Genetic Typing of *Candida albicans* Strains Isolated from the Oral Cavity of Patients with Denture Stomatitis before and after Itraconazole Therapy

Laura J. Cross, Marcello P. Riggio and Jeremy Bagg

University of Glasgow Dental School, Glasgow, Scotland

Correspondence to: L. J. Cross, Glasgow Dental Hospital and School NHS Trust, 378 Sauchiehall Street, Glasgow G2 3JZ, Scotland. Tel: +44 (0)141 211 9831; Fax: +44 (0)141 353 1593; E-mail: L.Cross@dental.gla.ac.uk

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This study determined, by molecular typing of *C. albicans* species isolated from denture stomatitis patients with a mycological relapse six months after successful itraconazole therapy, whether there had been recurrence of infection with the same strain(s), selection of particular strains or infection with new strains of *C. albicans*. Forty patients with long-standing *Candida*-associated denture stomatitis were assigned either cyclodextrin itraconazole solution or itraconazole capsules (100mg b.d. for 15 days). Palatal erythema was measured and imprint cultures undertaken at baseline and at 15 days, four weeks and six months after treatment commenced. Yeast isolates were formally identified and chromosomal DNA was extracted from pairs of isolates from those patients with *C. albicans* present at baseline and six months after treatment commenced. Southern blotting of *Eco*RI-digested chromosomal DNA was performed using the *C. albicans*-specific 27A repetitive element as a probe. Eighteen of 36 patients were infected with *C. albicans* at baseline and six months after treatment commenced. Overall, 13 genetically different strains of *C. albicans* were found. However, in 17 of 18 patients, the *C. albicans* strains isolated prior to itraconazole therapy and six months later were the same. Thus recurrence of denture stomatitis in these individuals was due to re-colonisation by the original strain, rather than re-infection with a different strain. *Key words*: *Genotyping*, *C. albicans*, *denture stomatitis*.

INTRODUCTION

Candida-associated denture stomatitis, characterised by erythema and oedema of the palatal mucosa beneath an upper denture, affects 50% of denture wearers (1). Nystatin, amphotericin B, miconazole and fluconazole have been used in the management of denture stomatitis, but the recurrence rate is high (2). Itraconazole has been effective in the management of oral candidosis among immunocompromised patients (3) and a pilot study showed itraconazole capsules to have comparable efficacy to fluconazole capsules in the management of denture stomatitis (4). Furthermore, both the new liquid formulation of itraconazole in hydroxypropyl-β-cyclodextrin and itraconazole capsules have been found to produce similar reductions in palatal erythema and yeast colonisation in patients with *Candida*-associated denture stomatitis, but rarely a long term mycological cure (submitted for publication). Molecular typing of yeasts isolated from denture stomatitis patients before and after antifungal therapy may permit a fuller understanding of the reasons for any relapses following treatment.

Methods of typing clinical isolates of *C. albicans* are useful in determining whether commensally carried strains are the source of infection, whether there are particular strains adapted to particular body niches and whether a new episode of the disease is caused by reinfection or relapse (5). It has been suggested that DNA typing is one of the methods with the greatest potential for discrimination (6). DNA fingerprinting was first applied to yeasts by Scherer and Stevens (7) who examined 17 isolates of *C. albicans*, 10 *C. tropicalis*, seven *C. parapsilosis*, one *C. krusei* and one *C. pseudotropicalis*. DNA typing of *C. albicans* by restriction fragment length polymorphisms (RFLP) and DNA probes have been well documented (8) and recently, the polymerase chain reaction (PCR) has been used to characterise *Candida* species (9). Mathaba et al. (10) determined the genotypic relationships of individual *C. albicans* isolates by hybridisation of a *C. albicans*-specific moderately repetitive sequence, 27A, to *Eco*RI-digested *C. albicans* chromosomal DNA. They concluded that denture stomatitis was due to the overgrowth of commensal strains in the oral cavity and that no one particular virulent strain of *C. albicans* was associated with denture stomatitis. Furthermore, in five of the 18 patients studied, *C. albicans* isolates were obtained from a secondary infection which occurred after antifungal therapy.

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It was concluded that this was due either to the re-emergence of the original infecting strain or the emergence of a new strain from an exogenous source, or both. The type of antifungal therapy used in this study was not specified.

In order to permit a fuller understanding of mycological relapse of denture stomatitis following antifungal therapy, *C. albicans* strains isolated from patients at baseline and six months after itraconazole treatment commenced were genetically typed to determine whether there was a recurrence of infection with the same strain, selection of particular strains or infection with new strains.

MATERIALS AND METHODS

Edentulous denture wearers with long-standing *Candida*-associated denture stomatitis were studied. Patients were recruited from the Prosthodontic and Oral Medicine Departments of Glasgow Dental Hospital and School NHS Trust. To be eligible for enrollment, patients had to be edentulous denture wearers with clinical evidence of denture stomatitis and positive yeast cultures from the palatal mucosa and dorsum of the tongue. Forty patients were enrolled and received either cyclodextrin solution of itraconazole (Sporanox Liquid®, Janssen Pharmaceuticals) at an oral dose of 100mg twice daily for 15 days, or itraconazole capsules (Sporanox®, Janssen Pharmaceuticals) at an oral dose of 100mg twice daily for 15 days. In addition, all patients were instructed in denture hygiene measures. At baseline, an objective measurement of palatal erythema was obtained by means of an electro-optical instrument, the *Erythema meter*, which provided an 'erythema index' (11). Imprint cultures (12) of the palatal mucosa, tongue and denture fitting surface and an oral rinse specimen (13) were collected and inoculated onto Sabouraud's dextrose agar (Life Technologies, Paisley, Scotland) and CHROMagar® *Candida* (Life Technologies, Paisley, Scotland). Microbiological samples were not taken from any other body sites. Clinical outcomes were assessed at 15 days, four weeks and six months after treatment commenced by recording changes in the 'erythema index'. The microbiological samples were repeated at the same time intervals.

One colony of each morphological type on CHROMagar® *Candida* was plated for purity, identified and stored at −70°C. For each patient, four further colonies were removed from the Sabouraud's plates, selected at random from the specimens available. Each isolate was formally identified and stored at −70°C for further investigation. *C. albicans* was identified on the basis of germ tube production. A sucrose assimilation test was performed on germ tube positive yeasts to distinguish *C. albicans* from *C. stellatoidea*. Germ tube negative yeasts were identified by the API 32C test (Bio-Mérieux S A, Marcy-l'Etoile, France).

DNA extraction

Pairs of isolates from those patients with *C. albicans* present at baseline and six months after treatment commenced, were selected at random, removed from storage at −70°C and plated onto Sabouraud's agar. The Sabouraud's plates were incubated at 37°C for 48 h. DNA was extracted using a method similar to one based partly on that originally devised by Scherer and Stevens (7), as described by Anthony et al. (14). Growth from a Sabouraud's plate was emulsified in 10ml YEPD broth (0.3% yeast extract, 1% mycopeptone, 2% dextrose) and incubated overnight at 30°C in an orbital incubator. Cells from 1.5ml of the broth were pelleted in a centrifuge, the supernatant removed, and the cells washed by resuspending in 1000µl 1M sorbitol. Cell walls were digested by re-suspending in 1M sorbitol plus 50mM K_2HPO_4 (pH 7.5) and adding to each tube 3µl β-mercaptoethanol and 20µl of freshly prepared 20mg/ml lyticase 20T (Sigma Chemical Company Limited, Dorset, UK) in 1M sorbitol and incubating at 37°C for 90 minutes. The resulting spheroblasts were pelleted by centrifugation at 13000 r.p.m., the supernatant removed and lysed by adding 0.5ml GES reagent (30g guanidium thiocyanate dissolved at 65°C in 20ml 0.5mol/l EDTA at pH 8 and then 2.5ml of 10% w/vol sarkosyl added, finally made up to 50ml with distilled water) (15). After 20 minutes at room temperature, samples were centrifuged for 3 minutes and the supernatant retained. The supernatant was extracted once with an equal volume of phenol:chloroform (1:1), potassium acetate was then added to a final concentration of 2.5M and the mixture extracted once with an equal volume of chloroform:pentanol (24:1). DNA was precipitated with an equal volume of isopropanol at −70°C for 30 minutes, pelleted and resuspended in TE buffer (10mM TrisHCL, 1mM EDTA, pH 7.5). RNA was removed by incubation with 5mg/ml RNAase A stock solution at 37°C for 15 minutes and DNA re-precipitated with isopropanol. DNA was quantitated by agarose gel electrophoresis.

Southern blotting

Two to three micrograms of *C. albicans* chromosomal DNA was digested with 30 units of *Eco*RI (Life Technologies, Paisley, UK) and electrophoresed on a 0.8% agarose gel in 1 × TBE buffer (10.8g Tris Base, 5.5g Boric acid, 0.746g EDTA). DNA fragments were transferred to positively-charged nylon membranes (Boehringer Mannheim, Lancs., UK) by Southern blotting (16). Briefly, gels were prepared for blotting by soaking in 250ml of denaturation solution (0.5M NaOH/1.5M NaCl) for 2 × 20 minutes followed by soaking in 250ml of neutralisation solution (0.5M tris-HCl, pH 7.4) for 2 × 20 minutes. DNA was transferred to membranes using a capillary blotting unit (Anachem Ltd., Luton, UK) using 20 × SSC (3.0M NaCl/0.3M sodium citrate, pH 7.0) as a transfer buffer. Follow-

ing transfer, membranes were rinsed in 20 × SSC and DNA immobilised by exposure to an optimal dose of UV energy in a cross-linker (UVC-508; Anachem Ltd., Luton, UK).

Following prehybridisation at 68°C for 4 hours in standard hybridisation solution (5 × SSC, 0.1% N-lauroylsarcosine, 0.02% sodium dodecyl sulphate, 1% blocking reagent), membranes were then hybridised with the *C. albicans*-specific 27A repetitive element labelled with digoxigenin (DNA labelling and detection kit; Boehringer Mannheim) at 25ng/ml in standard hybridisation buffer (17). The 27A repetitive element originally described by Scherer and Stevens (7) was kindly provided by Dr. B.B. Magee (University of Minnesota).

Following hybridisation, membranes were washed at room temperature in 2 × SSC/ 0.1% SDS for 2 × 5 minutes, and then 68°C in 0.1 × SSC/ 0.1% SDS for 2 × 15 minutes. Immunological detection of hybridised DNA fragments was carried out in accordance with the manufacturer's instructions using an anti-digoxigenin antibody conjugated to alkaline phosphatase and colorimetric detection with 4-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate as a colour substrate.

RESULTS

A total of 40 denture stomatitis patients entered the study. However, two patients from each treatment group failed to complete the course of itraconazole and were excluded from all efficacy analyses. Both forms of itraconazole resulted in a generalised reduction in erythema which was seen in most patients throughout the clinical trial (data submitted for publication).

C. albicans was isolated from all patients before treatment commenced. All of the *C. albicans* strains isolated at baseline were found to be susceptible to itraconazole (18) by means of a broth dilution assay (19). The mean itraconazole minimum inhibitory concentration (MIC) for the *C. albicans* species isolated at baseline was 0.16mg/L (range 0.06–0.25 mg/L).

A mycological cure was achieved, at all sites sampled, in 17 of the 36 patients on completion of the two week course of itraconazole. Four weeks after treatment commenced, 13 patients remained yeast free and seven were still mycologically cured six months after treatment commenced. Although no patients had an increased yeast count on completion of the itraconazole therapy, eight patients in total had higher yeast counts by four weeks and six months after treatment commenced than had been recorded at baseline (data submitted for publication).

Of the 36 patients who completed the clinical trial, 18 were colonised with *C. albicans* at baseline and six months after treatment commenced. Chromosomal DNA was extracted from 36 stored *C. albicans* isolates in total, two isolates from each of the 18 patients, one which had been collected at baseline and another collected six months after treatment commenced. Although *C. albicans* was isolated from these 18 patients at baseline and at the end of the trial, nine of these patients had been completely mycologically cured at either two weeks or four weeks after treatment commenced, five of the nine being completely yeast free at both of these visits. A further two of the 18 patients were free of *C. albicans* at two weeks and four weeks after itraconazole therapy, although other yeast species, *C. glabrata* in one case and *S. cerevisiae* in the other, persisted. Although *C. albicans* persisted in the remaining seven patients throughout the clinical trial, yeast counts were reduced following itraconazole therapy in six of these seven patients.

Hybridisation of the 27A repetitive element probe to the DNA samples digested with *Eco*RI allowed differentiation of the strains of *C. albicans* (Fig. 1). Use of the *C. albicans* 27A probe revealed only minor differences in band patterns between pre- and post-treatment isolates in all but one case, indicating that, in 17 of 18 patients, the *C. albicans* strains isolated prior to itraconazole therapy and six months after treatment commenced were the same. Any differences in band pattern between strains from the same patient were usually subtle when compared to the major differences seen among strains from different patients. For example, lanes 9 and 10 (Fig. 1) are *C. albicans* isolates taken from the same patient at baseline (lane 9) and six months after treatment commenced (lane 10). The band patterns of these two isolates are almost identical and were considered to be the same strain. However, comparison of isolates taken from different patients, for example, lanes 5, 9 and 11 in Fig. 1, reveals a significantly different band pattern in each lane implying that different strains of *C. albicans* have been isolated from each of these patients. Only one patient appeared to have a different strain of *C. albicans* isolated at baseline (lane 3) than at six months after treatment started (lane 4). Among this group of eighteen patients, thirteen completely different strains of *C. albicans* were detected. Of these thirteen strains, four were carried by more than one patient.

DISCUSSION

Progressive recolonisation of the mucosa and the denture fitting surface by yeasts and the consequent recurrence of denture stomatitis after completion of antifungal treatment has been reported (20–23). In the present study, both forms of itraconazole resulted in a reduction in erythema of the palatal mucosa, although the *Erythema meter* indices, in both treatment groups, remained higher after antifungal treatment than the indices found in patients with no history of denture stomatitis (4). Seven of 36 patients who completed the clinical trial remained mycologically cured after completing the course of itraconazole therapy until the end of the trial. A further patient who

was free of yeasts four weeks after treatment commenced remained so six months later. However, 50% of patients who had been mycologically cured at two or four weeks after treatment commenced were recolonised by yeasts at six months. Genetic typing of *C. albicans* strains isolated from these patients at baseline and six months after treatment has shown that, in all but one case, the same strain of *C. albicans* was present before treatment as at the end of the clinical trial.

The aim of this study was to determine, by molecular typing of *C. albicans* species isolated before and after treatment, whether there had been a recurrence of infection with the same strain, selection of particular strains or infection with new strains of *C. albicans*. In the past it was difficult to answer this type of question as strain comparisons were based on phenotypes rather than genotypes, with the assumption that the underlying genetic differences that produced the phenotypic changes were the same in different isolates (7). The advent of recombinant DNA technology has meant that genetic assessments of strain relatedness are now possible, overcoming the risk of grouping together genetically unrelated strains with similar phenotypes and separating related strains with different phenotypes. One of the most effective of these techniques has been Southern blot hybridisation with DNA probes containing moderately repetitive sequences (24). The *C. albicans* middle repetitive 27A sequence was chosen as this has been shown to be a very sensitive method of analysing DNA samples digested with restriction enzymes (5). *Eco*R1 was chosen as the enzyme for the digestion of the DNA because it has been suggested as the enzyme of choice for greatest discrimination when using the 27A probe (5, 14).

For every patient enrolled in the trial, at least five yeast isolates per visit had been stored at −70°C for further investigation. Although *C. albicans* was not the only species isolated from this study population it is considered to be the main pathogen in oral candidosis and, as at the time of this study corresponding species-specific repeat sequence probes had not been developed for other *Candida* species (25), it was decided to concentrate only on *C. albicans* for the molecular investigations. In this study there were 18 patients from whom *C. albicans* was isolated six months after treatment commenced as well as at baseline. Two isolates from each patient, one collected at baseline and one six months after treatment commenced, were selected at random and removed from storage. Although some studies have found that the majority of *C. albicans* isolates from different sites within the same oral cavity have been found to belong to the same genetic group (10), other

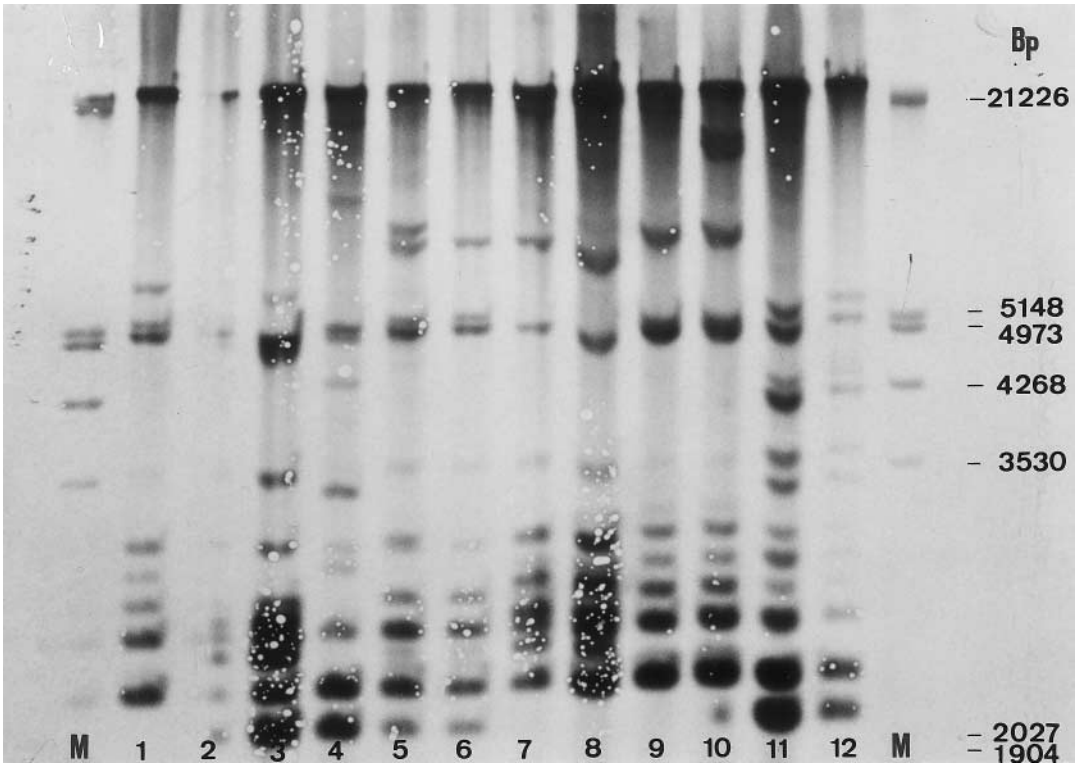

Fig. 1. Southern blot obtained, showing the *C. albicans*-specific 27A repetitive element probe hybridised to *Eco*R1 digested *C. albicans* DNA. Lanes 1 to12 show samples of digested DNA from *C. albicans* isolates taken from six of the study patients. Each pair of lanes represents *C. albicans* DNA extracted from isolates from the same patient. In any pair of lanes, the first lane represents digested DNA from yeasts isolated at baseline and the second lane represents DNA from yeasts isolated six months after treatment commenced. M is a standard base pair (Bp) marker for gel electrophoresis ie. λDNA digested with *Eco*RI and *Hind* III.

studies have reported that some patients may carry more than one strain of *C. albicans* (14). In this study, as far as possible, the isolate collected six months after treatment commenced was from the same intra-oral site as the baseline isolate with which it was to be compared. This was to overcome the chance of 'sampling error' which may have given the impression that a different strain was present at the six month review than at baseline, when actually multiple strains may have been present at different intra-oral sites for much of the time.

In this study, 13 different strains of *C. albicans* were isolated from the 18 patients. This genetic diversity is similar to that found in other studies of *C. albicans* strains isolated from the oral cavities of non-immunocompromised individuals (10, 24). For 17 of 18 volunteers with a mycological relapse, the strain of *C. albicans* isolated six months after itraconazole treatment commenced was considered to be genetically the same as the original strain isolated at baseline. The number of band differences judged acceptable for two isolates to be classified as identical strains has been interpreted differently by different workers (10, 14). Anthony et al. (14) identified minor differences between multiple isolates from a single patient, but these changes were subtle when compared to changes between strains from different patients. These authors concluded that restriction fragment length polymorphism types, differing by less than three bands, seen in isolates from individual patients were due to genetic recombination within a single strain. The same criteria have been used to interpret the genetic typing results found in this study.

In the study by Mathaba et al. (10) the *C. albicans* strains isolated from the dentures during the primary infection were the same as those isolated during the secondary infection, following a course of antifungal therapy, in nine out of 11 patients. This implies that the presence of *C. albicans* in these patients is typically due to re-colonisation by the original infecting strain rather than re-infection with a different strain. Although the primary reservoir of *Candida* species in healthy individuals is generally considered to be the gastro-intestinal tract (26), the patient's denture must be considered a potential source of re-infection, a view shared by other authors (22, 27, 28). This further supports the conclusion that the provision of new dentures is an important factor in the long-term resolution of denture stomatitis.

The variety of genotypes identified in this study, as in the study by Mathaba et al. (10), suggests that no one particular virulent strain of *C. albicans* is associated with denture stomatitis. However, it would be interesting to compare the genotypes of *C. albicans* isolates from denture wearers with healthy palatal mucosa and those from denture wearers with denture stomatitis, in an attempt to determine whether commensal strains of *C. albicans* are capable of causing an infection or whether, in the transition from a healthy to an infected mouth, there is strain substitution.

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