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Virulence-associated Genome Sequences of *Pasteurella canis* and Unique Toxin Gene Prevalence of *P. canis* and *Pasteurella multocida* Isolated from Humans and Companion Animals

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Background: Comparative analysis of virulence factors (VFs) between *Pasteurella canis* and *Pasteurella multocida* are lacking, although both cause zoonotic infections. We determined the virulence-associated genome sequence characteristics of *P. canis* and assessed the toxin gene prevalence unique to *P. canis* among clinical isolates of *P. canis* and *P. multocida*.

Methods: We selected 10 *P. canis* and 16 *P. multocida* whole-genome sequences (WGSs) from the National Center for Biotechnology database. The VFanalyzer tool was used to estimate *P. canis*-characteristic VFs. Amino acid sequences of VFs were compared with multiple-aligned sequences. The genome structure containing *P. canis*-characteristic and adjacent loci was compared to the corresponding *P. multocida* genome structure. After designing primer sequences and assessing their accuracy, we examined the gene prevalence of the *P. canis*-characteristic VFs using PCR among clinical isolates of *P. multocida* and *P. canis*.

Results: Using VFanalyzer, we found virulence-associated cytolethal distending toxin (*cdt*) A–*cdtB*–*cdtC* loci common to all *P. canis* WGSs that were not found in *P. multocida* WGSs. Similarities in the multiple alignments of CdtA–CdtB–CdtC amino acid sequences were found among the 10 *P. canis* WGSs. Shared or similar loci around *cdtA*–*cdtB*–*cdtC* were identified between the *P. canis* and *P. multocida* genome structures. The PCR-based *cdtA*–*cdtB*–*cdtC* prevalence differed for *P. canis* and *P. multocida* clinical isolates.

Conclusions: *P. canis*-specific *cdtA*–*cdtB*–*cdtC* prevalence was identified among clinical isolates. These three loci may be unique toxin genes and promising targets for the rapid identification of *P. canis* in clinical settings.

Key Words: *Pasteurella canis*, *Pasteurella multocida*, Genome sequence, Unique toxin gene, Cytolethal distending toxin, Japan, Korea, PCR, Zoonoses, Virulence factors

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INTRODUCTION

Pasteurella multocida was first isolated by Louis Pasteur in 1881 from samples received during an epidemiological survey of a fowl cholera outbreak. *Pasteurella canis* was previously classified as *P. multocida* biotype 6 or "dog type" and was then reclassified in 1985 based on DNA homology [1]. *P. canis* is a tiny, non-motile, facultatively anaerobic, gram-negative coccobacillus belonging to a species of G-Proteobacteria in the family Pasteurellaceae. *P. canis* forms smaller colonies as against *P. multocida* that forms larger colonies when the isolates are inoculated onto blood agar plates and incubated in 5% CO_2 at 37°C for 24 hours, suggesting that *P. canis* has relatively slower growth capacity than *P. multocida* under the same culture conditions [2]. With reclassification, *P. multocida* was divided into three subspecies: *P. multocida* subsp. *multocida*, *P. multocida* subsp. *septica*, and *P. multocida* subsp. *gallicida* [1]. Phylogenetic trees showing relationships between 16S rRNA sequences and fragment sequences (449–473 bp) of the *sodA* gene from type strains of *Pasteurella* demonstrated that *P. canis* is more closely related to *P. stomatis* and *P. dagmatis* than to *P. multocida* subspp. *multocida*, *septica*, and *gallicida* [3]. With respect to the biochemical properties of *P. canis*, the ornithine decarboxylation property is positive, whereas the urease test is negative, and variable reactions are obtained for fermentation of trehalose and D-xylose [1]. Biotype 1 isolates are positive for indole formation, whereas biotype 2 isolates are negative for indole formation.

P. canis biotype 1 is mainly observed in the oral cavities of dogs and is also often isolated from the wound sites of humans after receiving dog bites [1], whereas biotype 2 isolates are mainly recovered from calves. There are several reports of *P. canis*-infected humans with bacteremia [4], soft tissue infection [5], eye infection [6], respiratory infection [7], septic arthritis [8], osteomyelitis [9], gastrointestinal infection [10], breast implant infection [11], and peritonitis [12]. The Emergency Medicine Animal Bite Infection Study Group [13] performed a bacteriological assay of infected wound sites resulting from dog and cat bites, showing that *P. canis* was the most common species isolated from wounds caused by dog bites, whereas *P. multocida* subspp. *multocida* and *septica* were the most common bacteria isolated from wounds caused by cat bites. Basic investigations of *P. canis* isolates are scarce, especially studies on its virulence factors (VFs).

The whole-genome sequence (WGS) of a blood-origin *P. canis* isolate from a diseased male Saint-Bernard dog in Japan was recently reported [2]. No comparative genomic analysis of VFs between *P. canis* and *P. multocida* has been performed to date, although both cause zoonotic infections. This study aimed to compare the genome sequence characteristics of *P. canis* and *P. multocida* using the VF-detection tool VFanalyzer, which provides an automatic analysis pipeline (http://www.mgc.ac.cn/cgi-bin/VFs/v5/main.cgi?func=VFanalyzer) for the systematic screening of known or potential VFs in complete or draft bacterial genomes [14, 15]. We also assessed the prevalence of each unique VF gene for the two species (*P. canis* and *P. multocida*) among clinical isolates.

MATERIALS AND METHODS

P. canis and *P. multocida* WGSs

We extracted WGSs from *P. canis* (N=10) and *P. multocida* (N=16) deposited in the National Center for Biotechnology Information (NCBI) database (updated June 3, 2022) for this retrospective study. Sixteen *P. multocida* WGSs were selected covering various sequence types (including newly identified sequence types), demonstrating the high genomic diversity of this species. Among the 10 *P. canis* WGSs, seven are newly added, including three human isolates (two from Korea and one from Japan) and four dog isolates (three from Korea and one from Japan). Table 1 lists the characteristics of the *P. canis* and *P. multocida* WGSs (including the strain, host, isolation source, assembly level, collection date/geographic location, and GenBank accession number). Information on the host, isolation source, collection date, and geographic location was retrieved from the corresponding BioSample website or datasheets of collection companies. The included reference WGSs were *P. canis* National Collection of Type Cultures (NCTC) 11621(T), *P. multocida* subsp. *multocida* American Type Culture Collection (ATCC) 43137(T), *P. multocida* subsp. *septica* NCTC 11995(T), and *P. multocida* subsp. *gallicida* NCTC 10204(T).

Estimation of *P. canis*–characteristic VFs and multiple amino acid sequence alignment

To compare the VFs of *P. canis* and *P. multocida*, the VFanalyzer tool from the VF database (http://www.mgc.ac.cn/VFs/) was used. Any publicly available bacterial genome can be used as input for VFanalyzer, either from the pre-downloaded genome list or via any valid GenBank accession number. The genus *Haemophilus* was selected for the analysis because the option of *Pasteurella* was not available for selection. Each WGS file (complete or draft genome in GenBank format) from *P. canis* (N=10) and *P. multocida* (N=16) was entered into VFanalyzer, and the data of known or potential VFs, related genes, and corresponding loci

Table 1. Whole-genome sequences of *Pasteurella canis* and *Pasteurella multocida* available in the National Center for Biotechnology Information GenBank

Species or subspecies (sequence type)	Strain	Host	Isolation source	Assembly level	Collection date and location	GenBank accession number
P. canis	NCTC 11621(T)	Dog	Throat	Contig	1900/1983, unknown	UGTV00000000.1
P. canis	PA42*	Dog	Blood	Contig	2021, Japan	BPUX00000000.1
P. canis	HL_NV12211†	Dog	Pus	Complete	2020, Korea	CP085871.1
P. canis	HL_D3081	Dog	Pus	Complete	2019, Korea	CP085873.1
P. canis	HL_D1250	Dog	Throat	Complete	2018, Korea	CP085791.1
P. canis	PA57*	Human	Pus	Contig	2021, Japan	BQFX00000000.1
P. canis	NCTC 11650	Human	Dog bite	Contig	1900/1984, unknown	UATN00000000.1
P. canis	QBSD	Human	Pus	Contig	2019, China	WUMP00000000.1
P. canis	HL_1500	Human	Pus	Complete	2017, Korea	CP083396.1
P. canis	HL268	Human	Pus	Complete	2004, Korea	CP083262.1
P. multocida subsp. *multocida* (ST3)	NCTC 10322(T)/ATCC 43137(T)†	Porcine	Unknown	Complete	1900/1962, unknown	LT906458.1
P. multocida subsp. *gallicida* (ST42)	NCTC 10204(T)/ATCC 51689(T)	Cow	Unknown	Complete	1900/1960, unknown	LR134298.1
P. multocida subsp. *multocida* (ST135)	S298D	Dog	Oral swab	Contig	2016, Greece	PSQH00000000.1
P. multocida subsp. *septica* (ST30)	KVNON-213	Cat	Nasal cavity	Complete	2018, Korea	CP049756.1
P. multocida subsp. *gallicida* (ST25)	MSP58	Cat	Clinical sample	Contig	2019, USA	SJXC00000000.1
P. multocida subsp. *septica* (ST43)	NCTC 11995(T)/ATCC 51687(T)	Human	Abscess by cat bite	Contig	1900/1987, France	UGSV00000000.1
P. multocida subsp. *multocida* (ST131)	NCTC 10382	Human	Infected finger	Complete	1964, unknown	LS483473.1
P. multocida subsp. *multocida* (ST135)	PY81579	Human	Pus	Contig	2016, Greece	PSQI00000000.1
P. multocida subsp. *septica* (new ST; allele 18-21-28-15-48-17-45)	NCTC 11619	Human	Wound	Complete	1900/1983, unknown	LR134514.1
P. multocida subsp. *septica* (new ST; allele 11-21-32-16-18-17-55)	NCTC 11620	Human	Unknown	Contig	1900/1983, unknown	UGSW00000000.1
P. multocida subsp. *septica* (new ST; allele 18-56-37-16-18-18-55)	FDAARGOS_384	Human	Abscess	Complete	2015, USA	CP023516.1
P. multocida subsp. *septica* (new ST; allele 18-56-37-16-18-18-55)	FDAARGOS_385	Human	Wound	Complete	2015, USA	CP023972.1
P. multocida subsp. *gallicida* (ST128)	HuN001	Human	Unknown	Complete	2021, China	CP073238.1
P. multocida subsp. *septica* (ST137)	161215033201-1	Human	Right lung	Complete	2016, the Netherlands	CP026744.1
P. multocida subsp. *septica* (new ST; allele 20-58-28-15-18-17-24)	FDAARGOS_261	Human	Blood	Contig	2014, USA	NBTJ00000000.2
P. multocida subsp. *multocida* (new ST; allele 16-19-14-6-26-12-11)	SMC1	Human	Finger bone biopsy	Contig	2015, Malaysia	LNCO00000000.1

*Strains used as positive controls in PCR for clinical isolates; †Strains selected to construct genome structure graphics.

(along with their nucleotide positions/sequences) for each WGS were output. Putative *P. canis*-characteristic VFs that were not present among the *P. multocida*-characteristic VFs were estimated. All analyses were conducted at Ōmura Satoshi Memorial Institute, Kitasato University.

To evaluate the uniqueness of the estimated VFs, the corresponding nucleotide/amino acid (AA) sequences were inserted into the NCBI nucleotide/protein–nucleotide Basic Local Alignment Search Tool (BLASTn/BLASTp; https://blast.ncbi.nlm.nih.gov/Blast.cgi). To assess the percent similarities among deduced AA sequences obtained from the nucleotide sequences, multiple alignments were performed using the web-based tool ClustalW (https://www.genome.jp/tools-bin/clustalw).

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Genome structure comparison

The complete circular genomic structure was constructed for *P. canis* HL_NV12211 (CP085871.1) as a representative, based on the WGS data obtained in this study, which is now recommended in the NCBI database as the *P. canis* reference genome (https://www.ncbi.nlm.nih.gov/data-hub/taxonomy/753/) (Table 1). This isolate was recovered from a Korean canine pus sample (Table 1). The WGS from isolate ATCC 43137(T) (CP008918.1) was used as a representative *P. multocida* subsp. *multocida* genome (Table 1) to construct the comparative genomic structure. The gene information on HL_NV12211 and ATCC 43137(T) WGSs was obtained from GenBank in the NCBI database, including the directions and lengths of genes, locus tags, and gene–gene distances. The prokaryotic genome annotation pipeline was used as a tool for annotation revision. We assessed the shared, similar, and unique loci between HL_NV12211 and ATCC 43137(T) WGSs.

Assessing the accuracy of primer sequences for PCR

We designed PCR primer sets (forward and reverse) using the web-based Primer3Plus tool (https://www.primer3plus.com). The specificity of the primer sequences was examined by inserting them into NCBI BLASTn. To assess the accuracy of the primer sequences, we conducted simulated PCR assays using Serial Cloner 2.6 (http://serialbasics.free.fr/Serial_Cloner.html) based on the obtained WGSs [16, 17].

Prevalence of unique genes among isolates from humans and companion animals

Companion animal- and human-origin isolates from Japan were identified from PCR-based 16S rRNA sequencing data [18]. Thirty *P. canis* isolates from dogs (N=16), a cat (N=1), and humans (N=13) and 48 *P. multocida* isolates from dogs (N=5), cats (N=27), and humans (N=16) were included for this analysis. Isolation sources were mainly the pus/skin (N=44), upper/lower respiratory tract (N=25), and ear (N=6), along with the eye, blood, or pleural effusion (N=1 each). The collection years were mainly 2015–2019 (N=62) and 2021 (N=12), along with 1997, 1998, 2010, and 2013 (N=1 each). The geographic locations (prefectures in Japan) were mainly Chiba (N=40), Tokyo (N=15), Ibaraki (N=6), Saitama (N=5), Kanagawa (N=3), Aichi (N=3), Gifu (N=2), and Nagasaki (N=2), along with Okayama and Fukuoka (N=1 each).

We used primer sets for PCR amplification that were identical to the simulation PCR-based primer sets. One isolate (either PA42 or PA57) was used as a positive control (Table 1), and

DNase/RNase/protease-free water was used as a negative control in each PCR assay. PCR products were examined by 1.5% agarose gel electrophoresis in Tris-acetate–EDTA buffer. Direct sequencing of several PCR-positive products was performed on Applied Biosystems 3730xl DNA Analyzer with BigDye Terminator V3.1 (Thermo Fisher Scientific, Waltham, MA, USA). The amplification primers were used for the sequencing reaction. The prevalence of each estimated *P. canis*-unique VF gene was evaluated in all clinical isolates.

Ethics approval

The following ethics committees reviewed and approved our study design to maintain the privacy of humans and companion animals: Kitasato Institute Hospital (Tokyo, Japan; approval no. 21061), Kitasato University Medical Center (Saitama, Japan; approval no. 2021033), Sanritsu Zelkova Veterinary Laboratory (Tokyo, Japan; approval no. SZ20220525), Sanritsu Laboratory (Chiba, Japan; approval no. 22-01), and Chiba Kaihin Municipal Hospital (Chiba, Japan; approval no. 2021-02).

Background information (host species, isolation source, collection date, and geographic location) for the selected WGSs is publicly available from the online NCBI database.

Statistical analysis

Fisher's exact test (two-sided) was performed to analyze the significance of the association of the prevalence of each unique VF gene between *P. canis* and *P. multocida*. The associations of isolation sources (pus/skin, upper/lower respiratory tract, and ear) with host (animals, N=49; humans, N=29) or isolate (*P. canis*, N=30; *P. multocida*, N=48) were also determined using Fisher's exact test (two-sided). Statcel4 (OMS Publisher, Tokyo, Japan) was used for statistical analysis. $P < 0.05$ indicated statistical significance.

RESULTS

Estimation of *P. canis*–characteristic VFs

The VFs obtained from VFanalyzer in querying the *Haemophilus* genus included those related to adherence (e.g., HMW1, HMW2, hemagglutinating pili), endotoxin (lipooligosaccharide), immune evasion (e.g., exopolysaccharide, IgA1 protease, P2 porin), iron uptake (e.g., *Haemophilus* iron transport locus, heme biosynthesis, heme/hemopexin-binding complex), toxins (cytolethal distending toxin [Cdt] and hemolysin), cell-surface components (trehalose-recycling ABC transporter), nutritional virulence (pyrimidine biosynthesis from *Francisella*), secretion system (Hcp

CdtA

CdtA amino acid No.	44	45	46	47	48	49	50	51	59	60	61	62	63	64	65	71	72	73	74	75	76	77	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	200	201	202	203	204	205	206
HL_NV12211	I	Q	F	K	P	L	L	S	N	V	L	H	S	S	Y	K	E	K	T	P	E	P	L	T	Q	P	A	P	R	S	K	G	G	N	T	L	V	K	S	A	A	Q	G	N
NCTC11621(T)	I	Q	F	K	T	L	L	S	N	V	L	H	S	S	Y	K	E	K	T	P	E	P	L	T	Q	P	A	P	R	S	K	G	G	D	T	L	V	K	S	A	A	Q	G	N
NCTC11650	I	Q	F	K	P	L	L	S	N	V	L	H	S	S	Y	K	E	K	T	P	E	P	L	T	Q	P	V	P	R	S	K	G	V	N	T	L	V	K	S	A	A	Q	G	N
PA42	I	Q	F	K	P	L	L	S	N	V	L	H	S	S	Y	K	E	K	T	P	E	P	L	T	Q	P	A	P	R	S	K	G	G	N	T	L	V	K	S	A	A	Q	G	N
PA57	I	Q	F	N	T	L	L	S	N	V	L	H	S	S	Y	K	E	K	T	P	E	P	L	T	Q	P	A	P	R	S	K	G	G	D	T	L	V	K	S	A	A	Q	G	N
HL_268	I	Q	F	K	P	L	L	S	N	V	L	H	S	S	Y	K	E	K	T	P	E	P	L	T	Q	S	A	P	R	S	K	G	G	N	T	L	V	K	S	A	S	S	G	N
HL_1500	I	Q	F	K	P	L	L	S	N	V	L	H	S	S	Y	K	E	K	T	P	E	P	L	T	Q	S	A	P	R	S	K	G	G	N	T	L	V	K	S	A	S	S	G	N
HL_D1250	I	Q	F	K	T	L	L	S	N	V	L	Y	S	S	Y	K	E	K	T	P	E	P	L	T	Q	P	A	P	R	S	K	G	G	D	T	L	V	K	S	A	A	Q	G	N
HL_D3081	I	Q	F	K	T	L	L	S	N	V	L	H	S	S	Y	K	E	K	I	P	E	P	L	T	Q	P	A	P	R	S	K	G	G	D	T	L	V	K	S	A	A	Q	G	N
QBSD	I	Q	F	K	T	L	L	S	N	V	L	H	S	S	Y	K	E	K	T	P	E	P	L	T	Q	P	A	P	R	S	K	G	G	D	T	L	V	K	S	A	A	Q	G	N
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CdtB

CdtB amino acid No.	6	7	8	9	10	11	12	100	101	102	103	104	105	106	185	186	187	188	189	190	191	237	238	239	240	241	242	243	244	245	246	247	248	249	250	251	252	253
HL_NV12211	Q	L	S	L	I	F	C	R	P	N	T	V	Y	I	E	R	R	G	Y	S	W	A	I	L	R	D	A	N	L	L	P	R	R	E	Q	A	R	E	R
NCTC11621(T)	Q	L	S	L	I	F	C	R	P	N	T	V	Y	Y	E	R	R	R	Y	S	W	A	I	L	H	H	A	N	L	L	P	R	R	E	Q	A	R	E	R
NCTC11650	Q	L	S	L	I	F	C	R	P	N	I	V	Y	Y	E	R	R	G	Y	S	W	A	I	L	R	D	A	N	L	L	P	R	R	E	Q	T	R	E	R
PA42	Q	L	S	L	I	F	C	R	P	N	T	V	Y	I	E	R	R	G	Y	S	W	A	I	L	R	D	A	N	L	L	P	R	R	E	Q	A	R	E	R
PA57	Q	L	S	F	I	F	C	R	P	N	T	V	Y	I	E	R	R	R	Y	S	W	A	I	L	H	V	A	N	L	L	P	R	R	E	Q	A	R	E	R
HL_268	Q	L	S	L	I	F	C	R	P	N	T	V	Y	I	E	R	R	G	Y	S	W	A	I	L	H	D	A	N	L	L	P	R	R	E	Q	A	R	E	R
HL_1500	Q	L	S	L	I	F	C	R	P	N	T	V	Y	I	E	R	R	G	Y	S	W	A	I	L	H	D	A	N	L	L	P	R	R	E	Q	A	R	E	R
HL_D1250	Q	L	S	L	I	F	C	R	P	N	T	V	Y	I	E	R	R	G	Y	S	W	A	I	L	H	D	A	N	L	L	P	R	R	E	Q	A	R	E	R
HL_D3081	Q	L	S	L	I	F	C	R	P	N	T	V	Y	I	E	R	R	R	Y	S	W	A	I	L	H	D	A	N	L	L	P	R	R	E	Q	A	R	E	R
QBSD	Q	L	S	L	I	F	C	R	P	N	T	V	Y	I	E	R	R	G	Y	S	W	A	I	L	H	D	.	N	.	.	P	R	R	E	Q	A	R	E	R
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CdtC

CdtC amino acid No.	1	2	3	4	28	29	30	31	32	33	34	138	139	140	141	142	143	144
HL_NV12211	-	M	K	K	T	T	Y	P	D	V	E	R	D	F	A	D	L	N
NCTC11621(T)	M	M	K	K	T	T	Y	P	D	V	E	R	D	F	A	D	L	N
NCTC11650	M	M	K	K	T	T	Y	P	D	V	E	R	D	F	A	D	L	N
PA42	-	M	K	K	T	T	Y	P	D	V	E	R	D	F	D	D	L	N
PA57	-	M	K	K	T	T	Y	L	D	V	E	R	D	F	A	D	L	N
HL_268	-	M	K	K	T	T	Y	P	D	V	E	R	D	F	D	D	L	N
HL_1500	-	M	K	K	T	T	Y	P	D	V	E	R	D	F	D	D	L	N
HL_D1250	-	M	K	K	T	T	Y	P	D	V	E	R	D	F	A	D	L	N
HL_D3081	-	M	K	K	T	T	Y	P	D	V	E	R	D	F	D	D	L	N
QBSD	-	M	K	K	T	T	Y	L	D	V	E	R	D	F	D	D	L	N
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Fig. 1. Multiple amino acid (AA) sequence alignments of cytolethal distending toxin (Cdt)A, B, and C between HL_NV12211 (harboring CdtA–CdtB–CdtC of 251 AAs, 280 AAs, and 180 AAs, respectively) and the remaining nine whole-genome sequences using ClustalW version 2.1. "*" indicates positions having a single, fully conserved residue; ":" indicates that one of the following "strong" groups (STA/NEQK/NHQK/NDEQ/QHRK/MILV/MILF/HY/FYW) is fully conserved; and "." indicates that one of the following "weaker" groups (CSA/ATV/SAG/STNK/STPA/SGND/SNDEQK/NDEQHK/NEQHRK/FVLIM/HFY) is fully conserved. Gray shading shows AA substitutions compared to the consensus sequence of HL_NV12211.

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secretion island-1-encoded type VI secretion system [H1-T6SS] and *Legionella* vir homologs [Lvh] type IVa secretion system), stress adaptation (catalase from *Neisseria* and SodCI from *Salmonella*), and antiphagocytosis (capsular polysaccharide from *Vibrio* and capsule from *Klebsiella*). The prevalence and composition of VFs, related genes, and corresponding loci varied between the *P. canis* and *P. multocida* genomes. We identified *cdtA–cdtB–cdtC* sequences common to the 10 *P. canis* WGSs, which were absent among the 16 *P. multocida* WGSs; thus, the *cdtA–cdtB–cdtC* sequences were considered potential *P. canis*-characteristic VFs. The HL_NV12211 WGS included a *cdtA* locus of 756 bp (nucleotide positions 2,095,545 to 2,096,300), a *cdtB* locus of 843 bp (nucleotides 2,096,316 to 2,097,158), and a *cdtC* locus of 543 bp (nucleotides 2,097,169 to 2,097,711).

Multiple nucleotide/AA sequence alignment of the *cdtA–cdtB–cdtC* loci of the *P. canis* HL_NV12211 WGS showed high sequence similarities among *P. canis* populations but not among populations of *P. multocida* or other *Pasteurella* species. Fig. 1 shows the multiple AA sequence alignments of CdtA–CdtB–CdtC loci. High similarities (percentages) of deduced AA sequences were found between HL_NV12211 CdtA (251 AA), CdtB (280 AA), and CdtC (180 AA) and the sequences of the other *P. canis* genomes, with the lowest similarities of 98.8%, 98.6%, and 98.9%, respectively.

Genome structures of *P. canis* and *P. multocida*

Fig. 2 shows the genome structure containing the *cdtA–cdtB–cdtC* loci and adjacent loci in the *P. canis* HL_NV12211 WGS. Compared to that in the ATCC 43137(T) WGS, shared loci (*relA*,

recO, rsmE, ruvX/DR93_65, *eno*, and *pyrG*), similar loci (*rlmD/rumA* with AA sequence similarity of 67.3% and K7G93_001967/DR93_66 with AA sequence similarity of 89.2%), and loci were identified in the *P. canis* WGS (*cdtA–cdtB–cdtC* and K7G93_001965) but not in the *P. multocida* WGS. The loci K7G93_001965, K7G93_001967, and DR93_66 are predicted to encode hypothetical proteins.

Accuracy of primer sequences for PCR

Primer sequences are located within the corresponding open reading frames to amplify sequences with expected sizes of 693 bp, 582 bp, and 433 bp. Table 2 shows the oligonucleotide primers and their corresponding melting temperature (Tm) values. PCR was performed with 30 cycles consisting of denaturation at 98°C for 10 seconds, annealing at 52°C for 30 seconds, and extension at 72°C for 45 seconds to amplify *cdtA*; 30 cycles consisting of denaturation at 98°C for 10 seconds, annealing at 53°C for 30 seconds, and extension at 72°C for 40 seconds to amplify *cdtB*; and 30 cycles consisting of denaturation at 98°C for 10 seconds, annealing at 52°C for 30 seconds, and extension at 72°C for 30 seconds to amplify *cdtC*.

Prevalence of unique genes among isolates from humans and companion animals

We found no associations between the isolation sources and host (animals/humans) or pathogen species (*P. canis*/*P. multocida*): pus/skin vs. host/pathogen species $P=0.102$/$P=1.0$, respiratory tract vs. host/pathogen species $P=1.0$/$P=0.619$, and ear vs. host/pathogen species $P=0.079$/$P=0.397$.
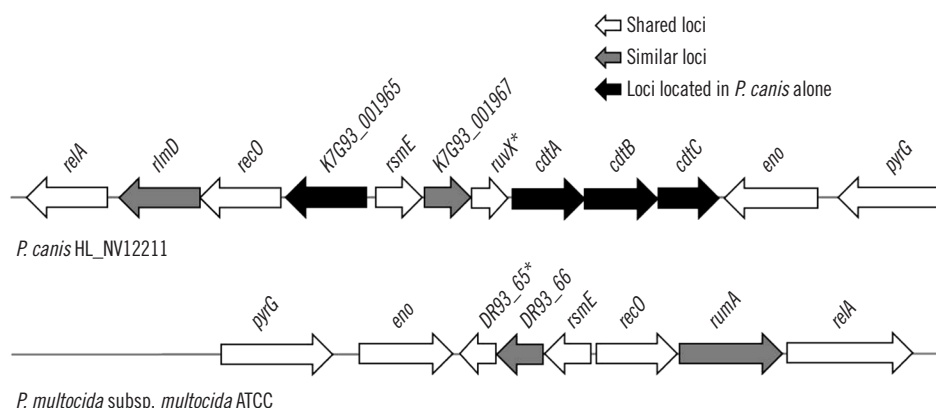

Fig. 2. Genome structure containing the cytolethal distending toxin (*cdt*)*A–cdtB–cdtC* loci of *Pasteurella canis* and adjacent loci from strain HL_NV12211 (GenBank accession no. CP085871.1) (upper) and the comparative structure from *Pasteurella multocida* subsp. *multocida* ATCC 43137(T) (GenBank accession no. CP008918.1) (lower). Asterisks show putative Holliday junction resolvase. K7G93_001965, K7G93_001967, and DR93_66 represent loci encoding hypothetical proteins.
Abbreviations: *relA*, GTP diphosphokinase; *rlmD* and *rumA*, 23S rRNA (uracil(1939)-C(5))-methyltransferase; *recO*, DNA repair protein; *rsmE*, 16S rRNA (uracil(1498)-N(3))-methyltransferase; *eno*, phosphopyruvate hydratase; *pyrG*, CTP synthase.

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Table 2. Oligonucleotide primers for targeted genes and their PCR amplicon sizes

Target gene (encoding protein)	Primer*	Direction	Sequence (5′→3′) (length, k-mer)	Tm (°C)†	Expected amplicon size
cdtA	Pc_cdtA_F	Forward	TCAGCAGATGTGTAATTGTCCTC (23)	54	693 bp
(cytolethal distending toxin A)	Pc_cdtA_R	Reverse	ATCGCAGTCGCATTTAATAGC (21)	54	
cdtB	Pc_cdtB_F	Forward	TCCAAGAGGCGGGTACTTTG (20)	54	582 bp
(cytolethal distending toxin B)	Pc_cdtB_R	Reverse	AACTGGCACCAATACGCTCA (20)	54	
cdtC	Pc_cdtC_F	Forward	GAGTTATCACCACCTCCACGT (21)	53	433 bp
(cytolethal distending toxin C)	Pc_cdtC_R	Reverse	GCGGTACTAAAATTTTACTTGGTCCA (26)	55	

*The same primers were used for both the PCR amplification and direct sequencing; †Tm values were calculated using the nearest-neighbor method.
Abbreviation: Tm, melting temperature.
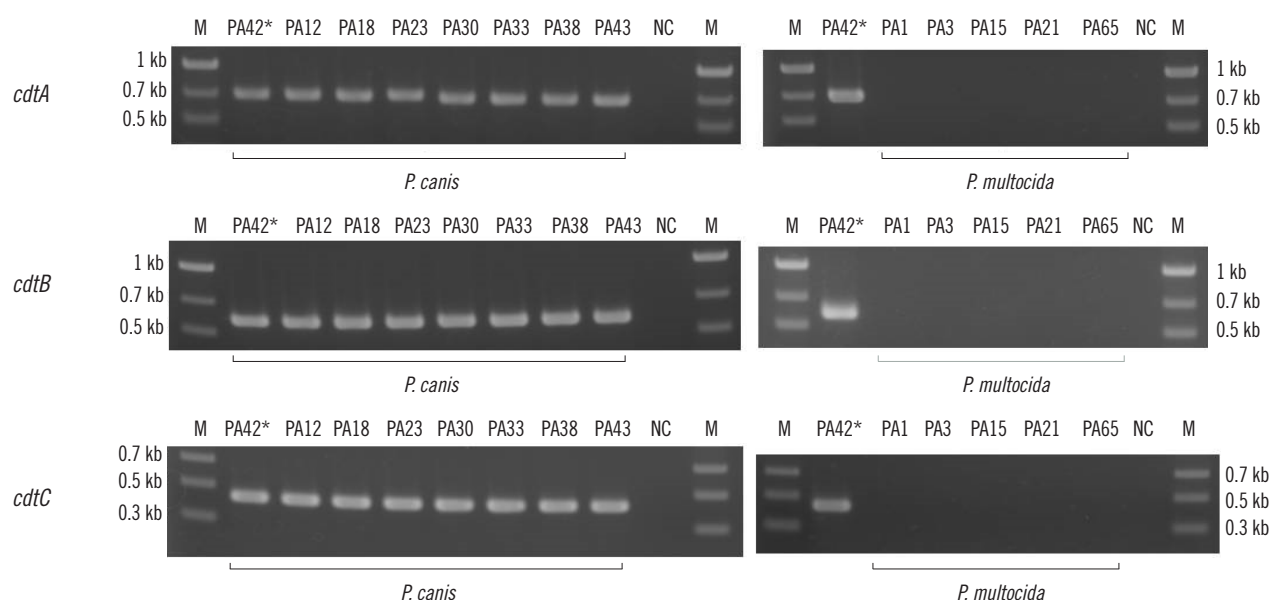
Fig. 3. Gel electrophoresis images of amplified *cdtA–cdtB–cdtC* products using DNA from clinical isolates. Asterisks indicate the positive control isolate (PA42).
Abbreviations: M, size marker; NC, negative control.

Table 3. Prevalence of cytolethal distending toxin (*cdt*) genes in *Pasteurella canis* and *Pasteurella multocida* isolated from different hosts

Target gene	*P. canis* isolates (N=30)		*P. multocida* isolates (N=48)		P*
	Dog/cat origin (N=17)	Human origin (N=13)	Dog/cat origin (N=32)	Human origin (N=16)	
cdtA	17/17	13/13	0/32	0/16	<0.01
cdtB	17/17	13/13	0/32	0/16	<0.01
cdtC	17/17	13/13	0/32	0/16	<0.01

*Fisher's exact test (two-sided) was used to assess the difference in the prevalence of *cdt* according to species.

Fig. 3 shows gel electrophoresis images of the amplified *cdtA–cdtB–cdtC* products using DNA extracted from the clinical isolates. Table 3 shows the prevalence of each *cdt* gene in *P. canis* and *P. multocida* isolates. High similarities (≥98.6%) were found between *cdtA* and *cdtB–cdtC* sequences using several PCR-positive products (N=11 for each gene) and reported HL_NV12211

cdtA–cdtB–cdtC sequences. The nucleotide sequences obtained by PCR amplification and direct sequencing in 11 isolates have been deposited in DDBJ/EMBL/GenBank under accession numbers LC716775–LC716807.

The prevalence of *cdtA–cdtB–cdtC* was significantly associated with *P. canis* rather than with *P. multocida* (P<0.01).

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DISCUSSION

We conducted a challenging study to find virulence-associated genome sequences specific to *P. canis*. Based on the "One Health" concept [19], which is a comprehensive health control strategy for humans, contact animals, and related environments, bacterial pathogens with VFs that may be circulating should be carefully monitored to maintain an environment of total health. Using VFanalyzer, we identified *cdtA–cdtB–cdtC* loci as potential *P. canis*-specific VFs, which were not identified in the WGSs of *P. dagmatis* NCTC 11617(T) (LT906448.1), *P. testudinis* NCTC 12150(T) (UGSY00000000.1), *P. skyensis* 95A1 (type strain) (CP016180.1), *P. bettyae* NCTC 10535(T) (UFRH00000000.1), and *P. bettyae* CCUG 2042 (AJSX00000000.1). These three loci may be unique toxin genes and promising targets for the rapid identification of *P. canis* in clinical settings.

Based on a search of the keywords "pasteurella canis, cytolethal distending toxin" or "pasteurella canis, cdt" in the PubMed database (https://pubmed.ncbi.nlm.nih.gov/), there were no hits for related manuscripts as of September 3, 2022. However, Fukushima, *et al.* [20] reported that *P. canis* isolates from the dental plaques of dogs with periodontal disease harbor the *cdtA–cdtB–cdtC* loci. Thus, our study provides a rare description of *cdt* genes in *P. canis* isolates from humans and companion animals in Korea and Japan.

Cdt was discovered in *Escherichia coli* in 1987 [21], and similar toxin activities have been documented in other enteric pathogens, including *Shigella* spp. and *Campylobacter* spp. [21, 22]. Cdt can modulate the host cell cycle by suppressing the G2/M transition. Bacterial genotoxins trigger single-strand and double-strand breaks in DNA of eukaryotic cells and are functionally homologous to mammalian DNase I, leading to alterations in the DNA damage response [23], resulting in cell aging, apoptosis, and genomic instability, which contribute to tumor initiation and progression. Three loci within an operon were identified: *cdtA–cdtB–cdtC* [24]. Among the three proteins encoded by *cdtA–cdtB–cdtC*, CdtB was shown to possess nuclease activity [25]. CdtA–CdtC is required for delivering CdtB into host cells, allowing CdtB to translocate to the nucleus, resulting in DNA damage [26]. CdtB nuclease activity in eukaryotic cells can induce an altered DNA damage response, which promotes genomic instability, disturbs the cell cycle, and establishes a chronic pro-inflammatory environment in the host [27].

Using BLASTp on the NCBI web server, we examined similarities in CdtA–CdtB–CdtC AA sequences between isolate HL_NV-12211 and other species. The CdtA AA sequence was similar to that of *Aggregatibacter actinomycetemcomitans* strain UP14 (220 AA and 66.5% similarity) and *Haemophilus ducreyi* strain 35000HP (223 AA and 65.1% similarity); CdtB was similar to *A. actinomycetemcomitans* NCTC_9710(T) (283 AA and 90.5% similarity) and *H. ducreyi* 35000HP (283 AA and 90.5% similarity); and CdtC was similar to *H. ducreyi* 35000HP (186 AA and 73.6% similarity) and *A. actinomycetemcomitans* strain RhAa3 (180 AA and 75.0% similarity). The family Pasteurellaceae mainly consists of the genera *Pasteurella, Haemophilus, Aggregatibacter,* and *Actinobacillus* as human pathogenic microorganisms. Thus, *P. canis* CdtA–CdtB–CdtC AA sequences are homologous to those of other species in the family Pasteurellaceae. For example, *A. actinomycetemcomitans*, a periodontal pathogen colonizing the human oral cavity, possesses CdtA–CdtB–CdtC as an exotoxin [28]. The possibility that *P. canis* is related to periodontal disease in dogs and cats has been reported [20]. A functional study of *H. ducreyi* CdtB and the endocytosis of CdtA dissociated from *H. ducreyi* CdtB–CdtC were recently reported [29, 30]. Further biological and functional analyses of *P. canis* CdtA–CdtB–CdtC are needed.

This study has two main limitations. First, we included five complete and five contig WGSs. We cannot deny the possibility that other characteristic genome sequences may be located within the gapped regions of contig WGSs. To enhance the quality of comparative genome analysis, the complete WGSs of *P. canis* should be determined and uploaded to the NCBI database. Second, we obtained limited information on host demographics (host species, isolation source, collection date, and geographic location). Detailed information on the underlying situation, diagnosis of infections, therapeutic strategies, and outcomes need to be collected from medical doctors and veterinarians.

Our observations suggest the prevalence of *cdtA–cdtB–cdtC* genes (as potential VFs) as a unique characteristic of *P. canis* isolates from humans and companion animals in Japan. Fukushima, *et al.* [20] found that supernatants from bacterial lysis had toxic and lethal effects on HeLa cells. We also plan to demonstrate the presence of CdtA–CdtB–CdtC within the supernatants of the isolate broth cultures and to evaluate the toxic and lethal effects on HeLa cells and other human cell lines. Future studies are also needed to monitor variations in *cdtA–cdtB–cdtC* prevalence among similar populations. The clinical significance of CdtA–CdtB–CdtC in humans and companion animals should be clarified to facilitate interpretation and clinical decision-making for medical doctors and veterinarians.

AUTHOR CONTRIBUTIONS

Conceptualization: Yoshida H and Takahashi T; Investigation: Yoshida H; Formal Analysis: Yoshida H, Kim J-M, Maeda T, Goto M, Kim J-S, and Okuzumi K; Resources: Tsuyuki Y, Shibata S, Shizuno K, Okuzumi K, and Takahashi T; Writing — Original Draft Preparation: Takahashi T; Writing — Review and Editing: Yoshida H.

CONFLICTS OF INTEREST

None declared.

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