Research Article
The Diagnostic Accuracy of Autofluorescence Microscopy of Pap Smears for Oral Candidal Hyphae
Fadi Titinchi, Jonathan Du Toit, Johannes J Hille, Greta Neethling

Abstract
Purpose: To evaluate the diagnostic performance of Paparnicolaou stained oral smears viewed under autofluorescence microscopy to detect oral Candida. Materials and Methods: A blinded cross-sectional study was designed to include patients (n=80) visiting two dental clinics. Patients were randomly selected and the presence of candidiasis was unknown to the researchers. Areas sampled were the dorsal tongue, buccal mucosa, and labial vestibulae. Specimens were collected by brush cytology. Two parallel exfoliative smears were prepared from each brushing sample. Each slide pair per patient was stained: one with PAS, the other with Pap. The PAS slides were then screened by cytologists with the results forming the control group. The randomized Pap slides were then viewed under autofluorescence microscopy by two independent observers. Results of the autofluorescence viewing were then correlated against the control group (PAS). Results: Paparnicolaou stained oral squamous cells and Candida autofluoresce bright green under autofluorescence microscopy. The diagnostic performance of autofluorescence microscopy for observer A was: sensitivity 39.4%, specificity 83.0%, diagnostic accuracy 63.8%; observer B: sensitivity 30.3%, specificity 95.7%, and diagnostic accuracy 67.5%. Conclusions: Orientation of the Candida hyphae and auto-fluorescing squamous cells affected screening results. Hyphae otherwise undetected under light microscopy may be identified under autofluorescence microscopy, and vice versa. Being an effective, rapid screening test for the diagnosis of fungi, autofluorescence microscopy was however inadequate in the detection of oral Candida.

Keywords: Candida; Oral Candidiasis; Paparnicolaou Smear; Stains; Fluorescence Microscopy; Periodic Acid-Schiff; Diagnostic Imaging.

Introduction
Oral candidiasis is the most prevalent opportunistic infection of the oral cavity most commonly caused by Candida albicans. In most individuals, Candida species are commensal organisms, though a change in the normal oral environment may lead to hyphae growth and clinical infection. It is highly underdiagnosed and common among elderly patients, denture wearers, impaired salivary gland function patients, medicated patients, diets high in carbohydrates, smokers, diabetics, Cushing's syndrome patients, oral malignancies, and in immunodeficiencies such as HIV infection. Literature reports on incidence rates ranging from 20%-75% in the general population, 30%-45% among healthy adults, 50%-65% among denture wearers, and as high as 75% among HIV positive individuals.

Diagnosis is most often made clinically by visual examination. Diagnosis may be confirmed microbiologically. Subclinical candidiasis or the carrier state is however diagnosed with the use of swabs, oral rinses, imprint/impression cultures and smears. When using the smear test, culture samples are collected using cytobrushes and smeared on glass slides. These are fixed and then commonly stained with Periodic Acid-Schiff (PAS) or Papanicolaou (Pap) stain, or Gomori methenamine silver stain. The silver stain is more complicated than PAS, and PAS is more complicated than Pap. Of these the PAS stain is most often used to identify fungal elements. PAS requires a relatively longer and more meticulous staining procedure than the Pap method. Viewing of PAS stained specimen has also shown to have lower sensitivity when compared to other methods of diagnosis.

Autofluorescence microscopy is the excitation of fluorophores intrinsic within cells by UV/LED radiation of suitable wavelength, resulting in fluorescence emission, rendering these viewable by microscope. The pros of autofluorescence microscopy for fungal screening include: no special staining procedures required; no
time delay - as is incurred with special stains;¹²⁻¹⁴ the ability to screen material at a relatively lower power magnification; the ability to tentatively diagnose fungi;¹³ and better pathogen discrimination against a dark background.¹²

Autofluorescence microscopy is also widely used for the rapid diagnosis of mycobacterium infection.¹⁴,¹⁵ The authors of this study hypothesized similarly that using autofluorescence microscopy in general practice oral pathology, oral medicine, and so forth, may be a rapid screening test for subclinical candidiasis or the carrier state, specifically significant to Candidiasis' association to HIV infection and the early detection thereof in previously undiagnosed patients. Due to the advantages of autofluorescence microscopy and of the Pap staining method, their possible combined efficacy formed the premise for this study. It has been shown in a number of studies (Table 1) that autofluorescence microscopy is a good, reliable technique for detecting fungi with Hematoxylin & Eosin (H&E)¹²,¹³,¹⁶,¹⁷ and Pap stains.¹⁸,¹⁹ In a recent study by Rao et al.¹⁷, the authors concluded that autofluorescence microscopy had a specificity of 100% and a sensitivity of 97.8% when diagnosing fungal infection. Conversely a study by Elston²⁰ reported that autofluorescence microscopy was of little benefit in identifying fungal organisms.

Since no study has previously assessed the accuracy of autofluorescence microscopy to detect oral Candidia spp., the aim of this study was thus to investigate the efficacy of screening Pap stained oral cytobrush material with autofluorescence microscopy to detect oral Candida hyphae; hypothesizing this technique to be of diagnostic value.

Materials and Methods
A blinded, cross-sectional study was designed and carried out from October 2009 to June 2010 at the University of the Western Cape's dental clinics in Cape Town, South Africa. The study's research proposal for the sampling and analysis of oral cytobrush samples obtained from the patients participating in this study was reviewed and received ethical clearance from University of the Western Cape's Senate Research Committee (approval number: 10/1/51) prior to commencing the project. Experimentation of biological matter and of patients was in strict accordance with the guidelines and principles stated in the World Medical Association Declaration of Helsinki.

The research study with its implications, obligations and the patients rights' were explained to each participant in his/her own language. Written and signed informed consent was volunteered by each of the eighty (80) patients partaking in the study.

Inclusion criteria stipulated adult patients for sampling, aged 18 or above - those attending the university's dental hospital. Patients attending the general screening clinic were sampled, at random, and over several weeks. No specific clinic, ie. an oral medicine clinic, was targeted. Candidiasis and HIV status of the patients was unknown to the researchers.

Exclusion criteria disallowed the sampling of minors due to consent issues. Sampling was done prior to any treatment to prevent any blood product or other contamination. The oral cavity areas sampled for each patient included the dorsal tongue, buccal mucosa, and labial vestibulae. Specimens were collected by brush cytology. Cytobrushing is typically superficial and harmless. No local or topical anaesthetic was required. Two parallel exfoliative smears were prepared from each brushing sample; depositing nearly identical sample specimen by rolling the cytobrush between two new microscopy glass slides (Star Frost® Braunschweig, Germany; approx. 76x 26 mm/3x1 inch). Slides were then immediately fixed with cytological fixative (Fencott® Clareinch, South Africa) containing ethyl alcohol with poly-ethylene-glycols. Each fixed slide pair per patient was thereafter stained; one with PAS and the corresponding slide with Pap stain.

The investigations consisted of two main phases. In the first phase, PAS stained slides were screened by medical cytologists using normal light microscopy. The presence of Candida hyphae constituted a positive result. The baseline screening results from this phase formed the control group for the study. Two independent research coordinators then managed the recording of results, the control group's records, the patient information, and the randomization details of the slides at each phase in an Excel® database.
Table 1: Diagnosis of fungal infections using autofluorescence microscopy tested in the available literature.

<table>
<thead>
<tr>
<th>Stain</th>
<th>Fungus / Fungal infection</th>
<th>Result</th>
<th>Authors</th>
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<tbody>
<tr>
<td>H&amp;E</td>
<td>Aspergillus, Candida and Zygomycetes</td>
<td>All organisms showed strong fluorescence</td>
<td>Rao et al. 17</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Chromomycosis, Sporotrichosis, Tinea nigra, Aspergillosis, Blastomycosis and Candidiasis</td>
<td>Most fungi showed little or no fluorescence; however Candida showed strong fluorescence</td>
<td>Elston 20</td>
</tr>
<tr>
<td>Pap</td>
<td>Aspergillus</td>
<td>Easily detected with autofluorescence microscopy</td>
<td>Hettlich et al. 18</td>
</tr>
<tr>
<td>Pap</td>
<td>Pulmonary fungi</td>
<td>Yield of positive results is higher when screening with both light and autofluorescence microscopy</td>
<td>Subramony et al. 19</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Blastomycosis</td>
<td>Infection previously misdiagnosed detected using autofluorescence microscopy</td>
<td>Margo and Bombardier 16</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Blastomyces, Cryptococcus, Candida, Aspergillus, Coccioidiodes</td>
<td>Fluorescence and identification of mentioned organisms</td>
<td>Mann 13</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Coccidioidomycosis, Candidiasis, Aspergillosis, Mucormycosis, and Histoplasmosis</td>
<td>Most useful in identifying Candida spp, Coccioidiodes inmites, and Aspergillus spp.</td>
<td>Graham 12</td>
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In the second phase of the study, the coordinators recompiled the Pap stained slides, mixed and reassigned them randomized numbers. The slides were then distributed to the two observers for diagnoses: A, an oral pathologist, and B, a medical cytologist. The second phase's autofluorescence microscopy made use of a Fraen AFTER® fluorescence LED module coupled to an Olympus CX31 microscope. Both observers viewed the Pap stained slides under the same conditions, in a darkened room, independently, using the same microscope. Results were compiled in the Excel® database by the independent coordinators. Using the results of the PAS stained material viewed under normal light microscopy in phase 1 (control group) as the gold standard, the diagnoses from the technique tested in phase 2 were then statistically analysed using SPSS statistical package (v14.0, SPSS Inc., Chicago, IL, USA). Indices of test validity used were sensitivity, specificity, diagnostic accuracy and positive predictive value.

Results
Results from the control group (phase 1) indicated a high prevalence of individuals in the carrier state, or with subclinical candidiasis (41.25%). The samples viewed under autofluorescence microscopy showed hyphae that autofluoresced bright green in colour against a dark background. Using this screening technique, only 55% of the diagnoses were correctly made by both observers of the same cases. Forty four out of 80 cases were accurately diagnosed by both observer A & B when compared with the gold standard (phase 1). The diagnostic performance of the technique as per each observer is summarized in table 2. Observer A correctly diagnosed 52 out of the 80 cases, while observer B correctly diagnosed 55 out of 80. Results showed a low sensitivity for both observers while the specificity for each was high. Diagnostic accuracy, similar for both observers, was low. The positive predictive values (posterior probabilities) of the test were higher for observer B than observer A.

<table>
<thead>
<tr>
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<th>Observer A (%)</th>
<th>Observer B (%)</th>
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<tr>
<td>Sensitivity</td>
<td>39.4</td>
<td>30.3</td>
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<tr>
<td>Specificity</td>
<td>83.0</td>
<td>95.7</td>
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<tr>
<td>Diagnostic Accuracy</td>
<td>63.8</td>
<td>67.5</td>
</tr>
<tr>
<td>Positive Predictive Value</td>
<td>61.9</td>
<td>83.3</td>
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Table 2: The sensitivity, specificity, diagnostic accuracy and positive predictive values as per each observer’s results.

Figure 1 compares the results of the observers against the gold standard (screening PAS stained oral cytology smears with normal light microscopy) to illustrate the respective false positives and false negatives. Observer A had diagnosed 25% of the cases, and observer B 28.7% of the cases, as false negatives. Conversely, observer A diagnosed more false positives than did observer B, 8 cases to 2. The concordance of results showed fair interobserver agreement when using this technique (kappa = 0.288). It was also found
that for observer A the marginal probability of error was 28.5%, which was statistically significant ($p < 0.05$) and for observer B was 8%, also statistically significant ($p < 0.05$).

Figure 1: Diagnostic performance of autofluorescence microscopy as compared with the gold standard.

**Discussion**

This work describes a relatively new method that has never been applied or tested in the detection of oral Candida species. The cost of adding LED devices to normal microscopes is relatively inexpensive and may have many added benefits, those being rapid screening for candida, and so forth. However, the hypothesis of screening Pap stained oral cytobrush material with autofluorescence microscopy as a good technique for the diagnosis of oral candidiasis was somewhat disproved. Worth noting, is that results were evaluated on the assumption that the screening of PAS stained oral cytobrush specimen under normal light microscopy is the gold standard and the baseline screening results to be 100% correct. The observers’ false positive diagnoses were rechecked by the medical cytologists from phase 1 to limit the impact of this assumption on the study. Those baseline screening results were verified as correct. Also, previous studies evaluating this diagnostic screening technique had assessed H&E stained material viewed under autofluorescence microscopy, and also to detect a variety of species of fungi. Rao et al.\(^{17}\) had investigated fungal infections of the sino-nasal tract. This study assessed Pap stained material to detect Candida hyphae only, or the carrier state, and of the oral cavity only.

Sensitivity referring to how well a test is at correctly identifying individuals who have the disease; this screening test did not perform well. The test on the other hand could quite accurately identify those who were not infected. The lack of sensitivity of autofluorescence microscopy noted in this study is at odds with the results of some previous reports. Elston’s\(^{20}\) results were similarly at odds with previous studies. Rao et al.\(^{17}\) had concluded that the use of autofluorescence microscopy was highly specific (100%) and highly sensitive (97.8%) in the diagnosis of fungi. Our study did not find this to be the case. Moreover, sensitivity and specificity alone do not give us the information needed to assess a test’s probability of giving a correct diagnosis. According to the positive predictive values only one of the observers was able to correctly identify the proportion of patients with positive test results with reasonable accuracy.

Adam et al.\(^{3}\) reported that the autofluorescence pattern in the yeast Candida albicans is largely dependent on the fixing method and on excitation wavelength. They were able to show that acetone fixation resulted in strong fluorescence of individual cells within a population of cells. The fixative used in this study contained ethyl alcohol though, which may or may not have had an impact on the potential fluorescence of the Candida hyphae.

Pap stained hyphae observed in this study autofluoresced bright green in colour against a black or dark background (Fig 2a & 2b). Both the Candidal hyphae and the oral squamous cells autofluoresce, and of the same colour, thereby obscuring the detection of pathogens if either is superimposed (Fig 3a & 3b). Elston\(^{20}\) reported the same finding when examining cutaneous candidal infections using autofluorescence microscopy. In such cases, it may be easier to detect hyphae using normal light microscopy. Conversely, hyphae not superimposed by squamous cells were rather distinct and easily detectable using autofluorescence microscopy. The two techniques may be said to be complimentary; possibly improving the accuracy of diagnosing candidiasis.

The authors of this study hypothesize that infection of cells may possibly have a negative effect on their intrinsic ability to autofluoresce. In this study, what appeared to be the infection of cells and the approximation of pathogen and cell were indistinguishable. In many cases oral squamous epithelial cells approximated to
the fungal hyphae were visible under normal light microscopy (Fig 4a), though interestingly appeared as ‘ghost cells’ - hidden when viewed under autofluorescence microscopy (Fig 4b).

The high prevalence of subclinical candidiasis or those in the carrier state among the sample population is alarming. Oral candidiasis has a strong correlation to HIV infection and is often an early oral manifestation among HIV positive individuals. Correctly diagnosing oral candidiasis in turn could aid in the diagnosis of HIV/AIDS in patients of previously unknown status, however, as a rapid screening test autofluorescence microscopy used alone for candidiasis diagnosis was found to be inadequate.

Conclusions

Pap stained oral cytobrush material viewed with autofluorescence microscopy is not sufficiently effective / reliable on its own when screening for oral Candida species. PAS stained oral cytology viewed with normal light microscopy remains the gold standard with regards to identifying oral candidiasis or the carrier state. The orientation of the hyphae in relation to the squamous cells may affect screening results significantly. The pathogens detectable by autofluorescence microscopy may be otherwise undetectable by normal light microscopy, and vice versa. Autofluorescence screening of Pap stained oral specimens may prove beneficial in cases where Candidal hyphae are otherwise undetected under normal light microscopy screening. Being an effective, rapid screening test for the diagnosis of fungi, autofluorescence microscopy was however inadequate in the detection of oral Candida.

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