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Recognition of Potentially Novel Human Disease-Associated Pathogens by Implementation of Systematic 16S rRNA Gene Sequencing in the Diagnostic Laboratory▽†

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Clinical isolates that are difficult to identify by conventional means form a valuable source of novel human pathogens. We report on a 5-year study based on systematic 16S rRNA gene sequence analysis. We found 60 previously unknown 16S rRNA sequences corresponding to potentially novel bacterial taxa. For 30 of 60 isolates, clinical relevance was evaluated; 18 of the 30 isolates analyzed were considered to be associated with human disease.

16S rRNA gene sequence analysis is broadly considered the "gold standard" in bacterial identification (6, 29). In daily clinical diagnostics, accurate bacterial identification is essential in judging whether a bacterial isolate is to be considered the causative agent of an infectious disease or merely a colonizer. In our study, we aimed to characterize the bacterial diversity encountered in a diagnostic laboratory by revealing potentially novel, clinically relevant species, according to the current species definition by the Clinical and Laboratory Standards Institute (22).

Routine 16S rRNA gene sequencing is implemented in our laboratory and is a fixed part of our diagnostic algorithms for identification of bacterial isolates (1, 2, 32). We retrospectively reanalyzed 16S rRNA gene sequences collected during 2004 to 2008 to identify potentially novel bacterial taxa of clinical relevance. The Institute of Medical Microbiology (IMM) serves the 850-bed University Hospital of Zurich and surrounding smaller hospitals. Bacterial isolates from blood, cerebrospinal fluid, wounds, joint aspirates, respiratory samples, genitourinary swabs, feces, and urine were recovered by culture on appropriate media according to standard procedures (19). Isolates that could not be identified by phenotypic methods underwent sequencing. 16S rRNA gene analysis was performed as previously described (1). Homology analyses were performed using the SmartGene Integrated Database Network System (IDNS) (24) and NCBI GenBank databases. For the first screening of our large data collection, we selected isolates with sequence homology of <99.0% to members of described taxa, regarding these as potentially novel species; isolates with sequence homology of <95% were regarded as representatives of a novel genus (2). The boundary for novel families was <87.5% homology and, for novel orders, <78.4% 16S rRNA sequence homology (30). After the first screening, we used more stringent cutoff values (<97.5% for species) for taxa with significant interspecies 16S rRNA divergence; i.e., members of the *Paenibacillaceae* family and the *Clostridiales* order (6, 25).

During the 5-year study period, 1,663 cultured isolates were subjected to 16S rRNA gene sequence analysis (Table 1). Of those, 60 isolates (0.4‰; see Table S1 in the supplemental material) had a 16S rRNA gene homology of <99% to members of accepted taxa on the date of the first interpretation. A total of 11 of the 60 sequences with a 16S rRNA homology of <99% in the first-time analysis could be allocated to a species established during the study term as a novel species by others: *Acinetobacter septicus* (16, 20), *Brevibacterium ravenspurgense* (17), *Corynebacterium freiburgense* (12), *Corynebacterium massiliense* ($n = 2$) (18), *C. mastitidis* (10, 18), *C. pyruviciproducens* (26), *C. ureicelerivorans* (11, 31), *Neisseria zoodegmatis* (28), *Paenibacillus barengoltzii* (21), and the reclassified *Campy-*

TABLE 1. Clinical bacterial isolates with 16S rRNA gene homology < 99% ($n = 60$)

Taxonomic group	No. of isolates with indicated 16S rRNA homology	
	<99% to >95%	<95%
Enteric Gram-negative rods	0	0
Fastidious Gram-negative rods	1	4
Gram-negative cocci	1	0
Gram-negative nonfermenters	7	1
Gram-positive cocci	12	2
Gram-positive rods	26	6
Total	47	13

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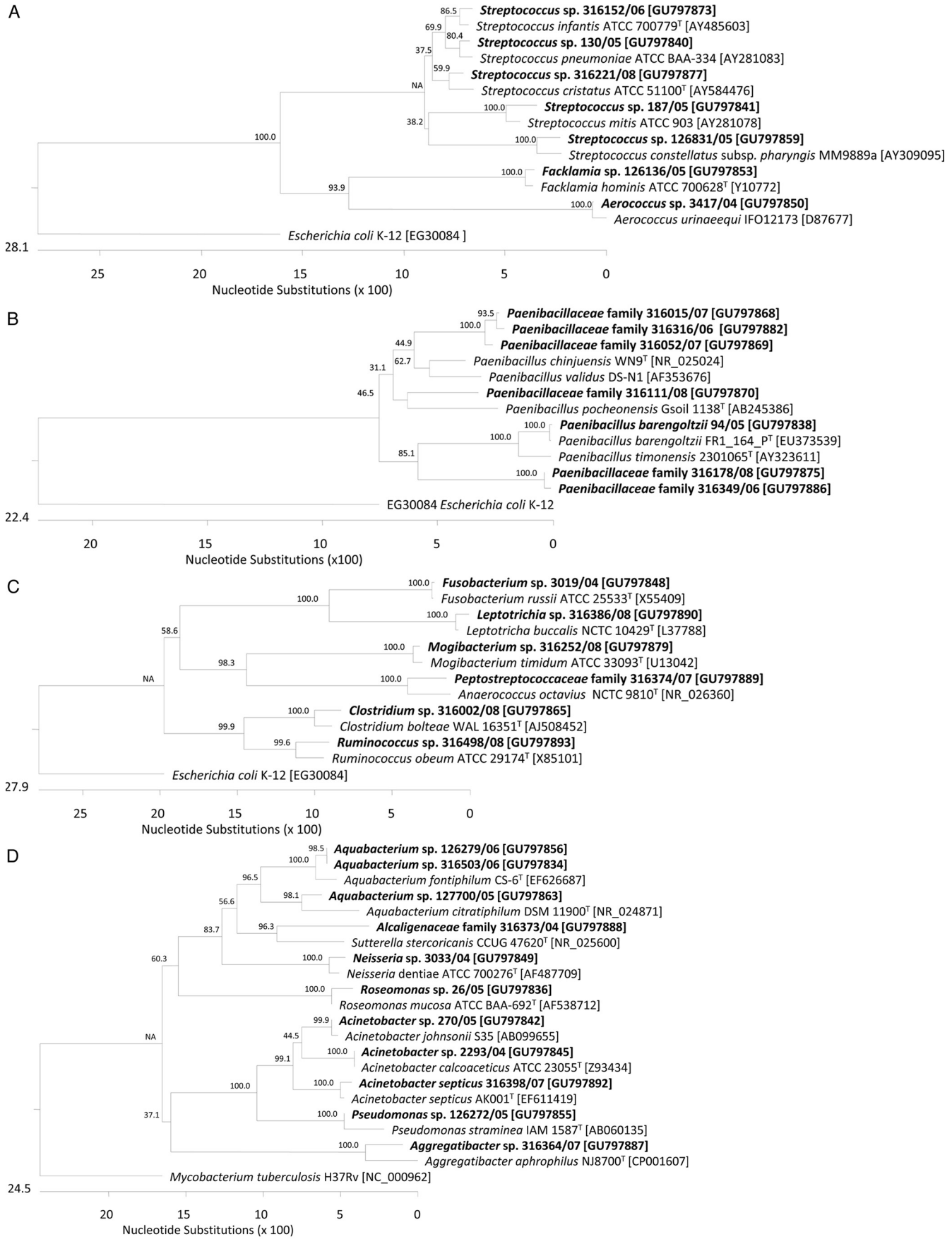
A
86.5
Streptococcus sp. 316152/06 [GU797873]
69.9
Streptococcus infantis ATCC 700779T [AY485603]
80.4
Streptococcus sp. 130/05 [GU797840]
37.5
Streptococcus pneumoniae ATCC BAA-334 [AY281083]
59.9
Streptococcus sp. 316221/08 [GU797877]
NA
Streptococcus cristatus ATCC 51100T [AY584476]
100.0
Streptococcus sp. 187/05 [GU797841]
38.2
Streptococcus mitis ATCC 903 [AY281078]
100.0
100.0
Streptococcus sp. 126831/05 [GU797859]
Streptococcus constellatus subsp. pharyngis MM9889a [AY309095]
100.0
Facklamia sp. 126136/05 [GU797853]
93.9
Facklamia hominis ATCC 700628T [Y10772]
100.0
Aerococcus sp. 3417/04 [GU797850]
Aerococcus urinaeequi IFO12173 [D87677]
Escherichia coli K-12 [EG30084]
28.1
25 20 15 10 5 0
Nucleotide Substitutions (x 100)
B
93.5
Paenibacillaceae family 316015/07 [GU797868]
100.0
Paenibacillaceae family 316316/06 [GU797882]
44.9
Paenibacillaceae family 316052/07 [GU797869]
62.7
Paenibacillus chinjuensis WN9T [NR_025024]
31.1
Paenibacillus validus DS-N1 [AF353676]
46.5
Paenibacillaceae family 316111/08 [GU797870]
Paenibacillus pocheonensis Gsoil 1138T [AB245386]
100.0
Paenibacillus barengoltzii 94/05 [GU797838]
100.0
Paenibacillus barengoltzii FR1_164_PT [EU373539]
85.1
Paenibacillus timonensis 2301065T [AY323611]
100.0
Paenibacillaceae family 316178/08 [GU797875]
Paenibacillaceae family 316349/06 [GU797886]
EG30084 Escherichia coli K-12
22.4
20 15 10 5 0
Nucleotide Substitutions (x100)
C
100.0
Fusobacterium sp. 3019/04 [GU797848]
100.0
Fusobacterium russii ATCC 25533T [X55409]
100.0
Leptotrichia sp. 316386/08 [GU797890]
58.6
Leptotricha buccalis NCTC 10429T [L37788]
100.0
Mogibacterium sp. 316252/08 [GU797879]
98.3
Mogibacterium timidum ATCC 33093T [U13042]
NA
100.0
Peptostreptococcaceae family 316374/07 [GU797889]
Anaerococcus octavius NCTC 9810T [NR_026360]
100.0
Clostridium sp. 316002/08 [GU797865]
99.9
Clostridium bolteae WAL 16351T [AJ508452]
99.6
Ruminococcus sp. 316498/08 [GU797893]
Ruminococcus obeum ATCC 29174T [X85101]
Escherichia coli K-12 [EG30084]
27.9
25 20 15 10 5 0
Nucleotide Substitutions (x 100)
D
98.5
Aquabacterium sp. 126279/06 [GU797856]
100.0
Aquabacterium sp. 316503/06 [GU797834]
96.5
Aquabacterium fontiphilum CS-6T [EF626687]
98.1
Aquabacterium sp. 127700/05 [GU797863]
56.6
Aquabacterium citratiphilum DSM 11900T [NR_024871]
83.7
96.3
Alcaligenaceae family 316373/04 [GU797888]
Sutterella stercoricanis CCUG 47620T [NR_025600]
60.3
100.0
Neisseria sp. 3033/04 [GU797849]
Neisseria dentiae ATCC 700276T [AF487709]
100.0
Roseomonas sp. 26/05 [GU797836]
Roseomonas mucosa ATCC BAA-692T [AF538712]
99.9
Acinetobacter sp. 270/05 [GU797842]
NA
44.5
Acinetobacter johnsonii S35 [AB099655]
100.0
Acinetobacter sp. 2293/04 [GU797845]
99.1
Acinetobacter calcoaceticus ATCC 23055T [Z93434]
100.0
100.0
Acinetobacter septicus 316398/07 [GU797892]
Acinetobacter septicus AK001T [EF611419]
37.1
100.0
Pseudomonas sp. 126272/05 [GU797855]
Pseudomonas straminea IAM 1587T [AB060135]
100.0
Aggregatibacter sp. 316364/07 [GU797887]
Aggregatibacter aphrophilus NJ8700T [CP001607]
Mycobacterium tuberculosis H37Rv [NC_000962]
24.5
20 15 10 5 0
Nucleotide Substitutions (x 100)

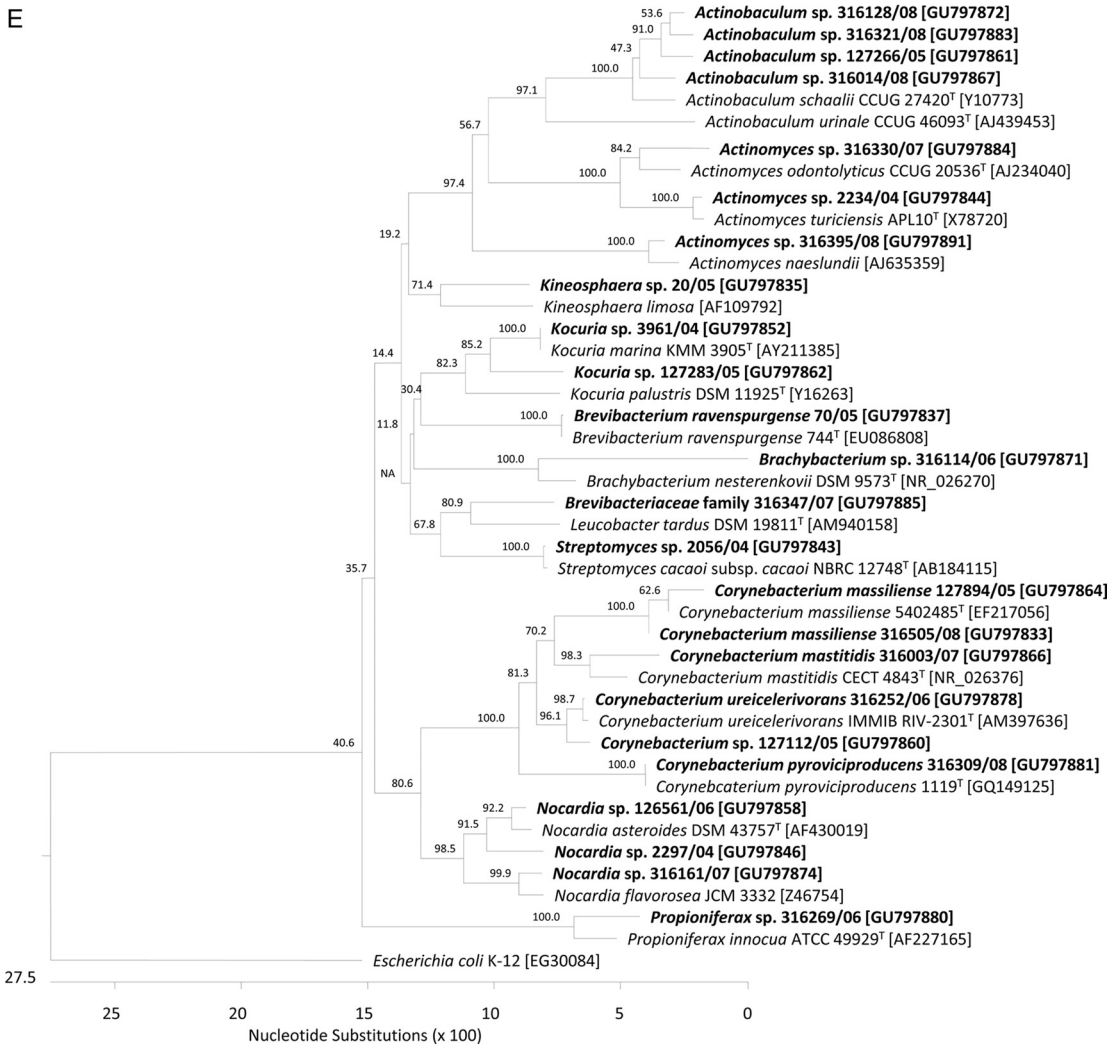

FIG. 1. Phylogeny of 55 of 60 cultured isolates recovered from clinical specimens with homology of <99% to 16S rRNA gene sequences of members of published taxa. The dendrograms were calculated using CLUSTAL V alignment and a matrix of Jukes-Cantor distances determined by the neighbor-joining method using DNASTAR Lasergene MegAlign 7.0 software. Taxonomic order adherence of 55 taxons identified in this study: (A) *Lactobacillales*; (B) *Bacillales*; (C) *Fusobacteriales* and *Clostridiales*; (D) *Pseudomonadales*; (E) *Actinomycetales*. Study isolates are shown in bold. Species in regular type were selected as (published) type strains of the different taxa. We used *Escherichia coli* K-12 *rrnA* [NCBI GenBank accession no. EG30084] and *Mycobacterium tuberculosis* H37Rv *rrs* [NC_000962] as outgroups.

lobacter ureolyticus (previously known as *Bacteroides ureolyticus*) (27).

We calculated dendrograms to assess phylogenetic relationships (Fig. 1). Potentially novel streptococcal species clustered with known pathogens. For example, within the streptococci, one isolate (GenBank accession no. GU797873) shared 98.8% sequence homology with *S. infantis*; another isolate (GU797840) shared 97.8% homology with *S. pneumoniae*. Within the *Bacillales* order (Fig. 1B), eight novel *Paenibacillus* sequence types were recovered: three of them (GU797882, GU797868, and GU797869) were distantly related (<95% sequence homology) to *Paenibacillus chinjuensis-P. validus*, and two isolates (GU797838 and GU797854) were related to *P. timonensis* (97.3% and 94.5% sequence homology). Several bacterial families are represented in the *Clostridiales* order. The novel sequences recovered in the *Clostridiales* order all belonged to different families (Fig. 1C). We found two representatives of the *Fusobacteriales* order (Fig. 1C): one isolate (GU797848) was related to *Fusobacterium russii* (sequence homology 98.8%), and one isolate (GU797890) was most closely related to *Leptotrichia buccalis* (98.9% sequence homology). Eleven novel sequences belonged to the *Pseudomonadales* order, and 3 of those (GU797845, GU797842, and GU797892) represented potential novel *Acinetobacter* spp. (Fig. 1D). The *Actinomycetales* order (Fig. 1E) comprises 25 potentially novel taxa (data for 24 taxa are given in the figure). Six corynebacterial isolates, i.e., *Corynebacterium freiburgense* (GU797839), *Corynebacterium massiliense* (GU797864 and GU797833), *C. mastitidis* (GU797866), *C. ureicelerivorans* (GU797878), and *C. pyruviciproducens* (GU797881), clustered with type strain sequences that were established as novel species during the study period. We recovered three *Nocardia* spp. (GU797846, GU797858,

and GU797874); two of them (GU797846 and GU797858) belonged to the *Nocardia asteroides* complex and one (GU797874) was related to *Nocardia flavorosea*. Four potentially novel *Actinobaculum* spp. (GU797861, GU797867, GU797872, and GU797883/GU797308) were attributed to the same taxonomic clade according to 16S rRNA sequence phylogeny results, with *Actinobaculum schaalii* as the nearest neighboring species (95.7 to 98.5% sequence homology).

To assess the clinical relevance of the microbiological findings, we selected 30 isolates for which sufficient clinical data were available and performed reviews of patient charts (Table 2). We reviewed the patient charts for clinical signs and symptoms of infection, inflammation parameters such as fever, leukocyte count, C-reactive protein (CRP) and procalcitonin levels, radiological and laboratory findings (serology and bacterial culture results), previous infections or bacterial isolates, antibiotic treatment, and clinical diagnosis (see Table S2 in the supplemental material). We established a clinical score incorporating the different parameters mentioned above to determine the likelihood (codified as "yes," "likely," "unlikely," "no") of an infectious disease in each case and to assess the association of the bacterial isolate found with disease.

Clinical relevance was attributed to 18 isolates. In 10 cases, patient history, laboratory findings, and clinical course following antibiotic therapy guided by the isolate's drug susceptibility testing results were compatible with a pathogenic role for the isolated microorganism. In eight cases, we concluded that the bacterial isolate was likely to have been the cause of an infection. The putative novel disease-associated species mostly belonged to the *Actinomycetales* order of Gram-positive rods. The recently described *Acinetobacter septicus* (GU797892) (16), two *Actinobaculum* spp. (GU797883 and GU797872), and a *Gardnerella* sp. (GU797857) were each identified as present in samples from patients with urinary tract infections. In one case, we recovered a potentially novel *Actinobaculum* sequence type (GU797872) from several blood cultures of a patient suffering from urosepsis, underlining the pathogenic potential of species of the *Actinobaculum* genus in ascending urinary tract infections. A potentially novel *Acinetobacter* sp. (GU797845), most closely related to *Acinetobacter calcoaceticus*, was identified in the peritoneal dialysate of a 22-year-old patient with kidney failure. He exhibited infective monobacterial peritonitis as a complication of continuous ambulatory peritoneal dialysis (CAPD). A previously unknown *Actinomyces* sp. (GU797891) was isolated from a 49-year old female patient suffering from severe, acute, suppurative parotitis. *Neisseria zoodegmatis* (previously *Neisseria* CDC EF-4 group [28]) was found in an isolate from a patient with a wound and a history of a cat bite (GU797849). A novel sequence type of *Paenibacillus barengoltzii* (21) was cultured from a central venous catheter in the jugular vein of a patient with a burn injury (GU797838). A potentially novel *Streptococcus* sp. (GU797859), belonging to the *Streptococcus anginosus* group, was found in an isolate from a septic patient suffering from cholangitis. A potentially novel *Streptococcus* sp. related to *Streptococcus oralis* (GU797840) was cultured from a hip joint aspirate of a patient with hip prosthesis infection. Bacterial isolates that were found to be irrelevant to a patient's clinical disease entity (12/30) were considered to represent skin flora (e.g., *Paenibacillus* spp. or *Ruminococcus* spp.) or to belong to environmental bacteria (e.g., *Aquabacterium* spp. or *Kineosphaera* spp.).

Broad-range 16S rRNA gene amplification readily allows the detection of members of as-yet-unknown bacterial taxa (4, 5, 9). Major hypervariable regions are present in the first 500 bp of the roughly 1,600 bp comprising the 16S rRNA gene downstream of the conserved primer target sites (5, 9, 29). Thus, analysis of this part of the gene sequence allows the recognition of potentially novel taxa based on previously established cutoff values of <99% homology for new species and <95% homology for new genera (1–3, 7). While such a general cutoff is appropriate for overall first analysis of large data sets, we note that the boundaries for species definition by 16S rRNA sequence homology may be different for different phyla (13, 25). A less stringent cutoff value (i.e., <99.6% homology) could have been used to delimit different species in bacterial groups such as the *Streptococcus mitis* group or nonfermenters (13). Conversely, for species belonging to the *Paenibacillaceae* family and the *Clostridiales* order, a more stringent cutoff value (i.e., 97.5% homology) is more appropriate (6) and was therefore applied after the first selection performed with the 99% cutoff value.

In 2008, the fraction of bacterial isolates submitted for molecular identification was 0.8%. Previous investigations reported rates of 0.5% to 1% for a similar study setup (7) and a rate of 14% for isolates restricted to aerobic Gram-positive rods (2). Gram-positive rods and Gram-negative cocci are overrepresented in the group of sequenced isolates in our comparative analysis of phenotypic and 16S rRNA-based identification methods. Of the 1,663 (3.7%) sequences determined during the study period, 60 were judged to be representatives of potentially novel species or novel genera. A recent review (29), summarizing 16S rRNA gene-based studies published from 2001 to 2007, calculated that 215 unique sequences recovered during this period from human specimens represented potentially novel species. Of the 215, 29 belonged to novel genera. During our study, the number of 16S rRNA sequences deposited in the NCBI nucleotide database increased by a factor of 15. The SmartGene IDNS 16S rRNA database, which is a curated database derived from the NCBI repository, increased in size by a factor of 4. Despite this increase in the number of sequences deposited, the recovery of sequences with <99% homology to members of established taxa in our data set during 2004 to 2008 remained relatively constant at between 2.4% and 5.1%. This finding may reflect the fact that many of the sequences deposited in public databases were the outcome of large-scale ecological or environmental (metagenomic) sequencing projects and did not include sequences of clinical laboratory isolates.

Bacterial taxonomic classification has advanced differently in various taxonomic groups (25): Phenotypic methods readily allow species determination below the resolution of 16S rRNA-based sequence analysis in studies of enteric Gram-negative bacteria (14, 15). In contrast, the *Actinomycetales* order is a rich but poorly investigated group (2). For example, within the *Corynebacterium* genus, 18 novel species were validly described from 2004 to 2009. A total of 5 of these, namely, *Corynebacterium freiburgense* (12), *Corynebacterium pyruviciproducens* (26), *C. mastitidis* (10, 18), *C. massiliense* (18), and *C. ureicelerivorans* (11, 31), were also identified in our study.

TABLE 2. Subset analysis of microbial and clinical data of 30 patients[a]

Source	16S rRNA-based identification	NCBI GenBank accession no.	Match with highest % homology	Homology of 1st match			Polymicrobial etiology	Clinical diagnosis	Clinical relevance
				No. of mismatches	%	Match length (bp)			
Urine	*Acinetobacter septicus*	GU797892	*Acinetobacter septicus*	0	100.0	531	No	Urinary tract infection (urothelial carcinoma)	Yes
Peritoneal dialysate	*Acinetobacter* sp.	GU797845	*Acinetobacter calcoaceticus*	10	98.3	574	No	Peritonitis (CAPD)	Yes
Urine	*Actinobaculum* sp.	GU797883, GU797308	*Actinobaculum schaalii*	26	96.6	768	No	Ascending urinary tract infection (pigtail catheter)	Yes
Blood culture	*Actinobaculum* sp.	GU797872	*Actinobaculum schaalii*	10	98.5	641	No	Urosepsis, urothelial carcinoma	Yes
Parotid gland aspirate	*Actinomyces* sp.	GU797891	*Actinomyces naeslundii*	15	97.4	573	Yes	Parotid inflammation	Yes
Urine	*Gardnerella* sp.	GU797857	*Gardnerella vaginalis*	6	98.9	527	No	Urinary tract infection	Yes
Tissue (hand)	*Neisseria zoodegmatis*	GU797849	*Neisseria zoodegmatis*	3	99.4	525	Yes	Cat bite	Yes
Central venous catheter	*Paenibacillus barengoltzii*	GU797838	*Paenibacillus barengoltzii*	3	99.4	505	No	Intravascular catheter-associated infection	Yes
Blood culture	*Streptococcus* sp.	GU797859	*Streptococcus constellatus*	8	98.6	571	No	Cholangitis, sepsis	Yes
Femur bone	*Streptococcus* sp.	GU797840	*Streptococcus oralis*	9	97.8	403	No	Hip prosthesis infection with soft tissue abscess	Yes
Pleural aspirate	*Actinomyces* sp.	GU797884	*Actinomyces odontolyticus*	17	96.6	496	Yes	Anastomosis insufficiency (pneumonectomy)	Likely
Blood culture	*Burkholderiales* order	GU797888	*Sutterella stercoricanis*	54	89.8	530	No	Small intestine ischemia	Likely
Corneal tissue	*Corynebacterium mastitidis*	GU797866	*Corynebacterium mastitidis*	0	100.0	463	Yes	Chronic blepharitis	Likely
Intravenous catheter	*Corynebacterium massiliense*	GU797833	*Corynebacterium massiliense*	1	99.8	556	No	Sepsis (unclear focus of infection)	Likely
Spongiosa tissue	*Mogibacterium* sp.	GU797879	*Mogibacterium timidum*	7	98.7	538	Yes	Maxillary bone necrosis	Likely
Sputum	*Nocardia* sp.	GU797874	*Nocardia flavorosea*	8	98.4	494	Yes	Upper lobe pneumonia (COPD)[b]	Likely
Deep wound swab	*Peptostreptococcaceae* family	GU797889	*Anaerococcus octavius*	28	94.7	530	Yes	Axillary abscess	Likely
Superficial wound	*Pseudomonas* sp.	GU797855	*Pseudomonas fulva*	10	98.1	526	Yes	Ulceration, digit II of right foot (diabetes mellitus)	Likely
Contact lens	*Kocuria* sp.	GU797852	*Kocuria marina*	26	96.1	668		Contact lens-associated ceratitis	Unlikely
Hip joint aspirate	*Paenibacillaceae* family	GU797869	*Paenibacillus chinjuensis*	34	92.9	480		Rheumatoid arthritis	Unlikely
Knee joint aspirate	*Paenibacillaceae* family	GU797870	*Paenibacillus pocheonensis*	35	93.8	563		Intravenous drug abuse, hepatitis C	Unlikely
Blood culture	*Propioniferax* sp.	GU797880	*Propioniferax innocua*	27	96.4	720		Aplastic anemia	Unlikely
Blood culture	*Aquabacterium* sp.	GU797863	*Aquabacterium citratiphilum*	22	95.8	520		Fever, AML[c]	No
Blood culture	*Campylobacter ureolyticus*	GU797876	*Campylobacter ureolyticus*	1	99.8	519		Fever, neutropenia	No
Bone biopsy	*Corynebacterium pyruviciproducens*	GU797881	*Corynebacterium pyruviciproducens*	0	100.0	745		Open bone fracture	No
Sputum	*Kineosphaera* sp.	GU797835	*Kineosphaera limosa*	24	95.6	549		Chronic bronchitis	No
urine	*Brevibacteriaceae* family	GU797885	*Leucobacter tardus*	57	92.2	734		Urinary tract infection	No
Bursa aspirate	*Paenibacillaceae* family	GU797882	*Paenibacillus validus*	32	93.5	493		Trochanteric bursitis	No
Blood culture	*Paenibacillaceae* family	GU797875	*Paenibacillus contaminans*	51	90.7	551		HIV infection, *Pneumocystis jirovecii* pneumonia	No
Blood culture	*Ruminococcus* sp.	GU797893	*Ruminococcus obeum*	22	95.9	539		HIV infection	No

[a] The likelihood of a relevant infectious disease associated with the microbiological findings was estimated after retrospective patient chart analysis.
[b] COPD, chronic obstructive pulmonary disease.
[c] AML, acute myelogenous leukemia.

When we calculated phylogenetic trees based on partial 16S rRNA sequences (Fig. 1), we found that differentiation was numerically strong (as measured by nucleotide substitutions of base pairs) in the *Actinomycetales*, *Clostridiales*, *Fusobacteriales*, and *Pseudomonales* orders whereas it was less profound in the *Lactobacillales* order and, more specifically, in the *Streptococcaceae* family. Regarding potentially novel *Streptococcus* spp., further molecular analysis of additional loci (by, e.g., *sodA*, *rpoB*, and *recA* sequence homology) would be required to determine exact phylogenetic relationships (8, 23).

In summary, out of 1,663 bacterial isolates subjected to 16S rRNA sequencing during a 5-year period, we recovered 60 clinical bacterial isolates that were indicative of the presence of putative novel bacterial species. Of these 60 isolates, 9 were established as novel pathogens in the literature during the period of the study. A total of 18 (60%) isolates showed clinical relevance in a subset analysis of 30 of the 60 isolates. Isolates with clinical implications are mostly representatives of genera that comprise known pathogens (i.e., *Streptococcus* spp., *Actinobaculum* spp., *Actinomyces* spp., and *Neisseria* spp.). Our findings underline the importance of 16S rRNA gene sequencing in routine identification algorithms designed to recognize novel pathogens in the diagnostic laboratory.

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