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Comparison of Three Antibiotic Susceptibility Tests for Viridans Group Streptococci

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Oral viridans streptococci are recognized as one of the etiological agents of a variety of infectious diseases such as dental caries and infective endocarditis. Although antimicrobial susceptibility tests for these fastidious bacterial species are now established and standardized, a comparison between the broth microdilution and broth macrodilution tests has not previously been performed. This comparison was performed in the present study using the tests adopted by the Clinical and Laboratory Standards Institute (CLSI) and seven clinical isolates of oral viridans streptococcal strains. A modified broth macrodilution susceptibility test method was also included in this analysis, in which the media was not supplemented with horse blood. The susceptibility interpretation category agreements were measured at 83% (broth microdilution versus broth macrodilution) and 71% (broth microdilution versus modified broth macrodilution). The interpretation category agreement between the broth macrodilution and modified broth macrodilution tests was also 83%. These data indicate that the interpretation of antibiotic susceptibility test results for oral viridans streptococci are influenced by the methods used.

Key words: antibiotic, streptococci, MIC, susceptibility

Introduction

Oral viridans streptococci are recognized as one of etiological agents of a variety of infectious diseases such as dental caries and infective endocarditis (Coykendall, 1989; Douglas *et al.*, 1993; Hamada and Slade, 1980; Takahashi and Nyvad, 2011).

Antimicrobial susceptibility testing can be done by several different methods. The disk diffusion procedures have been standardized primarily for testing common, rapidly growing bacteria (Clinical and Laboratory Standards Institute, 2009a). This method should not be used to evaluate antimicrobial susceptibilities of bacteria that show marked strain-to-strain variability in growth rates, e.g., some fastidious or anaerobic bacteria (Jorgensen, 2007). Even though the disk diffusion test method has been modified to allow reliable testing of certain fastidious bacteria, it is known that it is unreliable and should not be used for viridians group streptococci (Hinder, 2007).

It is generally considered that the agar dilution method is the reference against other methods (Baker *et al.*, 1991; Brown and Brown, 1991). However, the broth microdilution and the broth macrodilution method are routinely used in many clinical laboratories because the agar dilution method is time-consuming and inadequate for a routine testing (Brown and Brown, 1991; Mokaddas *et al.*, 2007).

Correlations between Minimal Inhibitory Concentration (MIC) values by microdilution broth method and macrodilution method for bacterial species other than oral streptococci such as *Escherichia coli* and *Staphylococcus aureus* have been reported to be between 85 and 96% (Harwick *et al.*, 1968; Marymont and Wentz, 1966). Very few studies have been carried out to compare the results of the broth microdilution method and the broth macrodilution method for testing antibiotic susceptibility of oral viridians group streptococcal isolates. The purpose of this study was to provide data comparing the broth microdilution antimicrobial susceptibility test for oral viridians streptococci with the broth macrodilution test.

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Materials and Methods

Isolation and identification of bacterial strains

Oral streptococcal strains were isolated from the supragingival plaque of healthy persons who have not taken antibiotics in recent 2 months. Supragingival plaque was transferred into a glass tube containing phosphate buffered saline (PBS) and glass beads. Dispersed plaque samples were diluted with PBS and plated on Mitis Salivarius agar plates (Becton, Dickinson and Company, Sparks, MD, USA). After incubating at 37°C for 48 h in aerobic condition supplemented with 5% CO_2, one bacterial colony from each plaque sample was isolated and grown in Brain Heart Infusion broth (Becton, Dickinson and Company). Further identifications were performed with the rapid API-20 Strep system and mini API reader (bioMerieux, Marcy-l'Etoile, France).

All volunteers who wanted to donate their plaques for this study were thoroughly informed about the procedure and gave written consent for inclusion in the study. This study was approved by the Institutional Review Board of Gangneung-Wonju National University Dental Hospital (IRB2011-2).

Bacterial strains

One streptococcal isolated strain of each species of viridans group streptococci obtained in this study was included for determination of MIC with broth microdilution susceptibility test and broth macrodilution test. *Streptococcus anginosus* KN427, *Streptococcus constellatus* KN436, *Streptococcus gordonii* KN180, *Streptococcus mitis* KN156, *Streptococcus mutans* KN405, *Streptococcus oralis* KN444 and *Streptococcus sanguinis* KN420 were used in this study.

Determination of MIC

To determine the MIC of the antibiotics, stock antibiotic solutions of penicillin G, ampicillin, streptomycin, gentamicin, amoxicillin and tetracycline were prepared. Antibiotics were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). The MICs were determined according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (Clinical and Laboratory Standards Institute, 2009b; Clinical and Laboratory Standards Institute, 2011) with 6 antimicrobial agents by a microdilution method and a macrodilution method in cation-adjusted Mueller-Hinton broth supplemented with lysed horse blood as recommended by CLSI.

Using streptococcal colonies taken directly from sheep blood agar plates (Hanil-KOMED, Sungnam, Korea) which were incubated at 37°C for 20 h in aerobic condition supplemented with 5% CO_2, a suspension equivalent to that of the 0.5 McFarland standard (approximately 1×10^8 CFU/ml) in cation-adjusted Mueller-Hinton broth were prepared. The bacteria were inoculated into serially diluted antibiotic solutions in 13 × 100 mm test tubes in macrodilution method and 96 well round bottom microtitration plates in microdilution method for final concentrations of 5×10^5 CFU/ml. The final volumes were 2 ml in macrodilution methods and 100 μl in microdilution methods. The microdilution trays and macrodilution tubes were incubated in an ambient air incubator at 37°C for 24 h.

In addition to the broth microdilution test and broth macrodilution test of CLSI, a modified broth macrodilution test was also performed. In a modified broth macrodilution method, the lysed horse blood was not added into cation-adjusted Mueller-Hinton broth and macrodilution tubes were incubated in aerobic condition supplemented with 5% CO_2 instead of ambient air. Other procedures in the modified macrodilution method were same as the macrodilution method of CLSI.

MICs were determined by interpretive standard concentrations from CLSI guidelines (Clinical and Laboratory Standards Institute, 2011). The tests were repeated at least twice. Range of concentrations tested for each antibiotic was from 0.001 μg/ml to 1024 μg/ml.

Results

Table 1 summarizes results of MIC and interpretation tested by broth microdilution method (CLSI), broth macrodilution method (CLSI) and modified broth macrodilution method for 7 oral streptococcal isolates to 6 different antibiotics. Susceptibility interpretation category agreement was 83% (broth microdilution versus broth macrodilution) and 71% (broth microdilution versus modified broth macrodilution). Category agreement between broth macrodilution and modified broth macrodilution was 83%.

Discussion

In this study, a comparison of the broth microdilution test and the broth macrodilution test of CLSI for 6 antibiotics was performed with 7 clinical isolates of oral viridians streptococcal strains. One recent study reported that there is a discrepancy in antimicrobial susceptibility test results obtained for oral streptococcal isolates with the E test and agar dilution method (Mokaddas *et al.*, 2007). They reported that the E Test appears to be as efficient as agar dilution method for susceptibility testing of viridans streptococci, except for vancomycin, where very major errors in the results were relatively high. This is the first reported comparison between the CLSI broth microdilution susceptibility test method and broth macrodilution method for viridians group streptococci.

The results of in vitro susceptibility test with broth microdilution and broth macrodilution procedures were found to be comparable except some antimicrobial-organism combinations. The reasons for these discrepancies are unclear. The discrepancy in test results from each method may be explained by the observation that results from the broth microdilution method are easier to read. Because lysed horse

Table 1. Comparison of MIC and interpretation among broth microdilution method (CLSI), broth macrodilution method (CLSI) and modified broth macrodilution method

Streptococcal Strain	Antibiotic	Microdilution (CLSI)		Macrodilution (CLSI)		Modified Macrodilution	
		MIC(μg/ml)	Interpretation	MIC(μg/ml)	Interpretation	MIC(μg/ml)	Interpretation
S. anginosus KN427	Pen	0.031	S	0.002	S	0.031	S
	Amp	2	I	1	I	1	I
	Amo	0.125	S	0.002	S	0.031	S
	Str	32	S	16	S	8	S
	Gen	4	S	2	S	1	S
	Tet	< 0.001	S	0.125	S	0.004	S
S. constellatus KN436	Pen	0.004	S	0.016	S	0.031	S
	Amp	0.125	S	1	I	2	I
	Amo	0.008	S	0.031	S	0.125	S
	Str	16	S	32	S	16	S
	Gen	1	S	4	S	2	S
	Tet	8	I	4	S	8	I
S. gordonii KN180	Pen	0.25	I	0.008	S	0.002	S
	Amp	16	R	0.5	I	0.125	S
	Amo	0.25	S	0.063	S	0.031	S
	Str	16	S	16	S	16	S
	Gen	64	R	8	I	8	I
	Tet	128	R	32	R	16	R
S. mitis KN156	Pen	0.031	S	0.125	S	0.008	S
	Amp	0.5	I	0.25	I	0.5	I
	Amo	0.016	S	0.004	S	0.008	S
	Str	32	S	64	S	32	S
	Gen	8	I	4	S	8	I
	Tet	64	R	64	R	64	R
S. mutans KN405	Pen	0.125	S	0.125	S	0.008	S
	Amp	8	R	2	I	0.5	I
	Amo	0.063	S	0.125	S	< 0.001	S
	Str	2	S	32	S	2	S
	Gen	8	I	4	S	0.125	S
	Tet	2	S	4	S	0.5	S
S. oralis KN444	Pen	0.125	S	0.25	I	0.125	S
	Amp	4	R	4	R	2	I
	Amo	0.125	S	0.25	S	0.125	S
	Str	8	S	32	S	8	S
	Gen	2	S	2	S	0.031	S
	Tet	64	R	512	R	16	R
S. sanguinis KN420	Pen	0.063	S	0.031	S	0.031	S
	Amp	4	R	16	R	1	I
	Amo	0.125	S	0.125	S	0.031	S
	Str	8	S	4	S	1	S
	Gen	2	S	2	S	0.125	S
	Tet	16	R	16	R	4	S

Pen: penicillin G; Amp: ampicillin; Amo: amoxicillin; Str: streptomycin; Gen: gentamicin; Tet: tetracycline; S: susceptible; I: intermediate resistant; R: resistant

blood was added in cation-adjusted Mueller-Hinton broth, the detection of bacterial growth in broth macrodilution tubes (reaction volume 2 ml) is often not easier than in broth microdilution trays (reaction volume 100 μl). The other

problem in the methods of CLSI was the difficulty of growth of some species of oral streptococci when incubating in an ambient air incubator without CO_2 supplement. CLSI method recommended that the trays and the tubes are incubated in ambient air at 37°C for 20 to 24 hours before reading the MICs (Clinical and Laboratory Standards Institute, 2009b). But, some strains did not grow enough to determine the MIC within 24 h in an ambient air incubator.

The modified broth macrodilution method was developed to overcome these problems of CLSI methods, in which lysed horse blood was not added and macrodilution tubes were incubated in aerobic condition supplemented with 5% CO_2. In the modified broth macrodilution method, bacteria grow well enough to determine MICs within 24 h and the detection of bacterial growth in macrodilution tubes were much easier than the CLSI macrodilution method. The modified broth microdilution method yielded overall results that were comparable to those of the broth microdilution method of CLSI.

The purpose of this study was to provide data comparing broth microdilution susceptibility tests for viridians group streptococci against 6 different antibiotics with the broth macrodilution methods. Because the results of this study were from a limited number of streptococcal strains, further studies will be necessary in which a large number of oral streptococcal isolates were included. However, it should be considered that the results of antibiotic susceptibility tests with oral viridans streptococci are influenced by test method.

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