Identification of oral micro biota of poor oral hygiene and evaluation of their drug's susceptibility

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ABSTRACT

Introduction: Poor oral hygiene is one of the major causes oforal infections and its related pathologies in the general population. Notwithstanding seemingly appropriate treatment, extreme orofacial infections can advance and lead to life-threatening complications. Another crucial concern is about the drug's sensitivity and resistance to oral microflora.This study was accomplished to evaluate the drug's sensitivity of microbial isolates from the oral cavity in hospitals of Odisha.

Methods: In this study total of 100 oral samples were collected from poor oral hygiene patients. Biochemical test characterization such as colony morphology, colour, texture, indole, methyl red, citrate etc. was determined to identify bacteria followed by antibiotic susceptibility test. Sabouraud Dextrose Agar (SDA) culture and Candida Differential Agar (CDA) test were also performed to identify candida species followed by antifungal susceptibility test was performed.

Results: Total of 90% (90) oral pathogens were recovered from total 100 patient's samples. Out of that 19 (21.11%) GPB(gram positive bacteria), 60(66.66%) GNB (gram negative bacteria), and 11(12.22%) budding yeast cells were isolated from the 90 patient's samples. S. epidermis was most common in GPC and showed maximum resistant 31.57% to oxacillin. Pseudomonas aeruginosa was the most common in GNB and showed maximum resistant (50.94%) to clarithromycin. In poor oral hygiene patients from 12.22% of candida yeast, we found that *Candida tropicalis* maximum in comparison to other species of candida. An antifungal sensitivity test showed that the maximum resistance was 38.88% with Fluconazole (FLC^{10}) then with Ketoconazole (KT50), 27.77% for *Candida tropicalis*. Minimum resistance (2.8%) was observed with Amphotericin B antifungal.

Conclusion: Study revealed that poor oral hygiene patients shown presence of maximum number of gram-negative bacteria in comparison to gram positive bacteria and candidal species. These microbes showed varies antibiotic and antifungal susceptibility. So, drug susceptibility should be done for effective treatment of microbes.

KEYWORDS: Oral microbiome, Drug sensitivity, Antifungals, Antibiotics.

INTRODUCTION

Many aerobic and anaerobic bacteria along with commensal fungi like candida reside in the oral cavity. Various conditions and environment of the Oro-pharyngeal area led to the existence of many microorganisms mainly anaerobic bacteria. Poor oral hygiene is one of the major causes of periodontal, gingival infection, dental caries, abscess, space infection, pericoronitis, candidiasis, etc. Infections need the use of antimicrobials (antibiotics and antifungal) as per requirement. The use of antimicrobials is depending on various factors like the type of infection, patient age, health allergic history, drug's plasma level, absorption ability, etc. All infectious foci together can disseminate these microorganisms if not treated with time with proper use of conventional antimicrobial agents. Poor oral hygiene condition also one of the causes of growth of non-oral bacteria in the oral cavity. Untreated disseminated infection can cause life threatening condition. [1-5]

There is an increase in resistance to antimicrobials agents may be due to production of biofilm and virulence factors as well as due to empirical use of it before any dental procedure.Another fact is long term use of antibiotics also can lead to growth of opportunistic oral fungal infection mainly, candidiasis and untreated cases can lead to candidemia, which is one of the causes of mortality. The percentage of resistance to antibiotics and antifungal is increasing more in recent years.[6,7] So this study was aimed to evaluate the drug's (antibiotics and antifungal) sensitivity of microbial isolates from the oral cavity of poor hygiene patients.

MATERIALS AND METHODS

With ethical permission (Ref. No. DMR/IMS.SH/SOA/180254) the present study was conducted in research laboratory of the Institute of Medical Science, Siksha O Anusandhan Deemed to be university, Bhubaneswar, Odisha, India. Samples were collected from; the patients who were having poor oral hygiene habit with and without oral candidiasis (Fig 1). Collected samples were transported from OPD to the lab safely and it was processed immediately by cultured on SDA, Blood agar, and MAC agar. The plates were incubated at 37^0C for 24 h.

Identification of bacteria and antibiotic susceptibility test-

For bacteria identification preliminary screening such as colony morphology, colour and gram staining were done. The bacterial colonies were identified as gram positive and gram-negative bacteria by gram staining. For gram positive bacteria isolates were subjected to catalase and coagulase testing. Gram negative bacteria further tested for lactose fermenting (LF) and non-lactose fermenting (NLF) bacteria. Further oxidase, methyl red (MR), Indole, citrate test, urase test and triple sugar iron (TSI) test performed for gram negative bacteria.[8]

All the strains of Gram-positive and negative bacteria were subjected to antibiotic sensitivity test by disc diffusion method. The Mueller-Hinton agar (MHA, HI media) plates were prepared and by using sterile spreader liquid bacterial culture was spreaded on plate and antimicrobial impregnated disk were placed over it then incubated for 24 hours. Different types of conventional antibiotic discs of different drugs groups like beta lactum, quinolones, aminocoumarin, macro ides, tetracycline, vancomycin, amino glycosides drugs etc were placed on the surface of MHA agar plate(Fig 2).[9]

Identification of Candida species and antifungal susceptibility test-

The collected swab samples were first inoculated on blood agar plates, MacConkey agar plate and SDA plates. Theses plates were incubated for 37^0 C for 24 h then growth of colonies was observed then these growths were further cultured on plates of nutrient agar to get a pure culture. For further biochemical testing single colony from pure culture was used.When we found culture of fungi candida on SDA then we did its species identification on CDA agar 37°C for 18-24 hours, by different colour pigmentation of candida species. (Table 3).

Similarly antifungal agents such as Fluconazole (FLC^{10}), Nystatin (NS^{100}), Amphotericin B(AP^{100}), Itraconazole (IT^{30}), Ketoconazole (KT^{50}), Miconazole (MIC^{50}), and Clotrimazole (CC^{10}) discs were placed on the surface of SDA plate. Then the plates were incubated at 37°C for 48hrs. The zones of inhibition were observed around the disc and the zones were measured by standard scale (Himedia) Fig 3. Basing upon the instructed size of zone of inhibition the result sensitive or resistant were documented.[10,11,12]

RESULTS

In present study 100 patients were enrolled for collecting oral samples in the year of 2021-22. Among 100 samples, 10 samples were excluded from study (3 samples due to contamination and 7 samples showed no growth. The age group of the patient was between 1-80. The maximum number of patients ranged between 40-60 age groups. We found, 60(66.66%) Gram-negative, 19(21.11%) were Gram-positive and 11(12.22%) candida species (Table 1).

Table 2 shows: Out of the total 90 patient, 60 (66.66%) were male and 30 (33.33%) were female. Four-gram negative bacteria were observed; as Pseudomonas aeruginosa (oxidase +ve) where as other four were *(E. coli, A. baumannii* and *K. pneumoniae*) oxidase negative. Three-gram positive bacteria were observed includes, *S. epidermidis* (catalase +ve, coagulase -ve), Staphylococcus aureus (catalase and coagulase positive), Streptococcus species (catalase -ve). Among Gram-positive bacteria maximum,11.11% (10) was S. epidermis (catalase-positive, coagulase-negative).

Among all the isolates of Gram-negative strain 50(55.55%) Pseudomonas aeruginosa,1(1.11%) E. coli, 3(3.33%) *A. baumannii* and 6(6.66%) *K. pneumoniae*. Whereas gram positive strain were 10(11.11%) *S. epidermidis*, 7(7.77%) *S. pyogen*,2(2.22%) *S. aureus*. Among candida species 8(8.88%) Candida tropicalis whereas 1(1.11%) of C. albicans, *C. glabrata*, *Candida krusei* each(Table 2).

Among Gram-negative isolates maximum Pseudomonas aeruginosa was isolated, it was 43.39% resistant to Amoxiclav (AMC), 35.84% resistant to Amoxicillin (AMX),49.05% were resistant to Oxacillin (OX)and 3.77% resistance to Ticarcillin (TI) that all comes under Beta-lactam group of antibiotics. In carbapenem group 1.88% resistant to cilastin (IC) by Pseudomonas aeruginosa and E. coli each and 3.77% resistant to *A. baumannii* and *K. pneumonia* eeach. From Quinolones group Levofloxacin (LE)resistance was 3.77% for Pseudomonas aeruginosa, and *A. baumannii* ,7.54% resistance to *E. coli* and Ciprofloxacin (CIP) resistance shows 11.32% by Pseudomonas auregenosa,1.88% in E. coli and 3.77% in *A. baumannii* and *K. pneumoniae*. In another case, the resistance percentage of Amikacin (AK) and Colistin (CL) was 9.43% for Pseudomonas aeruginosa. 3.77% was for *A. baumannii* and *K. pneumoniae*. Tobramycin (TOB) resistance was 1.88% in case of *P. aeruginosa*, *E. coli*, and *A. baumannii* and *K. pneumoniae*. Tetracycline

resistance was more in P. aeruginosa which is 15.09% and 1.88%resistance to *E. coli*, and *A. baumannii* (Table 4).

On the other hand, *S. pyogen* Gram-positive isolates showed 26.31% resistance to Amoxiclav (AMC), Ampicillin (AMP), Oxacillin (OX), Penicillin(P), Levofloxacin (LE), Nalidixic acid (NA), Novobiocin (NV), Tetracycline (TE), Vancomycin (VA). In the case of *S. aureus*, maximum resistance was 10.52% found in Erythromycin(E), Novobiocin (NA) Vancomycin (VA), Penicillin (P) and 5.26% resistance to Amoxiclav (AMC), Ampicillin (AMP), Oxacillin (OX), Ticarcillin (TI), Nalidixic acid (NA), Levofloxacin (LE), Azithromycin (AZM), Tetracycline (TE). For *S. epidermis* resistance was superlative in Oxacillin (OX) is 31.57%, Penicillin(P) resistance was 21.05% and for Erythromycin(E), Ampicillin(AMP), Vancomycin (VA) resistance was 15.78%whereas 10.52% resistance to Amoxiclav (AMC), Ofloxacin (OF),Nalidixic acid (NA), Chloramphenicol(C) (Table 5).

Similarly, an Antifungal sensitivity test was done (Fig 1) for fungus. Maximum resistance was seen 38.88% with Fluconazole (FLC^{10}), 27.77% with Ketoconazole (KT50) by *C. tropicalis*. 11.11% resistant to Miconazole (MIC^{50}) shown by *C. tropicalis*, *C. albicans* and by *C. krusei*. 11.11% Fluconazole (FLC^{10}) resistance was observed in *C. albicans* and in *C. Krusei*. 5.55% Itraconazole (IT^{30}) resistance was observed by *C. glabrata*, *C. krusei*. *C. tropicalis* showed least resistance to amphotericin B whereas *C. glabrata* showed least resistance to clotrimazole (3.8%). 5.55% Clotrimazole (CC10) resistance was shown by *C. albicans*, *C. tropicalis*. Overall different candida species showed least resistance to amphotericin B, Itraconazole and to clotrimazole. (Table 6).

DISCUSSION

Spread of oral microbiota can lead to life-threatening complications that include pneumonia, mediastinitis, thoracic empyema, intra-orbital infection, pericarditis, septic shock, and intracranial spread. Advancement can result from multiple factors, that including host immunologic status, virulence of organisms, resistance patterns of involved organisms, and management decisions[14-23]

Most bacterial isolates was Gram-negative *P. auregenosa*. [23]SouzaLC et al reported this gram negative bacteria in patients with chronic kidney disease and suggested that if presence of *P.*

aeruginosa detected in oral cavity so attention should be pay to evaluate presence of any systemic diseases. Similarly in Gram-positive bacterial infection in the oral biome, we got a maximum of *S. epidermidis* 10(11.11%), which showed 6(6.66%) in males and 4(4.44%) in females. we also found got *S. species* 7(7.77%) including 3(3.33%) in males and 4(4.44%) in females, followed by *S.aureus* 2(2.22%) only seen in female. This bacteria finding is concordance with studies done by Fritschi BZ et, Jackson MS et al and Persson GR et al[14-16.] The present study revealed that only 11 patients, showed presence of candidal species, were of old age group. 8(8.88%) of the strain were *C. tropicalis* (male6.66%, female 2.22%), *C. glabrata C. albicans,* and *C. krusei* were 1.11% of each. This is in concordance with the study of Darwezeh et al., 2003, who isolated Candida species from the oral cavity of denture wearer patients [24].

In our study *C. tropicalis* showed maximum38.88% resistance against fluconazole followed by 27.77% against ketoconazole, next to it 11.11% and 5.55% resistance showed against miconazole and clotrimazole respectively. Berkow and Lockhart, 2017 also elaborated fluconazole resistance in candida species. [25 In our study, *C. glabrata* gave maximum resistance data against ketoconazole i.e., 22.22%, and also it showed 5.55% resistance against both fluconazole and itraconazole. Similarly, in the case of Candida krusei, we found maximum resistance is 11.11% against two antifungals i.e., fluconazole and miconazole. Other than that, 5.55% resistance was found against another two antifungals ketoconazole and itraconazole. Sanglard D also documented emerging threats of antifungal drug resistance.[26]

For antibacterial screening, we used different groups of antibiotics for gram-positive bacteria. We found *S. epidermidis* showed maximum resistance (31.57%) to the β Lactam group of antibiotics specific to OX(Oxacillin). . Similarly, in the quinolones group against LE (Levofloxacin). , in macrolides groups against E(Erythromycin) and against vancomycin drug resistance of *S.epidermidis* was found 15.78%. We don't find any resistance strain of *S. epidermidis* against TE(Tetracycline). Another Gram-positive bacteria was Streptococcus pyogen which showed maximum resistance 26.31% against most of the antibiotics of different groups such as Amoxiclav (AMC), Ampicillin (AMP), Oxacillin (OX), Penicillin(P), Nalidixic acid (NA), Levofloxacin (LE), Novobiocin (NV), Tetracycline (TE), Vancomycin (VA) and Cefuroxime(C). Similarly, S. aureus also showed resistance against almost all antibiotics explained above except Ofloxacin (OF) Ciprofloxacin (CIP), Gentamycin (GEN), and Amikacin

(AK). The highest resistance was found against the antibiotics Penicillin 10 (P10), Novobiocin (NV), Erythromycin(E), and Vancomycin 30 (VA30). This is in concordance with the study explained by Smith SI et al.[27-32]

In this study, Gram-negative bacteria antibiotic screening was also performed using different antibiotics of various groups. Pseudomonas aeruginosa showed maximum resistance of 50.94% to Clarithromycin (CLR), followed by 49.05% resistance to Oxacillin (OX) and 43.39% to Amoxiclav (AMC). The lowest resistance was found against the Tobramycin and Imipenem (IC) (1.88%). *E.coli* showed the highest resistance of 9.43% to Clarithromycin. Acinetobacter baumanniishowed maximum resistance of 3.77% to antibiotics like Amoxyclav (AMC), Amoxicillin (AMX), Oxacillin (OX), Azithromycin (AZM), Clarithromycin (CLR), Imipenem (IC), Levofloxacin (LE), Ciprofloxacin (CIP) and Amikacin (AK). Klebsiella pneumoniaealso showed 5.66% resistance towards Amoxiclav (AMC), Amoxicillin and for clarithromycin in present study. These results are concordance with study done byKarakonstantis S et al.[33-38]We have found many gram negative (*P. aeruginosa*, E. coli, *A. baumannii*, *K. pneumoniae*) and positive bacteria (*S. epidermidis*, *S. pyogen* and *S. aureus*) in poor oral hygienepatents along with few candida species. We have also found antifungal drug resistance pattern of different candida species.[39,40] So antifungal and antibacterial stewardship is must to deal with resistant case.

As per antimicrobial Resistance Collaborators, the six leading pathogens(Escherichia coli, followed by Staphylococcus aureus, Klebsiella pneumoniae, S. pneumoniae, *A. baumannii*, and P. aeruginosa) were responsible for 929 000 (660 000-1 270 000) deaths attributable to antimicrobial resistance and 3·57 million (2·62-4·78) deaths associated with AMR in 2019.[41]

Gram-negative bacillus bacteria, K. pneumoniae (facultative anaerobic) is dominant in cases with removable maxillary prosthesis.[33]Presence of K. pneumoniae in oral cavity and risk of pneumonia by aspiration of these bacteria in people suffering from stroke.[42]Nakou et al identified P. aeruginosa in immunocompromised subjects. It can be important pathogen in gingivitis and periodontitis.[34,43]Some nonoral bacterial species are, Acinetobacter baumannii and Pseudomonas aeruginosa, both are obligate aerobic rod-shaped bacteria. They are seen with aggressive form of periodontitis.[35,36]Escherichia coli (facultative gram-negative bacteria) under nutritional friendly environment one of the dominated bacteria in oral polymicrobial biofilm.[4]

Ohara-Nemoto Y et al found gram positive S. epidermidis in saliva sample and dental plaque, they suggested it could be one of the causes of infective endocarditis. There has been seen association of another gram positive bacteria, S.Pyogen with oropharyngeal mild infection[31,32].Whereas Persson and Renvert found that Staphylococcus aureus in biofilm of patients with peri-implantitis. It's higher percentage also seen in aggressive periodontitis and in oral cavity of patients with rheumatoid arthritis.[16]

Non oral bacteria isolated from oral cavity, could be one of the causes of oral and systemic diseases. Oral microorganism has important impact for systemic health of human.[43-49]Poor oral hygiene cause orofacial infection which may cause from untreated odontogenic infection either of tooth or periodontitis. A person with weakened immunity is more likely to get a fungal infection of mouth especially if they have poor oral hygiene. So, maintenance of good oral hygiene is very important to keep away colonization of infectious microbiome.

CONCLUSION

Poor oral hygiene patient shown presence of maximum number of gram negativebacteria in comparison to gram positive bacteria and candidal species. These microorganisms showed varied antibiotic and antifungal drugs-resistant.Empirical use of antibiotic/antifungal may increase chance of resistant cases. So before start of therapy, culture sensitivity test should be carried out. In future it should be carried out with larger size samples to evaluate present of different fungal and bacterial species in head and neck infection with its antifungal and antibacterial drug's susceptibility.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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Table legends-

Table 1: Description of study population as per age and gender-wise.

Table 2: Total identified GN, GP and fungi, of the study as per gender wise.

Table 3: Colour identification of Candida species.

Table 4: Resistance percentage of gram-negative bacteria.

Table 5: Resistance percentage of Gram-positive bacteria.

Table 6: Description of Antifungal screening test.

Figure legend-

Fig 1: Showing oral Image of poor oral hygiene patients with teeth stain, gingival swelling and tongue coating.

Fig 2: Antibiotic and Fig 3: Antifungal susceptibility test by disc diffusion method.

Table 1: Description of study population as per age wise.

SN	Organism	Number	percentage	0-20yrs	20-40yrs	60-40yrs	60-80yrs
1	Gram -ve	60	66.66%	8	13	21	18
2	Gram +ve	19	21.11%	1	5	7	6
3	Fungi	11	12.22%	0	1	2	8
4	Total	90	100%	9	19	30	32

Table. 2: Total identified GN, GP and fungi, of the study as per gender wise.

Microorganism	**Total no. of strain**	**Male**	**Female**
Gram-negative Organism name			
Pseudomonas aeruginosa	50(55.55%)	37(41.11%)	13(14.44%)
Escherichia coli	1(1.11%)	1(1.11%)	0
Acinetobacter baumannii	3(3.33%)	1(1.11%)	2(2.22%)
Klebsiella pneumoniae	6(5.55%)	4(4.44%)	2(1.11%)
Gram-positive Organism name			
Staphylococcus. epidermidis	10(11.11%)	6(6.66%)	4(4.44%)
Streptococcus pyogen	7(7.77%)	3(3.33%)	4(4.44%)
Staphylococcus aureus	2(2.22%)	0	2(2.22%)
Fungi (Candida species found)			
Candida species name	**Total no. of strain**	**Male**	**Female**
Candida tropicalis	8(8.88%)	6(6.66%)	2(2.22%)
Candida albicans	1(1.11%)	1(1.11%)	0
Candida glabrata	1(1.11%)	1(1.11%)	0
Candida krusei	1(1.11%)	0	1(1.11%)
Total	90 (100%)	60(66.66%)	30 (33.33%)

Table 3: Colour identification of Candida species on Candida differential agar plate.

S.N.	Organisms	Colour
1.	Candida tropicalis	Blue to purple
2.	Candida albicans	Light green
3.	Candida glabrata	Creamy to pinkish-white
4.	Candida krusei	Purple

Table 4: Resistance percentage of gram -negative bacteria.

Organism	**β. Lactam**				**Macrolides**		**Carba penems**	**Tigecycline**	**Quinolones**		**Aminoglycoside**			**Polymyxin**	**Tetracycline**
	AMC	AMX	TI	OX	AZM	CLR	IC	TGC	LE	CLIP	AK	GEN	TOB	CL	TE
Pseudomonas aeruginosa	43.39%	35.84%	3.77%	49.05%	18.86%	50.94%	1.88%	3.77%	3.77%	11.32%	9.43%	9.43%	1.88%	9.43%	15.09%
Escherichia coli	0	5.66%	3.77%	0	3.77%	9.43%	1.88%	0	7.54%	1.88%	1.88%	0	1.88%	0	1.88%
Acinetobacter baumannii	3.77%	3.77%	1.88%	3.77%	3.77%	3.77%	3.77%	3.77%	3.77%	3.77%	3.77%	1.88%	1.88%	3.77%	1.88%
Klebsiella pneumoniae	5.66%	5.66%	0	3.77%	0	5.66%	3.77%	3.77%	0	0	3.77%	3.77%	3.77%	3.77%	0

AMC- Amoxiclav, AMX- Amoxicillin , TI- Ticarcillin , OX- Oxacillin ,AZM- Azitrhomycin , CLR- Clarithromycin ,IC- Cilastin , TGC- Tigecycline ,LE- Levofloxacillin ,CIP- Ciprofloxacin , AK- Amikacin ,GEN- Gentamycin , CL- Colistin ,TOB- Tobramycin ,TE- Tetracyclin

Table 5: Resistance percentage of Gram-positive bacteria

Organism	**β. Lactam**					**Quinolones**				**Amino couma rin**	**Macrolides**		**Tetracy cline**	**Vancomyci n**	**Aminoglycosid es**	
	AMC30	**AMP**	**OX1**	**P10**	**TI**	**OF**	**NA**	**CIP**	**LE**	**NV5**	**AZM1**	**E**	**TE**	**VA30**	**GEN10**	**C30**
Staphylococcus. epidermidis	10.52%	15.78%	31.57%	21.05%	5.26%	10.52%	10.52%	0	15.78%	0	0	15.78%	0	15.78%	0	10.52%
Streptococcus pyogen	26.31%	26.31%	26.31%	26.31%	5.26%	16.5%	26.31%	0	26.31%	26.31%	0	21.05%	26.31%	26.31%	0	26.31%

Staphylococcus aureus	5.26%	5.26%	5.26%	10.52%	5.26%	0	5.26%	0	5.26%	10.52%	5.26%	10.52%	5.26%	10.52%	0	0

AMC- Amoxiclav, AMP-Ampicillin, TI- Ticarcillin, OX- Oxacillin, P- Penicillin, TI- Ticarcillin, OF- Oflaxacin, NA- Nalidixic, CIP- Ciprofloxacin, LE- Levofloxacillin , NV- Novobiocin, AZM- Azithromycin , E- Erthromycin, TE- Tetracyclin, VA-Vancomycin, GEN-Gentamycin, AK-Amikacin.

Table 6: Description of Antifungal screening test.

Organisms	**AP^{100}**	**IT^{30}**	**KT^{50}**	**CC^{10}**	**NS^{100}**	**FLC^{10}**	**MIC^{50}**
	%	%	%	%	%	%	%
Candida tropicalis	2.8	4.2	27.77	5.55	22.8	38.88	11.11
Candida albicans	5.55	4.8	11.11	5.55	22.6	11.11	11.11
Candida glabrata	4.8	5.55	22.22	3.8	18.	5.55	8.9
Candida krusei	2.8	5.55	5.55	4.2	10.2	11.11	11.11

Fig 1: Showing oral Image of poor oral hygiene patients with teeth stain, gingival swelling and tongue coating.

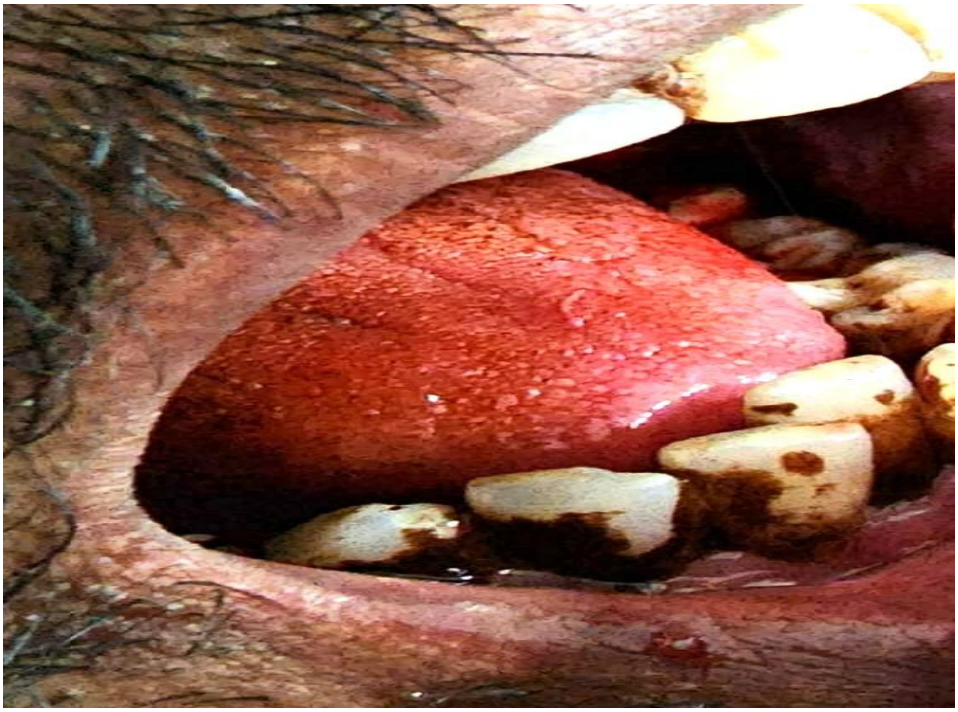

Fig 2: Antibiotic and Fig 3: Antifungal susceptibility test by disc diffusion method.