L).

Sekian, terima kasih.

Yang benar,

PROFESOR DR. ROSNAH MOHD ZAIN
Pengerusi
Jawatankuasa Etika Fakulti Pergigian

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APPENDIX B

DFP 234/02 (B)

PATIENT INFORMATION SHEET

Please read the following information carefully. Do not hesitate to discuss any questions you may have with your doctor.

Study Title

THE VIRULENCE DETERMINATION OF *CANDIDA* SPECIES IN THE ORAL CAVITY

Introduction

This information sheet explains about the research project and is hope to aid you in deciding whether or not you would like to participate in the research.

Candida species is a type of fungi organism which is able to attach to mucosal and tooth surfaces. These organisms have been found to cause oral infection such as oral candidiasis. This study attempts to look at the pattern of distribution of *Candida* species in subjects with healthy and diseased gum, as well in denture wearers. It also focus at virulent properties of *Candida* species as this information is important as to further understand the disease process.

What is the purpose of this study?

The aim of this study is to determine the pattern of distribution of *Candida* species in people with healthy and diseased gum, as well in denture wearers. The study will also look into *Candida* species' virulent properties as this information is important as to further understand the disease.

What are the procedures to be followed?

For this study, I seek your permission to allow me to obtain a plaque sample from your tooth/teeth, a saliva sample form floor of mouth and swab samples from your check mucosa, tongue and palate/ fitting surface of your denture. For the procedure, an excavator will be used to get into the sulcus/ pocket to obtain the plaque sample. Then, cotton pellets will be used to collect salivary sample. Finally, an interdental brush will be used to brush (swab) the check mucosa, tongue and palate /fitting surface of denture. All information collected and generated will be kept strictly confidential and any information that leaves the clinic or hospital will be anonymous so that you cannot be identified from it.

Who should not enter the study?

Every individual can volunteer to participate in the study except for pregnant and lactating mothers. No one will be discriminated from participating in the study.

What will be the benefits of the study:

(a) to you as a subject?

None

(b) to the investigator?

The findings from this study will provide understanding of disease process by *Candida* species. It would benefit the profession in diagnosing and managing oral candidiasis infection.

What are the possible drawbacks?

There may be some discomfort in the course of obtaining the plaque sample.

Can I refuse to take part in the study?

Yes, participation in this study is completely voluntary. It is up to you to decide whether or not you wish to take part.

Who shall I contact if I have additional questions during the course of the study?

If you need to require further questions regarding the study, do not hesitate to contact

Dr. Wan Himratul Aznita Binti Wan Harun (Phone): 79677415

Dr. Fathilah Abdul Razak (Phone): 79677416

Dr. Nor Adinar Baharuddin (Phone): 79674803

Dr. Ros Anita Omar (Phone): 79674542

Doctor's Name : Dr Nor Adinar Baharuddin — Tel. No.: 03-79674803

Dr Ros Anita Omar — Tel. No.: 03-79674542

APPENDIX C

CONSENT BY PATIENT FOR CLINICAL RESEARCH *FACULTY OF DENTISTRY, UM, K.L.*

I, ... Identity Card No. .....
(Name of patient)

of.....
(Address)

hereby agree to take part in the clinical research (clinical study) specified below :
Title of Study : THE VIRULENCE DETERMINATION OF *CANDIDA* SPECIES IN THE ORAL CAVITY .....

the nature and purpose of which has been explained to me by : Dr Nor Adinar Baharuddin / Dr Ros Anita Omar.....
(Name & designation of doctor)

and interpreted by.....to the best of his/her ability in.....
(Name & designation of interpreter)

language/dialect.

I have been told about the nature of the clinical research in terms of methodology, possible adverse effects and complications (as per the patient information sheet). After knowing and understanding all the possible advantages and disadvantages of this clinical research, I voluntarily consent of my own free will to participate in the clinical research specified above.

I understand that I can withdraw from this clinical research at any time without assigning my reason whatsoever and in such a situation shall not be denied the benefits of usual treatment by the attending doctors.

Date ..... Signature or thumbprint.....
(Patient)

IN THE PRESENCE OF

Name .....,

I/C No. ....., Signature .....
(Witness for signature of patient)

Designation .....

I confirm that I have explained to the patient the nature and purpose of the above mentioned clinical research.

Date ..... Signature .....
(Attending doctor)

CONSENT BY PATIENT FOR CLINICAL RESEARCH

R.N.
Name
Sex
Age
Unit

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