Prevalence of methicillin resistant *Staphylococcus aureus* isolated from saliva samples of patients with oral squamous cell carcinoma.

Prevalencia de *Staphylococcus aureus* resistente a la meticilina en muestras de saliva de pacientes con carcinoma oral de células escamosas.

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Abstract: Objectives: To study the prevalence of methicillin resistant *Staphylococcus aureus* (MRSA) in saliva samples of pre-surgical oral squamous cell carcinoma (OSCC) patients along with their resistance pattern to other antibiotics. Methods: Saliva samples of OSCC patients were collected and processed for isolation of MRSA. *Staphylococcus aureus* isolates were primarily identified using standard microbiological methods like biochemical assays, specialized media and latex agglutination test. Confirmation of MRSA strains was done by growing the isolates on MRSA agar and by using PCR to amplify two MRSA specific genes. All the isolated *Staphylococcus aureus* strains were subjected to antibiotic sensitivity tests. Results: A total of 17 *Staphylococcus aureus* strains were isolated from 50 saliva samples of pre-surgical OSCC patients of which 13 were confirmed to be MRSA. These MRSA strains were also found to be mostly resistant to other commonly used antibiotics. Univariate analysis revealed that most patients with MRSA infections had a prior history of hospitalization and surgery. Also, it was confirmed that patients with other comorbidities and infections were more prone to having MRSA present in the saliva. Conclusion: The majority of *Staphylococcus aureus* isolates from the saliva of OSCC patients were MRSA, and were resistant to several other commonly used antibiotics.

Keywords: Oral squamous cell carcinoma; methicillin-resistant Staphylococcus aureus; Antibiotic resistance; Oral cavity; MRSA.

Resumen: Objetivos: Estudiar la prevalencia de *Staphylococcus aureus* resistente a la meticilina (MRSA) en muestras de saliva prequirúrgicas de pacientes con carcinoma oral de células escamosas (COCE) junto con su patrón de resistencia a otros antibióticos. Métodos: Se recolectaron muestras de saliva de pacientes con COCE y se procesaron para el aislamiento de SARM. Los aislamientos de *Staphylococcus aureus* se identificaron principalmente mediante métodos microbiológicos estándar, como los análisis bioquímicos, los medios especializados y la prueba de aglutinación con látex. La confirmación de las cepas de SARM se realizó cultivando los aislados en agar SARM y utilizando PCR para amplificar dos genes específicos de SARM. Todas las cepas aisladas de *Staphylococcus aureus* se sometieron a pruebas de sensibilidad a los antibióticos. Resultados: Se aislaron un total de 17 cepas de *Staphylococcus aureus* a partir de 50 muestras de saliva de pacientes prequirúrgicos con COCE, de los cuales solo se confirmó que 13 eran SARM. También se encontró que estas cepas de SARM son resistentes a otros antibióticos de uso común. El análisis univariado reveló que la mayoría de los pacientes con infecciones por SARM tenían antecedentes previos de hospitalización y cirugía. Además, se confirmó que los pacientes con otras comorbilidades e infecciones eran más propensos a las infecciones por SARM. Conclusión: la mayoría de los aislamientos de *Staphylococcus aureus* de la saliva de los pacientes con OSCC fueron MRSA y fueron resistentes a varios otros antibióticos de uso común.

Palabras Clave: *Carcinoma oral de células escamosas; Staphylococcus aureus resistente a la meticilina; Resistencia a antibióticos; Cavidad oral; SARM.*

INTRODUCTION.

Methicillin resistant *Staphylococcus aureus* (MRSA) is gram-positive (GP) bacterium that can infect different parts of the body. In last three decades, the number of MRSA infections has increased worldwide and its treatment has become difficult because of its increasing resistance to commonly used antibiotics. MRSA can cause a variety of infections, ranging from mild infections on the skin, like sores or boils, to serious life-threatening skin infections or surgical wound, oral, bloodstream, respiratory and urinary tract infections.[1] Many public health reports have established that MRSA strains have spread across all sectors of the society, throughout the world. Today, MRSA is one of the major "superbugs" in the healthcare domain.[1,2]

Nearly 300 different types of bacteria are present in the human oral cavity. They are referred to as the normal microbiota and they help maintaining a healthy environment within the mouth. However, in unfavorable conditions, this balance gets upset and different type of bacteria may become opportunistic pathogens.[3]

Most patients with progressive malignant disorders have diminished saliva flow rate; elderly patients, hospitalized patients and patients in intensive care units are always at higher risk of MRSA infection as it is one the most common nosocomial pathogen. Porous surfaces of dentures and different types of devices in the mouth also facilitate the growth of MRSA.[4,5]

Oral squamous cell carcinoma (OSCC) is a cancer that affects the mucosal/squamous epithelial lining of the oral cavity. Nearly 275,000 OSCC cases are reported annually with 128,000 deaths each year.[6] Developing countries have a greater number of OSCC cases in comparison to developed countries. India accounts for 30% of the total OSCC cases worldwide, and within India OSCC is responsible for 22.9% of cancer-related deaths.[6] The major causative agents for oral cancer are tobacco and alcohol, along with dietary factors, human papilloma virus (HPV) infection, genetic factors and oral hygiene.[6,7]

Immunocompromised patients following treatment or surgery for oral cancer are always at risk of infection by multidrug-resistant opportunistic microbes including *Staphylococcus aureus*, *Streptococcus mutans*, *Pseudomonas aeruginosa*, *Porphyromonas gingivalis* and *Candida albicans*.[8] Until now no studies have recorded clear associations between MRSA colonization and OSCC, particularly in those patients who have no history of previous hospitalization or surgery.[9]

However, MRSA has been isolated from orofacial abscesses and other infections located on the gingival and corners of the mouth commonly associated with denture wear. Therefore, MRSA has become an important pathogen of concern as it can easily infect the oral cavity after any kind of surgery and it can gain entry to the oral cavity due to untrained health care personnel.[10] Few studies have reported on the isolation and identification of MRSA from OSCC patients, but mostly isolated from patients who had previously undergone hospitalization or any kind surgery for OSCC.[10]

This study attempts to understand the prevalence of MRSA strains in the saliva of pre-surgical OSCC patients and to find any correlation between MRSA and OSCC before surgery. Moreover, the pattern of resistance to other antibiotics is also reported.

MATERIALS AND METHODS.

Socio-demographic information, sample Collection, isolation and biochemical identification

A total of 50 saliva samples were obtained from 50 patients diagnosed with oral squamous cell carcinoma (OSCC) in their oral cavity, during a period of three months in the Dental OPD. The male to female ratio was 28:22.

There were 21 patients of both sexes between the age of 41 to 60, and 16 patients (both male and female) in the age group of 20-40 years old. Likewise, 12 patients were between the age of 61 to 100 and only 1 patient was in the age group of 1 to 10 years old. Regarding habits, 34 patients were addicted to alcohol and 47 of them had tobacco habits. The saliva sample was collected by making the patient squish with saline for 3 to 5 min and spit into a container. Nearly 4 to 5ml of saliva was collected from each patient.

The saliva samples were collected by making the patient swish with saline for 3 to 5min and spit into a container. Nearly 4 to 5ml of saliva/saline was collected from each patient. The saliva samples were cultured

on nutrient agar medium (NA) and mannitol salt agar medium (MSA) at 37ºC for 24hrs.

Subsequently, the Gram positive bacterial colonies were subjected to catalase and coagulase tests for assessing these biochemical characteristics.[11] A standard strain of *Staphylococcus aureus* (strain number 2275), obtained from the Microbial Type Culture Collection (MTCC), Imtech, Chandigarh, India was used as reference control for identification tests. This study was approved by the institutional ethical board and prior consent was taken from the patients before taking the saliva samples.

Latex agglutination test for confirmatory identification HiStaph Latex test Kit (HiMedia, Mumbai) was used for the confirmatory identification of *Staphylococcus aureus* colonies. The protocol was performed as per the manufacturer's instruction. Briefly 20µL of latex reagent was put on a clean grease-free slide and mixed with a pure colony obtained from the agar plate. The slide was rocked for two minutes and was then observed for agglutination.

Antibiotic susceptibility tests

Antibiotic susceptibility tests to 15 different antibiotics were performed by following Kirby Bauer's disc diffusion method.[12] The inhibition zone diameters were measured and assessed using the guidelines of the Clinical Laboratory Standards Institute 2017.[13]

Culture on MRSA Agar

All the Gram positive colonies with positive catalase, coagulase and latex agglutination tests were cultured on Hicrome MRSA agar (HiMedia, Mumbai). This medium is a highly selective medium which allows MRSA to grow, which can be identified by the characteristic blue colonies.[14]

DNA isolation and identification of MRSA using PCR

Genomic DNA was isolated from the *Staphylococcus aureus* pure cultures by using rapid genomic DNA isolation kit (HiPurA Bacterial Genomic isolation kit, Himedia, Mumbai). Multiplex PCR reaction using the *Staphylococcus aureus* specific nuc gene and methicillin resistant gene *mecA* for the identification of MRSA was employed.

For nuc gene, the primer set, F 5'- CGCG ATT GAT GGT GAT ACG GTT3' and R 5'- ACG CAA GCC TTG ACG AAC TAA AGC-3' was used whereas for *mecA* gene, F 5'-AAA ATC GAT GGT AAA GGT TGG-3' and R 5'- AGT TCT GCA CTA CCG GAT TTG C -3' was used.[15] The primers were provided by Genei laboratories, Bangalore, India. The PCR reaction mixture consisted of 3µL of genomic DNA, 12.5µL of 2X PCR master mix (Genei Laboratories, Bangalore, India), 1µL of each of the two primers and final volume made up to 20µL by adding of nuclease free water.

The amplification process was carried out in a thermocycler (LongGene, China) with an initial denaturation at 95ºC for 5min, followed by 30 cycles of denaturation at 95ºC for 1min, annealing temperature of primers was 55ºC for 45 seconds and extension at 72ºC for 1min. The amplified PCR products were resolved by electrophoresis in 1.8% agarose gel at 100 V for 30min, stained with ethidium bromide and finally visualized and documented under an UV trans-illuminator.[16]

RESULTS.

Isolation and identification of *Staphylococcus aureus*

All the 50 saliva samples obtained from oral cancer patients were cultured on nutrient agar plates. Of these, Gram-positive colonies from 38 saliva samples were further cultured on nutrient agar (NA) and mannitol salt agar (MSA) plates. A total of 17 colonies were positive for catalase and coagulase test as well as positive for latex agglutination test.

All other colonies were coagulase negative. The colony morphology of the 17 coagulase positive strains was yellow and mucoid on NA plates, whereas yellow colonies with yellow zone were observed on MSA plates. The 17 Gram positive colonies were identified as *Staphylococcus aureus* based on their colony morphology, gram staining, biochemical characteristics (Table 1) and latex agglutination assay. (Figure 1)

A total of 13 *Staphylococcus aureus* strains yielded blue colored colonies on MRSA chrome agar. The *Staphylococcus aureus* specific nuc gene of amplicon size 279-bp was successfully amplified from the genomic DNA of all isolated *Staphylococcus aureus* strains.

Out of 17 *Staphylococcus aureus* isolates, 13 were positive for *mecA* (532bp) indicating these *Staphylococcus aureus* isolates were resistant to methicillin.

Table 1. Microbiological characteristics of *Staphylococcus aureus.*

| Species	Media	Colony characteristics	Biochemical characteristics
Staphylococcus aureus Microbial Type Culture Collection (MTCC) strain2275	Nutrient Agar Mannitol salt agar MRSA	Yellow, mucoid LF, pink, mucoid Greenish –yellow colonies	Positive for catalase, coagulase and latex agglutination tests.

Table 2. Antibiotic sensitivity of *Staphylococcus aureus* isolates from OSSC.

Antibiotic Class/Group	Antibiotics	Number of susceptible isolates	Number of resistant isolates	Percentage of resistance (%)
Beta-lactams	Amoxicillin/clavulanic acid	8	9	52.94
	Oxacillin	4	13	76.47
Carbapenems	Imipenem	13	4	23.52
Cephalosporins	Cefoxitin	7	10	58.82
	Cefuroxime	8	9	52.94
Fluoroquinolones	Ciprofloxacin	5	12	70.58
	Levofloxacin	11	6	35.29
Glycopeptides	Vancomycin	7	10	58.82
	Teicoplanin	9	8	47.05
Lincosamide	Clindamycin	8	9	52.94
Macrolides	Azithromycin	6	11	64.7
	Erythromycin	5	12	70.58
Oxazolidinone	Linezolid	8	9	52.94
Rifamycin	Rifampicin	9	8	47.05
Sulfonamides	Nitrofurantoin	5	12	70.58
	Trimethoprim sulfamethoxazole	4	13	76.47
Others	Chloramphenicol	6	11	64.7
	Tetracycline	7	10	58.82

Table 3. Univariate analysis of MRSA positive and negative isolates *Staphylococcus aureus.*

Variables		MRSA positive	MRSA negative	*p*-value	Odd ratio	95% Confidence interval
Hospitalization or Surgery history	Yes	8	1	0.2232	4.80	0.3847 to 59.8973
	No	5	3			
Sex	Male	11	2	0.1765	5.50	0.4642 to 65.1649
	Female	2	2			
Comorbidities	Present	10	3	0.9368	1.11	0.0821 to 15.0370
	Absent	3	1			
Other infections	Present	7	1	0.3284	3.50	0.2838 to 43.1625
	Absent	6	3			

Figure 1. Confirmation of *Staphylococcus aureus* strains by latex agglutination assay.
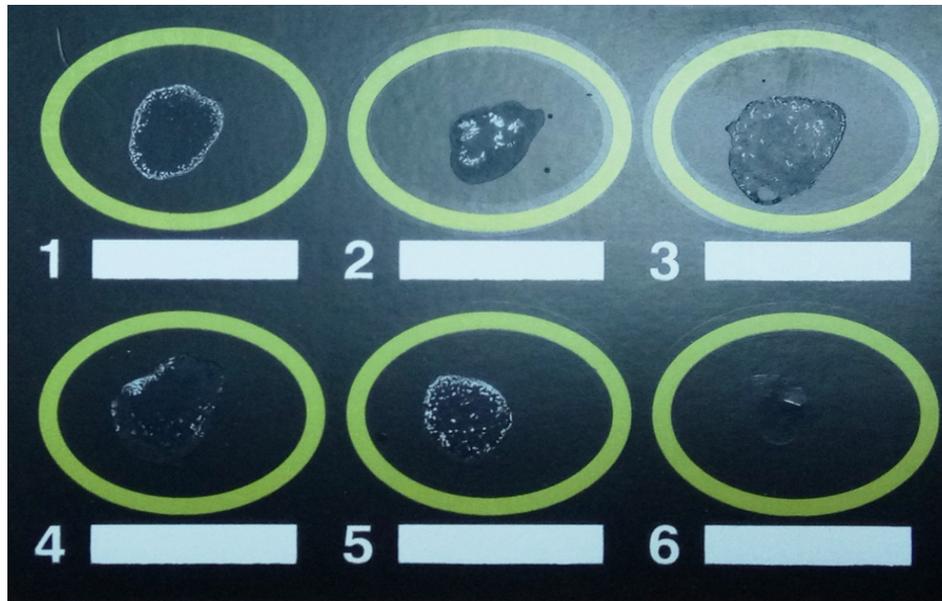
Therefore, from the antibiotic sensitivity culture on MRSA chrome agar and PCR amplification results, it was concluded that 13/17 *Staphylococcus aureus* isolates were MRSA.

Antibiotic susceptibility tests

Antibiotic susceptibility tests were evaluated by the disk diffusion method for all 17 isolates of *Staphylococcus aureus* and different degrees of resistance were recorded for the different antibiotics used. The resistance percentage of all the 17 *Staphylococcus aureus* isolates for 18 different antibiotics were of follows: amoxicillin and clavulanic acid 53%, oxacillin 75%, imipenem 24%, cefoxitin 59%, cefuroxime 53%, ciprofloxacin 71%, levofloxacin 35%, vancomycin 59%, teicoplanin 47%, clindamycin 53%, azithromycin 65%, erythromycin 71%, linezolid 53%, rifampicin 47%, nitrofurantoin 71%, trimethoprim-sulfamethoxazole 76%, chloramphenicol 65% and tetracycline, 59%. (Table 2)

Univariate analysis

In univariate analysis of MRSA positive and isolates of led to the conclusion that, Univariate analysis revealed that MRSA positive strains is 4.80 times more likely compared with non-MRSA *Staphylococcus aureus* in patients with a previous history of hospitalization or any kind of surgery. Furthermore, males were 5.5 times more likely to be positive for MRSA than females.

Patients with other comorbidities and non-infectious ailments had a 1.11- and 3.50-times higher chance, respectively, of being positive for MRSA. (Table 3)

DISCUSSION.

This study recorded a moderate percentage of MRSA isolates. All the antibiotics used in this study are the most commonly used antibiotics in dental practice. The great majority of MRSA isolates (96%) was resistant to oxacillin, and most isolates were resistant to amoxicillin/clavulanic acid and erythromycin.

The majority of isolates in our study (13/17 or 76%) carried the *mecA* gene. A study from United Kingdom reported 41.9% of total *Staphylococcus aureus* isolates from the oral mucosa and periodontal pockets of patients with gingivitis/periodontitis were *mecA*-positive.[17]

Another study, from the United States, reported a 20-35% prevalence of MRSA organisms in the nasal and oral cavities of nursing home residents.[18] In a Japanese study, 69.2% of *Staphylococcus aureus* isolates were MRSA, indicating that MRSA colonization is increasing among elderly Japanese population having oral cancer; however, they could not establish the role of MRSA in causing oral cancer.[10] Another study from China reveals the post-surgical occurrence of multidrug resistant microorganisms, including MRSA in oral cavity aged individuals diagnosed with oral cancer. The correlation between carcinogenesis

and bacteria was first recognized in the 1990s and the concept of microbial dysbiosis evolved.[19] This link between bacteria and OSCC has been assessed by several tecnhiques including cultivation, close-ended molecular techniques (e.g., DNA-DNA hybridization), and next-generation sequencing of the 16S rRNA gene.[20]

Association between host inflammation and microbial dysbiosis has been recognized to play a role in the etiology of various cancers like colon, gastric, esophageal, pancreatic, breast, and gall bladder carcinomas.[19] The existence of a symbiotic pro-inflammatory bacteriome within tissues of OSCC from a Yemeni patient cohort has been established and correlated with up-regulation in production of pro-inflammatory cytokines, illustrating the role of inflammation in cancer.[21,22] However, a very recent microbiome study of OSCC where functional prediction analysis was performed did not identify any bacterial inflammatory attributes, suggesting that bacteria-associated with OSCC may be a result of the tumor microenvironment, rather than an etiologic factor.

A range of factors in the tumor microenvironment, such as nutrient availability, pH, attachment ligands and immune elements, likely shape the arrangement and purpose of the microbial community within the tumor tissue leading to an enriched inflammatory surrounding promoting tumorogenesis.[22]

Saccharolytic and acid tolerant bacterial isolates like *Exiguobacterium oxidotolerans*, *Prevotella melaninogenica*, *Staphylococcus aureus* and *Veillonella parvula* have been reported from specific tumor tissues of oral cancer patients.[9]

Salivary markers of *Capnocytophaga gingivalis*, *Prevotella melaninogenica* and *Streptococcus mitis* have been identified in oral cancer patients, which was helpful for the early treatment and for reducing mortality rates. Specific bacterial markers in saliva in oral cancers can be used in planning of new treatment options for cancer prevention.[23]

Similarly, occurrence of MSRA in OSSC cases can be used as an early diagnostic marker for OSCC. However, more analysis and research is required in this subject to find a clearer association between MRSA or other bacterial species and OSCC.[9] However, the exact role of this *"superbug"* is not clear regarding OSCC, but it may become an important marker for the early detection of OSCC.

CONCLUSION.

To conclude, this study has provided information regarding the occurrence of MRSA in patients with OSCC and contributes information regarding the occurrence of antimicrobial resistance in OSCC. The majority of *Staphylococcus aureus* isolates from the saliva of OSCC patients were MRSA, and were resistant to several other commonly used antibiotics.

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