ORIGINAL ARTICLE

Molecular and serological typing of *Streptococcus mutans* strains isolated from young Galician population: relationship with the oral health status

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Abstract

The aim of this study was to determine the prevalence of *Streptococcus mutans* and its serotypes in samples from oral cavity of young Galician population and their relationship with the oral health state. The variables generally associated with dental caries, such as salivary flow rate, buffering capacity, eating habits, and lifestyle, were also analysed. No relationship was found between the variables studied and the presence of *S. mutans* in the oral cavity or the existence of dental caries. Presumptive strains of *S. mutans* were isolated from saliva samples from 48% of the analysed population. The use of conventional microbiological methods, API 20 Strep system, and species-specific polymerase chain reaction (PCR) allowed to substantiate the identity of the strains as *S. mutans*. Multiplex PCR protocols, developed in this study for the simultaneous detection of *S. mutans* and serotypes c, e, and f and for detection of *S. mutans* and serotype k, also confirmed this result and demonstrated that serotype c was predominant in the studied young Galician population (86%). Serotypes e (8%), k (3%), and f (2%) were also detected. Serotype c was detected in carious and caries-free subjects, while the remaining serotypes were only found in subjects with caries.

Keywords *Streptococcus mutans* · Oral health · Multiplex polymerase chain reaction · Serotyping

Introduction

Dental caries is a multifactorial infectious disease caused by specific types of acid-producing bacteria that cause demineralization and destruction of the teeth (Oong et al. 2008; Xu et al. 2011). *Streptococcus mutans*, a member of the mutans streptococci group (MS), is considered one of the primary causative agents of dental caries in human and one of the pathogens involved with bacteraemia and infective endocarditis (Banas 2004). Early detection and identification of the microorganisms is therefore important for diagnosing and preventing these infectious diseases. *S. mutans* identification is routinely performed by conventional microbiological methods, based on the isolation and phenotypic characterization of the isolated microorganism using standard bacteriological methods (Hirasawa and Takada 2003) or API 20 Strep system (Al-mudallal et al. 2008). Several sets of primers, targeting 16S rRNA, *gorESL*, *ddlA*, and *gtf* genes (Garnier et al. 1997; Hoshino et al. 2004; Hung et al. 2005; Oho et al. 2000), have been designed for the differentiation of oral streptococci using conventional PCR. Among them, the set of primers based on *gtfB* (Oho et al. 2000) *and gtfD* (Hoshino et al. 2004) genes that encode enzymes that synthesize extracellular polysaccharides, which facilitates the adhesion of oral bacterial cells to tooth surfaces, are the most frequently used. Quantitative PCR using species-specific primers based on *gtfB* gene (Childers et al. 2011; Sánchez et al. 2013; Yoshida et al. 2003) demonstrated that this technique was more sensitive than culture-based method for the detection and quantification of *S. mutans* from oral samples. *S. mutans* is classified into serotypes c, e, f, and k according to the composition of rhamnose-glucose polysaccharides of the cell-wall (Nakano et al. 2004; Shibata et al. 2003). Historically, immunological

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methods like immunodiffusion analysis or fluorescent-antibody technique have been used for serotyping. The studies based on the analysis of loci responsible for antigenicity allowed the design of specific primers for the identification of *S. mutans* serotypes without the need for specific antisera (Nakano et al. 2004; Nakano et al. 2013; Shibata et al. 2003). Previous studies reported that *S. mutans* strains isolated from the oral cavity of young and adult populations from different geographical areas showed differences in the predominant serotype regardless of the oral health status of the individuals analysed (Carletto-Körber et al. 2015; Hirasawa and Takada 2003; Rao and Austin 2014). On the other hand, the presence of multiple serotypes per individual have being suggested to be associated with a greatest experience in caries, in comparison with those infected by a single serotype (Seki et al. 2006; Shibata et al. 2003). Outside the oral cavity, the serotypes e, f, and k have been associated to cardiovascular disease (Nakano et al. 2007). There are studies that analyse the relationship between detection of *S. mutans* in the oral cavity and presence of caries within Spanish population (De La Higuera et al. 1999; Sánchez et al. 2013). However, to our knowledge, there is no data about the distribution and prevalence of *S. mutans* serotypes associated to the oral cavity in relation to the health status. Therefore, the main objective of this study was to determine the diversity and prevalence of serotypes of *S. mutans* among young adults of Galicia (Northwest of Spain) and to examine their relationship with the oral health state. Evaluation of different phenotypic and molecular methods for *S. mutans* characterization was also carried out.

Material and methods

Source of bacterial strains and maintenance conditions

For the detection of *S. mutans*, saliva samples were taken from 125 young Galicians (18–28 years old) in two different sampling periods. In the first sampling, saliva samples were taken from a group of 36 individuals. In the second sampling, saliva samples from 89 subjects were obtained. For the 125 individuals sampled, information was retrieved on their general and oral health status (decayed, missing, and full teeth (DMFT)), sex, and locality of origin. Fifty-four of 89 individuals of the second sampling group completed also a questionnaire about their eating habits and lifestyles. For these 54 subjects, the rate of saliva flow and its buffering capacity were also analysed. The 125 volunteers signed a written informed consent form, approved by the Bioethics Committee of the University of Santiago de Compostela.

To obtain saliva, donors chewed a paraffin pellets to transfer bacteria from the surface of the tooth to the saliva. Saliva was collected in sterile tubes for isolation of *S. mutans*,

measurement of buffering capacity, and determination of saliva flow rate. The stimulated saliva flow rate was measured as the volume of saliva extracted, while individuals chewed paraffin for 5 min. The values ≥ 1 ml/min were considered "normal" and the values < 1 as "low" (Tenovuo 1997). The buffering capacity of saliva was measured using CRT buffer strips (pH indicator) and interpreted using the CRT buffer chart.

For detection of *S. mutans*, samples of saliva were immediately inoculated on agar surfaces of CRT bacteria (Ivoclar Vivadent AG, Schaan, Liechtenstein), as recommended by the manufacturer. After 48 h of incubation at 37 °C, characteristic *S. mutans* blue-rough colonies were selected and isolated using Mitis Salivarius agar (Sigma, St. Louis, MO, USA), supplemented with bacitracin 0.2 U/ml (MSB), and then transferred to brain heart infusion (BHI) (Pronadisa, Madrid, Spain) agar and nutrient agar supplemented with sucrose 0.5% (*w/v*) for isolation and further characterization. For comparative purpose, reference strains of *Streptococcus mutans* ATCC 25175^{T} (serotype c) from the American Type Culture Collection (ATCC) and strains MT8148 (serotype c), MT703R (serotype e), OMZ175 (serotype f), and FT1 (serotype k) were included in all assays. Stock bacterial cultures were stored frozen at -80 °C in Microbank commercial medium (Pro-Lab Diagnostics, Ontario, Canada) until use. All strains were routinely cultured on BHI agar culture media in a 5% CO_2 incubator at 37 °C overnight prior to use in experiments.

Phenotypic characterization

BHI agar cultures (48–72 h) of the strains were examined macroscopically to determine colour, size, surface, elevation, and edges of the colonies. Cell morphology was observed using a phase contrast microscopy (Nikon eclipse 50i). Catalase and Gram reactions, capacity to ferment lactose, ribose, raffinose, sorbitol and mannitol, arginine and aesculin hydrolysis, methyl red, and Voges-Proskauer reactions were also tested using standard procedures (MacFaddin 2003). Biochemical profiles of the isolates were also evaluated using the API 20 Strep strips (BioMérieux, Marcy-l'Étoile, France). The reading of the strips was conducted after 24 h of incubation at 37 °C. Taxonomic identification was made with the api*web* software by analysing the numerical profiles obtained from the readings, as well as the identification table issued by the manufacturer.

Molecular characterization of *S. mutans*

DNA extraction

Total DNA was obtained with GenElute Bacterial Genomic DNA Kit (Sigma, St. Louis, MO, USA) according to the manufacturer recommendations for Gram-positive bacteria of the genus *Streptococcus*. DNA was quantified using the

fluorimeter Qubit 2.0 and the Qubit double-stranded DNA (dsDNA) BR assay kit (Invitrogen, CA, USA) according to the manufacturers' manual. The DNA was kept at − 30 °C until use in the PCR analyses.

Conventional PCR

For simultaneous identification of the species and the serotypes c, e, and f, a multiplex PCR protocol was developed, using the primers described by Yoshida et al. (2003) and Shibata et al. (2003). Due to the similar amplicon size of serotypes f and k, a duplex PCR protocol including primers for serotype k (Nakano et al. 2004) and species-specific primers was also developed. Gene targets, primers sequences, and product size are detailed in Table 1. Primers were synthetized by Integrated DNA Technologies (IDT) (IA, USA). For multiplex PCR amplification, each reaction contained 20 μl of reaction mixture including 4 μl of × 5 Phire reaction buffer (Thermo scientific, Vilnius, Lithuania); 0.4 μl of 10 mM dNTP (200 μM each) (Thermo scientific, Lithuania); 1 μl of each primer pair at 2 μM for serotypes c, e, and f and 10 μM of species-specific primers, 0.4 μl of Phire Hot Start II DNA polymerase; and 1 μl of DNA and sterile water. Amplification conditions were 1 initial cycle of denaturing at 98 °C for 3 min, 30 cycles of 30 s at 98 °C, 60 s at 60.3 °C, and 1 min at 72 °C. For serotype k, PCR reaction contained the above-described components except the primers for non-k serotypes. Reactions lacking or including DNA from the reference strains of each serotype were used as negative and positive controls, respectively. PCR products were electrophoresed (100 V for 60 min) in 1.7% (w/v) agarose gels in Tris acetate EDTA buffer (TAE, Bio-Rad Laboratories) with Nucleic Acid Staining Solution (× 20,000, iNtRON Biotechnology, Seongnam-si, Korea). A 100-bp GeneRuler DNA ladder (Thermo Fisher, CA, USA) was included as a molecular weight marker. PCR products were visualized using an ultraviolet light transilluminator (UV Transilluminator 2000, Bio-Rad, CA, USA). Molecular mass of the amplicons was determined using the Quantity One v 4.6.5 software (Bio-Rad Laboratories).

Sequencing

Eighteen representative strains isolated from saliva during the two screening periods were selected for sequencing. The first 527 bp fragment of the 16S rRNA gene was amplified using the primers 005-F and 531-R described by Fontana et al. (2005) (Table 1). Sequences were edited and assembled to obtain a consensus sequence using Sequencer 4.0 programme (Gene Codes Corporation, USA). The consensus sequences were compared with universal databases in the NCBI (National Centre for Biotechnology Information) data bank using the Blast tool.

Statistical analysis

The statistical analyses were performed with one-way ANOVA and chi-square test. A p value < 0.05 was considered to indicate statistical significance. Statistical analyses were

Table 1 Primers used in this study for *S. mutans* identification and serotype determination

Primers	Target*	Sequence 5′–3′	Product size (bp)	Reference
Sequencing				
005-F	16S rRNA	GGAGAGTTTGATCCTGGCTCAG	527	Fontana et al. (2005)
531-R		TACCGCGGCTGCTGGCAC		
Species-specific				
Smut3368-F	*gtfB*	GCCTACAGCTCAGAGATGCTATTCT	114	Yoshida et al. (2003)
Smut3481-R		GCCATACACCACTCATGAATTGA		
Serotype c				Shibata et al. (2003)
SC-F	*rgpF*-ORF12	CGGAGTGCTTTTTACAAGTGCTGG	727	
SC-R		AACCACGGCCAGCAAACCCTTTAT		
Serotype e				
SE-F	*rgpF*-ORF12	CCTGCTTTTCAAGTACCTTTCGCC	517	
SE-R		CTGCTTGCCAAGCCCTACTAGAAA		
Serotype f				
SF-F	*rgpF*-ORF12	CCCACAATTGGCTTCAAGAGGAGA	316	
SF-R		TGCGAAACCATAAGCATAGCGAGG		
Serotype k				
CEFK-Fb	*rgpF*	ATT CCC GCC GTT GGA CCA TTC C	294	Nakano et al. (2004)
CEF-Rc		CCG ACA AAG ACC ATT CCA TCT C		

*16S rRNA, 16S ribosomal gene; *gtfB*, glucosyltransferase B gene; *rgpF-ORF12*, gene located in the variable region among serotypes c, e, and f between *rgpF* and ORF12

performed using the SPSS statistical software package (SPSS Corporation, Chicago, IL, USA).

Results

Oral health status

A total of 125 subjects were sampled, the 75% of the young adults have had caries experience and present an average DMFT of 1.7 ± 1.0. The 67% were females and the 33% males; 75% live in urban locations and the remaining 25% in rural locations (Table 2). Bacterial colonies with typical characteristics of *S. mutans* were recovered from saliva samples from 60 of the 125 (48%) individuals analysed (Table 2). The stimulated salivation flow rate and saliva buffering capacity were evaluated as caries-protective factors in 54 randomly selected individuals from the second sampling period. The 79% of these individuals presented a normal salivary flow rate ≥ 1 ml saliva/min, while the 21% produced a low salivary flow rate < 1 ml saliva/min. The mean flow rate of stimulate salivation of the studied population was 1.79 ± 0.97 ml/min (Table 2). According to the pH values, the 69%, 22%, and 9% of the subjects presented high, normal, and low buffering capacity, respectively (Table 2). Data from lifestyle and eating habits of these 54 individuals were also recovered. Some of the subjects manifested to possess habits related with a bad oral health like smoking, alcohol, sugary drinks, and starchy products consumption (Table 2). Thus, the 56% of the subjects said they consumed sugary products (i.e. pastry) more than three times a week, and the 43% did it between meals (Table 2). There was no statistically significant association ($p < 0.05$) between oral health status and lifestyle or eating habits. None of the variables studied were related to a higher caries index or to the presence of presumptive *S. mutans*.

Phenotypic characterization of *S. mutans* strains

Initially, 95% of the isolates (187 of 197 strains tested) were presumptively assigned to the *Streptococcus* genus based on their cell and colony morphology, positive response to Gram stain, and to the absence of catalase enzymatic activity. Strains isolated in the present study and *S. mutans* reference strains were biochemically characterized by standard tests or by the API 20 Strep commercial test. The 100% of the strains tested by conventional methods were positive for acetoin production and produced acid from lactose and mannitol, and none of the strains fermented ribose or hydrolysed arginine.

Six different numerical profiles were obtained from the strains tested with the API 20 Strep according to the api*web* data base (Table 3). All the profiles obtained leaded to the identification of all strains as *S. mutans* with scoring values from 97.7 to 99.9% of identity. Different profiles and scores

resulted from the inability of some strains to produce acid from lactose, raffinose, and sorbitol, and to hydrolyse aesculin and starch (Table 3). In addition, contrary to conventional tests, three strains were negative to produce acid from lactose, raffinose, and sorbitol using the API 20 Strep.

Sequences analysis

The sequences from 18 strains were compared with the NCBI database sequences and classified according to the criteria of Clinical Laboratory Standards Institute (2008), in which positive identification of species by 16S region can be assigned when the similarity score is $\geq 99\%$, and the similarity score differences with the next closest species are greater than 0.5–0.8%. Thus, 15 strains showed similarity score $> 99\%$ of similarity with other *S. mutans* strains sequences and were identified as *S. mutans*, and 3 strains with similarity scores $< 99\%$ with *S. mutans* could not be differentiated beyond the genus and remained as *Streptococcus* spp.

PCR identification and serotyping

In the present study, a total of 187 strains isolated from 60 individuals were identified as *Streptococcus mutans* by the amplification of *gtfB* gene using conventional PCR, confirming the results of phenotypic characterization. No amplification was observed in the negative control, or when DNA from other species was used. Electrophoretic analysis of the PCR products showed an amplicon of expected size (114 bp).

The conventional multiplex PCR protocol developed in this work for the simultaneous detection of the species *S. mutans* and its serotypes c, e, and f produced well-defined single bands of 727 bp (serotype c), 517 bp (e), 316 bp (f), and 114 bp (species), when DNA from reference strains of each serotype was used. No amplification was observed in the negative control, or when DNA from other streptococci was used (Fig. 1). PCR for serotype k and species identification was carried out in a duplex PCR. The optimized multiplex and duplex PCR procedures were used to determine the serotype on the 187 strains previously identified as *S. mutans*. The most common serotype in the studied population was serotype c with a prevalence of the 87%, followed by 8% for serotype e, 3% for serotype k, and 2% for serotype f. There was only one individual with multiple serotypes (serotypes c and k) and a DMFT of 2, whereas the 98% of the population harboured only one serotype with a mean DMFT of 1.9. The serotypes e, f, and k were present only in carious individuals, while serotype c was present in both carious and caries-free subjects. The distribution of *S. mutans* serotypes among the young Galician population analysed is described in Table 4.

Table 2 Oral health status and eating habits from the sampled population

General data		No. of sampled subjects ($n = 125$)	No. of subjects with presumptive *S. mutans* ($n = 60$)
Caries	+	94 (75%)	51 (85%)
	−	31 (25%)	9 (15%)
Sex	F	84 (67%)	43 (72%)
	M	41 (33%)	17 (28%)
Locality	U	94 (75%)	46 (77%)
	R	31 (25%)	14 (23%)
Saliva measurements		No. of subjects analysed ($n = 54$)	No. of subjects analysed with presumptive *S. mutans* ($n = 33$)
Saliva flow rate (ml/min)	≥ 1, normal	43 (79%)	28 (85%)
	< 1, low	11 (21%)	5 (15%)
Saliva buffering capacity[a]	pH ≥ 6, high	37 (69%)	24 (73%)
	pH = 4.5–5.5, normal	12 (22%)	8 (24%)
	pH ≤ 4.0, low	5 (9%)	1 (3%)
Population unhealthy lifestyles and consumption frequencies of sugary products[b]			
Alcohol	+	13 (24%)	6 (18%)
Cigarettes	+	9 (17%)	6 (18%)
Starchy foods	+	13 (24%)	5 (15%)
Drinks	+	14 (26%)	6 (18%)
Sugary products	+	30 (56%)	18 (55%)
Between meals	+	23 (43%)	14 (42%)

+, positive; − negative; *F*, female; *M*, male; *U*, urban; *R*, rural

[a] Buffer capacity was measured according to Meurman & Rantonen (1994)

[b] Consumed more than three times a week

Discussion

Streptococcus mutans is one of the most prevalent bacteria of the human oral microbiota and is widely recognized as a key etiological agent of human dental caries, being detected in more than 70% of healthy people and in more than 90% of those with active caries (Hirasawa and Takada 2003). In addition, this organism occasionally causes bacteraemia, abscesses, and infectious endocarditis (Nakano et al. 2010; Nomura et al. 2009). Therefore, the precise identification and characterization of this bacterium in the oral cavity can be considered important for the prevention of the development of dental caries and other diseases associated with *S. mutans*. In this study, conventional and molecular methods were

Table 3 Predominant biochemical characters of *S. mutans* with API 20 Strep

Streptococcus strains	Numerical profile	Significative taxon	Identification (%)
Reference strains			
S. mutans ATCC25175[T](c)	5240770	*S. mutans*	Excellent (99.9)
S. mutans MT8148 (c)	5240770	*S. mutans*	Excellent (99.9)
S. mutans MT703R (e)	5240770	*S. mutans*	Excellent (99.9)
S. mutans OMZ175 (f)	5240770	*S. mutans*	Excellent (99.9)
S. mutans FTI (k)	5240770	*S. mutans*	Excellent (99.9)
This study			
$n = 11$	5240770	*S. mutans*	Excellent (99.9)
$n = 2$	5240750	*S. mutans*	Excellent (99.9)
$n = 2$	5240330	*S. mutans*	Very good (99.9)
$n = 1$	1240370	*S. mutans*	Very good (99.9)
$n = 2$	5240771	*S. mutans*	Good (97.7)
$n = 1$	5240570	*S. mutans*	Very good (99.9)
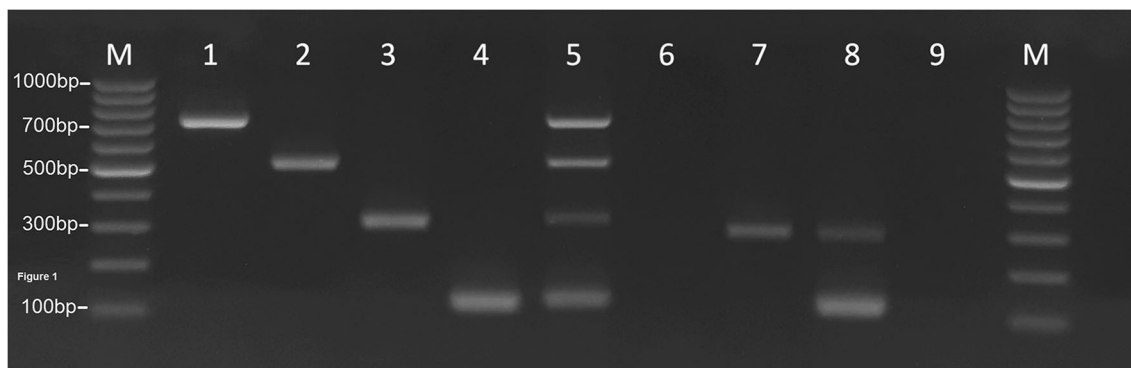
Fig. 1 PCR for the detection of *Streptococcus mutans* serotypes c (727 bp), e (517 bp), f (316 bp), and k (294pb), and species (114 bp). Lanes: M, 100 bp molecular weight marker; 1, strain ATCC 25175[T] (serotype c); 2, strain MT703R (e); 3, strain OMZ175 (f), 4, strain ATCC 25175[T]; 5, multiplex PCR for simultaneous detection of *S. mutans* and serotypes c, e, and f; 6, negative control; 7, strain FT1 (k), 8, duplex PCR of serotype k and *S. mutans*; 9, negative control

analysed to identify and characterize strains of *S. mutans* isolated from young Galician adults, as well as to determine if saliva properties and lifestyles have an impact on the presence of *S. mutans* and on the health status of the population. Saliva is important in oral functions such as mastication and swallowing, the buffering function of pH, antimicrobial activity, and cleaning action (Abelson 1989; Dodds et al. 2005). In this study, we have found that characteristics such us salivary flow rate and buffering capacity were not associated to dental caries or to the presence of *S. mutans* in young adults. Similar results were obtained by Cunha-Cruz et al. (2013), analysing the association between saliva characteristics and dental caries among adults from 18 to 49 years old. Although saliva production was in the normal range (1–3 ml/min) (Tenovuo 1997), and the buffering capacity was high among the population (69%), none of these factors were statistically associated with a better oral health or a lower presence of *S. mutans*.

Microbiological analysis of saliva samples showed that *S. mutans* was present in the oral cavity of 48% (60 of 125 individuals) of the sampled population, among which 85% (51 of 60 individuals) have had caries experience. This is consistent with the frequency in which *S. mutans* has been isolated in other studies, values ranging from 59 to 66% to up to even 100% (Krzyściak et al. 2017).

Table 4 *S. mutans* serotypes' presence in young adults

	Carious subjects (*n* = 51)	Caries-free subjects (*n* = 9)
Single serotype		
c	43	9
e	5	-
f	1	-
k	1	-
Multiple serotype		
c/k	1	-

The results of phenotypic characterization showed a good correlation between conventional microbiological tests and API 20 Strep, confirming the suitability of this commercial system for the identification of MS group. Although false negative reactions were detected in API 20 Strep for acid production from lactose, raffinose, and sorbitol, characters considered as differential for the species *S. mutans* (Vos et al. 2011), the isolates were correctly identified by the API system. Even though traditional microbiological methods are effective for the identification and quantification of *S. mutans*, they are time-consuming and have a lower sensitivity when compared with molecular methods (Childers et al. 2011). One of the most useful techniques for the precise identification of medically important bacteria both in clinical microbiology and in research laboratories is the sequencing of the 16S rRNA gene (Srinivasan et al. 2015; Woo et al. 2011). Since the 16S rRNA gene is not affected by phenotypic variation, it is considered a more objective identification that may reduce laboratory errors. In this study, amplification of the first 527 bp fragment of the 16S rRNA gene resulted in the correct identification at species level of 83% (15 of 18 isolates) of the strains tested. The remaining 3 strains that were correctly identified using phenotypic methods gave similarity values lower than 99% and could not be differentiated beyond the genus according to the criteria of the Clinical Laboratory Standards Institute (2008). As reported by Srinivasan et al. (2015) among others, the lack of high quality sequence, reliant on databases with sparse sequence coverage for these microorganisms, could explain the lack of relationship between phenotypic and molecular results observed in the present work.

Serotype classification of *S. mutans* (serotypes c, e, f, and k) is based on the chemical composition of the serotype-specific rhamnose-glucose polymers which are composed of a backbone of rhamnose polymers and sided chains of α- or β-linked glucosidic residues (Nakano and Ooshima 2009). Currently, there is a complete set of primers for *S. mutans* serotype detection, however, identifying and serotyping many

strains with PCR using single primer sets may be not only more expensive but also time-consuming. To reduce the number of tests in this study, we developed a conventional multiplex PCR assay for serotypes c, e, and f and species-specific detection in one step, and a duplex PCR assay for the simultaneous identification and typing of *S. mutans* of serotype k. Both procedures proved to be highly specific and were used to determine the serotype of the 187 strains previously identified as *S. mutans*. The predominant serotype found in this study was the serotype c (86%), followed by e (8%), k (3%), and f (2%). This distribution of the serotypes agree with other studies around the world in which serotype c represents 70 to 80% of the isolates, followed by 20% for serotype e, and 2 to 5% for serotypes f and k, respectively. In contrast, studies developed in Jordan, India, and the USA reported a higher prevalence of the serotype k with values ranging from 92 to 27% (Momeni et al. 2019; Rao and Austin 2014; Swedan et al. 2018). Differences regarding serotype k prevalence could be attributed to the population characteristics, differences in oral hygiene practices, use of different sampling sites, methods of cultivation, and isolation (Momeni et al. 2015; Rao and Austin 2014; Swedan et al. 2018). In addition, results of the present study demonstrated that serotype c was present in both carious and caries-free subjects, while the remaining serotypes were found only in subjects with caries. Further studies, with a representative number of strains of the *S. mutans* serotypes isolated from populations with different general health status and demographic characteristics, should be carried out in order to establish their relationship with caries activity. In addition, it should be considered that the use of *S. mutans* strains isolated from selective media (MSB) for serotyping studies could lead to an underestimation of the serotypes diversity as indicated by Momeni et al. (2019). Therefore, it is recommended that PCR typing with serotype-specific primers, using DNA from whole saliva samples, as a complementary method to improve serotype detection. On the other hand, PCR serotyping studies have shown that the infection of subjects with *S. mutans* multiple serotypes is not rare (Momeni et al. 2015; Nakano et al. 2007; Swedan et al. 2018). These studies found that the 18–92% of the populations analysed harboured more than one serotype in different combinations, being c/e, c/f, and c/k as the most frequently found. On the contrary, in the present study, most of the individuals harboured only one *S. mutans* serotype, and only in one individual, a combination of serotypes c/k were detected.

In conclusion, this study contributes with information on the most effective methods to identify and characterize *S. mutans* as well as its effect on oral health in the Galician population. In addition, the multiplex and duplex PCR developed in this study allowed the simultaneous detection of *S. mutans* and its serotypes, and to establish for the first time the prevalence of *S. mutans* serotypes c, e, f, and k, and their distribution within this population. Considering the association of the different serotypes with other diseases besides caries, this kind of study could lead to the detection of high-risk populations in the development of *S. mutans*–associated diseases.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Informed consent All volunteers signed a written informed consent approved by the Bioethics Committee of the Universidade de Santiago de Compostela. Each questionnaire and saliva sample obtained in this study was identified by a number, so that it could not be associated with any person.

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