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RESEARCH ARTICLE

COMPARATIVE EVALUATION OF ORAL MICROFLORA OF PATIENTS SUFFERING FROM ORAL SQUAMOUS CELL CARCINOMA IN THEIR PRE AND POST RADIOTHERAPEUTIC STATUS

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ABSTRACT

Background and Objective: The objective of the study was to identify the predominant oral microbes associated with oral squamous cell carcinoma in their pre and post radiotherapeutic status.
Materials and Methods: A total no of 40 patients were selected, having squamous cell carcinoma and not receiving any treatment. Swabs are collected from the ulcerated area and microbiological evaluation was performed. Swabs were again collected from the same patient receiving radiotherapy as treatment modality just one month ago. The microbes were identified by gram stain and biochemical test.
Results: The results suggested both aerobic and anaerobic bacteria were colonized over the surface of the ulcer, but the distinguishing feature between these two groups that after radiotherapy the % of bacterial count (especially the anaerobes) markedly diminished.
Conclusion: It was concluded from the results that, the diseased individuals suffering from Oral Squamous Cell Carcinoma in their pre-radiotherapeutic status showed the presence of *Streptococcus*, Staphylococcus, Pseudomonas, Proteus, Klebsiella and Bacillus subtilis, Actinomycetes, Fusobacterium in higher concentration, whereas in post radiotherapy phase more or less the same group of microbes were found, but in a significantly reduced amount. This was because of the fact- the removal of the tumour mass as well as effect of radiotherapy.

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INTRODUCTION

World Health Organization has designated CANCER as a complex disease in genes that encodes protein, that controls cell cycle, cell motility, cell survival and angiogenesis. (World Health Organization, 2006) Oral cancer is the sixth most common cancer worldwide. (Shah and Gil, 2009) In India the incidence of oral cancer is highest and more than 90% of all oral cancers are Oral Squamous Cell Carcinoma. (Attar *et al*., 2010; Bagan, G. Sarrion *et al*., 2010) The etiology of oral squamous cell carcinoma is multifactorial. The external factors like tobacco chewing, smoking, regular consumption of alcoholic beverages while the intrinsic factors include systemic or generalized states, such as generalised malnutrition or iron deficiency anaemia are responsible for the development squamous cell carcinoma. Ultraviolet light is a significant cause for squamous cell carcinoma of the skin and lip while microorganisms also play an important role in this disease process. The mouth harbours at least six billion bacteria representing more than 700 species as well as other types of microorganisms. The unique relationship of oral microflora and the host can break down in the mouth due to major changes to the biology of mouth from exogenous sources (antibiotic treatment or the frequent intake of fermentable carbohydrate in the diet) or from endogenous changes such as alterations in the integrity of the host defences following drug therapy which perturb the natural stability of microflora. Thus they become "Opportunistic Pathogens" and many microorganisms behave in this manner. (Parehitiyawa *et al*., 2010) Microflora found in Oral Squamous Cell Carcinoma predominantly are haemolytic *Streptococci*, *Neisseria* species, *Proteus*, *Klebsiella Pneumoniae*, *Staphylococcus Aureus*, *Haemophilus Influenzae*, *Pseudomonas* and *Candida Albicans*. (Muthu *et al*., 2004) From the different studies it has been demonstrated that the occasional presence of human papilloma virus (HPV) subtypes 16, 18, 33 in oral squamous cell carcinomas, suggesting a possible role for this virus in oral cancers. With the continuing advances in the techniques and modalities of radiation therapy a large percentage of head and neck cancers are treated by radiation alone or by a combination of radiation and surgery. But inspite of all these treatment modalities a substantial number of cases do recur and five years survival of advance stages of Oral Squamous Cell carcinoma is only 19%. Among all cancers, head and neck cancer is associated with a severe

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compromise of immunity and irradiation further depresses the immunologic systems, leading to infections, so much so that post operative sepsis is the leading cause of death in head and neck cancer patients, particularly those previously irradiated. (Panduranga kamath *et al*., 2002) As India is a cancer prone country and more than 90% of all oral cancers are Squamous Cell Carcinoma, it is very important to evaluate the status of microflora in pre and post irradiated patients with this disease process for proper management of the complications arising due to radiotherapy and improvement of patient's quality of life. But there is a paucity of work in this field specially in the eastern part of India. Keeping these considerations in mind, the present study has been designed to know the status of oral microflora in normal healthy individuals, to assess the oral microflora of patients suffering from oral squamous cell carcinoma in their pre radiotherapeutic and post radiotherapeutic phase. Finally to compare and corroborate the above microflora with a view to assess the possibility and nature of the microbial diseases, that may affect these individuals.

MATERIALS AND METHODS

A total number of 1000 patients attending the outpatient department of Guru Nanak Institute of Dental Sciences & Research were screened thoroughly for the presence of oral squamous cell carcinoma as per the clinical criteria laid down by Neville *et al*. In the process of this clinical screening a total number of 50 patients associated with oral squamous cell carcinoma were detected. All these patients were subjected to thorough medical check-up along with haematological and radiological evaluation. Among them 10 patients did not return with investigation reports. So, ultimately 40 patients were included for the invasive procedure to confirm the presence of oral squamous cell carcinoma. After taking written consent, the incisional biopsy was performed under local anaesthesia. The specimens were fixed, processed and stained with Haematoxylin and Eosin. The histological evaluation of the stained section revealed the diagnosis of squamous cell carcinoma. These 40 patients were included in the pre radiotherapeutic status group. After surgery the radiotherapy was given to these 40 patients in the Radiotherapy unit of Medical College and Hospitals Kolkata. These 40 patients were again included in the post radiotherapeutic status group.
This explorative study was conducted in the department of Oral and Maxillofacial pathology of Guru Nanak Institute of Dental Sciences and Research–Panihati, Kolkata in collaboration with Microbiology department of School Of Tropical Medicine, Kolkata and Radiotherapy department of Medical College & Hospitals, Kolkata during the period of June 2015 to July 2016.

Selection of sample

Inclusion criteria

- Patient selection was based on the clinical diagnosis of oral squamous cell carcinoma in their pre and post radiotherapeutic status.
- The patients who had not received any kind of treatment after diagnosis of squamous cell carcinoma were included in the pre radiotherapeutic status group.
- The patients who had received radiotherapy just before one month and did not use any kind of antiseptic mouth wash were included in the post radiotherapeutic status group.

Exclusion criteria

- Patients who had received radiotherapy were excluded from the preradiotherapeutic group of patients.
- Patients who had used any antiseptic mouth wash after radiotherapy were excluded.
- The patients suffering from Hepatitis-b or HIV infection were excluded from our study.
- Mentaly retarded patients were also avoided.

For the purpose of comparative evaluation 10 clinically normal healthy volunteers without any oral habits and diseases were also included in this study. The details of subjective and objective features of all the study subjects were recorded and written consents were received from all the selected patients in the specially prepared consent form.

Methods for bacteriological study

Before brushing of teeth sterile cotton swab was used to collect the specimen from the affected area of the patients mouth.The specimens were collected aseptically from the affected site or the ulcerated site of the squamous cell carcinoma, intraorally by the sterile swab stick. Contamination was carefully avoided by use of cotton rolls and saliva suction. Then, it was placed in a sterile containers under all aseptic conditions and proceed immediately. The swabs were directly inoculated onto i) Blood agar, ii) Nutrient agar, iii) MacConkey's agar for aerobic culture and fresh blood agar for anaerobic culture. After isolation of bacteria in pure culture from a specimen, it has to be identified phenotypically. The following studies are necessary to identify the bacteria -a) morphology of bacterial colony, b) staining (Gram's staning), c) motility test (hanging drop preparation), d) Biochemical test (oxidase test, triple sugar iodine test, urease test, citrate test, indole test.) After performing all the above mentioned tests, the most predominant bacteria has been identified in each patient both from pre and post radiotherapeutic groups. The resuts obtained are presented in tabular form showing the percentage of predominant group of bacteria in pre and post radiotherapeutic groups.

RESULTS

50 patients were selected for this study purpose.Among them 10 were considered as Control (not suffering from any dental infection, and their age group is under 30) and 40 patients were taken as test category (they were clinically screened, diagnosed by histology for the presence of oral squamous cell carcinoma). These 40 patients after receiving radiotherapy as treatment protocol were again taken as post radiotherapeutic group.

So, Total patients – 50
Control group - 10
Patient in pre-radiotherapeutic status – 40

Patient in post-radiotherapeutic status – same 40 patients after receiving radiotherapy. At first, from the control groups swabs were collected from the different sites of oral cavity like gingival sulcus, buccal vestibule, lingual vestibule, dorsum of

the tongue. These collected swabs then were immediately inoculated in the Blood agar, nutrient agar, Mackonkey's agar plate respectively. These plates were kept in the incubator at optimum temperature at 37 degree centigrade for 24 hours, bacterial colony was obtained from each culture plates, they were identified by gram staining, and biochemical reactions (oxidase test, citrate test, indole test, TSI, urease test). The most commonly isolated microorganisms were the normal oropharyngeal commensals i.e. *Streptococcus* and *Staphylococcus*, *Enterococcus*, *Neisseria*, *Vellionella*. Then, the swabs were collected from the patients, suffering from oral squamous cell carcinoma. The patients those who had not received antibiotics, chemotherapy, cytotoxic drugs or radiotherapy were selected. Samples were collected from affected mucosa and tongue mainly. The samples were inoculated into the culture plates (Blood, Nutrient, Mackonkey's agar), incubated at 37 degree centigrade for 24 hours. Bacterial colony obtained from the individual's plate and the bacteria were identified by gram staining and biochemical reactions. The most commonly isolated organisms were Aerobic *Streptococcus* (present in 55% cases), (Fig.1A) *Staphylococcus* (present in 55% cases), *Pseudomonas* (present in 30% cases), (Fig.1C) Klebsiella (present in 35% cases), Proteus (present in 40%cases), (Fig 1B) Bacillus subtilis (present in 45% cases). (Table-1, Histogram 1,2) Samples were then collected from the same patient for anaerobic culture.

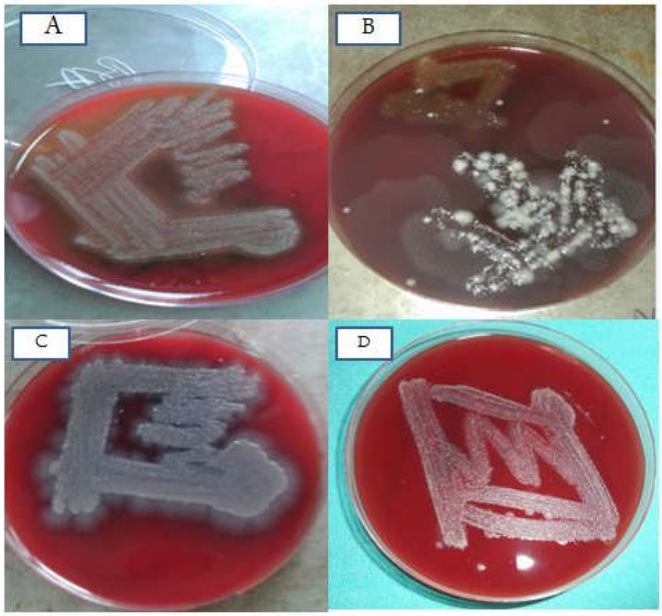
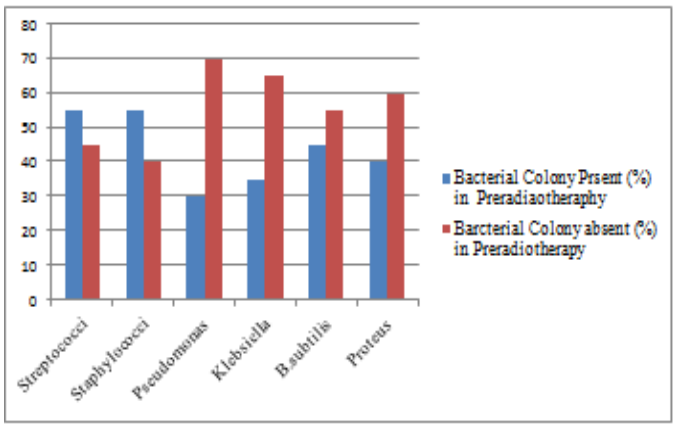


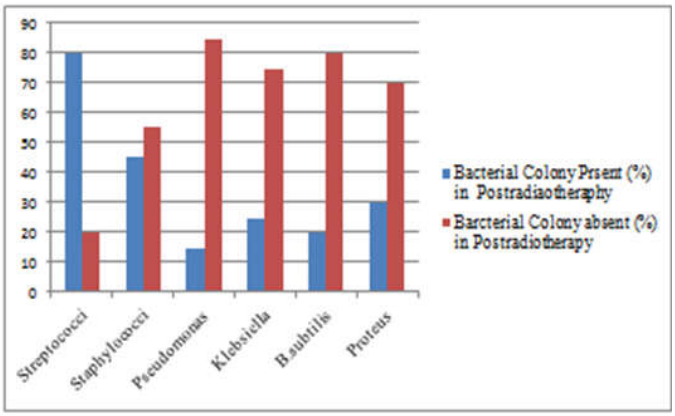
Figure 1. Culture plate containing blood agar showing [a] growth of *Streptococcus*, [b] swarming colony of Proteus, [c] growth of *Pseudomonas*, [d] growth of *Anaerobic bacteria*

Table 1. Distribution of different types of bacteria in study subjects in their pre and post radiotheraputic status

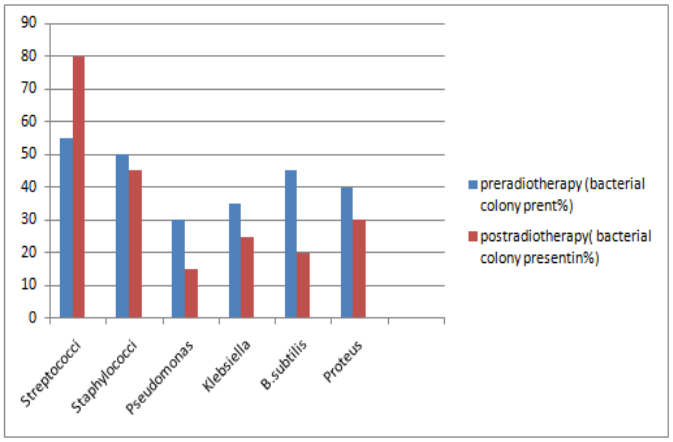
	Pre radiotherapy study subject (n=20)		Post radiotherapy study subject (n=20)	
	Present	Absent	Present	Absent
Streptococci	55%	45%	80%	20%
Staphylococci	55%	45%	45%	55%
Pseudomonas	30%	70%	15%	85%
Klebsiella	35%	65%	25%	75%
B.subtilus	45%	55%	20%	80%
Proteus	40%	60%	30%	70%
Actinomycetes	80%	20%	45%	55%
Fusobacterium	70%	30%	35%	65%



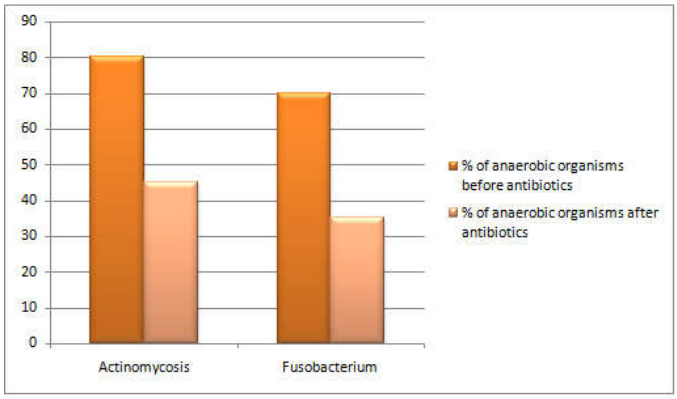
Histogram (1) Bacterial growth in pre radiotherapeutic status



Histogram (2) Bacterial growth in post radiotherapeutic status



Histogram (3) Distribution of different types of bacteria in study subjects in their pre & post radiotheraputic status



Histogram (4) Comparison of anaerobic bacterial % before & after radiotherapy

Samples were directly inoculated into fresh blood agar and immediately kept in anaerobic gas jar using gas-pak method. Bacterial colony was obtained after 3days, here the most commonly isolated organisms were Anaerobic (facaltative or obligate anaerobes) i.e. Actinomyces (present in 80%cases) (Fig.1D) and *Fusobacterium* (present in 70% cases). (Table-1, Histogram 4) Other samples were collected from the patients suffering from oral squamous cell carcinoma who had completed their radiotherapy course just one month ago. The taken swabs were inoculated in the same manner as previously and the bacterial colony that were found mainly *Streptococcus* (present in 80% cases), *Staphylococcus* (present in 45%cases), Bacillus subtilis (present in 20% cases), *Pseudomonas* (present in 15%cases), Proteus (present in 30%cases) and the number of total bacterial count were markedly reduced in post radiotherapeutic patients in all cases except Streptococci. Anaerobic Actinomycetes and Fusobacterium were also reduced in post radiotherapeutic group. (Table – 1, Histogram 4)

DISCUSSION

Cancer is the most common life threatening disease nowadays. Oral cancer is the sixth most common cancer worldwide. (Shah and Gil, 2009) Advances in the field of treatment modalities like chemotherapy, radiotherapy have resulted in improved survival of these patients. But it has been found that sepsis or infection is one of the most serious complications that has proved to play a significant role in death of oral cancer patients. Due to changes of pH, irregularity of lesion surface and broken defence mechanism of oral mucosa the status of microflora has been changed in oral cancer patients and these changes of microflora in oral carcinoma surfaces may lead to both local and systemic infections. (Milos Cankovic *et al*., 2013) Different bacteria have been proposed to induce carcinogenesis either through induction of chronic inflammation or by interference, either directly or indirectly with eukaryotic cell cycle and signaling pathways or by metabolism of potentially carcinogenic substances. It has been shown that several bacteria can cause chronic infections or produce toxins that disturb the cell cycle and lead to altered cell growth. It is also noted that *Streptococcus* species was predominant and present in 39.9% of oral squamous cell carcinoma patients, mainly *Streptococcus* alpha-haemolyticus and beta- haemolyticus. Pseudomonas aeruginosa and Klebsiella pneumonia were sporadically isolated in oral squamous cell carcinoma patients. (Milos Cankovic *et al*., 2013) In the present study, total 50 patients were selected. Among them 10 patients were in normal group, who had no oral diseases. Bacterial swabs were taken from buccal vestibule, floor of the mouth, labial mucosa. The swabs were subcultured on blood agar plate and incubated at 37 degree centigrade for 24 hours. Following this, the bacterial isolate was identified by gram staining and it showed the presence of normal commensals of oral mucosa, like *Streptococcus*, *Staphylococcus*, *Enterococcus*, *Neisseria*, *Vellionella* but in lower percentage. Recent research has confirmed earlier studies that the resident microflora of animals and human play a positive role in the normal development of the host.This resident microflora also plays an active role in the maintenance of the healthy state by contributing to the host defences and preventing colonisation by exogenous microorganisms. It has been estimated that the human body is made up of 10^4 cells of which only 10% are mammalian. The remainder are the microorganisms that make up the resident microflora of the host. Here, mainly 40 patients were taken who were suffering from oral squamous cell carcinoma and which was diagnosed by incisional biopsy and histological evaluation. These group of patients did not receive radiotherapy, chemotherapy or surgery as treatment modality. Bacterial swabs were collected from the ulcerated site in oral mucosa and then culture was done in blood agar, nutrient agar and mackonkey agar plate. After that microbial identification was made by gram staining and biochemical tests accordingly. The detectable microorganisms were *Streptococcus*, Staphylococcus, *Pseudomonas*, Proteus, Klebsiella and Bacillus subtilis. (Table–1, Histogram 1,2) These bacteriological findings were supported by the other previous studies. Here, *Streptococcus* and Staphylococcus were found in greater percentage than the normal individual. Different studies had shown that some normal bacterial flora became pathogenic if predisposing factors or conditions prevail. Head and neck cancer alters the immunological profile of the patient Previous studies found that IgA levels were elevated in oral cancer patients and this elevated IgA acts as blocking antibodies to the host immune response. This altered immunological system changes the profile of microbiologic flora in head neck cancer patients. (Parehitiyawa *et al*., 2010)

Several studies had shown that bacteria also has got specific role in carcinogenesis, like:

1. Microbial carcinogenesis may also involve nitrosation in which microbial cells catalyze the formation of N-nitroso compounds from the precursor's nitrite and amines, amides or other nitrosatable compounds. Several species of bacteria encompass strains capable of catalyzing nitrosation, in particular. This particular nitrosamine appears to be a relevant candidate for the cause of carcinoma, not only of the esophagus but also of other mucosal areas such as the oral cavity.
2. Chronic infections induce cell proliferation and DNA replication through activation of mitogen activated kinase (MAPK) pathways and cyclin D1 and increase the incidence of cell transformation and the rate of tumor development through increased rate of genetic mutation.
3. Several infections cause intracellular accumulation of the pathogen, leading to suppression of apoptosis primarily through modulation of the expression of Bcl-2 family proteins or by inactivation of retinoblastoma protein, pRb.This strategy provides a niche in which the intracellular pathogen can survive in spite of the attempts of the host immune system to destroy the infected cells by apoptosis. Thus, it allows the partially transformed cells to evade the self-destructive process and progress to a higher level of transformation, ultimately becoming tumorogenic.
4. Another possible mechanism is the metabolism of potentially carcinogenic substances by the bacteria. This is of relevance in the oral cavity, where the pre-existing local microflora may facilitate tumourogenesis by converting ethanol into its carcinogenic derivative, acetaldehyde to levels capable of inducing DNA damage, mutagenesis and secondary hyperproliferation of the epithelium. Also, this is evidential from the increased levels of microbial acetaldehyde production in heavy drinkers and smokers, supporting this concept.

Next the samples were again collected from the same 40 patients and were directly inoculated into the fresh blood agar.

After proper maintenance of anaerobiosis, the bacterial colony were picked up from individual culture plate and identification was done by gram staining. The most commonly isolated organisms Anaerobic Actinomycetes, Fusobacterium. (Table-1, Histogram 4) These anaerobes are the part of normal flora and act as defending barrier against pathogens by preventing colonization of exogenous species. Samples collection was done from the same 40 patients, who were suffering from oral squamous cell carcinoma and received radiotherapy as treatment modality just one month before. The bacterial culture was done by incubating culture plate at 37 degree centigrade for 24 hours. After that by gram staining and biochemical test the identification of organisms were made. Here, also *Streptococcus*, Staphylococcus and other pathogenic microbes like Pseudomonas, Klebsiella, Proteus were found but in a reduced percentage than preradiotherapeutic group. (Table – 1, Histogram -1) Anaerobic culture of the same patients showed the presence of Actinomycetes and Fusobacterium, but in reduced number than before radiotherapy. The growth of a wide variety of gram positive, gram negative, aerobic, anaerobic pathogens compared to control group suggest that irradiation has a marked effect on the oropharyngeal flora. Many studies have reported the effect of radiation on the immunological status of patients. High dose of irradiation of lymphoid tissues reduces the peripheral blood lymphocyte count also. This altered immunological status helps in growth of pathogenic organisms. (Chocolatewala *et al*., 2010) The reduced percentage of bacteria in post irradiation group compared to pre-radiotherapeutic group also proves the bactericidal effect radiotherapy. By knowing the specific kind of bacteria present in squamous cell carcinoma patient before and after radiotherapy, the control of infection can be done by prescribing proper medicines. As a result, we can reduce the infection caused by different types of bacteria in this manner. (Milos Cankovic *et al*., 2013)

Conclusion

In this study the presence of microflora associated with the squamous cell carcinoma patients had been proved and these were mainly *Streptococcus*, Staphylococcus, Pseudomonas, Proteus, Klebsiella and Bacillus subtilis, Actinomycetes, Fusobacterium in higher concentration, whereas in post radiotherapy phase more or less the same group of microbes were found, but in a significantly reduced amount. This was because of the fact- the removal of the tumour mass as well as effect of radiotherapy. This was also well established that most of the victims of Oral Squamous Cell Carcinoma in their post radiotherapeutic phase do develop various microbial diseases, namely dental caries, periodontal disease, chronic mucositis and osteoradionecrosis.These diseases are usually associated with mixed microbial infections. Accordingly, the assessment of post radiotherapeutic status of oral microflora is essential for treatment and management of radiotherapy induced complications. However, assessment of disease specific microflora in post radiotherapeutic group of patients and the antibiotic sensitivity test is advocated for better management and treatment.

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