JOURNAL OF CLINICAL MICROBIOLOGY, July 1998, p. 1907–1911 Vol. 36, No. 7
0095-1137/98/$04.00+0

Effects of Different Test Conditions on MICs of Food Animal Growth-Promoting Antibacterial Agents for Enterococci

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Received 23 September 1997/Returned for modification 31 December 1997/Accepted 7 April 1998

The influence of the addition of sheep blood to Mueller-Hinton II agar and the effects of aerobic incubation with or without CO_2 and of anaerobic incubation were tested with bacitracin, tylosin, avoparcin, virginiamycin, avilamycin, narasin, and flavomycin on enterococci. The antibacterial activity of bambermycin (Flavomycin) was strongly inhibited by the addition of blood, except with the species *Enterococcus faecium*, *Enterococcus mundtii*, *Enterococcus hirae*, *Enterococcus casseliflavus*, and *Enterococcus gallinarum*, which were not susceptible to this antibiotic on blood-free medium. With all other antimicrobials except avoparcin and tylosin, the presence of blood resulted in MIC increases of 1 to 3 $\log_2$ differences. Incubation in aerobic or anaerobic atmospheres enriched with CO_2 lowered the susceptibility of enterococci to tylosin and increased their susceptibility to avilamycin, narasin, and avoparcin. This effect was most pronounced in tests on blood-free media. Results of susceptibility tests incubated under anaerobiosis and in a CO_2-enriched atmosphere did not differ. For all enterococcal species, the preferred conditions for testing the susceptibility are Mueller-Hinton II medium supplemented with blood and incubation in a CO_2-enriched atmosphere. However, when only *E. faecium* and *Enterococcus faecalis* are being tested, Mueller-Hinton II medium without blood incubated aerobically gives satisfactory results.

Several enterococcal species belong to the normal intestinal flora of humans and animals. *Enterococcus faecalis*, *Enterococcus faecium*, and more rarely other species may be involved in human infections. Antibiotic therapy is problematic because of resistances. In particular, penicillin, aminoglycoside, and glycopeptide resistances cause problems (12, 15).

To date, in animals, enterococcal infections are not known to play an important role, possibly because geriatric medicine and intensive care are less well developed. Recently the susceptibility of these intestinal bacteria to food animal growth-promoting antimicrobial agents not used in therapy has become a subject of concern (1, 11, 18). However, few data on the in vitro susceptibility of intestinal bacteria to these agents are available (7). Enterococci from animals have been tested for susceptibility and resistance to growth promoters and therapeutic agents by the disc diffusion method (13), agar dilution tests in Mueller-Hinton agar (6), and the broth dilution method (18). These differences in test methods make test results difficult to compare.

Enterococci need rich media for rapid growth (3). Most probably because the usual standardized Mueller-Hinton II medium was unsatisfactory in this respect, a modification of culture conditions, consisting of the use of brain heart infusion, was suggested by the National Committee for Clinical Laboratory Standards (NCCLS) (standard M100-S7 for use with M7-A4) (14) for testing resistance of *E. faecalis* and *E. faecium* to gentamicin, streptomycin, and vancomycin. This complex medium has not been standardized for antibiotic susceptibility testing, and differences may occur between different manufacturers. Furthermore, several species of enterococci belonging to the normal flora of animals require special growth conditions. A CO_2-enriched environment is necessary for the growth of *Enterococcus columbae* and *Enterococcus cecorum* (4). Other species show enhanced growth when incubated in a CO_2-enriched environment. The natural habitat of the intestinal enterococci is, however, largely anaerobic, and these facultative anaerobic bacteria have a preference for anaerobic conditions (3). These special requirements imply a need for specialized culture conditions.

The activity of several antibiotics is known to be influenced by the composition of the medium and/or by the incubation conditions. The possible effects of the composition of the medium and/or incubation conditions are much less well-known for the growth-promoting antibacterials. The only well-known phenomenon involving these substances is the influence of CO_2 on the pH of the medium, which affects the activity of tylosin, a macrolide antibiotic (9, 10, 16).

Therefore, we wished to investigate the effects of different modifications of the standardized Mueller-Hinton II agar dilution procedure on inhibitory activity of growth promoters. Such modifications have been proposed by the NCCLS for use with fastidious bacteria (standard M100-S7 for use with M7-A4) (14). The effects of sheep blood (5%), CO_2 incubation, and anaerobic incubation on the MICs of seven growth-promoting agents used in Europe for collection strains of animal-associated species of the genus *Enterococcus* were determined.

MATERIALS AND METHODS

Strains. The tested collection strains of intestinal enterococci belonging to different species groups are shown in Table 1 (5, 21). The *E. faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 29213 control strains used for susceptibility tests were included in all tests.

Antibiotics. The following antibiotic preparations, manufactured for analytical purposes, were tested: avoparcin (American Cyanamid, Princeton, N.J.), bambermycin (Flavomycin; Hoechst, Frankfurt, Germany), virginiamycin (Pfizer, Rixensart, Belgium), bacitracin (67,000 U/mg) (Sigma, St. Louis, Mo.), tylosin (Sigma), avilamycin (Eli Lilly, Indianapolis, Ind.), and narasin (Eli Lilly). Antibiotics were dissolved in appropriate solvents to make stock solutions containing 1,000 μg/ml and then further diluted in sterile distilled water according to the

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TABLE 1. Enterococcal collection strains tested

Species group	Species	Strain[a]
E. faecalis group	*E. faecalis*	LMG 7937^T ATCC 29213 CCM 5613
E. faecium group	*E. faecium*	LMG 11423^T LMG 1620 LMG 10741
	Enterococcus mundtii	LMG 10748^T LMG 13044 LMG 12308
	Enterococcus hirae	LMG 6399^T NCFB 1632 LMG 11492
	Enterococcus durans	LMG 12691^T NCFB 498 LMG 122283
E. avium group	*Enterococcus avium*	LMG 10744^T LMG 12303 LMG 12304
	Enterococcus raffinosus	LMG 12300
	Enterococcus malodoratus	LMG 10747^T LMG 13602
E. gallinarum group	*Enterococcus casseliflavus*	LMG 10745^T LMG 16281 LMG 16285
	Enterococcus gallinarum	LMG 13129^T LMG 16204 LMG 16201
E. cecorum group	*E. columbae*	LMG 11740^T LMG 11747 LMG 12295
	E. cecorum	LMG 12902^T CCUG 27881 LMG 11743

[a] ATCC, American Type Culture Collection, Rockville, Md.; LMG, Collectie Laboratorium voor Microbiologie, Ghent, Belgium; CCM, Collection of Microorganisms, Brno, Czechia; NCFB, National Collection of Food-Borne Bacteria, now merged with National Collection of Industrial and Marine Bacteria, Aberdeen, United Kingdom; CCUG, Culture Collection of the University of Göteborg, Göteborg, Sweden.

methods recommended by the NCCLS (standard M100-S7 for use with M7-A4) (14).

Antimicrobial susceptibility tests. MIC tests were carried out on Mueller-Hinton II agar (Becton Dickinson, Cockeysville, Md.) with or without 5% sheep blood containing doubling dilutions of the antibiotics and used on the day of preparation. Appropriate antibiotic-free agar plates were included as controls. Inocula were prepared by diluting brain heart infusion (Oxoid, Basingstoke, United Kingdom) cultures in buffered saline overnight to a density of 0.5 on the McFarland turbidity scale and diluted 40-fold before inoculation. Plates were seeded with approximately 10^5 CFU.

Plates with or without sheep blood added were incubated aerobically, anaerobically (GasPak Plus; Becton Dickinson) with more than 4% but less than 10% CO_2, or in a CO_2 (5%)-enriched aerobic environment for 24 h.

RESULTS

Effect of blood supplementation. When plates were incubated aerobically, major differences between Mueller-Hinton media with and without blood were noted in bambermycin MICs for several strains (44% of the strains) (Table 2). This increase in MIC on blood-containing media was not evident in the range of concentrations tested with strains for which MICs obtained on blood-free media were high. These all belonged to the *E. faecium* and *E. gallinarum* species groups. Narasin MICs were almost systematically (72% of the strains) 2 or 3 doubling dilutions higher on blood-supplemented agar. With bacitracin, differences of 1 or 2 doubling dilutions were seen with most strains (72%), and for some strains, a much higher MIC was seen when blood was added. With virginiamycin and avilamycin, the MIC was 1 or 2 doubling dilutions higher for 48 and 44% of the strains, respectively. With avoparcin and tylosin, no important differences were found. It should be noted that none of the strains of the *E. cecorum* group grew in these aerobically incubated tests. The same was seen with certain of the *E. avium* group strains but only on blood-free media.

All strains grew when incubations were performed anaerobically (Table 2), and differences regarding blood supplementation effects were similar to those seen in the aerobically incubated tests. For bambermycin, major differences were seen in 61% of the strains tested. Virginiamycin, avilamycin, and bacitracin tended to have MICs 1 doubling dilution higher on blood-supplemented media for most of the strains (60, 80, and 76%, respectively). With narasin, the effect of blood was the same as that observed when plates were incubated aerobically. Tylosin MICs were only slightly different for 24% of the strains.

Upon incubation in a CO_2-enriched atmosphere (Table 2),

TABLE 2. Log_2 differences in the comparison of test results on Mueller-Hinton II medium either supplemented with 5% sheep blood or not supplemented

Incubation condition and antibiotic	n[a]	No. of strains with log_2 difference[b]:											
		−1	0	1	2	3	4	5	6	7	8	9	11
Aerobic													
Bambermycin	25		12		2			2	2	3	1	2	1
Virginiamycin	25		13	11	1								
Avilamycin	25		14	7	4								
Narasin	25		5	2	8	10							
Avoparcin	25	1	17	7									
Bacitracin	25		4	12	6	1	1		1				
Tylosin	25	1	16	6	2								
Anaerobic													
Bambermycin	33		13				1	1	9	6	3		
Virginiamycin	33	1	12	20									
Avilamycin	33		6	22	5								
Narasin	33		2	4	15	10	2						
Avoparcin	33		23	10									
Bacitracin	33	1	7	14	8	2			1				
Tylosin	33	1	24	6	1	1							
CO_2-enriched atmosphere													
Bambermycin	33		11	1	1		4	3	5	3	3	2	
Virginiamycin	33		14	17	1	1							
Avilamycin	33		8	16	9								
Narasin	33		1	13	15	1	3						
Avoparcin	33	2	16	15									
Bacitracin	33		7	15	7	4							
Tylosin	33	3	26	3	1								

[a] Number of strains tested.

[b] A positive difference is a higher MIC of the antibiotic incubated with 5% sheep blood; a negative difference is a higher MIC of the antibiotic incubated without 5% sheep blood. Strains were tested for log_2 differences of between −7 and 11; however, no differences of −7 to −2 or 10 were found.

TABLE 3. Log_2 differences in the comparison of test results from incubation occurring aerobically and anaerobically

Supplement and antibiotic	n^a	No. of strains with log_2 difference[b]											
		−7	−6	−4	−3	−2	−1	0	1	2	3	6	8
Blood													
Bambermycin	26						7	17	2				
Virginiamycin	26						3	17	6				
Avilamycin	26					5	3	15	3				
Narasin	26					1	16	6	3				
Avoparcin	26					1	8	14	3				
Bacitracin	26						3	21	1	1			
Tylosin	26						1	13	9	2	1		
None													
Bambermycin	25	1	1			1	4	14		2		1	1
Virginiamycin	25						3	19	1	1	1		
Avilamycin	25					3	13	8	1				
Narasin	25			1	1	5	14	4					
Avoparcin	25					1	12	12					
Bacitracin	25					1	7	11	6				
Tylosin	25						1	14	6	2	2		

[a] Number of strains tested.
[b] A positive difference is a higher MIC of the antibiotic incubated anaerobically; a negative difference is a higher MIC of the antibiotic incubated aerobically. Strains were tested for log_2 differences of between −7 and 11; however, no differences of −5, 4, 5, 7, or 9 to 11 were found.

TABLE 5. Log_2 differences in the comparison of test results from incubation occurring anaerobically and in a CO_2-enriched atmosphere

Supplement and antibiotic	n^a	No. of strains with log_2 difference[b]								
		−3	−2	−1	0	1	2	3	4	5
Blood										
Bambermycin	33		2	1	20	10				
Virginiamycin	33			1	25	7				
Avilamycin	33			1	22	5	5			
Narasin	33			9	20	4				
Avoparcin	33			2	28	3				
Bacitracin	33			3	27	2		1		
Tylosin	33			1	29	2		1		
None										
Bambermycin	33		1	4	20	6			1	1
Virginiamycin	33			3	22	6	2			
Avilamycin	33			1	26	4	2			
Narasin	33		1	2	19	7	3	1		
Avoparcin	33			3	17	13				
Bacitracin	33			6	18	6	2	1		
Tylosin	33	1	1	6	22	3				

[a] Number of strains tested.
[b] A positive difference is a higher MIC of the antibiotic incubated in a CO_2-enriched atmosphere; a negative difference is a higher MIC of the antibiotic incubated aerobically. Strains were tested for log_2 differences of between −7 and 11; however, no differences of −7 to −4 or 6 to 11 were found.

the same tendencies in MIC differences were seen as when incubation was performed anaerobically.

Anaerobic versus aerobic incubation. When blood-supplemented plates were used (Table 3), the antibiotics bambermycin, avilamycin, narasin, and avoparcin showed slightly higher MICs upon anaerobic incubation than upon aerobic incubation for 27, 30, 65, and 34% of the strains, respectively. With tylosin, the opposite was seen in 46% of the strains tested. For virginiamycin and bacitracin, there was an almost equal distribution between higher and lower MICs when the aerobic test results were compared with the anaerobic ones.

No uniform effect of anaerobic incubation was seen on Mueller-Hinton plates without blood supplement (Table 3). Avilamicin, narasin, and avoparcin had slightly lower MICs when incubated anaerobically for 64, 84, and 52% of the strains, respectively. Virginiamycin and bacitracin anaerobic and aerobic test results did not differ, while tylosin showed a tendency that was again opposite to that of the other antibiotics tested: MICs for 40% of the strains were higher when the strains were incubated anaerobically.

Aerobic incubation versus incubation in a CO_2-enriched atmosphere. On Mueller-Hinton plates supplemented with blood, no major differences were noted (Table 4). For avilamycin, narasin, avoparcin, and to a lesser extent bacitracin, there was a tendency towards slightly lower MICs on plates incubated in CO_2 with 46, 77, 58, and 31% of the strains, respectively. In tests with tylosin, the opposite was seen with 50% of the strains. Bambermycin and virginiamycin results did not differ when strains were incubated aerobically or in a CO_2-enriched atmosphere.

When Mueller-Hinton plates without blood were used (Table 4), differences between results obtained with aerobic incubation and in a CO_2-enriched atmosphere were relatively unimportant and similar to the ones noted in the comparative tests with anaerobic incubation.

Anaerobic incubation versus incubation in a CO_2-enriched atmosphere. Between anaerobic incubation or incubation in a CO_2-enriched atmosphere, there were no substantial differences in MIC results on plates with and without blood (Table 5).

MICs obtained in tests on Mueller-Hinton II with blood and incubated in 5% CO_2 are tabulated in Table 6.

TABLE 4. Log_2 differences in the comparison of test results from incubation occurring aerobically and in a CO_2-enriched atmosphere

Supplement and antibiotic	n^a	No. of strains with log_2 difference[b]										
		−7	−5	−4	−3	−2	−1	0	1	2	4	6
Blood												
Bambermycin	26							23	2	1		
Virginiamycin	26						4	19	3			
Avilamycin	26					5	7	8	3	3		
Narasin	26					7	13	6				
Avoparcin	26					1	14	11				
Bacitracin	26						8	17	1			
Tylosin	26							13	10	3		
None												
Bambermycin	25	1	1					2	13	3	3	1	1
Virginiamycin	25							5	18	2			
Avilamycin	25						1	14	10				
Narasin	25			2	1	4	7	10	1				
Avoparcin	25						20	5					
Bacitracin	25						16	8		1			
Tylosin	25							8	11	6			

[a] Number of strains tested.
[b] A positive difference is a higher MIC of the antibiotic incubated in an CO_2-enriched atmosphere; a negative difference is a higher MIC of the antibiotic incubated aerobically. Strains were tested for log_2 differences of between −7 and 11; however, no differences of −6, 3, 5, or 7 to 11 were found.

DISCUSSION

Blood supplementation of Mueller-Hinton plates has been shown to have an effect on the MICs of sulfonamide, trimethoprim, cephalosporins, novobiocin, and nafcillin for enterococci (22). The addition of blood reduces MICs of cephalosporins, but not all cephalosporins react in the same way:

TABLE 6. MICs of different growth-promoting antibiotics for enterococci tested on blood-supplemented Mueller-Hinton II agar and incubated in CO_2

Antibiotic and strain	n[a]	MIC (μg/ml)	
		Median	Range
Bambermycin			
E. faecalis ATCC 29212	4	12	8–16
S. aureus ATCC 29213	4	2	1–4
E. faecalis	2	32	32
E. faecium species group	12	>64	64–>64
E. gallinarum species group	6	32	32–64
E. avium species group	6	>64	>64
E. cecorum species group	6	1.5	0.5–4
Virginiamycin			
E. faecalis ATCC 29212	3	2	2–4
S. aureus ATCC 29213	3	0.25	0.25
Enterococcus (all species)[b]	32	1	0.12–4
Avilamycin			
E. faecalis ATCC 29212	3	0.5	0.5–2
S. aureus ATCC 29213	3	2	1–2
Enterococcus (all species)	32	0.5	0.06–2
Narasin			
E. faecalis ATCC 29212	3	0.5	0.25–0.5
S. aureus ATCC 29213	3	0.5	0.25–0.5
Enterococcus (all species)	32	0.5	0.06–1
Avoparcin			
E. faecalis ATCC 29212	3	2	2
S. aureus ATCC 29213	3	4	4
Enterococcus (all species)[c]	26	0.5	0.25–2
Bacitracin[d,e]			
E. faecalis ATCC 29212	3	4	4–8
S. aureus ATCC 29213	3	2	2
E. faecalis	2	5	2–8
E. faecium species group	11	8	0.25–8
E. gallinarum species group	6	8	8–16
E. avium species group	6	2	0.5–16
E. cecorum species group	6	≤0.03	≤0.03–0.12
Tylosin[d]			
E. faecalis ATCC 29212	3	2	1
S. aureus ATCC 29213	3	1	1–2
Enterococcus (all species)	24	2	0.5–2

[a] Number of repeats for *E. faecalis* ATCC 29212 and *S. aureus* ATCC 29213, and number of strains tested for the other enterococci.
[b] All *Enterococcus* species except *E. faecalis* ATCC 29212.
[c] All species except *E. faecalis* ATCC 29212 and the species from the *E. gallinarum* species group, which possess the *vanC* gene (8).
[d] Excluding strains with acquired resistance.
[e] The MIC of bacitracin is expressed in units/milliliter.

some remain unaffected, and for others the effect does not exceed 1 or 2 doubling dilutions. This is dependent on the chemical structure of the cephalosporins (2, 17). In the present study, major detrimental effects of blood on antibacterial activity were seen with bambermycin. In the original description of this antibiotic (20), which at that time was called moenomycin, it was indicated that serum inactivates its in vitro activity by 10 to 0.1% compared to protein-free medium. The effects of blood seen in the present tests were much more dramatic, at least on the inhibitory activity on species and strains which were susceptible to relatively low concentrations of this antibiotic. A similar effect of blood may possibly be found in higher concentration ranges for the other strains.

Few reports deal with the effects of anaerobic incubation on MIC results. The activity of aminoglycosides is significantly reduced with *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis* incubated anaerobically without CO_2 (19). The reason for the negative effect on tylosin activity is found in the presence of CO_2, which has an effect on the pH of the medium (9, 10, 16). The causes of the slightly positive effects of CO_2 and anaerobic incubation on the inhibitory activity of avilamycin, narasin, and avoparcin observed in the present investigation are unknown, though they are most probably found in the effect of CO_2 on the activity of the antibiotics. To our knowledge, nothing is known about a pH effect on the MICs of these growth promoters. The activity of bambermycin has been reported to be affected by pH increases (20). This was not evident in our tests on blood-containing media, not even with strains such as the *S. aureus* control strain and the few other strains that were inhibited by relatively low concentrations of bambermycin in this medium (Table 6). The effects of this antibiotic on blood-free medium were strongly variable and strain dependent. It should be noted that the comparisons between aerobic incubation results and the results obtained in aerobic or aerobic CO_2-supplemented atmospheres are somewhat hindered by the presence of *E. cecorum* and of *E. avium* group strains which did not grow or which grew poorly aerobically. These strains were not included in the comparative tables.

Many changes in MICs corresponded to the species groups, or to several species of a species group, indicating that there may be species-specific growth requirements and that the species-specific metabolism may play a role in the differences noted. For the capnophilic bacteria, CO_2 dependency is clearly species group related; none of the strains in the *E. cecorum* group grow without CO_2, and some of the strains in the *E. avium* group have such poor growth without CO_2 that MICs could not be evaluated. However, for the other species-related changes, no obvious reason can be found for the differences.

It can be concluded that Mueller-Hinton II agar can be used for testing of the inhibitory activity of growth-promoting agents on enterococci. The addition of blood decreases the activity of these products to various degrees. The addition of CO_2 under both aerobic and anaerobic conditions enhances the inhibitory effects of narasin, avoparcin, and avilamycin and is detrimental to the activity of tylosin. When CO_2 supplementation is necessary because capnophilic strains are to be tested, the choice between anaerobic incubation with 4 to 10% CO_2 in the jars or incubation in a CO_2 incubator can be left open. The preferred conditions for testing the susceptibility when all enterococcal species are being compared is Mueller-Hinton II medium supplemented with blood and incubated in a CO_2-enriched atmosphere. When only *E. faecium* and *E. faecalis* are being tested, Mueller-Hinton II medium incubated aerobically gives satisfactory results. It should be recognized, however, that the omission of blood strongly influences results obtained with certain antibiotics, especially bambermycin and narasin.

ACKNOWLEDGMENTS

This work has been supported by the Research Fund of the University of Ghent, Codenr. BOZF97/N2/022.

We thank A. Van de Kerckhove for her skilled technical assistance.

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