Isolation And Characterization Of A Klebsiella Strain From Smokeless Tobacco Consumers

D'Souza Racheal, Kazi Samina and Dr. Anupma Harshal

Abstract: Organisms that reside in the oral cavity become an important aspect of health and disease. Disruption of the oral health and hygiene has been attributed to the presence of abnormal microflora. Consumption of tobacco has been considered to be a leading cause for the presence of abnormal oral microflora, in turn disrupting the oral health. In this study, 45 respondents were targeted from South Mumbai who consumed tobacco in smokeless forms. Klebsiella pneumoniae strain was isolated from oral swabs of smokeless tobacco consumers and compared against controls. The strain was characterized using phenotypic and genotypic approaches. The presence of Klebsiella was reported in 24 of the 45 samples. Sequence comparison of 16S rRNA gene and phylogenetic analysis confirmed the presence of Klebsiella strain. The sequence obtained was submitted to the NCBI Gene bank that has been granted an accession number- KM186520. During the course of our study we observed Klebsiella pneumoniae to exhibit green fluorescent colonies under UV spectroscopy. λ_{max} of the fluorescent chromophore was estimated to be 220 nm with OD of 1.785. Our analysis provides evidence that Klebsiella pneumoniae in the oral micro flora of the tobacco consumers exhibits a novel phenomenon of fluorescence.

Index Terms: Klebsiella pneumoniae, oral cavity, smokeless tobacco, 16s rRNA, fluorescence, chromophore, UV spectroscopy

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1. INTRODUCTION

Organisms residing in the oral cavity and their collective genome–the oral micro biome–are critical components of health and disease. Disruption of the oral micro biome has been proposed to indicate, trigger, or influence the course of oral diseases[1]. Consumption of tobacco can cause the disruption of the oral micro biome. Smokeless tobacco is tobacco that is not burned. It is also known as chewing tobacco, oral tobacco, spit or spitting tobacco, dip, chew, and snuff [2]. There are 2 major types of smokeless tobacco products: chewing tobacco and snuff [3]. Chewing tobacco, which is available as loose leaves, plugs (bricks), or twists of rope. A piece of tobacco is placed between the cheek and lower lip, typically toward the back of the mouth. It is either chewed or held in place. Most people chew or suck the tobacco in their mouth and spit out the tobacco juices that build up [2]. In users of smokeless tobacco, nicotine in the tobacco is absorbed through the lining of the mouth. It is absorbed through the mouth tissues directly into the blood, where it goes to the brain. Even after the tobacco is removed from the mouth, nicotine continues to be absorbed into the bloodstream. Also, the nicotine stays in the blood longer for users of smokeless tobacco than for smokers. The level of nicotine in the blood depends on the amount of nicotine in the smokeless tobacco product, the tobacco cut size, the product's pH and other factors[2]. The major types of smokeless tobacco products consumed in India are in the form of Pan, Pan Masala, Ghutka, Masheri, Khaini, Beetle nuts, etc. The Europeans introduced tobacco into South Asia in the 1600s, for pipe smoking and probably also as snuff. Believed to have originated in prehistoric times, this practice extends eastwards as far as the South Pacific islands. After its introduction, tobacco soon became a new ingredient in betel quid (pan), which has become the most commonly used form of smokeless tobacco, although its use varies in different parts of the world. An estimate of the number of betel quid users globally is 600 million.[1] Smokeless tobacco users in India and Pakistan together have been estimated to number 100 million. Smokeless tobacco and snuff contain 3,000 chemicals including 28 carcinogens (cancer-causing agents) [4]. Klebsiella pneumoniae, a gram-negative rod-shaped bacillus from the genus Klebsiella are considered to be immobile in nature. They can be identified by its characteristic to produce a capsule made up of polysaccharide. Klebsiella pneumoniae grows at an optimum temperature of 37°C. The bacterium has been predominantly associated with oral health [5]. In chronic form, it has also been associated being the cause of Parenchymal scarring and Bronchiectasis [6]. Although found in the normal flora of the mouth, skin, and intestines[31]; Klebsiella pneumoniae can cause destructive changes to human lungs if aspirated, specifically to the alveoli resulting in bloody sputum. The organism has become important pathogens in nosocomial infections, and it is conditionally pathogenic and may produce pneumonia in man [7]. This study aims at isolating and characterizing one such strain of Klebsiella pneumoniae.

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2 PROCEDURE

2.1 Collection of Samples from the Smokeless Tobacco Consumers

This study was a random cross-sectional study conducted in South Mumbai, India. A convenient sampling method was used to recruit smokeless tobacco consumers from a mixed group consisting of taxi drivers, dabbawalas, local vendors and support staff at K.C. College. A total of 50 such people, male ranging between 20-60 years of age and above, participated in the study. Out of the 50 respondents, 45 were tobacco consumers and 5 were non-consumers. Unstimulated samples were collected using sterile cotton swabs, by swabbing it all round in the oral cavity. The swabs were dipped it in a tube containing 1.5ml of saline and further analyzed (not later than 24 hours).

2.2 Microbial analysis of the sample

Within 3-4 hours of collection, the samples were streaked on Nutrient Agar. The swab used for collection was used for streaking on plates. The plates were incubated at 37°C for 24-48 hours.

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2.3 Isolation and Characterization of the organisms

After incubation, the colonies observed on the plate were characterized by Hans Christian Gram's method of Gram Staining and observed under the light microscope (100X magnification-Oil immersion lens). Capsule staining was carried out by Maneval's method.

2.4 Examination of UV fluorescence and characterization of the strain

Nutrient Agar plates were observed under UV light. The colonies exhibiting fluorescence were used for scaling up to obtain more quantity of the pigment. The colonies were enriched in Nutrient Broth using a shaker incubator at 37°C. The flasks were observed for fluorescence in a dark room under UV light. The cells were pelleted from the suspension. The supernatant was discarded as it did not show any fluorescence. The pellets were washed in sterile saline and were re-suspended 0.4 N NaOH to dissolve the pellet. The extracted pigment was subjected to UV Spectroscopy using 0.4N NaOH as control.

3 RESULTS

3.1 Description of the colony

Twenty-four samples of the 50 exhibited the presence of green fluorescent colonies under the UV light (Fig 1).

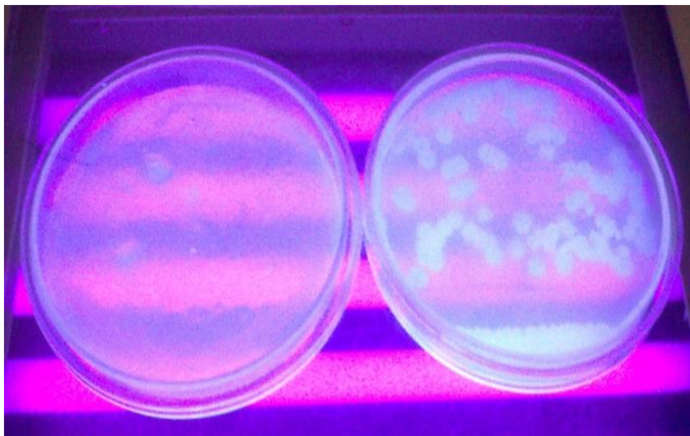

Figure 1: *Fluorescent colonies under UV light; control agar plate (left) test sample (right)*

On further characterization under the microscope, the fluorescent colonies were indicative of gram negative short rods present in singles and in chains. This colonies were stained to check for presence of a capsule. The colonies displayed presence of a capsule, confirming the probability of presence of a Klebsiella strain.

3.2 Characterization of the strain

The isolates were characterized using phenotypic and genotypic approaches. The colonies were observed at room temperature (Fig 2A) as well us under UV lights (Fig 2B) for a comparison. Fluorescent colonies were picked and scaled up in a nutrient broth flask. These colonies exhibited similar fluorescence (Fig 2C).

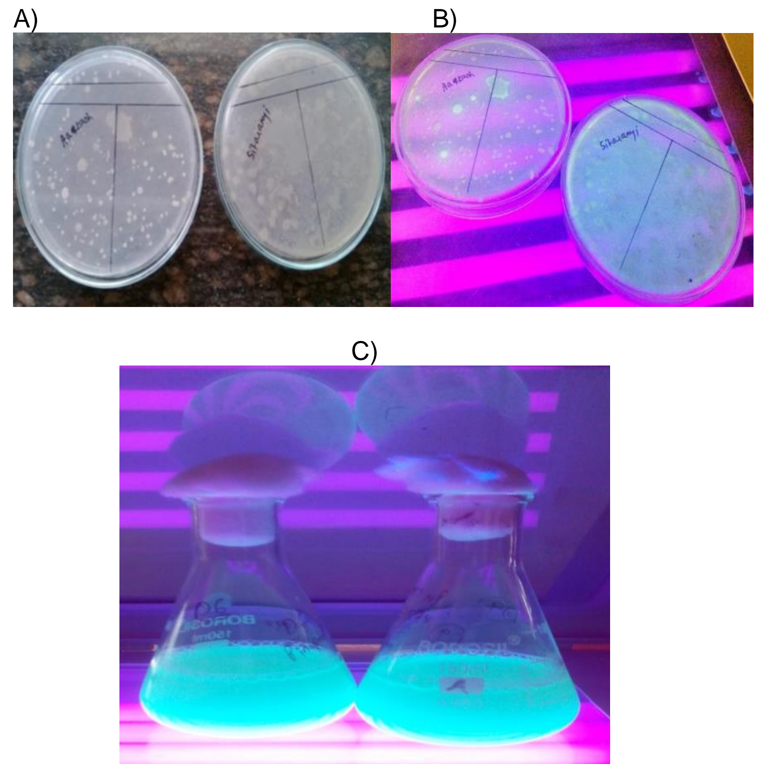

Figure 2: *A) Colonies growing on Veillonella Agar Plate. B)Colonies seen under UV light. C)Fluorescent colony inoculated and enriched in nutrient broth.*

λ max of the fluorescent chromophore was estimated to be 220 nm with OD of 1.785 with the help of spectrofluorometric analysis which further confirmed the presence of Klebsiella pneumoniae strain (Fig 3). Sequence comparison of 16S rRNA gene and phylogenetic analysis confirmed the presence of Klebsiella pneumoniae. The sequence thus obtained was submitted to the NCBI Gene bank that has been granted an accession number KM186520.

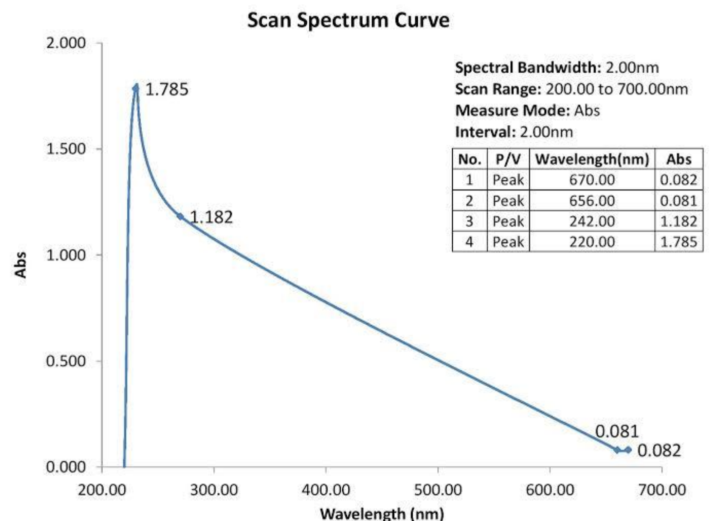

Figure 3: *Spectrofluorometric analysis of the fluorescent colony*

4. DISCUSSIONS:

In our study, the isolated fluorescent colony were found to be a strain of Klebsiella pneumoniae. These are gram negative short rods that are responsible for causing pneumonia. Gram-negative rods are unusual pharyngeal isolates in normal man, suggesting the presence of effective oral defense mechanisms against these organisms. Our findings have provided evidence for the presence of Gram-negative short rods, Klebsiella pneumoniae, in the oral flora of smokeless tobacco consumers. In previous studies, data generated using suspensions of Escherichia coli, Klebsiella pneumoniae, and Proteus mirabilis gargled by normal volunteers showed the most rapid decrease in buccal mucosa samples and the slowest decrease in tongue swabs of these organisms[7,8]. Although the oral cavity is considered to be a reservoir for saprophytic microorganisms, some of them may be pathogenic, especially those which are not part of the normal flora. K. pneumoniae infections are not frequent in the mouth [9]. Amongst the saliva of 50 respondents analyzed, 24 respondents showed the presence of the fluorescence strain. None of the controls harbored this strain. K. pneumoniae is also considered as a ubiquitous opportunistic pathogen that colonizes at the mucosal surfaces in humans and causes severe diseases, such as septicemia, pneumonia, urinary tract infections, and soft tissue infections[5]. Although Burnet et al., 1978 [10] consider K. pneumoniae to be a normal component of oral and nasal cavities, Trabulsi, 1991 [11] only referred to colonization of the nasopharynges by this bacterium. Other studies have shown that its presence is more common in the dental plaque of vegetarians [13] and in AIDS patients [14]. A study published in Dsouza et al [5] suggests most of the tobacco consuming respondents reported a problem of bad breath. Bad breath is a common phenomenon, usually the result of bacterial metabolism in the oral cavity. Goldberg S et al, 1997 [15] reported that isolates of Klebsiella and Enterobacter emitted foul odor in vitro which resembled bad breath, with concomitant production of volatile sulfides and cadaverine, both compounds related to bad breath. When incubated on a sterile denture, enterobacterial isolates produced typical denture foul odor. The results, taken together, suggest that Klebsiella and related Enterobacteriaceae may play a role in denture malodour[16]. Consumption of tobacco containing products have also been observed to have an impact on the total protein in the saliva thus affecting the oral microflora. Previous studies have reported that K.pneumoniae is responsible for a severe type of pneumonia, and for a large number of hospital-acquired infections. It is found in infection of the GI tract, peritonitis, enteritis, meningitis and septicemia, always associated with a suppurative process [18, 19] K. pneumoniae may also secondarily infect other areas from a primary focus of infection. In our study, chromophore was obtained in the supernatant whereas pellet did not show presence of the chromophore. We need to explore further, the pathway for formation of chromophore group leading to the fluorescence exhibited. The biochemical nature of fluorescent component in Klebsiella pneumoniae remains to be investigated. There have been previous references [10, 20-24] which say that consumption of tobacco may alter the microenvironment in the oral cavity. It may act as facilitator for non-resident bacteria and may cause changes in the adherence pattern of the micro flora. In our analysis, there must have been such a non-identified factor in oral swabs of tobacco consumers which may have played a role in adherence of Klebsiella pneumoniae, which needs further confirmation. Composition of various tobacco products is not yet defined, as a result is open for debate on different ingredients present in the product altering the microenvironment of the mouth. The presence of Klebsiella in these respondent samples needs to be correlated to any diseased state of the subjects. Long working hours leading to consumption of these products at a high frequency predisposes them to several dental complications and may even put them at a risk of oral cancer. The respondents need to be made aware of the implications of the findings of the study and its far-reaching effect on their health. This study may serve as a tool to detect the presence of Klebsiella species in oral isolates. This also has implications for a quick detection of presence of Klebsiella species using its ability to grow on Nutrient agar and possessing fluorescence under UV light. examples of units of measure.

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REFERENCES

[1] Patricia I. Diaz, Natalia I. Chalmers, Alexander H. Rickard, Colin Kong, Craig L. Milburn, Robert J. Palmer, Jr., Paul E., Molecular Characterization of Subject-Specific Oral Microflora during Initial Colonization of Enamel, Kolenbrander Appl Environ Microbiol. 2006 April; 72(4): 2837–2848. doi: 10.1128/AEM.72.4.2837-2848.2006

[2] National Cancer Institute. Smokeless Tobacco or Health: An International Perspective. Bethesda, MD: National Cancer Institute; 1992. Smoking and Tobacco Control Monograph 2.

[3] Community Dentistry Division, Faculty of Dentistry, King Abdulaziz University, Jeddah, Saudi Arabia ,D.E. Al Agili, Received:26/03/12;accepted:06/05/12 http://www.emro.who.int/emhj-vol-19-2013/8/oral-health-status-of-male-adolescent-smokeless-tobacco-users-in-saudi-arabia.html

[4] Gupta PC. Smokeless tobacco use in India. In: Smokeless Tobacco or Health—an International Perspective. USA Smoking and Tobacco Control Monograph 2. NIH Publication no. 92–3461, September 1992; 19–25.

[5] Racheal D'Souza, Anupma Harshal, "Epidemiological Study of Health Parameters in Smokeless Tobacco Consumers", International Journal of Science and Research (IJSR), Volume 9 Issue 2, February 2020, 1251 - 1255

[6] T Azar SL, Ebadi AR (2017) Examining the Patern of Susceptibility and Antibiotic Resistance in Klebsiella Pneumoniae Strains Isolated from Urine Samples of Children with Urinary Tract Infections from the Children's Hospital of Tabriz in 2015. Br Biomed Bull 5: 307

[7] Arnold, Walter, Andreas Rump, Werner Klipp, Ursula B. Priefer, and Alfred Pühler. "Nucleotide sequence of a 24,206-base-pair DNA fragment carrying the entire nitrogen fixation gene cluster of klebsiella pneumoniae ." Journal of molecular biology 203, no. 3 (1998): 715-738.

[8] Laforce FM, Hopkins J, Trow R, Wang WL, Human oral defenses against gram-negative rods, Am Rev Respir Dis. 1976 Nov;114(5):929-35.

[9] Antonio Luiz Barbosa PINHEIRO, André Carlos de FREITAS, A Rare Case of Lip Ulcer Infected by Klebsiella pneumoniae: Case Report, Braz Dent J (2000) 11(2): 161-165 ISSN 0103-6440
[10] Burnet GW, Sherp HW, Schuster GS: Microbiologia Oral e DoençasInfecciosas. 4th ed. Guanabara Koogan, Rio de Janeiro 1978
[11] Trabulsi LR: Microbiologia. 2nd ed. Atheneu, Rio de Janeiro 1991.
[12] Sedgley CM, Chu CS, Lo CM, Samaranayake LP: The oral prevalence of aerobic and facultatively anaerobic Gram-negative rods and yeasts in semi-reclused human vegetarians. Arch Oral Biol 41: 307-309, 1996
[13] Zambom JJ, Reynolds HS, Genco RJ: Studies of the subgingivalmicroflora in patients with acquired immunodeficiency syndrome. J Periodontol 61: 699-704, 1990
[14] Podschun, R; Ullmann, U (October 1998). "Klebsiella spp. as Nosocomial Pathogens: Epidemiology, Taxonomy, Typing Methods, and Pathogenicity Factors". Clinical Microbiology Reviews 11 (4): 589–603. PMC 88898.).
[15] Goldberg S[1], Cardash H, Browning H 3rd, Sahly H, Rosenberg M. Isolation of Enterobacteriaceae from the mouth and potential association with malodor. J Dent Res. 1997 Nov;76(11):1770-5.
[16] Yinnon AM, Butnaru A, Raveh D, Jerassy Z, Rudensky B, 1996.Klebsiella bacteraemia: community versus nosocomial infection. QJM89:933–941.
[17] D'Souza R, Kazi S, Harshal A. Effect of Consuming Smokeless Tobacco on Total Protein Content in Saliva. International Journal of Science and Research (IJSR). 2020 March; 9:1679. doi: 10.21275/SR20226203400.
[18] Gentry LO, Rodrigues GG: Oral ciprofloxacin compared with parenteral antibiotics in the treatment of osteomyelitis. Antimicr agents and chemoth 34: 40-43, 1990
[19] Hanna Jr CB: Cefadroxil in the management of facial cellulitis of odontogenic origin. Oral Surg Oral Med Oral Path 71: 496-498, 1991
[20] Scannapieco FA, Mylotte JM: Relationships between periodontal disease and bacterial pneumonia. J Periodontol 67 (suppl 10): 1114-1122, 1996
[21] Langford Jr, George C., John E. Faber Jr, and Michael J.Pelczar Jr. "The occurrence of anaerobic gram-negative diplococci in the normal human mouth." Journal of bacteriology 59, no. 3 (1950): 349
[22] Chocolatewala, Noureen, Pankaj Chaturvedi, and Rushikesh Desale. "The role of bacteria in oral cancer." Indian journal of medical and paediatric oncology: official journal of Indian Society of Medical & Paediatric Oncology 31.4 (2010): 126.
[23] Takahashi N. Acid-neutralizing activity during amino acid fermentation by Porphyromonas gingivalis, Prevotella intermedia and Fusobacterium nucleatum. Oral Microbiol Immunol.2003;18:109–13.
[24] Dr Gayathri Ramesh, Dr Smriti Chaubey, Dr Ramamurthy Sujatha, Dr Amrita Raj, DrRavi Kumar Seth, Dr Vikas Kumar Sant., Effect of Tobacco Chewing on Salivary and Tongue Coating pH and Assessment of Oral Microflora in Tobacco Chewers. Ijsrm.Human, 2017; Vol. 6(3): 119-128.

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