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Evaluation of the RapID ANA II and API ZYM Systems for Identification of *Actinomyces* Species from Clinical Specimens

MINNA A. BRANDER[1]* AND HANNELE R. JOUSIMIES-SOMER[2]†

Department in Lappeenranta, National Public Health Institute, Kahilanniementie 2, 53130 Lappeenranta,[1] and Anaerobe Reference Laboratory, National Public Health Institute, Helsinki,[2] Finland

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Classification and identification of fermentative actinomycetes are labor-intensive and problematic. In this study, we evaluated the applicability and reliability of the RapID ANA II system (Innovative Diagnostic Systems, Inc., Atlanta, Ga.) and the discriminatory value of the API ZYM system (Societes Analytab Products Inc., La Balme Les Grottes, France) in the identification of *Actinomyces*-like bacteria by using conventional methods as a reference. Eighty-five strains, including 71 isolates from mixed anaerobic infections and 14 reference strains, were tested. The RapID ANA II system correctly identified all *Actinomyces odontolyticus* strains and 65% of *Actinomyces israelii* strains. All *Arcanobacterium haemolyticum* strains were misidentified as *Actinomyces pyogenes*. The most common isolates in the study were *Actinomyces meyeri*-like organisms, 84% of which, however, were aerotolerant. The identification of these aerotolerant strains thus remains unresolved and warrants further studies. New characteristics and changes to the conventional API ZYM enzyme profiles are suggested. The API ZYM enzyme profiles of *A. odontolyticus* and *A. israelii* were very similar, the only discriminating enzyme being α-fucosidase. In differentiation between *A. pyogenes* and *Arcanobacterium haemolyticum*, the production of β-glucuronidase by the former and the production of acid phosphatase by the latter are suggested as new helpful characteristics for use in clinical laboratories. In summary, the RapID ANA II and API ZYM systems can be used as rapid preliminary methods in the identification of *Actinomyces* species but accurate identification requires supplementary conventional tests and gas-liquid chromatography.

With the exception of classical actinomycosis due to *Actinomyces israelii*, *Actinomyces* species are isolated usually from bacterial infections involving mixed floras. Thus, the clinical significance of individual species is still controversial (6–8, 15, 17). Conventional testing based on fermentation and biochemical reactions together with the determination of metabolic end products by gas-liquid chromatography is considered the most accurate and reliable approach for the identification of *Actinomyces* species (1, 9, 18). However, because these procedures are both labor-intensive and time-consuming and require special instrumentation, they are beyond the capabilities of many laboratories and the isolation and identification of these bacteria have remained a speciality of a few laboratories.

Recently, the trend in clinical anaerobic bacteriology has been towards the use of commercially available identification kits, including those detecting constitutive preformed enzymes. The performances of these kits are not dependent on active growth and require only 4 h of aerobic incubation. The differential charts of these systems also include *Actinomyces* species.

The purpose of this study was to evaluate the applicability, reliability, and accuracy of the RapID ANA II system and the discriminatory value of the API ZYM system in the identification of *Actinomyces* species isolated from clinical specimens by using conventional methods as a reference.

* Corresponding author.

† Present address: Microbial Disease Research Laboratory, 691/151J, VA Wadsworth Medical Center, Los Angeles, CA 90073.

MATERIALS AND METHODS

Bacterial strains. A total of 85 *Actinomyces* and *Actinomyces*-like bacterial strains were examined. These included 14 reference strains (13 from the American Type Culture Collection [ATCC] and 1 from the National Collection of Type Cultures, Central Public Health Laboratory, Colindale, London, England): *A. israelii* serotypes 1 (ATCC 10049; NCTC 10236) and 2 (*Actinomyces gerencseriae*; ATCC 23860), *Actinomyces odontolyticus* (ATCC 17929), *Actinomyces naeslundii* (ATCC 12104), *Actinomyces viscosus* serotype 1 (ATCC 15987), *Actinomyces meyeri* (ATCC 35568), *Actinomyces georgiae* (ATCC 49285), *Actinomyces humiferus* (ATCC 25174), *Actinomyces bovis* (ATCC 13683), *Actinomyces hordeovulneris* (ATCC 35275), *Actinomyces* sp. serotype WVA 963 (ATCC 49338), *Actinomyces pyogenes* (ATCC 19411), and *Arcanobacterium haemolyticum* (ATCC 9345). Here, *Actinomyces*-like bacteria were defined as gram-positive, non-spore-forming rods, most of which were branching and catalase negative and which in gas-liquid chromatography produced succinic acid and with the RapID ANA II system were identified as *Actinomyces* species.

All organisms except the reference strains were fresh clinical isolates obtained from patient specimens in 1990 and cultured and isolated at the Department of Lappeenranta, National Public Health Institute, Lappeenranta, Finland, mainly as part of mixed anaerobic floras. After isolation, the strains were examined by conventional Virginia Polytechnic Institute reference methods (9) and the RapID ANA II system. The strains were kept at −70°C in skim milk, thawed, and subcultured before being tested by the API ZYM system. The conventional methods included aerotolerance testing and determination of Gram reaction, cellular morphology, and colonial characteristics as well as patterns

TABLE 1. API ZYM reactions of the study strains identified by the RapID ANA II system as *A. meyeri*, *A. odontolyticus*, and *A. israelii*

Species	No. of strains	No. of strains positive by API ZYM in microtube[a]:																		
		2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
A. meyeri[b]	31	2	4	1	0	28	0	0	0	0	2	0	0	2	1	19	0	2	0	0
A. odontolyticus[c]	18	0	12	8	0	18	4	0	0	0	6	1	5	8	0	15	10	5	3	15
A. israelii[d]	14	0	14	8	0	14	5	0	0	0	1	0	3	6	1	5	3	0	0	0

[a] Reaction microtubes assayed for the following enzymes: 2, alkaline phosphatase; 3, esterase; 4, esterase-lipase; 5, lipase; 6, leucine arylamidase; 7, valine arylamidase; 8, cystine arylamidase; 9, trypsin; 10, chymotrypsin; 11, acid phosphatase; 12, naphthol-AS-BI-phosphohydrolase; 13, α-galactosidase; 14, β-galactosidase; 15, β-glucuronidase; 16, α-glucosidase; 17, β-glucosidase; 18, *N*-acetyl-β-glucosaminidase; 19, α-mannosidase; 20, α-fucosidase.

[b] Only two strains were obligate anaerobes.

[c] All strains keyed out identically by the conventional methods.

[d] Only eight strains keyed out identically by the conventional methods.

of fermentation of carbohydrates and production of indole, nitrate reductase, catalase, arginine dehydrolase, and urease, supplemented by the metabolic end product analysis with gas-liquid chromatography (20). Some of the strains were also tested for gelatin liquefaction, peptonization of litmus milk, and reverse CAMP reaction.

RapID ANA II system. The RapID ANA II system consists of 10 individual test wells (8 of which are bifunctional) containing dehydrated reactants designed to test 18 biochemical reactions. Substrates included in the 10 wells of the test panel are urea, *p*-nitrophenyl-β,D-disaccharide, *p*-nitrophenyl-α,L-arabinoside, *o*-nitrophenyl-β-D-galactoside, *p*-nitrophenyl-α,D-glucoside, *p*-nitrophenyl-β,D-glucoside, *p*-nitrophenyl-α,D-galactoside, *p*-nitrophenyl-α,L-fucoside, *p*-nitrophenyl-*N*-acetyl-β,D-glucosaminide, *p*-nitrophenylphosphate, leucine-glycine-β-naphthylamide, glycine-β-naphthylamide, proline-β-naphthylamide, phenylalanine-β-naphthylamide, arginine-β-naphthylamide, serine-β-naphthylamide, pyrrolidonyl-β-naphthylamide, and tryptophane. Pure cultures of the organisms were inoculated on chocolate agar (Oxoid). Plates were incubated in an anaerobic environment at 35 to 37°C for 48 h. The strains were suspended in RapID ANA II inoculation fluid, and the inoculum was adjusted to a turbidity of McFarland standard no. 3. The trays were inoculated according to the manufacturer's instructions and incubated aerobically at 35 to 37°C for 4 h. After incubation, reactions in the test wells were interpreted and recorded before and after the addition of reagents. A six-digit microcode was generated, and identification was determined by using the RapID ANA II code compendium (10). The *Actinomyces* species included in the code compendium are *A. bovis*, *A. israelii*, *A. meyeri*, *A. naeslundii*, *A. odontolyticus*, *A. pyogenes*, and *A. viscosus*. The reference strains were examined twice.

API ZYM system. The API ZYM gallery is composed of 20 microtubes, which contain 19 substrates in buffer. The first microtube serves as a control, and the enzymes determined in the other tubes are listed in Table 1, footnote *a*. Pure cultures of organisms were obtained and incubated as with the RapID ANA II system. The strains were suspended in sterile distilled water and adjusted to a turbidity of McFarland standard no. 5 or greater. The trays were inoculated according to the manufacturer's instructions and incubated aerobically at 35 to 37°C for 4 h. After incubation, reactions in the test cupules were interpreted and recorded on a report sheet after addition of reagents. A color intensity value from 0 to 5 was assigned for each reaction according to the color chart enclosed with the kit. Only colors showing an index of 2 or more were recorded as positive. The API ZYM system lacks an index book for specific bacterial identification. The reference strains were examined twice.

RESULTS

Sixty-three of the 71 clinical isolates studied were identified by the RapID ANA II system as *A. odontolyticus* (18 isolates), *A. israelii* (14 isolates), or *A. meyeri* (31 isolates). The reference strains for each were correctly identified; *A. gerencseriae* (*A. israelii* serotype 2) was also identified as *A. israelii*.

All of the 19 strains that were identified as *A. odontolyticus* by RapID ANA II also were identified as *A. odontolyticus* by the conventional methods; all were mannitol negative, 16 were raffinose and trehalose negative, and 9 were ribose negative. Twelve different microcodes were found, none of which was outstanding. Two microcodes, however, gave the identification probability belonging to *A. odontolyticus*, *Arachnia propionica* (*Propionibacterium propionicus*), *Lactobacillus acidophilus*, or *A. israelii*. As the study strains produced succinic acid and no propionic acid, the identification as *Actinomyces* species was accepted. Furthermore, as one of these two strains was α-fucosidase positive and the other produced red pigment, the final identification as *A. odontolyticus* instead of *A. israelii* was accepted.

Only 11 of the 14 strains identified as *A. israelii* by RapID ANA II were identified as *A. israelii* by conventional methods. (All strains were α-fucosidase and urease negative.) Ten different microcodes were obtained.

If obligately anaerobic growth (3) were used as a strict criterion, only 2 of the 31 clinical strains identified as *A. meyeri* in the RapID ANA II system and the type strain would have been identified as *A. meyeri* also by the conventional methods. Even these three strains (the two clinical isolates and the type strain) grew slightly in a 5% CO_2 atmosphere. The remaining 29 clinical isolates were aerotolerant; however, 3 of these grew very slowly in air. Three different microcodes were obtained; of these, 020671 was produced by 26 (84%) of the 31 strains. According to the RapID ANA II system code compendium, the probable identification given to microcode 020671 overlapped *A. meyeri* and *Propionibacterium granulosum*. As all the strains were catalase negative and produced succinic acid but no propionic acid, the identification as *A. meyeri* was accepted.

The API ZYM reactions of *A. odontolyticus*, *A. israelii*, and *A. meyeri* are summarized in Table 1.

Of the study strains, seven were identified as *A. pyogenes* by the RapID ANA II system. These strains were identified

TABLE 2. Reactivities of reference strains of *Actinomyces* spp. in the API ZYM system

Reference strain	Color intensity of API ZYM reaction in microtube[a]:																		
	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
A. meyeri	−	−	−	−	+	−	−	−	−	−	−	−	−	−	+	−	−	−	−
A. odontolyticus	−	−	−	−	+	−	−	−	−	−	−	−	+	−	−	−	−	−	+
A. israelii																			
ATCC 10049	−	−	−	−	+	−	−	−	−	−	−	w	+	−	w	+	−	−	−
NCTC 10236	−	−	−	−	+	−	−	−	−	−	−	w	+	−	w	+	−	−	−
Serotype 2, ATCC 23860	−	−	−	−	+	w	−	−	−	−	−	w	+	−	+	+	−	−	−
A. pyogenes	−	−	−	−	+	−	−	−	−	−	−	−	w	w	w	−	−	−	−
Arcanobacterium haemolyticum	−	−	−	−	w	−	−	−	−	+	−	−	−	−	−	−	w	−	−
A. naeslundii	−	−	−	−	+	−	−	−	−	−	−	w	+	−	−	w	−	−	−
A. viscosus	−	w	+	−	+	w	−	−	−	−	−	w	+	−	−	+	−	−	−
A. hordeovulneris	−	−	w	−	+	+	−	−	−	−	−	w	+	−	−	−	+	−	−
A. humiferus	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
A. georgiae	−	−	−	−	+	−	−	−	−	−	−	−	−	−	+	−	−	−	−
Actinomyces sp. serotype WVA 963	−	−	−	−	+	−	−	−	−	w	−	w	+	−	−	w	−	−	−
A. bovis	−	−	−	−	+	w	−	−	−	−	−	+	+	−	−	+	−	−	−

[a] For microtube numbering system, see Table 1, footnote *a*. −, w, and +, ranges of color intensity of 0 to 2, 2 to 3, and 3 to 5, respectively.

by the conventional methods as *Arcanobacterium haemolyticum*. All strains were reverse CAMP reaction positive (5), none liquefied gelatin, and only one strain fermented xylose slightly and was able to peptonize litmus milk (18). In the API ZYM system, all these strains produced at least some leucine arylamidase, β-galactosidase, and *N*-acetyl-β-glucosaminidase, all of which are enzymes produced by *A. pyogenes* according to *Bergey's Manual of Systematic Bacteriology* (18). In addition, all the strains produced acid phosphatase, which has not been reported as being produced by *A. pyogenes*. The API ZYM reactions of the type strain of *Arcanobacterium haemolyticum* were identical to the reactions of the seven strains; only the production of β-galactosidase was very weak. The type strain of *A. pyogenes* produced leucine arylamidase, β-galactosidase, β-glucuronidase, and α-glucosidase and a very small amount of *N*-acetyl-β-glucosaminidase but no acid phosphatase.

There was only one catalase-positive strain among the clinical strains tested. The RapID ANA II microcode obtained for this organism, which by the conventional methods most closely resembled *A. viscosus*, was not listed in the code compendium. Unlisted microcodes were also obtained for the two catalase-positive reference strains, *A. viscosus* ATCC 15987 and *A. hordeovulneris* ATCC 35275. The API ZYM reactions of these two ATCC strains as well as the other reference strains are summarized in Table 2.

DISCUSSION

The taxonomy of *Actinomyces* species is complicated, and classification and identification of fermentative actinomycetes still present problems despite the contributions of modern technology. In this study, two commercially available kits, the RapID ANA II system and the API ZYM system, were evaluated for the identification of *Actinomyces*-like bacteria and compared with the conventional Virginia Polytechnic Institute reference methods. In earlier evaluations of the RapID ANA II system, about 60% of the *Actinomyces* strains could be reliably identified to the genus level but not always to the species level (8). In those evaluations, however, only a few species and strains of these bacteria were included and they constituted only a minor fraction of the tested strains (4, 8, 12–14, 16, 19). Kilian (13) described the results for 162 strains of the *Actinomycetaceae* and related taxa for 20 different enzymatic activities, of which 10 were the same as in the API ZYM system. Our results concur with those of Kilian.

In the present study, *A. meyeri* was the most common identification given by the RapID ANA II system (31 of 71 study strains). By the conventional methods, all these strains would have received the same identification had 26 strains not grown aerobically. If the criterion of no aerobic growth (3) is obligatory, the identification of these strains remains unresolved. Whether these aerotolerant *A. meyeri*-like strains represent variants of *A. meyeri* or a new species warrants further studies. Recently, three of the strains have been investigated by Lillian V. H. Moore at Virginia Polytechnic Institute and State University, Blacksburg, and the strains were unlike any described species that she has studied (15a). It is also noteworthy that none of the 31 clinical *A. meyeri*-like strains nor the *A. meyeri* type strain produced valine arylamidase or cystine arylamidase, although these are listed in Bergey's manual as characteristic products of *A. meyeri*.

All 19 strains identified by the RapID ANA II system as *A. odontolyticus* were also identified identically by the conventional methods. The production of α-fucosidase was a unique reaction for *A. odontolyticus* strains, being produced by no other species in this study, a result similar to those of earlier studies (18). Only three of the *A. odontolyticus* strains did not produce any α-fucosidase. Although Kilian (13) reported that *A. viscosus* serotype 1 produced α-fucosidase in the API ZYM system, in the present study this reaction was negative for the type strain. The colonies of only 37% of *A. odontolyticus* strains developed red pigment. This percentage is somewhat lower than those reported earlier (2, 11, 15). Thus, the demonstration of pigment formation might not be as important in the identification of this *Actinomyces* species as has been thought. The specimens in the earlier studies have been mainly of dental origin, but this was not the case in the present study, as 83% were from nonoral sources. However, this difference in specimen source may not suffice to explain the divergent results. Some brownish red color could be seen in most of the ATCC strains included in this study, provided the strains were incubated long enough (5 to 7 days). Also, Johnson et al. could demonstrate typical red colonies in only 58% of the serotype I strains and 43% of the serotype II strains of *A. odontolyticus* (11).

According to Bergey's manual, there are only three enzymes produced by *A. odontolyticus* in the API ZYM system: leucine arylamidase, β-glucosidase, and α-fucosidase. The *A. odontolyticus* strains in the present study produced more enzymes, a result concordant with that of Kilian (13). More than half of our *A. odontolyticus* strains produced esterase and α-glucosidase, and at least 27% of the strains produced esterase-lipase, β-galactosidase, and *N*-acetyl-β-glucosaminidase. Two *A. meyeri*-like strains also produced some *N*-acetyl-β-glucosaminidase, an enzyme reported as being produced only by *A. bovis* and *A. pyogenes* according to Schaal (18). Of the reference strains, *A. bovis* did not produce that enzyme at all and *A. pyogenes* produced only a very small amount of it. The reference strains which produced the enzyme were *A. hordeovulneris* and *Arcanobacterium haemolyticum*.

We propose adding esterase, esterase-lipase, α-galactosidase, β-galactosidase, α-glucosidase, and *N*-acetyl-β-glucosaminidase to the list of enzymes produced by some strains of *A. odontolyticus*.

A total of 11 (all 3 reference strains and 8 clinical strains) of the 17 strains, including the reference strains identified as *A. israelii* by the RapID ANA II system, were identified correctly to the species level. Only three clinical strains gave the same reactions as the reference strains in the API ZYM system. The remaining five clinical strains did not produce any β-galactosidase or α- or β-glucosidase, enzymes produced by at least 90% of *A. israelii* strains (18). However, the carbohydrate fermentation results and a negative urease reaction were consistent with the characteristics of *A. israelii*. Most of the *A. israelii* strains in the study were smooth-colony variants and were aerotolerant. Only two study strains and ATCC strain 23860 did not grow aerobically.

According to our results and those of Kilian, the API ZYM enzyme profiles of *A. israelii* and *A. odontolyticus* are very similar, the only discriminating enzyme being α-fucosidase.

All seven strains identified as *A. pyogenes* by the RapID ANA II system were identified as *Arcanobacterium haemolyticum* by the conventional methods. The cell morphology and biochemical reactions of these two species very much resemble each other, and differentiation between them is difficult. Because the two species are nowadays considered to belong to two distinct taxa (18) and because *A. pyogenes* isolates are often of animal origin, the identification of *Arcanobacterium haemolyticum* as *A. pyogenes* is misleading in terms of both the source and the disease association of the bacterium and ought to be corrected. Comparing the type strains of these two species, we found that the hemolysis produced by *Arcanobacterium haemolyticum* was much stronger and developed more quickly (1-day incubation) than that produced by *A. pyogenes* (3-day incubation). *Arcanobacterium haemolyticum* also grew equally well in all three atmospheres, but *A. pyogenes* grew better in anaerobic and CO_2 atmospheres. Other important discriminative reactions were the reverse CAMP reaction, gelatin liquefaction, and xylose fermentation (5). In the API ZYM system, the most distinctive difference was between the production of acid phosphatase by *Arcanobacterium haemolyticum* and the production of β-glucuronidase by *A. pyogenes*, characteristics which could be of help to clinical laboratories in differentiating these two species.

The reactions observed with the RapID ANA II system were mostly easy to interpret, although borderline reactions occurred in the hydrolysis of naphthylamine substrates. In the API ZYM system, minor errors in interpreting the color intensities were possible. Although some of the reactions were included in both systems, the substrates were not consistently the same, nor were the inoculum densities equal. Therefore, direct comparison of the enzyme reactions was not considered justified.

In this study, the RapID ANA II system correctly identified to the species level all the *A. odontolyticus* strains and 65% of the *A. israelii* strains. However, all *Arcanobacterium haemolyticum* strains were misidentified as *A. pyogenes*. The enzyme reactions in the API ZYM system aided in the identification of the strains but did not always concur with those given in Bergey's manual for equivalent species; accordingly, additions to the enzyme profiles are presented. API ZYM profiles of some newer *Actinomyces* spp. not tested before are reported here. In conclusion, both rapid methods are useful aids in the identification of *Actinomyces*-like bacteria, although accurate identification will often require supplementary conventional methods.

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