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Molecular Typing and Detection of Collagen Binding Genes among *Streptococcus mutans* Isolated from Diabetic and Non-diabetic Individuals in Northern Jordan

Samer F. Swedan*, Heba M. Obeidat and "Muhamad Ali" K. Shakhatreh

Department of Medical Laboratory Sciences, Jordan University of Science and Technology, P.O. Box 3030, Irbid 22110, Jordan,

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Abstract

Streptococcus mutans, a cause of dental caries, is an opportunistic pathogen leading to subacute infective endocarditis and bacteremia. Bacterial attachment to the heart valves, requires the presence of collagen-binding proteins (CBP), such as Cnm and Cbm; encoded by *cnm* and *cbm* genes, respectively. Herein, the prevalent *S. mutans* serotypes and genes for CBPs among diabetics and non-diabetic controls from Northern Jordan were investigated. Teeth swabs were cultured on TYCSB agar for *S. mutans* isolation. PCR was used to confirm the isolates' identity, and to identify the isolates' serotypes and CBP genes. The most prevalent serotype among the diabetics was c (71.6 %), followed by k (43.2 %), f (32.1 %), and e (13.6 %). Among the diabetics, 44.4 % harbored two or more serotypes. The most prevalent serotype among the non-diabetics was k (92.1 %), followed by c (69.8 %), f (25.4 %), and e (15.9 %). Among the non-diabetics, 92.1 % harbored two or more serotypes. The prevalence rates for *cnm* and *cbm* were 75.3 % and 11.1 %, respectively among the diabetics' isolates, and 38.1 % each among the non-diabetics' isolates. Due to the high prevalence of serotype k and the isolates harboring CBP genes, the Jordanian population maybe at the risk of developing *S. mutans*-related complications, such as infective endocarditis.

Keywords: *Streptococcus mutans*, Prevalence, Serotypes, Collagen-binding protein, Diabetes

1. Introduction

The mouth of healthy humans is colonized by a huge number of microorganisms, the majority of which constitute the bacterial normal flora. These microorganisms compete with other microbes for food and colonization sites, and act as a part of the host immune defense (Aas *et al.*, 2005, Forssten *et al.*, 2010). The oral bacteria include streptococci, staphylococci, lactobacilli, and various anaerobes such as *Bacteroides* spp. (Aas *et al.*, 2005).

Streptococcus mutans is a Gram-positive facultative anaerobic bacterium capable of tolerating O_2. It is capable of surviving at a temperature range of 18-40 ^oC. It is an important member of the oral normal flora in most individuals. Colonization of the mouth, and more specifically, the dental plaque begins after the eruption of teeth (Liljemark and Bloomquist, 1996). *S. mutans* is capable of metabolizing sucrose to form high molecular weight polysaccharides using the glucosyltransferase enzyme, which allows the bacterium to initiate plague formation (i.e., biofilms) on the teeth's enamel surface. The enamel is the external visible part of the teeth, and the hardest substance in the human body as it contains a very high concentration of minerals. Unless quickly removed, plaques will grow in size and become very difficult to eliminate. The plaques are composed of food debris, bacteria (mainly *S. mutans*), and extracellular products. *S. mutans* is the leading cause of dental caries worldwide because it metabolizes several carbohydrates, creating lactic acid that demineralizes teeth enamel (Usha and R, 2009, Forssten *et al.*, 2010, Esberg *et al.*, 2017). Following the destruction of enamel, the bacteria utilize digestive enzymes to degrade the protein matrix found in the dentin and cement layers of teeth (Loesche, 1986).

S. mutans is classified into four main serotypes; c, e, f, and k, based on the identity of surface rhamnose-glucose polymers (RGPs). Serotype c strains are the most common in the oral cavity (Nakano *et al.*, 2007). The RGPs are important components of the cell wall of streptococci and other bacterial species. The polymers of α1,2- and α1,3-linked rhamnose units form the backbones of the RGPs and the polysaccharide antigens of Lancefield group A, C, and E streptococci. However, little is known about the mechanisms of synthesis of these polysaccharides (Shibata *et al.*, 2002).

* Corresponding author. e-mail: sfswedan4@just.edu.jo.

S. mutans is an opportunistic pathogen capable of causing infective endocarditis and bacteremia (Nakano *et al.*, 2007). Transient but harmless, bacteremia can occur following the brushing and flossing of teeth, tooth extractions, and other dental procedures (Forner *et al.*, 2006). The presence of *S. mutans* in the blood stream allows bacterial colonization of the inner lining of the heart, the endocardium, and the heart valves, potentially leading to damaged heart valves (endocarditis) and other complications (Nomura *et al.*, 2006). Endocarditis could lead to a progressive destruction and damage of the affected heart tissue, and may lead to other complications such as vertebral infections, bacterial pneumonia, and hemorrhagic stroke (Nakano *et al.*, 2006, Biswas *et al.*, 2010, Mansour *et al.*, 2017, St Michael *et al.*, 2017). Endocarditis, if untreated, is a life- threatening condition, therefore, individuals with damaged heart valves are instructed to take antimicrobials prophylactically before undergoing dental procedures or a surgery (Nakano *et al.*, 2007).

The bacterial binding and colonization of the extracellular matrix, is the first step in the invasion of host cells. The ability of *S. mutans* to attach to surfaces requires the presence of collagen-binding proteins (CBPs). To date, two CBPs, namely Cnm and Cbm, have been identified, with Cbm typically being more common than Cnm (Nomura *et al.*, 2013). Parts of the extracellular matrix that are most commonly colonized by bacteria are collagen, fibronectin, and laminin. The 120KD Cnm protein possesses binding activity to type I collagen and laminin (Nomura *et al.*, 2009, Abranches *et al.*, 2011). CBPs not only allow the efficient colonization of dental pulp, but possibly other tissues as well (Nomura *et al.*, 2016).

The CBPs are not only involved in teeth decay, but are now recognized as important virulence factors allowing the virulent strains to invade tissue, be attached to surfaces, invade endothelial cells, and cause infective endocarditis. In support for the increased virulence of strains expressing CBPs, it was found that CBP-expressing strains, such as most serotype k isolates ,were accumulated in damaged blood vessels and resulted in increased tissue damage when tested in a mouse cerebral hemorrhage model (Nakano *et al.*, 2007, Abranches *et al.*, 2011, Nakano *et al.*, 2011, Palmer *et al.*, 2013).

Diabetic patients have higher blood and saliva sugar levels than non-diabetics, which may potentially affect the constituents of the bacterial oral flora (Vernillo, 2003). Hence, the researchers hypothesized the presence of *S. mutans* serotype and genotype differences among diabetics and non-diabetic controls. Several studies have investigated the potential differences in *S. mutans* carriage among diabetics and non-diabetics (Hintao *et al.*, 2007, Nomura *et al.*, 2009, De *et al.*, 2016, Rezazadeh *et al.*, 2016). In this study the most frequent *S. mutans* serotypes among diabetic and non-diabetic individuals from Northern Jordan, and the molecular prevalence of *S. mutans* strains possessing collagen binding protein genes have been determined.

2. Materials and Methods

2.1. Sample Collection and Bacterial Isolation

The current study was approved by the Institutional Review Board of Jordan University of Science and Technology. One hundred diabetics (type 2), and seventy-seven non-diabetics, from both genders and all age groups were recruited for this study using judgmental sampling method. Informed written consents from the participants or their legal guardians, were obtained before enrollment in this study. A teeth surface material was obtained from each participant by swabbing the teeth surfaces using a sterile cotton swab. The swabs were passed on the teeth surfaces and buccal areas of molars to obtain the bacterial samples. The swabs were immediately cultured on tryptone-yeast-cysteine agar medium (Lab M, UK) supplemented with 20 % sucrose and 0.2 U/mL bacitracin (TYCSB agar); i.e., primary culture. The TYCSB agar medium is selective for *S. mutans* and provides excellent recovery of *S. mutans* from teeth swabs (Wan *et al.*, 2002). All the secondary subcultures of *S. mutans* were done utilizing Mueller Hinton agar media (Scharlan Microbiology, Spain). Since many *S. mutans* serotypes could colonize the same individual, several *S. mutans* colonies from each primary culture were processed during all cultural manipulations to identify all possible serotypes and CBP genes using the PCR protocols below. All primary and secondary cultures were incubated aerobically at 37 °C with 5% CO_2 for 48 h.

2.2. Streptococcus mutans Identification

Preliminary isolates identification following growth on TYCSB selective media was based on obtaining a negative catalase test and observing characteristic bacterial morphology and Gram-reaction. Definitive isolate identification was done using a commercially available kit (liofilchem® Strepto-System 9R, Italy) according to manufacturer instructions.

2.3. DNA Extraction

Bacterial suspensions were prepared for total (chromosomal and plasmid) DNA extraction of *S. mutans* isolates. Briefly, several pure bacterial colonies were inoculated into 3 mL of brain heart infusion (BHI) broth, and tubes were incubated aerobically overnight at 37 ºC in the presence of 5 % CO_2.

Genomic DNA extraction was performed from 1 mL overnight BHI cultures using E.Z.N.A® Bacterial DNA kit (OMEGA bio-tek, USA), in accordance with the manufacturer instructions. DNA was eluted using 50 µL of elution buffer. DNA was stored at -20 ºC for later use in PCR.

2.4. PCR Assays

Genomic DNA from all the isolates was subjected to multiple PCR assays to confirm the identity of isolates (using species specific genes; *gtfD* and *gtfB*), determine isolates' serotypes, and to identify the presence of CBP genes (*cbm* and *cnm*). Table 1 indicates the primers used for PCR.

Table 1. Primers used for PCR amplification.

Primer	Purpose	Sequence (5`-3`)	Amplicon size (bp)	Reference
gtfD-F	*S. mutans* detection	GGCACCACAACATTGGGAAGCTCAGTT	433	(Hoshino *et al.*, 2004)
gtfD-R		GGAATGCCGATCAGTCAACAGGAT http://jmm.sgmjournals.org/content/58/4/469/T1.expansion.html - ref-10		
gtfB-F	*S. mutans* detection	ACTACACTTTCGGGTGGCTTGG	517	(Oho *et al.*, 2000)
gtfB-R		CAGTATAAGCGCCAGTTTCATC		
SC-F	Serotype c detection	CGGAGTGCTTTTTACAAGTGCTGG	727	(Shibata *et al.*, 2003)
SC-R		AACCACGGCCAGCAAACCCTTTAT		
SE-F	Serotype e detection	CCTGCTTTTCAAGTACCTTTCGCC	517	(Shibata *et al.*, 2003)
SE-R		CTGCTTGCCAAGCCCTACTAGAAA		
SF-F	Serotype f detection	CCCACAATTGGCTTCAAGAGGAGA	316	(Shibata *et al.*, 2003)
SF-R		TGCGAAACCATAAGCATAGCGAGG		
CEFK-F	Serotype k detection	ATTCCCGCCGTTGGACCATTCC	294	(Nakano *et al.*, 2004)
K-R		CCAATGTGATTCATCCCATACC		
cbm-EF	*Cbm* detection	AGCTGAAGTTAGTGTTGTAAAACCTGCTTC	393	(Nomura *et al.*, 2012)
cbm-ER		TAGGATCATCAACCTTAGTCAAGTACACGA		
cnm-DF	*Cnm* detection	TGGAGGTTCAGGGCAAGTATGTTGGTGATT	579	(Nomura *et al.*, 2012)
cnm-DR		GTCTTTTGATCAGGATTGTCAACTTTAGTC		

2.4.1. PCR for Streptococcus mutans Identity Confirmation and Serotype k Detection

PCR confirmation of the *S. mutans* isolate identity was done using the species-specific *gtfB* and *gtfD* gene primers. For *gtfB* PCR, amplification conditions consisted of 95 ºC for four minutes; thirty cycles of 95 ºC for thirty seconds, 59 ºC for thirty seconds, and 72 ºC for one minute, and 72 ºC for seven minutes. For *gtfD* PCR, amplification conditions consisted of 98 ºC for four minutes; thirty cycles of 98 ºC for ten seconds and 70 ºC for one minute, and 70 ºC for seven minutes. For each of the two assays, each 25 µL PCR tube contained 12.5 µL of 2x PCR master mix solution (i-MAX II, iNtRON Biotechnology, South Korea), 3 µL of template DNA, 0.75 µL of each primer (10 pmoles/µL), and 8 µL of nuclease-free water. *S. mutans* ATCC 25175 was used as positive control. The negative control consisted of a reaction without the addition of template DNA.

PCR identification of *S. mutans* serotype k was done using the primer pair CEFK-F/K-R. Amplification conditions consisted of 95 ºC for four minutes, thirty cycles of 95 ºC for thirty seconds, 60 ºC for thirty seconds, and 72 ºC for thirty seconds, and 72 ºC for seven minutes. Each 25 µL PCR tube contained 12.5 µL of 2x PCR master mix solution (i-MAX II), 2 µL of template DNA, 0.75 µL of each primer (10 pmoles/µL), and 9 µL of nuclease-free water. The negative control consisted of a reaction without the addition of template DNA.

2.4.2. Multiplex PCR for Detection of Streptococcus mutans Serotypes c, e, and f

Multiplex PCR for the detection of *S. mutans* serotypes c, e, and f utilized the respective primers indicated in table 1. PCR consisted of 96 ºC for two minutes, twent-five cycles of 96 ºC for fifteen seconds, 61 ºC for thirty seconds, and one minute at 72 ºC, and 72 ºC for five minutes. Each 25 µL PCR tube contained 12.5 µL of 2x PCR master mix solution (i-MAX II), 3 µL of template DNA solution, 1.25 of each of the respective primers (10 pmoles/µL), and 2 µL of nuclease-free water. *S. mutans* ATCC 25175 was used as positive control. The negative control consisted of a reaction without the addition of template DNA.

2.4.3. Multiplex PCR for the Detection of Streptococcus mutans Collagen Binding Protein Genes

Multiplex PCR for the detection *S. mutans* CBP genes; *cbm* and *cnm* utilized the primers indicated in table 1. PCR consisted of 95 ºC for four minutes, thirty cycles of 94 ºC for thirty seconds, 60 ºC for thirty seconds, and 72 ºC for thirty seconds, and 72 ºC for seven minutes. Each 25 µL PCR tube contained 12.5 µL of 2x PCR master mix solution (i-MAX II), 3 µL of template DNA, 0.75 µL of each primer (10 pmoles/µL), and 6.5 µL of nuclease-free water.

2.4.4. Agarose Gel Electrophoresis

PCR amplification products were identified following electrophoretic separation on 2 % agarose gels containing ethidium bromide at 140 volts for forty minutes. Five microliters of each PCR product was used per gel lane. DNA bands were visualized using a UV transilluminator provided with a gel documentation system using the Quantity One software (Biorad, USA). Fragment sizes for each PCR product were determined by comparison with a 100 bp DNA ladder (Quick-load DNA ladder, Cat #N0467S, New England Biolabs, USA) and with the positive control when available.

2.5. Statistics

The Statistical Package for Social Sciences (SPSS) software (version 23) (IBM, USA) was used for the statistical analysis of data. Frequency results were compared using the Chi-Square test. A *P* value less than 0.05 was considered statistically significant.

3. Results

3.1. Identification of Streptococcus mutans

Eighty-one out of one-hundred samples among the diabetics, as well as sixty-three out of seventy-seven of the non-diabetics' samples, were positive for *S. mutans*. Identification was based on morphologic, phenotypic, biochemical characteristics, and the detection of the *S. mutans* specific genes *gtfB* and/or *gtfD*.

3.2. Study Participants

The eighty-one diabetics having *S. mutans* had a mean age of 52.9 years with a standard deviation of 11.7 years, and a range of twelve to sixty-nine years. The sixty-three non-diabetics having *S. mutans* had a mean age of thirty-four years with a standard deviation of 18.7 years, and a range of five to seventy-two years. Table 2 demonstrates the distribution of participants according to gender and arbitrary age groups.

Table 2. Gender and age group distribution of the study groups.

	Age	Age group (Years)		
Criteria	Mean (Years)	< 30 Count (%)*	30 - 59 Count (%)*	> 50 Count (%)*
Diabetics	Female 53	2 (2.5%)	29 (35.8%)	16 (19.7%)
	Male 52	1 (1.2%)	22 (27.2%)	11 (13.6%)
Non-diabetics	Female 32	15 (23.8%)	14 (22.2%)	5 (7.9%)
	Male 36	13 (20.6%)	11 (17.4%)	5 (7.9%)

* Percentages were calculated out of the total number among each study group.

3.3. Streptococcus mutans Serotypes and Collage Binding Protein Genes

Among the diabetics group, serotype c was the most frequently encountered (71.6 %), followed by k at 43.2 %, f at 32.1 %, and e at 13.6 %. No significant distribution differences were observed in the three indicated age groups (Table 3). *Cnm* had the highest prevalence rate at 75.3 %, followed by the *cbm* at 11.1 % (Table 3).

Among the non-diabetics group, serotype k was the most frequently encountered (92.1 %), followed by c at 69.8 %, f at 25.4 %, and serotype e at 15.9 %. A statistically significant difference (P = 0.028) in the distribution of serotype c was observed among the three age groups with prevalence occurring at a higher rate in the young and the old age groups compared to the middle age group (Table 4). *Cnm* and *cbm* were identified at the same rate (38.1 %) (Table 4).

A statistically significant difference in the distribution of serotype k, *cnm*, and *cbm* was observed among the two study groups. Serotype k and *cbm* were more prevalent among the non-diabetics group; 92.1 % vs. 43.2 %, and 38.1 % vs. 11.1 %, respectively. In contrast, *cnm* was more prevalent among the diabetics group; 75.3 % vs. 38.1 % (Table 5). Among the diabetics group isolates, *cnm* and *cbm* were most prevalent with serotype c. Among non-diabetics group isolates, *cnm* and *cbm* were most prevalent with serotypes k and c (Table 6). However, no statistically significant associations were observed between each of *cnm* and *cbm*, and each of the different serotypes, among each of the study groups (Table 6).

Table 3. Distribution of serotypes and collagen binding protein genes according to age among the diabetics group.

Criteria		Age group (Years) < 30 Count (%)*	30 - 59 Count (%)*	> 59 Count (%)*	*P* value	Total (%)
Serotype k	Absent	2	29	15	0.934	46
	Present (%)	1 (33.3%)	22 (43.1%)	12 (44.4%)		35 (43.2%)
Serotype c	Absent	1	14	8	0.961	23
	Present (%)	2 (66.7%)	37 (72.5%)	19 (70.4%)		58 (71.6%)
Serotype e	Absent	3	45	22	0.556	70
	Present (%)	0 (0%)	6 (11.8%)	5 (18.5%)		11 (13.6%)
Serotype f	Absent	2	37	16	0.489	55
	Present (%)	1 (33.3%)	14 (27.5%)	11 (40.7%)		26 (32.1%)
Serotype	c (%)	1	19	11	0.926	31 (49.2%)
	c + e + f (%)	0	1	0		1 (1.2%)
	C + e + f + k (%)	0	1	3		4 (4.9%)
	c + f (%)	0	1	0		1 (1.2%)
	c + f + k (%)	0	3	1		4 (4.9%)
	c + k (%)	1	12	4		17 (21%)
	e (%)	0	2	1		3 (3.7%)
	e + f (%)	0	1	1		2 (2.5%)
	e + k (%)	0	1	0		1 (1.2%)
	f (%)	1	5	2		8 (9.9%)
	f + k (%)	0	2	4		6 (7.4%)
	k (%)	0	3	0		3 (3.7%)
Cnm	Absent	1	14	5	0.643	
	Present (%)	2 (66.7%)	37 (72.5%)	22 (81.5%)		61 (75.3%)
Cbm	Absent	3	45	24	0.820	
	Present (%)	0	6 (11.7%)	3 (11.1%)		9 (11.1%)

* Percentages were calculated out of the total number for each age group.

Table 4. Distribution of serotypes and collagen binding protein genes according to age among the non-diabetics group.

Criteria		Age group (Years) < 30 Count (%)*	30 - 59 Count (%)*	> 59 Count (%)*	*P* value	Total (%)
Serotype k	Absent	2	2	1	0.960	5
	Present (%)	26 (92.9%)	23 (92%)	9 (90%)		58 (92.1%)
Serotype c	Absent	4	12	3	0.028	19
	Present (%)	24 (85.7%)	13 (52%)	7 (70%)		44 (69.8%)
Serotype e	Absent	25	18	10	0.074	53
	Present (%)	3 (10.7%)	7 (28%)	0		10 (15.9%)
Serotype f	Absent	23	17	7	0.466	47
	Present (%)	5 (17.9%)	8 (32%)	3 (3%)		16 (25.4%)
Serotypes	c (%)	1	1	0	0.353	2(3.2%)
	c + e + f + k (%)	1	1	0		2 (3.2%)
	c + f + k (%)	0	1	0		1 (1.6%)
	c + f + k (%)	2	0	0		2 (3.2%)
	c + k (%)	20	10	7		37 (58.7%)
	e + k (%)	2	5	0		7 (11.1%)
	f (%)	1	1	1		3 (4.8%)
	f + k (%)	1	6	2		9 (14.3%)
Cnm	Absent	16	14	9	0.136	39
	Present (%)	12 (42.9%)	11 (44%)	1 (10%)		24 (38.1%)
Cbm	Absent	14	16	9	0.079	39
	Present (%)	14 (50%)	9 (36%)	1 (10%)		24 (38.1%)

* Percentages were calculated out of the total number for each age group.

Table 5. Distribution of serotypes and collagen binding protein genes among study groups' isolates.

Criteria		Group Diabetics Count (%)*	Non-diabetics Count (%)*	*P* value
Serotype k	Absent	0	5	0.000
	Present (%)	35 (43.2%)	58 (92.1%)	
Serotype c	Absent	23	19	0.817
	Present (%)	58 (71.6%)	44 (69.8%)	
Serotype e	Absent	70	53	0.699
	Present (%)	11 (13.6%)	10 (15.9%)	
Serotype f	Absent	55	47	0.380
	Present (%)	26 (32.1%)	16 (25.4%)	
Cnm	Absent	20	39	0.000
	Present (%)	61 (75.3%)	24 (38.1%)	
Cbm	Absent	72	39	0.000
	Present (%)	9 (11.1%)	24 (38.1%)	

Table 6. Distribution of collagen binding protein genes among *S. mutans* serotypes of the study groups.

Criteria			*cnm* Absent Count	Present Count (%)*	*P* value	*cbm* Absent Count	Present Count (%)*	*P* value
Diabetics	Serotype k	Absent	11	35	0.852	39	7	0.178
		Present (%)	9	26 (42.6%)		33	2 (22.2%)	
	Serotype c	Absent	6	17	0.854	21	2	0.663
		Present (%)	14	44 (72.1%)		51	7 (77.7%)	
	Serotype e	Absent	19	51	0.197	62	8	0.819
		Present (%)	1	10 (16.4%)		10	1 (11.1%)	
	Serotype f	Absent	17	38	0.059	48	7	0.501
		Present (%)	3	23 (37.7%)		24	2 (22.2%)	
Non-diabetics	Serotype k	Absent	5	0	0.068	4	1	0.385
		Present (%)	34	24 (100%)		35	23 (95.8%)	
	Serotype c	Absent	12	7	0.893	11	8	0.667
		Present (%)	27	17 (70.8%)		28	16 (66.7%)	
	Serotype e	Absent	35	18	0.120	34	19	0.398
		Present (%)	4	6 (25%)		5	5 (20.8%)	
	Serotype f	Absent	29	18	0.955	29	18	0.955
		Present (%)	10	6 (25%)		10	6 (25%)	

***** The percentages were calculated out of the total number for each collagen binding protein gene.

4. Discussion

Oral health is one of the determinants of the quality of life. It limits and prevents oral diseases that arise from different microbial species colonizing the oral cavity. These organisms tend to form dental plaque biofilms. *S. mutans* is the most frequent species in dental plaques. It has several common serotypes, namely c, e, f, and k. Some isolates also carry genes for CBPs, which enable the bacterial binding and colonization of the heart endocardium and damaged heart valves, leading to infective endocarditis and bacteremia. In this study, the prevalent serotypes and the major virulence CBP genes of *S. mutans* isolated from the teeth of diabetic and non-diabetic subjects from Northern Jordan were investigated.

Teeth of diabetics and non-diabetics were swabbed, and the swabs were used to inoculate TYCSB medium, one of the best media for isolation of *S. mutans* (Wan *et al.*, 2002). After growth and identification, multiple bacterial colonies were taken from each plate to assure the recovery of multiple serotypes, if present. Bacterial DNA was extracted and used in several PCR assays using primers specific for *S. mutans*, its serotypes, and CBP genes.

Among the diabetics group, 81 % (81/100) of the subjects sampled were positive for *S. mutans*. All isolates were positive for at-least one of the species-specific *S. mutans* genes (*gtfB* and *gtfD*). The most prevalent serotype among this group was serotype c (71.6 %), followed by k (43.2 %), f (32.1 %), and e (13.6 %). Many subjects harbored several *S. mutans* serotypes, namely, thirty-six subjects (44.4 %) had two or more serotypes. No significant serotype distribution differences were observed in the three indicated age groups. A recent study from Italy reported serotype c as the major serotype among diabetics at 82 %, which is consistent with the findings of the current study, followed by e at 11.8 %, and f at 5.9 % (De *et al.*, 2016). However, the study from Italy utilized a small sample size (seventeen total isolates). Hence, the reported results may not be highly representative.

Among the non-diabetics group, 81.8 % (63/77) of the subjects sampled were positive for *S. mutans*. All isolates were positive for at-least one of the species-specific *S. mutans* genes (*gtfB* and *gtfD*). The most common serotype among the participants was serotype k (92.1 %; 58/63), followed by c (69.8 %), f (25.4 %; 16/63), and e (15.9 %; 10/63). Most subjects harbored several *S. mutans* serotypes; namely, fifty-eight subjects (92.1 %) had two or more serotypes. A statistically significant difference (P = 0.028) in the distribution of serotype c was observed among the three age groups with prevalence occurring at a higher rate in the young and the old age groups compared to the middle age group. Such differences may be related to dietary differences or differences in oral-hygiene practices according to age.

Studies from Japan, Europe, and North America, which investigated the prevalence of *S. mutans* serotypes among healthy individuals, reported that serotype c was the most-frequent, occurring at a prevalence rate of 70-80 % (Nomura *et al.*, 2009). Similarly, serotype c was also present in the current study at rates similar to those reported worldwide (i.e., 71.6 % and 69.8 % among the diabetics and non-diabetics groups, respectively) (Nomura *et al.*, 2009). In contrast to other reports, serotype k was the most frequently identified serotype among the non-diabetics group (92.1 %), and the second most frequent among the diabetics at 43.2 %. The difference in serotype k prevalence among the two groups was statistically significant. Differences observed regarding serotype k prevalence from that of the previous reports could be attributed to population, socioeconomic, and life-style differences, use of different sampling sites, methods of cultivation and isolation, differences in sugars intake and eating habits, and differences in oral hygiene practices. Furthermore, serotype k was more prevalent among the controls than the diabetics. This difference could be attributed to the higher blood sugar levels (and consequently higher saliva sugar levels), and other clinical and physiological differences among the diabetics, compared to the non-diabetics.

A strong relationship has been reported between serotype k and infective endocarditis. This may be attributed to a reduction in the content of glucose side chains in its RGPs, leading to less solubility in blood and higher resistance to phagocytosis by neutrophils (Nakano *et al.*, 2004). Studies from Japan and the UK, reported the entry of serotype k *S. mutans* to the circulation following dental procedures, when no antimicrobial agents were administered for prophylaxis (Nakano *et al.*, 2004, Nakano *et al.*, 2007, Biswas *et al.*, 2010). Therefore, Jordanians having this serotype are potentially at a higher risk of developing *S. mutans*-induced heart-related complications.

Among the diabetic's group isolates, the CBP gene *cnm* was the most prevalent at 75.3 %, followed by *cbm* at 11.1 %. In contrast, among the non-diabetics group isolates, *cnm* and *cbm*, were identified at the same rate (i.e., 38.1 %). Following entry into the circulation, the attachment of *S. mutans* to the exposed collagen tissue is an important step for the initiation of infective endocarditis. Recent studies in Thailand, Fenland, and Japan had shown that the collagen binding proteins encoded by *cnm* and *cbm*, mediate bacterial binding to type I collagen and enhance bacterial virulence (Nakano *et al.*, 2004, Abranches *et al.*, 2011). These genes were predominantly frequent among k and f serotypes, and less frequently among c, and e serotypes. Furthermore, these genes appear to be common worldwide (Abranches *et al.*, 2009, Nomura *et al.*, 2012). Interestingly, *cnm* and *cbm* were most prevalent with serotypes k among diabetic's group isolates, which is consistent with what was reported by others. However, contrary to the previous reports, *cnm* and *cbm* were also prevalent with serotype c isolates among both study groups. Nonetheless, no statistically significant associations were observed regarding the distribution of each of *cnm* and *cbm* with the various serotypes, which is likely due to the small sample size. Although, the serotype k isolates that express CBPs are associated with infective endocarditis, hemorrhagic stroke, and inhibition of platelets aggregation, nonetheless, the relationship between clinical conditions and *S. mutans* serotype remains unclear, as well as the association between *S. mutans* and systemic disease (Nakano *et al.*, 2011, Aikawa *et al.*, 2012).

A study in Japan, reported the presence of *cnm* among approximately 10 % of isolates (Aikawa *et al.*, 2012). *Cnm* in the current study was identified at a much higher frequency than that in Japan. This difference is likely due to inherent differences in predominant strains and genotypes.

Since the current study utilized a small-sized sample, the researchers suggest performing future larger-scale studies in Jordan to investigate the prevalent *S. mutans* serotypes and genotypes among diabetic and non-diabetic individuals, in addition to subjects diagnosed with infective endocarditis. This would yield more representative data for the Jordanian population, and facilitate the identification of potential associations between the serotypes and virulence genes, in addition to the association with clinical diseases.

5. Conclusions

The diabetics and the non-diabetics groups demonstrated similar prevalence of serotype c, e, and f isolates, but significant differences in prevalence of serotype k isolates. Due to the high prevalence of the serotype k isolates among the two study groups, and the high percentage of isolates harboring CBP virulence genes, the Jordanian population are at the high risk of developing *S. mutans*-related complications, such as infective endocarditis.

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