Ureaplasma canigenitalium sp. nov., Isolated from Dogs

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Ureaplasma strains isolated from dogs (*Canis familiaris*) were characterized and compared with the type strains of five previously described species of the genus *Ureaplasma*, *Ureaplasma urealyticum* (isolated from humans), *Ureaplasma diversum* (isolated from cattle), *Ureaplasma gallorale* (isolated from chickens), *Ureaplasma cati* (isolated from cats), and *Ureaplasma felinum* (isolated from cats). The canine strains hydrolyzed urea but not arginine or glucose, were membrane bound, lacked a cell wall, passed through 450-nm-pore-size membrane filters, required cholesterol for growth, and formed minute colonies (diameter, 20 to 140 µm) on agar medium. These canine ureaplasma strains have been reported to be members of four serovars. The four serovars of canine strains fell into a single group on the basis of their genomic properties, as determined by DNA-DNA hybridization. On the basis of these findings, we propose that ureaplasmas with these characteristics belong to a new species, *Ureaplasma canigenitalium*, with strain D6P-C (= ATCC 51252) as the type strain.

The genus *Ureaplasma* comprises those members of the family *Mycoplasmataceae* that hydrolyze urea. Ureaplasmas have been isolated from a variety of animal species, including humans (23), cattle (28), nonhuman primates (29), goats (7), sheep (19), dogs (29), cats (27), mink (6), raccoon dogs (16), pigs (26), and fowl (25). Previously the following five species of the genus *Ureaplasma* have been described: *Ureaplasma urealyticum* (type strain, T960 [= ATCC 27618]), isolated from a patient with nongonococcal urethritis (24); *Ureaplasma diversum* (type strain, A417 [= ATCC 43321]), isolated from cattle with pneumonia (10); *Ureaplasma gallorale* (type strain, D6-1 [= ATCC 43346 = NCTC 11707]), isolated from the oropharynx of a chicken (17); and *Ureaplasma felinum* (type strain, FT-2B [= ATCC 49229 = NCTC 11709]) and *Ureaplasma cati* (type strain, F2 [= ATCC 49228 = NCTC 11710]), both of which were isolated from the oral cavities of cats (8). There are at least 14 serovars of *U. urealyticum* and three serovars of *U. diversum*. We isolated ureaplasma strains from the prepuces, oral cavities, and nasal cavities of dogs (*Canis familiaris*); these strains were serologically distinct from the previously described *Ureaplasma* species (13), and we classified them into four serovars (18). A considerable amount of data (see below) has been accumulated to support the proposal that these canine ureaplasmas should be given taxonomic status as a new species in the genus *Ureaplasma*.

MATERIALS AND METHODS

Ureaplasma strains. *U. urealyticum* 7, 23, 27, 58, 354, Pi, Co, and T960^T (T = type strain) (serovars I to VIII) were obtained from D. K. Ford, University of British Columbia, Vancouver, Canada, and *U. urealyticum* Vancouver, Western, K2, U24, U38, and U26 (serovars IX to XIV) were obtained from J. A. Robertson, University of Alberta, Edmonton, Canada (22). *U. diversum* A417^T, T315, and T44 were obtained from R. N. Gourlay and C. J. Howard,

Institute for Research on Animal Diseases, Compton, England (11). The sources of representative canine ureaplasma strains DIM-C (serovar A), D29M (serovar B), D11N-A (serovar C), and D6P-C^T (serovar D) have been described previously (18) and are shown in Table 1, together with the sources of human and bovine strains and other animal ureaplasmas used for comparative studies. All four canine ureaplasma strains were cloned three times on agar plates before characterization.

Culture media. The media used for cultivation of ureaplasmas have been described previously (8). The liquid medium used (T-broth) was composed of 70 ml of PPLO broth (Difco Laboratories, Detroit, Mich.), 20 ml of horse serum, 10 ml of 25% (wt/vol) yeast extract prepared from bakery yeast (Nitten Co., Tokyo, Japan), 1 ml of a solution containing 100,000 U of penicillin G per ml, 1 ml of 10% urea, and 0.004% phenol red; the pH was adjusted to 6.0 ± 0.2 by using 1 N hydrochloric acid. The solid medium used (T-agar) consisted of the same ingredients except that 2.5 ml of phosphate buffer (1.5 M KH₂PO₄-1.5 M Na₂HPO₄, 7:3) and 0.8% purified agar (Oxoid) were added and the pH was adjusted to 6.5. Broth cultures were incubated aerobically in tightly capped flasks at 37°C, whereas inoculated solid media were incubated at 37°C in a 10% CO₂ incubator.

Morphology and ultrastructure. Mid-log-phase cells collected by centrifugation at 10,000 × g for 30 min with a Hitachi model 20PR centrifuge from broth cultures were stained with Gram stain and examined by light microscopy. For electron microscopy of ultrathin sections with a Hitachi model HU-12 electron microscope, pelleted cells obtained by centrifugation at 10,000 × g for 30 min were fixed with uranyl acetate and lead citrate.

Reversion studies. Each canine ureaplasma strain was serially subcultured three times in broth medium without antimicrobial agents, inoculated onto antimicrobial agent-free solid medium, and observed for the appearance of colonies.

Filtration studies. Overnight broth cultures of canine strains DIM-C, D29M, D11N-A, and D6P-C^T were passed in

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TABLE 1. Ureaplasma strains used in this study

Species	Strain(s)	Origin	Source(s)	Reference(s)
<i>U. urealyticum</i>	7, 23, 27, 58, 354, Pi, Co, T960 ^T	Human	Urogenital tract	24
	Vancouver, Western, K2, U24, U38, U26	Human	Urogenital tract	22
<i>U. diversum</i>	A417 ^T , T315, T44	Cattle	Lung, urogenital tract	10, 11
<i>U. gallorale</i>	D6-1 ^T	Chicken	Oropharynx	17
<i>U. felinum</i>	FT2-B ^T	Cat	Oral cavity	8
<i>U. cati</i>	F2 ^T	Cat	Oral cavity	8
<i>Ureaplasma</i> sp.	D6P-C ^T	Dog	Prepuce	18
	D1M-C, D29M	Dog	Oral cavity	18
	D11N-A	Dog	Nasal cavity	18

sequence through sterile membrane filters with average pore diameters of 650, 450, and 200 nm (Millipore Corp., Bedford, Mass.). The titers of the ureaplasmas in the resulting filtrates were determined by inoculating the filtrates into broth medium and preparing 10-fold dilutions and were expressed as numbers of color-changing units per milliliter.

Sterol requirement. The growth response of ureaplasma strains DIM-C, D29M, D11N-A, and D6P-C^T to cholesterol was examined by using a technique that was modified slightly from the recommended direct broth culture method (21). Twelve flasks containing base medium (T-broth lacking horse serum) were enriched with various supplements (bovine albumin fraction V, palmitic acid, cholesterol, pleuropneumonia-like organism [PPLO] serum fraction, horse serum) to give the final concentrations shown in Table 2. The inocula were prepared from cultures of test strains grown in 50-ml portions of basal medium containing 1% PPLO serum fraction. Each medium preparation was then distributed into a series of eight test tubes (1.8 ml per tube). The first tube was inoculated with 0.2 ml of test inoculum, and serial 10-fold dilutions were prepared up to a dilution of 10⁻⁸. The tubes were incubated aerobically at 37°C for several days. End points of growth were determined by examining the tubes for a color change in the broth. The last tube in which such a change was observed was recorded as the number of color-changing units of organisms per milliliter.

The four canine ureaplasma strains were also examined to determine whether growth on agar medium was inhibited by disks containing an ethanolic solution of digitonin (1.5%, wt/vol) (5).

Biochemical tests. The ability to catabolize urea and the ability to catabolize arginine were determined in broth

cultures by inoculating strains into broth medium containing either 0.05% urea or 0.5% arginine and into broth medium lacking urea and arginine. The inoculated broth media and uninoculated controls were incubated at 37°C and observed for 2 weeks for alkaline color change, which indicated catabolism of either compound. The ability to ferment glucose was examined in broth medium (pH 7.7) containing 0.5% glucose.

Serological tests. The methods used to prepare antigens for immunizing rabbits and antisera for serological tests have been described previously (18). The antigenic properties of the canine strains were compared with those of the previously described species by using the indirect immunoperoxidase test (14).

High-performance liquid chromatography. The base compositions of the canine ureaplasma strains were estimated by high-performance liquid chromatography. About 10 µl of a 1-mg/ml DNA solution prepared by the hydroxyapatite batch elution method (4) was suspended in 150 µl of 88% formic acid, and the preparation was sealed in vials and hydrolyzed at 150°C for 1 h. The hydrolysate was evaporated to dryness under a stream of nitrogen gas and then resuspended in 20 µl of 0.1 N HCl, as described previously (9). About 20 µl of the hydrolyzed sample was applied to a Shodex HC125 column (Showa-Denko Co., Tokyo, Japan) and eluted in 50 mM ammonium formate buffer (pH 2.5). The column was run at ambient temperature (22 to 24°C) at a pressure of 50 kg/cm² by using a Constametric model IIG pump (Milton Roy Co., Riviera Beach, Fla.). The bases were detected by their A₂₆₀, as measured with a Spectromonitor II instrument (Atto Co., Tokyo, Japan).

[³H]DNA probe synthesis. Ureaplasma DNA was extracted from a cell pellet by the hydroxyapatite batch elution technique (4). Purified native DNA was labeled by the nick translation method (20) and was processed for hybridization as described by Aulakh and Gallo (1). The specific activity of the resulting single-stranded DNA probe was approximately 1.2 × 10⁷ cpm/µg of DNA.

DNA-DNA hybridization procedure. DNA-DNA hybridization was performed by the method of Aulakh and Gallo (1). Each hybridization mixture contained 100 µg of unlabeled, sheared DNA per ml, 1.0 × 10⁵ cpm of [³H]DNA probe per ml, 0.2% sodium dodecyl sulfate (SDS), 1 mM EDTA, and 0.48 M neutral phosphate buffer (an equimolar mixture of NaH₂PO₄ and Na₂HPO₄, pH 6.8). The reaction mixtures were denatured at 105°C for 5 min and incubated at 65°C to a C₀t value of more than 100 (3). Hybridized DNA was adsorbed to a hydroxyapatite column equilibrated at 60°C with 0.12 M neutral phosphate buffer containing 0.2% SDS.

TABLE 2. Effects of cholesterol, PPLO serum fraction, and horse serum on the growth of the four canine ureaplasma strains

Addition to base medium	No. of color-changing units per ml			
	Strain D1M-C	Strain D29M	Strain D11N-A	Strain D6P-C ^T
None ^a	10 ²	10 ^{1b}	10 ¹	10 ¹
Supplements ^c	10 ²	10 ¹	10 ¹	10 ¹
Supplements plus cholesterol (20 µg/ml)	10 ⁵	10 ²	10 ²	10 ²
PPLO serum fraction (1%)	10 ⁶	10 ⁴	10 ²	10 ⁶
Horse serum (20%)	10 ⁶	10 ⁶	10 ³	10 ⁶

^a Serum-free base medium alone.

^b The color change in the 10¹ tubes may have been due to the urease enzymes present in the organisms in the inoculum.

^c Base medium supplemented with 0.5% bovine albumin fraction V and 10 µg of palmitic acid per ml.

TABLE 3. Levels of antigenic relatedness among previously described *Ureaplasma* species and members of four canine serogroups

Host	Species	Strain	Reciprocal immunoperoxidase titers determined by using antisera prepared against the following strains:								
			D1M-C	D29M	D11N-A	D6P-C ^T	7	23	27	58	354
Canine	<i>Ureaplasma</i> sp.	D1M-C	12,800	— ^a	—	—	—	—	—	—	—
		D29M	—	25,600	—	—	—	—	—	—	—
		D11N-A	—	—	12,800	—	—	—	—	—	—
		D6P-C ^T	—	—	12,800	25,600	—	—	—	—	—
Human	<i>U. urealyticum</i>	7	—	—	—	—	204,800	3,200	—	6,400	—
		23	—	—	—	—	25,600	51,200	—	25,600	102,400
		27	—	—	—	—	—	—	25,600	—	—
		58	—	—	—	—	3,200	400	—	12,800	—
		354	—	—	—	—	100	3,200	—	50	12,800
		Pi	—	—	—	—	12,800	—	1,600	6,400	12,800
		Co	—	—	—	—	—	—	—	—	3,200
		T960 ^T	—	—	—	—	—	200	3,200	50	51,200
		Vancouver	—	—	—	—	200	50	—	3,200	6,400
		Western	—	—	—	—	—	—	100	50	50
		K2	—	—	—	—	—	—	800	50	200
		U24	—	—	—	—	12,800	6,400	200	51,200	400
		U38	—	—	—	—	12,800	3,200	—	12,800	—
		U26	—	—	—	—	200	—	800	—	—
Bovine	<i>U. diversum</i>	A417 ^T	—	—	—	—	—	—	—	—	—
		T315	—	—	—	—	—	—	—	—	—
		T44	—	—	—	—	—	—	—	—	—
			—	—	—	—	—	—	—	—	—
Avian	<i>U. gallorale</i>	D6-1 ^T	—	—	—	—	—	—	—	—	—
Feline	<i>U. felinum</i>	FT2-B ^T	—	—	—	—	—	—	—	—	—
	<i>U. cati</i>	F2 ^T	—	—	—	—	—	—	—	—	—

^a —, less than 50.

RESULTS AND DISCUSSION

The optimal pH for growth of strains D6P-C^T, D1M-C, D29M, and D11N-A was determined by inoculating 10² to 10³ color-changing units into broth media with the pH adjusted to values varying by 0.5-pH unit from pH 5.0 to 8.0. Growth was observed within 1 day in broth media at pH 6.0 to 7.0. No growth was observed in broth media at pH 5.0 or 8.0. Colonies grown on agar medium had a fried egg appearance and were about 20 to 140 µm in diameter (Fig. 1).

Smears prepared from sedimented broth cultures of strain D6P-C^T and stained with Gram stain contained gram-negative round or coccobacillary organisms. Electron microscopic examination of thin sections of the cell pellets revealed pleomorphic organisms with a unit membrane and no evidence of a cell wall outside the membrane (Fig. 2). At

higher magnifications, the membrane appeared to be a trilaminar unit structure.

None of the canine strains showed reversion to the bacterial form when the strains were serially subcultured in antimicrobial agent-free broth and agar media. Canine strains D1M-C, D29M, D11N-A, and D6P-C^T passed through membrane filters with pore sizes of 450 nm or greater but failed to pass through filters with pore sizes of 200 nm or less.

The growth responses of canine strains D1M-C, D29M,

FIG. 1. Colony of canine ureaplasma strain D6P-C^T grown on agar medium after 3 days of incubation at 36°C in a 10% CO₂ incubator. Bar = 100 µm.

FIG. 2. Thin-section electron micrograph of canine ureaplasma strain D6P-C^T. Note the three-layer membrane and the lack of a cell wall. Bar = 200 nm.

TABLE 3—Continued

Reciprocal immunoperoxidase titers determined by using antisera prepared against the following strains:														
Pi	Co	T960 ^T	Vancouver	Western	K2	U24	U38	U26	A417 ^T	T315	T44	D6-1 ^T	FT2-B ^T	F2 ^T
—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	1,600	—	800	25,600	25,600	6,400	—	—	—	—	—	—
—	—	—	6,400	100	3,200	51,200	12,800	800	—	—	—	—	—	—
—	—	—	100	6,400	6,400	6,400	1,600	102,400	—	—	—	—	—	—
—	—	—	100	400	6,400	12,800	3,200	—	—	—	—	—	—	—
—	—	100	6,400	—	1,600	—	—	—	—	—	—	—	—	—
12,800	—	6,400	12,800	6,400	12,800	12,800	3,200	25,600	—	—	—	—	—	—
—	25,600	51,200	51,200	1,600	6,400	3,200	400	800	—	—	—	—	—	—
—	12,800	51,200	25,600	25,600	51,200	25,600	12,800	—	—	—	—	—	—	—
100	6,400	25,600	25,600	3,200	12,800	12,800	3,200	200	—	—	—	—	—	—
—	200	25,600	12,800	6,400	6,400	12,800	6,400	—	—	—	—	—	—	—
50	—	51,200	6,400	12,800	51,200	25,600	12,800	—	—	—	—	—	—	—
50	—	12,800	12,800	51,200	6,400	102,400	12,800	—	—	—	—	—	—	—
—	—	6,400	6,400	12,800	12,800	51,200	102,400	200	—	—	—	—	—	—
—	—	—	50	—	—	50	—	102,400	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	25,600	400	—	—	—	—
—	—	—	—	—	—	—	—	—	3,200	102,400	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	6,400	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	51,200	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—	12,800	—
—	—	—	—	—	—	—	—	—	—	—	—	—	—	25,600

D11N-A, and D6P-C^T to cholesterol and to various supplements added to the basal medium are shown in Table 2. Little growth was apparent in serum-free basal medium or in basal medium containing bovine albumin fraction V and palmitic acid, but marginal growth occurred when both cholesterol and other supplements were incorporated into the basal medium. However, significantly increased multiplication was observed when 1% PPLO serum fraction or 20% horse serum was added to the basal medium, thus confirming the requirement for serum.

Growth on agar medium of all four canine ureaplasma strains studied was inhibited by 1.5% digitonin. The inhibition zones around the disks containing digitonin ranged from 4 to 6 mm wide. No inhibition of growth was seen adjacent to disks lacking digitonin.

All four canine ureaplasma strains produced a slight alkaline color change which represented an increase in pH from 6.3 to 6.7 in broth medium without added urea. In broth medium containing 0.05% urea, there was a marked alkaline color change and an increase in pH from 6.3 to 7.8, indicating that urea was hydrolyzed. There was no evidence of arginine hydrolysis or glucose fermentation in broth medium lacking urea.

The four canine ureaplasma strains did not react with the hyperimmune sera prepared against the feline *U. cati* and *U. felinum* strains, the 14 human *U. urealyticum* serovars, or the three bovine *U. diversum* serovars in the immunoperoxidase test. Conversely, the hyperimmune sera prepared against the four canine ureaplasma strains reacted with homologous strains. The hyperimmune serum prepared against strain D11N-A reacted with strain D6P-C^T and with strain D11N-A to the same degree (Table 3).

The genome size of strain D6P-C^T has been reported to be

860 kbp (15). The guanine-plus-cytosine contents of canine ureaplasma strains D6P-C^T, D1M-C, D29M, and D11N-A were 28.7, 29.4, 28.3, and 29.1 mol%, respectively. These values are within the range of values reported previously for members of the genus *Ureaplasma* (2, 12).

The results of DNA-DNA hybridization experiments in which [³H]DNA probes prepared from four unclassified canine ureaplasma strains and the type strains of *U. urealyticum*, *U. diversum*, *U. gallorale*, *U. cati*, and *U. felinum* were used are shown in Table 4. The data show that the human, bovine, avian, feline, and canine ureaplasma strains tested are genomically distinct. The four canine ureaplasma

TABLE 4. Hybridization of [³H]DNAs from the canine ureaplasma strains with unlabeled DNAs from the human, bovine, avian, feline, and canine ureaplasma strains

Source of unlabeled DNA	% Homology with [³ H]DNA probe from:			
	Strain D1M-C	Strain D29M	Strain D11N-A	Strain D6P-C ^T
<i>U. urealyticum</i> T960 ^T	32 ^a	26	27	33
<i>U. diversum</i> A417 ^T	27	23	30	28
<i>U. gallorale</i> D6-1 ^T	34	21	22	25
<i>U. felinum</i> FT2-B ^T	31	23	25	24
<i>U. cati</i> F2 ^T	29	22	21	26
<i>Ureaplasma</i> sp. strain D1M-C	100	54	49	52
<i>Ureaplasma</i> sp. strain D29M	49	100	46	50
<i>Ureaplasma</i> sp. strain D11N-A	62	51	100	63
<i>Ureaplasma</i> sp. strain D6P-C ^T	56	41	53	100

^a Homologous values were normalized to 100% and represent the averages of the results from two separate experiments.

strains exhibited from 21 to 34% homology with the human, bovine, and avian strains and from 41 to 63% homology with each other; thus, the hybridization values obtained with the canine strains were about twice as high as the values obtained with the previously described *Ureaplasma* species.

We propose the canine strains, which were frequently isolated from genitals as well as from oral and nasal cavities, should be designated *Ureaplasma canigenitalium*. A cloned line of strain D6P-C, the type strain of this species, has been deposited in the American Type Culture Collection as strain ATCC 51252.

Description of *Ureaplasma canigenitalium* sp. nov. *Ureaplasma canigenitalium* (ca.ni.ge.ni.ta'li.um. L. n. canis, dog; M. L. pl. n. genitalia, genitals; M. L. pl. gen. n. canigenitalium, of canine genitalia).

Morphological and physical characteristics. Pleomorphic cells are bounded by a single unit membrane. Colonies have a typical fried egg appearance on agar. Organisms are filterable through 450-nm-pore-size membrane filters and are resistant to penicillin.

Growth characteristics. Requires sterol. Inhibited by digitonin. Optimal growth occurs at 36 to 37°C under aerobic and anaerobic conditions.

Metabolic characteristics. Urea is hydrolyzed; glucose is not metabolized; and arginine is not hydrolyzed.

Serological characteristics. Serologically distinct from all other *Ureaplasma* species.

DNA base composition. The DNA base composition is 28.7 mol% guanine-plus-cytosine.

Habitat. The habitat is canine prepuces, vaginas, and oral and nasal cavities.

Type strain. The type strain is strain D6P-C (= ATCC 51252).

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