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Dentistry Section

Effect of Heavy Metal Ions on *Candida* Isolated from HIV Positive Patients

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ABSTRACT

Introduction: Over 90% of AIDS patients and 1/3rd of HIV seropositive patients are affected by Oral candidiasis. Moreover, the frequency of HIV related oral candidiasis is increased when CD4 count falls <400/mm^3 of blood. The widespread use of antifungal drugs in People Living with HIV/AIDS (PLHA) has led to emergence of drug-resistant strains of *Candida* species.

Aim: To detect the effect of heavy metal ions on *Candida* species and also to find the relationship between CD4 count and oral candidiasis in PLHA.

Materials and Methods: A total number of 25 HIV positive patients were studied after taking written informed consent. From each patient oral swabs from tongue and hard palate were collected and thus a total of 50 specimens were processed for isolation of *Candida* as per conventional methods. Effects of heavy metal ions like Lead, Zinc, Silver, Mercury and Cadmium on 26 *Candida* strains isolated were studied by agar dilution method. Percentages and proportions were used using frequency tables.

Results: All 26 (100%) *Candida* strains were resistant to Zinc ions with 100 mM concentration whereas all of these 26 (100%) *Candida* strains were sensitive to Cadmium ions even with 1 mM concentration. Maximum 8 (32%) PLHA from whom *Candida* species was isolated had CD4 count 300-399/mm^3 of blood.

Conclusion: Though heavy metal salts can be used for therapeutic use at very high dilutions heavy metals toxicity can result in long-term exposure. Heavy metal contacts with the skin also cause toxicity to different organ and damage to Central Nervous System (CNS), erythematous area over the skin, hyperpigmentation and argyria. Further study with animal experiment and human volunteers is required.

Keywords: CD4 count, Heavy metal ions, Isolation of candida, Oral candidiasis

INTRODUCTION

Globally the estimated number of people living with HIV/AIDS (PLHA) was 36.9 million at the end of 2017 [1] while in India the PLHA were 2.14 million, with 0.22 % of adult HIV prevalence [2]. Mortality among PLHA is mostly due to various opportunistic infections. Candidiasis has been found to be one of the most common HIV related oral lesions serving as a marker of immunodeficiency and HIV disease progression. Oral candidiasis affects approximately 1/3rd of HIV seropositive patient with AIDS. Oral candidiasis is an opportunistic mucosal infection caused, in most cases, by *Candida albicans*, but which can be caused by other species such as *C glabrata, C tropicalis and C krusei* [3]. The frequency of HIV related to oral candidiasis is notably increased when CD4 T-Lymphocyte count falls to <400 cells/cumm. The increased frequency and severity of candidal infections in HIV infected individuals has prompted the wide use of antifungal agents such as Amphotericin B, Ketoconazole and Fluconazole, resulting in the emergence of drug resistant strains of *Candida albicans* [4].

It has been found that heavy metals particularly Silver and Mercury have a variety of applications in controlling microbial infections, e.g., *Pseudomonas aerunginosa, Staphylococcus aureus* [5]. Toxic effects of Silver ions on *Candida albicans* and Mercury ions on *Candida albicans* and other *Candida* species [6]. Resistance to Cadmium ions was also studied in *Pseudomonas aerunginosa, Staphylococcus aureus, Alcaligenes species, Bacillus species* etc., [7-10]. Oral candidiasis is considered as most common opportunistic infection among People Living with HIV/AIDS (PLHA) and there is an increase in the risk of antifungal resistance and many people are suffuring from drug-resistant fungal infections. In order to develop better methods to prevent and control drug-resistant fungal infections. Hence, a need was felt to detect the effect of heavy metal ions on *Candida* isolated from PLHA and to find relationship between CD4 T lymphocyte count and oral candidiasis in PLHA so that we can consider these heavy metals ions for therapeutic use.

MATERIALS AND METHODS

The present cross-sectional study was conducted from March 2009 to November 2009. A total number of 50 Specimens were collected from which 25 HIV seropositive patients (two specimens from each patient) who were admitted in Community Care Centre of Acharya Vinoba Bhave Rural Hospital, Wardha, India were studied. Consent of subjects and ethical committee approval was taken for this study.

The inclusion criteria for the patients were: i) Patients who have been diagnosed as HIV seropositive Integrated Counselling and Testing Centre (ICTC) among age group ≥18 year; (ii) Patients having CD4 lymphocyte count <500 cells/cu.mm; (iii) Absence of antifungal treatment within last three months. Patients with xerostomia and salivary gland disease, pregnant and nursing woman and also those who did not give consent were excluded from the study.

Data Collection

A proforma was used to collect information just before collecting the specimen. Written informed consent was taken from each subject. Confidentiality was maintained. Extraoral examination was done which includes examination of regional group of lymph nodes. Intraoral examination includes examination of lips, buccal mucosa, tongue, hard palate and soft palate for any association of lesions. Information including date of HIV antibody testing as per National AIDS Control Organisation (NACO) guidelines and CD4 count of the patients was noted with date of examination.

Microbiological Procedures

Oral swab was collected from each site of hard palate and tongue from all 25 patients studied. From each site, two swabs were collected. One swab was used for Gram's staining and another swab was used for culture on Sabouraud's dextrose agar with chloramphenicol. The inoculated culture plates were incubated overnight at 37°C. If no growth appeared on Sabouraud's dextrose agar with chloramphenicol after overnight incubation, the inoculated plates were further incubated for 24 hours at 37°C. Very smooth, cream to buff-coloured colonies of *Candida* were observed on Sabouraud Dextrose Agar plate and identified up to the species level as per conventional methods. Effect of heavy metal ions on *Candida* species was determined by using agar dilution method described by Riley TB and Mee BJ [11]. Plates containing 20 mL Sabouraud Dextrose Agar (SDA) with chloramphenicol and 3 graded final concentrations of different metal ions were prepared. The concentrations were 100 mM, 10 mM and 1 mM for Lead ions. Also, same 3 graded concentration (100 mM, 10 mM, 1 mM) prepared for Zinc, Silver, Cadmium and Mercury ions.

For Lead ion, Lead acetate, for Cadmium ion Cadmium acetate, for Zinc ion, it is Zinc pure metal and for Mercury is Mercury chloride. All the solutions of different metal ions were sterilised by membrane filtration. The SDA with chloramphenicol was sterilised by autoclaving and was cooled to around 45-50°C and then sterile solutions of different metal ions in 3 graded concentrations (100 mM, 10 mM and 1 mM) were added to each SDA plate with chloramphenicol respectively. The different Candida species isolated was inoculated as per 1x104 Colony Forming Unit (CFU)/spot on those culture plate containing different concentration of metal ions. The inoculum size was adjusted to 1×104 CFU/spot. Each plate was divided and the inoculum was applied and one strain was put in each sector. The plates were read after incubating at 37°C for two days. The presence of growth indicated that the strain is resistant to that particular concentration of metal ion and the absence of the growth indicated the susceptibility of the strains.

STATISTICAL ANALYSIS

Data were analysed by using Microsoft Excel. Data were tabulated using frequency distribution tables. Frequency of laboratory findings were expressed as proportions (%).

RESULTS

Swabs from dorsum of tongue and hard palate were taken from each of 25 HIV seropositive patients. Thus a total of 50 specimens were collected for culture [Table/Fig-1]. One swab was used for Gram staining and other swabs for culture on Sabourad's dextrose agar with Chloramphenicol. A total of 15 (30%) specimens were showing gram-positive budding yeast cells with pseudohyphae. A total of 26 (52%) specimens were positive for *Candida* species by culture [Table/Fig-2]. Out of these 26 specimens, 19 (38%) were *Candida albicans*, 2 (4%) were *Candida krusei* and 3 (6%) were *Candida glabrata* and 2 (4%) were *Candida dubliensis*. In 10 (40%) patients *Candida* species were isolated from both sites i.e., dorsum of tongue and hard palate whereas in 6 (24%) patients *Candida* species were isolated from tongue only. The relationship between

Total No. of Patients	Total No. of Specimens collected	Total No. of Specimens isolated *Candida*	Percentage of *Candida* Isolation
25	50	26	52

[Table/Fig-1]: Prevalence of *Candida* species isolated from specimens (n=50).

CD4 count (cells/mm³)	Specimens Collected from PLHA		Specimens Positive for *candida*	
	No.	Percentage	No.	Percentage
<100	8	16	8	30.8
100-199	14	28	10	38.5
200-299	12	24	5	19.2
300-399	16	32	3	11.5
Total	50	100	26	100

[Table/Fig-2]: Relationship between CD4 count and *Candida*.

CD4+ T lymphocyte and growth of *Candida* species was also studied. Eighteen specimens were positive for growth of *Candida*, collected from patients having CD count <200 cells/mm³ while only 3 specimens positive for *Candida* had CD count >300 cells/mm³ [Table/Fig-2]. It was found that all *candida* strains were resistant to Zinc ions even with 100 mM concentration compared to Cadmium ions which showed 100% sensitivity with as low as 1 mM concentration. 88% of *Candida* strains were susceptible to Mercury ions with 1 mM concentration [Table/Fig-3].

Metal ions	Percentage of *Candida* strains susceptible to different heavy metal ions in 3 graded concentrations		
	1 mM	10 mM	100 mM
Zinc	0	0	0
Lead	11	58	100
Silver	46	77	100
Mercury	88	100	100
Cadmium	100	100	100

[Table/Fig-3]: Antifungal activity of heavy metal ions against *Candida* strains (n=26).

DISCUSSION

The association between oral candidiasis and CD4 count is important because it suggests that oral candidiasis could be used as a marker of immune status when CD4+ T lymphocyte counts are not readily available [5]. Resistance to mucosal candidiasis appears largely dependent on cell-mediated immunity and CD4+ T lymphocytes [6]. A study of liu X et al., stated that asymptomatic oral Candidal colonisation is not related to CD4 lymphocyte count in individuals with HIV infections [12]. Another similar study by Torssander J et al., positive carriage rate of *C. albicans* in HIV-seropositive men was significantly higher than that in HIV-seronegative men; however, they noted that no correlation with CD4 cell counts [13]. On the contrary Fetter A et al., found that *C. albicans* colonisation of the oral cavity was significantly higher in i.v. drug users, CDC Group IV, subjects with lymphocytopenia, CD 4 cell number below 400/μL [14]. However, in the present study, all 25 patients from whom *Candida* species was isolated had CD4 count below 400/mm³. The reason may be that, all 25 patients were on anti-retroviral therapy under community care centre of the present hospital and because of that, they did not have any frank oral lesion.

Very little information is available in the literature, regarding the action of heavy metals against fungal infections. As a topical antimicrobial agents silver salt alone or in combination with other drugs have a great potential [8-10]. Also, Matsumura Y et al., found that Silver ion plays an important role for the bactericidal action by inhibiting several functions in the cell and generating reactive oxygen species through the inhibition of respiratory enzymes [15]. Vishnu Prasad S et al., reported that there is least or no relationship seen in between the antibiotic and heavy susceptibility of *Pseudomonas aerunginosa* and in vitro. And heavy metal ions like Silver and Mercury salts are found to be very effective and can be used for topical treatment instead of antibiotic creams which may induce resistance in *Pseudomonas aeruginosa* when applied in subinhibitory concentrations [16]. The most common species implicated in acute and or chronic heavy metal toxicity are Lead, arsenic and Mercury. Heavy metals bind to oxygen, nitrogen and sulfhydryl group in protein resulting in alterations of enzymatic activity. Though the exact mechanism of action of heavy metal ions on fungus is not known, it has been found that Mercury and Silver both inhibit yeast respiration. No specific target for Mercury has been defined but Brunker RL, in 1976 has reported that on exposure to Mercury ATP content of the yeast cell is rapidly depleted [17]. In the *Escherichia coli* cell, collapse of the proton motive force results after the binding of silver and phosphate [18].

Metal ions including Cadmium, Mercury, Cobalt, Nickel also inhibit plasma membrane ATPase of eukaryotic cells by means of various binding interactions [19]. Heavy metal toxicity represents an uncommon, yet clinically significant medical condition. Metal toxicity depends (whether acute or chronic) on exposure route, duration and dose. Heavy metal toxicity can result in significant morbidity and mortality. Prolonged exposure to heavy metals such as Cadmium, Copper, Lead, Nickel and Zinc if unrecognised or inappropriately treated, can cause deleterious health effects in humans, Yang HC et al., found in the decreasing order the toxicity of metal ions to yeast cells ranked as: Hg>Ag>Au>Cu, Ni, Co, Zn. Mostly the degree of toxicity correlates with human cells [20]. Dawson DC and Ballatori N, have also reported that Silver and Mercury have relatively high affinities for reduced thiol groups in cellular constituents [21]. Zhang S and Crow SA Jr, have experimentally proved the effects of Silver ion and Mercury ion on membrane potential and integrity of cells of *Candida albicans* and other *Candida* species with the flow cytometric procedure [10]. The membrane potential of cells of *Candida* species were reduced rapidly within 15 minutes of exposure to Silver ions whereas *Candida* species lost membrane potential gradually in presence of Mercury ions.

Even in the present study, the present authors found 77% and 100% *Candida* strains were susceptible to just 10 mM concentration of Silver ions and Mercury ions respectively. A 100% *Candida* strains were also susceptible to 1 mM concentration of Cadmium ions.

LIMITATION

Heavy metals are natural components of the Earth's crust. They cannot be degraded or destroyed. To a small extent, they enter our bodies via food, drinking water and air. However, at higher concentrations, they can Lead to poisoning. A Large number of samples not included in the present study.

CONCLUSION

In the present study, authors found that *candida* strains were susceptible to Silver, Mercury, and Cadmium ions. Though heavy metal salts can be used for therapeutic use at very high dilutions, heavy metals toxicity can result in long-term exposure. Heavy metal contacts with the skin also cause toxicity to different organ and damage to central nervous system, erythematous area over the skin, hyperpigmentation and argyria. Prolonged exposure to heavy metals can cause deleterious effects in humans. Molecular understanding of the long-term effects of plant metal accumulation might not be yet known. Further study with animal experiment and human volunteers is required.

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