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Cytological Patterns and Molecular Detection of Methicillin Resistance Staphylococcus Aureus in Buccal Mucosa among Local Tobacco Users

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ABSTRACT

Objectives: This study was carried out to assess the cytological changes and to detect methicillin resistant *staphylococcus aureus in oral smears* among local tobacco users in Port Sudan city, Sudan, using Cytological Pap smear and PCR technique to help in detection of MRSA among local tobacco users. **Materials and Method:** One hundred buccal smears and oral swabs were obtained randomly from local tobacco users. The specimens were cytologically prepared and examined, swabs were cultured on Blood Agar with novobiocin disc and Mannitol Salt Agar for primary isolation of Pathogen.Then mecA gene detection was done used PCR technique. **Result:** 32% subjects were identified with keratinization, 21% of the users were identified with bacterial infection and moniliasis, inflammation was present in 18% of the users in the Pap smear. A 70 *Staphylococcus aureus* was isolated from the mouth swab, and 14 MRSA was isolated out of this 70 *Staphylococcus aureus*, 1 out of this were positive for mecA gene. Cytological changes and MRSA isolate was found to be high among Tobacco consumer for long period using rather than those who tobacco consuming for short period. The result revealed that the antimicrobial resistance of *S. aureus* for Methicillin was (20%). **Conclusion:** This study concluded that there is correlation between tobacco use and duration of use and increasing susceptibility to MRSA infection and cytological changes.

Keywords: Cytological patterns, Local Tobacco, MRSA, *staphylococcus aureus, Oral cavity*

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I. INTRODUCTION

Globally Tobacco use is a major health problem and one of the main causes of death overall worldwide. [1] Local Tobacco (Toombak snuff) was introduced into Sudan approximately 400 years ago. The use of Toombak has been stated to play a major role in the etiology of oral cancer and infections in Sudan[2]. The consuming of tobacco is common overall world-wide, but People of many regions, including southern parts of United States, Scandinavian countries, southern African countries and the Sudan in northeast Africa, have a long history of tobacco use. [3] Tobacco in the Sudan known as Toombak and mad up from fermented ground tobacco mixed with sodium carbonate.[3] The Snuff is usually placed in the buckle lower or labial vestibules of the oral cavity, in long period users leading to cause various diseases including periodontal diseases, cancer of the oral cavity and pharynx.[3,4,5] Periodontal diseases is a chronic infectious disorders caused primarily by bacteria.[4] Tobacco is containing a numbers of carcinogens substance that make the tobacco is most important risk factor for cancer.[2]

Antimicrobial resistance is a major worldwide health problem concern, and drug resistant *Staphylocccus aureus* is a serious threat. [6] *S. aureus* is the most clinically significant species responsible for skin, soft tissue, bacteremia and endocarditis and this infections ranging from relatively mild to life-threatening condition. [7,8] Methicillin Resistant *Staphylococcus aurous* (MRSA),

a potential dangerous type of staph bacteria that causes sever morbidity and mortality worldwide. [9] The first strains of MRSA emerged during the 1960s. [10] Unlike the penicillin resistance in *S.aureus*, this resistance was mediated by hyper production of beta-lactamases, modification of normal PBPs, Methicillin resistance is due to the acquisition of gene encoding a unique penicillin-binding protein PBP2a , its encoded by the mecA gene. [11,12] The Centers for Disease Control and Prevention (CDC) has concluded that MRSA is an important etiological agent that responsible for both hospital and Community onset disease and remains to be done to further decrease risks of developing this infection [13] The harmful effect of tobacco use lead to health problem in the mouth and developed to be complicated, infection of Staph in tobacco users characterized by the presence of ulcer in the lip that is red and warm to the touch. The harmful effect of MRSA infection is un-response to treatment and leading to cause fatal condition. MRSA infection among tobacco users is not investigated and researched enough, the purpose of this study was to examine the relationship among MRSA infection and tobacco using.

II. MATERIALS AND METHODS

A cross-sectional study was conducted from February to June in 2018 among local tobacco users in Port Sudan City. Port Sudan is a district of the Red Sea state, which is located in the north-east of the Republic of Sudan. Local Tobacco consumer adult male aged 19 years old to 55years old were included into study. Data were collected by structured questionnaire.

III. EXPERIMENTAL WORK

(A) Sample collection

A multistage random sampling technique was used to select the study location. One hundred samples were obtained from various local tobacco users and collected from the dip site area from buccal mucosa by using a flat sterile wooden tongue depressor and swabs. All smears were fixed immediately by conventional fixative for Pap smears (95% ethyl alcohol) while they were wet for 15 minutes and then stained in Pap protocol. The swabs were transported to the laboratory directly and inoculated onto plates of Blood Agar with Novobiocin disc and Mannitol Salt Agar (MSA) and then incubated aerobically at 37 ℃ for 24h.

Cytological Interpretation in Pap smear

The presence of two or more of the following features indicated the presence of epithelial atypia which are nuclear enlargement associated with increased nuclear cytoplasmic ratio; hyperchromatism, chromatin clumping with moderately prominent nucleolation ; irregular nuclear borders; bi or multinucleation, increased keratinization; scantiness of the cytoplasm and variations in size and or shape of the cells and nuclei. For each of these features, three possible grades were provided (mild-moderate-severe) [15,16].

Bacterial identification:

The isolated *Staphylococcus.aureus* identified by phenotypic method such as colony morphology and by performing Gram's stain and biochemical tests.

(B)Colonial Morphology

Mannitol Salt Agar: Yellow colony (mannitol ferment), circular, small in size, smooth, convex, moist.

Blood Agar: Beta hemolytic, Creamy colony, circular, small in size, smooth, convex, moist and sensitive to Novobiocin disc.

Gram stain: The aim of this stain to distinguish between gram positive and gram negative bacteria. Thin smear of the isolates was prepared on a clean glass slides by using sterile loop. The slides dried by air and then heat fixed by passing it through the flame and gram stain was performed, by covering the smear with crystal violet stain for 30 second and washed with water. Flood with Lugol's iodine for 30 second and then washed with water, then decolorized by 95% alcohol for 3 second and washed with water. The slide was counter stained with suffranin stain for 1 minute and then washed with water. The slides were air dried and then examined under oil immersion lens 100 x.

(C)Biochemical tests

Catalase test: This test was performed to distinguish between Staphylococci from Streptococci. Catalase is an enzyme that breakdown hydrogen peroxide into water and oxygen. A small amount of colony taken by wooden stick and immersed in 3% H2O2 Solution in test tube. The production of gas bubbles indicated as a positive test.

***Coagulase test*:** This test was used to differentiate *Staphylococcus aureus* from coagulase-negative staphylococci. *Staph.aureus* produces two forms of coagulase bound coagulase and free coagulase Bound coagulase, otherwise known as "clumping factor", can be detected by carrying out a slide coagulase test, and free coagulase can be detected by using a tube coagulase test. The coagulase test was performed by the fibrinogen slide method, two drops of saline were putted onto the slide labeled with sample number, Test (T) and control (C). The two saline drops are emulsified with the tested organism by wooden stick. A drop of plasma (rabbit plasma anti coagulated with EDTA was used) placed on the inoculated saline drop corresponding to test, and mixed well, then the slide was rocked gently for about 10 seconds. The positive, macroscopic clumping was observed in the plasma within 10 seconds.

DNAse test: This test was performed to confirm staphylococci aureus colonies. Aloop was sterilized and allowed to cool, then a part from the colony was taken and

inoculated as a line in DANse media and incubated at 37℃ for 18h, in the second day 1% N HCL was added in surface of media. The clear zone around the colony indicated as a positive test.

(D)Antibiotic Susceptibility Testing

This test was performed to determine the sensitivity of isolates to antibiotics. The resistance of *Staph.aureus* against Methicillin was observed by disk diffusion method in this Study. Suspension from *Staph.aureus* in broth medium with a turbidity equivalent to 0.5 McFarland stander (0.5 mL barium chloride + 99.5 mL of sulfuric acid) was prepared, from each inoculated suspension was cultured on Mueller Hinton agar by cotton swab and the Disc of Methicillin (5µg)was applied to the surface of agar plate. Then the plates were incubated at 37 ℃ for 18h. Zone of inhibition was measured by a ruler in millimeter.

The result were interpreted according to CLSI guidelines 2013. An inhibition zone diameter of ≤ 9 mm was reported as methicillin resistant and ≥ 14 mm was reported as methicillin sensitive.

(E)Molecular study using polymerase chain reaction

DNA Extraction: The targeting DNA was extracted from bacterial cell by boiling method. To an Eppendorf tube 200µl of DW was added, then by using sterile loop two colonies was taken, and putted into the Eppendorf tube, then was dissolved by shaking and then 10µl from Lysosome enzyme solution was added to Eppendorf tube and incubated at 37 ℃ in water bath for 30 minute. The Eppendorf tube then was transmitted to another water bath for boiling, boiled at 100 ℃ for 30 minute, then was centrifuged at 12.000 RPM for 15 minute, with caution all the supernatant was transferred to new Eppendorf tube by using Automatic pipette and was stored at -20 ℃ for PCR technique.

PCR Amplification and Product Detection
Primes Design

Forward and reverse primers targeting mecA gene were designed used software program.

PCR Amplification and Product Detection

All extracted 16 MRSA sample were amplified by using PCR technique. Ready-mixed master mix tubes used with 5µl volume, 5µl of DNA template was added to each PCR tubes, then 2µl of forward and reverse primers were added, and 13µl of distil water, then was mixed well by gentle vertexing. PCR protocol was run: First DNA was initially denatured at 94°c for 5 minute, followed by 30 second, then primer annealing at 55°c for 30 seconds, and extension at 72°c for 30 second and final extension for 5 minute. This process repeated for 40 cycle.

Then PCR product along with 5o bp ladder control were detected using agarose gel diffusion (1%), and examined by gel documentation machine.

IV. RESULTS

Cytological Pap smear: Thirty two (32%) subjects from the cases were identified with keratinization; hence, not found in the control normal smears (Non Users). Toombak dipping with infection is a major risk factor for the occurrence of the keratinization in the oral mucosa. This was found to be statistically significant P < 0.0001. In regard to the infection, 21 (21%) users were identified with bacterial infection and moniliasis. The inflammation was present in 18 (18%) of the users and users were more susceptible to infections and inflammation.

Bacterial identification and sensitivity testing: The result showed that 70 out of 100 sample (70%) gave positive result for *Staphylococcus aureus* (able to grow on mannitol salt agar and the gram stain reaction is gram positive cocci arrangement in cluster).(table 1) Biochemical tests were used to confirm the identification of S.aureus isolated like; Catalase, Coagulase and DNase, were100% positive. Fourteen samples were resistant to Methicillin and 56 were sensitive. The percent of Methicillin resistant was 20% (table 2) and the maximum zone of inhibition of Methicillin against *S.aureus* was 6 mm and most frequent zone of inhibition was 8 mm, and the incidence of MRSA among tobacco users different age groups slightly higher in older age who used tobacco for long period of time (figure1).

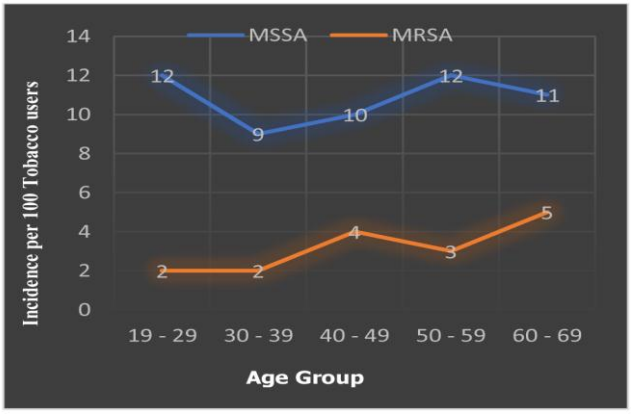
Table 1: Age groups and Percentage of Isolated Organisms

Sensitivity against Methicillin disc		Percentage of Sensitive	Percentage of Resistance
Sensitive	Resistance		
56	14	80%	20%

Table 2: Percentage of Sensitivity against Methicillin disc for staphylococcus isolates

Age	Mean Period of tobacco use	No of Sample taken	No of S.aureus isolate	No of Other isolate	Percentage S.aureus	Percentage of Other
19 - 29	4.7	20	14	6	14%	6%
30 - 39	7.9	17	11	6	11%	6%
40 - 49	13.5	18	14	4	14%	4%
50 - 59	28.2	23	15	8	15%	8%
60 - 69	32	22	16	6	16%	6%
Total	86.3	100	70	30	70%	30%

Fig: 1 Incidence of MSSA and MRSA among different age groups of tobacco users.



Molecular detection of mecA gene:

One sample out of 14 methicillin resistant *staphylococcus aureus* was positive for mecA gene used MA1 and MA2 (table3), with band length of 210 bp (figure 2).

Table 3: Oligo nucleotide primer used in the study:

Name	Sequence	Target
MA1	5'-TGCTATCCACCCTCAAACAGG-3'	MecA gene
MA2	5'-AACGTTGTAACCACCCCAAGA-3'	MecA gene

Figure 3: Agarose gel analysis of PCR products (50 bp ladder):

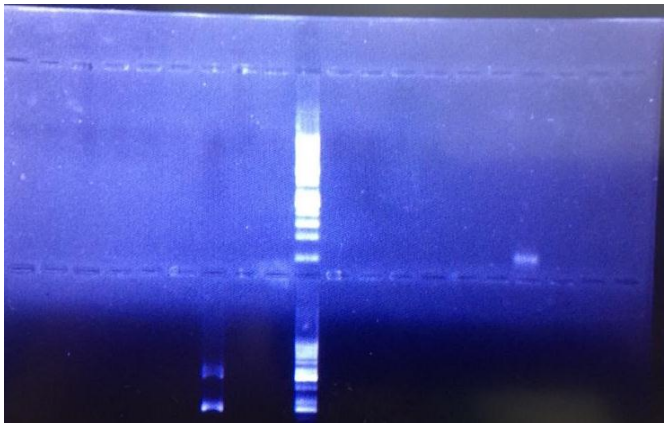
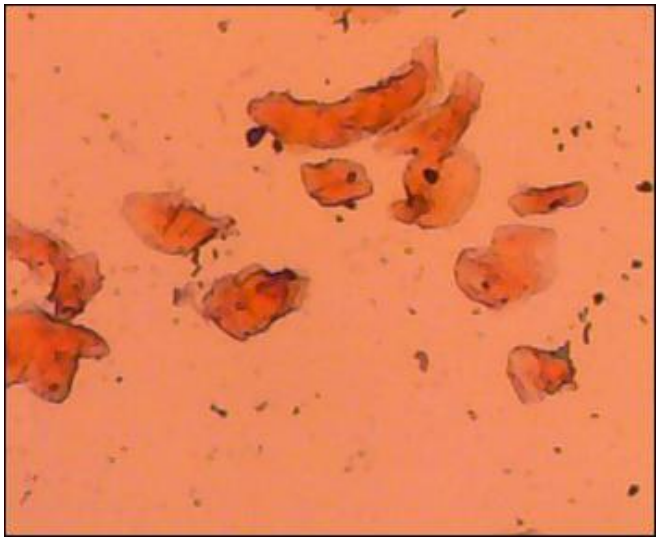


Figure 4: Buccal cells from the dipping area showing the keratinization. A nucleated cells appeared in the field (Pap stain 100 X).



V. DISCUSSION

Thirty two (32%) subjects from the cases in this study were identified with keratinization. Since keratinization with a nucleation may lead to premalignant lesion called leukoplakia, there is a strong association between cancers of the oral cavity and pharynx and Toombak use. The use of Toombak has shown to produce a variety of oral mucosal changes such as dysplasia and hyperkeratosis and infections. [17] The keratinization with infection is a direct proportion to increase in the age of the cases and this due to prolonged exposure to the Toombak use. In regard to the infections and inflammatory conditions, cases were more susceptible than controls, and this was found to be statistically significant ($p < 0.003$). The Erosions and exposure of the oral mucosa to the Toombak irritating substances are the major causative factors. This study is first study carried in Port Sudan city using this technique to detect the incidence of MRSA among tobacco users. No similar studies were carried out at the population in Sudan or other near places. The most studies that have been carried out were detection of MRSA among ciggerate smoker's and nasal carrier. In this study the total incidence of S.aureus were 67 and the MRSA were 16 among local tobacco users in Port Sudan city, Sudan.

VI. CONCLUSION

Oral exfoliative cytology using Pap smear is useful in evaluation of epithelial atypia, inflammation and infections. There is a correlation of tobacco using and the period of using with MRSA infection and Cytological changes. Alarming and highlights the need for adjusted infection control measures to prevent MRSA.

VII. RECOMMENDATIONS

A. Perform campaigns awareness to community about the effect of toombak use.
B. Ask the government to make some strict rules about toombak sale in order to reduce the number of tobacco users.
C. Conduct a further study on S.aureus isolation and Methicillin and vancomycin sensitivity testing.

VIII. ACKNOWLEDGMENT

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