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 actinomycetemcomitans*) 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 pittmaniae*, 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

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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[a,b]	16S rRNA Gene Analysis Results[c]	Difference Between Max Scores[d]	NCBI–BLAST Species Interpretation	Vitek 2 NH Identification[e]
A. ureae	NCTC 10219^T	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^T	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^T	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	CCUG 14858^T	710 / 675 (*H. parainfluenzae*) // 413	35	Confirmed	Correct
A. aphrophilus/paraphrophilus	CCUG 49494	818 / 789 (*H. parainfluenzae*) // 468	29	Confirmed	Correct
A. segnis	ATCC 33393^T	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	CCUG 19190	657 / 657 (*C. cynodegmi*) // 492	0	Probable	Unidentified
C. canimorsus	CCUG 19141	857 (*C. cynodegmi*) // 854 (*C. canimorsus*) // 494	-3	Possible	Unidentified
C. canimorsus	CCUG 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	CCUG 9715^T	893 / 852 (*C. granulosa*) // 503	41	Confirmed	Unidentified
C. ochracea	SSI: 3435/04	859 / 852 (*C. sputigena*) // 491	7	Probable	Correct
C. sputigena	CCUG 9714^T	785 / 765 (*C. ochracea*) // 481	18	Confirmed	Unidentified
C. hominis	CCUG 31207	841 / 753 (*C. valvarum*) // 496	88	Confirmed	Correct
C. hominis	CCUG 2711^T	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^T	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^T	856 / 856 (*H. influenzae*) // 500	0	Probable	Misidentified
H. influenzae	NCTC 8143^T	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^T	838 / 838 (*A. pleuropneumoniae*) // 486	0	Probable	Correct
H. parahaemolyticus	CCUG 48512	901 / 893 (*A. pleuropneumoniae*) // 499	8	Probable	Misidentified
H. parainfluenzae	NCTC 7857^T	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 Designations[a,b]	16S rRNA Gene Analysis Results[c]	Difference Between Max Scores[d]	NCBI–BLAST Species Interpretation	Vitek 2 NH Identification[e]
H. parainfluenzae	CCUG 49489	929 / 837 (*A. paraphrophilus*) // 445	92	Confirmed	Correct
H. pittmaniae[f]	CCUG 48703^T	686 / 676 (*H. parainfluenzae*) // 400	10	Probable	Low Discrimination
K. denitrificans	CCUG 6516^T	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 303^T	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 353^T	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 Designations[a, b]	16S rRNA Gene Analysis Results[c]	Difference Between Max Scores[d]	NCBI –BLAST Species Interpretation	Vitek 2 NH Identification[e]
N. cinerea	CCUG 2156^T	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 30802^T	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 26876^T	585 / 562 (*N. cinerea*) // 329	23	Confirmed	Correct
N. gonorrhoeae	SSI: 189/2006	904 / 850 (*N. meningitidis*) // 501	54	Confirmed	Correct
N. gonorrhoeae	SSI: 196/2006	915 / 861 (*N. meningitidis*) // 507	54	Confirmed	Correct
N. gonorrhoeae	SSI: 199/2006	805 / 760 (*N. meningitidis*) // 447	45	Confirmed	Correct
N. gonorrhoeae	SSI: 218/2006	823 / 773 (*N. meningitidis*) // 456	50	Confirmed	Low Discrimination
N. gonorrhoeae	SSI: 223/2006	921 / 866 (*N. meningitidis*) // 510	55	Confirmed	Correct
N. gonorrhoeae	SSI: 253/2006	854 / 800 (*N. meningitidis*) // 487	46	Confirmed	Low Discrimination
N. gonorrhoeae, proA[f] 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 5853^T	717 / 675 (*N. polysaccharea*) // 406	42	Confirmed	Correct

Table 1b. contd….

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	CCUG 3269^T	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	893 / 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 I
N. meningitidis	SSI: 109/1998	913 / 888 (*N. polysaccharea*) // 501	25	Confirmed	Correct
N. sicca	CCUG 23929^T	859 / 854 (*N. pharyngis*) // 483	5	Probable	Correct I
N. sicca	SSI: "19343"	884 / 845 (*N. pharyngis*) // 498	39	Confirmed	Correct

[a-e], see Table 1a.
[f] proA: proline A arylamidase.

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 actinomycetemcomitans, 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 Max-score 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 $\leq$ 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 $\leq$ 15 Maxscore bit difference to the best taxon match) or 4) 'misidentified' (if the conventional phenoptypis 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

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 Designations[a,b]	16S rRNA Gene Analysis Results[c]	Differences Between Max Scores[d]	NCBI - BLAST Species Interpretation	Vitek 2 NH Identification	Vitek 2 NH Interpretation
A. hominis	NCTC 11529^T, 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 19975^T	861 / 843 *(M. lacunata*) // 473	18	Confirmed	Low discrimination	Correct genus included
M. osloensis	ATCC 19976^T	746 / 601 (*M. canis*) // 413	145	Confirmed	Low discrimination	Correct genus not included
N. animaloris (CDC EF-4a)	NCTC 12228^T	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 13120^T	654 / 643 (*N. flava*) // 491	11	Probable	Low discrimination	Correct genus included
N. mucosa	CCUG 26877^T	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[e]	Misidentified	Misidentified	Misidentified
N. polysaccharea	CCUG 18030^T	886 / 883 (*N. meningitidis*) // 505	3	Possible	Low discrimination	Correct genus included
N. weaveri	SSI: 3667B/1997	802/708 (*N. subflava*) // 442	94	Confirmed	Misidentified	Misidentified
N. weaveri	SSI: 4194/1998	889/778 (*N. meningitidis*) // 489	112	Confirmed	Misidentified	Misidentified
N. weaveri	SSI: AB 2363	898/787 (*N. meningitidis*) // 494	111	Confirmed	Low discrimination	Correct genus included
N. zoodegmatis (CDC EF-4b)	NCTC 12230^T	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 10322^T	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-e], 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. parahaemolyticus* (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 unidentified: 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 unidentified, 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

Table 3. Vitek 2 NH Identification Results and Quality of Identification for all 100 Strains Included in the Study. No. of Strain(s) in Brackets

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 [b] (1)
A. ureae (2)	*A. ureae*, excellent (1); *H. influenzae*, excellent (1)
A. actinomycetemcomitans (3)	*A. actinomycetemcomitans*, excellent (2) *A. actinomycetemcomitans* 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 [b] (1); *H. influenzae* or *H. haemolyticus*, LD [b] (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. sputigena (1)	unidentified
C. hominis (3)	*C. hominis*, excellent (2), acceptable (1)
E. corrodens (3)	*E. corrodens*, excellent (3)
H. haemolyticus (1)	*H. parainfluenzae*, good (1)
H. influenzae (3)	*H. influenzae*, excellent (3)
H. parahaemolyticus (2)	*H. parahaemolyticus*, excellent (1); *H. parainfluenzae*, good (1)
H. parainfluenzae (3)	*H. parainfluenzae*, excellent (2), very good (1)
H. pittmaniae [c] (1)	*A. aphrophilus/paraphrophilus* or *A. segnis*, LD [b] (1)
K. denitrificans (2)	*K. denitrificans* or *N. cinerea*, LD [b] (1); *K.denitrificans* or *N. menigitidis*, LD [b] (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 [b] (1)
M. non-liquefaciens [a] (1)	*M. catarrhalis* or *N. gonorrhoeae*, LD [b] (1)
M. osloensis[a] (1)	*Campylobacter fetus* or *Campylobacter coli*, LD [b] (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 [b] (1)
N. elongata ssp. e*longata* (3)	*N. elongata*, excellent (2); *N. elongata*, acceptable (1)
N. flavescens[a] (1)	*N. elongata* or *K. denitrificans* or *N. cinerea*, LD [b] (1)
N. gonorrhoeae, proA [d] positive (7)	*N. gonorrhoeae*, excellent (5); *N. cinerea* or *N. gonorrhoeae*, LD [b] (1); *N. gonorrhoeae* or *N. cinerea* or *N. elongata*, LD [b] (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 [b] (1)
N. mucosa [a] (2)	*N. elongata*, excellent (1); *N. sicca* excellent (1)
N. pharyngis [a] (1)	*N. sicca*, excellent (1)
N. polysaccharea [a] (1)	*N. sicca* or *N. meningitidis* LD [b] (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 [b] (1)
*N. zoodegmatis (*CDC EF-4b) [a] (3)	*N. elongata*, excellent, (2); *K. denitrificans*, good (1)
P. canis [a] (1)	*H. parainfluenzae*, good (1)
P. dagmatis [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. gonnorhoeae*, the haemolytic *Haemophilus spp.,* the *Kingella spp.* and *A. segnis*. There were four misidentified strains with the epithet 'excellent', three gonococci and one *A. ureae*, 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. gonnorhoeae, H. haemolyticus, H. parahaemolyticus, A. ureae* 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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