JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 2000, p. 3652–3655 Vol. 38, No. 10
0095-1137/00/$04.00+0

Evaluation of D-Xylose and 1% Methyl-α-D-Glucopyranoside Fermentation Tests for Distinguishing *Enterococcus gallinarum* from *Enterococcus faecium*

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Received 17 April 2000/Returned for modification 29 May 2000/Accepted 21 July 2000

To determine the validity of the rapid xylose and methyl-α-D-glucopyranoside (MDG) fermentation tests in distinguishing *Enterococcus gallinarum* from *Enterococcus faecium*, 156 well-characterized clinical isolates of enterococci (55 *E. gallinarum*, 91 *E. faecium*, and 10 *Enterococcus faecalis* isolates) known to be of different clones were examined in a blinded fashion. Species identification was confirmed by PCR of the *ddl* ligase genes of *E. faecium* and *E. faecalis* and the *vanC1* gene of *E. gallinarum*. Xylose tests were performed with D-xylose tablets by using a heavy bacterial suspension and were interpreted after 2 h of incubation. Standard MDG fermentation tests were read after 24 h of incubation. The xylose fermentation test had a sensitivity of 98% (54 of 55) and a specificity of 99% (100 of 101) in distinguishing *E. gallinarum* from *E. faecium* and *E. faecalis*. The standard MDG test had a sensitivity of 100% (55 of 55) and a specificity of 95% (96 of 101) after 24 h. The xylose fermentation test is a simple method, easily incorporated into laboratory protocols, that distinguishes *E. gallinarum* from *E. faecium* with high sensitivity and specificity in 2 h. The standard MDG test has high sensitivity and can be useful in ruling out the presence of *E. gallinarum* but requires overnight incubation.

Enterococci are increasingly common causes of hospital-acquired infection and have become progressively more resistant to antibiotics (4, 8, 18, 38). Until 1990, at least 90% of enterococcal infections were due to *Enterococcus faecalis*, and infections due to ampicillin- or vancomycin-resistant enterococci were rare (14, 33). However, in the last decade, the proportion of infections due to *Enterococcus faecium* has increased, ampicillin resistance in *E. faecium* has become common, and vancomycin-resistant enterococci have become endemic in many hospitals (8, 17–19, 21).

The most common clinically important enterococci are *E. faecalis* and *E. faecium*. In these species, vancomycin resistance is associated with the *vanA*, *vanB*, *vanD*, or *vanE* gene cluster (9, 12, 13, 23, 30). The *vanA* and *vanB* gene clusters are acquired through the transfer of plasmids or transposons (9, 13). *E. faecalis* and *E. faecium* spread rapidly in hospitals, and outbreaks with vancomycin-resistant strains are being described with increasing frequency (2, 24, 26, 40). In contrast, *Enterococcus gallinarum* and *Enterococcus casseliflavus* possess intrinsic, nontransferable vancomycin resistance encoded by *vanC1* and *vanC2* ligase genes, respectively (1). These species seldom cause infection and are rarely associated with transmission and hospital outbreaks (20, 32, 35).

Hence, for infection control purposes and prevention of person-to-person transmission, vancomycin-resistant *E. faecium* and *E. faecalis* need to be rapidly and accurately distinguished from *E. gallinarum* and *E. casseliflavus* (4). Isolates of *E. casseliflavus* are easily distinguished by their yellow pigment production (11). However, automated detection systems commonly fail to accurately differentiate between *E. gallinarum* and *E. faecium* (5, 15). Motility has been used as a distinguishing feature, but up to 8% of *E. gallinarum* strains are nonmotile (B. M. Willey, E. O. Petrof, L. Louie, I. Campbell, H. Dick, S. Richardson, A. McGeer, and D. E. Low, Abstr. 36th Intersci. Conf. Antimicrob. Agents Chemother, abstr. D31, 1996). Another test described in the literature for discriminating between enterococcal species involves acidification of methyl-α-D-glucopyranoside (MDG) (6). This fermentation test however, requires an overnight incubation step. All enterococci ferment xylose, although *E. gallinarum*, having performed enzyme, does so more quickly. The objective of this study was to determine the validity of the rapid xylose fermentation test compared with PCR in distinguishing between *E. gallinarum* and *E. faecium*. Standard and modified MDG tests were also examined.

(Part of this work was presented at the 39th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, Calif., 26 to 29 September, 1999.)

MATERIALS AND METHODS

Strains. In total, 156 clinical isolates of enterococci, from diverse geographic locations, were studied. All had been previously identified to the species level by Gram staining and conventional biochemical testing (pyrrolidonyl arylamidase, pigment production, and fermentation of mannitol, arabinose, sorbose, sorbitol, raffinose, and pyruvate) (10). Motility was determined by using motility test medium containing triphenyl-tetrazolium chloride (PML Microbiologicals, Wilsonville, Oreg.) incubated at 30°C for 18 h. All vancomycin-resistant isolates were further characterized by determining antibiotic susceptibility profiles (broth microdilution MICs of vancomycin, teicoplanin, ampicillin, gentamicin, and streptomycin), *vanA* and *vanB* genotypes, and clonal relatedness using *Sma*I pulsed-field gel electrophoresis (25, 27–29). Strains confirmed to contain *vanA* or *vanB* resistance genes were separated into distinct clones based primarily on the published consensus guidelines of Tenover et al. (34). However, this assessment was also influenced by the epidemiological origin of each isolate as well as the considerable intercluster band differences known to be associated with the enterococci (22). The species, vancomycin resistance genotypes, and clonal relatedness of the enterococcal isolates selected for this study are described in Table 1.

PCR-based identification of enterococci to the species level. PCR analysis using previously described primers targeted to the *ddl* genes of *E. faecalis* and *E. faecium* and the *vanC1* ligase gene of *E. gallinarum* confirmed the identities of each of the 156 enterococcal isolates, including all 20 nonmotile *E. gallinarum* strains (7). Crude DNA extracts were obtained by boiling a 10-μl loopful of fresh overnight culture of each enterococcal isolate in 200 μl of lysis buffer containing 100 mM NaCl, 10 mM Tris-HCl (pH 8.0), and 1% Triton X-100 for 10 min. The multiplex PCR mixture contained 2 μl of supernatant from the DNA extract, 2.5

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TABLE 1. Characteristics of 156 enterococcal isolates studied

Species	No. of strains	No. of PFGE[a] clones
E. faecium	91	40[b]
Vancomycin resistant	65	40
vanA	39	29
vanB	26	11
Vancomycin susceptible	26	Not done
E. gallinarum	55	40
E. faecalis	10	7
Vancomycin resistant	8	6
vanA	3	2
vanB	5	4
Vancomycin susceptible	2	Not done

[a] PFGE, pulsed-field gel electrophoresis.
[b] Forty clones out of 65 vancomycin-resistant strains that were examined by PFGE.

μl of 10× PCR buffer, 100 μM each deoxynucleotide, 100 pmol of each oligodeoxynucleotide primer, and 5 U of *Taq* DNA polymerase. After the initial denaturation at 94°C for 2 min, 35 amplification cycles were completed in a Perkin-Elmer 9600 thermal cycler (Perkin-Elmer Applied Biosystems, Mississauga, Ontario, Canada). Each cycle consisted of 94°C for 15 s, 56°C for 15 s, and 72°C for 15 s. The extension step of the last cycle was prolonged by 10 min. Amplicons were examined by agarose gel electrophoresis, and gels were stained with ethidium bromide. Control strains consisting of *E. gallinarum* ATCC 35038, *E. faecium* SH 228, and *E. faecalis* ATCC 51299 were included with each PCR assay.

Rapid xylose fermentation test. For the rapid xylose fermentation test, a 10-μl loopful from a fresh overnight culture of each isolate was emulsified in 500 μl of sterile 0.45% saline to obtain a turbidity equivalent to a 3 McFarland standard. A 5-μg D-xylose tablet (Rosco Diagnostic Tablets, Taarstrup, Denmark; obtained from Prolab Diagnostics, Richmond Hill, Ontario, Canada) was then added to each tube, and the tubes were incubated in a 37°C water bath for 2 h. Acidification, indicated by a yellow or yellow-orange color change, was interpreted as a positive result, and no color change (red) was interpreted as negative.

MDG test. MDG (Sigma Chemicals, St. Louis, Mo.) was dissolved in distilled water, filter sterilized, and added to an autoclaved phenol red broth base (Difco Laboratories, Detroit, Mich.) to a final concentration of 1%. The standard MDG test was performed in 2 ml of medium, whereas accelerated tests were performed by modifying the standard method to use aliquots of 200 μl of medium dispensed into 96-well plates. Both standard and accelerated methods involved inoculation using a 10-μl loopful of organisms taken from a fresh overnight culture, and tests were incubated at 37°C. Standard tests were read after 24 h. Accelerated tests were read hourly for 4 h and then after 18 and 24 h of incubation. A positive result was indicated by a color change from orange-red to yellow.

Blinding. Each isolate was coded to blind its identity. Rapid xylose and MDG testing, and PCR detection of *ddl* and *vanC1* genes, were performed by different technicians, each blinded to the results of the other tests. When the results of either rapid xylose or MDG tests were discordant with PCR results, an investigator blinded to all previous test results repeated the MDG and rapid xylose fermentation tests for confirmation.

TABLE 3. False-positive results for enterococcal isolates with the standard MDG test interpreted at 24 h

Species	PFGE[a] type	MIC (μg/ml) of:		Time to positive test (h)
		Vancomycin	Teicoplanin	
E. faecium	NA[b]	<1	<0.5	24
	A	256	<0.5	24
	NA	<1	<0.5	24
E. faecalis	B	16	<0.5	24
	C	512	>32	24

[a] PFGE, pulsed-field gel electrophoresis (Ontario clone designations, Mt. Sinai Hospital).
[b] NA, not applicable for vancomycin-susceptible enterococci.

RESULTS

The xylose fermentation test (Table 2) had a sensitivity of 98% (54 of 55) and a specificity of 99% (100 of 101). The one false-positive result with the xylose fermentation test was for *vanA E. faecium* isolated from a stool specimen. The false-negative result was for an *E. gallinarum* strain isolated from a rectal swab. Its antibiotic profile revealed typical MICs: vancomycin, 8 μg/ml; teicoplanin, <0.5 μg/ml; ampicillin, 2 μg/ml; piperacillin, >32 μg/ml; gentamicin, <500 μg/ml; streptomycin, <2,000 μg/ml.

The MDG test was interpreted after 24 h (standard test). The standard MDG test showed a sensitivity of 100% (55 of 55) and a specificity of 95% (96 of 101) (Table 2). The five false-positive results with the standard MDG fermentation test included two susceptible and one *vanB E. faecium* isolate as well as one *vanA* and one *vanB E. faecalis* isolate (Table 3).

The accelerated MDG test was interpreted hourly for 4 h and then after 18 and 24 h of incubation (accelerated test; Table 2). All five false-positive results that occurred with the accelerated MDG test interpreted at less than 24 h of incubation were for *E. faecalis* isolates. Incubation times of less than 18 h did not yield acceptable test characteristics (i.e., sensitivity and specificity greater than 95%).

DISCUSSION

This is the first published report demonstrating the validity of a rapid xylose fermentation test as a method for distinguishing *E. gallinarum* from *E. faecium*. The xylose fermentation test is simple to perform, its results are available in 2 h, and these data demonstrate that it has excellent operating characteristics. The required reagents are commercially available, allowing the test to be easily incorporated into laboratory protocols (39).

TABLE 2. Comparison of the operating characteristics of rapid xylose and MDG fermentation tests for enterococcal isolates

Method	No. of indicated results for:						Test operating characteristic	
	E. gallinarum (n = 55)		*E. faecium* (n = 91)		*E. faecalis* (n = 10)			
	Positive	Negative	Positive	Negative	Positive	Negative	Sensitivity (%)	Specificity (%)
Rapid xylose test (2 h)	54	1	1	90	0	10	98	99
MDG test, standard (24 h)	55	0	3	88	2	8	100	95
MDG test, accelerated								
2 h	9	46	0	91	1	9	16	99
3 h	9	46	0	91	1	9	16	99
4 h	26	29	0	91	1	9	47	99
18 h	52	3	0	91	5	5	95	95
24 h	54	1	4	87	7	3	98	89

Because the xylose fermentation test yields rapid results, unnecessary patient isolation and other infection control measures for *E. gallinarum* (xylose positive) misidentified as vancomycin-resistant *E. faecium* might be avoided. If the xylose fermentation test is negative and the *Enterococcus* species is vancomycin resistant, infection control precautions for vancomycin-resistant enterococci may be implemented promptly while species identification and susceptibility are confirmed.

Vancomycin-resistant *E. gallinarum* strains possessing the *vanA* gene cluster have been described, raising the possibility of transferable *vanA* vancomycin resistance from *E. gallinarum* species. To date, however, this remains only a theoretical concern, given the rarity of these isolates. In addition, dissemination of *E. gallinarum* in clinical settings, despite a relatively high prevalence in stool specimens, has not been observed (18, 20, 32, 35, 37). Hence, for infection control purposes at this time, the rapid xylose test is useful for distinguishing *E. gallinarum* from *E. faecium.*

The MDG test has excellent sensitivity when carried out in standard 2-ml tubes and incubated overnight. These findings are consistent with previous reports in the literature, although others have not noted false-positive results with the MDG test (3, 6, 16, 31, 36). However, only two of these reports used examination of nucleic acid as a "gold standard" for identification of enterococcal species (31, 36). Our attempt to make the test more practical by shortening the incubation time and reducing reagent volumes was not successful. Others have attempted to accelerate the MDG test by utilizing an increased concentration of MDG (up to 7.5%), without success (16).

In summary, our rapid xylose fermentation test is a simple method, easily incorporated into laboratory protocols, which reliably distinguishes *E. gallinarum* from *E. faecium*. The MDG test is equally useful but requires overnight incubation.

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