Use of the Phoenix Automated System for Identification of *Streptococcus* and *Enterococcus* spp.

Gioconda Brigante,[1] Francesco Luzzaro,[1] Alessia Bettaccini,[1] Gianluigi Lombardi,[1]
Francesca Meacci,[2] Beatrice Pini,[1] Stefania Stefani,[3] and Antonio Toniolo[1]*

*Laboratory of Medical Microbiology, Ospedale di Circolo and University of Insubria, Varese,[1] Department of Molecular Biology,
University of Siena, Siena,[2] and Department of Microbiological Sciences, University of Catania, Catania,[3] Italy*

The Phoenix system (Becton Dickinson Diagnostic Systems, Sparks, MD) was evaluated for identification (ID) to the species level of streptococci and enterococci. Two hundred clinical isolates were investigated: beta-hemolytic streptococci ($n = 50$), *Streptococcus pneumoniae* organisms ($n = 46$), viridans group streptococci ($n = 31$), *Enterococcus faecium* ($n = 36$), *Enterococcus faecalis* ($n = 25$), and other catalase-negative cocci ($n = 12$). The API system (bioMérieux, Marcy l'Étoile, France) was used as a comparator. Molecular methods (sequencing of 16S rRNA and *zwf* and *gki* genes and *ddl* gene amplification) were used to investigate discordant results. Upon resolution of discrepancies, correct species ID was achieved by the Phoenix system for 121/129 (93.8%) streptococci and 63/70 (90.0%) enterococci. Excellent results were obtained for *S. pneumoniae* (45/45) and beta-hemolytic streptococci (49/50). With regard to viridans streptococci, the accuracy of the Phoenix system was 83.9%. Among the latter organisms, the best performance was obtained with isolates of the *Streptococcus sanguinis* group and *Streptococcus anginosus* group; problems were instead encountered with the *Streptococcus mitis* group. Four *E. faecium* and three *E. faecalis* isolates were misidentified as *Enterococcus casseliflavus*/*Enterococcus gallinarum* or *Enterococcus durans*. Thus, these isolates were identified only at the genus level. Compared with commercially available systems, the Phoenix system appears a reliable diagnostic tool for identifying clinically relevant streptococci and enterococci. The SMIC/ID-2 panel proved particularly effective for beta-hemolytic streptococci and pneumococci.

Catalase-negative, gram-positive cocci are a heterogeneous group of 17 genera that include streptococci, enterococci, and nonstreptococcal, nonenterococcal species (9, 10). Over 70 streptococcal and enterococcal species have been implicated in human disease (10, 27). Of these, only a few are known to cause important infections (e.g., *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Enterococcus faecalis*, *Enterococcus faecium*, and viridans group streptococci).

A number of manual, semiautomated, and automated systems are reported to produce acceptable identification (ID) results for *S. pneumoniae*, beta-hemolytic streptococci, and enterococcal species (26, 27). These systems, however, were shown not to be sufficiently accurate in identifying streptococci of the viridans group (13, 20), organisms of complex taxonomy (2, 10, 26). The performance of some automated systems has been evaluated with regard to catalase-negative, gram-positive cocci (7, 13, 14, 17, 24). Reproducibility and accuracy of results, turnaround time, availability of data for epidemiological monitoring, and cost-effectiveness constitute the main reasons supporting the choice of automated systems.

Becton Dickinson (BD Diagnostic Systems, Sparks, MD) has introduced the Phoenix automated microbiology system for ID and antimicrobial susceptibility testing (AST) of human pathogenic bacteria, including enterobacteria, nonfermenting gram-negative bacteria, staphylococci, and enterococci (5, 8,

11, 25). Recently, the SMIC/ID-2 panel, dedicated to ID and AST of streptococcal species, was launched (15, 18). This study was designed to evaluate the performance of the Phoenix system for identification of streptococcal and enterococcal isolates at the species level.

MATERIALS AND METHODS

Clinical isolates. Clinical isolates were obtained from routine clinical specimens at the Microbiology Laboratory of the Ospedale di Circolo, Varese, Italy. A total of 200 nonduplicated isolates of gram-positive, catalase-negative cocci were studied. The following strains were investigated: *S. pneumoniae* ($n = 46$), *S. pyogenes* ($n = 15$), *S. agalactiae* ($n = 15$), *Streptococcus dysgalactiae* subsp. *equisimilis* ($n = 20$), viridans group streptococci ($n = 31$), *E. faecium* ($n = 36$), *E. faecalis* ($n = 25$), other enterococcal species ($n = 9$), and other catalase-negative, gram-positive cocci ($n = 3$). Isolates were stored at $-70°C$ in Todd-Hewitt broth containing 20% glycerol. Before ID assays were performed, all strains were passed twice on Mueller-Hinton agar containing 5% sheep blood (Oxoid SpA, Milan, Italy) to get them to an active-growth stage following metabolic inactivity while frozen.

Phoenix system procedures. The Phoenix system uses different panels for gram-positive cocci. The SMIC/ID-2 panel is dedicated to streptococci and the PMIC/ID-14 panel to enterococci and staphylococci. All panels include two separate sections: wells on the left contain ID substrates, and wells on the right side are dedicated to AST. Panel inoculation was performed according to the manufacturer's instructions. Both panel sections were inoculated, but only ID results have been taken into consideration for this study. After overnight culture, bacteria were suspended in the ID broth. Turbidity was adjusted to a 0.5 McFarland standard by using the CrystalSpec Nephelometer (Becton Dickinson). Panels were then sealed, logged, loaded into the instrument, and incubated at 35°C. Kinetic, colorimetric, and fluorescent signals were automatically collected by the instrument every 20 min until results were completed.

Comparator biochemical ID method. Two different API ID systems (bioMérieux, Marcy l'Étoile, France) were used to identify streptococcal and enterococcal isolates at the species level. The API 20 Strep system was used for beta-hemolytic streptococci. The rapid ID 32 Strep system was used for entero-

* Corresponding author. Mailing address: Laboratory of Medical Microbiology, University of Insubria and Ospedale di Circolo e Fondazione Macchi, Viale Borri 57, 21100, Varese, Italy. Phone: 39-0332-278.309. Fax: 39-0332-260.517. E-mail: antonio.toniolo@ospedale.varese.it.

cocci and non-beta-hemolytic streptococci. Inoculation, reading, and interpretation of panels were performed according to the manufacturer's instructions.

Data analysis and resolution of discrepancies. Isolates that were equally identified at the species level by both the API and the Phoenix systems were considered to be correctly identified and included in the "concordant ID" category. Due to the inability of the Phoenix system to discriminate between *Enterococcus casseliflavus* and *Enterococcus gallinarum*, the classification of an isolate as *E. casseliflavus/E. gallinarum* by the Phoenix system was considered concordant when the isolate was identified by the API system as either *E. casseliflavus* or *E. gallinarum*. Isolates with discordant species ID (i.e., an ID produced by the Phoenix system that differed from that obtained with the API system) were retested using both the Phoenix and the API systems. When discrepant results persisted, bacterial ID was investigated by molecular methods.

Amplification and sequencing of the 16S rRNA gene. Bacterial DNA was extracted from pure cultures by using a QIAamp DNA mini kit (QIAGEN, Basel, Switzerland). DNA eluted in Tris-EDTA was stored at −80°C. ABI 2400 thermal cyclers and PCR reagents were from Applied Biosystems (Foster City, CA). AmpliTaq Gold polymerase, PCR buffer II, standard deoxynucleoside triphosphate mixture, and the universal 16S rRNA gene primers 8f (5′-GAGA GTTTGATCCTGGCTCAG-3′) and 1492r (5′-TACGGCTACCTTGTTACGA CT-3′) were used to produce a 1,498-bp amplicon (23). Amplification products were purified using a Mini Elut PCR purification kit (QIAGEN) and directly sequenced using an ABI 310 genetic analyzer (Applied Biosystems). Sequences for both DNA strands were determined, each by using the product of a different PCR as a template. Analysis and comparison of sequence data were carried out at the BLAST interface (http://www.ncbi.nlm.nih.gov/BLAST/) and ClustalW interface (http://www.ebi.ac.uk/clustalw/) websites.

Amplification and sequencing of housekeeping genes of viridans group streptococci. Samples were amplified with degenerate primers specific for the internal fragments of the *zwf* (encoding glucose-6-phosphate dehydrogenase) and *gki* (encoding glucose kinase) streptococcal genes (21). Two primer pairs were used: 5′-CCG(T/G)ATCGACCATTA(T/C)CTTGG(T/C)AAGG-3′ and 5′-TC(A/T) GTCAG(T/A)CGTTTACCTGT(A/G)CGGA-3′ for the *zwf* gene and 5′-GGC ATTGGAATGGGATCACCAGG-3′ and 5′-CCGATAA(C/T)TCCAGCGTCA TTTCC-3′ for the *gki* gene. Amplicons of 453 bp (*zwf*) and 624 bp (*gki*) were directly sequenced.

Amplification of housekeeping genes in enterococci. The ID of *E. faecium* and *E. faecalis* isolates was confirmed by amplification of a fragment internal to the *ddl* gene encoding a D-Ala-D-Ala ligase (4, 6). The reaction mixture contained 250 ng of DNA as a template, 50 pmol of each primer, 200 pmol per liter of each deoxynucleoside triphosphate (dATP, dCTP, dGTP, and dTTP), 10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 50 mM KCl, and 2 U of AmpliTaq Gold. Upon electrophoresis on a 2% agarose gel, ethidium bromide-stained DNA fragments were visualized under UV light with a Kodak CF440 camera (NEN Life Science Products, Boston, MA).

Quality controls. The following strains were included in each run: *S. pneumoniae* ATCC 49619, *S. agalactiae* ATCC 13813, and *E. faecalis* ATCC 29212. The identification results obtained with the above-mentioned reference strains were consistently satisfactory.

RESULTS

Biochemical identification of clinical isolates. Compared with the API system, the Phoenix system correctly identified 180/200 (90.0%) test organisms at the species level: 116/129 (89.9%) streptococci, 63/70 (90%) enterococci, and 1/1 *Aerococcus viridans* isolate. Results are summarized in Table 1.

Concordant ID for *S. pneumoniae* was obtained in 45/46 cases, since one isolate (identified as *S. pneumoniae* by the API system) was classified as a "not identified organism" by the Phoenix system. After resolution of discrepancies, however, this isolate was ultimately defined as *Streptococcus mitis* by sequencing the 16S rRNA gene. Fourteen out of 15 (93.3%) *S. pyogenes* and 21/31 (67.7%) viridans streptococci isolates were correctly identified by the Phoenix system. Among the latter, 8/8 members of the *Streptococcus anginosus* group, one *Streptococcus mutans* isolate, 3/4 members of the *Streptococcus sanguinis* group, and 9/17 members of *S. mitis* group were correctly identified. Correct species ID was obtained for 90% of the

TABLE 1. Results of biochemical identification of streptococci and enterococci by the Phoenix system, with the API system used as a comparator[a]

Species identified by API system	No. of isolates	No. (%) of Phoenix system results that were:	
		Concordant	Discordant
Streptococcus pneumoniae	46	45 (97.8)	1 (2.2)
Streptococcus pyogenes	15	14 (93.3)	1 (6.7)
Streptococcus agalactiae	15	15 (100)	0
Streptococcus dysgalactiae subsp. *equisimilis*	20	20 (100)	0
Viridans streptococci (*n* = 31)			
Streptococcus mitis group	17	9 (53.0)	8 (47.0)
S. mitis	9	4	5
S. oralis	7	4	3
S. cristatus	1	1	0
Streptococcus anginosus group	8	8 (100)	0
S. anginosus	7	7	0
S. intermedius	1	1	0
Streptococcus sanguinis group	4	3 (75.0)	1 (25.0)
S. sanguinis	2	1	1
S. parasanguinis	1	1	0
S. gordonii	1	1	0
Streptococcus salivarius	1	0	1
Streptococcus mutans	1	1	0
Enterococcus faecium	36	32 (88.9)	4 (11.1)
Enterococcus faecalis	25	22 (88.0)	3 (12.0)
Other enterococcal species (*n* = 9)			
Enterococcus gallinarum	4	4	0
Enterococcus durans	3	3	0
Enterococcus casseliflavus	1	1	0
Enterococcus avium	1	1	0
Other catalase-negative cocci (*n* = 3)			
Streptococcus equinus	1	0	1
Streptococcus bovis	1	1	0
Aerococcus viridans	1	1	0
Total	200	180 (90.0)	20 (10.0)

[a] Results refer to the biochemical identification obtained by the API system. Results obtained with the Phoenix system are shown as concordant or discordant with API results, without taking into account the resolution of discrepancies by the molecular methods reported in Table 2.

enterococci. Four *E. faecium* and three *E. faecalis* isolates were misidentified as *E. casseliflavus/E. gallinarum* or *Enterococcus durans*. Thus, these isolates were identified only at the genus level. Overall, the Phoenix system correctly identified all isolates of the following species: *S. pneumoniae*, *S. agalactiae*, *S. dysgalactiae* subsp. *equisimilis*, and *Streptococcus bovis*.

Analysis of discrepancies. Species ID of 20 discordant isolates was investigated by molecular methods. As shown in Table 2, sequencing of the 16S rRNA gene assessed that one *S. mitis* isolate was not identified by either the API or the Phoenix system. One *S. pyogenes* isolate (not identified by the Phoenix system) was correctly identified by the API system. Five iso-

TABLE 2. Analysis of 20 discordant isolates[a]

API system result (no. of isolates)	Discrepant result provided by the Phoenix system (no. of isolates)	Species (no. of isolates) identified by molecular method			
		16S rRNA gene sequencing	*zwf* and *gki* gene sequencing	*ddl* gene amplification	ID by molecular analysis
S. pneumoniae (1)	NI	*S. mitis*	—	—	*S. mitis*
S. pyogenes (1)	NI	*S. pyogenes*	—	—	*S. pyogenes*
S. mitis (5)	*S. parasanguinis*	*S. parasanguinis*	—	—	*S. parasanguinis*
	S. parasanguinis	*S. parasanguinis*	—	—	*S. parasanguinis*
	S. oralis	*S. mitis, S. oralis, S. pneumoniae*	*S. mitis, S. pneumoniae*	—	Unresolved
	S. sanguinis	*S. sanguinis*	—	—	*S. sanguinis*
	NI	*S. mitis, S. oralis, S. pneumoniae*	*S. mitis, S. oralis, S. pneumoniae*	—	Unresolved
S. oralis (3)	*S. sanguinis*	*S. sanguinis*	—	—	*S. sanguinis*
	S. cristatus	*S. mitis, S. oralis, S. pneumoniae*	*S. mitis, S. oralis, S. pneumoniae*	—	Unresolved
	S. pneumoniae	*S. mitis, S. oralis, S. pneumoniae*	*S. mitis, S. oralis*	—	Unresolved
S. salivarius (1)	*S. gordonii*	*S. gordonii*	—	—	*S. gordonii*
S. sanguinis (1)	NI	*S. gordonii*	—	—	*S. gordonii*
S. equinus (1)	*S. mitis*	*S. mitis, S. oralis, S. pneumoniae*	*S. mitis, S. pneumoniae*	—	Unresolved
E. faecium (4)	*E. casseliflavus/ E. gallinarum* (2)	—	—	*E. faecium* (4)	*E. faecium* (4)
	E. durans (2)	—	—		
E. faecalis (3)	*E. casseliflavus/ gallinarum* (3)	—	—	*E. faecalis* (3)	*E. faecalis* (3)

[a] Molecular methods were used in an attempt to resolve discrepant identification results provided by the two biochemical methods, i.e., the API system and the Phoenix system. *zwf*, glucose 6-phosphate dehydrogenase gene; *gki*, glucose kinase gene; *ddl*, D-Ala-d-Ala ligase gene of *E. faecium* and *E. faecalis*; NI, not identified; —, not done.

lates (which had been assigned to the *S. mitis* or *Streptococcus salivarius* group by the API system) did belong to the *S. sanguinis* group (*Streptococcus parasanguinis*, n = 2; *S. sanguinis*, n = 2; and *Streptococcus gordonii*, n = 1). The above-mentioned five isolates were correctly classified at the species level by the Phoenix system, not by the API system. One discordant isolate (resolved by 16S rRNA gene sequencing as *S. gordonii*) was not identified by the Phoenix system and was identified only at the group level by the API. Sequencing of the 16S rRNA and the housekeeping *zwf* and *gki* genes failed to resolve five additional isolates at the species level. The most probable molecular ID for those five isolates appeared to be *S. mitis*, *Streptococcus oralis*, or *S. pneumoniae* (species belonging to the *S. mitis* group) (26).

Concerning seven enterococci identified by the Phoenix system as *E. casseliflavus/E. gallinarum* (n = 5) or *Enterococcus durans* (n = 2), PCR analysis of the *ddl* gene confirmed the IDs

TABLE 3. Analysis of 20 discordant isolates

Molecular identification at the species or group level (no. of isolates)	No. of isolates for which biochemical identification matched identification by:		
	API system	Phoenix system	Neither
Species ID			
S. pyogenes (1)	1		
S. sanguinis (2)		2	
S. parasanguinis (2)		2	
S. gordonii (2)		1	1
S. mitis (1)			1
E. faecium (4)	4		
E. faecalis (3)	3		
Group ID			
S. mitis group (5)	4[a]	4[a]	1

[a] Identification by the API and the Phoenix systems gave results that were concordant at the group level but discordant at the species level.

given by the API system (four *E. faecium* and three *E. faecalis* isolates).

The performance of the Phoenix and API systems with regard to discordant isolates is summarized in Table 3. Of 13 streptococci, 9 were correctly identified at the species or group level by the Phoenix system and 5 by the API system. Three isolates could not be identified by either system. The correct IDs for seven discordant enterococci were given by the API system, not by the Phoenix system. Of the streptococci that could not be resolved at the species level by molecular methods, 4/5 were identified at the group level by both the API and the Phoenix systems. Overall, taking into consideration species IDs given by molecular methods, the accuracy of the Phoenix system in identifying streptococci rose from 89.9% to 93.8%. For enterococci, accuracy of ID at the species level remained at 90%.

DISCUSSION

Automated systems may have significant diagnostic impact on diseases caused by streptococci and enterococci, especially with regard to aggressive infections and drug-resistant isolates (26).

The Phoenix automated system did agree with the API system for 89.9% of streptococcal IDs. Upon resolution of discrepancies, accuracy for streptococci rose to 93.8%. The performance of the new SMIC/ID-2 panel dedicated to streptococci was excellent for beta-hemolytic streptococci (49/50) and *S. pneumoniae* (45/46). Only one *S. pyogenes* isolate and one *S. pneumoniae* isolate were not identified. It should be noted that the latter isolate (reported by the API system as *S. pneumoniae*) was ultimately identified as *S. mitis* by molecular methods. This brings the accuracy for *S. pneumoniae* to 100% and underlines difficulties that may be encountered in the biochemical identification of streptococcal isolates by commercial methods (3, 16, 22).

Taken together, the results confirm the documented ability of automated systems in identifying beta-hemolytic streptococci and *S. pneumoniae* (18, 24). Kanemitsu et al. (18) reported that the Phoenix system performed satisfactorily with regard to beta-hemolytic streptococci (>90% concordance with a manual biochemical test supplemented by hemolysis data and serological grouping) and behaved less brilliantly with *S. pneumoniae* (85.9% concordance). Better performances with the Phoenix system were reported by Hirakata et al. (15); concordance with the comparator (phenotypic tests and serological grouping) was >90% for *S. pneumoniae* and >95% for beta-hemolytic streptococci.

With regard to viridans group streptococci, the performance of automated systems has been reported as problematic; only 55% (6/11) of *S. bovis* isolates and 40% (6/15) of viridans group streptococci were correctly identified at the species level by the VITEK 2 system (13). On the other hand, the cited Japanese studies on Phoenix panels evaluated IDs of viridans streptococci only at the group level (*S. anginosus* group or *S. mitis* group) (15). The performance of the Phoenix SMIC/ID-2 panel for species ID of viridans streptococci was evaluated for the first time by this study. The results for the Phoenix system were in agreement either with the API system or with molecular methods for 26/31 viridans streptococci (83.9%). Discrepancies between the Phoenix and the API systems were encountered especially within the *S. mitis* group, possibly due to close genetic relations among members of this group (19). Four of eight discordant isolates belonging to the *S. mitis* group were not resolved by molecular methods. The remaining four isolates were correctly identified exclusively by the Phoenix system (*S. parasanguinis*, n = 2; *S. sanguinis*, n = 2). Thus, the Phoenix system appeared to correctly identify 8/9 members of the *S. sanguinis* group.

Among enterococci, correct IDs were achieved in 90% of cases by the Phoenix system. Discrepancies were limited to *E. faecalis* and *E. faecium*. Problems in identifying enterococci with automated systems have already been reported. For instance, the VITEK 2 system failed to identify substantial numbers (9% to 37%) of *E. faecium* and *E. faecalis* isolates (1, 7, 12). The latter isolates were most frequently identified as *E. casseliflavus/E. gallinarum*. Similarly, the Phoenix system has been reported to misidentify *E. faecium* and *E. faecalis* as *E. casseliflavus/E. gallinarum* (5, 11). The present results show that automated ID of enterococci remains a problem. In a clinical laboratory, however, the simple motility test usually allows for discrimination of *E. casseliflavus* and *E. gallinarum* from other enterococci (27), thus improving ID accuracy.

In conclusion, the Phoenix system appears a reliable tool for identification of clinically relevant streptococcal and enterococcal species. The new SMIC/ID-2 panel proved particularly effective for beta-hemolytic streptococci and pneumococci. Though not perfect, ID performance with viridans group streptococci appeared to be superior to those of currently available systems.

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