Fastidious Gram-Negatives: Identification by the Vitek 2 Neisseria-Haemophilus Card and by Partial 16S rRNA Gene Sequencing Analysis

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Abstract: Taxonomy and identification of fastidious Gram negatives are evolving and challenging. We compared identifications achieved with the Vitek 2 Neisseria-Haemophilus (NH) card and partial 16S rRNA gene sequence (526 bp stretch) analysis with identifications obtained with extensive phenotypic characterization using 100 fastidious Gram negative bacteria. Seventy-five strains represented 21 of the 26 taxa included in the Vitek 2 NH database and 25 strains represented related species not included in the database. Of the 100 strains, 31 were the type strains of the species. Vitek 2 NH identification results: 48 of 75 database strains were correctly identified, 11 strains gave ‘low discrimination’, seven strains were unidentified, and nine strains were misidentified. Identification of 25 non-database strains resulted in 14 strains incorrectly identified as belonging to species in the database. Partial 16S rRNA gene sequence analysis results: For 76 strains phenotypic and sequencing identifications were identical, for 23 strains the sequencing identifications were either probable or possible, and for one strain only the genus was confirmed. Thus, the Vitek 2 NH system identifies most of the commonly occurring species included in the database. Some strains of rarely occurring species and strains of non-database species closely related to database species cause problems. Partial 16S rRNA gene sequence analysis performs well, but does not always suffice, additional phenotypical characterization being useful for final identification.

Keywords: Evaluation, fastidious Gram negatives, 16S rRNA gene sequencing, Vitek 2 NH.

INTRODUCTION

Fastidious Gram-negative bacteria comprise a number of different genera and species that may cause serious systemic infections. Their fastidious nature often makes identification a challenge in the routine microbiology laboratory, and their ability to cause invasive disease makes correct identification important. Their ability to cause endocarditis (HACEK group of bacteria: Haemophilus spp, Aggregatibacter spp, Cardiobacterium species, Eikenella corrodens, and Kingella kingae), animal-bite infections (e.g. Capnocytophaga spp., Neisseria weaveri, and Pasteurella spp.) and their role in abscess formation (E. corrodens, Aggregatibacter actinomy-cetemcomitans) illustrates their importance [1]. Other diagnostic challenges are that their taxonomy, including nomenclature [e.g. 2, 3], as well as the recognition of their etiologic possibilities, are continuously evolving; an example of the latter is the possibility of infective endocarditis when Neisseria elongata is isolated from blood cultures.

Conventional identification of fastidious Gram-negative bacteria is at times cumbersome, often requiring special media and phenotypic tests, plus specialist knowledge. As far as we know, there are only two fully automated identification systems for identification of fastidious Gram negative bacteria on the market, Vitek 2 Neisseria-Haemophilus (NH) (bioMérieux, Marcy L’Etoile, France) and Phoenix (Becton Dickinson, Cockneyville, MD, USA). BioMérieux has developed a card for the identification of 26 taxa of fastidious Gram-negatives, including Neisseria, Haemophilus, and the HACEK bacteria, for use in the Vitek 2 system. The card consists of 30 biochemical tests that are monitored up to 8 hours. The purpose of this study was to evaluate the utility of the Vitek 2 system in a clinical microbiology laboratory by comparing it with identification by conventional methods. In addition, it was attempted to assess the value of partial 16S rRNA gene sequence analysis (526 bp stretch) for identification within this group of bacteria.

MATERIALS AND METHODS

Bacterial Strains

The 100 bacterial strains examined in this study are shown in Tables 1a, 1b, and 2. They comprise clinical strains received for species identification or for research and monitoring purposes at the reference laboratories at Statens Serum Institut (SSI), supplemented with strains from various culture collections. The latter included 31 type strains, of which one is the type strain of Haemophilus paraphrophilus, now part of the new species Aggregatibacter aphrophilus [2], and another the type strain of Haemophilus pituitamia, a new species comprising some haemolytic strains of Haemophilus [4]. The strains comprised two groups: a group of 75 strains representing 20 of the 26 taxa included in the Vitek 2 database (the three Campylobacter taxa plus
Table 1a. Identification Results Obtained By partial 16S rRNA Gene Sequence Analysis and by Vitek 2 NH Characterization of 45 Strains Included in the Vitek 2 NH Database

| Strains                  | Strain Designations<sup>a,b</sup> | 16S rRNA Gene Analysis Results<sup>c</sup> | Difference Between Max Scores<sup>d</sup> | NCBI–BLAST Species Interpretation | Vitek 2 NH Identification<sup>e</sup> |
|--------------------------|------------------------------------|---------------------------------------------|-------------------------------------------|----------------------------------|--------------------------------------|
| A. ureae                 | NCTC 10219<sup>T</sup>            | 893 / 879 (A. arthritidis) // 502            | 14                                        | Probable                         | Misidentified                       |
| A. ureae                 | SSI: P 524                         | 881 / 877 (A. arthritidis) // 498            | 4                                         | Probable                         | Correct                             |
| A. actinomycetemcomitans | NCTC 9710<sup>T</sup>             | 755 / 704 (A. aphrophilus) // 425            | 51                                        | Confirmed                        | Low Discrimination                  |
| A. actinomycetemcomitans | HK 666                             | 830 / 731 (H. influenzae) // 463              | 99                                        | Confirmed                        | Correct                             |
| A. actinomycetemcomitans | HK 1662                            | 805 / 713 (H. segnis) // 448                  | 92                                        | Confirmed                        | Correct                             |
| A. aphrophilus/paraphrophilus | NCTC 5906<sup>T</sup>        | 762 / 739 (H. parainfluenzae) // 431          | 41                                        | Confirmed                        | Correct                             |
| A. aphrophilus/paraphrophilus | SSI: P 536, AB 1635                   | 706 / 673 (A. actinomycetemcomitans) // 407 | 33                                        | Confirmed                        | Correct                             |
| A. aphrophilus/paraphrophilus | CCGU 14858<sup>S</sup>            | 710 / 675 (H. parainfluenzae) // 413          | 35                                        | Confirmed                        | Correct                             |
| A. aphrophilus/paraphrophilus | CCGU 49494                      | 818 / 789 (H. parainfluenzae) // 448          | 29                                        | Confirmed                        | Correct                             |
| A. segnis                | ATCC 33393<sup>T</sup>            | 856 / 740 (Pasteurella aerogenes) // 477      | 116                                       | Confirmed                        | Unidentified                        |
| A. segnis                | SSI: P 1292                        | 847 / 782 (H. influenzae) // 497              | 65                                        | Confirmed                        | Low Discrimination                  |
| A. segnis                | SSI: P 1351                        | 825 / 782 (H. influenzae) // 499              | 43                                        | Confirmed                        | Low Discrimination                  |
| C. canimorsus            | CCGU 19190                         | 657 / 657 (C. cynodegmi) // 492              | 0                                         | Probable                         | Unidentified                        |
| C. canimorsus            | CCGU 19141                         | 857 (C. cynodegmi) // 854 (C. canimorsus) // 494 | -3                                        | Possible                         | Unidentified                        |
| C. canimorsus            | CCGU 19140                         | 829 / 661 (C. cynodegmi) // 485              | 168                                       | Confirmed                        | Correct                             |
| C. canimorsus            | SSI: 4642/2006                     | 838 / 751 (C. cynodegmi) // 488              | 87                                        | Confirmed                        | Misidentified                       |
| C. canimorsus            | SSI: 140/2006                      | 587 / 533 (C. cynodegmi) // 365              | 54                                        | Confirmed                        | Misidentified                       |
| C. canimorsus            | SSI: 187/2006                      | 856 / 780 (C. cynodegmi) // 485              | 76                                        | Confirmed                        | Misidentified                       |
| C. gingivalis            | CCGU 9715<sup>T</sup>             | 893 / 852 (C. granulosa) // 503              | 41                                        | Confirmed                        | Unidentified                        |
| C. ochracea              | SSI: 3435/04                       | 859 / 852 (C. sputigena) // 491              | 7                                         | Probable                         | Correct                             |
| C. sputigena             | CCGU 9714<sup>T</sup>             | 785 / 765 (C. ochracea) // 481               | 18                                        | Confirmed                        | Unidentified                        |
| C. hominis               | CCGU 31207                         | 841 / 753 (C. valvarum) // 496               | 88                                        | Confirmed                        | Correct                             |
| C. hominis               | CCGU 2711<sup>T</sup>             | 893 / 761 (C. valvarum) // 502               | 132                                       | Confirmed                        | Correct                             |
| C. hominis               | SSI: AB 2089                       | 708 / 663 (C. valvarum) // 407               | 45                                        | Confirmed                        | Correct                             |
| E. corrodens             | ATCC 23834<sup>T</sup>            | 780 / 646 (N. denitrificans) // 436          | 134                                       | Confirmed                        | Correct                             |
| E. corrodens             | SSI: 13794/1992                    | 623 / 541 (K. denitrificans) // 416          | 82                                        | Confirmed                        | Correct                             |
| E. corrodens             | SSI: 13897/1992                    | 747 / 659 (K. denitrificans) //425           | 88                                        | Confirmed                        | Correct                             |
| H. haemolyticus          | NCTC 10659<sup>T</sup>            | 856 / 856 (H. influenzae) // 500              | 0                                         | Probable                         | Misidentified                       |
| H. influenzae            | NCTC 8143<sup>T</sup>             | 832 / 736 (H. haemolyticus) // 462           | 96                                        | Confirmed                        | Correct                             |
| H. influenzae            | SSI: P 1227                        | 756 / 680 (H. haemolyticus) // 418           | 76                                        | Confirmed                        | Correct                             |
| H. influenzae            | ATCC 49247                         | 906 / 837 (H. haemolyticus) // 508           | 69                                        | Confirmed                        | Correct                             |
| H. parahaemolyticus      | NCTC 8479<sup>T</sup>             | 838 / 838 (A. pleuropneumoniae) // 486       | 0                                         | Probable                         | Correct                             |
| H. parahaemolyticus      | CCGU 48512                         | 901 / 893 (A. pleuropneumoniae) // 499       | 8                                         | Probable                         | Misidentified                       |
| H. parainfluenzae        | NCTC 7857<sup>T</sup>             | 889 / 841 (A. paraphrophilus) // 481         | 48                                        | Confirmed                        | Correct                             |
| H. parainfluenzae        | SSI: P 1538                        | 870 / 756 (A. paraphrophilus) // 471         | 114                                       | Confirmed                        | Correct                             |
Table 1a. contd…

| Strains     | Strain Designationsa,b     | 16S rRNA Gene Analysis Resultsc | Difference Between Max Scoresd | NCBI-BLAST Species Interpretation | Vitek 2 NH Identificatione |
|-------------|----------------------------|---------------------------------|------------------------------|-----------------------------------|-----------------------------|
| *H. parainfluenzae* | CCUG 49489               | 929 / 837 (A. paraphrophilus) // 445 | 92              | Confirmed Correct                 |                             |
| *H. pittmaniae* | CCUG 48703f              | 686 / 676 (H. parainfluenzae) // 400 | 10              | Probable Low Discrimination       |                             |
| *K. denitrificans* | CCUG 6516f              | 838 / 838 (N. weaveri) // 469     | 0               | Probable Low Discrimination       |                             |
| *K. denitrificans* | CCUG 14999             | 883 / 767 (N. elongata) // 512    | 116             | Confirmed Low Discrimination      |                             |
| *K. kingae* | SSI: A 303T             | 886 / 762 (N. weaveri) // 495     | 114             | Confirmed Unidentified           |                             |
| *K. kingae* | CCUG 13025             | 879 / 758 (N. weaveri) // 499     | 111             | Confirmed Unidentified           |                             |
| *K. kingae* | SSI: 4541/05        | 904 / 785 (N. weaveri) // 509     | 119             | Confirmed Correct                |                             |
| *M. catarrhalis* | CCUG 353f               | 816 / 758 (M. canis) // 462       | 58              | Confirmed Correct                |                             |
| *M. catarrhalis* | SSI: RH 56295/84     | 805 / 747 (M. canis) // 460       | 58              | Confirmed Correct                |                             |
| *M. catarrhalis* | CCUG 11766           | 760 / 729 (M. nonliquefaciens) // 424 | 31              | Confirmed Low Discrimination     |                             |

a ATCC, American Type Culture Collection, Bethesda, Md., USA; CCUG, Culture Collection of the University of Göteborg, Sweden; HK, Mogens Kilian, Institute of Microbiology, Aarhus, Denmark; NCTC, National Collection of Type Cultures; SSI, Statens Serum Institut, Copenhagen, Denmark.
b T denotes type strain.
c Max score best taxon match / Max score next best taxon match (taxon of next best match) // base pairs examined. If best taxon match 16S identification is not identical to gold standard identification, the 16S rRNA identification is given before the first /
d Difference between best taxon match (most commonly same identification as gold standard identification) and next best match; in cases when gold standard identification was the same as next best match, the difference is negative.
e See Table 3 for details of Vitek 2 NH card examination.
f Formerly *H. parahaemolyticus*.

Table 1b. Identification Results Obtained by Partial 16S rRNA Gene Sequence Analysis and by Vitek 2 NH Characterization of 30 *Neisseria* Species Strains Included in the Vitek 2 NH Database

| Strains     | Strain Designationsa,b      | 16S rRNA Gene Analysis Resultsc | Difference Between Max Scoresd | NCBI–BLAST Species Interpretation | Vitek 2 NH Identificatione |
|-------------|----------------------------|---------------------------------|------------------------------|-----------------------------------|-----------------------------|
| *N. cinerea* | CCUG 2156f              | 904 (N. meningitidis) / 897 (N. polysaccharea) / 893 (N. cinerea) // 504 | -11             | Confirmed Correct                 |                             |
| *N. cinerea* | CCUG 346             | 758 / 753 (N. meningitidis / N. polysaccharea) // 420 | 5               | Confirmed Correct                 |                             |
| *N. cinerea* | CCUG 5746            | 924 (N. meningitidis) / 915 (N. cinerea) // 515 | -9              | Confirmed Low Discrimination      |                             |
| *N. elongata subsp. elongata* | CCUG 30802f | 693 / 625 (N. subflavia / N. animalis) // 384 | 68              | Confirmed Correct                 |                             |
| *N. elongata subsp. elongata* | CCUG 9686 | 879 / 774 (N. animalis) // 492 | 105             | Confirmed Correct                 |                             |
| *N. elongata subsp. elongata* | SSI: AB 2895  | 909 / 798 (N. animalis) // 502 | 111             | Confirmed Correct                 |                             |
| *N. gonorrhoeae* | CCUG 26876f         | 585 / 562 (N. cinerea) // 329 | 23              | Confirmed Correct                 |                             |
| *N. gonorrhoeae* | CCUG 189/2006     | 904 / 850 (N. meningitidis) // 501 | 54              | Confirmed Correct                 |                             |
| *N. gonorrhoeae* | CCUG 196/2006     | 915 / 861 (N. meningitidis) // 507 | 54              | Confirmed Correct                 |                             |
| *N. gonorrhoeae* | CCUG 199/2006     | 805 / 760 (N. meningitidis) // 447 | 45              | Confirmed Correct                 |                             |
| *N. gonorrhoeae* | CCUG 218/2006     | 823 / 773 (N. meningitidis) // 456 | 50              | Confirmed Low Discrimination      |                             |
| *N. gonorrhoeae* | CCUG 223/2006     | 921 / 866 (N. meningitidis) // 510 | 55              | Confirmed Correct                 |                             |
| *N. gonorrhoeae* | CCUG 253/2006     | 854 / 800 (N. meningitidis) // 487 | 46              | Confirmed Low Discrimination      |                             |
| *N. gonorrhoeae, proA neg.* | SSI: 177/2002   | 675 / 643 (N. cinerea) // 374 | 32              | Confirmed Misidentified          |                             |
| *N. gonorrhoeae, proA neg.* | SSI: 67/2002   | 765 / 729 (N. meningitidis) // 428 | 36              | Confirmed Misidentified          |                             |
| *N. gonorrhoeae, proA neg.* | SSI: 52/2002   | 904 / 850 (N. meningitidis) // 501 | 54              | Confirmed Misidentified          |                             |
| *N. lactamica* | CCUG 5853f               | 717 / 675 (N. polysaccharea) // 406 | 42              | Confirmed Correct                 |                             |
Suttonella indologenes, Gardnerella vaginalis and Oligella urethralis were not included); and another group of 25 non-database strains representing 14 species of the same genera as those included in the database (Actinobacillus hominis, Moraxella spp. and Neisseria spp., i.a. animal bite species (Neisseria weaveri, Neisseria animaloris, and Neisseria zoodegmatis) plus Pasteurella spp.). The strains had been stored as either lyophilized or at -70 °C until the present study. All strains were sent from SSI under code numbers to the Clinical Microbiology Department at Hillerød, so that the investigators were blinded with respect to species identification.

The new validly published genus name Aggregatibacter [2] was used in the present study for the following species given as such in the Vitek database: Haemophilus actinomycetencomitans, Haemophilus aphrophilus / paraphrophilus and Haemophilus segnis.

Identification of Strains

Conventional phenotypic identification comprised extensive characterization by the various reference laboratories at SSI according to conventional biochemical methods [1, 5, 6]. The final identification reached was considered to be the ‘gold standard’ with which identifications obtained by partial 16S rRNA gene sequence analysis and the Vitek 2 NH system were compared.

Partial 16S rRNA gene sequence analysis followed by blast examination was performed [7] using two amplification primers, BSF 8 and BSF 534, producing a 526 base pair (bp) fragment; these fragments were sequenced both ways. The edited sequences were compared to deposited sequences in the NCBI “bacteria” database (BLAST examination) and evaluated for the best and second best taxon matches taking into consideration the % identity (number of identical bases between the query and the subject sequence in the database), Maxscore bit (indication of alignment concordance) and E-values (indication of statistical significance of a given alignment). Thereby, the following results could be obtained by partial 16S rRNA gene sequencing/BLAST examination: 1) ‘confirmed’ (best species match was identical to the gold standard phenotypic identification with a distance in Maxscore bits to next best taxon match of > 15), 2) ‘probable’ (best species match was identical to the gold standard identification, but with a Maxscore bit difference to next best taxon match of ≤ 15), 3) ‘possible’ (best species match was not identical to the gold standard identification, but the gold standard identification was among closely related taxons, which means a ≤ 15 Maxscore bit difference to the best taxon match) or 4) ‘misidentified’ (if the conventional phenotypic species identification was not listed among the closely related species/taxons).

Vitek 2 NH system testing was done by a microbiologist without expert knowledge of fastidious Gram-negative bacteria and was performed according to the manufacturer’s recommendations. Supplementary tests for strains identified with Low Discrimination were not done for two reasons: i) some of the supplementary tests were unavailable to us; and ii) results of these tests would tend to confuse identifications further since 25 % of the tested strains were not included in the Vitek 2 NH database.

Interpretation was done on the basis of results provided from the software (EX: excellent, VG: Very Good, GI: Good, AC: Acceptable, LD: Low Discrimination (between 2-3 identification choices), INC: Inconclusive (> 3 identification choices), and UNI: Unidentified (atypical biopattern)). The categories of results in the present study were defined as

| Strains          | Strain Designations\(^a, b\) | 16S rRNA Gene Analysis Results\(^c\) | Difference Between Max Scores\(^d\) | NCBI –BLAST Species Interpretation | Vitek 2 NH Identification\(^e\) |
|------------------|------------------------------|-------------------------------------|-----------------------------------|---------------------------------|-------------------------------|
| N. lactamica     | SSI: BH 67320                | 794 / 791 (N. polysaccharea) // 518 | 3                                 | Probable                        | Correct                       |
| N. meningitidis  | CCGU 3269\(^2\)             | 937 / 913 (N. polysaccharea) // 507 | 24                                | Confirmed                       | Correct                       |
| N. meningitidis  | SSI: 17/2006                 | 937 / 904 (N. cinerea) // 513       | 33                                | Confirmed                       | Correct                       |
| N. meningitidis  | SSI: 18/2006                 | 828 / 798 (N. polysaccharea) // 452 | 30                                | Confirmed                       | Correct                       |
| N. meningitidis  | SSI: 19/2006                 | 924 / 889 (N. cinerea) // 503       | 35                                | Confirmed Low Discrimination    |                               |
| N. meningitidis  | SSI: 20/2006                 | 902 / 878 (N. polysaccharea) // 501 | 24                                | Confirmed                       | Correct                       |
| N. meningitidis  | SSI: 21/2006                 | 913 / 889 (N. polysaccharea) // 504 | 24                                | Confirmed                       | Correct                       |
| N. meningitidis  | SSI: 50/2006                 | 895 / 863 (N. cinerea) // 489       | 30                                | Confirmed                       | Correct                       |
| N. meningitidis  | SSI: 23/2006                 | 918 / 900 (N. cinerea) // 503       | 18                                | Confirmed                       | Correct                       |
| N. meningitidis  | SSI: 60/2004                 | 915 / 880 (N. cinerea) // 502       | 35                                | Confirmed Correct 1            |                               |
| N. meningitidis  | SSI: 109/1998                | 913 / 888 (N. polysaccharea) // 501 | 25                                | Confirmed                       | Correct                       |
| N. sicca         | CCGU 23929\(^3\)            | 859 / 854 (N. pharyngis) // 483     | 5                                 | Probable                        | Correct 1                     |
| N. sicca         | SSI: “19343”                | 884 / 845 (N. pharyngis) // 498     | 39                                | Confirmed                       | Correct                       |

\(^a\), see Table 1a.

\(^b\) proA: proline A arylamidase.
Table 2. Identification Results Obtained by Partial 16S rRNA Gene Sequence Analysis and by Vitek 2 NH Characterization of 25 Strains not Included in the Vitek 2 NH Database

| Strains                 | Strain Designationsa,b | 16S rRNA Gene Analysis Resultsc | Differences Between Max Scoresd | NCBI - BLAST Species Interpretation | Vitek 2 NH Identification | Vitek 2 NH Interpretation |
|-------------------------|------------------------|---------------------------------|---------------------------------|-------------------------------------|---------------------------|--------------------------|
| A. hominis              | NCTC 11529T, SSI: P 578 | 865 / 852 (A. suis) // 496       | 13                              | Probable                           | Low discrimination        | Correct genus not included |
| A. hominis              | SSI: P 575             | 836 / 816 (A. suis) // 495       | 20                              | Confirmed                          | Misidentified             | Misidentified            |
| A. hominis              | SSI: P 880             | 812 / 809 (A. suis et A. equuli) // 477 | 3                              | Probable                           | Low discrimination        | No identification to genus level |
| M. non-liquefaciens     | ATCC 19973T            | 861 / 843 (M. lacunata) // 473   | 18                              | Confirmed                          | Low discrimination        | Correct genus included    |
| M. osloensis            | ATCC 19976T            | 746 / 601 (M. canis) // 413      | 145                             | Confirmed                          | Low discrimination        | Correct genus included    |
| N. animaloris (CDC EF-4a) | NCTC 12228T            | 778 / 765 (N. canis) // 413      | 13                              | Probable                           | Misidentified             | Misidentified            |
| N. animaloris (CDC EF-4a) | CCUG 1976             | 865 / 859 (N. canis) // 413      | 6                               | Probable                           | Unidentified              | Correct                  |
| N. animaloris (CDC EF-4a) | SSI: P 669            | 855 / 836 (N. canis) // 455      | 19                              | Confirmed                          | Unidentified              | Correct                  |
| N. flavescens           | ATCC 13120T            | 654 / 643 (N. flava) // 491      | 11                              | Probable                           | Low discrimination        | Correct genus included    |
| N. mucosa               | CCUG 26877T            | 806 (N. pharyngis)/ 791 (N. mucosa) // 499 | -15                             | Possible                           | Misidentified             | Misidentified            |
| N. mucosa               | SSI: 10496/78          | 795 (N. pharyngis)/ 780 (N. mucosa) // 496 | -15                             | Possible                           | Misidentified             | Misidentified            |
| N. pharyngis            | SSI: Piot 1268         | 822 (N. flavescens)/ 802 (N. subflava) // 504 | Not given                       | Misidentified                     | Misidentified             | Misidentified            |
| N. polysaccharea        | CCUG 18030T            | 886 / 883 (N. meningitidis) // 505 | 3                               | Possible                           | Low discrimination        | Correct genus included    |
| N. weaver               | SSI: 3667B/1997        | 802/708 (N. subflava) // 442     | 94                              | Confirmed                          | Misidentified             | Misidentified            |
| N. weaver               | SSI: 4194/1998         | 889/778 (N. meningitidis) // 489 | 112                             | Confirmed                          | Misidentified             | Misidentified            |
| N. weaver               | SSI: AB 2363           | 898/787 (N. meningitidis) // 489 | 111                             | Confirmed                          | Low discrimination        | Correct genus included    |
| N. zoodegmatis (CDC EF-4b) | NCTC 12230T            | 836 / 782 (N. canis) // 476      | 54                              | Confirmed                          | Misidentified             | Misidentified            |
| N. zoodegmatis (CDC EF-4b) | SSI: P 1168           | 834 / 810 (N. dentiae) // 498    | 24                              | Confirmed                          | Misidentified             | Misidentified            |
| N. zoodegmatis (CDC EF-4b) | SSI: P 983            | 868 / 809 (N. canis) // 498      | 59                              | Confirmed                          | Misidentified             | Misidentified            |
| P. canis                | SSI: P 824             | 838 / 803 (P. dagmatis) // 501   | 35                              | Confirmed                          | Misidentified             | Misidentified            |
| P. dagmatis             | SSI: P 1533            | 857 / 839 (P. stomatis) // 501   | 18                              | Confirmed                          | Misidentified             | Misidentified            |
| P. multocida            | NCTC 10322T            | 854 / 776 (P. pneumotropica) // 495 | 78                             | Confirmed                          | Misidentified             | Misidentified            |
| P. multocida            | SSI: P 1367            | 892 / 816 (P. pneumotropica) // 497 | 76                             | Confirmed                          | Misidentified             | Misidentified            |
| P. multocida            | SSI: P 1320            | 838 / 762 (P. pneumotropica) // 469 | 76                             | Confirmed                          | Unidentified              | Correct                  |
| P. stomatis             | SSI: P 716             | 796 / 774 (P. pneumotropica) // 455 | 22                             | Confirmed                          | Unidentified              | Correct                  |

*a, b, see Table 1a.

follows: (i) Correct identification was species identification identical to the ‘gold standard’ with the quality epithets EX, VG, GI and AC, except for the four *Capnocytophaga* species, where identification to the genus level was considered correct; (ii) Low discrimination (LD) between two or three species; (iii) Unidentified (included both INC and UNI); and (iv) Misidentification was identification with the epithets EX, VG, G and AC to a different species.
RESULTS

Partial 16S rRNA Gene Sequence Analysis Identifications

The identifications achieved by partial 16S rRNA gene sequence analysis of the 100 strains are shown in Tables 1a, 1b, and 2. Phenotypic and sequence analysis identifications to the species level were identical for 76 strains, resulting in ‘species confirmed’. For 23 strains sequence analysis identifications resulted in either ‘species probable’ (n = 16) or ‘species possible’ (n = 7): 2 of 3 A. hominis strains, 2 of 6 C. canimorsus strains, 1 of 2 K. denitrificans strains, 1 of 2 N. lactamica strains, 1 of 2 N. sicca strains and 2 of 3 N. animaloris strains; and all included strains of the following species: Actinobacillus ureae (2), C. ochracea (1), H. para-haemolyticus (2) and H. pittmanniae (1), N. cinerea (3), N. flavescens (1), N. mucosa (2) and N. polysaccharea (1). Only for the Neisseria pharyngis strain was the result of 16S rRNA gene sequence analysis in conflict with the conventional phenotypic identification, where the “gold standard” species was not among the listed taxon matches. Of the 24 strains where phenotypic and sequence analysis identifications were not identical, 12 were type strains.

Where several strains of the same species were examined, score bit differences among the different strains were about the same size for most of the species. However, for two of the six C. canimorsus strains (CCUG 19190 and CCUG 19141) the differences between first and second best taxon match were very small (0 and 3 respectively), while they were between 54 and 168 for the remaining four strains. The same applied to the two K. denitrificans strains (0 and 116). Remarkable variations in score bit differences between strains belonging to the same species were seen for all the strains of A. segnis, C. hominis, H. parainfluenzae and K. kingae. Of the 24 strains where the result was not ‘species confirmed’ by sequencing, 13 were Vitek database strains. Of these, seven were correctly identified by the Vitek 2 NH card.

Vitek Identifications of Vitek 2 NH Database Strains

Vitek 2 NH results for the 75 examined strains included in the Vitek 2 NH database are shown in Table 1a and 1b. Epithets of ‘acceptable’ or better were obtained for 57 (76%) of the strains. Of these, 48 (64%) were correctly identified, while 9 (12%) were misidentified. The risk of misidentification seems to be related to the epithets, as 4 of the 45 with ‘excellent’ identification, 0 of 2 with ‘very good’ identification, 2 of 4 with ‘good’ identification and 3 of 6 with ‘acceptable’ identification were misidentified (Table 3). The nine misidentified strains comprised three of nine Capnocytophaga strains identified as Neisseria elongata, three proline-arylamidase (proA) negative Neisseria gonorrhoeae identified as Moraxella catarrhalis, one each of Haemophilus haemolyticus (type strain) and H. parahaemolyticus, both identified as Haemophilus parainfluenzae, and one A. ureae identified as H. influenzae.

In 11 (15%) instances where ‘low discrimination’ between 2 or 3 species was obtained, the correct species was included among the suggested species for 9 strains (Table 3). For the 4 ‘low discrimination’ Neisseria spp., 3 were identified correctly to the genus level (Table 3). If one disregards the recent taxonomic changes within the genus Haemophilus (Materials and Methods), 4 of 4 ‘low discrimination’ strains of former and present Haemophilus spp. were identified correctly to the genus level (Table 3).

There were no strains where an ‘inconclusive’ result was obtained. Seven strains (9%) were unidentifiable: four of nine strains of Capnocytophaga spp., including the type strains of C. gingivalis and C. sputigena; two of three Kingella kingae strains, including the type strain; and the type strain of A. segnis.

Vitek Identification of Non-Vitek 2 NH Database Strains

Table 2 shows results for the 25 examined strains not included in the Vitek 2 NH database. Of these, 14 (56%) were identified with epithets of ‘excellent’ (7), ‘good’ (4), and ‘acceptable’ (3). All of these were by definition misidentified. Four strains were unidentifiable, which in this context is the correct result; and ‘Low discrimination’ was obtained for seven strains (Table 3).

DISCUSSION

Analysis (and comparison) of 16S rRNA gene sequences has revolutionized bacterial taxonomy and identification [9]. For strains difficult to identify by conventional phenotypic identification 16S rRNA gene sequencing is especially in focus [8]. Among the 100 strains studied, only a N. pharyngis strain obtained sequencing analysis results in conflict with the conventional phenotypic identification, as the “gold standard” species was not among the listed possible taxon matches. Importantly, the 16S rRNA gene sequence analysis results obtained did not result in misidentifications, but for 24 strains the need for further characterization was evident. This could consist of sequencing of longer bp stretches of the 16S rRNA gene, sequencing of other genes, or more extensive phenotypic characterization.

The obtained results thus illustrate both the strengths and weaknesses of the use of 16S rRNA gene sequence analysis for identification. There are, as yet, no generally accepted guidelines for correct genus and species identification, as it has not been possible to reach a consensus on threshold values like there is for DNA–DNA hybridization (Petti, 2007 [9], Stackebrandt & Goebel, 1994 [10], Janda & Abbott, 2007 [11]). In addition, different studies have identified groups of bacteria for which 16S rRNA gene sequences are less discriminative, as seen in this study for the 23 strains resulting in either species probable or possible.

Sequence divergence may vary considerably within genera and must ideally be assessed for each genus. We have attempted to elucidate the 16S rRNA gene sequence identification process by using standardized quantitative criteria for all the studied taxa (see Materials and Methods) and reporting the data in Tables 1 and 2 together with the species of the best and next best taxon match. This in order to document the 16S rRNA gene sequence identification process.

Great variation in score bit differences was seen within strains of A. segnis, C. canimorsus, C. hominis, H. parainfluenzae, K. denitrificans, and K. kingae. This might be an expression of great variation within the individual species, it may illustrate that taxonomic subgroups exist, or it could be caused by deposition of unvalidated sequences. Whether
| Strains (no. of Strains) | Vitek 2 NH Results and Quality of Identification |
|--------------------------|-------------------------------------------------|
| A. hominis \(^a\) (3)   | H. influenzae, good (1); H. parahaemolyticus or A. aphrophilus/paraphrophilus; LD\(^b\) (1); H. parahaemolyticus or A. aphrophilus/paraphrophilus or H. parainfluenzae, LD\(^3\) (1) |
| A. ureae (2)            | A. ureae, excellent (1); H. influenzae, excellent (1) |
| A. actinomycescomitans (3) | A. actinomycescomitans, excellent (2) A. actinomycescomitans or A. segnis, LD\(^b\) (1); |
| A. aphrophilus/paraphrophilus (4) | A. aphrophilus/paraphrophilus, excellent (2), good (1), acceptable(1) |
| A. segnis (3)           | A. segnis or H. parainfluenzae, LD\(^3\) (1); H. influenzae or H. haemolyticus, LD\(^3\) (1), unidentified (1) |
| C. canimorsus (6)       | Capnocytophaga spp., good (1); N. elongata, acceptable (3); unidentified (2) |
| C. gingivalis (1)       | Unidentified |
| C. ochracea (1)         | Capnocytophaga spp, excellent (1) |
| C. spatigera (1)        | C. hominis, excellent (2), acceptable (1) |
| C. hominis (3)          | E. corrodens, excellent (3) |
| H. haemolyticus (1)     | H. parainfluenzae, good (1) |
| H. influenzae (3)       | H. parahaemolyticus, good (1) |
| H. parahaemolyticus (2) | H. parahaemolyticus, excellent (1); H. parainfluenzae, good (1) |
| H. parainfluenzae (3)   | H. parainfluenzae, excellent (2), very good (1) |
| H. pittmanae \(^c\) (1) | A. aphrophilus/paraphrophilus or A. segnis, LD\(^3\) (1) |
| K. denitrificans (2)    | K. denitrificans or N. cinerea, LD\(^3\) (1); K.denitrificans or N. meningitidis, LD\(^3\) (1) |
| K. kingae (3)           | K. kingae, acceptable (1); unidentified (2) |
| M. catarrhalis (3)      | M. catarrhalis, excellent (1), very good (1); N. cinerea or M. catarrhalis or N. meningitidis, LD\(^3\) (1) |
| M. non-liquefacien\(^a\) (1) | M. catarrhalis or N. gonorrhoeae, LD\(^3\) (1) |
| M. ostensis\(^a\) (1)   | Campylobacter fetus or Campylobacter coli, LD\(^3\) (1) |
| N. animaloris (CDC EF-4a) \(^a\) (3) | N. elongata, acceptable (1); unidentified (2) |
| N. cinerea (3)          | N. cinerea, excellent (2); N. cinerea or K. denitrificans, LD\(^3\) (1) |
| N. elongata spp. elongata (3) | N. elongata, excellent (2); N. elongata, acceptable (1) |
| N. flavescens\(^a\) (1) | N. elongata or K. denitrificans or N. cinerea, LD\(^3\) (1) |
| N. gonorrhoeae, proA\(^d\) positive (7) | N. gonorrhoeae, excellent (5); N. cinerea or N. gonorrhoeae, LD\(^3\) (1); N. gonorrhoeae or N. cinerea or N. elongata, LD\(^3\) (1) |
| N. gonorrhoeae, proA negative (3) | M. catarrhalis, excellent (3) |
| N. lactamica (2)        | N. lactamica, excellent (2) |
| N. meningitidis (10)    | N. meningitidis, excellent (9); N. meningitidis or N. sicca, LD\(^3\) (1) |
| N. mucosa \(^a\) (2)    | N. elongata, excellent (1); N. sicca excellent (1) |
| N. pharyngis\(^a\) (1)  | N. sicca, excellent (1) |
| N. polyacaehaurea \(^a\) (1) | N. sicca or N. meningitidis LD\(^3\) (1) |
| N. sicca (2)            | N. sicca, excellent (2) |
| N. weaveri \(^a\) (3)   | N. elongata, excellent (2); N. cinerea or N. elongata or M. catarrhalis, LD\(^3\) (1) |
| N. zoolegmnatis (CDC EF-4b) \(^a\) (3) | N. elongata, excellent, (2); K. denitrificans, good (1) |
| P. canis \(^a\) (1)     | H. parainfluenzae, good (1) |
| P. dagnatis \(^a\) (1)  | H. parainfluenzae, good (1) |
| P. multocida \(^a\) (3) | H. influenzae, acceptable (1); H. parainfluenzae, acceptable (1); unidentified (1) |
| P. stomatis \(^a\) (1)  | unidentified (1) |

\(^a\) Strains not included in the Vitek 2 NH database.
\(^b\) LD: low discrimination.
\(^c\) Formerly H. parahaemolyticus.
\(^d\) ProA: proline A arylamidase.
sequencing the whole 16S rRNA gene would have resulted in a confirmed species designation for the 23 probable and possible strains is not known. Of these 23 strains, 12 were type strains, six were culture collection strains and the remaining five were from well known reference laboratories.

Identification with the Vitek 2 NH card is, as with the whole Vitek 2 system, easy to handle. Correct identification (including Capnocytophaga to the genus level) was achieved for 48 of 75 (64%) strains in the Vitek 2 NH database, while 9 (12%) were misidentified. Identification problems, i.e. low discrimination and non- or misidentification of strains, were mainly connected with the Capnocytophaga spp., proA-negative N. gonorrhoeae, the haemolytic Haemophilus spp., the Kingella spp. and A. segnis. There were four misidentified strains with the epithet ‘excellent’, three gonococci and one A. uraeae, which means that this epithet is not a guarantee of correct identification. It must, however, be borne in mind that the three misidentified gonococci were proA-negative, a clone with this characteristic appearing most commonly in Scandinavia.

Our finding of 64% of correctly identified strains appears to be at variance with the findings of Valenza et al. [12], who found that 91% of their 188 strains were correctly identified without supplementary tests. This difference is most readily explained by differences in the qualitative and quantitative composition of the examined strains in the two studies. Valenza et al. examined no strains of proA-negative N. gonorrhoeae, H. haemolyticus, H. parahaemolyticus, A. uraeae or A. segnis; and only one strain each of Capnocytophaga spp. and Kingella spp. This is in contrast to our nine strains of Capnocytophaga spp. and five strains of Kingella spp. However, these taxa represent some of the most difficult with regard to conventional identification, making it extra desirable that automatic identification results in reliable identifications. Disregarding these problematic strains, results of the two studies are similar. With regard to the 49 remaining strains in the present study we found no un- or misidentified strains compared to five unidentified and one misidentified strains among the 126 remaining strains in the study of Valenza et al.

Our results also appear to disagree with the recently published multicenter study by Rennie et al. [13], where 371 clinical strains were tested. They found 97% overall correct identification, including among the correctly identified strains 10% with low discrimination where the correct identification was among the suggested choices. Again, the variance is probably explained by the different quantitative composition of the strains examined in the two studies. Of the strains examined in the study of Rennie et al., 35% were ‘easy-to-identify’ H. influenzae and H. parainfluenzae, in contrast to only 6% in the present study. Also, their study did not comprise proA-negative N. gonorrhoeae. The conclusion drawn from the three studies is thus that the Vitek 2 system correctly identifies almost all strains of H. influenzae, H. parainfluenzae, C. hominis, E. corrodens, N. meningitidis and the four apathogenic Neisseria species included in the database.

As done previously by others [14, 15], we did not limit our study to strains included in the Vitek 2 database. This was done in order to evaluate the ability of the Vitek 2 NH card in a setting most closely emulating the diagnostic challenges in clinical microbiology laboratories. As seen under Results, 56% of these strains were erroneously ‘correctly identified’ with epithets of acceptable or better, half of them ‘excellent’. Only four strains were correctly found to be unidentified and seven showed ‘low discrimination’. This is not satisfactory.

In conclusion, the Vitek 2 NH card was found to be an easily used tool in the laboratory, being able to identify the most commonly occurring species in the database correctly. The system would benefit from including tests in the card that ensures that apparent “correct identifications” of bacteria not in the database kept at a minimum. And conversely, including tests that enable difficult bacteria such as Capnocytophaga and Kingella to be identified correctly.

CONFLICT OF INTEREST

Funding from external sources has not been received. None of the authors has any associations that can pose a conflict of interest.

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