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Fusobacterium canifelinum sp. nov., from the Oral Cavity of Cats and Dogs

Georg Conrads[1,2], Diane M. Citron[1], Reinier Mutters[3], Spencer Jang[4], and Ellie J.C. Goldstein[1]

[1] R.M. Alden Research Laboratory, Santa Monica, California
[2] Lehr- und Forschungsgebiet Orale Mikrobiologie und Immunologie der Klinik für Zahnerhaltung, Parodontologie und Präventive Zahnerhaltung und Institut für Medizinische Mikrobiologie, Universitätsklinikum (RWTH), Aachen, Germany
[3] Institute for Medical Mikrobiology and Hygiene, Philipps University Marburg, Marburg, Germany
[4] Veterinary Medical Teaching Hospital, University of California at Davis, Davis, California

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Summary

Fourteen strains of Gram-negative, anaerobic, fluoroquinolone-resistant, non-sporulating rods were isolated from various infections in cats and dogs, as well as from wounds in humans after cat- or dog–bites. These strains were characterized by sequencing of the 16S–23S rDNA internal transcribed spacer (ITS) regions, 16S rDNA, DNA-DNA hybridization, phylogenetic analysis, and phenotypic tests. The results indicate that the novel strains belong to a distinct species, closely related to *Fusobacterium nucleatum*. The species *Fusobacterium canifelinum* sp. nov. is proposed, with strain ATCC BAA 689^T as the type strain.

Key words: *Fusobacterium* – cats – dogs – 16S–23S rDNA ITS – fluoroquinolone-resistance

Introduction

Fusobacteria are obligately anaerobic non-spore-forming, nonmotile, pleomorphic rod-shaped bacilli. Although they stain Gram-negative, they are allied with the Gram-positive phylum. The DNA base composition within this genus is heterogeneous with the majority of strains having a restricted range of 26 to 34 mol% G+C. *Fusobacterium prausnitzii* belongs to a highly distantly related taxonomical group [3, 4, 5, 20], that has now been reclassified as *Faecalibacterium prausnitzii* [6]. Another new species, *Fusobacterium equinum* from the oral cavity of horses, was recently proposed [5].

During studies of the microflora of infected cat and dog bite wounds in humans, 25 isolates were preliminarily characterized as *Fusobacterium nucleatum*. Nine of 16 isolates that were tested for fluoroquinolone susceptibility were found to be resistant (levofloxacin MIC >4 µg/ml), unlike human strains which are typically susceptible. In addition, 10 strains of *F. nucleatum* like-organisms from cat- and dog-infections were tested for fluoroquinolone susceptibility and four were found to be resistant. The quinolone resistant strains were further characterized.

We sequenced the 16S–23S rDNA internal transcribed spacer (ITS) of all 13 isolates mentioned above plus the only other fluoroquinolone resistant *Fusobacterium* in our collection (RMA 11693), isolated from an upper left leg soft tissue infection of a diabetic patient. Upon further investigation, we discovered that this patient had a pet dog that had frequently licked his legs, thus establishing an animal source for this strain as well.

We chose ITS as the taxonomical marker in addition to DNA-DNA hybridization, as we recently showed that this sequence is highly informative and suitable for discrimination between fusobacterial species and sub-species [4].

GenBank Accession Nos: ITS: AF342829-342861, AY125473-125486; 16S: AY162219-162222

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Materials and Methods

Bacterial strains and culture media

All strains described in this study, including ITS sequence similarities to *F. nucleatum* subsp. *nucleatum* ATCC 25586^T are shown in Table 1, and the phenotypic characteristics of all fusobacterial species and subspecies [7, 9, 10, 12, 13, 18] are given in Table 2.

The bacterial strains were either directly received from a type culture collection or phenotypically characterized at the R.M. Alden Research Laboratory according to the original Bergey's Manual description. Fusobacterial strains and *Leptotrichia buccalis* were cultivated at 37 °C on supplemented Brucella agar (Anaerobe Systems) in an anaerobic chamber.

Cellular fatty acid analysis

Cellular fatty acids were extracted from *F. nucleatum* subsp. *nucleatum* ATCC 25586^T, *F. nucleatum* subsp. *vincentii* ATCC 49256^T, *F. nucleatum* subsp. *animalis* ATCC 51191^T and *F. nucleatum* subsp. *polymorphum* ATCC 10953^T and all 14 strains of the new species and determined with an automated GC following the methods outlined by the manufacturer (Microbial Identification System, MIDI Inc).

Antimicrobial susceptibility testing

Susceptibility tests were performed by the agar dilution method as described in the NCCLS M11-A5 document [15]. Briefly, the test medium was Brucella agar supplemented with vitamin K_1, hemin, and laked blood. The inoculum was prepared directly from 48 h plates and applied to plates containing serial two-fold dilutions of the drugs, at a final concentration of 10^5 cfu/spot. After 48 h incubation at 37 °C under anaerobic conditions, the plates were examined for growth. The MIC was defined as the lowest concentration of drug that significantly reduced growth as compared to the drug-free growth control plate.

Table 1. Distinctive genotypic characteristics based on 16S–23S Internal Transcribed Spacer analysis of type and reference strains, and 14 isolates of *Fusobacterium canifelinum* sp. nov. in the genus *Fusobacterium*.

Fusobacterium canifelinum			No. of bands	Short version [bp]	Long version [bp]	16S part [bp]	Spacer [bp]	23S part [bp]
1. RMA	1036^T	H, from dog-bite	3	760	1050	112	154	494
2. RMA	1072	H, from dog-bite	3	760	1050	112	154	494
3. RMA	1079	H, from dog-bite	3	760	1050	112	154	494
4. RMA	7631	H, from dog-bite	3	760	1050	112	154	495
5. RMA	7654	H, from cat-bite	3	760	1050	112	154	495
6. RMA	7723	H, from cat-bite	3	760	1050	112	154	495
7. RMA	7782	H, from dog-bite	3	760	1050	112	154	495
8. RMA	7897	H, from cat-bite	3	760	1050	112	154	495
9. RMA	7903	H, from cat-bite	3	760	1050	112	154	495
10. RMA	11693	H, diabetic wound	3	760	1050	112	154	494
11. RMA	12701	F, from bronchus	3	760	1050	112	154	494
12. RMA	12702	F, from ear	3	760	1050	112	154	494
13. RMA	12703	F, tracheal wash	3	760	1050	112	154	494
14. RMA	12708	C, from abscess	3	760	1050	112	154	494
Reference strains								
ATCC	23726	*F. nucleatum* subsp. *nucleatum*	3–4	800	1050	112	124	496
ATCC	25586^T	*F. nucleatum* subsp. *nucleatum*	3–4	810	1050	112	135	498
ATCC	51190^T	*F. nucleatum* subsp. *fusiforme*	3	800	1050	112	123	496
RMA	7159	*F. nucleatum* subsp. *polymorphum*	1	850–920	–	112	151	479
ATCC	10953^T	*F. nucleatum* subsp. *polymorphum*	1	850–920	–	112	151	479
ATCC	49256^T	*F. nucleatum* subsp. *vincenti*	3	800	1050	112	121	496
RMA	6840	*F. nucleatum* subsp. *animalis*	3	810	1050	112	132	497
RMA	6681	*F. nucleatum* subsp. *animalis*	3	810	1050	112	132	498
ATCC	51191^T	*F. nucleatum subsp. animalis*	3	810	1050	112	132	497
ATCC	25557^T	*F. mortiferum*	3–4	830	1050	112	168	501
NCTC	12111^T	*F. ulcerans*	3–4	830	1050	112	171	496
ATCC	8501^T	*F. varium*	3–4	830	1050	112	168	498
ATCC	25556^T	*F. necrogenes*	3–4	880	1080	112	208	490
ATCC	33568^T	*F. simiae*	3	810	1050	112	132	497
ATCC	33693^T	*F. periodonticum*	1	900	–	112	186	498
ATCC	25832^T	*F. naviforme*	1	800	–	112	123	498
ATCC	25533^T	*F. russii*	1–2	800	–	112	115	497
ATCC	51357^T	*F. necrophorum* subsp. *funduliforme*	1	830	–	112	139	495
ATCC	25286^T	*F. necrophorum* subsp. *necrophorum*	1	830	–	112	139	495
ATCC	25563^T	*F. gonidiaformans*	3–4	800	1000	112	168	462
ATCC	29250^T	*F. perfoetens*	1	830	–	112	115	518
ATCC	14201	*L. buccalis*	3	810	1100	112	108	496

Data for *Leptotrichia buccalis* ATCC 1420 are used as an outgroup; H = Human; F = Feline, C = Canine.

Table 2. Characteristics of *Fusobacterium* species and subspecies including *Fusobacterium canifelinum* sp. nov.

Test	*F. equinum*	*F. gonidiaformans*	*F. mortiferum*	*F. naviforme*	*F. necrogenes*	*F. necrophorum* ssp. *fundiliforme*	*F. necrophorum* ssp. *necrophorum*	*F. nucleatum* ssp. *nucleatum*	*F. nucleatum* ssp.*fusiforme*	*F. nucleatum* ssp. *polymorphum*	*F. nucleatum* ssp. *vincentii*	*F. nucleatum* ssp. *animalis*	***F. canifelinum***	*F. perfoetans*	*F. periodonticum*	*F. russii*	*F. simae*	*F. ulcerans*	*F. varium*
Cellobiose	–	–	w⁻	–	w⁻	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Esculin hyd.	–	–	+⁻	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Fructose	–	–	Wᵃ	–	Wᵃ	–ʷ	–ʷ	w⁻	w⁻	w⁻	w⁻	w⁻	w⁻	w	w⁻	–	w	–	Wᵃ
Glucose	–	w⁻	Aʷ	–ʷ	Wᵃ	–ʷ	–ʷ	–ʷ	–ʷ	–ʷ	–ʷ	–ʷ	–ʷ	w	w⁻	–	w	Aʷ	Wᵃ
Lactose	–	–	Wᵃ	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Maltose	–	–	w⁻	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Mannose	–	–	Wᵃ	–	Wᵃ	–	–	–	–	–	–	–	–	–	–	–	–	Aʷ	w
Raffinose	–	–	v	–	–ʷ	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Sucrose	–	–	v	–	–ʷ	–	–	–	–	–	–	–	–	w	–	–	–	–	–
Gelatin	–ʷ	–	–	–	–	v	v	–ʷ	–ʷ	–ʷ	–ʷ	–ʷ	–ʷ	–	–	–	–	–	–
Indole	+	+	–	+	–	+	+	+	+	+	+	+	+	–	+	–	+	–	+⁻
Nitrate	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
Bile growth	+	–	+	–	+	v	v	–	–	–	–	+	–	–	–	–	+	+	+
Lipase	+	–	–	–	–		+⁻	–	–	–	–	–	–	–	–	–	w	–	–ʷ
Beta-hemolysis	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
Gas produced in agar deep	nt	+	+	–	+	+	+	–	–	–	–	–	–	+	–	–	–	+	+
Lactate → propionate	+	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
Threonine → propionate	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+
Alpha-glucosidase	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Beta-glucosidase	–	–	+⁻	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Alpha-galactosidase	–	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Beta-galactosidase	–	–	+	–	+	–	–	–	–	–	–	–	–	+	–	–	–	–	–
Beta-glucoronidase	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Alkaline-phosphatase	–	–	+	–	+	–	+	–	–	–	–	–	–	–	–	w	–	–	w
Acid-phosphatase	+	w	+	–	+	+	+	–	–	–	–	–	–	w	–	+	–	+	w
N-acetyl-glucosaminidase	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
Arginine aryl amidase	nt	nt	+	nt	nt	–	–	–	–	–	–	–	–	nt	–	nt	nt	+	+
Serine aryl amidase	nt	nt	+	nt	nt	–	–	–	–	–	–	–	–	nt	–	nt	nt	+	+
Esterase (C4)	+	–	–	–	–	w	w	–	–	–	–	–	–	–	–	–	w	–	–
Esterase (C8)	+	–	–	–	–	w	w	–	–	–	–	–	–	–	–	–	–	–	–
Fluoroquinolone-resistance	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–
Original source	E	HNO	HNO	HO,S	HNO	HO	HO,C	HO	HO	HO	HO	M	C,D	H,P	HO	HNO	M	HNO	HNO
ITS % relative similarity to *F. nucleatum* ATCC 25586	nt	75	78	93	72	77	77	100	94	90	92	95	92	77	83	84	92	79	79

W: weakly positive; W⁻: most strains weakly positive, some negative; –ʷ: most strains negative, some weakly positive; Aʷ: strongly acid with some weakly acid reactions; Wᵃ: usually weakly acid with occasional strong acid reactions; E: horse; HO: human, oral; HNO: human, non oral; S: sheep; C: cat; D: dog; M: monkey; P: pigs, nt: not tested. Methods for testing are according to the Wadsworth-KTL Anaerobic Manual, 6th edition, 2002.

ITS and 16S rDNA directed PCR amplification and sequence analysis

DNA was extracted using the DNeasy Tissue Kit (QIAGEN Inc.). For ITS sequencing a 16S primer (3′ end of the gene) and a 23S primer (5′ end of the gene) were used. The 16S primer SPF (5′ GTA CAC ACC GCC CGT CAC ACC 3′ *E.coli* position 1391–1411) was designed on the basis of universal 16S rRNA/DNA sequence information (RDP, Ribosomal Database Project II, Il, USA). The target sequence of the degenerated 23S primer SPR [5′ GGT (TG)CT TTT C(GA)C CTT TCC 3′, *E. coli* position 468–485] was highly conserved among bacterial large subunit sequences (RDP). PCR and sequencing of 16S–23S rDNA internal transcribed spacer were carried out as described previously [4].

Sequences were assembled using the program Vector NTI Suite (InforMax) and aligned using the program GeneDoc [16]. A phylogenetic tree was constructed by the neighbour-joining method and the program Clustal W [11] using *Leptotrichia buccalis* as an outgroup.

Approximations of ITS lengths were obtained from agarose gels. Table 1 gives the number and lengths of different amplicons found in each species and strain tested.

In addition to ITS-amplification and sequencing, around 100 ng of purified DNA from strains RMA 1036 (ATCC BAA 689^T, DSM 15542^T, dog isolate), RMA 11693 (human with dog contact, from diabetic wound), RMA 12701 (ATCC BAA 690, DSM 15543, cat isolate), and RMA 12708 (dog isolate, from abscess) were used in a PCR reaction to amplify the 16S rDNA. Amplification primers (taken from *E. coli* sequence [8]) were 5′- AGA GTT TGA TCC TGG CTC AG- 3′ (forward, positions 8–27) and 5′- AAG GAG GTG ATC CAG CCG CA- 3′ (reverse, positions 1542–1522). All 16S rDNA-amplicons were directly sequenced without cloning procedures by the dye terminator cycle-sequencing method (Applied Biosystems) and an automatic capillary DNA sequencer (API PRISM 310, Applied Biosystems). The 16S directed PCR primers plus additional internal sequences [8, 21] served as sequencing primers.

DNA-DNA hybridization

DNA-DNA hybridizations were performed by the spectrophotometric and micro-well methods with renaturation under optimal conditions [1, 14], and using the representatives RMA 1036^T, 7654, 11693, 12701, 12708 as well as *F. nucleatum* subsp. *nucleatum* ATCC 25586^T, a closely related type strain.

Results and Discussion

The biochemical and other phenotypic characteristics of all 13 cat- , dog-, and bite- isolates, plus the human diabetic leg wound, principally matched *Fusobacterium nucleatum*. However, the main differences with *F. nucleatum* species were colony morphology and the resistance to fluoroquinolones (minimum inhibitory levofloxacin concentration of >4 µg/ml), which is intrinsic and stable, as isolates RMA 1036^T, 1072 and 1079 were isolated between 1977 and 1984, before fluoroquinolones were introduced into the market. The quinolone resistance found in *F. canifelinum* is determined by multiple point mutations in the *gyr* genes, which serve as reliable phylogenetic marker [2, 17]. Although *Fusobacterium* spp. is a frequent isolate in clinical specimens and testing for fluoroquinolone susceptibility is performed in many laboratories, no other fluoroquinolone-resistant fusobacterial strain has been described so far. However, if quinolone resistance is observed in other fusobacterial species in the future, DNA probes and primers, deduced from our 16S rDNA or ITS data, can be used for species identification.

Fatty acid compositions from the 14 strains used in this study were compared to those of closely related *F. nucleatum* subspecies. With the exception of minor differences in the mean percentages of the fatty acids, the profiles were similar. The characteristics are in close agreement with those previously reported [19]. The minor differences we found were: *F. nucleatum*: 12:0 FAME: 1.30 [standard deviation: 0.25], 16:0 3-OH FAME: 4.10 [0.56] versus *F. canifelinum*: 12:0 FAME: 2.38 [0.66], 16:0 3-OH FAME: 3.48 [0.30].

Sequencing of the purified ITS amplicons using SPF and SPR as primers was performed in duplicate and led to ambiguity-free sequence determination by comparing both runs and directions. A similarity matrix table and an alignment file of DNA-DNA hybridization data for all 32 taxa sequenced are available from the corresponding author.

The phylogram deduced from the ITS sequences is shown in Fig. 1. The different strains of the new, quinolone-resistant species matched at a 99–100% level. In contrast, ITS-sequences of the thirteen quinolone-susceptible *F. nucleatum*-like strains from cats and dogs, and bites in humans were identical to *F. nucleatum* subspec. *nucleatum*, subspec. *animalis*, or subspec. *polymorphum*.

The five subspecies of *Fusobacterium nucleatum* along with *F. naviforme*, *F. simiae* and *F. periodonticum* were found to be closely related (85–93%) to *F. canifelinum*. A similar relationship, but with reduced phylogenetic resolution (96–98%), was found by analyzing 16S rRNA gene sequence information. Thus, ITS information makes differences between species and especially subspecies more obvious.

To validate *F. canifelinum* as a new species, and not just as a subspecies of *F. nucleatum*, DNA-DNA hybridization was performed and showed 75 to 92% identity among all *F. canifelinum* strains. In contrast, *F. canifelinum* RMA 1036^T shared only 44% with the genome of *F. nucleatum* subspec. *nucleatum* ATCC 25586^T (Table 3).

As discussed previously [4], ITS data have the potential to support the validity of species or subspecies within the *F. nucleatum*-branch. However, the ITS sequences in this cluster, which includes *F. simiae* and *F. naviforme*, show a “fan-like” branching. This fusobacterial branch seems to have an increased potential for genetic diversity. Further diversification will likely be evident after additional strains from other animals are sequenced.

Obviously, the ancestor in the evolution of *Fusobacterium nucleatum* was very successful in colonizing and infecting a variety of animals and, finally man. With exposure to new hosts and new ecological niches, additional new species and subspecies could evolve. However, as we found for *F. canifelinum*, these species and subspecies may possess the potential to cause infections in other hosts, e.g. after being transferred during a bite.

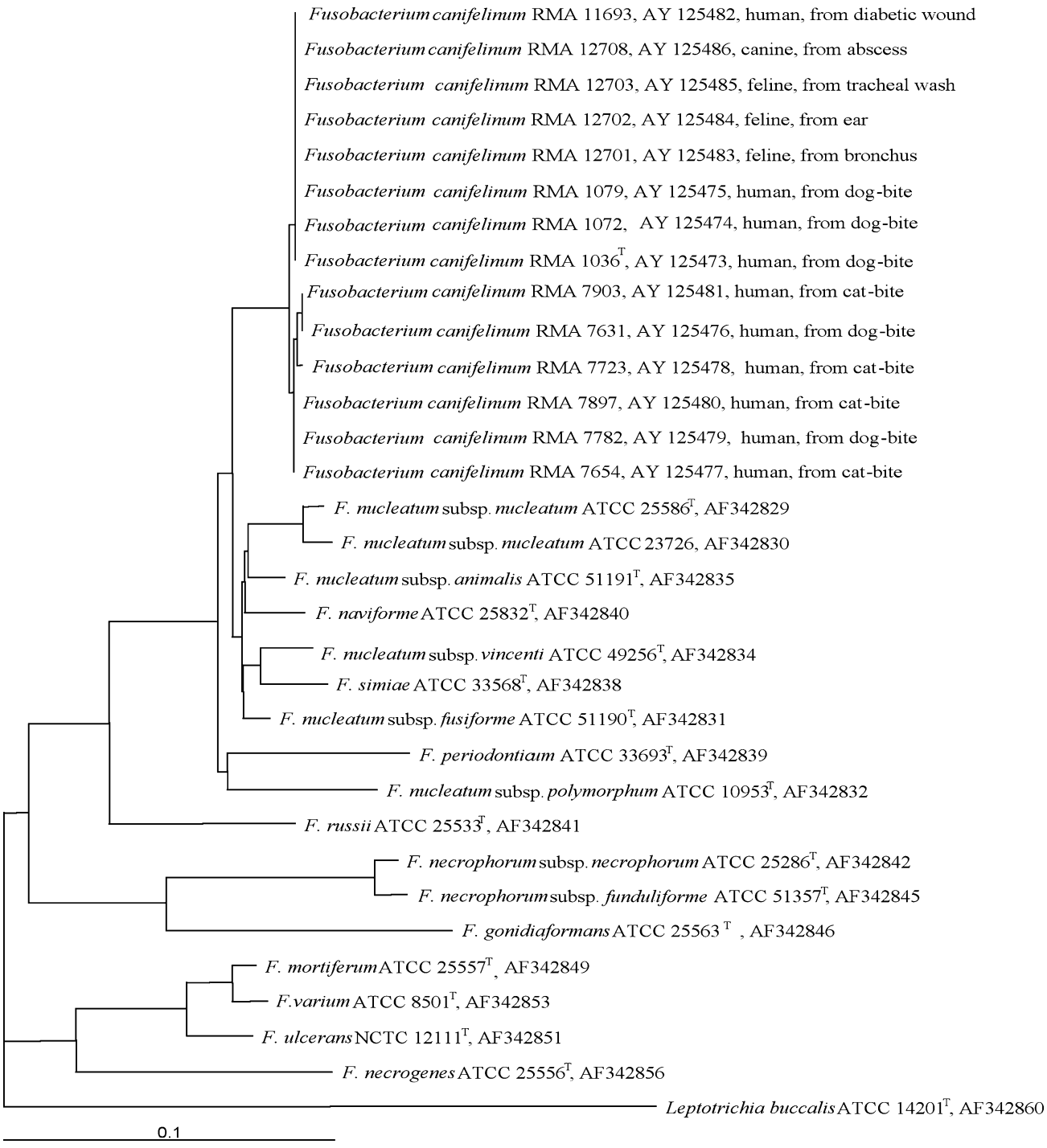

Fig. 1. Phylogram (neighbour-joining method) showing the genetic relationships among fusobacterial species and *Fusobacterium canifelinum* sp. nov. based on the DNA sequences of their short 16S–23S rDNA spacer regions (GenBank accession numbers included). The scale-bar represents 10% differences in nucleotide sequences.

Table 3. Percentages of DNA-DNA similarity of representative *Fusobacterium canifelinum* strains and the type strain *F. nucleatum* subspec. *nucleatum*. Standard deviations are given in parenthesis.

Source	*F. canifelinum*				*F. nucleatum*
	RMA 7654	RMA 11693	RMA 12701	RMA 12708	**ATCC 25586^T**
RMA 1036^T	92 (5.8)	83 (1.0)	99 (4.9)	75 (5.0)	44 (3.9)
RMA 7654		76 (3.3)	90 (12.4)	80 (3.1)	
RMA 11693			85 (3.3)	81 (10.2)	
RMA 12701				78 (3.8)	

In conclusion, the results show that all 14 strains of *F. canifelinum* are identical at the 99 to 100% level by ITS- and 16S rDNA-data, and at the 75 to 92% level by DNA-DNA-hybridization to each other, but distinct from all other currently known fusobacterial species. Because of the clinical relevance and the various sequence data we propose a new species, *Fusobacterium canifelinum* sp. nov., for which the following description is given.

Description of *Fusobacterium canifelinum*

Fusobacterium canifelinum sp. nov. (ca. ni. feli´num., L. gen. pl. n. canium, of dogs; L. gen. pl. n. felium, of cats; N.L. nom. sg. n. canifelinum, of dogs and cats).

After incubation on supplemented Brucella blood-agar for 2d, colonies are convex, 1–2 mm in diameter with a slightly lobate margin, white, opaque with a granular internal appearance. Gram stains of single colonies on Brucella agar plates or from growth in chopped meat or thioglycolate broths reveal slender Gram-negative rods with pointed ends. Glucose and fructose are fermented weakly with a terminal pH 5.7 to 6.0. End-product analysis of PY-glucose by gas-liquid chromatography reveals acetic and butyric acids. Threonine but not lactate is converted to propionate. The isolates produce indole, fail to grow in bile, do not hydrolyse esculin, and do not produce acid from lactose, maltose, mannose, raffinose and sucrose. All strains were weakly positive in the phosphoamidase test in the API ZYM system (bioMerieux) but all other reactions were negative. On agar dilution sensitivity tests, all isolates were susceptible to penicillin G, and metronidazole. All strains were resistant (MIC >4 µg/ml) to levofloxacin, moxifloxacin, gatifloxacin, gemifloxacin, and other fluoroquinolones. The G + C content of DNA is 26–28 (thermal denaturation method). The type strain is ATCC BAA 689^T (DSM 15542^T, RMA 1036^T) , which was isolated in 1977 from a purulent dog-bite wound in a human patient.

Fusobacterium canifelinum can be distinguished phenotypically from other fusobacterial species by the resistance to fluoroquinolones and by genetic characteristics listed in Table 1.

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Corresponding author:
Georg Conrads, Division of Oral Microbiology and Immunology, University Hospital (RWTH), Pauwelsstrasse 30, 52057 Aachen, Germany
Tel.: ++49-241-8088448; Fax ++49-241-8082483;
e-mail: gconrads@ukaachen.de