ORIGINAL ARTICLE

A new biotyping method for *Streptococcus mutans* with the API ZYM system

Clin Microbiol Infect 1999; 5: 88–91

Angustias de La Higuera, José Gutiérrez, José Liébana, Antonio Garcia-Mendoza and Ana Castillo

Department of Microbiology, Odontology and Medicine Sections, University of Granada, Spain

Objective: To test a new system for the biotyping of *Streptococcus mutans*, based on the measurement of enzyme activity, and to investigate the relationship between biotype and in vitro susceptibility to seven clinically useful antibiotics.

Methods: In total, 160 oral isolates of *S. mutans* were classified into different biotypes with the APIZYM test for enzyme activity, excluding results that were positive or negative in >80% of the strains. The susceptibility of all 160 strains to amoxycillin, cefazolin, erythromycin, clindamycin, vancomycin, teicoplanin and imipenem was tested by dilution in a solid medium. Statistical analysis of susceptibility (mean minimum inhibitory concentrations (MICs)) was based on chi-squared tests.

Results: Eight different biotypes (1–8) were identified on the basis of three kinds of enzyme activity: valine aryl amidase, acid phosphatase and α-galactosidase. Biotype 5 was found to be the most common. The mean MIC values showed strains belonging to biotype 4 to be the most susceptible to amoxycillin, cefazolin and erythromycin, whereas biotype 1 was the least susceptible to teicoplanin.

Conclusions: The proposed biotyping method, which is relatively fast and simple to perform, provided reproducible results, and may contribute to clinically effective treatment of *S. mutans* infections.

Key words: *Streptococcus mutans*, biotype, antibiotic susceptibility

INTRODUCTION

Streptococcus mutans is a microorganism that may be involved in localized disease such as dental caries [1–5], systemic infections resulting from dental interventions, or bacteremia. In some patients with predisposing factors, *S. mutans* can lead to subacute endocarditis [6–8]; in rare cases it has been associated with other severe infectious diseases [9]. Since the appearance of Keyes's study in 1960 [10], several studies have confirmed that *S. mutans* is transmissible [11–13]. For these reasons, accurate typing is essential in order to establish differences in antibiotic susceptibility and provide effective treatment. Moreover, correct typing can shed light on the precise origin and incidence of different associated infections, including caries and bacteremia, thereby helping health professionals to assess the effectiveness of prophylactic measures in breaking the chain of infection.

Various typing methods have been proposed to date for *S. mutans*; some are based on the antigenic heterogeneity of the cell wall carbohydrates [14–17], while others detect differences in bacteriocin susceptibility [11,12,18,19]. A more complex ribotyping method has also been described [20]. However, developing an accurate biotyping procedure (i.e., one capable of distinguishing types within a species on the

Corresponding author and reprint requests:

José Liébana Ureña, Cátedra de Microbiología, Facultad de Medicina, Avda. de Madrid 11, E-18012 Granada, Spain

Tel: +34 958 243549 Fax: +34 958 246119

E-mail: josegf@goliat.ugr.es

Revised version accepted 5 August 1998

basis of differences in biochemical behaviour) is especially difficult in the case of *S. mutans*, because conventional assays are unable to establish distinctions among the limited variety of its biochemical processes and distinguish corresponding biotypes [21–23]. Further complications arise from the fact that the term '*S. mutans* biotype' [24–27] may be used in relation to subspecies included previously in the *mutans* group on the basis of either genotype, serotype or phenotype [8,27–29], and the designation 'biotype I' is sometimes applied to *S. mutans* itself. The current trend, however, is to consider these bacteria not as a subtype, but rather as an independent species [21–23,30].

This paper focuses on the development and application of an accurate new methodology for biotyping *S. mutans* based on enzymatic activity; similar earlier studies have been reported [31]. After evaluation of the reproducibility of the results of the biotyping method, the comparative susceptibility of the biotypes identified was tested in vitro against seven different antibiotics.

MATERIALS AND METHODS

In total, 160 strains of *S. mutans* were isolated from clinical oral samples (saliva or supragingival plaque) from different subjects and identified by conventional methods. Cultured microorganisms were visualized after staining by Gram's method and subjected to the following tests: arginine hydrolysis; esculin hydrolysis in the presence and absence of bile; mannitol, raffinose and inulin fermentation; and resistance to bacitracin. The culture media, laboratory procedures and interpretation of results have been described by Barrow and Feltham [21]. Identification criteria used were those of Maiden et al [23] (arginine hydrolysis negative; esculin hydrolysis negative in the presence of bile and positive in the absence of bile; mannitol, raffinose and inulin fermentation positive; and resistance to 2U of bacitracin).

The 160 strains were then classified according to biotype obtained with the APIZYM system for enzyme activity (bioMérieux; Marcy-l'Etoile, France), used according to the manufacturer's instructions. However, indications of enzyme activity that were positive or negative in >80% of the strains were excluded. Tests were read after incubation for 4 h at 36±1°C. Enzyme activity, reflected by a change in the color intensity of the substrate, was considered positive if a reading between 1 and 5 was obtained; a reading of 0 was considered negative. The test was carried out in triplicate for each strain, and each set of tests was scored by a different observer.

The susceptibilities of all 160 strains were then tested against amoxycillin, cefazolin, erythromycin, clindamycin, vancomycin (Sigma, St Louis, MO, USA), teicoplanin (Merrell Dow, Spain) and imipenem (Merck, Germany) by dilution in a solid medium [32], with concentrations between 0.003 and 1.0 mg/L, except for vancomycin and teicoplanin, which were tested at a range of 0.007–2.0 mg/L. The statistical significance of the differences in antibiotic susceptibilities of the biotypes was determined, based on the mean MIC (minimum inhibitory concentration) values with chi-squared tests.

RESULTS AND CONCLUSIONS

The new biotyping method was arrived at by excluding the results of enzyme activities that were positive or negative for >80% of the strains, leaving three enzymes to be considered: valine aryl amidase, acid phosphatase and α-galactosidase. The three API ZYM assays performed gave reproducible readings. Different combinations of the presence or absence of these three activities led to the identification of eight different biotypes of *S. mutans*, designated biotypes 1–8 (Table 1). The biotype that appeared most frequently (31.5% of strains) was biotype 5 (valine aryl amidase negative, acid phosphatase positive, α-galactosidase positive), followed by biotype 1 (+,+,+; 17.5% of strains). The least frequent was biotype 3 (+,−,−), which was represented by only four (2.5%) strains.

Table 1 Subdivision of 160 strains of *S. mutans* into eight biotypes

	Valine aryl amidase	Acid phosphatase	α-galactosidase	*n*	% of total
Biotype 1	+	+	+	28	17.5
Biotype 2	+	+	−	10	6.25
Biotype 3	+	−	−	4	2.5
Biotype 4	+	−	+	22	13.75
Biotype 5	−	+	+	50	31.25
Biotype 6	−	+	−	12	7.5
Biotype 7	−	−	+	24	15
Biotype 8	−	−	−	10	6.25

Table 2 Mean MIC, MIC range and MIC_{50} values (mg/mL) for selected antibiotics, associated with different biotypes of *S. mutans* (mg/L)

	Biotype							
Antibiotic	1	2	3	4	5	6	7	8
Amoxycillin								
MIC range	0.015–0.50	0.015–0.50	0.03–0.25	0.015–0.03	0.007–0.25	0.015–0.50	0.003–0.06	0.015–0.06
MIC_{50}	0.03	0.06	0.03	0.015	0.03	0.03	0.015	0.03
MIC mean	0.076	0.13	0.033	0.017	0.052	0.11	0.022	0.039
Cefazolin								
MIC range	0.03–1	0.03–0.25	0.007–0.06	0.015–0.06	0.007–0.25	0.015–0.50	0.003–0.06	0.015–0.06
MIC_{50}	0.06	0.06	0.007	0.015	0.03	0.03	0.03	0.03
MIC mean	0.16	0.10	0.033	0.025	0.049	0.19	0.032	0.027
Clindamycin								
MIC range	0.015–1	0.015–0.06	0.015–0.03	0.015–0.06	0.007–0.50	0.015–1	0.015–0.06	0.015–0.06
MIC_{50}	0.03	0.03	0.015	0.06	0.03	0.03	0.03	0.03
MIC mean	0.16	0.033	0.022	0.05	0.06	0.19	0.039	0.03
Erythromycin								
MIC range	0.015–0.50	0.015–0.12	0.015–0.12	0.015–0.12	0.07–1	0.015-0.25	0.015–0.12	0.03–0.06
MIC_{50}	0.06	0.03	0.015	0.03	0.06	0.03	0.03	0.06
MIC mean	0.16	0.048	0.067	0.041	0.11	0.069	0.051	0.048
Imipenem								
MIC range	0.007–0.25	0.015–0.50	0.25–0.50	0.007–0.25	0.007–0.50	0.007–1	0.003–0.03	0.007–0.03
MIC_{50}	0.03	0.03	0.25	0.03	0.015	0.015	0.015	0.015
MIC mean	0.094	0.212	0.375	0.083	0.077	0.22	0.015	0.016
Vancomycin								
MIC range	0.03–1	0.03–1	0.50–1	0.25–1	0.03–1	0.03–1	0.03–1	0.03–0.50
MIC_{50}	0.50	0.50	0.50	0.50	0.50	0.06	0.50	0.50
MIC mean	0.56	0.51	0.55	0.52	0.442	0.35	0.41	0.312
Teicoplanin								
MIC range	0.12–1	0.12–1	0.25–0.50	0.06–1	0.12–1	0.50–1	0.12–1	0.12–1
MIC_{50}	0.50	0.50	0.25	0.50	0.50	0.50	0.25	0.50
MIC mean	0.69	0.474	0.375	0.56	0.625	0.58	0.375	0.57

Comparisons of the mean MICs for each antibiotic, the MIC range and the MIC_{50} value (Table 2) showed that biotypes had different degrees of antibiotic susceptibility. As indicated by the mean MIC values, strains belonging to biotype 4 were the most susceptible to amoxycillin ($p<0.001$), cefazolin ($p<0.001$) and erythromycin ($p<0.01$), a finding supported by the MIC_{50} values. The mean MIC values showed biotype 1 to be the least susceptible to teicoplanin ($p<0.05$). According to the MIC_{50} values, imipenem appeared to be more effective against biotypes 5–8, while cefazolin was most effective against biotype 3 (Table 2).

An important advantage of this methodology for biotyping *S. mutans* in comparison with other approaches is its reproducibility. Since the method is based on a commercial product, and provides clear positive or negative readings, the results obtained are remarkably consistent. Moreover, it is a relatively fast and simple procedure, and should thus prove valuable in laboratory studies involving *S. mutans.*

Acknowledgments

This study was partially supported by the Andalusian Regional Government through the research project 'Microbiology, Immunology and Epidemiology of Oral Diseases.'

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