

Caries and genetic variability of *Streptococcus mutans*.

Caries y variabilidad genética de *Streptococcus mutans*.

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Abstract: In the last two decades, the increase in population genetics studies has contributed to elucidating important questions about the evolution of the pathogenesis of bacteria of clinical interest. The objective of this study is to revise and update the knowledge of the last fifteen years regarding the genetic variability of *Streptococcus mutans* and their association with dental caries. *Streptococcus mutans*, one of the most widely distributed bacteria in the world, are heavily associated with this condition. This research shows the results of numerous studies carried out in various countries that, using molecular and biochemical methods, revealed associations between different serotypes and caries activity. In addition, it is reported that the population genetics structure of *Streptococcus mutans* in Argentina is highly recombinant, which reflects the largest waves of human immigration that occurred in the 19th and 20th centuries. On the other hand, demographic analysis suggests that these bacteria experienced a population expansion that coincided with the beginning of agricultural development.

Keywords: *Streptococcus mutans*; dental caries; serogroup; genetics, population; emigration and immigration; Argentina.

Resumen: En las últimas dos décadas el incremento de los estudios de genética de poblaciones ha contribuido a dilucidar cuestiones importantes sobre la evolución de la patogénesis de bacterias de interés clínico. El objetivo de este trabajo es realizar una actualización sobre los conocimientos de los últimos quince años referidos a la variabilidad genética de *Streptococcus mutans* y su relación con la caries dental. *Streptococcus mutans*, de amplia distribución mundial, es una de las bacterias más fuertemente asociada a dicha enfermedad. En este trabajo se muestran resultados de numerosos estudios realizados en diferentes países que utilizando métodos moleculares y bioquímicos revelaron asociaciones entre diferentes serotipos y la actividad de caries. Además, se reporta que la estructura genética poblacional de *Streptococcus mutans* de Argentina es de alto nivel recombinante, lo que reflejaría las grandes oleadas inmigratorias humanas ocurridas en los siglos 19th y 20th. Por otra parte, los análisis demográficos sugieren que esta bacteria experimentó una expansión poblacional coincidente con el comienzo del desarrollo de la agricultura.

Palabra Clave: *Streptococcus mutans*; caries dental; serogrupo; genética de población; emigración e inmigración; Argentina.

INTRODUCTION.

The appearance of new diseases or the re-emergence of others reveals changes in the epidemiology of pathogenic organisms. Despite numerous technological advances, in many cases, it has not yet been possible to explain the mechanisms by which microorganisms cause diseases, the factors that determine the emergence of pathogenic bacteria, why certain clones of bacteria are pathogenic and others are not, or why non-pathogenic strains possess genes associated with virulence.¹

Although causes are multifactorial, population genetics studies can contribute to the understanding of the mechanisms involved, providing information about the nature of the genetic variation that exists in natural populations. Bacteria exhibit rapid evolutionary changes due to their short generation times and their enormous population sizes. Variations in the genetic composition of populations depend on processes such as mutation, recombination, migration, genetic drift, and natural selection. For this reason, studies on genetic structure and population dynamics try to explain the mechanisms by which a species evolves, and also answer specific questions about the history of the species and their populations.^{1,2}

Infectious diseases have become a challenge for the development of new diagnostic techniques with high sensitivity and specificity. Population genetics studies are useful to characterize strains of pathogenic bacteria, a central aspect of the epidemiology of infectious diseases. Within the same bacterial species, it is important to understand the genetic connections between pathogenic strains that cause the disease and those that do not. Comparing them can help us explain the origins of the pathogenic strains and identify the genetic differences between the two. In this context, population genetics studies were carried out on bacteria that cause infectious diseases such as *Vibrio cholerae*;³ *Chlamydia trachomatis* and *Neisseria gonorrhoeae*,⁴ and *Bordetella pertussis*.⁵

Dental caries is one of the most widespread diseases in the world and is frequently diagnosed in children. It is associated with different bacteria that should be studied with a population genetics approach. Caries is a chronic infection of multifactorial etiology, characterized by the demineralization of dental tissues as a consequence of acid products generated by bacterial metabolism.

Dental caries, in general, especially severe, especially severe cases of early childhood caries, negatively affect the quality of life of children and their families and has been directly associated with sociodemographic

variables such as a low educational level, a sugar-rich diet and poor oral hygiene.^{6,7}

The ecological plaque hypothesis proposes that dental caries are the consequence of changes in the natural balance of the oral microbiota that occur as a reaction to an alteration in the conditions of the oral environment, leading to an increase in the proportion of acidogenic and aciduric bacteria.⁸

Streptococcus mutans is the species that is most commonly associated with dental caries. It belongs to the Streptococcaceae family, which also includes other species such as *Streptococcus gordonii*, *Streptococcus sanguinis* and *Streptococcus sobrinus*, the latter has been identified, alongside with *Streptococcus mutans*, as the species associated with caries (Figure 1).^{9,10}

Streptococcus gordonii is among the first colonizing species of the oral cavity, and it can decrease the production of virulence factors in *Streptococcus mutans*. *Streptococcus gordonii* produces a protease that can degrade the competence-stimulating peptide, which is a regulator of quorum-sensing in *Streptococcus mutans* related with bacteriocin production, which allows survival in highly acidic environments.¹¹

Streptococcus mutans levels in the oral cavity have been linked to the presence of caries in the past and to risk of future caries. In 2009, Acevedo *et al.*,¹² found that the mere presence of *Streptococcus mutans* was not sufficient evidence to predict dental caries. In another study, which was carried out in Sudan on 12-year-old children, a high presence of *Streptococcus mutans* was identified in caries-free children (97%), and in those with carious lesions (98.2%).

This study reported that *Streptococcus sobrinus* isolates were always found alongside *Streptococcus mutans*, and never on their own.⁶ The cariogenic potential of *Streptococcus mutans* is attributed to virulence factors such as the ability to metabolize carbohydrates as they produce acids like lactate (acidogenesis), which is their main source of energy in anaerobiosis.⁸

Streptococcus mutans are also able to survive in acidic environments, stimulate enamel hydroxyapatite adhesion and bacterial co-adhesion, which favors biofilm formation.¹¹ On the other hand, they are not only capable of producing bacteriocins that inhibit the metabolism and growth of other bacterial species, they also produce exoenzymes and proteases capable of destroying elements related to the host's immune response.¹³

This bacterial family's cell wall contains proteins that play an important role in adhesion, aggregation, and

co-aggregation phenomena, as well as polysaccharides such as RGP (rhamnose-glucose polysaccharide) that display specific antigens. Various polysaccharides and proteins that characterize different bacterial serotypes have been identified. Therefore, serotypes c, e, f and k have been identified in *Streptococcus mutans*, while serotypes d and g have been identified in *Streptococcus sobrinus*.^{14,15}

Among the characteristics of *Streptococcus mutans* genotypes, the following stand out: high diversity (number of genotypes in a certain population), homo-

geneity (number of shared genotypes in a certain population) and stability (persistence of genotypes throughout time in a certain population). Children tend to have fewer genotypes (less diversity) compared to adults, suggesting that new strains of *Streptococcus mutans* are acquired over the years.¹⁰

The aim of this study is to revise and update the concepts related to the geographical distribution of the different genetic variants of *Streptococcus mutans* and their association with dental caries.

Figure 1. Morphology of *Streptococcus mutans* via Optic Microscopy.

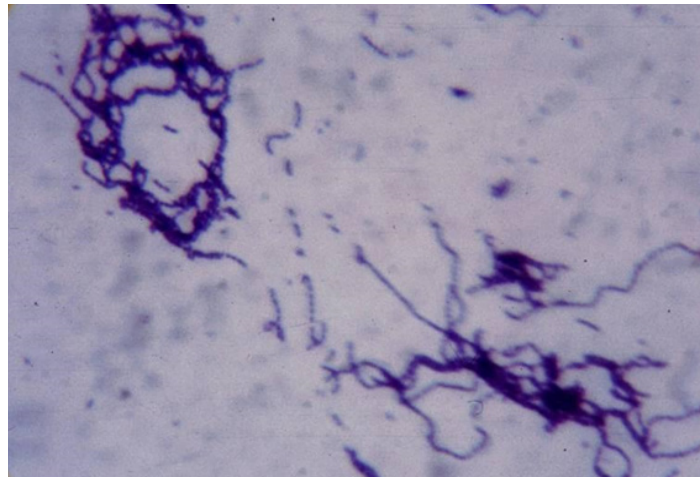


Figure 2. Distribution of the different *Streptococcus mutans* serotypes in the total population and in the SiC population.²⁴

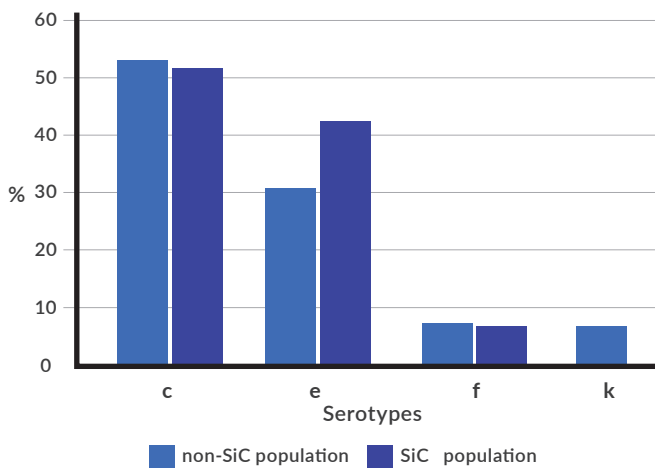
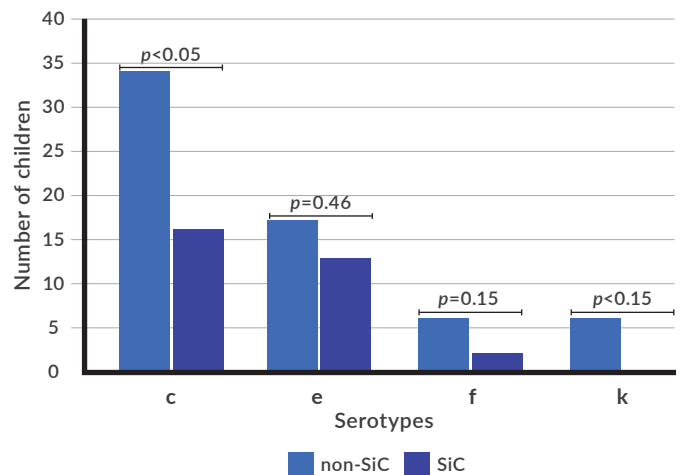


Figure 3. Comparison of the number of children with different *Streptococcus mutans* serotypes in the SiC and non-SiC populations, using the statistical test of homogeneity of proportions.²⁴



Genetic characterization of *Streptococcus mutans*

This bacterial species has been identified as the main microorganism associated with dental caries. Using biochemical methods, approximately 66-80% of the strains present in the oral cavity worldwide were classified, belonging to serotype c, followed by e (20%) and f (10%).¹⁵

In addition, it was detected that some strains were not reactive with antigens extracted from these serotypes. These strains were designated as serotype k, which has a global prevalence of less than 5%.¹⁶

Knowing the relative proportion of each serotype is important because serotype c is detected more frequently in healthy patients, while non-c serotypes or a mixture of multiple serotypes are more frequent in subjects affected by heart disease or those who underwent surgery.^{17,18}

Dental surgical procedures (including tooth extraction, filling, and root canal treatments) are believed to cause the spread of *Streptococcus mutans* in the bloodstream, leading to transient bacteremia.¹⁹

Once in the bloodstream, serotypes f and e are capable of invading endothelial cells in primary human coronary arteries, whereas serotype c strains are not invasive.²⁰

This difference could be explained by the specific ability of this serotype's polysaccharides to bind to fibronectin, laminin, and type I collagen, which are exposed in damaged heart tissue.²¹

The incorporation of new techniques such as PCR (polymerase chain reaction) have made it possible to identify different *Streptococcus mutans* serotypes (c, e, f and k). Two comprehensive studies were conducted in Japan and Thailand to determine circulating serotypes.

Figure 4. Bayesian cluster analysis of *Streptococcus mutans* strains from Argentina, Thailand, Japan and Finland. Each strain within a country is represented by a thin vertical line, divided into segments that represent the probability of belonging to one of the three estimated genetic groups. *: Strains with mixed ancestry following the criteria defined by Vähä and Primmer (2006).³²

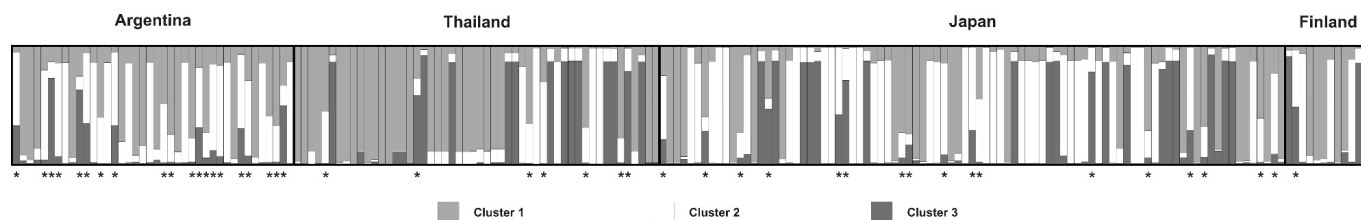
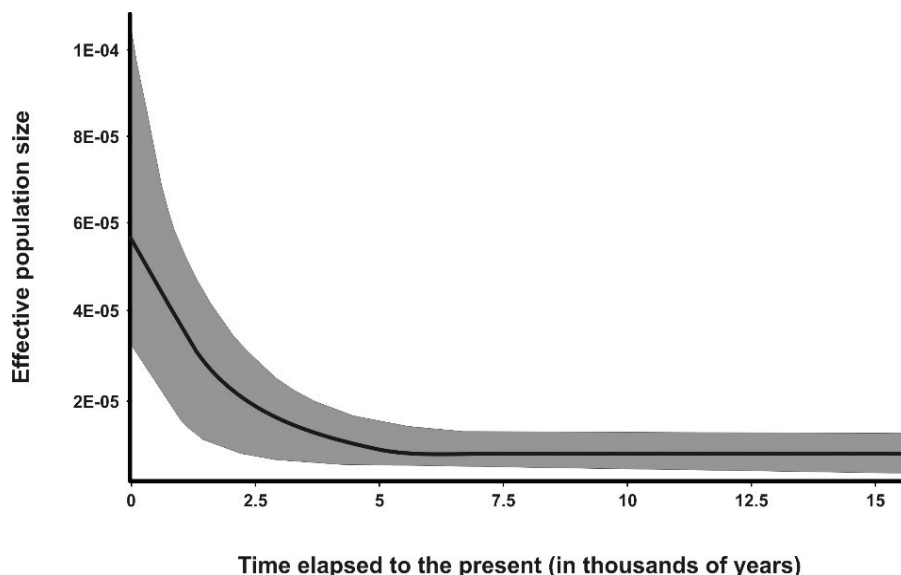


Figure 5. Analysis known as “Extended Bayesian Skyline Plot” that represents the demographic history of *Streptococcus mutans* in the last thousands of years.



The "y" axis represents the effective population size and the x axis represents time.

The black lines represent the median p-value and the gray lines represent the 95% upper and lower confidence intervals

Table 1. Comparison of paired FST between countries based on 193 *Streptococcus mutans* strains.

Argentina	Thailand	Japan	Finland
Argentina	---		
Thailand	0.035**	---	
Japan	0.022*	0.037**	----
Finland	0.02	0.009	0.006

*: $p < 0.05$. **: $p < 0.01$ (p is the statistical significance of the pairwise comparison after 1000 iterations, using the Arlequin 3.5.1.2 software)

In preschool children from Fukuoka (Japan), Serotypes c (84.8%; 178 isolates), e (13.3%; 28 isolates) and f (1.9%; 4 isolates)²² were detected.

In Osaka (Japan), 69 healthy children exhibited the following 84 strains: serotypes c (75%; 63 strains), e (17.86%, 15 strains), f (4.76%; 4 strains) and k (2.38%; 2 strains).¹⁴ Finally, in Bangkok (Thailand), in a sample group comprised of children and adults, serotypes c (70%; 175 isolates), e (22.8%, 57 isolates), c (4.4%, 11 isolates) and k (2.8%; 7 isolates) were detected.²³

In a study by Carletto-Körber *et al.*,²⁴ which was carried out on healthy children attending four different schools in the Córdoba province (Argentina), serotypes c (53.2%), e (31.9%), f (8.5%) and k (6.4%) were identified (Figure 2).

The distribution of serotypes in this population is different from the previous biochemical and molecular findings. This study was the first analysis of circulating serotypes in Córdoba, one of the most populated provinces in Argentina.

Recently, Momeni *et al.*,²⁵ studied the prevalence of *Streptococcus mutans* serotypes using PCR detection in two cohorts of African American children from rural Alabama (USA), using three types of samples: saliva, dental plaque, and *Streptococcus mutans* isolates. They detected a single serotype in 52 children and multiple serotypes in 77 children, with a relative frequency of 98% for serotype c, 26% for serotype e, 7% for serotype f and 52% for serotype k. They also found a statistical association between serotype c and high incidence of caries in children aged 11-12 years old, while serotype k showed a significant association with the female gender.

Although serotype c has been recognized in various studies as the serotype with the highest distribution per population, new research areas suggest the importance of serotype k.¹⁶ Nakano *et al.*,¹⁷ reported that strains of serotype k had lower susceptibility to phagocytosis and a high capacity for the invasion of the human endothelium,

suggesting that their presence could be considered a risk factor for the development of infectious endocarditis. Special care should be taken in children with cardiac disorders and in children with Down syndrome that are going to receive dental treatment while possessing serotype k strains, due to possible disorders in the functions of their polymorphonuclear leukocytes.

Because of this, pre-treatment prescription of antibiotics is recommended. On the other hand, Carletto-Körber *et al.*,²⁴ analyzed which serotypes are associated with greater caries prevalence in a population. No significant differences were found when considering the mean number of caries for the different serotypes; as a result, the idea that non-c serotypes were associated with a greater health risk could not be confirmed. However, the third of the population that had the greatest caries prevalence, identified as the "SiC population", exhibited differences with the "non-SiC population". In the "SiC population", serotype k was absent, and the presence of serotype c was significantly lower. Furthermore, serotype k was significantly associated with the "non-SiC population", a population with healthier oral cavity conditions (Figure 3).

This result is similar to other studies, where serotype c is the most prevalent in healthy subjects.^{14,22} The low levels of serotype c in Córdoba, Argentina (53.2%), compared to the 84.8% and 75% registered in Japan^{14,22} and the 70% reported in Thailand,²³ could probably be attributed to dietary habits.

In the last 20 years, Argentina has experienced dietary changes: its diet is now characterized by high consumption of meat, saturated fats and refined sugars, with relatively low consumption of fiber and complex carbohydrates, contrary to the healthier diet that the population had in previous years.²⁶

In contrast, Japan and Thailand are characterized by a high consumption of rice and fish, which could be considered as a healthier diet.²⁷

Genetic structure of the population: recombinant or clonal?

Living organisms have the peculiarity of transferring their genes from generation to generation through sexual and asexual reproduction, but bacteria can acquire genes and/or genetic variation through horizontal gene transfer by transduction, conjugation, and transformation.²⁸

Cornejo *et al.*,²⁹ identified common genes for all *Streptococcus mutans* strains (core genome) and genes only present in some strains (dispensable genome components) that together constitute the pan-genome.

These expendable genes can be spread through horizontal gene transfer. Studies that used the MLST technique reached different conclusions about the population genetics structure of *Streptococcus mutans*: Nakano *et al.*,¹⁴ and Lapidattanakul *et al.*,³⁰ detected a clonal structure, while Do *et al.*,³¹ identified genetic recombination in these bacteria.

In a study conducted by González-Iltig *et al.*,³² that used the MLST technique and applied different statistical methods, a comparison was carried out between the sequences obtained from Argentina and those retrieved from Japan, Thailand and Finland, which were reported by Nakano *et al.*,¹⁴ and Lapidattanakul *et al.*³⁰ The results of analyzing the 193 strains obtained from these four countries were that 137 sequence types (STs) were detected. In the population genetics structure, the majority of these analyses revealed an absence of recombination at the intra-gene level and the presence of recombination at the inter-gene level; however, this process may be different in each country. Low recombination levels were detected in *Streptococcus mutans* strains from Japan, Thailand, and Finland, which was a similar result to those obtained in the aforementioned studies.^{14,30}

On the other hand, recombination levels were very high in *Streptococcus mutans* strains from Argentina. Therefore, it is not surprising that, in the studies carried out by Do *et al.*,³¹ and González-Iltig *et al.*,³² the phylogenetic trees obtained using different software (*e.g.* eBURST and START2) show various groupings of STs, all with very low statistical support.

In relation to this, the Bayesian analysis carried out using Structure software,³³ detected that 45 strains had a mixed ancestry, a product of genetic recombination between strains of pure ancestry with groups 1, 2 and 3 (Figure 4).

Considering the models proposed by Maynard-Smith *et al.*,² global results reinforce the idea that the genetic structure of the *Streptococcus mutans* population is one

where genetic recombination is the most important evolutionary process occurring in these bacteria.

Historical demography of *Streptococcus mutans*

In a study conducted by González-Iltig *et al.*,³² it was detected that many STs were exclusive to each country and that F_{ST} values (an estimator of the variance in allele frequencies and genetic differentiation between countries) showed significant differences between Argentina, Japan and Thailand (Table 1).

However, Finland was the only country that recorded non-significant values for this index, which could be a statistical artifact caused by its small sample size ($n=12$), since 10 of the 12 STs were exclusive to that country.

The analysis that was carried out using Structure software³³ (Figure 4) revealed that the four countries have strains that largely belonged to groups 1, 2 and 3. However, the frequency of each group was very different between countries, which reinforces the idea that the homogenizing effect of gene flow is not that important. The amount of strains with mixed ancestry was also very different: 50% in Argentina, 13.5% in Thailand, 19.1% in Japan and 8.3% in Finland (Figure 4).

All of these results reflect the history of the human population: the degree of immigration and racial mixing that have occurred in the last 500 years in Argentina are much higher than those in Finland, Japan, and Thailand.

According to Didelot *et al.*,³⁴ recombination in bacteria can be high during the adaptation period to new environments or lifestyles, followed by low recombination levels once the adaptation is complete. This could be the case for *Streptococcus mutans* in Argentina, where the mixture of strains of different geographical origin (brought by immigrants) and the change to a meat-rich diet could have favored the high recombination levels detected. Meanwhile, in the other countries analyzed, *Streptococcus mutans* strains could have already undergone an adaptation due to the longer-lasting environmental stability.

It is important to note that there are major deficiencies in the sampling of *Streptococcus mutans* strains around the world, which prevents an accurate estimation of gene flow levels. However, if the current gene flow is not as high as what was inferred in the study, then, why do *Streptococcus mutans* strains differ so little at the nucleotide level?

A possible explanation for this could be that all strains have a very recent common ancestor. Cornejo *et al.*,²⁹ suggested that *Streptococcus mutans* began exponentially expanding approximately 10,000 years ago, coinciding with the emergence of agriculture. In a study conducted

by González-Iltig *et al.*,³² this hypothesis was analyzed using different statistical tests. Results showed that the expansion of *Streptococcus mutans* began about 5000 years ago from a small number of ancestral strains (Extended Bayesian Skyline Plot analysis applied using the Beast software; (Figure 5),³⁵ a value that is within the 95% confidence interval of Cornejo *et al.*²⁹

These results are consistent with data extracted from ancient human bone remains: in general, hunter-gatherer humans had very low caries levels, and these levels increased notably in societies where agriculture was incorporated, as it resulted in a diet rich in carbohydrates.^{36,37} In turn, the oral microbiota remained constant between the Neolithic and the Middle Ages, after which cariogenic bacteria became predominant. After medieval times, the prevalence of *Streptococcus mutans* increased significantly and became dominant during the industrial revolution.

According to Adler *et al.*,³⁷ the demographic expansion of *Streptococcus mutans* may have occurred due to two important dietary changes: the first involved a carbohydrate-rich diet associated with agriculture, while the second one was associated with the recent development of industrially processed flour and sugar. However, both Cornejo *et al.*,²⁹ and González-Iltig *et al.*³² recognized a single stage of *Streptococcus mutans* demographic expansion related to the establishment and worldwide development of agriculture.

CONCLUSION.

Genetic studies carried out on *Streptococcus mutans* combined with new knowledge of the structure of biofilm open new research areas regarding the prevention and treatment of dental caries, and the prophylaxis of systemic diseases such as infective endocarditis.

Results from numerous studies carried out in different countries using molecular methods revealed associations between different serotypes and caries activity.

Serotypes C and K were more frequent in individuals with low caries activity. Serotype c was the most common in the Argentine population; however, the ratio was lower when compared to other countries such as Japan and Thailand.

Results obtained from the population genetics structure of *Streptococcus mutans* in Argentina were of the recombinant type.

The demographic analysis supported the hypothesis that these bacteria underwent a population expansion in the last 5,000 years that coincided with the beginning of agricultural development. The high amount of

recombinant strains in Argentina demonstrates the effect of the large human migratory waves that occurred in the 19th and 20th centuries, from various geographical regions. These findings may contribute to bolster the understanding of the epidemiology of dental caries.

Molecular biology has been incorporated in clinical practice. The numerous advantages of molecular techniques over classical techniques have encouraged the implementation of molecular biology laboratories, both in public and private health systems.

Despite this growth, several challenges must be addressed to ensure accurate and quality diagnostics. On the other hand, it is important to instruct and educate future health professionals about these types of techniques, indicating their advantages and limitations. The idea is to promote the development of multidisciplinary teams that, in the future, can design, implement, standardize, control, and interpret these diagnostic tools.

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