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Serotype diversity of Streptococcus mutans and caries activity in children in Argentina

ABSTRACT

Aim *The purpose of this study was to analyse the serotype distribution of S. mutans and their association with caries activity in school children from Córdoba, Argentina.*

Materials and methods *Clinical examination was performed in 133 children. The dmft+DMFT and Significant Caries (SiC) indices were calculated to identify individuals with high caries activity. After DNA extractions of S. mutans strains, serotypes were determined by PCR amplifications. The median caries activity of each serotype group was compared using a non-parametric Kruskall-Wallis test.*

Results *We obtained S. mutans strains from stimulated saliva of 94 children. The mean dmft+DMFT was 4.14 and the mean SiC index was 8.65. Serotype c was the most frequent (53.2%), followed by e (31.9%), f (8.5%) and k (6.4%). The comparison between the SiC and Non-Sic groups showed significant differences in the frequency of serotypes c and k. The median caries activity was non-significant in the different serotypes.*

Conclusion *The difference between the serotype frequencies detected in Argentina compared to those of other countries could be related with contrasting dietary habits. The results obtained in the present study would increase the knowledge about the epidemiology of dental caries in children from Argentina.*

Keywords *Argentina; Serotype diversity; Streptococcus mutans.*

Introduction

The diverse ecosystems present in the oral cavity contribute to the complexity of the oral microbiota, which allows different communities of microorganisms to coexist. Each microorganism with its own metabolic characteristic is in equilibrium with other species of the community and with the host [Zijnge, 2010]. Most of the oral bacteria are compatible with the host health; however, special circumstances of the oral environment, along with changes in the virulence of microorganisms, can disrupt the equilibrium between microbiota and tissues [Whiley and Beighton, 1998]. Dental caries is a chronic infectious disease with multifactorial aetiology, characterised by demineralisation of the dental tissues by means of acids produced by bacterial metabolic activity [Liébana Ureña, 2002]. Worldwide, the main species associated with caries in humans is Streptococcus mutans, which is part of the "mutans streptococci group", including species with different natural hosts, such as S. mutans and S. sobrinus (humans), S. cricetus (hamsters), S. rattus and S. ferus (rats), and S macacae and S. downei (monkeys) [Hung et al., 2005; Ota et al., 2006; Kanasi et al., 2010]. The cell wall of this bacterium contains proteins involved in processes such as adhesion, aggregation and co-aggregation, as well as polysaccharides showing different antigenic specificities (they play important roles in adherence to monocytic and fibroblastic cells); both proteins and polysaccharides characterise different serotypes: S. mutans (serotypes c, e, f and k), S. sobrinus (d and g), S. cricetus (a), S. rattus (b), S. ferus (c), S. macacae (c) and S. downei (h) [Nakano et al., 2007; Nakano and Ooshima, 2009]. Historically, serotypes were detected by immunodiffusion methods [Nakano and Ooshima, 2009; Sabelli et al., 1978; Borgarelli et al., 1985; Nakano et al., 2004a]; however, Shibata et al. [2003] and Nakano et al. [2004b] developed the PCR (polymerase chain reaction)-based methods for serotype detection in S. mutans, which are more sensitive than previous techniques. Based on this new method, Nakano et al. [2004b] and Nakano and Ooshima [2009] determined that serotype c was the most frequent in Japan, followed by e, f and k. In Thailand, Lapirattanakul et al. [2009] detected a similar pattern with serotype c as the most common. In a population-based genetic study of S. mutans, Do et al. [2010] serotyped 135 strains from Japan, Brazil, South Africa, USA, UK, Turkey, New Guinea, Iceland and China; the majority were serotype c with fewer strains identified as serotypes e or f and only 1 isolate was found to be serotype k. In Argentina, Borgarelli et al. [1985] identified four groups of "mutans streptococci" using an immunodiffusion method: group 1 serotypes a, d and g; group 2: e and f; group 3: c; and group 4: b. In addition, in a comparative study on experimental decay, Sabelli et al. [1978] observed that serotype c was the least pathogenic. Since then, no new studies have been performed in Argentina regarding serotype determination. Therefore, the main objectives of this study were to analyse the diversity of serotypes of S. mutans in Córdoba (the second most populated province of Argentina), by using molecular methods and to explore possible associations between different serotypes and caries activity in school children.

Material and methods

Study population

The study protocol was approved by the Institutional Committee of Ethics in Health Research from the Faculty of Dentistry, Universidad Nacional de Córdoba. Each school principal gave expressed formal approval to conduct this investigation, and student parents gave their written informed consent to participate in the study, in accordance with the Helsinki Declaration [Williams, 2008].

For the calculation of the minimum sample size we considered: an expected proportion of children with presence of Streptococcus mutans of 0.4 according to our previous study [Carletto Körber et al., 2010], a variance of the sample proportion of 0.05 and a significance level of 5%. We also assumed a population of 400 children with ages ranging from 6 to 8 years attending the four schools. Using these values, the minimum sample size was 190 children using the procedure proposed by Cochram [1977] for sampling the binomial distribution (1 presence - 0 absence of S. mutans). If we considered a variance of the sample proportion of 0.06 and a 90% confidence in the estimate, the minimum sample size is 124 children. In this study, we could collect saliva samples from 133 children of both sexes between 6 and 8 years old, attending urban-marginal schools from three localities of Córdoba province, Argentina: in Córdoba city, Luciani (n=33) and Cabanilla (n=31) schools; in Valle Hermoso city, Zevallos school (n=35); and in Alta Gracia city, Yrigoyen school (n=34).

Clinical examination and saliva collection

The dental clinical examination of children was conducted by one trained examiner. The teeth were cleaned and dried with a cotton-wool roll; only a mouth mirror in a well-lit environment was used. The caries index for the primary and permanent teeth in each child was expressed in terms of the dmft+DMFT, which was calculated as the total number of decayed, extracted due to caries and filled teeth following the criteria of the World Health Organization [1997]. Stimulated saliva samples were obtained in the morning two hours after breakfast by direct salivation in a calibrated polyethylene sterile tube; to stimulate the production of saliva each subject chewed a paraffin capsule for 5 minutes. The saliva samples were kept at -20°C until processing. After completing the procedure, children were allowed to brush their teeth.

Genetic and microbiologic study of S. mutans

Saliva samples were cultivated in Mitis Salivarius Agar (Difco Laboratories, Detroit, USA), with the addition of 0.281 mg/ml Bacitracin (ICN Biomedicals, 71% activity) and incubated at 37°C under microaerophilic conditions for 48 h; this medium is very selective and highly sensitive for the growth of Streptococcus mutans and S. sobrinus. The number of colony forming units (CFU) of S. mutans per ml of saliva was recorded in each child according to the method described by Jordan et al. [1987]. In children where S. mutans was not recovered, we repeated the procedure of isolation three times. Morphological analysis of colonies was performed by Gram stain and optical microscopy [Ruoff, 1995]. Typifications were made using the commercial biochemical Api 20 Strep kit (BioMérieux, France); it is a standardised method which combines 20 biochemicals tests allowing the differentiation between Streptococcus mutans from Streptococcus sobrinus. The culture plate of each child was divided into six areas; one colony from of each area was chosen for serotyping; they were cultivated in 5 ml of brain-heart broth and incubated at 37°C for 48 h; then DNA was extracted following the method of Bollet et al. [1991] with phenol-chloroform-isoamyl alcohol purification (25:24:1; Sigma-Aldrich, Buenos Aires, Argentina). The DNA pellet was resuspended in 50 µl of TE buffer and stored at -20°C. Serotypes c, e and f were amplified according to the conditions described in Shibata et al. [2003] using three primers simultaneously (multiplex PCR). In each PCR we included a negative control (sterile water was added instead of the DNA) and a positive control (strain ATCC 25175, corresponding to serotype c). Serotype k was amplified according to Nakano et al. [2004b] in a standard PCR, using only negative controls. Amplifications were observed through electrophoresis in agarose gels at 1%, stained with 0.5µg/ml of ethidium bromide and visualised under ultraviolet light.

Statistical analyses

The experience of dental caries in each individual was obtained by calculating the number of decayed, missing and filled teeth in primary (dmft index) and permanent (DMFT index) dentition. In addition, the Significant Caries Index (SiC Index) was calculated to identify individuals with highest caries activity: individuals were sorted according to their dmft+DMFT values, the third of the population with the highest caries scores was selected and the mean dmft+DMFT for this subgroup was considered the SiC index [Bratthall, 2000]; this last index was calculated with the software SiC version 1.0 [Nishi, 2001]. The remaining 2/3 was considered the Non-SiC population. To assess any significant difference in the serotype distribution between the SiC and the Non-SiC populations, the homogeneity test of proportions was performed using the professional Infostat software [InfoStat User Manual, 2011].

Quantitative data of the caries component were described by the median and percentile values. Data obtained were statistically processed using the non-parametric Kruskall Wallis test for unpaired samples. Differences were considered significant at $p<0.05$. Data analyses were performed with the same software.

Results

A total of 133 children from four schools in three localities of Córdoba province, Argentina, were clinically examined. Results of the quantitative variables, gender distribution, caries-free children, dmft and DMFT are shown in Table 1. We recovered S. mutans from 70.7% (n = 94) of the school children; in the remaining children (n=39) S. mutans was not recovered. The number of isolates recovered from children in each school was low; for that reason, we considered all S. mutans strains as a single population. According to the dental clinical examination of these children, 19 were caries-free, 23 presented between 1 and 2 caries activity, and 21 presented between 3 and

a				b						
n	Male	Female	Caries-free children	dmft	DMFT	S. mutans strains	Serotypes			
							c	e	f	k
94	45	49	19	3.69	0.44	94	50	30	8	6

DMFT: decayed missing filled teeth (permanent teeth); dmft: decayed missing filled teeth (primary teeth); D-d: decayed; M-m: missing; F-f: filled

TABLE 1 a) Clinical examination of children from Córdoba province, Argentina; b) Microbiological and genetic studies of S. mutans in saliva samples.

5 caries activity. The SiC group, which is composed of individuals with highest number of caries, included 31 children (33%) presenting from 6 to 14 caries activity. The mean dmft+DMFT of the 94 children was 4.14 ± 3.75 and the SiC index of the children with highest caries activity was 8.65 ± 2.42.

In the study sample, serotypes c, e, f and k were detected (Table 1). The relationship between the SiC group and the different serotypes showed a different distribution of serotypes c, e, f and k compared with the "Total population" (SiC + Non SiC). The percentages of serotypes c and f were similar in the "Total population" compared with the "SiC population", but serotype e was lower in the "Total population" than in the "SiC population" and serotype k was absent in the "SiC population" (Fig. 1). The median caries activity of the different serotypes of S. mutans is shown in Table 2. The Kruskall Wallis test revealed non-significant differences of the caries activity among the four serotypes. The homogeneity test of proportions between the "SiC population" and the "Non-SiC population" revealed significant differences in serotypes c and k, which were higher in the "Non-SiC population" (Fig. 2).

Serotype	Minimum	P(25)	P(50)	P(75)	Maximum
c (n=50)	0	1	3	8	14
e (n=30)	0	1	4	6	13
f (n=8)	0	1	4	5	10
k (n=6)	0	0	2	2	5

P: percentile; P(50): median caries activity

TABLE 2 Median caries activity of the different serotypes of S. mutans in children from Córdoba, Argentina.

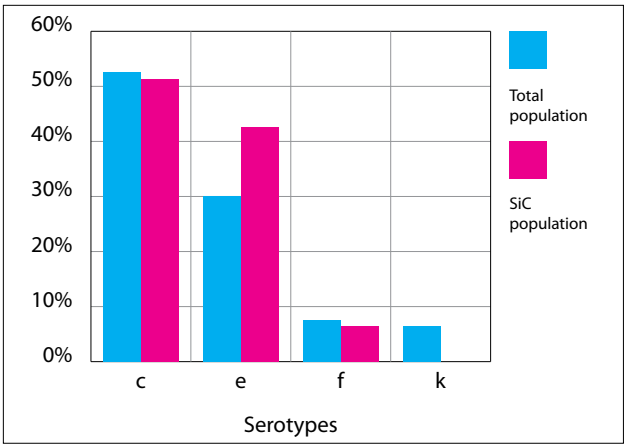

FIG. 1 Distribution of the different serotypes of S. mutans in the Total population and in the SiC population.

Discussion

Dental caries is the most recurrent infection worldwide and is one of the most common diseases in the oral cavity. One of the primary pathogens in humans is the bacterium Streptococcus mutans, which presents serotypes c, e, f and k [Nakano and Ooshima, 2009]. Worldwide, approximately 66-80% of the strains present in the oral cavity were classified as serotype c using biochemical methods, followed by e (20%), f (10%) [Nakano and Ooshima, 2009]. Some strains were not reactive with antigens extracted from serotypes c, e or f; these strains were designated as a novel serotype k, which worldwide has a frequency of less than 5% [Nakano et al., 2004a].

Knowing the relative proportion of each serotype is important because serotype c is detected more frequently in healthy patients, whereas non-c serotypes or a mixture of multiple serotypes are more frequent in subjects affected by cardiac diseases or that underwent surgery [Nakano et al., 2004b; Nakano et al., 2009]. Dental surgical procedures (including tooth extraction, scaling, polishing, filling procedures and root canal treatments) are thought to cause the dissemination of S. mutans into the bloodstream, leading to transient bacteremia [Sonbol et al., 2009]. Once in the bloodstream, serotypes f and e are able to invade primary human coronary artery endothelial cells, whereas serotype c strains are not invasive [Abranches et al., 2009]; this difference may be due to the capacity of specific serotype polysaccharides to bind to fibronectin, laminin and collagen type I, which are exposed on damaged heart tissues [Nagata et al., 2006]. Since the implementation of molecular methods for serotype detection, two exhaustive studies in Japan and one in Thailand have been performed to study the serotype composition of oral cavities mostly in healthy children. In pre-school children from Fukuoka (Japan) serotype c (84.8%; 178 isolates), e (13.3%; 28 isolates) and f (1.9%; 4 isolates) were detected [Shibata, 2003], and in Osaka (Japan) 84 strains from 69 healthy children presented serotypes c (75%; 63 strains), e (17.86%, 15 strains), f (4.76%; 4 strains) and k (2.38%; 2 strains) [Nakano et al., 2007]. Finally, in Bangkok, Thailand, in a population involving children and adults, serotypes c (70%; 175 isolates), e (22.8%, 57 isolates), f (4.4%, 11 isolates) and k (2.8%; 7 isolates) were detected [Lapirattanakul et al., 2009]. In the present study, we focused our attention in healthy children attending to four different schools in Cordoba (Argentina), Province. We recovered serotype c (53.2%) followed by e (31.9%), f (8.5%) and k (6.4%) (Fig. 1); the percentages obtained are different from previous biochemical and molecular findings. Moreover, we performed statistical analyses to detect which serotypes were associated with the population with the highest caries scores. When considering the median number of caries activities of the different serotypes we could not find significant differences, which do not allow us to affirm that non-c serotypes would be related to higher risk for health. However, we did find some

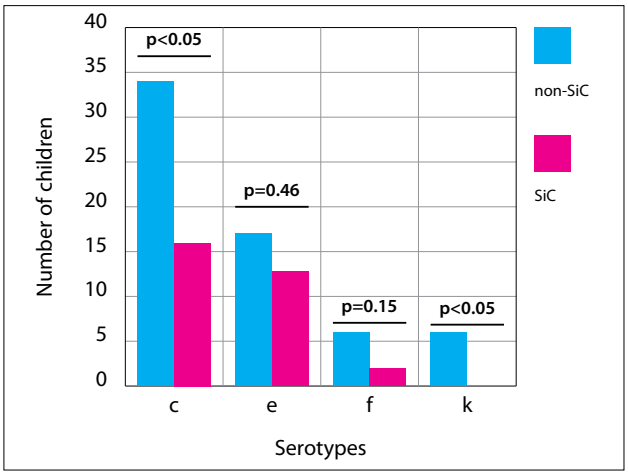

FIG. 2 Comparison of the distribution of the different serotypes of S. mutans in the SiC and Non-SiC populations using the homogeneity test of proportions.

differences among the "SiC population" and the "Non-SiC population": serotype k was absent and serotype c was significantly lower in the "SIC population" (Fig. 2). This last result is similar to other ones, in which serotype c is the most prevalent in healthy subjects. On the other hand, strains of serotype k shows unique characteristics involving a drastic reduction of glucose side chains, associated with a lack of glucosyltransferase activity from rgpE gene, leading to inhibition of glucose polymer production by serotype-specific polysaccharides and commonly possess defects of major surface proteins, such as protein antigen (PA) and glucan-binding proteins (Gbps), which are known to play a role in the glucan-binding properties of S. mutans in the presence of sucrose [Lapirattanakul et al., 2009]. Therefore, it is not surprising that in our study serotype k was significantly associated with the "Non-SiC population", a population with healthier conditions in the oral cavity (Fig. 2). If serotype c is associated with better oral conditions, the low levels of this serotype in Córdoba, Argentina (53.2%), compared with the 84.8%-75% in Japan [Shibata et al., 2003; Nakano et al., 2007] and the 70% in Thailand [Lapirattanakul et al., 2009], could probably be attributed to the diet. In the last 20 years, Argentina has experienced changes in the diet as a consequence of the increase in life expectancy, declining birth rates and infectious diseases. The nutrition is now characterised by a high consumption of meat, saturated fats, refined sugars and relatively low consumption of fiber and complex carbohydrates, contrary to the healthier diet the population had in previous years [Quinteros, 2009]. In contrast, Japan and Thailand, are characterised by the consumption of high amounts of rice and fish, which could be considered a healthier diet [World Health Organization, 2003]. In summary, the present study provides insights into the most important cariogenic microorganism related to dental caries in relation to the epidemiology of S. mutans, which expands our understanding of the progression of the disease. Moreover, this study is the first report of the circulating serotypes in Córdoba, one of the most populated provinces of Argentina; we detected the four serotypes described in S. mutans but with different frequencies compared with Japan and Thailand, which could be associated with the differences in the diets of these countries.

Conclusions

This is the first study that analysed the diversity of serotypes of S. mutans in Córdoba (one of the most populated provinces of Argentina) by using molecular methods. We evaluated the associations between different serotypes and caries activity in school children. Serotypes c and k were more frequent in children with low caries activity. Serotype c was the most common in the present study population; however, the percentage was lower compared with other countries like Japan and Thailand. The findings of this study would increase the knowledge amongst paediatric dentists about the aepidemiology of dental caries in children from Argentina.

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