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FREQUENCY OF *SELENOMONAS NOXIA* IN ORAL MICROBIOTA OF OBESE AND NORMAL WEIGHT PEOPLE IN DUHOK-IRAQ

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ABSTRACT:
Obesity represents one of the major problematic health issues worldwide. Recent evidences suggest that obesity is related with the alteration of the oral microbiome. The aim of this study was to measure the salivary bacterial *Selenomonas noxia* in Duhok population. A total of 155 saliva samples were collected from individuals (aged between 19-35 years) of both genders (86 females and 69 males). The individuals were divided into three groups (obese, overweight, and normal weight) based on their body mass index. Bacterial genomic DNA was extracted from saliva samples. Molecular detections of *Selenomonas noxia* were performed by the polymerase chain reaction. Among the 155 participants, 34.1% were obese, 26.4% overweight and 39.3% normal weight individuals. The prevalence rate of oral *S. noxia* among all people was 82.6%. The highest rate of *S. noxia* was in obese people (86.8%), followed by overweight (85.4%) and normal weight people (77%). The prevalence of *S. noxia* in overweight people was statistically significant in compare with the normal weight people ($p<0.0001$). Moreover, the oral carriage of *S. noxia* was highest among the overweight females (94.5%) followed by obese females (88.9%). However, no significant difference was found compared to males. The result revealed that it is possible to assume that the expansion of *S. noxia* in saliva is due to obesity. Moreover, the composition of salivary microbiome may lead to the risk that the overweight group is at risk of future obesity. However, further investigations are required with larger sample and participants with different socioeconomic status in order to address the exact link between obesity and oral bacteria. This could lead to a new and promising therapeutic way for improving human's health.

KEYWORDS: Obesity, *Selenomonas noxia*, BMI, Duhok, Iraq.

1. INTRODUCTION

Obesity is considered one of the major health risks worldwide and the incidence of obesity in developed countries is approximately more than 20% (Hruby and Hu, 2015). Studies revealed that obese people have high ratio of Firmicutes to Bacteroidetes (F/B) bacteria profile in their gut in compare with normal weight people (Ley *et al*., 2005). Oral microbiome is comprised of a vast array of bacterial species that interact in complex ways, which can influence oral health and disease. Researchers found that oral microbiome is a significant risk factor and it plays an important role in oral diseases and other disease such as diabetes mellitus cardiovascular diseases and bacteremia (Makkar *et al*., 2018). However, few studies have investigated the overall characterization salivary microbiome profile of individuals with obesity.

Selenomonas are a family group of anaerobic, gram-negative oral and gastrointestinal bacteria. *Selenomonas noxia* is one the five new species of the genus Selenomonas order (selenomonadales) that belongs the phylum Firmicutes. This bacterium was described for the first time by Moore *et al*., (1987). It is a gram negative rod, anaerobic, non spore-forming, motile bacteria (Moore *et al*., 1987). Several *Selenomonas* species have been recently associated with periodontal disease and poor oral health in human population (Craig *et al*., 2001). Studies support the hypothesis that microbial ecology and the prevalence of Firmicutes, particularly *S. noxia* can be an important component of our understanding of the regulation of weight and body composition (Boutaga *et al*., 2007).

An association between obesity and oral bacterial profile was first studied by Godson *et al*., (2009). Many researchers have found that the salivary microbiome have a higher phylogenetic diversity in obese people (Piombino *et al*., 2014; Takeshita *et al*., 2016; Wu *et al*., 2018). The aim of this study was to evaluate the prevalence of *S. noxia* in saliva samples from normal weight, overweight and obese people in Duhok city, Kurdistan region-Iraq utilizing polymerase chain reaction (PCR).

2. MATERIAL AND METHODS

2.1 Study design

The study was conducted in the period between September 2018 and May 2019 in Duhok city, Kurdistan region, Iraq. A total of 155 saliva samples were collected from overweight, obese and normal body weight healthy adults (aged between 19-35 years) from both genders (86 females and 69 males).

2.2 Samples collection

Samples were collected based on previously described work by Wu *et al*., (2018). Briefly, unstimulated saliva was collected from participants between 9:00 and 12:00 am in separate area in Duhok city. Participants were requested to refrain from drinking, eating, and tooth brushing about 1 hr before sampling. Additionally, any food residue was removed from the mouth by rinsing with water. After 10 min, 5 ml of saliva was spitted into a 50 mL DNA-free sterile container labelled with identification number, age, gender, date and time of

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collection. The samples were then transported directly to the laboratory for further investigation (Wu *et al*., 2018).

2.3 Questionnaire and body mass index (BMI) test

Participants completed a brief questionnaire to find out whether they had any oral issues, such as gum disease and bleeding when brushing. The exclusion criteria involved: existence of any systemic disease, use of medications, smoking, pregnancy/lactation, used antibiotics (in the last three months), any chronic disease such as psychiatric disorders, anorexia, acute relapse etc. Also, insufficient quantity (<2 mL) or insufficient quality (concentrated) saliva samples were excluded. The body mass index (BMI) of all people was calculated as weight in kg divided by height (in cm)-squared. Next, the participants were grouped into three categories according to their BMI based on the WHO guidelines (WHO, 2018). These include normal individuals (BMI between 18.5 and 24.9 kg/m2), overweight individuals (BMI between 25.0 and 29.9 kg/m2) and obese individuals (BMI ≥30.0 kg/m2).

2.4 Genomic DNA extraction

Bacterial genomic DNA from saliva samples was extracted using a commercial DNA purification kit (Promega, USA) according to the manufacturer's recommendations. In brief, 2 ml of saliva sample was centrifuged at 13,000×g for 2 min. Next, the cell pellet was suspended in 480µl of 50mM EDTA. Then, 120 µl of lysozyme (10mg/ml) was added and gently mixed by pipetting. The sample was incubated at 37°C for 40 min then centrifuged for 2 min. The pellet was suspended in 600 µl of nuclei lysis solution and incubated at 80°C for 5 min. After that, 3µl of RNase solution was added and incubated at 37oC for 40 min. Then, 200µl of protein precipitation solution was added and mixed vigorously for 20 seconds. The samples were cooled on ice for 5 min and centrifuged for 3 min. The genomic DNA (supernatant) was concentrated and desalted by adding 600 µl of isopropanol. The tube was gently mixed by inversion until forming the thread-like strands of DNA. The sample was then centrifuged for 2 min and the supernatant was transferred to a new tube. Then, 600µl of 70% ethanol was added and the sample was centrifuged for 2 min. Finally, 100µl of DNA rehydration solution was added and the sample incubated overnight at 4°C. The rehydrated DNA was stored at −20°C until used for PCR.

2.5 DNA concentration and purity

The extracted genomic DNA was measured using a NanoDrop spectrophotometer (Thermo scientific, USA). This work was done in the PCR department in the public central laboratories of Duhok. The spectrophotometer calculates the concentration of the DNA based on the 260/280 absorbance ratio. Samples were generally accepted as pure DNA when the ratio ranged between 1.8-2.0.

2.6 Polymerase Chain Reaction (PCR)

Molecular detections of *Selenomonas noxia* from all collected saliva samples were performed by Polymerase Chain Reaction. Two species-specific oligonucleotide primer pairs were used. *S. noxia* Forward primer- SNF1: 5'TCTGGGCTACACACGTACTACAATG3', and *S. noxia* Reverse prime- SNR1, 5'GCCTGCAATCCGAACTGAGA3' with amplicon lengths of 97bp (Cruz *et al*., 2015; Bui *et al*., 2017).

The reactions of PCR amplification were achieved in 20 µl as a final volume. Each PCR reaction contained 1µl primers (forward and revers) at a final concentration of 10 pmol/ µl each; 10 µl of deoxyribonucleotide master mix (Promega, USA); 1 µl of extracted DNA at a final concentration of 25-50ng/µl; and 7 µl of nuclease-free water. PCR reactions were carried out in a C1000 thermal cycler (Bio-Rad) using the following conditions: 95°C for 5 min as a denaturation step, followed by 35 cycles of denaturation at 94°C for 30 s; 20 s at 62°C for annealing; and 72°C for 30 s extension. Finally, extension step at 72°C for 5 min.

2.7 Agarose gel electrophoresis

1% Agarose gel was prepared for separating fragments of the amplified PCR products according to their size. Electrical current was used to separate amplified DNA in 1x TBE buffer. The electrical power was turn on 45 V. for 15 min and then risen to 65 V. for 40 min. In order to stain the agarose gels, they were dipped in distilled water containing ethidium bromide at a final concentration of 5 µg ml-1 for 30-45 min. The DNA bands were visualised using U.V illumination at 366nm wavelengths (HVD life science, Austria). The bands sizes were estimated by comparison to the bands of the 100 bp DNA ladder (1500 bp -100 bp) (Atom Scientific, UK).

2.8 Statistical Analysis

Statistical analysis has been conducted utilizing Chi square test. The statistical analysis was carried out with Minitab 18. $p < 0.05$ values were considered as significant.

3. RESULTS

The results revealed that among the 155 participants, three BMI groups were identified including 53 (34.1%) obese people with BMI (≥30.0 kg/m2); 41 (26.4%) Overweight people with BMI (between 25.0 and 29.9 kg/m2) and 61 (39.3%) normal weight (control) individuals with BMI (between 18.5 and 24.9 kg/m2) (Table 1). The highest BMI rate was found in obese female (42%).

Table 1. The study population according to BMI category.

	Normal weight	Overweight	Obese
Male	29 (42%)	23 (33.3%)	17 (25%)
Female	32 (37.2%)	18 (20%)	36 (42%)
Total	61 (39.3%)	41 (26.4%)	53 (34.1%)

Salivary bacterial populations in 155 individuals (53 Obese, 41 overweight and 61 normal weight) measured targeting *Selenemonas noxia* by PCR assay. DNA was successfully extracted from all saliva samples. Each of the DNA was then processed using PCR. The species-specific primers SNF1 and SNR1 were utilized to detect *S. noxia*. The PCR product with the expected size of 97 bp was considered as positive for *S. noxia* as it is shown in figure 1 (Cruz *et al*., 2015).

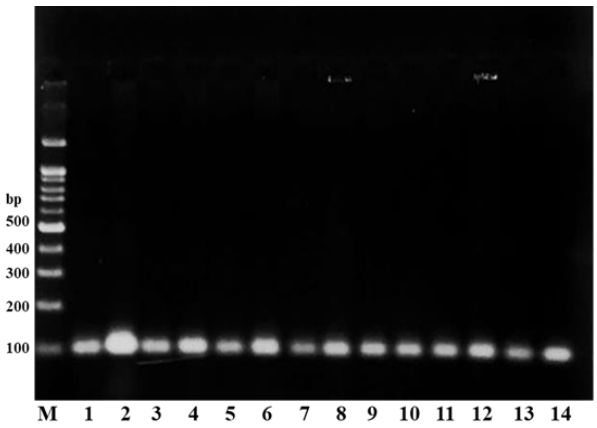

Figure 1. % Agarose gel analysis showing the amplicon bands from PCR product for *S. noxia*. Lanes: M, 100 bp DNA marker; 1-14, ~ 97 bp fragment amplified using SNF1 and SNR1 primers for different screened DNA samples.

The analysis of this screening on agarose gel revealed that the prevalence rate of *S. noxia* among all people was 82.6% (128/155) (Table 2). The prevalence rates of oral *S. noxia* in normal weight (control), overweight and obese people were 77% (47/61), 85.4% (35/41) and 86.8% (46/53) respectively.

Table 2: Distribution of oral *S. noxia* in people with different BMI in both sexes.

BMI groups	Gender	*S. noxia*	
		Positive	Negative
Normal weight	Male	22 (75.9%)	7 (24.1%)
	Female	25 (78.1%)	7 (21.9%)
	Total	47 (77%)	14 (23%)
Overweight	Male	18 (78.3 %)	5 (21.7%)
	Female	17 (94.5%)	1 (5.6%)
	Total	35 (85.4%)	6 (14.6%)
Obese	Male	14 (82.4%)	3 (17.6%)
	Female	32 (88.9%)	4 (11.1%)
	Total	46 (86.8%)	7 (13.2%)

The highest level of the total salivary bacterium *S. noxia* was recorded in obese people group, followed by the overweight and normal weight people.

The prevalence of *S. noxia* in overweight people was higher in compare with the normal weight (control) people but this rate was statistically not significant ($p=0.2996$). However, the carriage rate of oral *S. noxia* in obese people was higher in compare with the normal weight (control) people and this rate was statistically significant ($p<0.0001$). Moreover, the prevalence rate of *S. noxia* in obese people was higher than the rates in overweight people. However, this differences was statistically not significant ($p= 0.8425$).

It was observed that *S. noxia* microbial profiles according to the gender of the people differed between the groups. However, the statistical analysis shows lack of significant differences ($p>0.01$) among overweight and obese people when compared with normal weight (control) group. The carriage rate of *S. noxia* was highest amongst the overweight females (94.5%) followed by obese females (88.9%). However, this difference was statistically not significant.

4. DISCUSSION

The occurrence of obesity has grown significantly in the last decades. According to the WHO, in 2016 approximately more than 1.9 billion adults globally were overweight and more than 650 million were obese (WHO, 2018). The data of this study revealed that there were a high percentage of overweight and obese people in Duhok population. Moreover, the highest percentage of BMI (obesity) was found in female population. Although tremendous research focused on the causes of obesity, there is still misunderstanding of its exact mechanism (Komaroff, 2016). The interactions between genetics and environment is the most important factor that contributes to obesity as well as it is the result of complex pathological adaptations of body cells (Bouchard and Tremblay, 1990; Williams, 2012).

During the previous decade, the role of microbiome in obesity has aroused curiosity and many original articles were published about this area (Piombino *et al*., 2014). In addition to host genotype and age, the composition of gut microbiota within an individual and moreover the dynamic changes is also associated by external factors such as diets, drugs, and anthropometric measures, which may result in dysbacteriosis (Rothschild *et al*., 2018). Recently, an association between the salivary bacterial profile and obesity was reported in many studies (Goodson *et al*., 2009; Zeigler *et al*., 2012). There are huge evidences that the prevalence of Firmicutes, particularly *S. noxia* can play an important role in the regulation of weight and body composition (Boutaga *et al*., 2007; Goodson *et al*., 2009).

The purpose of this study was to investigate the carriage rates of *S. noxia* in saliva of people with different BMI in Duhok city using molecular methods. In general, this study showed that *S. noxia* had high level of existence among healthy people in this region (82.6%). Since the new nature discovery of these obligatory anaerobic bacteria, little is known about the oral prevalence of *S. noxia* with healthy oral populations to establish basic knowledge of their epidemiology. Because they are fastidious anaerobic bacteria, culture techniques of *Selenomonas* spp. are rarely found in microbiological laboratories and probably time consuming. However, rapid polymerase chain reaction (PCR) assay specific for this organism broke this barrier through several studies conducted to detect this bacteria (Cruz *et al*., 2015).

Although most studies have focused on intestinal microbiota, all gastrointestinal bacteria at a specific point in time enter through the oral cavity and some of these transients can be localized in there. Goodson *et al*., (2009) provided evidence that *S. noxia* from saliva could be the only Firmicutes that has a role in developing adiposity. Studies showed that obese people have relatively higher Firmicutes compared to Bacteroidetes (Koliada *et al*., 2017). Almost one gram of oral bacteria, containing about 10^{11} cells, is ingested daily with 500-1500 ml of saliva (Socransky and Haffajee, 2005). Metabolites of the microbiota of the oral cavity enter the bloodstream and the human body will be in an inflammatory state. Then it contributes to the development of different chronic diseases of the digestive system (Abed *et al*., 2016).

Data of this study revealed that the highest level of the total salivary bacterium *S. noxia* was recorded in obese (86.8%) followed by overweight (85.4%) and then normal weight (control) people (77%). This prevalence was significantly increased in obese people in compare with the normal weight people but it was not significant compared with overweight people. This result is compatible with the study made by Jeelani *et al*., 2013 as reported that levels of *S. noxia* in the oral cavity in obese people are higher than normal weight individuals (Jeelani *et al*., 2013).

It is known that the appearance of specific microbes in the gut, such as Firmicutes can promote the absorption of monosaccharides and play a role in the development of obesity (DiBaise *et al*., 2008; Tehrani *et al*., 2012). Other theories about the mechanisms by which intestinal microbiome increase metabolic disturbances involve: increase the permeability of the intestinal, high generation of short chain fatty acids, decrease angiopoietinlike protein 4, de novo lipogenesis, AMP activated kinase, and maintenance of a subclinical inflammatory status (Moreno-Indias *et al*., 2014). Macrophages are activated to produce a number of proinflammatory cytokines such as interleukin-1, prostaglandins, and tumor necrosis-α (Mazumdar *et al*., 2009). The latter is produced by infected periodontal tissue, which may be a major inflammatory cytokine contributing to obesity. Studies showed that periodontal bacteria can induce the generation of inflammatory cytokines, like TNFα, which alter

the metabolism of energy to synthesis of lipid and can contribute to obesity (Iwamoto *et al*., 2001; Goodson *et al*., 2009).

Although, multiple researchers are focusing on the intestinal microbiota, little studies shed light on the oral cavity bacterial profile and its association with overweight (McDermott, 2016). Oral studies through saliva screening also demonstrated the impact of oral microbiome on obesity (Goodson *et al*., 2009; Abkar *et al*., 2019). The importance of oral microbiome in systemic body inflammation is not less than the gut microbiota, because the infected oral tissue by microbiota also increase circulating proinflamatory cytokines and promote insulin resistance, thereby we can associate the oral microbiome to obesity.

Evidences support indirectly the hypothesis that alteration of the oral flora could be associated with obesity. The role of microbiota in regulating bodyweight was came from studies of animal model, where transplantation of gut flora through fecal samples from conventional mice in to free germ mice resulted more than 50% increase in body weight (Ellekilde *et al*., 2014). Several *Selenomonas* species have been more recently associated with periodontal disease and poor oral health in human population (Craig *et al*., 2001).

Although the level of *S. noxia* was highest among the overweight females followed by obese females, the level of the bacterium according to gender distribution had no significant differences among all people groups. This finding was in agreement with a study achieved by Goodson *et al*., (2009). As he demonstrated that 98.4% of the overweight females carried *Selenomonas noxia* in their salivary microbiological composition (Goodson *et al*., 2009).

There are several patholophysiological mechanisms behind systemic metabolic dysfunctions contributed to obesity such as insulin resistance, hypertension and dyslipidaemia (Ouchi *et al*., 2011). One of these mechanisms is that obesity leads to low grade systemic inflammation in obese people mediated by bacteria (Alabdulkarim *et al*., 2005; Hotamisligil, 2006). In addition to fat storage, adipose tissue also considered an endocrine organ that secretes at least 30 biological peptides and proteins in which some of them play a key role in the integration of systemic metabolism and inflammatory processes (Ahima and Flier, 2000). Moreover, it is hypothesized that oral bacteria can have a role in obesity by three approaches, first they redirect energy metabolism by increasing insulin resistance in response to increasing tumor necrosis factor (TNF). Secondly, bacteria increase metabolic efficiency (consuming even small amounts of calories) that causes the body to gain weight without changing the exercise and diet. Third, they can increase the appetite of the host, although there is no research to support this theory (Goodson *et al*., 2009; Abkar *et al*., 2019).

5. CONCLUSION

To conclude, an increase level of *S. noxia* was observed in saliva of obese and overweight people compared with normal weight. Thus, from our study we can conclude that in Duhok population, there is an expansion in the profile of *S. noxia* in higher BMI compared to normal category. Our findings provide clues that oral flora could be involved in mechanism that cause obesity. However, to investigate this relationship, further examination a lager more sample with different socioeconomic status may be necessary. If the oral microbiome confirmed to have a role in obesity, then new and promising therapeutic methods could be applied such as probiotics or prebiotics. This will provide a new target for improving the physical state of humans in future.

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