

2770 2780 2790 2800 2810 2820 2830 2840 2850 2860 2870 2880
 OCTGETTCTTGTGAGGAGGTGAAGCTTCTGTCTCGAGTTCCTTAAGGAAACAGAAAGATCGTCTGGCTTCCAACAGTTCCTGGTCCCTCTGGGAGAGAGTTCCTAGCGGCAAA
 P G [S C E E] V K L R V L Q F L K E T E E I V L A S H S S W F P L G E S F L A A K
 2890 2900 2910 2920 2930 2940 2950 2960 2970 2980 2990 3000
 GGAATCTGGCTGADGATGAGGAGCTCTCCCTTCTCGACTCTCCATCCGGGAGACTTCTCAGAGAAGTTTCTGAGTGGAGGCGATTATGCCCTTCGTTGGCAGCTCAGTCTACC
 [G I W L [T D] E E] L S L P R L S P S R E [T F S E] K F L [S [G G D] Y A L] R L A A Q [S T
 3010 3020 3030 3040 3050 3060 3070 3080 3090 3100 3110 3120
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 L D I F Y O R R G F L L G D S T R T S A L L R P [V P Y V P Q C D V W G G W E P V Q]
 3130 3140 3150 3160 3170 3180 3190 3200 3210 3220 3230 3240
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 C H A R T G Y C W C V D G K G] E Y V P A S L T A R S P R I L R C P [T A C E] K S R
 3250 3260 3270 3280 3290 3300 3310 3320 3330 3340 3350 3360
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 A S [G L L S S W] K O A G S O G N P [S P K D] L [F I P [T C L E] T G E F A R R O E S E]
 3370 3380 3390 3400 3410 3420 3430 3440 3450 3460 3470 3480
 GGGGGACCTGGTGTGGACCCAGGCTGGGGGGCGGCGAGGCCCCCGGACAGGACAGCAGTGGCCGTCGCCAGGCTCTGTGAAGAGCTCTCGAGTGGGACCTCTCCAGGAGGCG
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 3490 3500 3510 3520 3530 3540 3550 3560 3570 3580 3590 3600
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 S S G [Y S P A C R A E D G G F S P V Q C D L A Q [G S C W C V] L D [S G] E E] V P [G T
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 R V A [G] S O L A C] E S P R C P L P F N I [T D V D] G [G V I V C E] R A S G P G G A P
 3730 3740 3750 3760 3770 3780 3790 3800 3810 3820 3830 3840
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 V O R C D L L C R R G Y R S A F L P G P L V C [S L E E] G R W L S O P P Q P Q A C
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 Q R P H L W O T F Q T O G D F O L O L P P G K M C S A D Y A [G L L P A F] Q V F L
 3970 3980 3990 4000 4010 4020 4030 4040 4050 4060 4070 4080
 GCAGACGAGCTCGAGGCGCTGGTGGTGTGAGATCCAGGCAAGACTTTGGGACCCCAATTCACCTTCTGCTGCGACAGCTCCACGGTGCAGGTGGAGTGTGACCCGAGAGGCT
 A D E L E A R G F C O I O A K T L [G T P I S I] P V C D S S T V O V E C V T G E R
 4090 4100 4110 4120 4130 4140 4150 4160 4170 4180 4190 4200
 TTAGAGTGAATGTACAGTGGAAAGTTCACAGTGGAGGATGCCAGCGGCTTCTCTGATTGCGATGACATCGAGGAGGCTTAGTGGCAAGGATCTCATTGAGCGCTCACGAGT
 L [G V N V T W] K L H L E D V P P A [S L P D] L H D I E E A L V G K D L I G R F T D
 4210 4220 4230 4240 4250 4260 4270 4280 4290 4300 4310 4320
 CTGATCCAGAGTGGAGGTTCCAGCTCACCTGGATTCTAAGACTTCCAGCAGACCTCCATCTACTTCCAGGGGGACCGCTTCGCGACCTCTCCAGGCGATGGTTGGGTGC
 L I D S G G F Q L H L D S K T F P A D I S I Y F L Q G D R F G T S P R A F W [G C
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 TTGGAAGGATTCACCAAGTCTGGCTCCAGCAATGCCCTCAGGACCAATGGGGTGTAAAGTGTCCGAAAGGAGCTATTTTCAGAAAGGAGATTTGCATCCCTGTCTGTGGGA
 L E A G F] H O V L A P S N A P O D P W G C V K C P E G S Y F O K E I C I P C P V G
 4450 4460 4470 4480 4490 4500 4510 4520 4530 4540 4550 4560
 TTCTACCAAGAACAGGCGAGGATGGACTGTGCCATGTCCGTTGGGCGAGAAGCATTCCCTGGAGCTTTCAGCCACAGCAGTGTGTACCGACTGTCCAGAGGAGTGGAGTG
 F Y O E O A [G S M D C V] P C P V G R T T I S P G A F S H T H C V T D C O R S E V]
 4570 4580 4590 4600 4610 4620 4630 4640 4650 4660 4670 4680
 GGCCTGCGTGTGACCCAGGCGAGTACCGAGCCAGGCGAGGAGCGGCGCGGGGAGGCGCTTCTGTGTGAGCGGCGAGGCGGCGGCTGCCCTGGTCCGAAACGGAGGCTCCG
 [G L R C D O D] [G O Y R A] [S] O R D] G A G G K A F C V D G E G] R R L P W [S E T E] A P
 4690 4700 4710 4720 4730 4740 4750 4760 4770 4780 4790 4800
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 L T D S O C O M M O K F E K A P D S K V V F S A N V T A V G R S K V S G S G S P
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 T T S A D H O D T L [G N S E A T] S F [G S L R C O] M T V R S [G A O D S L] A V Y L K
 5050 5060 5070 5080 5090 5100 5110 5120 5130 5140 5150 5160
 AAAGCCAAAGAAATCACCCCAAGTTCAGAAAGGCTTCGAGCAACAGGTTCCAAAACATGCTCTTGGACTGTACCAGGCGCTCATATCCAGCCTCAGGAGCTGATCTGACTGCC
 K [G O E F T T] T S O K S F E O T G F D N M L S G L Y R P V I F P A [S [G A D] L T A]
 5170 5180 5190 5200 5210 5220 5230 5240 5250 5260 5270 5280
 GCTCATCTTCTGTCTGCTGGCTGTGACGCTGATTCATGCTGTGACGGCTTCACTTCCAGCTCCAAAGGAGGCGGCTCATCTGTGGTGTGCTGAGCTCCCGGATGCTACTT
 A H L F C L L A C D R D [S C C D] G F I L A Q L O G G P V I C G L L [S S P D] V L L
 5290 5300 5310 5320 5330 5340 5350 5360 5370 5380 5390 5400
 TGCCATGTCAAAGACTGGAGGGACCCACTGAAGCCAGGCTAATGCCACCTGTCCGTTGTGACATATGACCGGGGAGCGCGCAGGCTGCGTCTGGGAGCCAGGAGTTCAGG
 C H V K D W R D P T E A O A N A T C P G V T Y D R [G S R Q A T] L R L G G O E F R
 5410 5420 5430 5440 5450 5460 5470 5480 5490 5500 5510 5520
 GTCCGCTAGCCCTGGAAGAACTTCGGGACCGTTCAGGCTCCAGCGGTTTATCTCGAAAGAGTGTGACATGGGATCTCGGCTGAGTCTATGGATGTAGGAGAGCATGGAA
 V P V A L E [G T S [G T V] T S F] O R V Y L W K E S D M G [S R S E] S M G C R R D M E
 5530 5540 5550 5560 5570 5580 5590 5600 5610 5620 5630 5640
 CCAAGGCCAGCATCCAAACAGAAACAGATTGACAACAGAGCTTTTCCCTGTCGACCTGACAGGTCATTGTCAATGGAAGCAATCCCTGCCAGCCAGCAGCAGCTGCTTTTC

Fig. 1. (Continued)

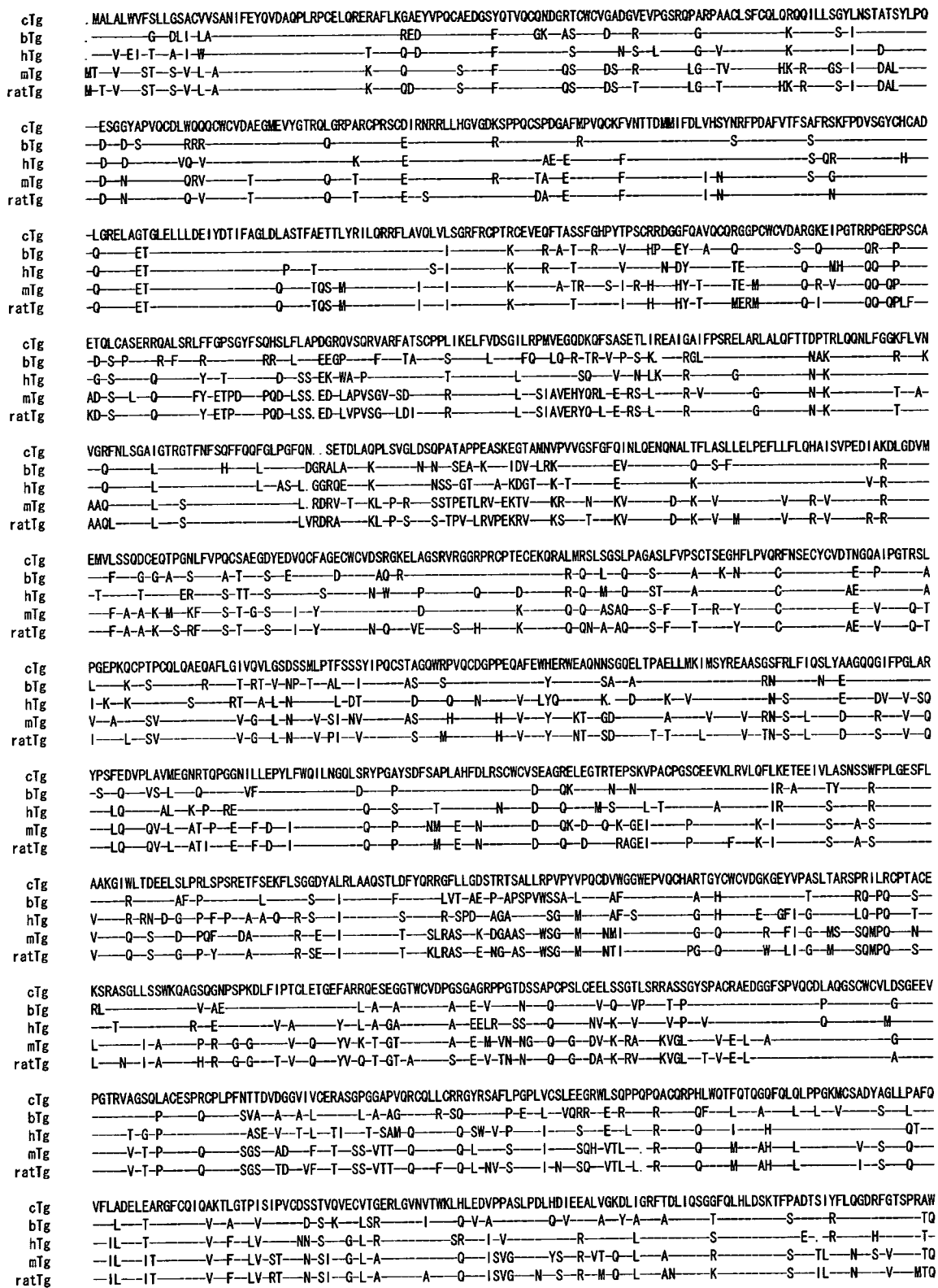


Fig. 2. Alignment of canine, bovine, human, murine, and rat thyroglobulin. The dash indicates the conserved amino acid compared with canine Tg, and dots indicate positions where the amino acid residues are missing.

cTg	FGQLGEGHQVLAPSNAPQDPWGCVKCEPGSYFQKEICIPCPVGFYQEQAGSMDCVPCVGRRTISPGAFSHTHCYVTDORSEVGLRCDQDGOYRASORDGAGGKAFQVDGEGRRLPWSET
bTg	GR-V-A-D-S-AL-D-Q-A-LA-E-VYA-Q-KN-Q-S-RTS-T-A
hTg	S-Y-TS-E-S-GL-S-D-E-LA-Q-N-A-Q-N-K-RGS-W
mTg	L-M-YR-PTT...R-AL-FS-DGR-T-A-T-SA-I-R-TT-K-KN-A-Q-N-Q-KNRDS-EV-S-K-Q-L-Q
ratTg	L-YR-STT...S-L-FS-DGK-T-A-T-G-SA-I-R-TIT-K-D-A-Q-N-Q-N-K-MDS-EV-S-Q-Q-L-Q
cTg	EAPLTDSCQMMKQFEKAPDSKVVFSANVTAVGRSKVSGSGSPLLQCLTDCALDETCFLAVSTAGSEVSCDFYGTSDSICACTTSAHQDTLGNSEATSFGLRQCMTVRSGAQDSLAV
bTg	V-A-LV-R-L-E-I-D-AVMV-E-P-E-S-M-A-A-G-T-A-A-GRSE-A-T-Q-Q-VK-REG-P
hTg	E-L-V-E-I-D-APVAV-PD-EF-VM-TE-A-FT-TEP-I-A-NV-M-D-KR-A-K-VK-HG-P
mTg	G-SE-L-IR-D-E-I-D-SPVIVK-S-PSAD-V-N-A-T-ME-S-R-NF-V-D-E-AM-SLK-VK-MSGK
ratTg	G-SE-L-R-E-I-D-SSPVIK-R-PSAN-V-A-D-A-VT-SMS-L-S-R-NF-V-D-EE-AVDSLKE-VK-MSGK
cTg	YLKKGQEFETTSSQKFEQTFQNLHSLGLYRYPVIFPASGADLTAHLFLCLACDRDSCDGFILAQLGGPVI CGLLSSPDVLLCHVKDWRDPTEADANATCPGVTYDRGSRQATRLRGGQ
bTg	I-G-R-SA-M-S-T-S-S-AEV-H-V-V-LL-R-A-S-OD-V
hTg	GS-L-R-P-N-IV-S-D-L-V-T-V-AI-S-N-M-S-W-QE-H-VI-D
mTg	V-Y-S-AAG-P-V-S-V-S-DT-IY-N-IT-VK-T-I-IN-TSAT-A-Q-M-S
ratTg	V-H-ASG-P-V-SS-V-S-L-T-DT-F-Q-VT-VKE-T-A-I-V-IN-ASDT-G-A-Q-M-MS
cTg	EFRVPVALEGTSGLTVTSFQRVYLWKESDMGRSSESMGCRDMEPRPASPTETDLTTELFSPVLDQVI VNGSQSLPSQQHMLFKHLFSQQANLWCLSRCVQEPSFQQLVEITDSAPLY
bTg	I-GLTP-QD-L-Q-D-T-S-G-I-D-NV-L-AG-A-V-E
hTg	EFIKSLTP-QD-F-N-Q-D-P-K-TV-AG-N-N-S-K-A-H-A-E-S
mTg	EFLQGLAL-QDSF-Q-D-P-E-G-V-SDF-G-MA-IT-T-H-Y-T-AE-A-I-AD-K-SS
ratTg	EFLQGLTL-QDSFI-Q-D-I-P-G-G-V-KSEA-EGA-MA-IT-T-H-Y-ST-AE-A-V-AD-ME-SS
cTg	FTCALYPEARVCDVSMESPCKGRLILPHRPETLFRKVKILRDKVKNFYTRLPFKLIMGISIRNKMPMSEKISNGFFECERLCCADPCCTGFGFLNVSQLTGGVETCLTLNLSLGLQTC
bTg	T-Q-DIL-R-SA-Y-V-Q-R-N-T-V-D-S-M-K
hTg	T-Q-DI-NAQ-QM-KA-E-V-R-A-K-I-M
mTg	F-Q-N-NA-N-SO-Q-TA-R-V-N-R-T-D-V-G-L-R-Q-M-I-N
ratTg	S-Q-NDV-NA-N-SO-RQ-TA-OR-V-N-R-S-DRI-L-R-MQ-M-M-I
cTg	EENMGAWRILDCGSPDTEVRTYFQWYQKPAARNADSPFCVPTTP.SLPEKVTLSGWSLAPSAAVVDSSIRNFDVAHVSTATTNDFSDARDFLCCESRHOACLVTTLRAQPGAVRCV
bTg	YG-V-VSPS-S-AL-A-T-N-A-D-L-S-IV-P-I-T-AVG-N-A-R-W-D-OT-M
hTg	G-I-H-I-Q-N-L-VL-T-S-D-L-S-V-P-H-T-A-SN-AV-L-S-Q-E-I-OT-M
mTg	SGAT-E-H-VWS-T-SAALQSLTE-SD-T-L-S-IV-P-KH-I-T-A-SN-M-Q-QQ-D-QI-V
ratTg	GAT-E-H-VWS-SAALQSLTE-A-D-T-L-S-I-P-KH-I-SA-RN-L-Q-Q-D-QI-Q-V
cTg	FYADAQICTHSLQAQNCQLLREEATHIYRKLNIPLLS.FGTSVPSVTITPHGQLLGRSQAIVQVTSNWKQVDQLGVYATPPLAESFRFAPELWNTGSHDATKPRASCSQWPGTRTLES
bTg	T-S-R-H-Y-P-PG-S-P-AT-P-A-G-K-H-EA-R-I-PTP
hTg	T-S-G-R-PG-S-YEA-P-ST-R-A-R-Q-AS-STP
mTg	P-I-N-I-RSHT-W-H-Y-SG-VQSDV-T-R-DSF-Q-G-V-K-A-YR-A-DN-V-A-PTP
ratTg	P-I-S-E-RSKT-W-H-AY-SGA-HOSD-I-T-H-DSF-Q-G-VVK-A-Y-A-N-Q-V-A-L-S-PTP
cTg	PGVDEDCLYLNVFQNVAPNASVLVFFHNTLEGRGSEGLAIDGSLYLAAGNLIVVTAGYRVGIFGLSSGSELGSMNGLLDQLAALTWQVTHIVGFQDPRRVTLAADRRGADVASI
bTg	S-M-AA-K-GDRP-V-F-V-S-T-S-VV-QA-I
hTg	S-I-MDREE-WP-F-V-S-V-V-V-V-RG-S
mTg	QIN-E-LVS-M-ME-G-Q-T-I-V-F-N-L-V-D-VA-V-S-A-Q-S
ratTg	QIS-E-LVS-V-ME-G-Q-N-I-V-N-L-V-D-VA-V-A-Q
cTg	HLLTTRAADSRLFRRAILMGGSFAFSPAVVISQGRAQEQAAALAEICGPTSSSTQELVSLCRKQPAI LNDAGTKLLAVSGPFHYWGPVVDGOYLREAPARALQRTLRAKVDLLIGSSQDD
bTg	V-N-V-L-A-RPE-RO-K-V-S-V-M-E-R-T-V-AP-V
hTg	A-TN-Q-V-L-A-HE-Q-I-K-VS-M-S-V-NV-I-HF-P-K-S-WVE
mTg	IS-PTRLQ-K-L-L-AI-PE-Q-K-V-I-V-N-L-S-R-K-P-PV-G
ratTg	I-PTRLQ-K-L-L-AI-PD-Q-K-V-N-V-F-N-E-L-S-R-K-P-PV-G
cTg	GLINRAKAVKQFEESQGRSSKTAFYQALQNSLGGEDADAGVRAAAATWYYSLEHSTDDYASFSRALENATRDYFICTPVI DMASHWARRARGHVFMHAPESYDHSLELLADVQYAFGL
bTg	A-Q-DS-Q-I-TV-S-S-T-L
hTg	R-S-R-E-I-I-A-K-N-G-G-F-L
mTg	N-S-RIL-V-I-MVN-L-T-V-G-G
ratTg	N-S-RIL-I-IVN-L-T-V-G-G
cTg	PFYVYEGQFTLEEKSLSLKIMQFFSNFVRSNGPNYPHEFSRKASELAAPWPDFVPRVTGGESYKEFSVLLPNRGLKSDCSFWAKYIQSLKAVADEAKGELSAESEEDDEPADSGLIGE
bTg	A-Y-I-R-P-F-D-A-L-K-S-S-T-DGP-D-E-Q-G-TED
hTg	A-S-Y-H-I-Y-VPTF-T-A-N-E-K-S-S-S-G-GGQ-EELT-G-RED
mTg	SA-Q-ST-Q-V-Y-I-A-F-T-I-GA-L-AQ-Q-S-T-G-DAQLTK-E-LEVGP-EED
ratTg	SA-Q-Y-ST-Q-V-Y-I-Q-A-F-T-GA-L-AQ-K-S-T-G-DAQLTK-G-E-LEVGP-SEED
cTg	PG.....SKSYSK
bTg	LLGLPELA-T
hTg	LLSLQEPG-T
mTg	LSGSLEPVP
ratTg	FSGSLEPVP

Fig. 2. (Continued).

formation. Under physiological conditions, only four tyrosines are available for iodination and hormonogenesis and these tyrosines are designated A–D [13,15,19]. These four major hormonogenic sites are highly conserved among the species. In the cTg sequence, these sites are also identified (Fig. 1). The hormonogenic site A is usually located within 30 amino acids, like as the first Tg-type 1 repeat [20,21]. Sites B–D are located at 2573, 2760 and 1309 amino acid, respectively. Analysis for internal homologies confirmed the presence of one domain, about 65 amino acids, repeated 11 times (Fig. 1). This domain, known as thyroglobulin type-1, also is described in the bovine and human Tg sequence and identified in other proteins of different origin and function [22]. It likely represents the duplication of ancestral genes [20,21], and can act as proteinase inhibitors in other proteins [22,23]. It is hypothesized that the type-1 repeats in Tg could act as pH-dependent blinders and reversible inhibitors of the proteases implicated in Tg degradation and T₄ release [24]. However, the definite function for this type of repeat in Tg has not been established yet. Sequence databases searches revealed the strongest match between cTg and acetylcholinesterase (31% identity). Acetylcholinesterase has the strongest match, with 31% identity with cTg. The native structure of the AChE-like region functions as a dimerization domain, facilitating intracellular transport of Tg to the site of thyroid hormonogenesis [25]. However, the putative function of AChE-like region in Tg is not clear, but as acetylcholinesterase interacts with cell membranes in the nervous system a similar role for the homologous domain at the carboxy-terminal end of the Tg molecule has been proposed for apical membrane interaction. In *cog/cog* mice, Tg secretion defect is associated with a single L2263P substitution that falls within the AChE homology domain [26]. Unfortunately, the mutation in *cog/cog* mice located in this domain leads to ER storage of apparently misfolded Tg molecules [26] and therefore does not directly contribute to the elucidation of the function of this domain. Patients with congenital goitrous hypothyroidism and defective Tg, as well as animal models of the human diseases, have been recognized for many years. The thyrocytes in such patients typically exhibit a dilated ER lumen [27] with little or no secreted Tg [28]. Mutations in a variety of Tg regions can result in goitrous hypothyroidism as a result of deficiency of Tg folding [16,29]. The homologous regions of cTg and acetylcholinesterase, AChE-like region, may be the target epitopes of autoimmune thyroid diseases [14,30].

In the pathogenic mechanism of autoimmune diseases, T- and B-cell epitopes have important roles. Several T-cell epitopes were reported in human and rat Tg peptides [31–34]. About B-cell epitopes, Saboori and others reported conformation-dependent epitopes [35–38]. Previously, we have identified tryptic peptides bearing non-conformational epitopes, suggesting that specific peptides, not conformational, were involved in the TgAA induction in dog as being compared with human Tg [39]. However, we have not yet analyzed these tryptic peptides, and have not enough information as to the localization of epitopes on cTg.

Presently, the predicted cDNA sequence of cTg was registered in the data bank from canine genome project (XM_539168). We compared it with our complete cDNA sequence. In the analysis results, although there was 99% homology between two sequences, we found several missing and varying amino acids sequences (data not shown). We could not determine whether these unmatched sequences were simple analytic errors or not. But, the present complete cDNA sequence was analyzed directly by RT-PCR method from mRNA. However, it is believed that these two sequences from different sources could provide available information about mutation, substitution or deletion on cTg.

In conclusion, we analyzed the complete primary structure of cTg, deducing it from the cDNA sequence, and to compare it with human and other Tg sequences to identify canine specific areas. Defects in the structure of cTg are considered linked to the existence of hereditary thyroid diseases in dog, and knowledge of the primary structure of cTg and the corresponding gene facilitates the development of studies on the structural bases of defects in Tg production. These results could be useful to further research about mutations of cTg, epitopes mapping associated with autoimmune thyroid diseases and structure–function relationship on cTg.

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—Research Note—

Expression of Uterine Sensitization-Associated Gene-1 (USAG-1) in the Mouse Uterus During the Peri-Implantation Period

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Abstract. Rat uterine sensitization-associated gene-1 (USAG-1) mRNA is expressed in the uterus during the peri-implantation period, and its mRNA expression in uterine epithelial cells is highest on day 5 of pregnancy. On the other hand, since changes in USAG-1 mRNA expression in the mouse uterus are not seen during the estrous cycle, USAG-1 expression might be specifically regulated by embryonic factors rather than by the maternal environment. However, the expression pattern and function of USAG-1 in the mouse uterus have not been determined. Thus, we examined the tissue-specific USAG-1 mRNA expression in the uteri of ICR mice during peri-implantation using real-time quantitative PCR. Uterine tissues, such as the myometrium, luminal epithelium, and stroma, were collected by laser capture microdissection at 3.5–6.5 dpc. USAG-1 mRNA was expressed in the uteri of pregnant mice from 3.5 dpc to 6.5 dpc, and the highest level of expression was seen at 4.5 dpc ($P < 0.01$). Significantly high USAG-1 mRNA expression was detected in the luminal epithelium at 4.5 dpc ($P < 0.05$). The stroma and myometrium exhibited unchanged expression levels of USAG-1 mRNA at 3.5–5.5 dpc. USAG-1 mRNA was undetectable in blastocysts and implanting embryos. Expression of USAG-1 mRNA appears to be associated with blastocyst implantation to the luminal epithelium, suggesting that physiological or biochemical contact of the blastocyst to the uterus is required for USAG-1 expression.

Key words: Implantation, Mouse, mRNA, Uterine sensitization-associated gene-1 (USAG-1), Uterus (J. Reprod. Dev. 53: 931–936, 2007)

For successful establishment of pregnancy, blastocyst development must proceed beyond the attachment stage and result in endometrial decidualization and subsequent placental formation. Estrogen and progesterone regulate the events that lead to the receptivity and sensitization of the endometrium for the decidual cell reaction [1]. In rodents and humans, implantation is

initiated with an apposition between the trophoblast and apical surface of the luminal epithelium. This is followed rapidly by adhesion of the conceptus to the luminal surface and then penetration through the luminal epithelium to the underlying stroma, which initiates the endometrial decidualization reaction [2, 3]. Uterine sensitization-associated gene-1 (USAG-1), a gene of unknown function during conceptus development, is upregulated in the sensitized endometrium of the rat uterus during the peri-implantation period [4].

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This molecule is expressed as an antagonist to bone morphogenetic protein (BMP) in teeth [5] and the kidney renal tubules [6] during late embryogenesis. The homology of rat USAG-1 cDNA to those of mouse and human is 96% and 91%, respectively [7]. We previously identified USAG-1 mRNA expression in mouse uteri during the estrous cycle [8]. In contrast to the temporally regulated expressions of COX-1, COX-2, EGF, LIF, and other growth factors/cytokines, USAG-1 mRNA expression did not change during the estrous cycle [8]. However, the expression pattern and function of USAG-1 in the mouse uterus during the peri-implantation period have not been studied. Thus, we examined tissue-specific expression of USAG-1 mRNA in pregnant uteri and blastocyst stage embryos following dissection of various uterine tissues through laser microdissection.

Materials and Methods

Animals

ICR and (C57BL/6J × C3H/HeN)F1 mice at 8–10 weeks of age were purchased from a commercial supplier (CLEA Japan, Tokyo, Japan) and maintained in the animal facility of the National Research Center for Protozoan Diseases at Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan. All animals were housed in

polycarbonate cages and maintained in a specific pathogen-free environment in light-controlled (lights on from 07:00 to 19:00) and air-conditioned rooms (temperature of 24 ± 1 C, humidity of $50 \pm 10\%$). They had access to standard laboratory chow (CE-2; CLEA Japan) and water *ad libitum*. The animals used in this study were cared for and used under the Guiding Principles for the Care and Use of Research Animals set forth by the Obihiro University of Agriculture and Veterinary Medicine.

Natural mating and tissue and embryo collection

Female mice were mated with fertile ICR males or vasectomized B6C3F1 males. The morning a vaginal plug was discovered was designated 0.5 days post coitum (dpc). Plug-positive females were sacrificed by cervical dislocation at 3.5–6.5 dpc. Five animals were sacrificed each day. The uteri of the animals were excised immediately immersed into liquid nitrogen, and stored at -80 C until use. For total RNA extraction from blastocysts, 200 blastocysts were collected from the uteri of naturally mated females at 3.5 dpc, immediately immersed in liquid nitrogen, and stored at -80 C until use.

Laser capture microdissection (LCM) of uterine tissues

Frozen uterine tissue from pregnant and pseudopregnant animals were embedded in OCT

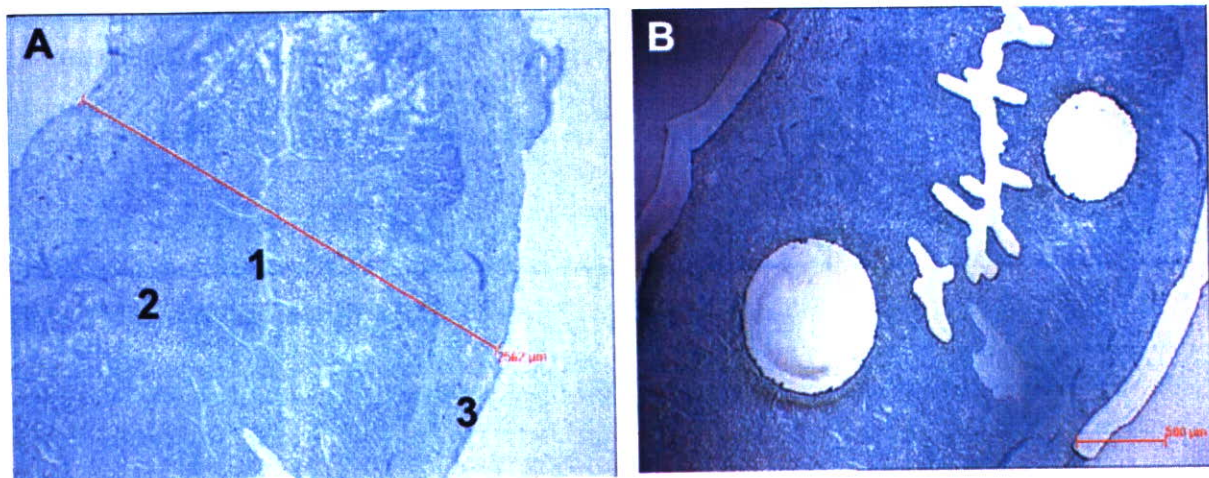


Fig. 1. Laser capture microdissection (LCM) of uterine tissues. LCM was performed using an AS LMD laser microdissection system (Leica) to separately collect attached blastocyst, myometrium, luminal epithelium and stromal tissue. The collected tissues were subjected to real-time quantitative PCR for detection of USAG-1 mRNA expression. A: before LCM. B: after LCM. 1. Luminal epithelium. 2. Stroma. 3. Muscle.

Table 1. Primer/probe sequence used for real-time quantitative PCR

Transcript	Primer/probe sequence (5' to 3')	GenBank accession number
USAG-1	F GAGGCAGGCATTTCAGTAGCA	NM_025312
	R TGTATTGGTGGACCGCAGTT FAM-TCGAAACAGTCGAGTTCA-MGB	
Beta-Actin	F GCTCTGGCTCCTAGCACCAT	NM_007393
	R GCCACCGATCCACACAGAGT FAM-ATCAAGATCATTGCTCCTC-MGB	
18S rRNA	VIC-ABI (Part No. 4308329)	
GAPDH	F ABI (Cat No. 430448604022)	
	R ABI (Cat No. 430449004024) VIC-ABI (Part No. 4308313)	
	F ABI (Cat No. 0412021)	
	R ABI (Cat No. 430410906026)	

compound (Tissue-Tech, Sakura, Tokyo, Japan). Serial cryostat sections were cut to 12–16 μm in thickness and mounted on autoclaved polyethylene naphthalate (PEN) foil on a glass slide (Leica, Wetzlar, Germany). The slides were fixed in 70% ethanol : acetic acid (19:1) for 3 min and briefly rinsed with RNase-free DEPC-treated water for 1 min. They were then stained with 0.05% toluidine blue (Merk, Darmstadt, Germany) dissolved in RNase-free DEPC-treated water for 1 min at room temperature and washed twice with RNase-free DEPC-treated water for 1 min each. The slides were then air-dried for 5 min and stored at -80 C .

LCM was performed using an AS LMD laser microdissection system (Leica) to collect the attached blastocyst, myometrium, luminal epithelium, and stromal tissues separately [9]. PEN foil slides were mounted on an AS LMD laser microdissection system with the section facing downwards. After adjusting the intensity, aperture, and cutting velocity, the pulsed UV laser beam was carefully directed along the borders of the cell layer (Fig. 1). A 20x objective was used for the laser microdissection system along with the following settings: intensity was set to 45, aperture was set to 10, speed was set to 6, and offset was set to 39. The separated tissues were transferred by gravity alone into a microcentrifuge tube cap containing TRI reagent (Sigma, St. Louis, MO, USA) that was placed directly underneath the sections.

Real-time quantitative PCR

Total RNA was extracted from frozen reproductive tissues and blastocysts by means of a TRI Reagent Kit (Sigma) according to the

manufacturer's protocol. The extracted total RNA was then subjected to real-time PCR analysis. Primers and a TaqMan probe for the USAG-1 gene and beta-actin [8] were designed using the primer design software Primer Express version 1.5 (Applied Biosystems, Foster City, CA, USA). Primers and a TaqMan probe for 18S rRNA and GAPDH were purchased from a commercial supplier (Applied Biosystems). The GenBank Accession numbers of all the cDNA sequences are summarized in Table 1. Quantification of all gene transcripts was carried out with an ABI Prism 7900 HT (Applied Biosystems). Templates for real-time PCR were obtained by reverse transcriptase reaction using total RNA. For the RT-PCR reaction, a TaqMan One-Step RT-PCR Master Mix Reagents Kit (Applied Biosystems) was used with 20 μl /tube as follows: the template (20 ng) was mixed with 2 \times Master Mix without UNG, 40 \times MultiScribe and RNase Inhibitor Mix, 200 nM TaqMan Probe, and 900 nM of each primer. The reaction conditions were 1 cycle at 48 C for 30 min, 1 cycle at 95 C for 10 min, and then 45 cycles of the amplification step (95 C for 15 seconds and 60 C for 1 min). The gene expression levels of USAG-1 were calculated as gene expression rates, as reported previously [10]. Briefly, the amounts of USAG-1, 18S rRNA, GAPDH and beta-actin mRNA in the samples were estimated with standard curves representing the log of the input amount (the log starting with cDNA molecules) as the X axis and the threshold cycle as the Y axis. A relative standard curve (SC) for real-time PCR was used as a common set of samples that linked the experimental PCR plates together and permitted overall analysis of the

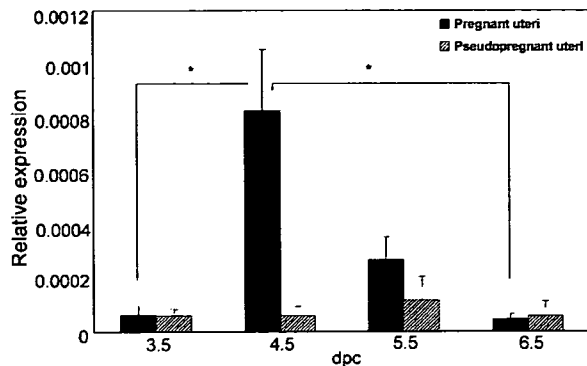


Fig. 2. Expression pattern of USAG-1 mRNA in the uteri of pregnant and pseudopregnant mice. Total RNA was extracted from the uteri of pregnant and pseudopregnant mice and subjected to real-time PCR analysis. The results are shown as the mean \pm SE. Asterisks above the error bar indicate significant difference between the experimental groups. *: $P < 0.05$.

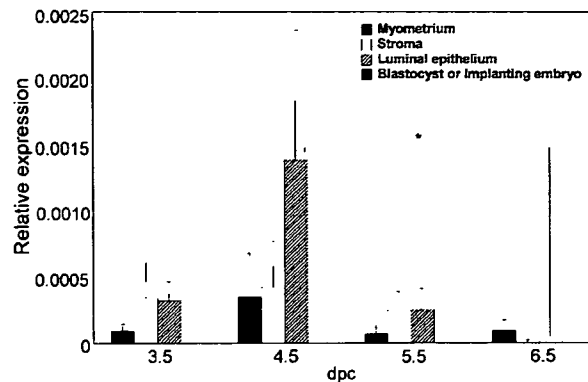


Fig. 3. Expression pattern of USAG-1 mRNA in the uterine tissues, blastocysts, and implanting embryos of pregnant mice. An LCM was used to separately collect uterine tissue samples from pregnant mice, and then total RNA was extracted from the tissue samples and subjected to real-time PCR analysis. The results are shown as the mean \pm SE. Asterisks indicate significant differences between the experimental groups. *: $P < 0.05$. The expression level of USAG-1 mRNA in the luminal epithelium at 4.5 dpc was significantly higher than that at 6.5 dpc ($P < 0.05$).

samples. Preparation and utilization of this SC as a quality control of the efficiency of amplification of the PCR plate is described elsewhere [11]. The gene expression rate was obtained by normalizing the amount of USAG-1 with that of a housekeeping gene. Kidney mRNA was used as the standard.

Statistical analysis

All data are expressed as the mean \pm standard error (SE). Statistical comparisons of the relative mRNA expressions of each gene between experimental groups were analyzed by two-way analysis of variance (ANOVA) followed by a *post hoc* test using StatView 4.5 (Abac Concepts, Berkeley, CA, USA). In all statistical tests, differences were considered significant when P was < 0.05 .

Results

Expression profiling of housekeeping genes in the pregnant uteri of the mice

The geNorm software (<http://medgen.ugent.be/jvdesomp/genom>) was used to select the most appropriate housekeeping gene as an internal control [12]. The internal control gene-stability

measure (M) was calculated using geNorm. Since the M values of beta-actin, 18S rRNA, and GAPDH in the pregnant uteri were 4.134, 4.823 and 7.527, respectively, beta-actin was selected as the internal control gene for further experiments.

Expression pattern of USAG-1 mRNA in the uteri of the pregnant and pseudopregnant mice during the peri-implantation period

As shown in Fig. 2, USAG-1 mRNA expression in the uteri of the pregnant mice dramatically increased from 3.5 dpc to 4.5 dpc ($P < 0.05$) and subsequently declined at 5.5 and 6.5 dpc ($P < 0.05$). Although the differences were not statistically significant, the expression level of USAG-1 mRNA in the pregnant uterus was higher than that in the pseudopregnant uterus at 4.5 dpc. This difference may have been caused by the presence or absence of embryos in the reproductive tract.

Expression of USAG-1 mRNA in the myometrium, stroma, and luminal epithelium tissue of pregnant uteri during the peri-implantation period and in blastocysts and implanting embryos

The combination of LCM and real-time quantitative PCR revealed that the significant increase in USAG-1 mRNA seen in the pregnant

uterus at 4.5 dpc was localized mainly to the luminal epithelium (Fig. 3). On the other hand, the uterine stroma and myometrium exhibited minimal changes in USAG-1 mRNA expression at 3.5–5.5 dpc. As shown in Fig. 3, USAG-1 mRNA expression was undetectable in blastocysts collected from the uterus at 3.5 dpc and implanted embryos at 4.5, 5.5 and 6.5 dpc.

Discussion

USAG-1 expression in the reproductive tract was first found in the rat [4]. Induction of USAG-1 mRNA is restricted to the uterine glandular epithelial cells of the pregnant and pseudopregnant uteri on Day 5 [4]. Given the remarkably tight localization of its expression, USAG-1 might be involved in the onset of endometrial receptivity for implantation. It has been demonstrated that the expression of implantation-associated genes is influenced by ovarian hormones such as estrogen and progesterone [1]. We have previously examined the expression pattern of 16 implantation-associated genes, including USAG-1, during the estrous cycle in mice [8]. Expression of the majority of genes involved in the implantation process is temporally regulated during the estrous cycle [8]. However, interestingly, USAG-1 mRNA expression does not change during the estrous cycle in mice [8]. The results from these reports suggest that USAG-1 expression could be regulated by an embryonic signal(s) rather than an ovarian hormone(s).

The present study revealed that USAG-1 mRNA was expressed in both pregnant and pseudopregnant uteri throughout the peri-implantation period, i.e., from 3.5 dpc to 6.5 dpc, and was upregulated in pregnant animals on 4.5 dpc (Fig. 2). Furthermore, USAG-1 mRNA was localized exclusively in the luminal epithelium of

the pregnant uteri at 4.5 dpc (Fig. 3). The expression of USAG-1 in blastocysts at 3.5 dpc and implanting embryos at 4.5 and 5.5 dpc was not detected by analysis using real-time quantitative PCR (Fig. 3). Furthermore, USAG-1 expression was not upregulated in pseudopregnant uteri during the peri-implantation period. These results indicate that the presence of embryos and/or a factor(s) from the embryos were an essential for upregulation of USAG-1 expression in the luminal epithelium at implantation in the mice. Stimuli from implanting embryos may influence uterine endometrial tissues and subsequently upregulate USAG-1 gene expression. We believe that blastocysts make their first molecular contact with the luminal epithelium for establishment of implantation and decidualization in conjunction with the expression of USAG-1. It is known that Mif is released from the luminal epithelium and superficial glandular epithelium by IFN-tau, which is produced by trophoblast cells in the cow [13], and that the epithelial cell-derived secreted adhesion proteins are regulated by progesterone and/or IFN-tau in sheep [14]. Although mice deficient in USAG-1 appear to be fertile [15] and the physiological function/s of USAG-1 for uterine receptivity and the process of implantation are still unclear, further studies using of gene targeting should clarify the role of USAG-1 in mammalian implantation.

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Effect of pressure at primary drying of freeze-drying mouse sperm reproduction ability and preservation potential

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Abstract

Freeze-dried spermatozoa are capable of participating in normal embryonic development after injection into oocytes and thus useful for the maintenance of genetic materials. We recently reported that long-term preservation of freeze-dried mouse spermatozoa by conventional methods requires temperatures lower than -80°C . Successful permanent preservation of mouse spermatozoa at much higher temperatures requires thorough investigation of the freeze-drying procedure. Thus, we examined the relationship between the pressure at primary drying and the preservation potential of freeze-dried mouse spermatozoa. Three different primary drying pressures were applied to evaluate the effect of pressure on freeze-dried spermatozoa under varying storage conditions and the rate of development measured. The developmental rate of embryos to the blastocyst stage from intracytoplasmic sperm injection by freeze-dried spermatozoa at pressures of 0.04, 0.37, and 1.03 mbar without storage were 59% (337/576), 71% (132/187), and 33% (99/302) respectively. When stored at 4°C for 6 months, the rate was 13% (48/367), 50% (73/145), and 36% (66/182) respectively. These results show that primary drying pressure is an influential factor in the long-term preservation of freeze-dried mouse spermatozoa.

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Introduction

In the last decade, numerous reports have shown that freeze-dried mouse spermatozoa are capable of participating in normal embryonic development after injection into oocytes (Wakayama & Yanagimachi 1998, Kusakabe *et al.* 2001, Kaneko *et al.* 2003a,b, Ward *et al.* 2003, Kawase *et al.* 2005). Freeze-dried mouse spermatozoa are most efficiently stored for extended periods, several to tens of decades, at temperatures lower than -80°C (Kawase *et al.* 2005). However, maintaining such low temperatures over a long period of time puts the samples at risk of loss from technical difficulties (e.g. power failure) and requires a relatively high initial investment. Although freeze-dried spermatozoa can be stored for 1.5 years (Ward *et al.* 2003), this length of time is insufficient for either the maintenance of genetically modified mouse strains or mutant mice in saturation mutagenesis projects. Long-term preservation at ambient temperatures would be ideal. The freeze-drying process is of importance, especially the primary drying process; however, no study of pressure at primary drying of

spermatozoa has been reported. Here, we focused on the pressure at primary drying and found 0.37 mbar to be the optimum pressure for preservation of freeze-dried mouse spermatozoa at much higher temperatures.

Materials and Methods

Animals

F1 (B6C3F1) mice were purchased from Clea Japan (Tokyo, Japan). All the mice were housed in polycarbonate cages and maintained under a specific pathogen-free environment in light-controlled lights on from 0500 to 1900 h and air-conditioned rooms (temperature, $24 \pm 1^{\circ}\text{C}$; humidity, $50 \pm 10\%$). The mice had free access to standard laboratory chow (CE-2, Clea Japan). The Institutional Animal Care and Use Committee of Chugai Pharmaceutical reviewed the protocols and confirmed that the animals used in this study were cared for and used under the Guiding Principles for the Care and Use of Research Animals promulgated by Chugai Pharmaceutical.

Freeze-drying and preservation of spermatozoa

The procedure for freeze-drying was essentially the same as that described by Kaneko *et al.* (2003a) and Kawase *et al.* (2005). The six epididymides from three B6C3F1 male mice were removed and a dense sperm mass was squeezed out of each cauda epididymis from a cut made with scissors. The total sperm mass was gently placed in 9 ml EGTA Tris-HCl-buffered solution (50 mM EGTA, 50 mM NaCl, and 10 mM Tris-HCl, pH 8.0; Kaneko *et al.* 2003a) in a tube (352059, Becton Dickinson Labware, NJ, USA) and kept at 37 °C for 10 min. The sperm suspension at a concentration of $15\text{--}38 \times 10^6$ cells/ml was divided into 18 aliquots. Each aliquot of 500 μ l was put into an amber vacuum vial for freeze-drying (V-2B, Nichiden-rika Glass Co. Ltd., Kobe, Japan). The vials were plunged into liquid nitrogen (~ -196 °C) for 5 min and then transferred to a programmable freeze-dryer (BETA2-16, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany), which had been pre-cooled to -30 °C. The freeze-drying conditions consisted of primary drying at a pressure of 0.04 mbar for 8 h, 0.37 mbar for 13 h, or 1.03 mbar for 13 h and secondary drying at a pressure of 0.001 mbar for 6 h. More time is required for complete drying when lower pressures are provided at primary drying. The inside pressure of the vials at the time of sealing was 0.001 mbar and pressure reduction was within 5 min. The vials were stored at 4 or 30 °C until use. Immediately before ICSI, the vials of freeze-dried spermatozoa were unsealed, and the spermatozoa were hydrated by adding 500 μ l sterile distilled water. To maintain a similar composition of sperm suspension before and after freeze-drying, distilled water was added rather than a medium such as Hepes-buffered culture medium.

Comet assay for DNA damage

DNA damage of the spermatozoa from freeze-drying and subsequent preservation was assessed by single-cell gel electrophoresis (comet assay; Hughes *et al.* 1997, Steel *et al.* 1999, Cho *et al.* 2003). Evaluation of the shape of the DNA 'comet' tail and migration pattern gives an assessment of DNA damage. The sperm suspension was suspended in Comet LMAgarose (1% low-temperature melting agarose, Trevigen, Gaithersburg, MD, USA) at a ratio 1:10 (v/v). With the addition of molten LMAgarose at 37 °C, 25 μ l sperm suspension was immediately placed on a CometSlide (Trevigen). The slides were placed flat in a refrigerator at 4 °C for 10 min and then submerged in 23 ml lysis solution (Trevigen) at 4 °C for 60 min. Next, 2.5 ml 10 mM dithiothreitol was added and the slides were then incubated for 30 min at 4 °C, followed by the addition of 2.5 ml of 4 mM LIS (lithium diiodosalicylate) and incubation for 90 min at room temperature. The slides were then kept in an alkaline solution ($> \text{pH } 13$) for 20 min at room temperature in the

darkness. The slides were subjected to electrophoresis in $1 \times \text{TBE}$ (Tris-borate EDTA) buffer at 25 V for 10 min, stained with SYBR Green (Trevigen), and analyzed under a microscope (IX-70, Olympus Co., Tokyo, Japan). DNA damage of freeze-dried spermatozoa was assessed by Comet assay twice per experimental group. Comet tail length is the distance the damaged DNA has migrated from the sperm head and gives an estimate of the extent of the damage.

Preparation of oocytes

Mature B6C3F1 females were induced to superovulate by i.p. injections of 5 IU equine chorionic gonadotropin (Serotrophin, Teikokuzoki Co., Tokyo, Japan) and 5 IU human chorionic gonadotropin (hCG; Puberogen, Sankyo Co., Tokyo, Japan) 48 h later. Freshly ovulated oocytes were collected from oviducts 15–16 h after being injected with hCG. The oocytes were treated with 0.1% hyaluronidase (280 units/mg; H-3506, SIGMA Chemical Co.) in Whitten's medium (Whitten 1971) supplemented with 100 μ M ethylene diamine tetraacetic acid disodium salt (EDTA; Abramczuk *et al.* 1977) to remove cumulus cells.

Intracytoplasmic sperm injection

After rehydration of the freeze-dried spermatozoa as described above, one part of the sperm suspension was thoroughly mixed with nine parts 0.9% NaCl solution (saline) containing 12% (w/v) polyvinyl pyrrolidone (PVP, No. 99219, Mt. 360 000, Irvine Scientific, Santa Ana, CA, USA). Two drops (~ 5 μ l each) of 12% PVP saline and two drops of 20 mM Hepes-buffered Whitten's medium containing 0.1% polyvinyl alcohol (PVA, P-8136, MW 30 000–70 000, Sigma) were linearly placed on the injection chamber (Kawase *et al.* 2001) and then covered with mineral oil (M-8410, embryo tested, Sigma). The first drop of 12% PVP saline was used to wash the injection pipette, and added to the second drop was 1–2 μ l of the diluted sperm suspension. The first drop of the medium was used to remove spermatozoa that had attached to the surface of the injection pipette. The cumulus-free oocytes were placed in the second drop of Hepes-buffered Whitten's medium. The injection chamber with the spermatozoa and oocytes was transferred onto the stage of an inverted microscope maintained at ~ 18 °C (MATS-555RSP, Tokai Hit, Shizuoka, Japan).

The procedure for micromanipulation of the sperm for ICSI was essentially the same as that described previously (Kimura & Yanagimachi 1995, Kawase *et al.* 2001). The sperm head was separated from the tail by applying three or four piezo pulses (controller setting: speed 2, intensity 2) to the head–tail junction of the spermatozoon. In the same manner, a total of 3–5

Table 1 Effect of vacuum pressure at primary drying on the *in vitro* development of embryos generated by ICSI of freeze-dried, non-stored spermatozoa.

Vacuum pressure (mbar)	No. of oocytes injected	No. (%) of oocytes survived	No. (%) of oocytes fertilized ^a	No. (%) of embryos developed to two-cell ^b	No. (%) of embryos developed to blastocyst ^b
0.04	864	608 (70) ^a	576 (95) ^a	557 (97) ^a	337 (59) ^a
0.37	253	194 (77) ^{ab}	187 (96) ^a	181 (97) ^a	132 (71) ^b
1.03	404	317 (78) ^b	302 (95) ^a	288 (95) ^a	99 (33) ^c

Different superscript letters within a column indicate significantly different values ($P < 0.05$).

^aPercentage of oocytes survived. ^bPercentage of oocytes fertilized.

isolated sperm heads were lined up in the pipette. Several piezo pulses (controller setting: speed 2, intensity 2) were applied to advance the tip of the injection pipette to the surface of zona pellucida; the pipette was advanced mechanically while applying slightly negative pressure. The oolemma was punctured using 1–2 piezo pulses (controller setting: speed 1, intensity 1). A single sperm head was then expelled into the ooplasm accompanied with a minimum amount of medium. Following retrieval of as much as possible of the medium, the injection pipette was withdrawn while applying negative pressure to the pipette.

Culture of oocytes and embryos transfer

Sperm-injected oocytes were incubated and cultured in Whitten's medium supplemented with 100 μ M EDTA at 37.5 °C in 5% CO₂ and 95% air. After 6-h ICSI, live oocytes showing two distinct pronuclei and a second polar body were considered fertilized. The fertilized eggs were further cultured in Whitten's medium supplemented with 100 μ M EDTA for 96 h at 37.5 °C in 5% CO₂ 95% air. About 96 h after ICSI, the blastocysts were transferred into the uteri of pseudopregnant ICR recipients (CLEA Japan) 2.5 days *postcoitum* (dpc) using the embryo transfer method described by Suzuki *et al.* (1994). The recipient females were killed on 18.5 dpc to determine the number of implantation sites by macroscopic check and the number of term fetuses.

Statistical analysis

Data presented in this study were analyzed statistically by the χ^2 -test and Tukey's test for non-parametric multiple comparisons (SAS version 6.12, SAS Institute, Cary, NC, USA). In all statistical tests, the difference was considered significant when $P < 0.05$.

Table 2 Effect of vacuum pressure at primary drying on the *in vitro* development of embryos generated by ICSI of freeze-dried spermatozoa stored at 30 °C for 3 days.

Vacuum pressure (mbar)	No. of oocytes injected	No. (%) of oocytes survived	No. (%) of oocytes fertilized ^a	No. (%) of embryos developed to two-cell ^b	No. (%) of embryos developed to blastocyst ^b
0.04 ^c	251	175 (70) ^a	170 (97) ^a	162 (95) ^a	34 (20) ^a
0.37	161	117 (73) ^a	113 (97) ^{ab}	111 (98) ^a	61 (54) ^b
1.03	187	145 (78) ^a	132 (91) ^b	129 (98) ^a	25 (19) ^a

Different superscript letters within a column indicate significantly different values ($P < 0.05$).

^aPercentage of oocytes survived. ^bPercentage of oocytes fertilized. ^cData from Kawase *et al.* (2005).

Results

The developmental rates to the blastocyst stage of embryos from ICSI by freeze-dried spermatozoa without storage, with storage at 30 °C for 3 days, and at 4 °C for 6 months are shown in Tables 1–3. All the three storage conditions showed the highest rate of embryonic development when primary drying was performed at 0.37 mbar. In addition, the number of live-term fetuses produced was higher at 0.37 mbar than at other pressures for groups non-stored and stored at 4 °C for 6 months although the difference was not significant (Table 4). All fetuses were morphological normal – some of the mice have since been mated and delivered normal offspring. A numerical indicator for overall efficiency of mouse production after ICSI using freeze-dried mouse spermatozoa was determined by dividing the ratio of the number of embryos that developed blastocysts to the number of fertilized oocytes, by the ratio of the number of live-term fetuses to the number of blastocysts transferred (Fig. 1). With storage at 4 °C for 6 months, the overall efficiency from a pressure of 0.37 mbar (10.3) or 1.03 mbar (8.8) was significantly higher than 0.04 mbar (1.1). Moreover, with a pressure of 0.37 mbar (25.7) non-stored, the overall efficiency after ICSI was significantly higher than for 0.04 mbar (17.5) or 1.03 mbar (6.62). The results showed that blastocyst formation was significantly higher when primary drying was performed at 0.37 mbar than at 0.04 and 1.03 mbar irrespective of the storage conditions (non-stored, 30 °C for 3 days, or 4 °C for 6 months; Tables 1–3, Fig. 1).

Percentages of spermatozoa with comet tails freeze-dried at 4 °C for 6 months were 100 and 96%, and stored at 30 °C for 3 days were 4 and 100% respectively. Although there was no significant difference between the percentages of spermatozoa with damaged DNA at 0.04 mbar stored at 30 °C for 3 days and of those without

Table 3 Effect of vacuum pressure at primary drying on the *in vitro* development of embryos generated by ICSI of freeze-dried spermatozoa stored at 4 °C for 6 months.

Vacuum pressure (mbar)	No. of oocytes injected	No. (%) of oocytes survived	No. (%) of oocytes fertilized ^a	No. (%) of embryos developed to two-cell ^b	No. (%) of embryos developed to blastocyst ^b
0.04 ^c	522	404 (77) ^a	367 (91) ^a	346 (94) ^a	48 (13) ^a
0.37	213	156 (73) ^a	145 (93) ^{ab}	142 (98) ^a	73 (50) ^b
1.03	267	187 (70) ^a	182 (97) ^b	179 (98) ^a	66 (36) ^c

Different superscript letters within a column indicate significantly different values ($P < 0.05$).

^aPercentage of oocytes survived. ^bPercentage of oocytes fertilized. ^cData from Kawase *et al.* 2005.

Table 4 Effect of vacuum pressure at drying on the *in vivo* development of embryos generated by ICSI using freeze-dried spermatozoa.

Vacuum pressure (mbar)	Storage temperature (°C)	Sperm storage time (months)	No. of blastocysts transferred	No. (%) of implantation sites	No. (%) of live-term fetuses
0.04	RT	non-stored	194	137 (71) ^a	58 (30) ^a
0.37	RT	non-stored	132	93 (70) ^a	48 (36) ^a
1.03	RT	non-stored	99	70 (71) ^a	20 (20) ^a
0.04	4	6	48	28 (58) ^a	4 (8) ^a
0.37	4	6	73	39 (53) ^a	15 (21) ^a
1.03	4	6	66	56 (85) ^b	16 (24) ^a

Values within a column with the same superscript are not significantly different ($P > 0.05$).

storage, developmental rates to the blastocyst stage after ICSI was significantly reduced with storage. In contrast, spermatozoa freeze-dried at 0.37 mbar and then assessed immediately (non-stored) or after storage at 4 °C for 6 months did not have comet tails (Fig. 2, Table 5). At the primary drying pressure of 0.04 mbar, the average comet tail length of spermatozoa stored at 30 °C for 3 days and at 4 °C stored for 6 months was 0.7 ± 3.4 and 20.2 ± 5.0 μm ($P < 0.05$), and at 1.03 mbar, average tail length was 23.4 ± 6.2 and 23.0 ± 8.4 μm ($P > 0.05$) respectively (Table 5).

Discussion

Preservation of freeze-dried spermatozoa for extended periods of time, potential 100 years or more, by current methods would require storage temperatures lower than -80 °C (Kawase *et al.* 2005). Successful long-term preservation of mouse spermatozoa at higher temperatures requires investigation and modification of the current freeze-drying method. Although the primary drying phase is one of the most important processes in the freeze-drying process of spermatozoa, there have been no studies on the primary drying pressure. Nail *et al.* (2002) reported that the pressure in the freeze-dryer has to be lower than the vapor pressure of ice at the temperature of the product, and pressures of 0.04, 0.37, and 1.03 mbar are appropriate for materials stored at -50 , -30 , and -20 °C respectively. Thus, in this study, we selected primary drying pressures of 0.37 and 1.03 mbar, in addition to the commonly used 0.04 mbar. Primary drying is characterized by the specimen undergoing rapid shrinkage as the ice sublimates (ice forming water vapor and leaving the

specimen). In this study, ~98% of the total volume of water (0.51 g wet weight) was lost. During this step, evaporative cooling keeps the temperatures low. When most ice has sublimed, heat is no longer lost by evaporative cooling and the temperature of the product usually increases sharply toward shelf temperature. Since the driving force for freeze-drying is the vapor pressure of the ice, from the standpoint of process efficiency, it is important to keep the product temperature as high as possible during primary drying (Nail *et al.* 2002). As we reported recently, freeze-dried sperm kept at a pressure of 0.04 mbar and then stored at 30 °C for 3 days or at 4 °C for 3 months had the same rate of blastocyst formation (Kawase *et al.* 2005). Although a pressure of 0.37 mbar gave the best results when

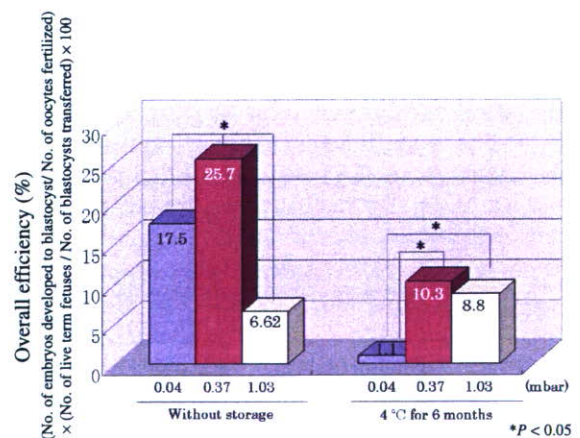


Figure 1 Overall efficiency of mouse production from ICSI using freeze-dried spermatozoa. Sperm were dried at 0.04 mbar (blue bar), 0.37 mbar (purple bar), or 1.03 mbar (yellow bar) and then used for ICSI non-stored (left) or after storage at 4 °C for 6 months (right).

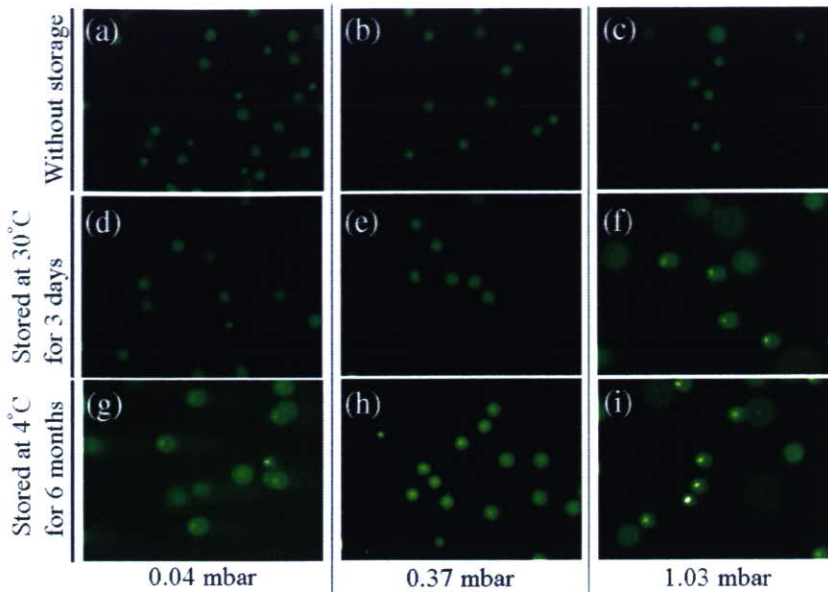


Figure 2 Comet assay of freeze-dried spermatozoa. Microscopy of spermatozoa freeze-dried at 0.04 mbar (left column), 0.37 mbar (middle column), or 1.03 mbar (right column), with assessment immediately (top row), after storage at 30 °C for 3 days (middle row) or after storage at 4 °C for 6 months (bottom row). The presence of comet tails (halo effect) in (f), (g), and (i) indicates the presence of fragmented DNA. Original magnification $\times 200$.

compared with 0.04 and 1.03 mbar, a decrease in developmental rates to blastocysts and live born was seen after storage, compared with freshly freeze-dried samples (Tables 1–4). When comparing sperm dried using three pressures, it seems that sperm dried at 0.04 mbar deteriorate significantly during storage both at 30 and 4 °C ($P < 0.05$); sperm dried at 0.37 mbar lowers its potentials similarly during storage at 30 and 4 °C ($P < 0.05$); and sperm dried at 1.03 mbar deteriorate slightly at 30 °C ($P < 0.05$), but maintains its reproductive potentials when stored at 4 °C ($P > 0.05$). Although precise mechanisms affecting storage potential in pressure at primary drying of freeze-drying mouse spermatozoa was not clarified in this study, differences of an alteration of tertiary structure in seminal proteins by freeze-drying might influence their potential (Jeyendran *et al.* 1983). Sperm freeze-dried at 1.03 mbar yielded the same proportion of development to blastocysts and live born after transfer when injected immediately after preservation (no storage) and after 6 months storage (Tables 1, 3, and 4). However, overall efficiency at

1.03 mbar was not significantly different between the experimental groups.

Comet assay, the evaluation of the shape of the DNA 'comet' tail and migration pattern, is widely used to measure DNA damage in many different cell types, including sperm from laboratory animals and humans (Haines *et al.* 1998). In this study, we performed a comet assay using alkaline electrophoresis to measure single-stranded DNA breaks and alkali-labile sites to assess the DNA integrity of individual freeze-dried sperm. Assessment of DNA damage is determined by the percentage of spermatozoa with tails and the average comet tail length. Comet tails of substantial average length were detected by comet assay at 0.04 and 1.03 mbar stored at 4 °C for 6 months and at 1.03 mbar stored at 30 °C for 3 days (Table 5), suggesting that storage induces DNA damage in freeze-dried spermatozoa.

The overall efficiency determined from the data in this study clearly indicates that the pressure at primary drying is an important factor affecting the outcome after storage. Modifications to the freeze-drying method, with

Table 5 DNA damage (comet assay) of spermatozoa after freeze-drying and subsequent preservation.

Primary pressure	0.04 mbar		0.37 mbar		1.03 mbar		Fresh	
	No. of sperm with tail/total (%)	Average of tail length (μm)	No. of sperm with tail/total (%)	Average of tail length (μm)	No. of sperm with tail/total (%)	Average of tail length (μm)	No. of sperm with tail/total (%)	Average of tail length (μm)
Without storage	0/56 (0) ^a	0	0/62 (0) ^a	0	0/55 (0) ^a	0	0/76 (0) ^a	0
Stored at 30 °C for 3 days	2/55 (4) ^a	0.7 \pm 3.4 ^a	0/54 (0) ^a	0	50/50 (100) ^b	23.4 \pm 6.2 ^a	–	–
Stored at 4 °C for 6 months	55/55 (100) ^b	20.2 \pm 5.0 ^b	0/55 (0) ^a	0	49/51 (96) ^b	23.0 \pm 8.4 ^a	–	–
Stored at 37 °C for over 1 week	–	–	–	–	–	–	63/64 (98) ^b	32.7 \pm 6.8

Different superscript letters between same primary pressure indicate significantly different values ($P < 0.05$).

attention to the pressure at primary drying, will contribute to the success of permanent preservation of mammalian spermatozoa stored at higher temperatures.

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A Rapid Agglutination Assay for Canine Brucellosis Using Antigen Coated Beads

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ABSTRACT. *Brucella canis* is the causative agent of canine brucellosis and facultative intracellular pathogen. The diagnosis of canine brucellosis is based on bacteriological examination and serological methods including agglutination and gel diffusion tests. In this study, crude antigens were extracted from *B. canis* using hot saline, coated on to latex beads and their usefulness in the serological diagnosis of canine brucellosis was examined. Mixing the antigen coated latex beads with the sera of dogs infected with *B. canis* produced clear agglutination, but this was not so for *B. canis* free dog sera. N-terminal amino acid sequence analysis of the crude hot saline extracts, showed that they contained copper-zinc superoxide dismutase, ribose ABC transporter and hypothetical protein of *Brucella* as antigens. A serological survey of canine serum samples conducted by means of an agglutination test using the antigen coated latex beads, showed that this method was more specific than the tube agglutination test using whole bacterial cell antigens. Although these results suggest that our method in which crude hot saline extracted antigens are coated on to latex beads would be useful in the serological diagnosis of canine brucellosis, we need further investigation using more serum samples to confirm the usefulness of our method.

KEY WORDS: agglutination test, brucellosis, canine, diagnosis, latex beads.

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Canine brucellosis is widely distributed around the world and this is an important disease due to the economic losses in animal production, and its risks for human health [8]. Reproductive disorders, such as abortions and premature births, are the clinical signals of this bacterial disease in pregnant animals. Diagnosis of the disease is based on bacteriological examination and serological tests [4]. Serological diagnosis is usually performed by the tube agglutination test, rapid slide agglutination test, and gel immunodiffusion test [4, 5, 7]. However, there is the problem that agglutination tests sometimes give false-positive reactions due to cross-reactions with other pathogens, and a general strategy for eliminating cross-reactions is to use purified antigen with unique epitopes in the serological tests. Bacterial cell wall antigens of *B. canis* can be prepared by hot saline extraction, and have been shown to be useful in serological diagnosis [12]. The characteristics of the several proteins, in such hot saline extracts, however, are still unclear.

This paper describes the characterization of crude hot saline extracts, including identification of the gene encoding the antigens, and provides evidence that for the usefulness of these antigens in the serological diagnosis of canine brucellosis. It also describes a serological investigation of exposure to *B. canis* in Japan conducted by means of an agglutination test using the latex beads.

MATERIALS AND METHODS

Antigen preparation: *Brucella canis* QE-13 was cultured in Brucella broth at 37°C, with shaking, and harvested dur-

ing the stationary phase of growth. The bacterial cells were washed twice with saline and resuspended in saline to a final concentration of 10% (w/v). This suspension was autoclaved at 105°C for 15 min and centrifuged at 10,000 × g for 10 min to eliminate bacterial debris. The supernatant was frozen at -20°C, melted, and centrifuged at 1,000 × g for 10 min to remove the small aggregates. Next, the supernatant was dialyzed against a solution of polyethylene glycol 6000 (15%) to a 10-fold concentration and then against saline. The antigen solution was kept frozen at -20°C.

Coating of antigens on to latex beads: Equal volumes of antigen solution (protein concentration of 300 µg/ml that was measured by Lowry methods) and 1% latex beads (diameter 1 µm, Sekisui Chemical Co.) were mixed for 16 hr at room temperature with stirring. Next, the latex beads were washed three times with washing buffer (phosphate-buffered saline (PBS) containing 1% bovine serum albumin, 0.01% Tween 20, 0.1% polyethylene glycol 6000, 0.2% sucrose) and then blocked with washing buffer for 16 hr at room temperature with stirring. The latex beads were then adjusted to a final concentration of 1% (w/v) with PBS and kept at 4°C.

Agglutination test: Equal volumes (50 µl) of the latex bead solution and canine serum diluted 10-fold with PBS were mixed on a glass slide and incubated at room temperature for 15 min without shaking. Samples showing agglutination were considered to be positive.

The tube agglutination test was performed as follows. Heat-inactivated *B. canis* QE-13 whole-cell antigens were obtained from Kitasato Laboratories. Equal volumes (0.5