Sequence-Based Identification of Gram Negative Nonfermenters Using Commercial 16S rDNA Databases

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Abstract

Background: Identification of gram negative nonfermenters (NF) from cultured human specimens is often difficult using traditional phenotypic methods. Partial 16S rDNA sequencing to identify NF depends on the completeness of the comparative database. Public databases lack quality control, but are routinely updated as sequences are published. Commercial databases are often incomplete and lack routine updates. In this study, the validity of the databases from two commercial systems were compared for the identification of NF. **Methods:** 100 NF clinical isolates identified by phenotypic methods were included. The 16S rDNA sequence for the first 500 bases was determined for each isolate. Consensus sequences were compared to the MicroSeq500 (Applied Biosystems) and Virodec (Roche Diagnostics) databases. Sequence-based identifications were determined from the closest matched sequences in each database and then compared to the phenotypic identification. **Results:** There was 50% species agreement among the two databases and phenotypic testing. In 28 cases (28%) phenotypic testing did not match either of the database results while in 12 and 10 cases phenotypic testing matched with MicroSeq and Virodec, respectively. Phenotypic identification systems and MicroSeq500 were found to include out-dated organism nomenclature whereas Virodec included more recent taxonomic changes. 55 isolates showed agreement between the two databases. Of these, 21 (38%) were exact matches to sequences in Virodec while 3 (5%) matched exactly to sequences in MicroSeq500. Higher similarity scores were achieved using Virodec for 41 of the remaining 45 isolates. Virodec and MicroSeq500 showed >/= 99% similarity in 93 and 66 cases, respectively. **Conclusion:** The Virodec database is more precise than the MicroSeq500 database for identifying NF since it contains more exact matches and includes more recent taxonomic changes.

Methods

Samples

100 isolates were recovered from clinical samples and identified using phenotypic methods by the Cleveland Clinic Foundation (Cleveland, OH), the University of Texas Medical Branch (Galvaston, TX), Cook Childrens Medical Center (Fort Worth, TX), and the University of Nebraska Medical Center (Omaha, NE). The isolates were sent to the ARUP Institute for Clinical and Experimental Pathology in Salt Lake City, UT between May and August of 2005 for sequencing and analysis.

Phenotypic/conventional Identification

Phenotypic Identification was obtained using one or more of the following methods:

- Colony morphology and pigmentation
- Routine biochemicals (i.e. oxidase)
- Microscan Neg Combo panel type 32
- Vitek
- API 20 NE
- Unknown (sent to reference lab for ID)

Sequencing

DNA Preparation

A loopful of bacterial cells was suspended in distilled water to a 1.0 McFarland standard.
The suspension was centrifuged for 1 min at 6,000 xg.
The pellet was suspended in 200 ul PrepMan Ultra reagent (Applied Biosystems, Foster City, CA).
The suspension was vortexed for 1 min, boiled for 5 min, then centrifuged for 1 min at 6,000 xg.
4 ul of the supernatant was used in each PCR reaction.

PCR Primers

Forward: 16S-27for 5'-AGAGTTTGATCMTGGCTCAG [A. Mellman et al., 2003, Int. J. Mol. Microbiol. 293(5):359-70]
Reverse: 16S-519rev 5'-GWATTACCGCGGCKGCTG [A. Mellman et al., 2003, Int. J. Mol. Microbiol. 293(5):359-70]

PCR Reaction

Mixture of 40 ul total volume containing:
- 1X FastStart DNA Master Plus Sybr Green (Roche Diagnostics Corp., Indianapolis, IN),
- 500 nM each primer
- 4 mM final Mg++ concentration
- 4 ul DNA preparation

Thermal Cycling Reactions
- RotorGene 3000 real-time PCR instrument (Corbett Research, Sydney, Australia)
  - Protocol:
    - initial denaturation (10 min at 95C)
    - 35 cycles of denaturation (30 s at 95C), annealing (20 s at 55C), and extension (30 s at 72C)
    - single final extension (2 min at 72C)
    - Melt (75-99C), hold 45 secs on 1st step, hold 5 secs on next steps

Positive PCR products showed a melting peak at approximately 92C

PCR Product Cleanup

Positive PCR products were purified with Microcon-100 microconcentrator columns (Amicon, Beverly, MA)

Sequencing Reactions

ABI Prism BigDye Terminator v3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems)
- 0.5 ul of premix from the kit
- 1.8 ul Tris-HCl/$MgCl_2$ buffer (400 mM Tris-HCl; 10 mM $MgCl_2$)
- 10 pmol of sequencing primer (same as PCR primers)
- 2 ul of cleaned PCR product

Sequencing Product Cleanup

Sequencing products were cleaned using pre-made columns of Sephadex G-50 (Amersham-Pharmacia)
[Cloud et al., 2002, J Clin Microbiol. 40(2):400-406]

Sequencing: ABI Prism 3100 - by the ARUP Institue for Clinical and Experimental Pathology

Sequence Analysis

DNAStar (Lasergene, Madison, WI) - trim, edit forward and reverse; obtain consensus sequence
MicroSeq500 v. 1.4.3 software (check version)
Virodec v. 6 software (check version)

Results

Table 1. All identifications comparing phenotypic methods with 16S sequencing using two database

Isolate No.	Conventional ID method	Traditional ID	MicroSeq500	%	Virodec	%	Phenotype Agrees With...	Notes for Discrepancies	System Most Likely to Give Correct Species (best judgement)
1	Vitek	A. baumannii	S. maltophilia	97.1	Stenotrophomonas sp. 45	98.1	Neither Sequence Database	Low sequence similarity scores; neither database includes this sequence.	All - unable to identify
2	Vitek	A. baumannii	A. baumannii	99.3	A. calcoaceticus subsp. anitratus	99.6	MicroSeq Database	Higher sequencing score with Virodec; check references for valid species	*MicroSeq and Phenotype; Virodec not valid
3	Vitek	A. baumannii	A. baumannii	99.6	A. calcoaceticus subsp. anitratus	99.8	MicroSeq Database	Higher sequencing score with Virodec; check references for valid species	*MicroSeq and Phenotype; Virodec not valid
4	Microscan	A. baumannii	A. baumannii	99.9	A. calcoaceticus subsp. anitratus	99.8	MicroSeq Database	Higher sequencing score with Virodec; check references for valid species	*MicroSeq and Phenotype; Virodec not valid
5	Microscan	A. baumannii	A. baumannii	99.9	A. calcoaceticus subsp. anitratus	99.8	MicroSeq Database	Higher sequencing score with Virodec; check references for valid species	*MicroSeq and Phenotype; Virodec not valid
6	Microscan	A. baumannii	A. calcoaceticus	98.5	Acinetobacter sp. RUH53T	100.0	Neither Sequence Database	Virodec has perfect score, but with un-named species.	All-Genus only - unable to speciate
7	Vitek	A. baumannii	A. baumannii	99.8	A. calcoaceticus subsp. anitratus	100.0	MicroSeq Database	Higher sequencing score with Virodec; check references for valid species	*MicroSeq and Phenotype; Virodec not valid
8	Vitek	A. baumannii	Acinetobacter genomosp. 3	99.8	A. calcoaceticus	100.0	Neither Sequence Database	Virodec has perfect score for A. calcoaceticus ; check references for valid species	*MicroSeq and Phenotype; Virodec not valid
9	Microscan	A. baumannii / A. haemolyticus	Acinetobacter genomosp 13	99.7	Acinetobacter sp. AU783	99.8	Neither Sequence Database	No specific identification with phenotypic or sequencing methods.	All-Genus only - unable to speciate
10	Microscan	A. baumannii / A. haemolyticus	Acinetobacter genomosp 14	98.2	Acinetobacter sp.	97.4	Neither Sequence Database	No specific identification with phenotypic or sequencing methods.	All-Genus only - unable to speciate
11	Microscan	A. baumannii-calcoaceticus complex	Acinetobacter genomosp. 3	99.8	A. calcoaceticus	100.0	Neither Sequence Database	Virodec has perfect score for A. calcoaceticus ; check references for valid species	*MicroSeq and Phenotype; Virodec not valid
12	Ref lab for ID	A. junii	A. haemolyticus	96.6	A. calcoaceticus	99.4	Neither Sequence Database	Highest score with Virodec, but is unpublished with 492 bases (short).	All-Genus only - (Virodec not valid)
13	Vitek, API 20 NE	A. lwoffi	A. junii	99.8	A. junii	99.8	Neither Sequence Database	Both MicroSeq and Virodec equal with pretty good scores.	MicroSeq and Virodec
14	Vitek	A. lwoffii	A. haemolyticus	96.7	A. calcoaceticus	99.4	Neither Sequence Database	No perfect scores by sequencing. Highest score with Virodec.	All-Genus only - unable to speciate
15	Microscan	A. xylosoxidans	S. maltophilia	99.9	S. maltophilia	99.6	Neither Sequence Database	Both MicroSeq and Virodec equal with pretty good scores.	MicroSeq and Virodec
16	Vitek, API 20 NE	A. xylosoxidans	A. xylosoxidans xylosoxidans	99.7	A. xylosoxidans	99.8	Both Sequence Databases	Both MicroSeq and Virodec fairly equal for same species as identified phenotypically.	All
17	Vitek, API 20 NE	A. xylosoxidans	A. xylosoxidans xylosoxidans	99.8	A. xylosoxidans	100.0	Both Sequence Databases	Both MicroSeq and Virodec fairly equal for same species as identified phenotypically.	All
18	Microscan	A. xylosoxidans	A. xylosoxidans xylosoxidans	99.9	A. xylosoxidans	99.8	Both Sequence Databases	Both MicroSeq and Virodec fairly equal for same species as identified phenotypically.	All
19	Vitek	A. xylosoxidans xylosoxidans	A. xylosoxidans xylosoxidans	99.8	A. xylosoxidans	100.0	Both Sequence Databases	Both MicroSeq and Virodec fairly equal for same species as identified phenotypically.	All
20	Microscan	Acinetobacter species/ P. oryzihabitans	Acinetobacter genomosp. 3	99.7	A. calcoaceticus	99.6	Neither Sequence Database	Virodec has high score for A. calcoaceticus ; check references for valid species	*MicroSeq and Phenotype; Virodec not valid
21	Microscan	Acinetobacter spp.	Acinetobacter genomosp. 3	99.8	A. calcoaceticus	100.0	Neither Sequence Database	Virodec has perfect score for A. calcoaceticus ; check references for valid species	*MicroSeq and Phenotype; Virodec not valid
22	Microscan	Alcaligenes spp.	B. pertussis/ B. parapertussis/ B. bronchiseptica	96.8	B. petrii	99.0	Neither Sequence Database	Sequencing suggests Bordetella species; Virodec was close to a species, but needs validity check.	Virodec (B. petrii valid)
23	Microscan	Alcaligenes spp.	A. faecalis faecalis	99.7	A. faecalis	99.4	Both Sequence Databases	Phenotypic identification not to species level; sequencing not perfect, but both databases agree.	MicroSeq and Virodec
24	Microscan	Alcaligenes spp.	A. faecalis faecalis	99.7	A. faecalis	99.4	Both Sequence Databases	Phenotypic identification not to species level; sequencing not perfect, but both databases agree.	MicroSeq and Virodec
25	Microscan	Alcaligenes spp.	A. faecalis faecalis	99.4	A. faecalis	100.0	Both Sequence Databases	Phenotypic identification not to species level; sequencing by both databases agree, Virodec perfect.	MicroSeq and Virodec
26	routine biochemical	B. diminuta	B. diminuta	99.6	B. diminuta	100.0	Both Sequence Databases	Virodec has perfect score, close to MicroSeq	MicroSeq and Virodec
27	API 20 NE	C. meningosepticum	C. meningosepticum	97.1	C. meningosepticum	99.8	Both Sequence Databases	Phenotypic identification not to species level; MicroSeq gave low score while Virodec almost perfect.	Phenotype and Virodec
28	Microscan	Chryseobacterium spp.	C. indologenes	99.9	C. indologenes	99.8	Both Sequence Databases	Phenotypic identification not to species level; sequencing not perfect, but both databases agree.	MicroSeq and Virodec
29	Vitek, API 20 NE	D. acidovorans	D. acidovorans	99.8	D. acidovorans	100.0	Both Sequence Databases	Phenotypic identification not to species level; sequencing by both databases agree, Virodec perfect.	All
30	API 20 NE	F. oryzihabitans	F. oryzihabitans	99.6	P. psychrotolerans	99.8	MicroSeq Database	Virodec has higher score for P. psychrotolerans, which is not in the MicroSeq database.	Virodec (new species, 2004)
31	Vitek, API 20 NE	B. cepacia	B. cepacia	99.3	B. cepacia	99.8	Both Sequence Databases	Virodec has better score (probably contains more genomospecies).	All
32	Vitek, API 20 NE	R. pickettii	R. pickettii	97.3	R. insidiosa	100.0	MicroSeq Database	Virodec perfect score with a species that is not in the MicroSeq (and probably Vitek) database.	Virodec (new species, 2003)
33	Vitek	S. maltophilia	S. maltophilia	98.6	Stenotrophomonas sp. 45	98.8	Both Sequence Databases	Virodec has higher score with an unnamed species; neither score is great. Should just report Genus.	All-Genus only - unable to speciate
34	Vitek	S. maltophilia	S. maltophilia	98.3	Stenotrophomonas sp. 45	98.8	Both Sequence Databases	Virodec has higher score with an unnamed species; neither score is great. Should just report Genus.	All-Genus only - unable to speciate
35	Vitek	S. maltophilia	S. maltophilia	99.1	S. maltophilia	99.0	Both Sequence Databases	Neither sequencing score is great. Should just report Genus.	All-Genus only - unable to speciate
36	Vitek	S. maltophilia	S. maltophilia	98.4	S. maltophilia	99.2	Both Sequence Databases	Neither sequencing score is great. Should just report Genus.	All-Genus only - unable to speciate
37	Microscan	S. maltophilia	S. maltophilia	99.4	S. maltophilia / Methylobacterium sp. 4BR9	99.2	Both Sequence Databases	Neither sequencing score is great. Should just report Genus.	All-Genus only - unable to speciate
38	Vitek	S. maltophilia	S. maltophilia	98.6	S. maltophilia	99.4	Both Sequence Databases	Neither sequencing score is great. Should just report Genus.	All-Genus only - unable to speciate
39	Vitek	S. maltophilia	S. maltophilia	99.0	Stenotrophomonas sp. 45	99.4	Both Sequence Databases	Neither sequencing score is great. Should just report Genus.	All-Genus only - unable to speciate
40	Microscan	S. maltophilia	S. maltophilia	99.1	Pseudomonas sp. An18	99.4	MicroSeq Database	Neither sequencing score is great. Virodec also shows S. malto 99.0%, (published)	MicroSeq and Phenotype-Genus only
41	Vitek	S. maltophilia	S. maltophilia	99.7	S. maltophilia	99.6	Both Sequence Databases	Both sequence database with pretty good scores.	All
42	Microscan	S. maltophilia	S. maltophilia	99.9	S. maltophilia	99.6	Both Sequence Databases	Both sequence database with pretty good scores.	All
43	Microscan	S. maltophilia	S. maltophilia	98.6	S. maltophilia	99.8	Virodec Database	Virodec almost perfect score, MicroSeq lower.	Phenotype and Virodec
44	Microscan	S. maltophilia	S. maltophilia	98.2	S. maltophilia	99.8	Virodec Database	Virodec almost perfect score, MicroSeq lower.	Phenotype and Virodec
45	Microscan	S. maltophilia	S. maltophilia	98.6	S. maltophilia	99.8	Virodec Database	Virodec almost perfect score, MicroSeq lower.	Phenotype and Virodec
46	Microscan	S. maltophilia	S. maltophilia	98.6	S. maltophilia	99.8	Virodec Database	Virodec almost perfect score, MicroSeq lower.	Phenotype and Virodec
47	Vitek	S. maltophilia	S. maltophilia	99.7	S. maltophilia	99.8	Both Sequence Databases	Both sequence database with pretty good scores.	All
48	Microscan	S. maltophilia	S. maltophilia	99.7	S. maltophilia / Methylobacterium sp. 4BR9	99.8	Both Sequence Databases	Both sequence database with pretty good scores.	All
49	Microscan	S. maltophilia	S. maltophilia	99.6	S. maltophilia	99.9	Both Sequence Databases	Both sequence database with pretty good scores.	All
50	Microscan	S. maltophilia	S. maltophilia	99.7	S. maltophilia	99.9	Both Sequence Databases	Both sequence database with pretty good scores.	All
51	API 20 NE	S. paucimobilus	S. sanguinis	99.0	S. yabuuchiae / S. echinoides	98.5	Neither Sequence Database	Low sequence similarity scores; neither database includes this sequence.	All-Genus only - unable to speciate
52	Microscan	NF, unable to ID	B. intermedia / B. vesicularis	99.7	B. nasdae	99.6	Neither Sequence Database	Both MicroSeq and Virodec equal with pretty good scores, but do not agree with each other.	MicroSeq and Virodec-Genus only
53	Ox./ pigment/ morpho	P. aeruginosa	P. aeruginosa	98.3	P. aeruginosa	99.0	Neither Sequence Database	Low sequence similarity scores; neither database includes this sequence.	All-Genus only - unable to speciate
54	Ox./ pigment/ morpho	P. aeruginosa	P. aeruginosa	99.7	P. aeruginosa	99.8	Both Sequence Databases	Both sequence database with pretty good scores.	All
55	Vitek	P. aeruginosa	P. aeruginosa	99.9	P. aeruginosa	99.8	Both Sequence Databases	Both sequence database with pretty good scores.	All
56	Microscan	P. aeruginosa	P. aeruginosa	99.8	P. aeruginosa	100.0	Both Sequence Databases	Both sequence database with good scores, Virodec perfect.	All
57	Microscan	P. aeruginosa	P. aeruginosa	99.8	P. aeruginosa	100.0	Both Sequence Databases	Both sequence database with good scores, Virodec perfect.	All
58	Microscan	P. aeruginosa	P. aeruginosa	99.8	P. aeruginosa	100.0	Both Sequence Databases	Both sequence database with good scores, Virodec perfect.	All
59	Ox./ pigment/ morpho	P. aeruginosa	P. aeruginosa	99.8	P. aeruginosa	100.0	Both Sequence Databases	Both sequence database with good scores, Virodec perfect.	All
60	Ox./ pigment/ morpho	P. aeruginosa	P. aeruginosa	99.8	P. aeruginosa	100.0	Both Sequence Databases	Both sequence database with good scores, Virodec perfect.	All
61	Ox./ pigment/ morpho	P. aeruginosa	P. aeruginosa	99.8	P. aeruginosa	100.0	Both Sequence Databases	Both sequence database with good scores, Virodec perfect.	All
62	Ox./ pigment/ morpho	P. aeruginosa	P. aeruginosa	99.8	P. aeruginosa	100.0	Both Sequence Databases	Both sequence database with good scores, Virodec perfect.	All
63	API 20 NE	P. aeruginosa	P. aeruginosa	99.8	P. aeruginosa	100.0	Both Sequence Databases	Both sequence database with good scores, Virodec perfect.	All
64	Vitek	P. aeruginosa	P. aeruginosa	99.8	P. aeruginosa	100.0	Both Sequence Databases	Both sequence database with good scores, Virodec perfect.	All
65	Vitek	P. aeruginosa	P. aeruginosa	100.0	P. aeruginosa	100.0	Both Sequence Databases	Both sequence database with good scores, Virodec perfect.	All
66	Vitek	P. aeruginosa	P. aeruginosa	100.0	P. aeruginosa	100.0	Both Sequence Databases	Both sequence database with good scores, Virodec perfect.	All
67	Microscan	P. aeruginosa	P. aeruginosa	99.8	P. aeruginosa	100.0	Both Sequence Databases	Both sequence database with good scores, Virodec perfect.	All
68	routine biochemical	P. alcaligenes	A. piechaudii	99.3	Streptomyces MSU 2261 / Alcaligenes SGE1	99.6	Neither Sequence Database	Low sequence similarity scores; neither database includes this sequence.	All (none) - unable to identify
69	Vitek, API 20 NE	P. flourescens / P. putida grp	P. asplenii / P. putida	95.1	P. oryzihabitans / P. putida	98.8	Neither Sequence Database	Low sequence similarity scores; neither database includes this sequence.	All-Genus only - unable to speciate
70	routine biochemical	P. fluorescens	P. asplenii / P. putida	98.7	P. putida	99.6	Virodec Database	Virodec much higher score than MicroSeq, but with invalid strain.	*MicroSeq and Phenotype; Virodec not valid
71	routine biochemical	P. fluorescens	P. fulva	99.3	Pseudomonas sp. ACP14	99.6	Neither Sequence Database	Low sequence similarity scores; neither database includes this sequence.	All-Genus only - unable to speciate
72	routine biochemical	P. fluorescens	P. pseudoalcaligenes pseudoalcaligenes	98.2	P. putida / P. plecoglossicida	99.8	Virodec Database	Virodec almost perfect score; check validity of both species.	Virodec, P. plecoglossicida only
73	routine biochemical	P. fluorescens	P. pseudoalcaligenes pseudoalcaligenes	98.3	P. putida / P. plecoglossicida	99.8	Virodec Database	Virodec almost perfect score; check validity of both species.	Virodec, P. plecoglossicida only
74	Vitek, API 20 NE	P. fluorescens	P. synxantha	99.6	Pseudomonas sp. 7:3	99.8	Virodec Database	Virodec almost perfect score, MicroSeq lower but with different species.	All-Genus only - unable to speciate
75	routine biochemical	P. fluorescens	P. pseudoalcaligenes pseudoalcaligenes	98.1	P. putida / P. plecoglossicida	100.0	Virodec Database	Virodec almost perfect score; check validity of both species.	Virodec, P. plecoglossicida only
76	Vitek, API 20 NE	P. fluorescens	P. pseudoalcaligenes pseudoalcaligenes	98.3	P. putida / P. plecoglossicida	100.0	Virodec Database	Virodec almost perfect score; check validity of both species.	Virodec, P. plecoglossicida only
77	Vitek, API 20 NE	P. fluorescens	P. fluorescens A (bt)	99.8	Pseudomonas sp. G2	100.0	MicroSeq Database	Virodec has perfect score, but with un-named species.	All-Genus only - unable to speciate
78	Vitek, API 20 NE	P. fluorescens	P. synxantha / P. mucidolens	99.8	Pseudomonas sp. 7:3	100.0	Neither Sequence Database	Virodec has perfect score, but with un-named species.	All-Genus only - unable to speciate
79	Microscan	P. fluorescens/putida	P. pseudoalcaligenes pseudoalcaligenes	98.2	P. putida / P. plecoglossicida	99.8	Virodec Database	Virodec almost perfect score; check validity of both species.	Virodec, P. plecoglossicida only
80	Microscan	P. fluorescens/putida	P. pseudoalcaligenes pseudoalcaligenes	98.2	P. putida / P. plecoglossicida	99.8	Virodec Database	Virodec almost perfect score; check validity of both species.	Virodec, P. plecoglossicida only
81	Microscan	P. fluorescens/putida grp.	S. maltophilia	99.7	S. maltophilia	99.8	Neither Sequence Database	Both sequence database with good scores.	MicroSeq and Virodec
82	Vitek	P. multocida	P. multocida septica	99.6	P. multocida septica	99.6	Both Sequence Databases	Both sequence database with good scores.	All
83	Vitek	P. multocida	P. multocida septica	99.9	P. multocida septica	100.0	Both Sequence Databases	Both sequence database with good scores, Virodec perfect.	All
84	Vitek	P. putida	P. asplenii / P. putida	97.7	P. oryzihabitans / P. putida	98.4	Neither Sequence Database	Low sequence similarity scores; neither database includes this sequence.	All-Genus only - unable to speciate
85	Vitek, API 20 NE	P. putida	P. pseudoalcaligenes pseudoalcaligenes	95.0	P. putida / P. plecoglossicida	99.6	Virodec Database	Virodec almost perfect score; check validity of both species.	Virodec, P. plecoglossicida only
86	Vitek, API 20 NE	P. putida	P. pseudoalcaligenes pseudoalcaligenes	97.9	P. putida / P. plecoglossicida	99.8	Virodec Database	Virodec almost perfect score; check validity of both species.	Virodec, P. plecoglossicida only
87	routine biochemical	P. putida	P. pseudoalcaligenes pseudoalcaligenes	98.1	P. putida / P. plecoglossicida	100.0	Virodec Database	Virodec almost perfect score; check validity of both species.	Virodec, P. plecoglossicida only
88	Vitek, API 20 NE	P. putida	P. pseudoalcaligenes pseudoalcaligenes	98.1	P. putida / P. plecoglossicida	100.0	Virodec Database	Virodec almost perfect score; check validity of both species.	Virodec, P. plecoglossicida only
89	routine biochemical	P. putida	P. pseudoalcaligenes pseudoalcaligenes	98.3	P. putida / P. plecoglossicida	100.0	Virodec Database	Virodec almost perfect score; check validity of both species.	Virodec, P. plecoglossicida only
90	routine biochemical	P. putida	P. pseudoalcaligenes pseudoalcaligenes	98.3	P. putida / P. plecoglossicida	100.0	Virodec Database	Virodec almost perfect score; check validity of both species.	Virodec, P. plecoglossicida only
91	Vitek, API 20 NE	P. putida	P. citronellolis	99.4	Pseudomonas sp. AT2	100.0	Neither Sequence Database	Virodec has perfect score, but with un-named species.	All-Genus only - unable to speciate
92	Vitek, API 20 NE	P. putida	P. fulva	99.5	P. parafulva / P. fulva	100.0	Neither Sequence Database	Virodec has perfect score, but not specific; MicroSeq with good score.	All-Genus only - unable to speciate
93	API 20 NE	P. putida	P. pseudoalcaligenes pseudoalcaligenes	98.2	P. putida / P. plecoglossicida	99.8	Virodec Database	Virodec almost perfect score; check validity of both species.	Virodec, P. plecoglossicida only
94	API 20 NE	P. putida	P. pseudoalcaligenes pseudoalcaligenes	98.2	P. putida / P. plecoglossicida	99.8	Virodec Database	Virodec almost perfect score; check validity of both species.	Virodec, P. plecoglossicida only
95	Vitek, API 20 NE	P. stutzeri	P. stutzeri	99.4	P. stutzeri	99.0	Both Sequence Databases	Good, but lower sequence similarity scores; neither database includes this sequence perfectly.	All-Genus only - unable to speciate
96	Vitek	P. stutzeri	P. pseudoalcaligenes pseudoalcaligenes	99.6	P. pseudoalcaligenes pseudoalcaligenes	99.6	Neither Sequence Database	Both sequence database with good scores.	MicroSeq and Virodec
97	Vitek, API 20 NE	P. stutzeri	P. stutzeri	99.7	P. stutzeri	99.8	Both Sequence Databases	Both sequence database with good scores.	All
98	Vitek, API 20 NE	P. stutzeri	S. maltophilia	99.0	Stenotrophomonas sp. 45	99.8	Neither Sequence Database	Both sequence database with good scores.	MicroSeq and Virodec-Genus only
99	Vitek, API 20 NE	P. stutzeri	P. stutzeri	99.8	P. stutzeri	100.0	Both Sequence Databases	Both sequence database with good scores, Virodec perfect.	All
100	Vitek, API 20 NE	P. stutzeri	P. aeruginosa	99.8	P. aeruginosa	100.0	Neither Sequence Database	Both sequence database with good scores (Virodec perfect).	MicroSeq and Virodec

*** Without adequate checking for species validity (i.e. accepted by IJSEM), Virodec results would have been reported in error.**

IJSEM - International Journal of Systematic and Evolutionary Microbiology.

Abbreviations

Achromobacter piechaudii	A. piechaudii
Achromobacter xylosoxidans	A. xylosoxidans
Acinetobacter baumannii	A. baumannii
Acinetobacter calcoaceticus	A. calcoaceticus
Acinetobacter haemolyticus	A. haemolyticus
Acinetobacter junii	A. junii
Alcaligenes faecalis	A. faecalis
Bordetella parapertussis	B. parapertussis
Bordetella pertussis	B. pertussis
Bordetella petrii	B. petrii
Bordetella bronchiseptica	B. bronchiseptica
Brevundimonas diminuta	B. diminuta
Brevundimonas intermedia	B. intermedia
Brevundimonas nasdae	B. nasdae
Brevundimonas vesicularis	B. vesicularis
Burkholderia cepacia	B. cepacia
Chryseobacterium indologenes	C. indologenes
Chryseobacterium meningosepticum	C. meningosepticum
Flavimonas oryzihabitans	F. oryzihabitans
Pasteurella multocida	P. multocida
Pseudomonas asplenii	P. asplenii
Pseudomonas citronellolis	P. citronellolis
Pseudomonas flourescens	P. flourescens
Pseudomonas fulva	P. fulva
Pseudomonas oryzihabitans	P. oryzihabitans
Pseudomonas parafulva	P. parafulva
Pseudomonas plecoglossicida	P. plecoglossicida
Pseudomonas putida	P. putida
Pseudomonas pseudoalcaligenes	P. pseudoalcaligenes
Pseudomonas psychrotolerans	P. psychrotolerans
Pseudomonas stutzeri	P. stutzeri
Pseudomonas synxantha	P. synxantha
Ralstonia insidiosa	R. insidiosa
Ralstonia (Pseudo) pickettii	R. pickettii
Sphingomonas paucimobilus	S. paucimobilus
Sphingomonas sanguinis	S. sanguinis
Sphingomonas yabuuchiae	S. yabuuchiae
Sphingomonas echinoides	S. echinoides
Stenotrophomonas maltophilia	S. maltophilia

Table 2. An ambiguous base at position 12 (position 15 in the MicroSeq system) causes discrepancies, especially with the MicroSeq software.

Issues with Base 12 Ambiguity		
C at base 12 (default MicroSeq Primer sequence)	AGAGTTTGATCCTGGCTCAG	no effect on % similarity score
Seq--A for C at base 12 (within primer)	AGAGTTTGATCATGGCTCAG	0.2% less similarity
Seq--M for C at base 12 (within primer)	AGAGTTTGATCMTGGCTCAG	0.1% less similarity
Isolate numbers 65 and 66 (short sequenced, therefore no issue)	agagtttgatcCTGGCTCAG	no effect on % similarity score

The MicroSeq (MacIntosh-based) software system adds sequence of universal primers to the beginning and end of the sample sequence. Non-fermenters show a variable base at position 15 that is not always the same base in the universal primer. In this study, the forward primer begins 3 bases downstream making this position number 12.

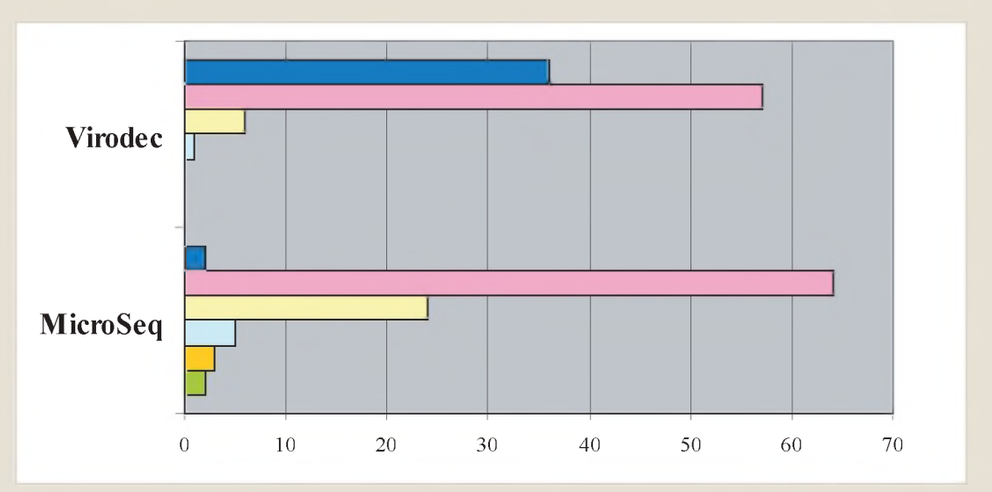
Table 3. Discrepancies between Virodec "perfect matches" and MicroSeq500 scores for the same isolates/sequences.

Isolate No.	Virodec	%	MicroSeq500	% without base 12 ambig.	Reason for discrepancy
6	Acinetobacter sp. RUH53T	100.0	A. calcoaceticus	98.7	Sequence un-named; sequence not in MicroSeq database
7	A. calcoaceticus subsp. anitratus	100.0	A. baumannii	100.0	A. calcoaceticus subsp. anitratus not a valid species (strain unpublished)
8, 11, 21	A. calcoaceticus	100.0	Acinetobacter genomosp. 3	100.0	A. calcoaceticus from Virodec is not a valid species (strain unpublished)
17, 19	A. xylosoxidans	100.0	A. xylosoxidans xylosoxidans	100.0	no discrepancy
25	A. faecalis	100.0	A. faecalis faecalis	99.4	Ambiguous bases in 4 positions prevent perfect matches with MicroSeq since there is only one type strain sequence.
26	B. diminuta	100.0	B. diminuta	99.8	Virodec has additional invalid sequence with base 12 ambiguity to make perfect match. Ambiguities allowed for multiple gene copies.
29	D. acidovorans	100.0	D. acidovorans	100.0	no discrepancy
32	R. insidiosa	100.0	R. pickettii	97.5	R. insidiosa is a valid species, but is not in the MicroSeq database
56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 100	P. aeruginosa	100.0	P. aeruginosa	100.0	no discrepancy
75, 76, 87, 88, 89, 90	P. putida / P. plecoglossicida	100.0	P. pseudoalcaligenes pseudoalcaligenes	98.5	P. putida not valid in Virodec; P. plecoglossicida valid species, but not in MicroSeq database
77	Pseudomonas sp. G2	100.0	P. fluorescens A (bt)	100.0	species non-specific
78	Pseudomonas sp. 7:3	100.0	P. synxantha / P. mucidolens	100.0	species non-specific
83	P. multocida septica	100.0	P. multocida septica	100.0	no discrepancy
91	Pseudomonas sp. AT2	100.0	P. citronellolis	99.6	Sequence un-named; sequence not in MicroSeq database
92	P. parafulva / P. fulva	100.0	P. fulva	99.7	P. parafulva not in MicroSeq database; strain of P. fulva not valid in Virodec
99	P. stutzeri	100.0	P. stutzeri	100.0	no discrepancy

Virodec uses an online system and is continually updated as new GenBank submissions occur. GenBank submissions are not quality-controlled, so are not necessarily the gold standard. However, more recent taxonomical changes and validated new species are peer-reviewed and accepted by IJSEM. GenBank results (and Virodec) have links to publications for a quality-control step to decide whether the Virodec answer is acceptable.

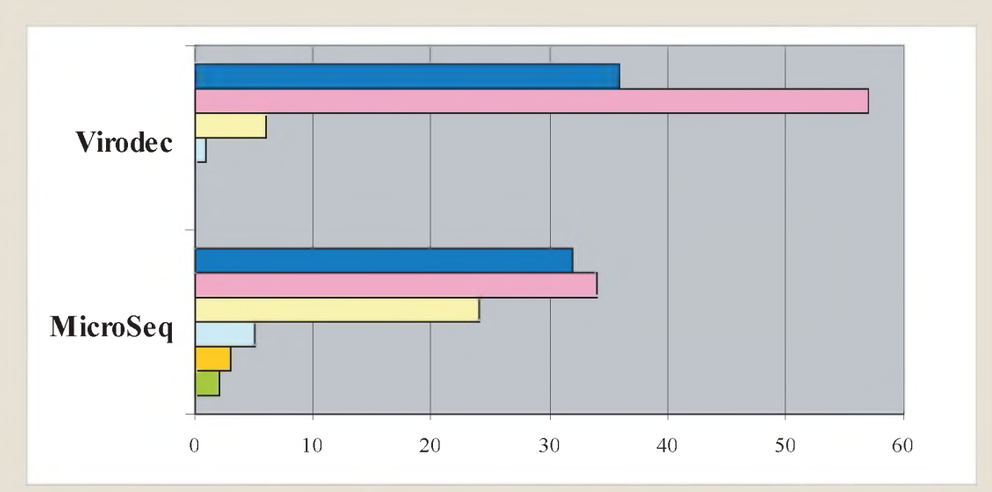
Figure 1. Comparison of Sequence Databases (MicroSeq vs. Virodec)

With base 12 ambiguity



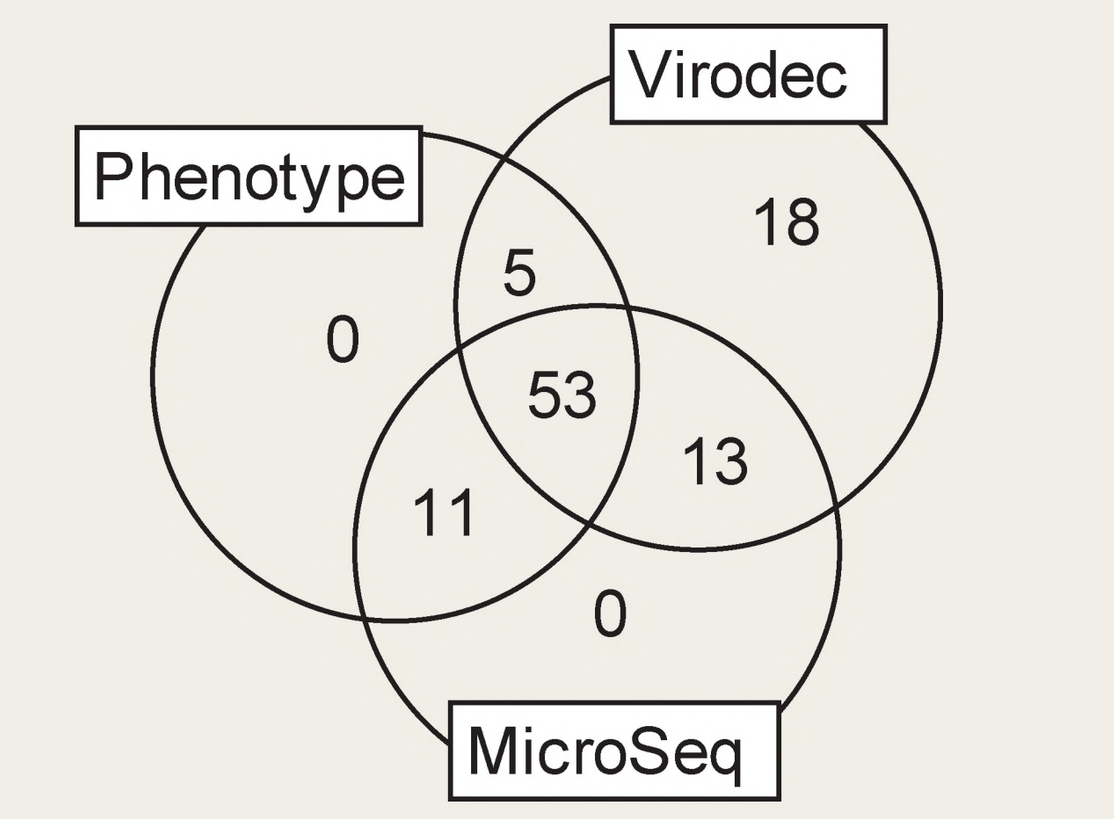
	MicroSeq	Virodec
100%-perfect match	2	36
99 to 99.9%	64	57
98 to 98.9%	24	6
97 to 97.9%	5	1
96 to 96.9%	3	0
95 to 95.9%	2	0

Without base 12 ambiguity



	MicroSeq	Virodec
100%-perfect match	32	36
99 to 99.9%	34	57
98 to 98.9%	24	6
97 to 97.9%	5	1
96 to 96.9%	3	0
95 to 95.9%	2	0

Figure 2. Summary of Results for Identification: Phenotype vs. Virodec vs. MicroSeq500.



Conclusions

Sequence-based identifications, as well as phenotypic identifications, require regular database updates to keep abreast of the newest taxonomy.

Phenotypic methods often have outdated databases and could assign species that are less accurate than sequence-based identifications.

The MicroSeq500 database is quality-controlled, but consists mainly of type strains and lacks recently described species.

The MicroSeq BLAST (FULL) analysis results in more matches for non-fermenters if the forward primer region (base 15 variability) is eliminated.
- **However, if primer region is eliminated then**
  - **(a) uniformity in sequence size will be lacking, making it difficult to compare scores between isolates to derive at a cutoff value**
  - **(b) clinical laboratories will need to develop a QC program mandating a certain size sequence**

The Virodec database does not allow for uniformity in sequence size (see above comments).

There is a GenBank number and often a published reference associated with Virodec matches to help verify species validity.

The Virodec database is continually updated as new GenBank submissions occur.

Virodec has more sequences in the database that are not quality-controlled, including un-named species.
- **However,**
  - **(a) users may be able to develop a customized database within the Virodec system**
  - **(b) clinical laboratories can develop a QC program that will filter out the invalid sequences**