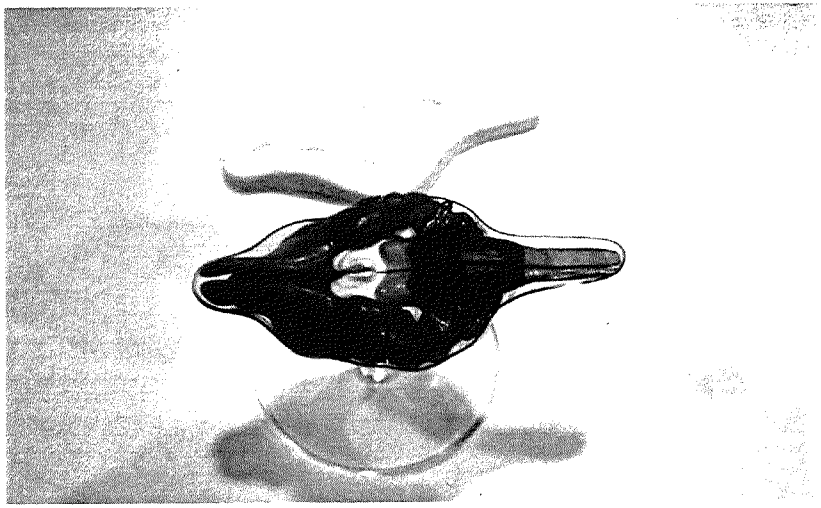
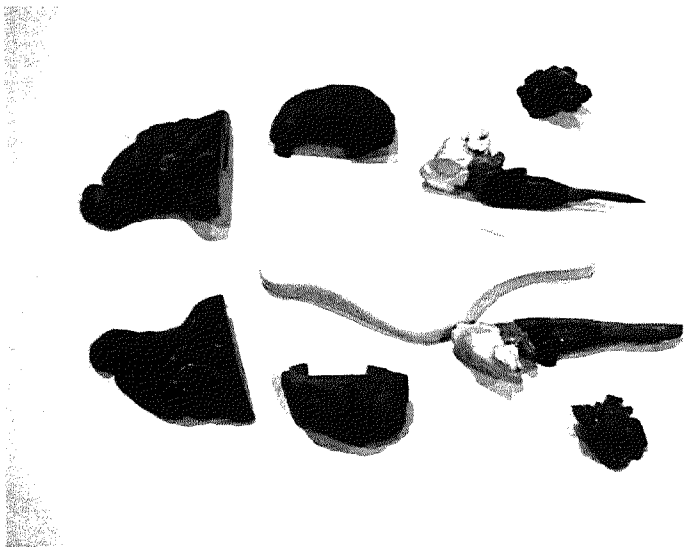
図3. 完成モデルと材質　脳モデル



脳モデル裏　材質はエポキシ

脳モデル表



脳モデル分解状態　置き台（白）はFRP

収納カップの材質は塩ビとアクリル

図4a. 解剖手技ビデオの制作

★検体の脳取り出し手順をビデオで撮影し映像コンテンツとしてまとめる

★ハイビジョンカメラで撮影した立体視版と、片側のカメラ映像のみを編集した通常版を制作

★マスターDVDのプロトタイプを1枚作成する

制作・放映スケジュール

2008年10月10日	東京都城南島動物愛護相談センター解剖室で手技を撮影
2008年10月23日	国立感染症研究所にてゲラ版チェック
2008年10月29日	Ver1．0編集
2008年10月30日	東京都城南島動物愛護相談センターにて試写
2008年10月31日	北里大学白金キャンパスで開催された平成20年度動物由来感染症技術研修会ホールにて試写
2008年12月8日	国立感染症研究所にて、Ver1．0のテロップ修正
2008年12月22日	新しいテロップとCGを盛り込んだVer2．0立体版と通常版の編集
2008年12月25日	国立感染症研究所にて、Ver2．0チェック、通常版DVDマスター第1版納品
2008年12月26日	タイトルを編集したVer3．0編集
2009年1月13日	国立感染症研究所班会議にて、研究成果発表
2009年1月13日	通常版DVDマスター第2版納品
2009年1月29日	平成20年度全国動物管理関係事業所協議会の中国・四国ブロック会議（倉敷）にて試写

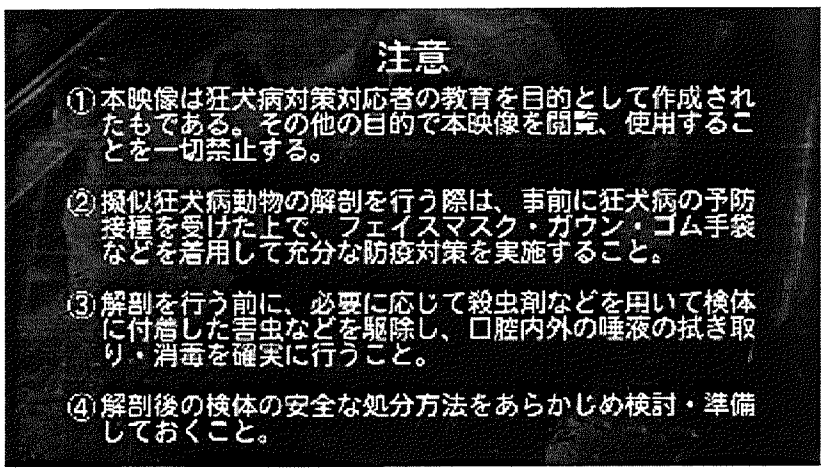
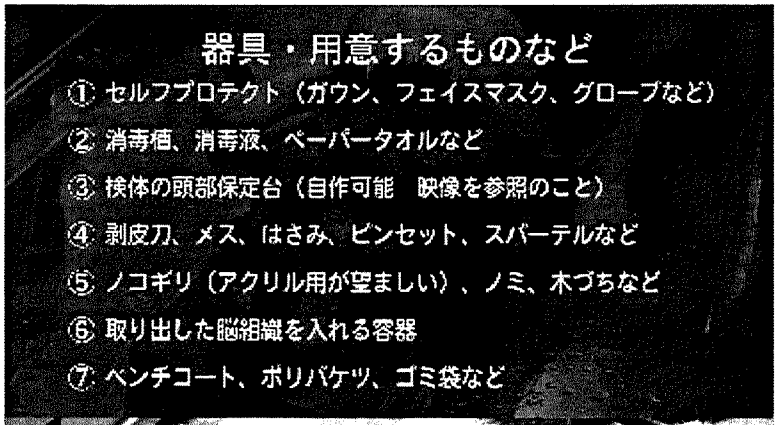
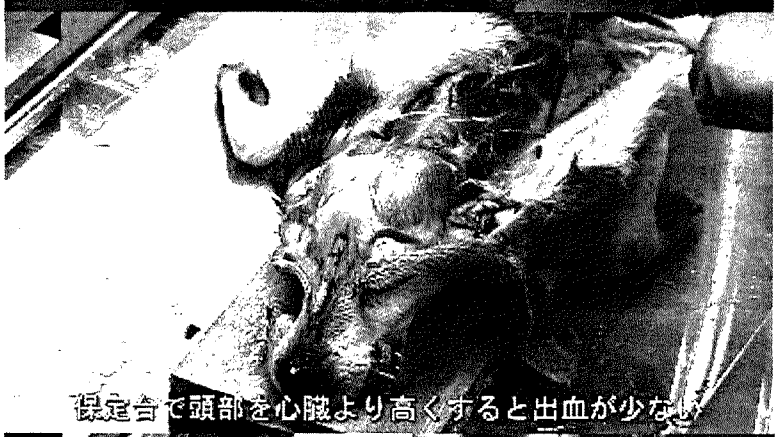


図4b. 解剖手技ビデオの制作





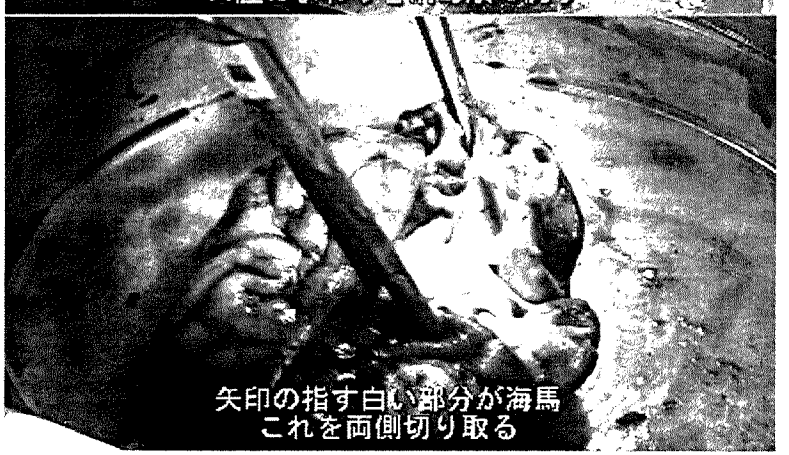


図5. 自治体等関係者への立体視による頭部解剖ビデオ試写とプロトタイプモデル紹介

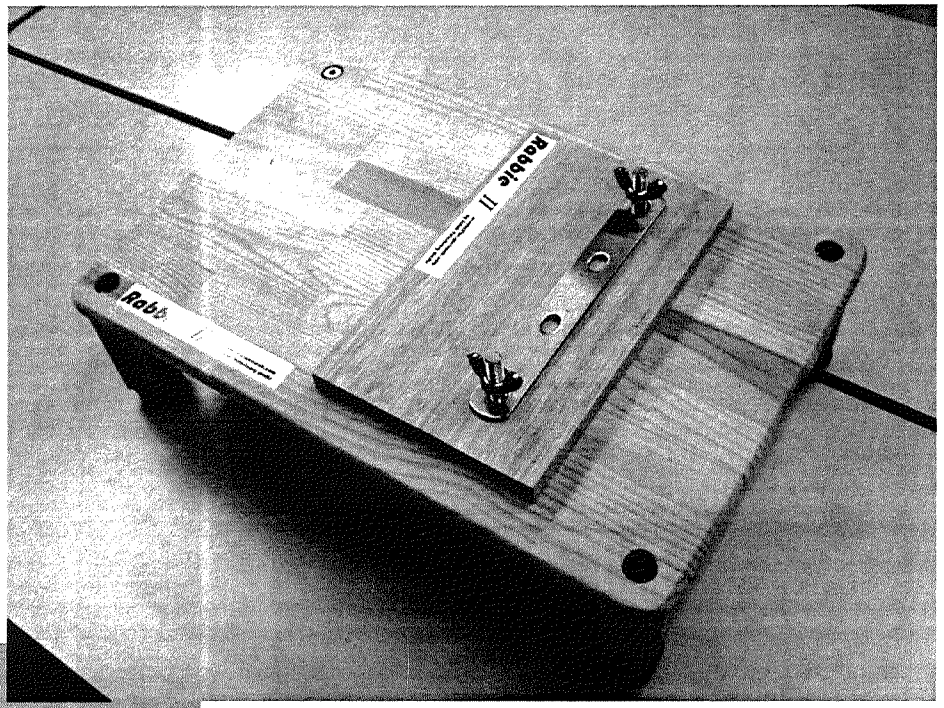
日時	内容
2008年10月30日 13:00～	場所　東京都城南島動物愛護相談センター2階会議室 名称　東京都動物愛護相談センター職員研修会 参加　東京都動物愛護相談センター職員　約20名 内容　頭部解剖の立体視ビデオ(Ver1. 0)の試写と解説 アペックス(3名立会い):機器のセッティング、立体視メガネの配布 試写後、解剖実習
2008年10月31日 9:30～17:15	場所　北里大学白金キャンパス　薬学部コンベンションホール 名称　平成20年度動物由来感染症技術研修会 参加　全国の動物由来感染症に関わる施設の職員　約300名 内容　会場入り口ホールにて、解剖の立体視ビデオの試写、モデルプロトタイプ紹介(解剖手順立体CG、解剖手技モデルプロトタイプ)、簡易解剖台の展示 本研究の説明ポスター掲示 アペックス(3名立会い):機器のセッティング、立体視メガネの配布、インタラクティブCGのオペレート、解剖手技モデルプロトタイプの解説
2009年1月13日 13:00～	場所　国立感染症研究所会議室 名称　班会議 参加　班員等、約15名 内容　頭部解剖の立体視ビデオ試写、各プロトタイプモデルの紹介 アペックス(2名立会い):機器のセッティング、立体視メガネの配布、モデル教材の準備
2009年1月29日 15:00～17:00 2009年1月30日 10:00～12:00	場所　倉敷市アパホテル会議室 名称　平成20年度全国動物管理関係事業所協議会中国・四国ブロック会議 参加　中国・四国地区の動物愛護相談センターなどの職員　約40名 内容　狂犬病対策の一環として、擬似狂犬病動物検体取り出しについての講義、解剖立体視ビデオ試写 解剖モデルプロトタイプ3種を紹介(実際に手に取ってもらい評価、意見交換) アペックス(2名立会い):機器のセッティング、立体視メガネの配布、モデルの準備と解説 骨切断モデルを実際にノコギリで切断する試みを行った

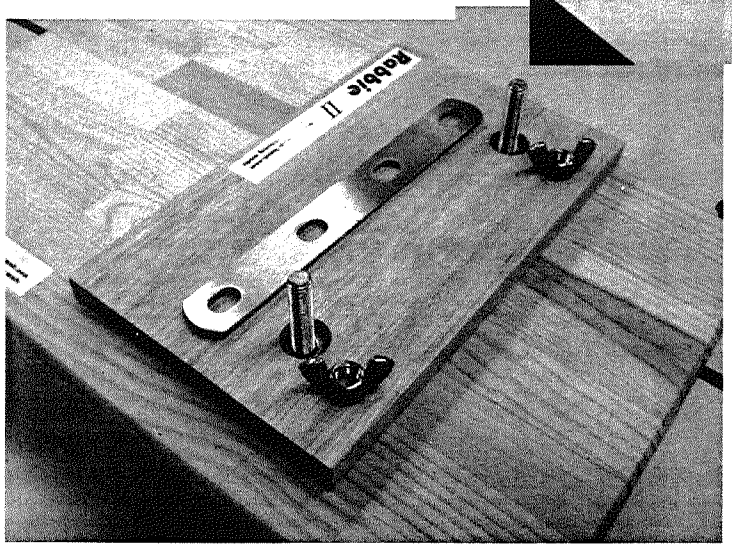
図6a. 関連資料


タイで麻痺型狂犬病と診断された
イヌの臨床経過（1例）
disc
2002年11月
for Windows
厚生科学研究費補助金新興・再興感染症研究事業
「動物由来感染症対策としての新しいサーベイランスシステムの開発に関する研究」
狂犬病のサーベイランス及び診断に関するワーキンググループ
2002 Ministry of Health, Labour and Welfare. All Rights Reserved.
Made in Japan.


狂犬病
DVD

図6b. 関連資料





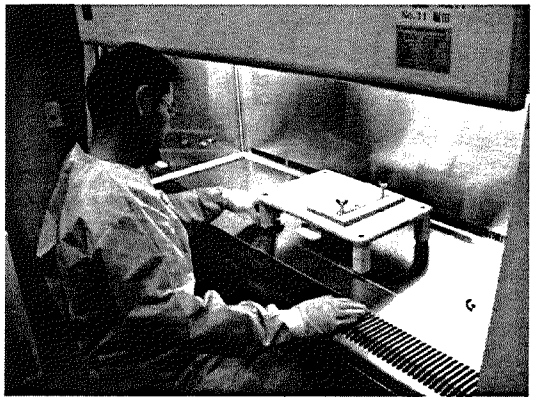
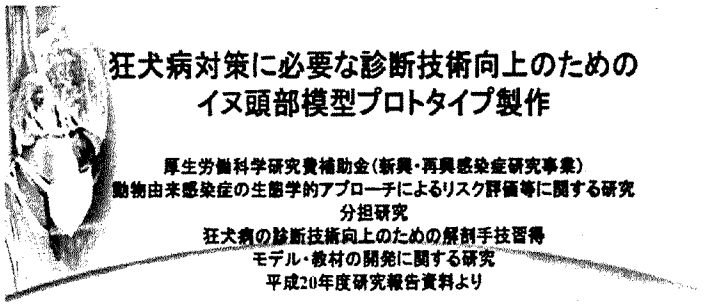
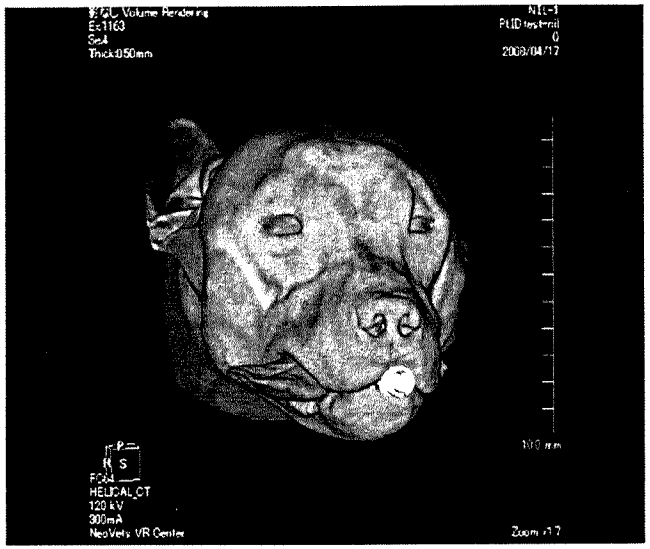


図6c. 関連資料

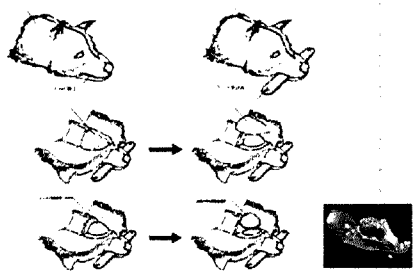


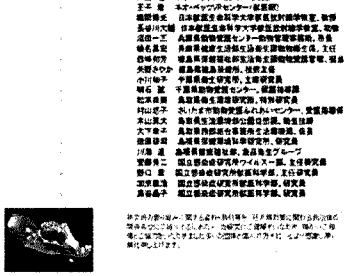
狂犬病対策に必要な診断技術向上のための
イヌ頭部模型プロトタイプ製作

厚生労働科学研究費補助金（新興・再興感染症研究事業）
動物由来感染症の生態学的アプローチによるリスク評価等に関する研究
分担研究
狂犬病の診断技術向上のための解剖手技習得
モデル・教材の開発に関する研究
平成20年度研究報告資料より

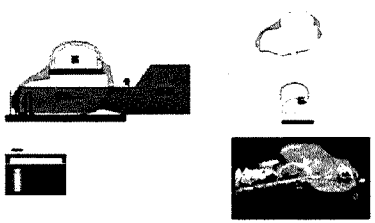


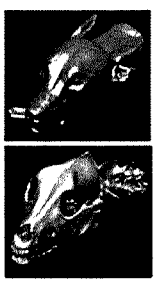
①解剖手技モデル



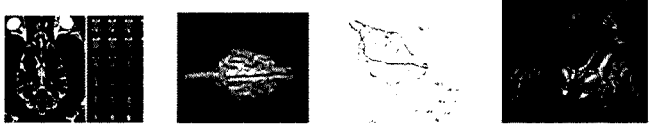


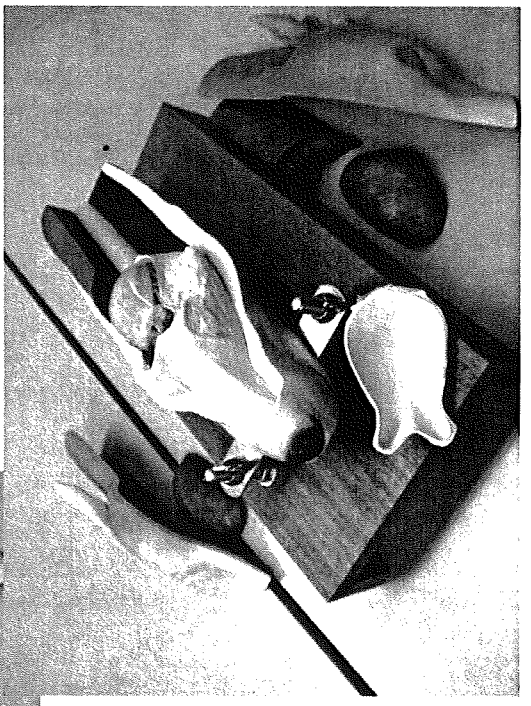
②骨切断モデル

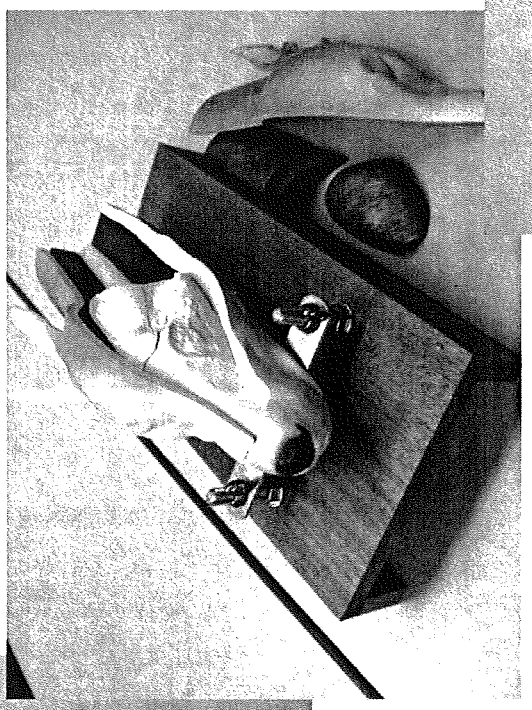


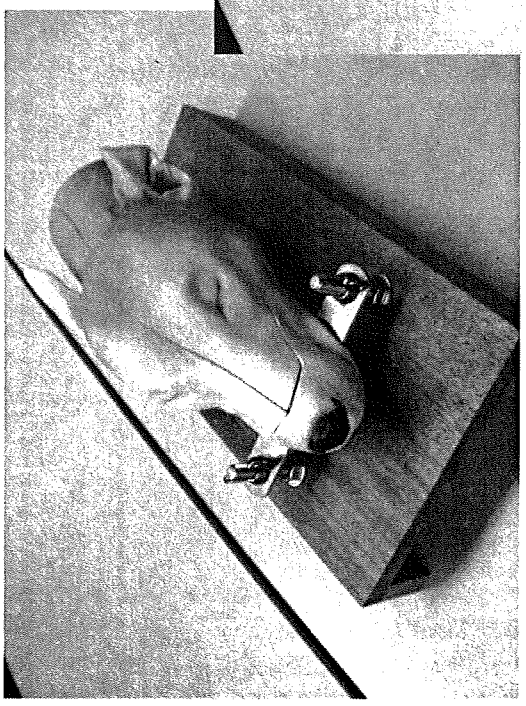


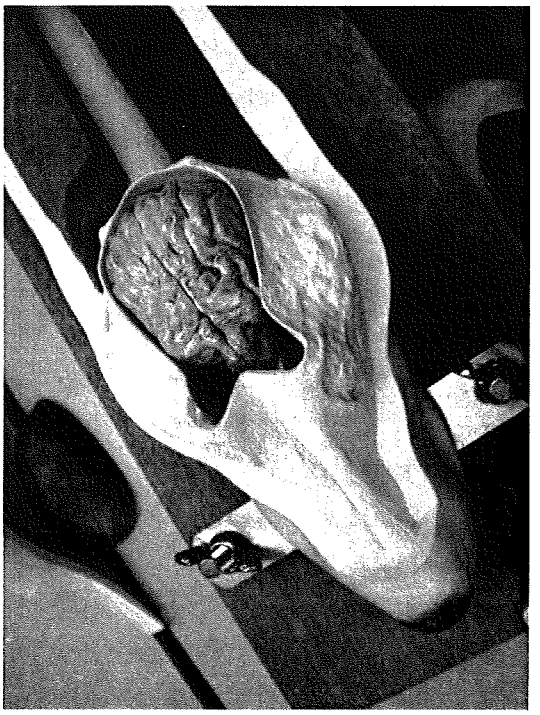
③脳モデル











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川端寛樹	野兎病菌	バイオメデゥカルサイエンス研究会	バイオセーフティの辞典-病原微生物とハザード対策の実際-	みみずく舎/医学評論社	東京	2008	181-182
川端寛樹	ボレリア・ブルクドルフェリ抗体	櫻林郁之助，熊坂一成	新・臨床検査項辞典	医歯薬出版	東京	2008	664-665
今岡浩一	ブルセラ病とその検査	日本獣医師会	感染症検査実習マニュアル	日本獣医師会	東京	2008	95-108
今岡浩一	ブルセラ	バイオメディカルサイエンス研究会	バイオセーフティの事典	みみずく舎・医学評論社	東京	2008	169-171
井上 智、佐藤 克、梅田浩史、衛藤真理子	狂犬病 (Rabies)		JRA特別振興事業（ウエストナイルウイルス感染症等特別対策事業)	社団法人全国家畜畜産物衛生指導協会		2008	
井上 智	人獣共通感染症	和田 攻	産業保健ハンドブック	財団法人産業医学振興財団		2008	p32
井上 智	動物由来感染症	和田 攻	産業保健ハンドブック	財団法人産業医学振興財団		2008	p32-33
井上 智	狂犬病ウイルス	バイオメディカルサイエンス研究会	バイオセーフティの辞典	医学評論社		2008	p258-259

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Seto Y, Komiya T, Iwaki M,Kohda T, Mukamoto M, Takahashi M, Kozaki S	Properties of Corynephage attachment site and molecular epidemiology of Corynebacterium ulcerans isolated from humans and animals in Japan	Jpn. J. Infectious Diseases	61	116-122	2008
Hanaoka N, Sakata A, Takano A, Kawabata H, Watanabe H, Kurane I, Kishimoto T, Ando S.	Development of a pUC19-based recombinant plasmid to serve as a positive control in PCR for *Orientia tsutsugamushi*.	Microbiology and Immunology			Accepted
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Kubo M, Uni S, Agatsuma T, Nagataki M, Panciera R,Tsubota T, Nakamura S,Sakai H, Masegi T, Yanai T	*Hepatozoon ursi* n. sp. (Apicomplexa: Hepatozoidae) in Japanese black bear (*Ursus thibetanus japonicus*)	Parasitology International	57	287-294	2008
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井上　智	狂犬病の診断技術向上のためのイヌの頭部解剖手技の習得モデルと教材開発の紹介	LABIO 21	34	33-35	2008
井上　智、野口　章	リッサウイルス感染症	検査と技術	36	1465-1467	2008
井上　智	人獣共通感染症が侵入・発生した場合の動物側の対応	JVM	61	901-907	2008
井上　智	世界・日本の現状と獣医師の役割	MVM	110	6-7	2008

Jpn. J. Infect. Dis., 61, 116-122, 2008

Original Article

Properties of Corynephage Attachment Site and Molecular Epidemiology of *Corynebacterium ulcerans* Isolated from Humans and Animals in Japan

Yukiji Seto[1,2], Takako Komiya[1], Masaaki Iwaki[1], Tomoko Kohda[2], Masafumi Mukamoto[2], Motohide Takahashi[1] and Shunji Kozaki[2]*

[1]*Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases, Tokyo 208-0011, and* [2]*Department of Veterinary Science, Graduate School of Life and Environmental Science, Osaka Prefecture University, Osaka 599-8531, Japan*

(Received October 17, 2007. Accepted January 7, 2008)

SUMMARY: Sporadic reports of *Corynebacterium ulcerans* infection in humans and animals have become increasingly common throughout the world. Between 2001 and 2006, five human cases, in addition to isolation of the bacterium from the carcasses of *Orcinus orca* and *Panthera leo*, were reported in Japan. While an isolate from *P. leo* generated only phospholipase D (PLD), the other isolates produced both PLD and diphtheria-like toxin (DLT). Pulsed-field gel electrophoresis analysis showed that isolates from *P. leo* and humans were genetically homologous. Southern blotting found that a human isolate was lysogenized by two corynephages coding DLT. Sequence analysis of the region of the DLT gene revealed that the integration in *C. ulcerans* occurred in the same manner as that in *C. diphtheriae*.

INTRODUCTION

Corynebacterium ulcerans was first isolated from human throat lesions in 1926 (1), and has recently been recognized as a distinct species within the genus *Corynebacterium* by DNA-DNA hybridization (2). *C. ulcerans* can carry the corynephage that codes for diphtheria toxin gene (*tox*), and toxigenic strains produce diphtheria-like toxin (DLT), which may be associated with classical pharyngeal diphtheria in humans. The organism also causes respiratory symptoms in macaques (3), mastitis in cattle (4), and caseous lymphadenitis in sheep and goats (5). Since there have been some cases in pets, such as chronic labial ulceration and rhinorrhea in dogs (6) and bilateral nasal discharge in cats (7,8), it has been suggested that clinical cases in humans are usually derived from infections in household pets (9). Sporadic cases in humans and animals have recently been reported throughout the world (10-17), and many reports have originated in the United Kingdom (7,19). In Japan, 5 cases in humans were reported between 2001 and 2006 (12,14). During the same period, *C. ulcerans* was identified from the carcass of *Orcinus orca* (killer whale) and *Panthera leo* (lion).

C. ulcerans is a member of the so-called "diphtheria group", which includes *C. diphtheriae* and *C. pseudotuberculosis* (4). Toxigenic strains of these bacteria can produce diphtheria toxin (DT) (*C. diphtheriae*) or DLT (*C. ulcerans* and *C. pseudotuberculosis*). The production of urease and a failure to reduce nitrates are characteristics of *C. ulcerans* that are not shared with other members of the diphtheria group (4). The characteristics of members of the group have been of special interest to those studying the epidemiology of diphtheria, since it was reported that diphtheria-like organisms had been isolated from cases of acute sore throat and from healthy carriers (4). Diphtheria is an acute infectious disease caused by the toxin-producing *C. diphtheriae*. The disease is typically characterized by local infection of the upper respiratory tract and occasionally the skin. Systemic manifestations can affect, in particular, the heart, kidneys, and peripheral nerves because of DT, which is a cytotoxic protein that inhibits cellular protein synthesis in eukaryotes by inactivating elongation factor 2 through ADP ribosylation (19). *C. ulcerans* also produces phospholipase D (PLD), which is a known virulence factor in *C. pseudotuberculosis* (4,20). Lipsky et al. have shown that there are three toxigenic groups in *C. ulcerans* based on the production of DLT and PLD, in which the three groups respectively produce DLT, PLD, or both (4). Another report indicated that PLD production was a feasible marker for distinguishing between *C. ulcerans* and *C. diphtheriae* (21). However, little recent attention has been directed at PLD production by *C. ulcerans*.

Toxin production by *C. diphtheriae* depends on infection of an organism with a beta corynephage encoding *tox*. In strains of *C. ulcerans* and *C. pseudotuberculosis*, phages are also able to induce the production of DLT. In toxigenic *C. diphtheriae*, site-specific recombination occurs between a phage attachment (*attP*) site and a bacterial attachment (*attB*) site (22). Certain strains of *C. diphtheriae* contain two *attB* sites (*attB*1 and *attB*2) in their chromosome, and the corynephage can integrate into either *attB*1 or *attB*2 with equal frequency. Lysogens containing two phage copies have also been isolated and were shown to contain either two phages tandemly integrated at the same *attB* site (unstable conformation) or one phage integrated in each of the two *attB* sites (stable conformation). The production of DT by a given lysogen is proportional to the number of prophages integrated into its chromosome (22,23). The sequence homologous to the beta corynephage integration site in *C. diphtheriae* is conserved in members of the genus *Corynebacterium*. The sequence of the *attB* site was also found in *C. ulcerans* (24,25). Maximescu et al. showed that *C. ulcerans* (strains 40c, A238, 298G, and 9304) could be lysogenized and converted with phage W from *C. diphtheriae* PW8; moreover, *C. ulcerans*

*Corresponding author: Mailing address: Department of Veterinary Science, Graduate School of Life and Environmental Science, Osaka Prefecture University, 1-1 Gakuen-cho, Naka-ku, Sakai, Osaka 599-8531, Japan. Tel: +81-72-254-9504, Fax: +81-72-254-9499, E-mail: kozaki@center.osakafu-u.ac.jp

phage "h" induced the production of DLT in *C. ulcerans* (strains 40 and A238) (26). Although the occurrence of phage conversion and the presence of the *attB* site in *C. ulcerans* have been demonstrated, the details of the relationship between the *attB* site and *tox* in the bacterium are not well understood in comparison with those of *C. diphtheriae*.

The aims of this study were to determine the toxigenicity and genetic relatedness among isolates in Japan using restriction fragment length polymorphisms of rRNA genes (ribotyping) with *Bst*EII and pulsed-field gel electrophoresis (PFGE) with *Sfi*I. We found a correlation between the *attB* site and *tox* in *C. ulcerans* using Southern blot analysis with specific probes for *tox*, the *attB* site, and the PLD gene (*pld*).

MATERIALS AND METHODS

Bacterial strains and DNAs: We used seven strains isolated from humans and animals (Table 1). Strain 0509 was obtained from a swab from a 57-year-old man, whose chief complaint appeared to develop after the death of his dog. Another strain, 0607, was isolated from swab used to obtain a sample from a 58-year-old woman, who also kept a dog. The immunization status of these persons with diphtheria toxiod was not known. We encountered an isolate of the organism, strain Ran, from a female *O. orca* that had manifestations of decreased appetite and fever at day 4 post-partum, and the animal died the next day. The strain designated O-9 was obtained from the carcass of a male killer whale that had been one-half of a pair of *O. orca* from the same pool. The strain lion was isolated from a blood sample from a female *P. leo*, and the sample was simultaneously contaminated with two *Staphylococcus* spp. and a *Streptococcus* sp. *C. ulcerans* ATCC®51799 (identical to CCUG 2708, NCTC 7910, and DSM 46325), and *C. diphtheriae* PW8 and ATCC 700971 were used as reference strains in this study. The total DNA was isolated as described previously by Michel et al. (18) and was used for ribotyping, Southern blotting, and sequence analysis of 16S rRNA, *tox*, *pld* genes, and the *attB* site. For polymerase chain reaction (PCR) diagnosis to detect *tox* and *pld*, DNA was purified using a QIAamp DNA Mini Kit (Qiagen, Tokyo, Japan).

Biotyping, toxigenicity testing, and sequencing: Biotyping of isolates was performed using API *Coryne* (BioMérieux, Marcy-l'Etoile, France) according to the manufacturer's instructions. Sequencing of 16S rRNA gene of the isolates was carried out with purified DNA and the following primers: 16S-fD1 (5′-AGAGTTTGATCCTGGCTGAG-3′) and 16S-rP2 (5′-ACGGCTACCTTGTTACGACTT-3′) (27). Using a modified Elek test, we also examined the production of DT by the isolates (28) and an in vitro cytotoxicity assay (29,30) was carried out using the culture supernatants of the Elek broth (2% [wt/vol] proteose peptone [Oxoid, Basingstoke, UK], 0.1% [wt/vol] yeast extract [Oriental Yeast Co., Ltd., Tokyo, Japan], 0.26% NaCl, pH 7.4) for 24 h at 37°C with shaking (19). The culture supernatants were prepared by removing bacterial cells by centrifugation at 4,000 × *g* for 10 min. PLD activity was assayed a modified Zaki method (20,31). Briefly, isolates were grown in Brain heart infusion broth (Oxoid) for 24 h at 37°C with shaking. Bacterial cells were eliminated by centrifugation at 4,000 × *g* for 10 min. Sheep erythrocytes in Alsevers solution were washed three times in 10 volumes of buffer-saline (10 mM Tris-HCl, 10 mM $MgCl_2$, 0.85% [wt/vol] NaCl, 0.1% [wt/vol] bovine serum albumin [Sigma-Aldrich Japan K.K., Tokyo, Japan], pH 7.3). Dilutions of the culture supernatants in buffer-saline (100 μl) were incubated with 1.5% washed sheep erythrocytes (100 μl) at 37°C for 45 min in a 96-well microtiter plate (Asahi Glass, Co., Ltd., Tokyo, Japan). *Staphylococcal* sphingomyelinase C (Biomol. International, L. P., Pa., USA) (0.01 U/50 μl) was added to each well. After incubation of the plate at 37°C for 60 min, it was allowed to stand on ice for 60 min. Zaki unit values of the samples were calculated from the dilution in which sheep blood cells were no longer protected from lysis by sphingomyelinase C. PCR was performed using TaKaRa *ExTaq* polymerase (Takara, Kyoto, Japan) according to the manufacturer's instructions. For PCR diagnosis, a fragment (248 bps) of *tox* in the isolates was detected by the method of Nakao et al. using the primers Tox1 and Tox2 (32). *C. diphtheriae* PW8 was used as a positive control. As a negative control, no templates were added to the reaction mixture. PCR diagnosis of *pld* was performed with the following primers based on published *pld* sequences (pldFw [5′-AACATCTCTCCGTGAAAAAT-3′; position, -157 to -137 base] and pldRv [5′-AGCTCAATCGCACCGTT GTC-3′; position, 1,165 to 1,180 base]). For the sequencing of *tox*, *pld*, and *attB* site-related genes, we carried out amplification using toxFw (5′-CCATGTAACCAATCTATCAA-3′; position, -179 to -159 base) and toxRv (5′-GTGCGCCCG GAGGGA-3′; position, 1,720 to 1,735 base) based on our sequence data, pldFw and pldRv, and CU-Rfw (5′-CGCGGA TCCGCGTACTCTCCGGGGCA-3′; position, 833 to 851 base on DLT) and attBRv (5′-CCGCAAGCACGAAGAAAC

Table 1. Clinical isolates of *C. ulcerans* from Japan

Strain	Date of isolation	Age (years)/sex	Source	Clinical details	Reference
Isolated from human					
0102	2001/Feb	52/F	Throat swab	Sore throat	13
0509	2005/Sep	57/M	Throat swab	Fever, partitas	This study
0510	2005/Oct	51/M	Lung	Lung lesion	25
0607	2005/Nov	58/F	Throat swab	Pseudomembrane	This study
Isolated from killer whale					
Ran	2004/Aug	16/F	Blood, Lung	Bacteremia, Purulent pneumonia	This study
O-9	2004/Sep	15-16/M	Blood, Lung	Bacteremia, Purulent pneumonia	This study
Isolated from lion					
lion	2005/Dec	6/M	Blood	Sepsis	This study

F, female; M, male.

CCCAGCCCACA-3′), respectively. The sequence data were analyzed by GENETYX-MAC 10.0 and DNASIS-Mac v3.2.

Ribotyping and PFGE: For the detection of genetic correlations, isolates were analyzed by ribotyping (7,33) and PFGE (34,35). For ribotyping, 15 μg of total DNA was cleaved with 40 units of *Bst*EII (New England Biolabs Inc., Beverly, Mass., USA). The digested fragments were subjected to 1% agarose gel electrophoresis for 15 h and the samples were then blotted onto Hybond-N$^+$ membranes (GE Healthcare, Buckinghamshire, UK) with a Capillary Blotting Unit (Scotlab, Ltd., Strathclyde, UK). The efficiency of DNA transfer was confirmed by staining the gels with ethidium bromide, and the DNA fragments on the membranes were fixed with 0.4 N NaOH. Each membrane was further incubated at 37°C for 30 min with DIG Easy Hyb (Roche Diagnostics K.K., Tokyo, Japan), and hybridization was performed for 15 h at 37°C with fresh DIG-Easy Hyb containing DIG-labeled Oligo5Mix (100 pmol each) (33), 25 mg/ml fish DNA (Roche Diagnostics), 10 mg/ml Poly (A) (Roche Diagnostics), and 5 mg/ml Poly d (A) (Roche Diagnostics). After the samples were rigorously washed twice with 2 × SSC-0.1% SDS for 5 min at room temperature and treated twice with 0.5 × SSC-0.1% SDS for 15 min at 37°C, the bands were visualized with alkaline phosphatase-conjugated anti-DIG antibody (Roche Diagnostics) and detected using CDP-Star (GE Healthcare) and Hyperfilm ECL (GE Healthcare) in a cassette with an enhancing screen. For PFGE, each strain was grown on a sheep-blood agar plate for 24 h at 37°C. The cells were harvested and suspended in 0.5 ml PIV buffer (1 M NaCl, 10 mM Tris-HCl, [pH 7.5]). A portion (0.25 ml) of the suspension was mixed with 0.25 ml 1.6% Seakem® Gold Agarose (Cambrex Bio Science, Rockland, Maine, USA) at 50°C and then pipetted into a plug mold (Bio-Rad, Hertfordshire, UK). The agarose blocks were incubated overnight at 37°C in lysis buffer (6 mM Tris-HCl [pH 7.5], 1 M NaCl, 0.1 M EDTA, 0.5% Briji 58 [Sigma-Aldrich Japan], 0.2% deoxycholate [Wako Pure Chemical Industries, Ltd., Kyoto, Japan], 0.5% sodium lauroyl sarcosine, 1 mg/ml lysozyme [Nacalai Tesque, Kyoto, Japan]), followed by deproteination in proteolysis buffer (0.5 M EDTA [pH 8.0], 1% sodium lauroyl sarcosine [Sigma-Aldrich Japan], 2 mg/ml Proteinase K [Nacalai Tesque]) for 48 h (35). The DNA in the plug was cleaved with *Sfi*I (New England BioLabs), and PFGE was carried out in 1% agarose gel with 0.5 × Tris-borate-EDTA buffer at 14°C using CHEF Mapper (Bio-Rad). PFGE profiles were analyzed using Fingerprinting™ II software (Bio-Rad).

Southern blotting: *Bam*HI-digested DNA fragments were applied directly to 1.0% agarose gel and electrophoresis was performed with 1 × Tris-acetate-EDTA buffer for 14 h. The fractionated DNA was then transferred to a Hybond-N$^+$ membrane as described above. The membrane was incubated at 37°C for 30 min with DIG Easy Hyb, and hybridization was performed at 37°C for 15 h with fresh DIG-Easy Hyb (Roche Diagnostics) containing 20 ng/ml DIG-labeled probes. After the membrane was washed, bands were detected with alkaline phosphatase-conjugated anti-DIG antibody. The *tox*, *pld*, and *attB* site probes were obtained from a DIG PCR synthesis kit (Roche Diagnostics) using *C. diphtheriae* PW8 with Tox1 and Tox2 primers, strain ATCC®51799 with pldFw and pldRv primers, and strain ATCC®51799 with attBFw (5′-GCGCC CGTAGCTCAACGGATAGAGCA-3′) and attBRv primers, respectively.

RESULTS

Biotyping, sequencing of DLT and PLD, and toxigenicity: Isolates from Japan were identified as *C. ulcerans* using the API *Coryne* system (API code 0111326). Biochemical characteristics of the isolates were also confirmed by partial sequencing of the 16S rRNA gene, which was almost identical (99.9%) to that of strain ATCC®51799 (GenBank accession no. X84256) (27). To determine the toxigenicity of the isolates, we performed a diagnostic PCR using primers Tox1 and Tox2 for the detection of *tox*. Strains ATCC®51799 and lion did not harbor *tox* (nontoxigenic strains); however, strains 0102, Ran, O-9, 0509, 0510, and 0607 retained *tox* (toxigenic strains). We also determined the DLT production of the isolates using an Elek test and a cytotoxicity assay using Vero cells, and the results were consistent with the PCR findings (Table 2). Diphtheria antitoxin completely neutralized the culture supernatants of strains 0102, Ran, O-9, 0509, 0510, and 0607 (data not shown). We then sequenced the complete *tox* genes (1,683 bp) of strains Ran, O-9, 0509, 0510, and 0607, and compared these sequences with that of *tox* from strains 0102 (GenBank accession no. AB304278), A6361 (GenBank accession no. AY141014) (37), and *C. diphtheriae* (GenBank accession no. K01722) (36) (Table 3). Strains Ran and O-9 possessed identical *tox* sites (GenBank accession no. AB304279). The *tox* genes of strains 0509 and 0606 were

Table 2. Toxigenicty of *C. ulcerans* and *C. diphtheriae*

Strain	DLT			PLD	
	PCR	Cytotoxicity[1] (CD_{50}/25 μl)	Elek test	PCR	Zaki assay[2] (U/ml)
C. ulcerans					
ATCC®51799	−	ND	−	+	20
0102	+	362	+	+	40
Ran	+	256	+	+	20
O-9	+	362	+	+	20
lion	−	ND	−	+	160
0509	+	512	+	+	40
0510	+	512	+	+	80
0607	+	256	+	+	320
C. diphtheriae					
PW8	+	3,200	+	−	ND
ATCC700971	+	128	+	−	ND

[1]: Cytotoxicities of culture supernatants measured by pH color change methods.
[2]: *Staphylococcal* sphingomyelinase C inhibition activity in cultured supernatants.
+, positive; −, negative; ND, not detected.

Table 3. Nucleotide sequence and deduced amino acid residue homology of diphtheria-like toxin produced from *C. ulcerans*

Isolate	% Homology for nucleotide (roman) and deduced amino acid (*italic*) sequence[1]				
	C. ulcerans				*C. diphtheriae*
	0102	O-9	0510	A6361	DT
0102		*100.0*	*99.10*	*100.0*	*95.00*
O-9	99.70		*99.10*	*100.0*	*95.00*
0510	98.57	98.51		*99.10*	*94.82*
A6361	100.0	99.70	98.57		*95.00*
DT	95.18	95.24	95.18	95.18	

[1]: Sequences were aligned using GENETYX-MAC 10.0, and percent identities were determined by DNASIS-Mac v3.2 without gaps as the denominator.

Table 4. Nucleotide sequence and deduced amino acid residue homology of phospholipase D produced from *C. ulcerans*

Isolate	% Homology for nucleotide (roman) and deduced amino acid (*italic*) sequence[1)]						
	ATCC®51799	0102	O-9	lion	0510	0607	ATCC739
ATCC®51799		*99.69*	*97.39*	*99.02*	*97.72*	*99.02*	*98.04*
0102	98.05		*96.74*	*99.67*	*97.72*	*99.67*	*98.04*
O-9	96.75	96.96		*97.06*	*98.37*	*97.06*	*96.74*
lion	98.16	99.89	97.07		*98.74*	*100.0*	*98.37*
0510	96.96	97.18	98.18	97.29		*98.74*	*98.37*
0607	99.05	99.78	96.96	99.89	97.18		*98.37*
ATCC739	97.40	96.86	96.10	96.96	98.26	96.86	

[1)]: Sequences were aligned using GENETYX-MAC 10.0, and percent identities were determined by DNASIS-Mac v3.2 without gaps as the denominator.
GenBank accession nos. ATCC®51799, AB304281; 0102, AB304282; O-9, AB304283; lion, AB304284; 0510, AB304285; 0607, AB304286.

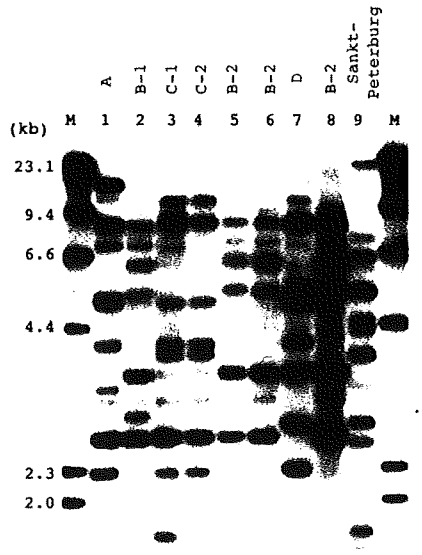

Fig. 1. *Bst*EII rRNA gene profiles of isolates and ATCC strains. M, molecular size standard (sizes are indicated on the left); lane 1, ATCC®51799; lane 2, 0102; lane 3, Ran; lane 4, O-9; lane 5, lion; lane 6, 0509; lane 7, 0510; lane 8, 0607; lane 9, *C. diphtheriae* ATCC 700971.

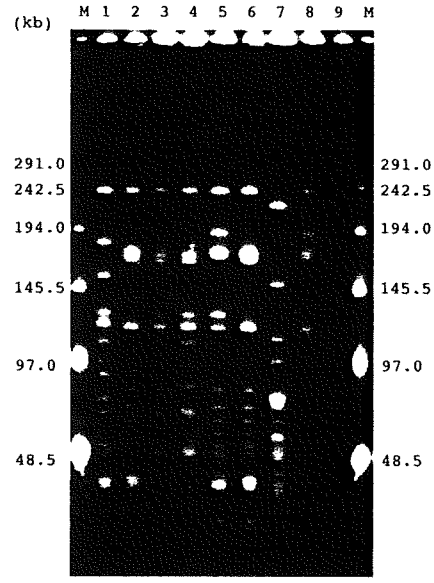

Fig. 2. *Sfi*I PFGE profiles of *C. ulcerans* isolates from Japan and ATCC strains. M, lambda concatemer as a size standard (sizes are indicated on the left); lane 1, ATCC®51799; lane 2, 0102; lane 3, Ran; lane 4, O-9; lane 5, lion; lane 6, 0509; lane 7, 0510; lane 8, 0607; lane 9, *C. diphtheriae* ATCC 700971.

indistinguishable from that of strain 0102; however, that of strain 0510 (GenBank accession no. AB304280) differed by 5 amino acid residues from that of strain 0102. Although the majority of *C. ulcerans* strains possess the PLD gene, confinement data on the PLD production of isolates were not available. When we determined by PCR analysis that the isolates in Japan had *pld* and that they yielded PLD in the Zaki assay (Table 2), strains 0102, Ran, O-9, 0509, 0510, and 0607 were found to produce both DLT and PLD, whereas strain lion generated PLD only, as seen in strain ATCC®51799. We sequenced the *pld* gene of the isolates in order to examine their similarity, because they could potentially be of use as a distinctive marker within the genus *Corynebacterium*. The sequences closely resembled (over 95% similarity) that of *C. ulcerans* ATCC 739 (GenBank accession no. L16585) (20) (Table 4).

Ribotyping and PFGE analysis: We determined the genomic relationship of isolates using ribotyping and PFGE. Figure 1 shows that six ribotype patterns (ribotype A, B-1, B-2, C-1, C-2, and D) were identified among the nine strains; ribotype B-1 was identical to ribotype B-2, with the exception of one band, and ribotype C-1 contained one band more than ribotype C-2. Ribotype groups B (B-1 and B-2) and C (C-1 and C-2) exhibited high similarity, despite their different sources. There were two unique ribotypes in the reference strain ATCC®51799 (ribotype A) and strain 0510 (ribotype D). PFGE using *Sfi*I was performed in order to further discriminate between ribotypes (Fig. 2). Four types (P1, P2, P3, and P4) of PFGE pattern were found among the nine strains using cluster analysis (Fig. 3). P2 consisted of a cluster and was classified as ribotype group B. There was high (97.5%) relatedness between strains Ran and O-9 (P3). The PFGE patterns of strains ATCC®51799 (P1) and 0510 (P4) were distinct from each other, as were the ribotype patterns. Ribotype and PFGE type had the same groupings as those of the nine strains.

Southern hybridization and sequencing of the *attB* site of isolates: To determine the location of *tox* and the *attB* site in the isolates, we performed a Southern blot analysis with *tox* and *attB* sites probes. *Bam*HI-digested DNA was used because the *tox* genes did not contain any *Bam*HI sites. Of the eight strains tested, only strain 0102 contained two frag-

ments hybridizing with *tox*-probe (*tox* fragment). The other toxigenic strains had one *tox* fragment, and, as expected, no reactive bands were observed in strains ATCC®51799 and lion (Fig. 4A). One *attB* homologous site was present in the nontoxigenic strains; however, 2 or 3 *attB* homologous sites were present in toxigenic strains (Fig. 4B). Southern blotting was also carried out with a *pld*-specific probe, which demonstrated that each strain possessed a single *pld* copy (Fig. 4C). The toxigenic strains had one or two bands that reacted with both the *tox* and *attB* site probes; however, no *pld* genes were colocated with any *attB* homologous regions.

The Southern blots indicated that both the *tox* gene and *attB* site were located together in single *Bam*HI-digested fragments. PCR analysis with the primers attBRv and CU-Rfw was performed to clarity the relevance of these sequences. These primers amplified a region of 635 bps in all toxigenic strains (data not shown). Figure 5 shows the alignment of regions downstream of the *tox* and *attB* sites of strains 0102, O-9, 0510, known *attB*-related sites of *C. ulcerans* and *C. diphtheriae*, and the *attP* site of corynephage. The regions of strains 0102 and O-9 were identical to those of strains 0509 and 0607, and that of strain Ran, respectively. There were major portions of the *attP*/*attB* common core, which was created by site-specific recombination between the *attP* and *attB* sites (24), located 83 bps after the *tox* stop codon. The regions were more similar to the *attP* site than to the *attB*-related site.

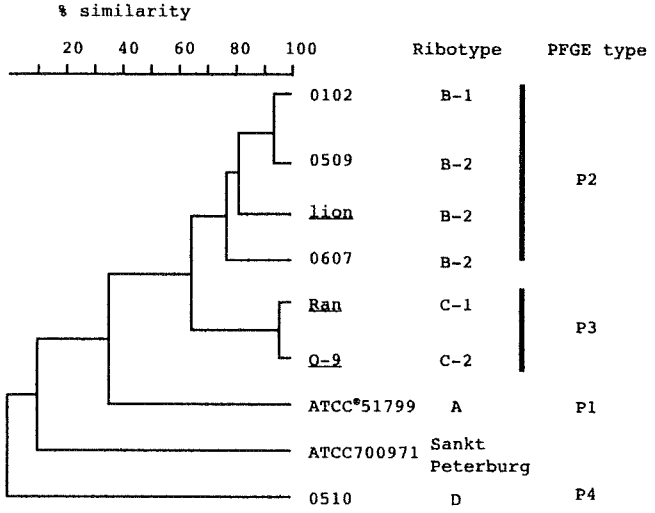

Fig. 3. Dendrogram showing the genetic relationships of *C. ulcerans* isolates in Japan. The isolates obtained from animals are underlined.

DISCUSSION

We described here the first cases of *C. ulcerans* isolated from *O. orca* and *P. leo*, the utility of ribotyping and PFGE for epidemiological surveillance, the similarity of *tox* and *pld* sequences between these strains, and the properties of the corynephage attachment site.

Although person-to-person spread of toxigenic *C. ulcerans* was not detected, it is thought that diseased animals were associated with the *C. ulcerans* infection of patients in some cases (12). To clarify the source of the bacterium, epidemiological surveillance is needed. The utility of subtyping of *C. ulcerans* has not been investigated in nearly as much detail as has that of *C. diphtheriae*. Subtyping methods include serotyping, phage typing, ribotyping, and PFGE (38-42). We carried out ribotyping and PFGE using the same protocols as those used for examination of *C. diphtheriae*. Interestingly, the lion strain and certain other clinical isolates (strains 0509 and 0607) shared similar ribotypes and PFGE type patterns. It is possible that the isolates were genetically homologous. The data indicated the possibility of the association of some diseases in animals with *C. ulcerans* infection in humans in Japan, which has also been observed in European countries. Moreover, *tox* and *pld* gene sequencing, Southern blot, ribotyping, and PFGE analyses demonstrated that two of the isolates from *O. orca* corresponded to a single strain. This finding pointed out the possibility of ease of transmission due to close contact with animals. Strains Ran, O-9, and lion were isolated from one facility. This finding suggested that there were both toxigenic and nontoxigenic bacteria present at the same facility. Because we were unable to use European strains in this study, there are no definite conclusions regarding differences between Japanese and European strains. However, these isolates in Japan are likely to be characteristic of those previously observed in Japan based on comparisons with

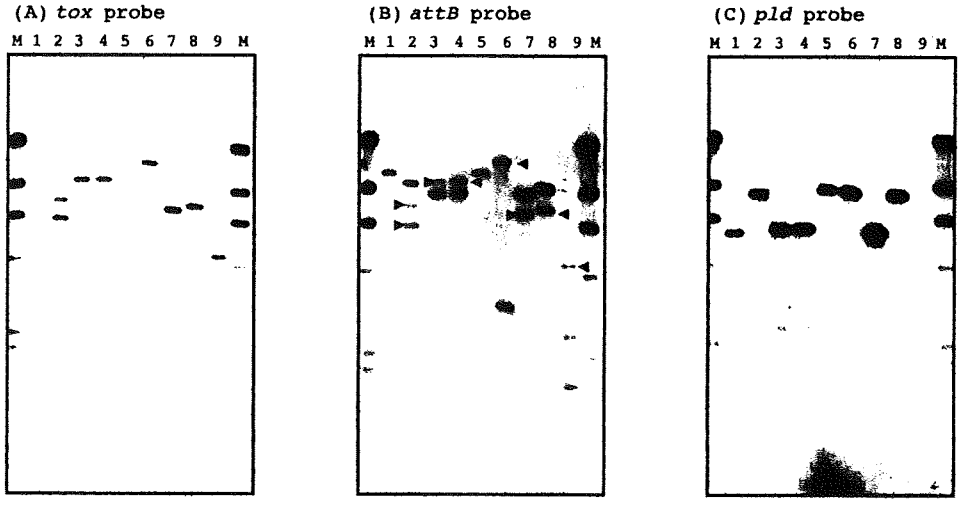

Fig. 4. Southern blot analysis of *C. ulcerans* and *C. diphtheriae* genomic DNA with DIG-labeled (A) *tox*, (B) *attB* site, and (C) *pld* specific probes. M, DIG-labeled molecular standard marker; lane 1, ATCC®51799; lane 2, 0102; lane 3, Ran; lane 4, O-9; lane 5, lion; lane 6, 0509; lane 7, 0510; lane 8, 0607; lane 9, *C. diphtheriae* ATCC 700971. Arrows indicate the bands that are the same in *tox* fragments.

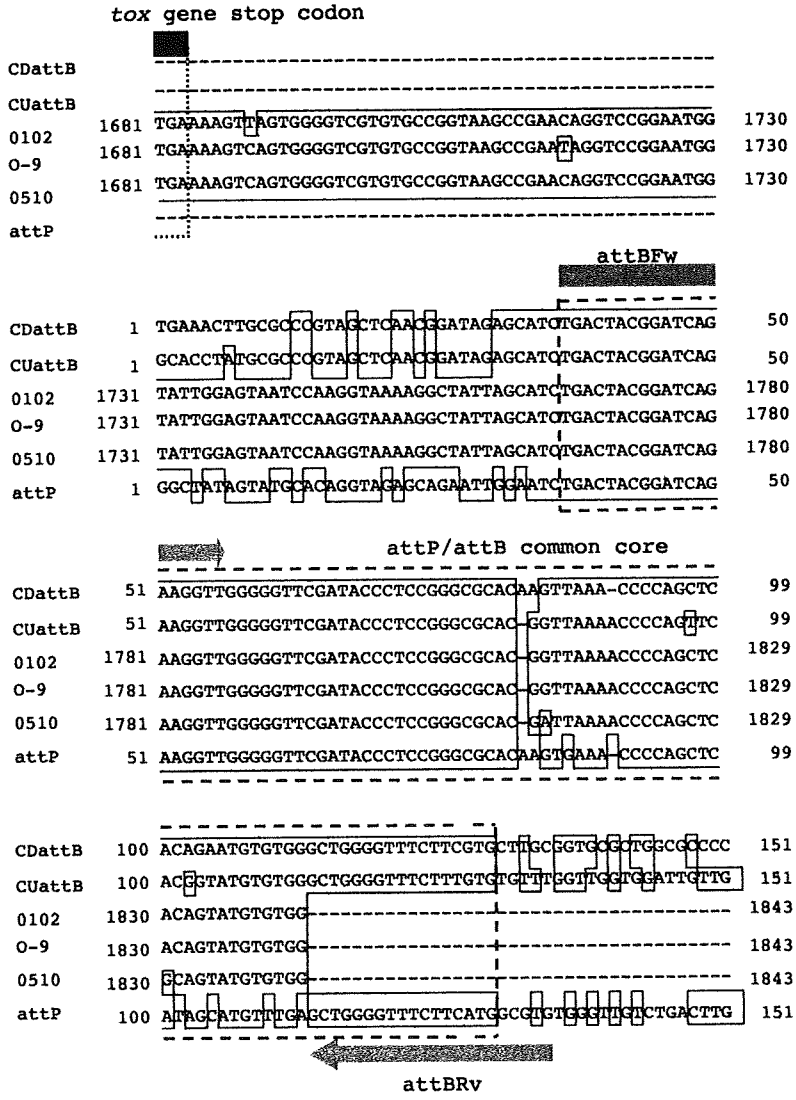

Fig. 5. Alignment of the deduced nucleotide sequences of attachment site homologous genes from *C. ulcerans* strains 0102, O-9, 0510, *C. ulcerans attB*-related site, *C. diphtheriae attB*-related site, and corynephage-gamma DNA for the *attP* site.

the ribotyping data from UK-derived isolates (7). The dendrogram derived from the PFGE pattern was same as that obtained from the ribotype grouping in our study. However, a larger number of isolates will be needed to determine the comparative usefulness of molecular epidemiological typing in *C. ulcerans*.

The results of the Southern blot analysis revealed that the *tox* genes were close to the *attB* sites in these isolates. Strain 0102 was a tandem double lysogen (unstable conformation) and the other strains were single lysogens. The production of DT by *C. diphtheriae* was shown to be proportional to the number of prophages integrated into its chromosome. The culture supernatant of strain 0102 had equivalent cytotoxic activity to that of the single lysogen strains under the conditions used here. We also demonstrated that the *attP/attB* common core was located downstream of the *tox* gene. The present results suggest that the number of *attB* sites in *C. ulcerans* differs from that in *C. diphtheriae*; however, the integration system appears to function in the same manner.

There have been few investigations of the pathogenicity of PLD production by *C. ulcerans*. We cannot exclude the possibility that differences in toxin production may exert an influence on *C. ulcerans* infection. Due to PLD production and the high level of similarity between *pld* genes among the isolates, PLD may be useful as a marker of *C. ulcerans* (21). It was moreover noteworthy that the DLT sequences of four toxigenic strains were identical to that of a German isolate, A6361 (Table 3). The identity of these DLT sequences suggests that there is a high degree of conservation in DLT. We are currently investigating differences in the immunological and cytotoxic activities of DLT and DT.

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NOTE *Public Health*

Evaluation of a Microplate Agglutination Test (MAT) for Serological Diagnosis of Canine Brucellosis

Masanobu KIMURA[1], Koichi IMAOKA[1]*, Michio SUZUKI[1], Tsuneo KAMIYAMA[1] and Akio YAMADA[1]

[1]*Department of Veterinary Science, National Institute of Infectious Diseases, 1–23–1 Toyama, Shinjuku-ku, Tokyo 162–8640, Japan*

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ABSTRACT. A microplate agglutination test (MAT) was compared with the tube agglutinin test (TAT), a standard test for the diagnosis of *Brucella canis*, in terms of the sensitivity and specificity. The results showed that MAT was more sensitive, simpler to perform and easier to read the results than TAT. On top of that the MAT allows us to handle a larger number of samples at once. Using this method we conducted sero-surveillance of the prevalence of *B. canis* in dogs kept in an Animal Shelter located in Kanagawa Prefecture. Twelve of 485 (2.5%) showed seropositive against *B. canis*. These results indicate that *B. canis* infection in dogs is still occurring in Japan.
KEY WORDS: *Brucella canis*, canine brucellosis, microplate agglutination test.

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Brucellosis, one of the major zoonoses worldwide, is caused by a bacteria belonging to the genus *Brucella* [4]. Among many species of the genus *Brucella, B. melitensis, B. abortus, B. suis* and *B. canis* are known to result in human brucellosis. Although *Brucella* spp. with smooth-type lipopolysaccharides (LPS), such as *B. melitensis, B. abortus* and *B. suis*, are known to infect several domestic animals, such as cows, sheep, goats and pigs, *B. canis*, one of *Brucella* spp. with rough LPS, infects a limited host range, such as dogs and wild canidae. *B. canis* infection in dogs is usually asymptomatic but can sometimes cause contagious abortion, epididymitis, testicular atrophy and infertility [3]. Most canine infections occur by direct contact with lochia at the time of abortion or vaginal discharges in infected female dogs. Semen and urine from infected male dogs have also been implicated as sources of infection [7]. Drug therapy for *B. canis* infection requires an appropriate regimen of antibiotic combination, but relapse may ensue, because *B. canis* often persists within macrophages or other type of cells [3]. Humans are rarely infected with *B. canis*. Most human infections are asymptomatic; however, several clinical symptoms, which are milder than those observed with other *Brucella* spp. , are sometimes noticed [12].

In Japan, *B. canis* infection was first reported in a breeding colony of beagles in 1972 [20]. Several epidemiological studies of canine brucellosis in Japan were conducted in the 1970s and 1980s [10, 11, 15–18], but there have only been a few reports since then. In 2003 and 2006, canine brucellosis emerged as outbreaks in large breeding colonies, suggesting that *B. canis* infection is still enzootic in Japan. To assess the possible risk of *B. canis* on human, determination of the prevalence of *B. canis* in the dog population in Japan seemed helpful.

Although tube agglutination test (TAT) is the most widely used laboratory test for the detection of *B. canis* antibodies in both humans and canines, it is time-consuming and cumbersome in terms of performance and measurement of results [2]. On the other hand, microplate agglutination test (MAT) described for *B. canis* [5] and *B. abortus* [1, 6] appeared advantageous, because a larger number of samples can be processed simultaneously by this method. In the present study, we attempted to evaluate whether the use of MAT with safranine-stained bacterial cells as antigens could serve as a substitute for TAT to conduct sero-epidemiologic investigations of canine brucellosis in Japan.

TAT was carried out by placing 0.5 m*l* of 2-fold serially diluted sera and an equal volume of *B. canis* antigen solution (OD_{600}=1) purchased from the Kitasato Institute (Tokyo, Japan) in glass tubes. After incubation at 50°C for 24 hr, the agglutination titer was determined and expressed as a reciprocal of final serum dilutions, which gave rise to agglutination as observed in the 50% control tube. Titers of 160 or higher were considered positive. Anti-*B. canis* antibody was prepared in our laboratory by immunizing a rabbit with inactivated *B. canis* whole antigen and was included as a reference.

MAT was performed as follows. First, serum samples, 2-fold serially diluted in phosphate-buffered saline, were prepared in a 96-well U-bottom microplate. Then, an equal volume (25 μ*l*) of *B. canis* antigen solution (Kitasato Institute), which is same as used in TAT, containing 0.005% safranine solution (2% of Favor G®, Nissui Pharmaceutical Co., Tokyo, Japan) was added to each well. The sealed plates were mixed gently for 20 sec and incubated at 50°C for 24 hr in a humid atmosphere. The titers were expressed as a reciprocal of the highest dilution of sera showing agglutination. Safranine-stained antigens made it possible to judge the results more easily and objectively. An agglutination titer greater than 160 was considered positive.

We have experienced an outbreak of *B. canis* infection in 2003 [8]. Sera obtained from dogs involved in the outbreak were examined for the presence of anti-*Brucella* antibody by TAT. Fifty-one of 110 sera tested positive for antibody against *B. canis*. These sera were subjected to MAT for

* CORRESPONDENCE TO: IMAOKA, K., Department of Veterinary Science, National Institute of Infectious Diseases, 1–23–1 Toyama, Shinjuku-ku, Tokyo 162–8640, Japan.
e-mail: imaoka@nih.go.jp

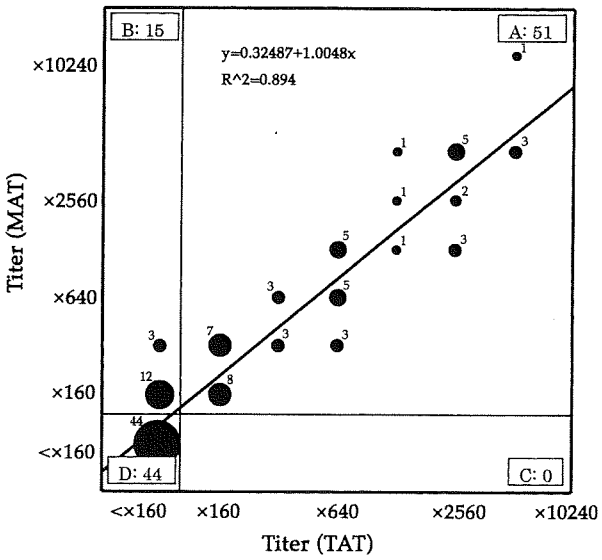

Fig. 1. Correlation of antibody titers determined by TAT and MAT. Sera were obtained from 110 dogs in the outbreak of *B. canis* infection in 2003. The titers are the reciprocals of the highest dilution of serum showing agglutination. Pearson's product-moment correlation coefficient (r) was determined to be 0.945, $p<0.01$ by regression analysis. A-D shows each area and the number of dogs of each area.

determining its specificity and sensitivity. As shown in Fig. 1, 15 sero-negative samples (1:80) by TAT became positive (1:160 and 1:320) when MAT was employed. Combinatorial polymerase chain reaction method [9] showed that *B. canis*-specific gene segments were present in the sera of those 15 dogs [8], which indicate that they were infected with *B. canis* (data not shown). Therefore, MAT appeared superior to TAT in terms of sensitivity as shown in the previous report by Dump *et al.* [5]. The titers determined by two methods correlated well (R^2 = 0.894) as shown in Fig. 1.

We have therefore decided to apply MAT in the sero-surveillance of *B. canis* in Japan. During the period from February 2003 to December 2006, 485 serum samples were obtained from dogs in an animal shelter in Kanagawa Prefecture (Table 1). The dogs were categorized into three groups according to their origin: pets, strays, and unknown.

The results are summarized in Table 1. Of 485 dogs, 12 (2.5%) tested positive for antibody against *B. canis*. Sero-prevalence in this study (2.5%) seemed slightly lower than those of 1970's (0.8%–21.7%) [10], but there was no apparent difference. This indicated that the disease was enzootic but not epizootic in Japan. Of the 12 sero-positive dogs, 3 were pets and 9 were strays. The reason why the apparent prevalence of infection was higher in strays (5.7%) than in pets (0.9%) was unknown, but it was likely that stray dogs had more opportunities to encounter other dogs, fomites or environments contaminated by bacteria. No differences attributed to the sex (Table 1) or breed (data not shown) of dogs were observed in the prevalence of infection.

Although symptomatic infections of *B. canis* in humans are rarely reported, Lucero *et al.* [12] pointed out that the possibility of *B. canis* infection in humans may be more widespread than speculated. Recently, an unusually severe form of human brucellosis caused by *B. canis* was reported [13]. Because human infections commonly occur after contact with the blood, semen, or placenta of infected dogs [19], an understanding of the prevalence of *B. canis* infection in dogs may help in inferring preventive measures for reducing human exposure to the bacteria. The results of the present sero-epidemiologic study showed that *B. canis* infection is still enzootic in Japan. Moreover, human brucellosis cases reported in 2002, 2005 and 2006 were probably caused by *B. canis* [14]. It therefore seems, prudent that individuals at high risk of infection such as veterinarians, breeders and pet owners, be advised of possible *B. canis* infection in dogs.

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Table 1. Sero-prevalence of antibodies against *B. canis* in dogs determined by MAT

Year	Male		Female		Total	
	Negative	Positive	Negative	Positive	Negative	Positive
2003	67	0	24	1	91	1
2004	82	3	38	2	120	5
2005	103	3	74	1	175	4
2006	52	2	29	0	81	2
Total	304	8 (2.6%)	165	4 (2.4%)	467	12 (2.5%)

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