Molecular and histological association between *Candida albicans* from oral soft tissue and carious dentine of HIV-positive children.

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Abstract

and caries are frequently investigated among healthy and immunosuppressed individuals. The objective of this study was to demonstrate the presence of *#* on both oral soft and hard tissue and to investigate, at molecular level, the genetic subtype of the organism from the two oral sites. Tongue swabs and dentine scrapings from 362 HIV-positive children, referred for extraction of carious primary teeth, were cultured on CHROMagar and identified to species level with ID32C. Histological staining of extracted carious teeth was also done. In patients with positive *#* cultures from both the tongue and carious dentine, DNA fingerprinting of such paired isolates were performed, using Southern blot hybridisation with the Ca3 probe. Yeasts were cultured from the tongue of 151 (41.7%) individuals and 57 (37.7%) simultaneously yielded positive *#* cultures from carious dentine. Nine different yeast spp. were identified from the tongue using the ID32C commercial system but *#* was the only species recovered from carious dentine and histological investigation demonstrated fungal elements penetrated into the dentine and not limited to superficial debris on the floor of the cavity. Twelve of 13 paired isolates of *#* revealed identical fingerprinting patterns. The findings from this study demonstrated that in a particular individual, the same genetic subtype of *#* was capable of colonizing both oral soft tissue and carious dentine. This renders carious teeth a constant source, or reservoir, of potentially infectious agents and, particularly among immunosuppressed individuals, should therefore not be left unattended.

Introduction

The involvement of *#* in caries has been widely researched through both *·* and *·* studies [1-5]. Because *#* is the yeast spp. most frequently encountered in the mouth of both healthy [6] and immunosuppressed individuals, it is also the species most commonly studied in this regard. A high prevalence of caries [7, 3] and Candida carriage [8] has been reported

among HIV-infected individuals, particularly children, including the fact that oral candidiasis remains the most frequently encountered oral soft tissue infection in these individuals [9].

The plasticity, or adaptability of # ˙ to extreme environmental conditions is well known [10] and much has been published on gene expression or phenotypic characteristics displayed under conditions of biofilm formation [11]. The interactions of # ˙ with several other oral microorganisms is summarised in a recent review by Krom ˙ [12]. # ˙ is also known to utilise a wide variety of substrates and to produce various metabolites, amongst others acids, a fact that, for many years, constituted the basis of fungal biotaxonomy [13, 14]. The various commercially available yeast identification systems, amongst others Vitek and ID32C [15] currently used for clinical diagnostic purposes, are derived from these extensive sets of substrates of biotaxonomy.

˙ has furthermore been delineated into a number of well-defined genetic subgroups, also referred to as clades [16-19]. While antifungal resistance has been demonstrated to occur more frequently among clade II and clade SA isolates [20, 21], conclusive evidence has yet to emerge of hyper-virulent strains through the use of accepted DNA fingerprinting techniques with high discriminatory power [22].

Significant numbers of children, vertically infected with the HI-virus, are dependent on public health services in South Africa [23, 24], with the two most prevalent oral complications among these children being oral candidiasis and dental caries [25, 26].This study was undertaken to determine whether # ˙ occurs both on oral soft tissue and in carious teeth of HIV-positive children and if so, to determine the genetic relatedness of paired isolates of # ˙ from the two oral sites in a particular individual.

Materials and Methods

Approval was obtained from the CEO of the hospital to publish data collected in the process of delivering a routine oral health service to HIV- patients, provided that individual patient

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confidentiality is respected. Patients attending academic health facilities also sign consent for the anonymous use of data and material for research purposes. All children included in the study were referred for extractions by independent clinicians and no invasive procedures were performed solely for research purposes.

Confirmed HIV-positive children attending a paediatric outpatient clinic and referred for extraction of one or more carious primary teeth, were included in the study. Patients presenting with abscesses and who were not already on antibiotic coverage were prescribed 200mg/5ml metronidazole, 125mg/ml of amoxicillin and 125mg/5ml paracetamol, three times daily for five days with extractions performed between days 3 to 5. Parents or caregivers were instructed on the appropriate use of the prescribed medication. Patients presenting with oral candidiasis were first prescribed antifungal treatment and multiple extractions were performed only when the oral candidiasis was cleared, or in the case of severely immunocompromised patients, only once the patient was responding to antiretroviral treatment (ART). Patients who had received antifungal therapy up to 4 weeks prior to referral for extraction, were excluded from the study. A swab was taken of the tongue in a uniform manner with four strokes on the dorsal surface, as well as passing the swab once around the lateral border of the tongue while twirling the swab. Extracted teeth were placed in a sterile specimen container, sealed and together with the tongue swab immediately transported to the laboratory. Infection prevention and control measures related to clinical specimens were applied throughout. Swabs were cultured on CHROMagar [27], again by twirling the swab while stroking across the surface of the agar and incubated for 48 hours at 30°C. Readily removable carious material and debris from the cavity floor of an extracted tooth was disposed of before fragments of the remaining intact dentine were scooped out with a sterile excavator and suspended in a tube with 2.5ml sterile saline. The specimens were vortexed for 30 seconds and 0.5 ml of the supernatant was plated on one half of a CHROMagar plate and spread with a sterile

nichrome inoculation loop, follow by placing dentine fragments from the same tube on the other half of the agar plate. Plates were incubated similar to those of tongue swabs and after 48 hours the germ tube test was performed on colonies that were presumptively identified as *# ˙* [28] On plates that yielded confluent growth, single colonies were obtained by collecting a loop full of yeast with a sterile nichrome inoculation loop and again streaked on a CHROMagar plate. Where more than one yeast species was cultured as evident from different colony colours on CHROMagar, a representative colony from each colour was selected for definitive identification to species level using the ID32C commercial identification system (bioMerieux, France) according to the manufacturer's instructions [29]. Approximately 5 colonies were selected respectively off each CHROMagar plate of patients that harboured *# ˙* on both the tongue and in the dentine of extracted teeth, suspended in sterile water and stored at room temperature until required for DNA fingerprinting.

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In instances requiring the extraction of more than one carious tooth during the same visit, a second tooth was placed in formalin. The teeth from the first 10 patients from whom paired *# ˙* cultures were obtained, were processed for histological examination.

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Teeth were fixed whole in 10% neutral buffered formalin solution for at least 24 hours followed by decalcification in a routinely used decalcifying solution (880 ml distilled water, 70 ml nitric acid, 50 ml hydrochloric acid). Decalcification was followed by rinsing in tap water for 1 hour prior to processing. The teeth were embedded in paraffin wax and 3 micron sections were cut of each tooth using a Leica semi-automated microtome. Sections were stained with Haematoxylin and Eosin, Grocott's Methenamine Silver (Grocott) and Periodic Acid Schiff (PAS) using standard protocols.

) V° ˙

The first 13 pairs of definitively identified *#* isolates obtained from both the tongue and

carious dentine, and from patients who had a second extracted tooth stored in formalin, were

selected for DNA fingerprinting.

In brief, stored paired isolates were revived in Yeast Potato Dextrose (YPD) broth and fresh, 24 hour

cultures were used to extract genomic DNA. DNA was purified and digested with EcoR1 prior to

electrophoresis on 0.8% agarose gels.[30] DNA from the reference strain 3153A was run in the two

outermost lanes of each gel. Following electrophoresis the separated DNA fragments were

transferred from the gel through capillary blotting to Hybond N$^+$ membranes (Amersham,

Piscataway, NJ). Blots were hybridized overnight with randomly primed ^{32}P-labelled Ca3 probe [31]

and autoradiographed on XAR-S film (Eastman Kodak Co., Rochester, NY) with Cronex Lightning-Plus

intensifying screens (Dupont Co., Wilmington, DE.).

Results

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Demographic data and yeast culture results are summarised in Table I.

Positive yeast cultures were obtained from the tongue swabs of 151 children (41.7%), and nine

different species were conclusively identified with the ID32C commercial system following

presumptive identification on colony colour on CHROMagar. In addition to *#* which

constituted the major isolate (80%) from the tongue, eight other yeast species also obtained from

the tongue were definitively identified with the ID32C commercial identification system. These spp.

were *#* (1%), *#* (1%), *#* (1%), *#* (1%) *#* (1%), *#*

(1%) *#* (1%), and *#* (12%) Yeasts were simultaneously cultured from

carious dentine of 57 (37.7%) of the 151 children and *#* was the only spp. cultured from

carious dentine.

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Table I: Summary of demographic and yeast culture results of 362 HIV-positive children referred for extraction of one or more carious teeth under local anaesthesia.

V	*)*
Gender	Male 177 (48.5%); Female 185 (51.5%)
Mean age	4.5 y (3.5 – 6.5)
Anti-retroviral treatment (ARV)	176 (48.6%)
Positive yeast culture tongue	151 (41.74%)
Positive yeast culture tongue and carious dentine	57 (37.74%)
Patients carrying more than one yeast spp.	7 (4.63%)
Number of different yeast spp. identified from tongue	9

Figs. 1a and b: PAS staining of decalcified and sectioned carious primary teeth of HIV-positive children referred for extractions, demonstrating the presence of fungal elements on the surface of the carious lesion and hyphae penetrating into the dentinal tubules (original magnification X40 and X100 respectively).
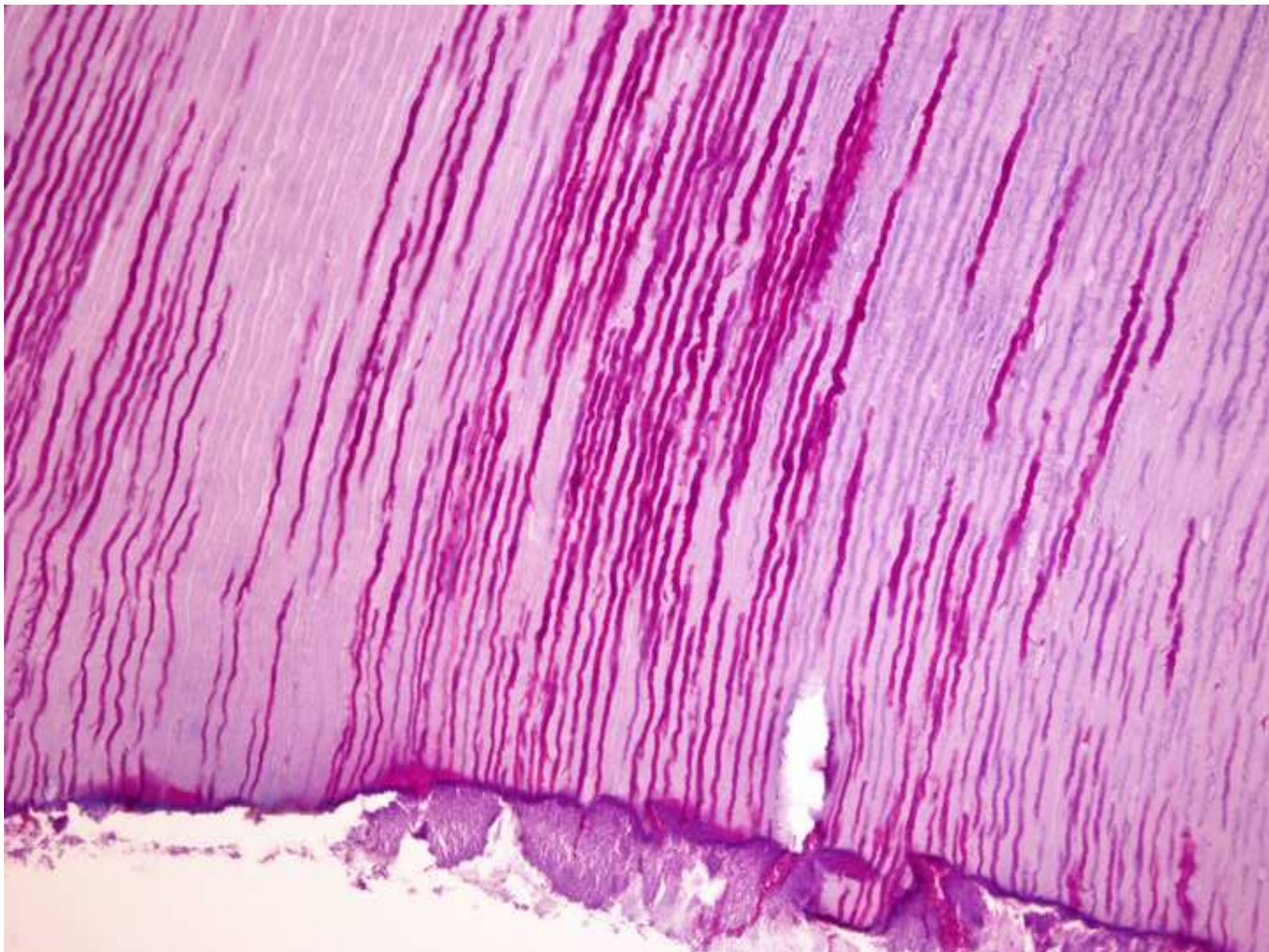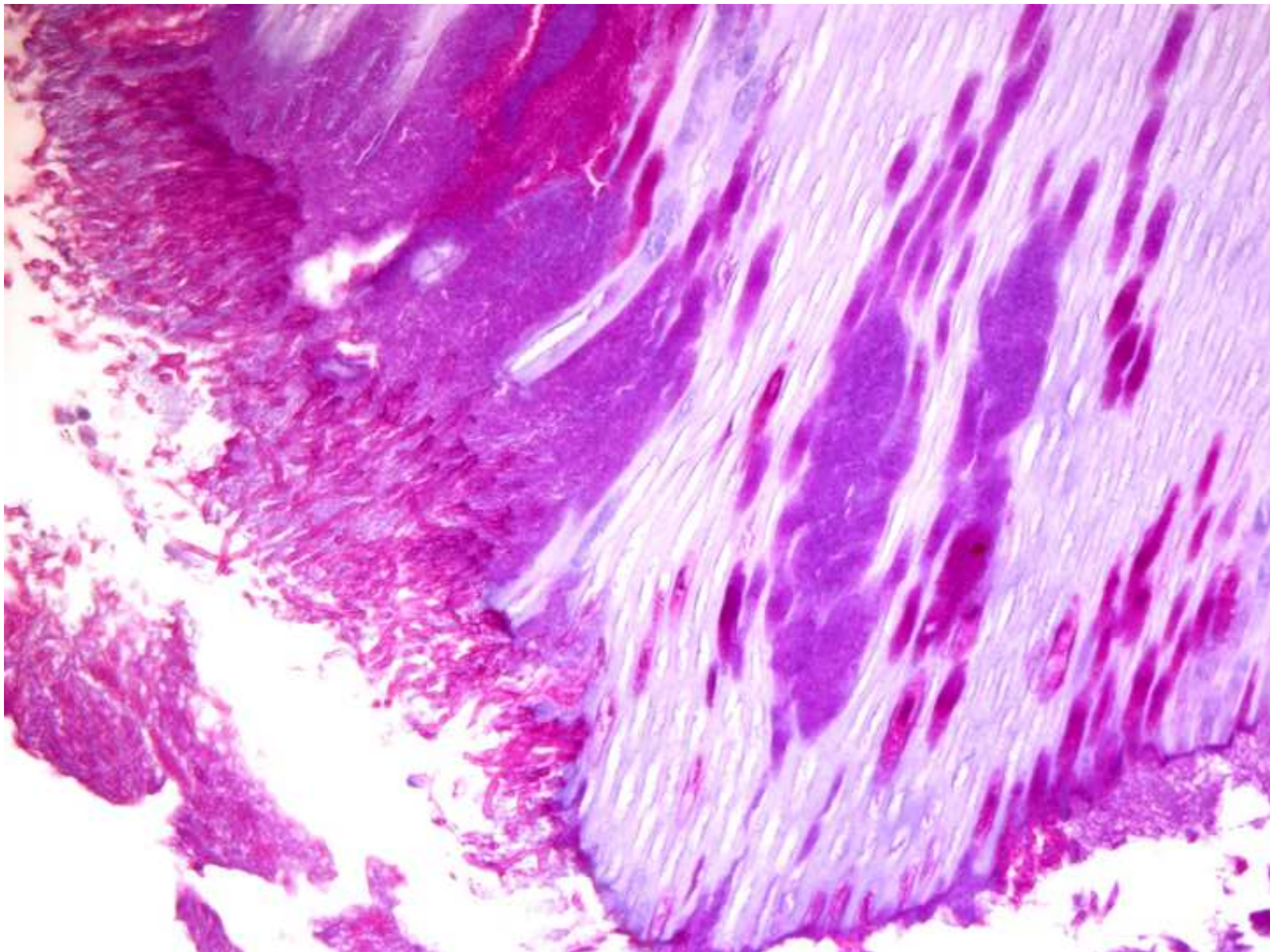Figure 1a

Figs. 2a and b: Grocott's silver stained sections of decalcified teeth confirming the presence of fungal elements on the surface and penetrating along the dentinal tubules (original magnification X40 and X100 respectively).
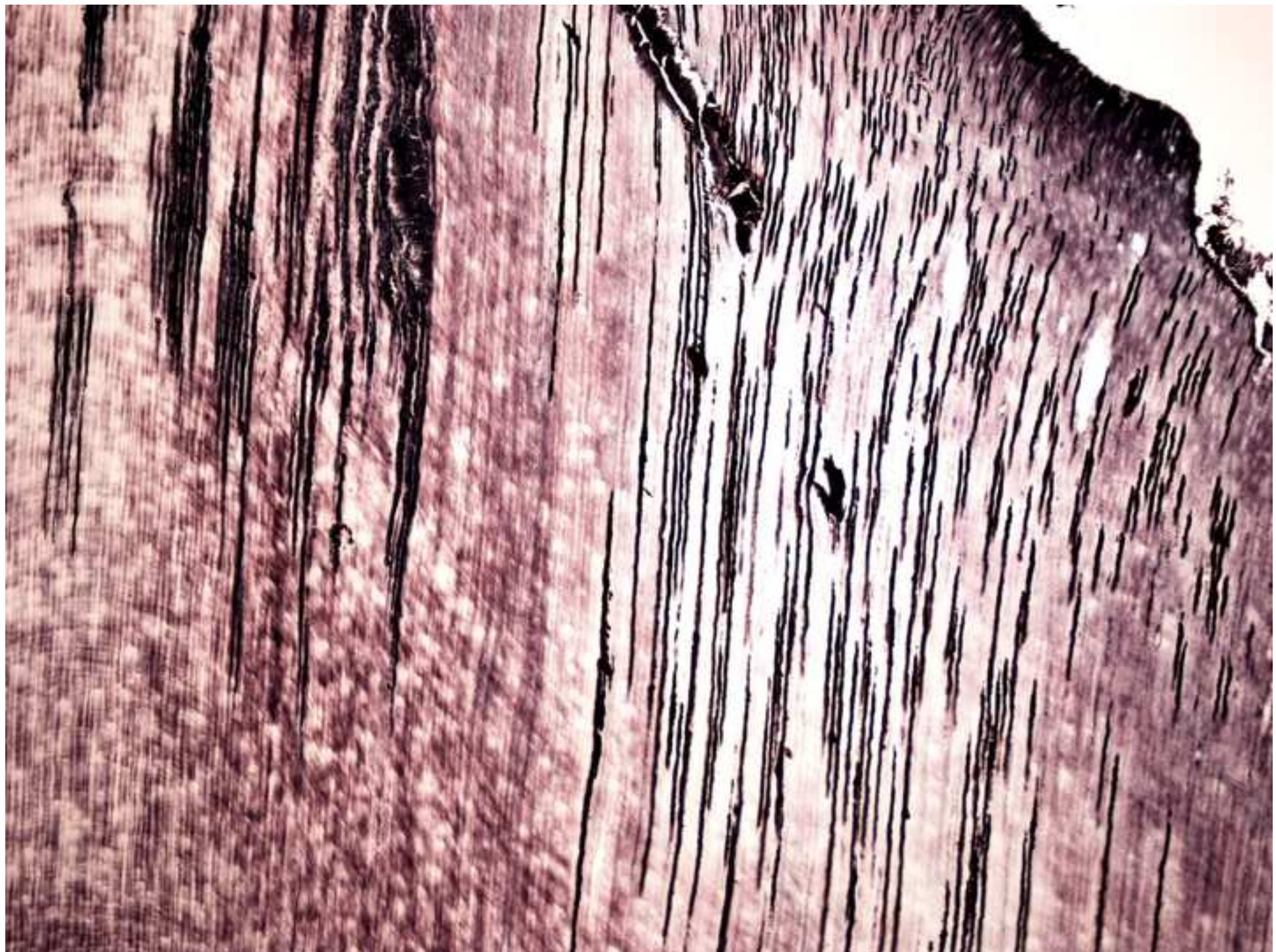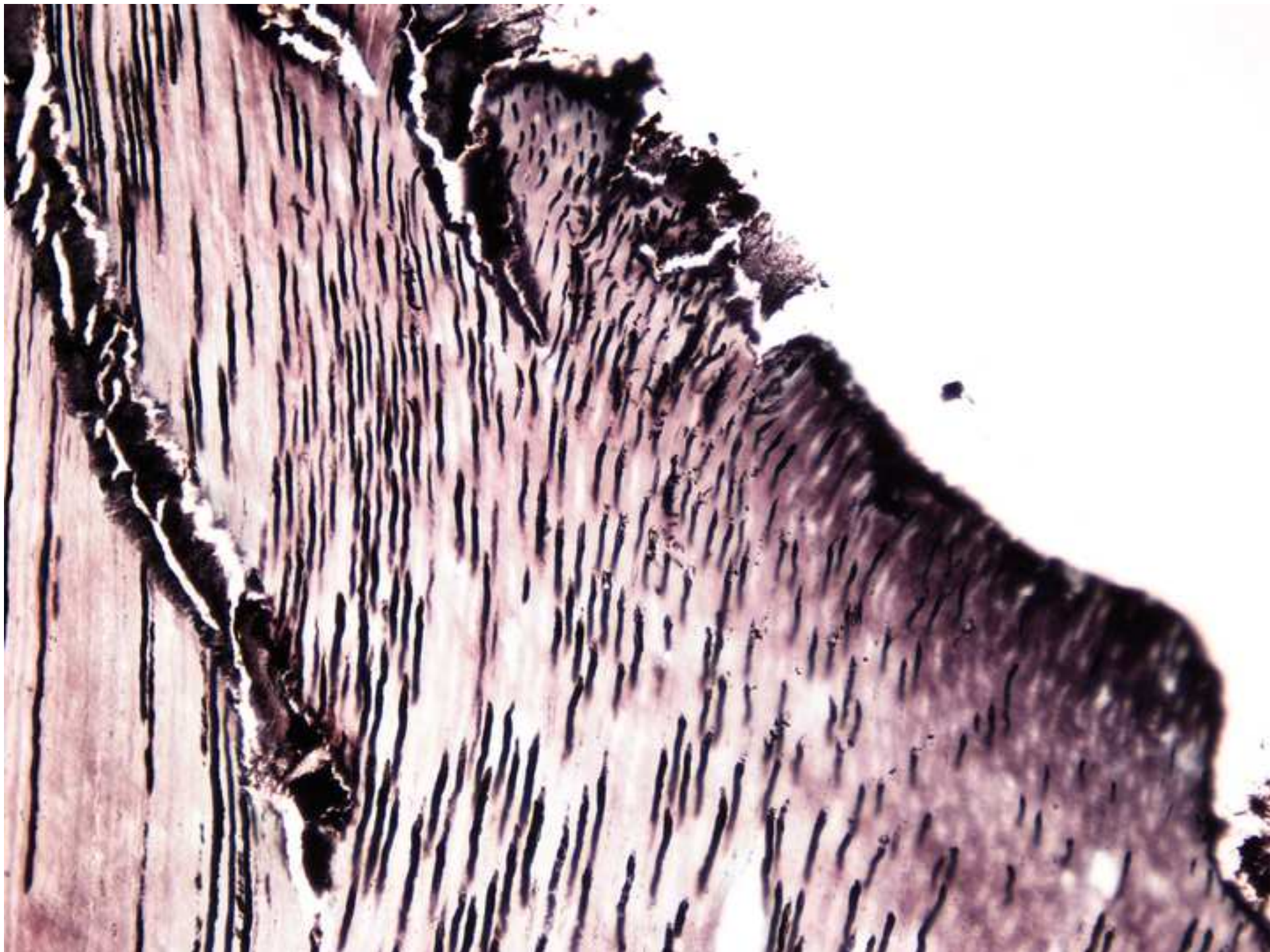
Figure 2a

Figs. 3a and b: Southern blots of paired isolates of C. albicans obtained from the tongue and carious dentine of children, demonstrating identical fingerprinting patterns, except isolates 5a and 5b. The reference strain 3153A is in the two outermost lanes, and comparing the patterns to a computer reconstruction of fingerprinting patterns in Fig. 4[32], isolate 5a is identified as belonging to clade SA while 5b is similar to clade I.
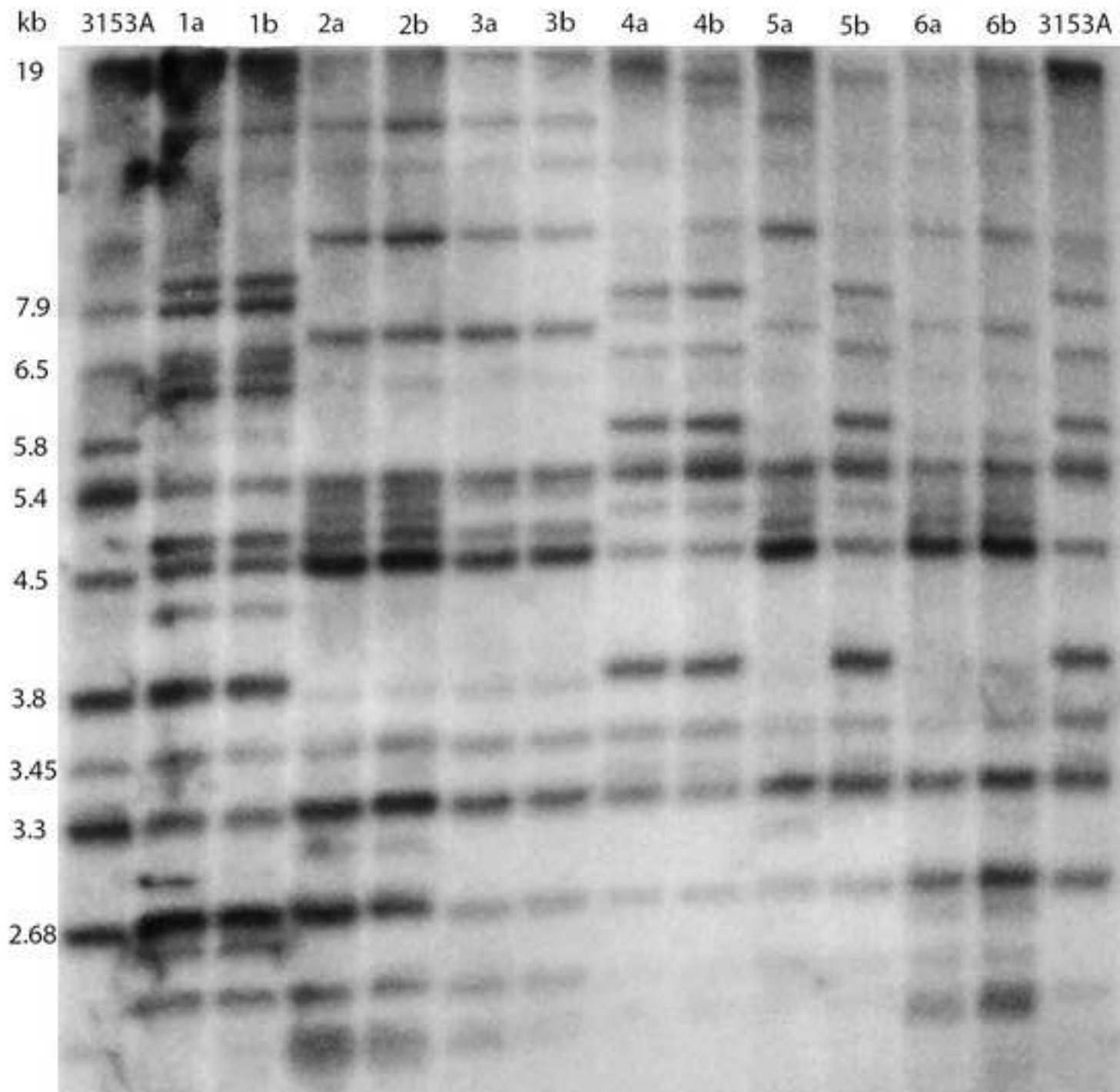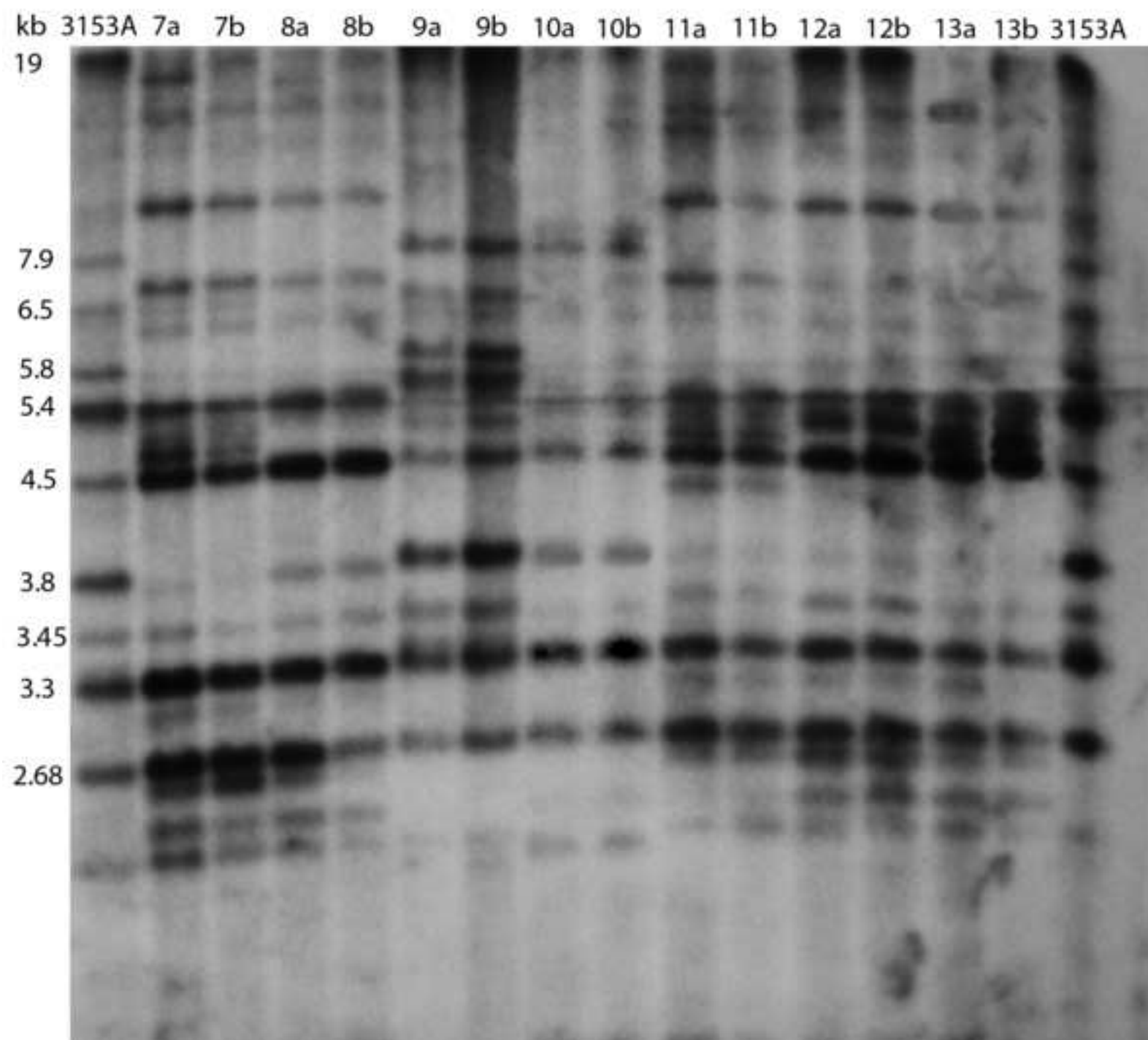
Figure 3a

Figure 3b

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The Grocott and PAS stains demonstrated fungal hyphae on the floor of the carious lesions as well as penetration across some distance into the dentine in a striated manner suggestive of penetration along the dentinal tubules (Figs. 1a & b and 2a & b).

DNA fingerprinting

Considering the two Southern blots (Figs.3a and b) it is seen that the DNA fingerprinting patterns of all the paired isolates are identical, except for isolates 5a and b which differ. Comparing the fingerprinting patterns of the Southern blots from this study with computer reconstructed patterns of the different *C. albicans* groups or clades in figure 4 [32], it is evident that the fingerprinting pattern of isolates 2a & b, 5a, 6a & b, 7a & b, 11a & b, 12a & b and 13a & b demonstrated the same dense banding pattern in the 5.8 to 4.5 kb region characteristic of Group SA. The fingerprinting pattern of isolate 5b has the characteristic appearance of two well demarcated bands in the 7.9 and 6.5 kb region, with no other bands in the immediate vicinity, similar to the reference strain 3153A in the two outermost lanes, which corresponded to that of Group I. The distribution of the remaining isolates among the other genetic subgroups were 30.7% belonging to Group III, 15.3% to Group I and 11.5% to Group II.

Children, at the time of being diagnosed as HIV-positive, often present simultaneously with extensive caries and pseudomembranous candidiasis as depicted in Figs. 5a and b. Before ART was available to children, these individuals had to receive repeated topical antifungal treatment due to the pseudomembranous candidiasis recurring within 4-5 weeks of treatment.

Discussion

Using a DNA fingerprinting method with high discriminatory power, this study is the first to conclusively demonstrate the colonisation of oral soft tissue and the penetration into dentine by the same genetic subgroup of *C. albicans*. The 151 (41%) patients from whom yeasts were isolated in this study were fewer than the 62% reported for Brazilian children, all of whom were

Fig. 4: Computer reconstructed fingerprinting patterns of representative C. albicans isolates labelled with the Ca3 probe and grouped into 4 clades as initially described for South African C. albicans oral isolates, namely Clades I, II, III and SA.[32]

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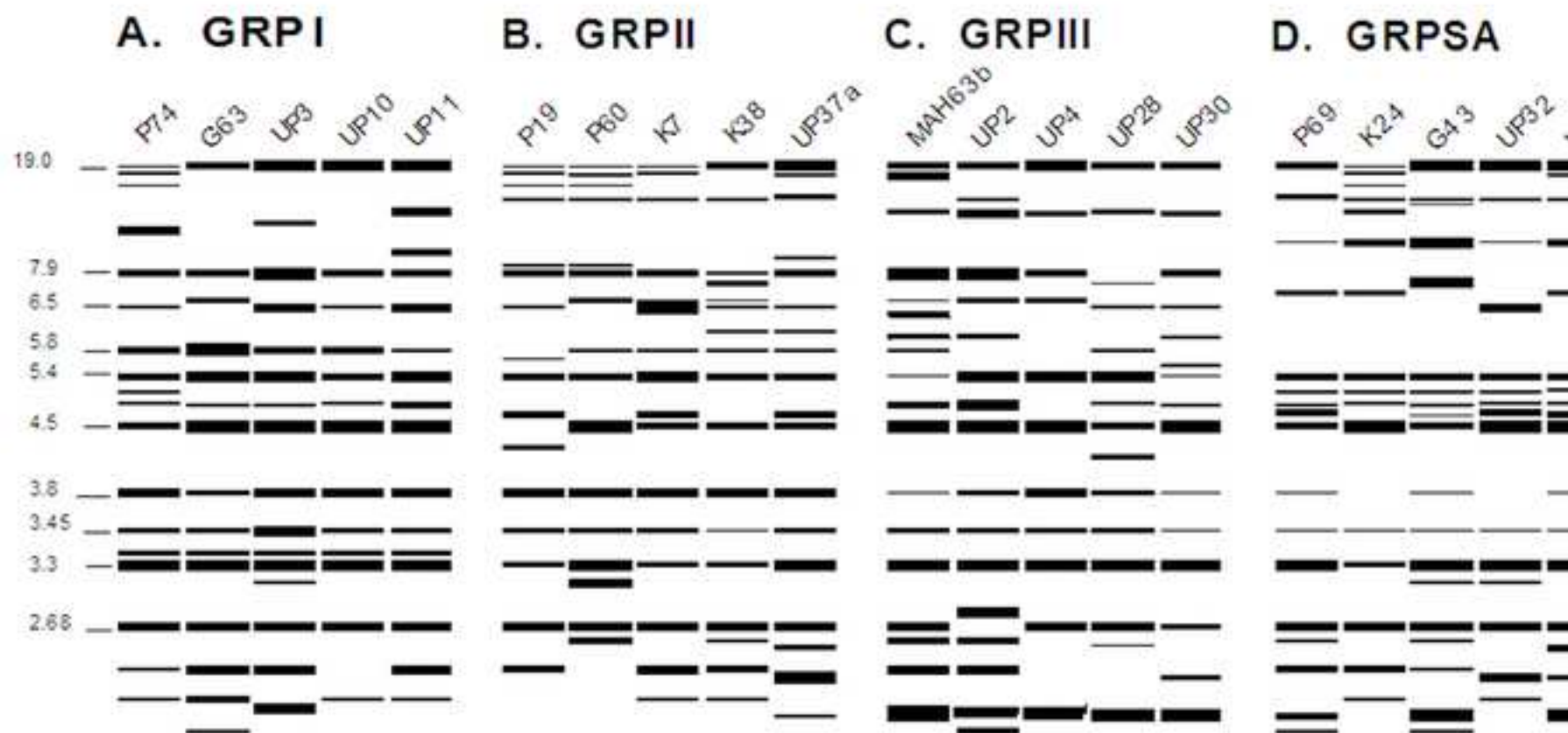
Figs. 5a and b: Clinical presentation of a newly diagnosed 6 year old HIV-positive patient presenting simultaneously with rampant caries of the primary dentition and extensive pseudomembranous candidiasis.

Figure 5a

immunosuppressed and diagnosed with AIDS [8]. The lower prevalence in this study could be attributed to the fact that the children were either receiving ART or not sufficiently immunosuppressed to qualify for ART [33]. Children who received antifungal therapy at the time of referral, or who had completed antifungal therapy four weeks prior to referral were excluded from the study, while nevertheless having the teeth extracted that they were originally referred for. It was therefore unlikely that prior antifungal treatment could have been responsible for the lower prevalence of oral yeast carriage. Health care professionals only referred children for extractions when the children complained of tooth ache or if they presented with dental abscesses. The only treatment available to patients dependent on public dental services is extractions. While the exact extent of caries among this cohort was not noted, by the time children were referred for extractions, they presented with at least two carious teeth. Caries is prevalent among South African children and when the previous National Oral Health Survey was performed approximately 15 years ago caries among 6-year old South African children was 60.3% [34]. A survey conducted in 2006 among children from a nearby community to where the current study was performed, found the prevalence of early childhood caries among 4 and 5 year old children to be 55.8% and 53.4% respectively [35].

The reason that none of the other non-*C. albicans* spp. were isolated from carious dentine is not clear. Non-*C. albicans* spp. were isolated from only seven (4.6%) patients and in each case these spp. occurred together with *C. albicans* which predominated as evidenced by the colony colour on CHROMagar. Because the objective of the study was to determine the similarity of yeast spp. from the two oral sites and not a quantitative assessment of yeast growth, swabs were taken from the tongue in a semi-quantitative manner only to standardise the sampling between patients and not for the purpose of determining colony forming units (CFUs). Similarly, no attempt was made to weigh or quantitate the dentine fragments from the carious teeth but merely to culture possible yeasts that were penetrated into the dentine. It can be stated with a high degree of certainty that the yeast elements that were demonstrated on histological sections with both Grocott's and PAS staining of carious teeth was *C. albicans* as this had been confirmed by four generally accepted yeast

identification methods. Fluid from vortexed dentine fragments, yeast growth from or immediately adjacent to dentine fragments placed on CHROMagar plates [28], with further confirmation done with the germ tube test, followed by a commonly utilised commercial yeast identifications system, namely ID32C [15], and finally with DNA fingerprinting.

Taking the degree to which *C. albicans* can adjust to a wide range of environmental conditions , also referred to as plasticity, into account [36], the ability of the organism to penetrate a considerable distance from the surface of the carious lesion into the dentine was not surprising. The reasons for Maijala and co-workers not being able to demonstrate *C. albicans* in carious dentine is unclear [2].

Selecting Southern blot hybridised with the Ca3 probe as fingerprinting method for this study [37], was due to the fact that the similarity in fingerprinting patterns of a relatively small number of isolates of *C. albicans* were readily visible on the Southern blots. In addition, this method was previously used to fingerprint a large number (361) of South African oral yeast isolates [32], thus facilitating comparison of subsequent fingerprinting patterns of *C. albicans* oral isolates. Multilocus sequence typing (MLST) with 8 – 10 household genes, is the method preferred by European researchers to study the genetic similarity of *C. albicans* isolates from that region [18, 17]. This method, however, delineates *C. albicans* into 17 genetic groups [17] as opposed to 5 according to the Ca3 fingerprinting method [16]. Together with the fact that MLST was not available for this study, it has not been applied to large numbers of South African *C. albicans* oral isolates and information to enable comparison of subsequent *C. albicans* oral isolates is therefore not available. Visual comparison of the fingerprinting patterns of isolates from this study is facilitated by the inclusion of the computer reconstruction of the fingerprinting patterns obtained from the previous study (Fig 4) in which isolates from the various genetic subgroups were clustered in order to obtain an oversight of the characteristic fingerprinting patterns of the different genetic subgroups [32]. Despite a relatively small number of isolates having been fingerprinted, it is important to note that 50% of these fingerprinted isolates are readily distinguishable as belonging to clade or Group SA,

which corresponds to the 53% of isolates from the previous DNA fingerprinting study on South African oral yeast isolates [32]. This again confirms the geographic predilection of certain clades [16].

This study further confirmed the absence of a particular genetic group or clade of *C. albicans* being associated with colonisation of a particular subgroup of *C. albicans* [32, 38]. Neither soft tissue nor dentine were preferentially colonised or penetrated by a particular genetic subgroup and representatives from all the known genetic groups of *C. albicans* that occur in South Africa are capable of existing in both niches. Similarly the results from a previous DNA fingerprinting study conducted among 170 healthy individuals and 79 patients with oral candidiasis conclusively demonstrated that no particular genetic group of *C. albicans* was associated with oral candidiasis [32]. By not taking the *C. albicans* clade distribution in a particular region into account [16], and not comparing isolates from diseases sited to the distribution of isolates from healthy individuals from the same region, it might well appear as if particular genetic subgroups of the organism predominate in certain disease conditions.   In this study more than one *C. albicans* colony was selected and stored for fingerprinting and it was unlikely that it could be attributed to chance that 12 of the 13 paired isolates were identical. Demonstration of the same genetic subtype from a particular patient's oral soft tissue also colonising that patient's carious dentine, is similar to the findings of another study in which identical *C. albicans* genetic subgroups were demonstrated to colonise two different anatomical sites [39].

It was not attempted to determine whether oral bacteria co-existed with *C. albicans* or that they possibly facilitated the penetration of the organism deep into the dentine, but from previous *in vitro* studies it would be feasible that it can be the case [40]. No claims are made based on the results of this study regarding the cariogenic potential of *C. albicans.* The high degree of plasticity that the organism is capable of is again emphasised and demonstrating the mere presence of the organism in a particular anatomical site, without the direct demonstration of putative pathogenic traits

expressed at the time [41], is not sufficient to assign a pathogenic role to the organism. Prominent researchers in the field of *C. albicans* have furthermore expressed reservations about extrapolating *in vitro* research findings on artificial media to include the *in vivo* behaviour of the organism [36].

Having demonstrated fungal elements penetrating into dentine and culturing only *C. albicans* from this dentine, together with demonstrating that the *C. albicans* isolates from dentine and oral soft tissue were of the same genetic subtype, warrant serious consideration in the clinical management of the oral health of immunosuppressed children. It is not possible to deduct from this study which of oral soft tissue or carious teeth was first colonized by *C. albicans* but the fact that the organism is not only present in debris on the surface of carious lesions increases the possibility that it might survive treatment of oral candidiasis with routinely prescribed topical antifungal agents and that carious teeth may therefore serve as a reservoir of a potentially pathogenic agent in an immunocompromised host. In the case of the children from this cohort who may wait up to a year before they have multiple carious teeth extracted under general anesthesia, serious consideration ought to be given to the prevention of caries or the early management of carious teeth. Although not eliminating *C. albicans* completely, Starr and co-workers have found the timely treatment of carious teeth to reduce oral yeast carriage over a 3 year period [42].

Conclusion

The findings from this study demonstrated that all the known genetic groups or clades of *C. albicans* that occur in South Africa were able to colonize both oral soft tissue and carious dentine of HIV-positive children and that in more than 92% of these individuals, isolates from the two sites were genetically identical. The numbers of the various genetic groups found in this study were representative of the distribution of previously identified South African *C. albicans* oral isolates. Carious teeth can therefore be regarded as potential reservoirs of *C. albicans* which at some stage may result in infection in immunocompromised individuals. Carious teeth should therefore not be

regarded as completely harmless, and left unattended until pain is experienced or abscesses develop.

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