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Oropharyngeal candidiasis among HIV seropositive patients in Chennai, South India: An evaluation of Polymerase chain reaction-Restriction Fragment Length polymorphism for speciation and Antifungal drug resistance

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Abstract:

CONTEXT: Oropharyngeal candidiasis is a common opportunistic manifestation of HIV/acquired immunodeficiency syndrome. Non-albicans Candida and drug-resistant strains are on the rise leading to treatment difficulties.

AIMS: The present study aims to speciate, perform antifungal susceptibility, and also to evaluate the significance of antifungal drug resistance by correlating the prior exposure to Fluconazole and CD-4 counts.

SETTINGS AND DESIGN: This was a cross-sectional study.

MATERIALS AND METHODS: A total of 115 HIV patients with clinical evidence of oropharyngeal candidiasis were included in the study. Two swabs were collected from the lesions. Speciation and antifungal susceptibility by microbroth dilution method for fluconazole, itraconazole, and amphotericin B were done as per the standard microbiological techniques. Polymerase chain reaction and restriction fragment length polymorphism (PCR and RFLP) were performed for speciating the Non-albicans candida. The association between prior exposure to Fluconazole, CD-4 counts, and antifungal resistance was assessed (Fisher's exact test, Chi-square test $P < 0.05$ was considered as statistically significant).

RESULTS: Among the 115 patients, 60 (52.17%) showed culture positivity for Candida species. *Candida albicans* 35 (56.45%) was the predominant species isolated followed by *Candida tropicalis* 12 (19.30%). PCR RFLP produced specific products for 19 of the non albicans Candida. A resistance of 17 (27.41%) and 18 (29.00%) was seen to fluconazole and itraconazole, respectively. A significant association was observed between prior exposure to Fluconazole and drug resistance ($P = 0.0201$).The correlation between CD-4 counts and antifungal resistance was not statistically significant ($P = 0.595$).

CONCLUSION: Antifungal susceptibility testing should be performed routinely along with species-level identification whether it is the first or recurrent episode of oral candidiasis and therapy should be modified accordingly to prevent the morbidity and mortality.

Keywords:

Antifungal susceptibility pattern, non-albicans Candida

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Introduction

Infection with HIV and the opportunistic infections occurring in its end stage acquired immunodeficiency syndrome (AIDS) is one of the major public health problems in the present scenario. Majority of the patients do not have access to therapy, and among some patients, despite therapy with highly active antiretroviral therapy (HAART), the incidence of opportunistic infections remains the same due to the lack of response to antiretroviral drugs, poor adherence and drug toxicities.

Oropharyngeal candidiasis is a common opportunistic infection of AIDS and occurs in about 95% of patients with advanced disease.[1,2] Although not associated with considerable morbidity and mortality, severe oropharyngeal candidiasis can interfere with the administration of drugs and food intake. Frequent recurrences can lead to repeated exposure to antifungal drugs. *Candida albicans* is the common species implicated in the aetiology of oral candidiasis among HIV-infected patients, but there has been a rising incidence of non-albicans Candida over the past two decades from 3.4% to 16.8%.[3]

Fluconazole is the first-line drug used in the treatment of oropharyngeal candidiasis among HIV/AIDS patients. Studies have estimated the incidence of fluconazole resistance as 5%–36%.[2] Repeated exposure to azoles can lead to the emergence of species such as *Candida krusei* and *Candida glabrata* with innately reduced susceptibility to fluconazole.[1,4] Other drugs such as amphotericin B and echinocandins are used for the treatment of azole-resistant and invasive candidiasis. Hence, species identification along with the antifungal susceptibility pattern is of paramount importance. The conventional phenotypic method of identification requires at least two to three days or even more time for species-level identification. Molecular methods such as polymerase chain reaction and restriction fragment length polymorphism (PCR RFLP) can facilitate the identification of the isolates within a few hours.

The present study aims to isolate and to identify the candida species causing oropharyngeal candidiasis in HIV/AIDS patients to determine their antifungal susceptibility pattern and to correlate prior exposure to fluconazole and CD-4 counts with antifungal drug resistance.

Aims and objectives

1. To isolate and identify the candida species causing oropharyngeal candidiasis in HIV/AIDS patients by conventional phenotypic method and by PCR RFLP method
2. To perform antifungal susceptibility testing of the isolates by microbroth dilution method for fluconazole, itraconazole and amphotericin B
3. To evaluate the risk factors for antifungal drug resistance by correlating the prior exposure to fluconazole and CD-4 counts.

A total of 125 HIV positive patients with clinical evidence of oropharyngeal candidiasis such as presence of white patch/plaque, erythematous and ulcerative lesion, difficult and painful swallowing were included in the study. This was a cross-sectional study conducted for six months from January 2013 to June 2013. Patients who were on antifungal therapy within the past seven days were excluded. The information regarding the symptoms, treatment details, CD-4 counts and previous history of exposure to azoles was obtained and documented in the form of questionnaire. Patients not willing to participate in the study were excluded from the study. The study was approved by the Institutional Ethical Committee

Two sterile swabs were used to collect the specimen from the lesions in the oropharyngeal region. Direct gram staining was done with one swab and fungal culture was performed with the other. Inoculation was done on Sabouraud dextrose agar (SDA) with chloramphenicol and incubated at 24°C for 48 h. Gram staining to look for the presence of yeast cells was done on all cultures that showed a growth of white creamy colonies on SDA. Germ tube test was performed to differentiate *C. albicans* from non-albicans Candida. Further speciation was done by colony morphology on CHROM agar (colour of the colony), Cornmeal agar (to identify morphological forms such as chlamydospore, blastospore and mycelium), sugar fermentation test, sugar assimilation test and the presence of growth at 45°C (to differentiate *Candida dubliniensis* and *Candida albicans*) as per the standard microbiological techniques.[5-7] Non albicans Candida isolates that showed varying morphological features in CHROM agar, sugar fermentation and assimilation tests were subjected to PCR RFLP for the confirmation of species. ATCC *Candida albicans* (90028) was used as control strains.

Polymerase chain reaction-Restriction fragment length polymorphism

DNA extraction

The isolates were subcultured onto SDA, and four to five colonies were scraped and mixed in 0.5 ml of normal saline in an Eppendorf tube and centrifuged at 6000 rpm for ten minutes. The pellet was suspended in 200 µl of phosphate buffered saline. Fifty µl of Lysozyme was added and incubated at 37°C for 15 min. A volume of 400 µl of Lysis buffer and Proteinase K [40 µl] was added and incubated in a water bath at 70°C for 10 min. The whole lysate was transferred into a spin column. The Eppendorf tube was centrifuged for one minute at

10000 rpm (rotations per minute). 500 µl of Wash Buffer 1 was added and centrifuged for one min at 10000 rpm. Wash Buffer-2 (500 µl) was added and centrifuged for one minute at 10000 rpm. Washing step was repeated once more, and 100 µl of Elution Buffer was added for DNA elution. For PCR amplification one µl of the DNA was used. ITS1-F-5′-TCCGTAGGTGAACCTGCGG-3′ and ITS 4-R-5′-TCCTCCGCTTATTGATATGC-3′primers were used.

Polymerase chain reaction procedure

PCR Master Mix contains 10X Taq buffer, 2 mM Mgcl2, 0.4 mM dNTPs mix and 2U Taq DNA polymerase. The PCR vial contains Master Mix 25 µl, ITS Forward primer and ITS reverse primer oneµl each, extracted DNA 5 µl and nuclease-free water 18 µl. The vial was mixed gently and spins down briefly. The PCR tube was placed into PCR machine and thermal cycling conditions for 35 cycles were set as Initial Denaturation: 94°C for 3 min, Denaturation: 94°C for 30 s Annealing: 56°C for 30 sec 35 cycles, Extension: 72°C for 30 sec Final extension: 72°C for 5 min.[8-10]

Restriction fragment length polymorphism

The PCR amplification products were purified using PCR clean-up kit and digested with *MspI* restriction enzyme. The total volume of RFLP tube is 50 µl (*Nuclease-free water* 23 µl, 10X reaction buffer 5 µl, Purified PCR product 20 µl, *MspI* Enzyme 2 µl). The RFLP tube was incubated at 37°C for 2 h.

Loading

After the amplification procedure, the PCR products and RFLP Products were loaded into 2% agarose gel along with 10 µl of HELINI 100 bp DNA Ladder and visualised under UV light and results interpreted.

Antifungal susceptibility testing was performed by microbroth dilution methods, and the minimum inhibitory concentration (MIC) was determined. Results were interpreted as per CLSI guidelines (Clinical laboratory and Standards Institute 2009) for Fluconazole, itraconazole and amphotericin B.[11,12]

Statistical methods

The significance of the association between drug resistance pattern and the prior history of usage of azoles and the CD-4 counts were analysed by Chi-square test and Fisher's exact test. Graph pad Software Quick Cals software was used for statistical analysis (GraphPad Software, La Jolla California USA, www.graphpad.com) ($P < 0.05$ was considered statistically significant).

Results

A total of 125 patients with clinical evidence of oropharyngeal candidiasis were recruited for the study out of which ten patients who were on antifungal therapy were excluded, and the rest 115 were included in the study. The present study showed a male preponderance 82 (71.3%) with the majority of the patients, i.e., 42 (36.5%) being in the age group of 31–40 years [Table 1]. Painful swallowing, cough and fever were the most common presenting complaints and presence of white plaque/patch in the oral cavity was the common sign elicited [Table 2].

Direct Gram staining showed the presence of Gram-positive yeast cells and pseudohyphae in 66 (57.3%) cases. Culture positivity was observed in 60 (52.17%) patients. *C. albicans* 35 (56.45%) was the most frequently isolated species followed by *Candida tropicalis* 12 (19.30%), *C. krusei* 6 (9.67%). A mixture of *C. albicans* and *C. tropicalis* was isolated from two specimens. The species-wise distribution of the isolates is depicted in Figure 1. *C. dubliniensis* comprised of three (4.8%) of the isolates [Figure 1]. The three isolates of *C. dubliniensis* were identified based on the dark green in CHROM agar, the absence of growth at 45°C elsius and a positive xylose fermentation test [Figure 1].

Out of the 27 non albicans Candida and 19 of the isolates that showed varying morphological features in CHROM agar, sugar fermentation and assimilation tests were subjected to PCR RFLP for confirmation of species. The PCR products were digested with *Msp1* enzyme and each species showed products with specific base pairs (b p).

Table 1: Age and sex wise distribution of the patients

	n (%)
Age distribution (years)	
20-30	24 (20.86)
31-40	42 (36.52)
41-50	31 (26.95)
51-60	18 (15.65)
Sex wise distribution	
Male	82 (71.30)
Female	33 (28.69)

Table 2: Distribution of the presenting complaints and clinical signs of the patients

	n (%)
Clinical feature	
Fever	68 (59.13)
Cough	86 (74.78)
Weight loss	48 (41.73)
Diarrhoea	25 (21.7)
Burning micturition	28 (24.34)
Painful swallowing	75 (65.21)
Difficulty in swallowing	12 (10.43)
Signs	
Presence of white patches in the oral cavity	89 (77.39)
Presence of ulcer	26 (22.60)
Presence of erythematous lesions	32 (27.82)

Figure 2 shows the PCR product and RFLP product alternatively. Lane 1 and 2 shows PCR product and RFLP digestion product of *Candida parapsilosis*, respectively (520 bp). *Candida parapsilosis* produces only one band for both PCR and RFLP product. Lane 3 and 4 shows ATCC *C. albicans* 90028 (PCR product amplicon size 535 bp, RFLP-297 bp and 238 bp). Lane 5 and 6 shows *C. tropicalis* (PCR product 524 bp, RFLP product 340 and 184 bp). Figure 3 shows the RFLP products after digestion with *Msp1* enzyme. Lane 2 shows *Candida kefyr* with a single digestion product of 722 bp. Lane 3 shows *C. glabrata* (557 and 314 bp), Lane 4 and 6 shows *Candida parapsilosis* and Lane 7 shows *C. tropicalis*.

By microbroth dilution method, *C. albicans* and *C. tropicalis* showed a resistance of 7 (20.00%) and 4 (33.30%) to fluconazole, (one drug missing), respectively. *C. tropicalis* and *C. krusei* showed a resistance of 5 (41.60%) and 5 (83.30%) for itraconazole, respectively. One (1.61%) *C. albicans* was resistant to amphotericin B. All non-albicans Candida isolates were sensitive to amphotericin B. There was an overall resistance of 17 (27.40%) and 18 (29.00%) to fluconazole and Itraconazole, respectively [Table 3].

Non-albicans Candida showed a higher percentage of resistance 10 (37.00%) to fluconazole and itraconazole compared to *C. albicans 7* (20.00%) In case of itraconazole the overall resistance was 7 (20.00%) for *C. albicans* versus 11 (40.7%) for non albicans Candida [Table 4].

There was a significant association between prior exposure to fluconazole and the presence of fluconazole resistance (P = 0.0201) (Fisher exact test) [Table 5]. Majority of the patients in the present study had CD-4 counts in the range of 51–200 cells/mm^3 [Table 6]. The CD-4 counts and fluconazole resistance were compared and there was no significant association (P = 0.595 Chi-square test) [Table 7]. Out of the 60 patients who were culture positive, 28% were on HAART therapy.

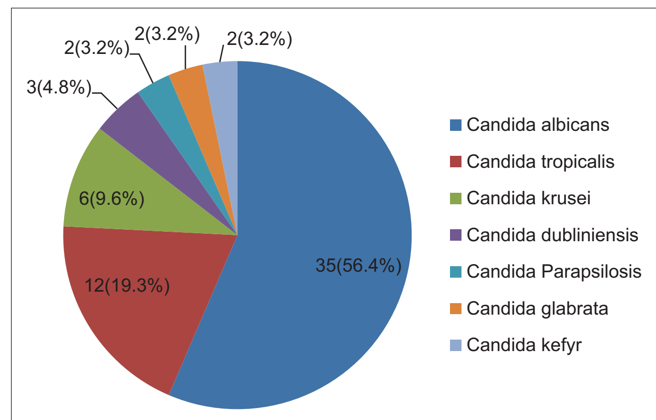

Figure 1: Mixed isolation of two species was obtained from two patients (*Candida albicans and Candida tropicalis*)

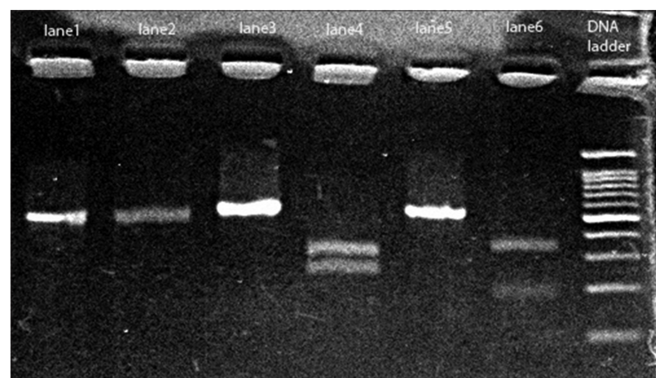

Figure 2: Polymerase chain reaction and restriction fragment length polymorphism products of the *Candida* isolates. Lane 1 and 2: Polymerase chain reaction and restriction fragment length polymorphism product of *Candida parapsilosis*, Lane 3 and 4: polymerase chain reaction and restriction fragment length polymorphism product of ATCC *Candida albicans*, Lane 5 and 6: Polymerase chain reaction and restriction fragment length polymorphism product of *Candida tropicalis,* Lane 7: DNA Ladder

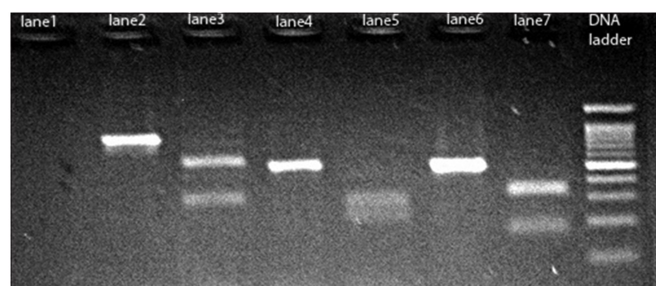

Figure 3: Restriction fragment length polymorphism products of the Candida isolates. Lane 2: *Candida kefyr*, Lane 3: *Candida glabrata*, Lane 4 and 6: *Candida parapsilosis*, Lane 7: *Candida tropicalis,* Lane 8: DNA Ladder

Discussion

Oropharyngeal candidiasis is one of the most common manifestations in patients infected with HIV and it reveals a deteriorating immune status and progression to AIDS. Among patients with oropharyngeal candidiasis, frequent recurrences will occur leading to considerable mortality and morbidity.[13] *C. albicans* 35 (56.40%) was the most common species isolated similarly to Shafir and Mane.[14,15] Non-albicans Candida comprised of 27 (43.50%) isolates. Among non-albicans Candida, *C. tropicalis* 12 (19.3%) was the predominant isolate followed by *C. krusei* 6 (9.67%) similar to other studies in the literature.[15-17] Three isolates of *C. dubliniensis* were also obtained. *C. dubliniensis* and *C. albicans* shares similar phenotypic and genotypic characters. Similar to *Candida albicans*, *C. dubliniensis* is also germ tube test positive but differing in the colony morphology in chrom agar (Dark green colonies of *C. dubliniensis* versus light green of *C. albicans*), assimilating xylose and absence of growth at 45°C. Reports of isolating *C. dubliniensis* from the oral cavity of HIV positive patients have been reported.[14,17,18]

Table 3: Species wise distribution of antifungal susceptibility pattern by microbroth dilution method

Species	MIC range				
	Fluconazole resistant (>8 µg/ml), *n* (%)	Fluconazole SDD (16-32 µg/ml), *n* (%)	Itraconazole resistant (>0.125 µg/ml), *n* (%)	Itraconazole SDD (0.25-0.5 µg/ml), *n* (%)	Amphotericin B resistant percentage (<1 µg/ml), *n* (%)
C. albicans (*n*=35)	7 (20)	1 (2.85)	7 (20)	Nil	1 (2.85)
C. tropicalis (*n*=12)	4 (33.33)	Nil	5 (41.66)	Nil	Nil
C. krusei (*n*=6)	5 (83.33)	1 (2.85)	5 (83.33)	1 (2.85)	Nil
C. glabrata (*n*=2)	1 (50)	Nil	1 (50)	Nil	Nil
Overall resistance percentage	17 (27.41)	2 (3.33)	18 (29)	1 (1.61)	1 (1.61)

All the *C. parapsilosis*, *C. dubliniensis* and *C. kefyr* isolates were susceptible to Fluconazole, Itraconazole and Amphotericin B. SDD: Susceptible dose dependent; MIC: Minimum inhibitory concentration; *C. albicans*: *Candida albicans*; *C. tropicalis*: *Candida tropicalis*; *C. krusei*: *Candida krusei*; *C. glabrata*: *Candida glabrata*; *C. parapsilosis*: *Candida parapsilosis*; *C. dubliniensis*: *Candida dubliniensis*; *C. kefyr*: *Candida kefyr*

Table 4: Antifungal susceptibility pattern by Microbroth dilution method *Candida albicans* versus non-*Candida albicans*

Antifungal drug	*C. albicans* (*n*=35)			Non-*C. albicans* (*n*=27)		
	Sensitive (%)	SDD (%)	Resistant (%)	Sensitive (%)	SDD (%)	Resistant (%)
Fluconazole	27 (77.1)	1 (2.85)	7 (20)	16 (59.25)	1 (3.70)	10 (37.03)
Itraconazole	28 (80.00)	Nil	7 (20)	15 (55.55)	1 (3.70)	11 (40.74)
Amphotericin B	34 (97.14)	Nil	1 (2.8)	27 (100)	Nil	Nil

SDD: Susceptible dose dependent; *C. albicans*: *Candida albicans*

Table 5: Significance of fluconazole resistance among patients with prior usage of fluconazole

	Presence of fluconazole resistance (*n*=17)	Absence of fluconazole resistance (*n*=45)
Prior usage of fluconazole present (*n*=24)	11	13
Prior usage of fluconazole absent (*n*=36)	6	30

The two-tailed *P*=0.0201 (Fisher's exact test) is considered to be statistically significant

Table 6: Distribution of CD4 counts among the patients

CD4 counts (cells/mm^3)	*n* (%)
<50	16 (13.91)
51-200	64 (55.65)
201-350	26 (22.60)
351-500	5 (4.34)
>500	4 (3.47)

Table 7: Comparison of fluconazole resistance with the CD4 counts

CD4 counts	Resistant isolates	
	C. albicans	Non-*C. albicans*
<50	3	6
51-200	2	3
201-300	2	1

P=0.595 (not significant) by Chi-square test. *C. albicans*: *Candida albicans*

Due to the increase in the number of cases and shift in the epidemiology of Candida infection, rapid species identification becomes imperative for choosing empirical therapy for inherently azole-resistant species like *C. krusei*. Certain inaccuracies can occur while presumptively identifying the species by conventional phenotypic methods, and in such situations, the results of morphology on corn meal agar, sugar assimilation and fermentation tests should be correlated which can be time-consuming. Molecular tests like PCR-RFLP can be more accurate in species identification with a fast turnaround time.[9,10,19] In the present study, we have confirmed the species of the 19 non-albicans isolates by PCR-RFLP method along with two control strains. Each species produces a specific banding pattern which helps in the identification. Further, the time taken for species identification is in hours compared to days (minimum of 2–3 days) for conventional methods. The other added advantage is that a very little amount of culture is sufficient and viability of the culture does not affect the procedure.

A study by Vijay Kumar *et al*. have documented that PCR-RFLP can be a valuable diagnostic technique for species identification of Candida isolates from bloodstream infection in an Intensive Care Unit setting.[10] Roshan and Shokoi *et al*. have used PCR-RFLP with *MspI* for speciation of Candida isolates in vulvovaginal candidiasis and cancer patients, respectively.[8,19,20] PCR-RFLP is a fast, sensitive and specific method, which can provide with valuable information regarding the species and assist the clinicians to choose an empiric antifungal drug for therapy, especially in cases of immunocompromised individuals and invasive infections such as candidaemia.

Knowledge regarding the antifungal resistance is of significance due to intrinsic drug resistance showed by certain species such as *Candida krusei* and *C. glabrata*.

Studies have documented fluconazole resistance to be ranging from 6% to 37% worldwide.[21-24] In the Indian scenario fluconazole resistance is being reported as 5%–34%.[15,17,25-28] In the present study, we have reported a resistance percentage for fluconazole and itraconazole as 17 (27.41%) and 18 (29.00%), respectively. This might be attributed to the replacement of previously drug-sensitive strains by resistant ones due to the repeated indiscriminate usage of azoles. Studies have also documented that repeated exposure to antifungal agents like fluconazole for prophylaxis and treatment of oral candidiasis might be a reason for the replacement of *C. albicans* by non-albicans species with a more resistant phenotype.[4] In the present study, non-albicans Candida has shown more resistance compared to *Candida albicans*. Higher resistance has been recorded for non-albicans Candida by different studies.[23,24]

The association between prior usage of fluconazole and antifungal resistance was statistically significant ($P = 0.0201$) similar to Hamza *et al*. and Mane *et al*.[15,16] The higher percentage of resistance among *Candida albicans*, for fluconazole and itraconazole in patients previously treated with azoles indicate the emergence of an acquired pattern of drug resistance. Immunosuppressed status of the patients infected with HIV as indicated by the CD-4 counts is an important parameter in deciding HAART therapy and also is a predictor of opportunistic infections. In the present study, CD-4 counts and fluconazole resistance had no significant association ($P = 0.556$ Chi-square test). Independent of previous exposure to antifungal drugs, the immunosuppressed status might be an indirect risk factor for the emergence of antifungal drug resistance since immunosuppression leads to the recurring episodes of oropharyngeal candidiasis exposing the patients to antifungal drug therapy, especially fluconazole which is being used as a first drug for prophylaxis.

The present study emphasizes the importance that in patients with oropharyngeal candidiasis in spite of the first episode/recurrent episode, the Candida isolate should be identified to species level, and antifungal drug susceptibility testing should be performed routinely as for bacterial infections and treatment protocols should be modified according to the drug susceptibility pattern to prevent the morbidity associated with candidiasis.

The limitation of the present study is that the genetic relatedness among the Candida strains isolated in individuals with previous exposure to azoles was not ascertained. It is of substantial importance to study for the genetic relatedness among the Candida strains by molecular studies, in cases of recurrent oral candidiasis to analyse the acquisition of drug resistance and the source of infection. Surveillance programs should be formulated to detect changes in the species distribution and the drug susceptibility pattern in a particular area to monitor the trends and help in the improved management of HIV/AIDS patients.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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