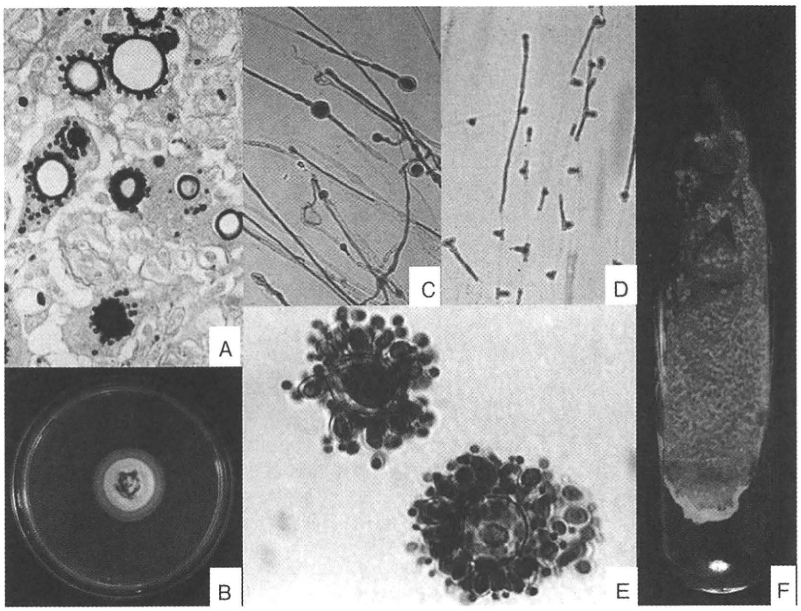

図3 *Paracoccidioides brasiliensis*
(A) 感染組織内での操舵輪状の構造物，Grocott渡銀染色。
(松下記念病院 中島善洋先生よりご提供)
(B) 菌糸形集落，ポテト・デキストロース寒天平板培地，25℃，60日間。
(C) 厚膜胞子。
(D) アレウリオ型分生子。
(E) 酵母様細胞の多極性出芽。
(F) 酵母様集落，1%ブドウ糖添加ブレインハートインヒュージョン寒天斜面培地，35℃，7日間。

色で検出されることから，リンパ節の腫脹，肉芽腫性病変を呈する腫瘍との鑑別が可能である(図3A)。

原因菌は*Paracoccidioides brasiliensis*で温度依存性の二形性真菌で，室温ではきわめて遅い菌糸形発育をし，集落は変化に富む。初め無毛，やがて中心部からビロード状，羊毛状から綿毛状，培養が進むにつれて褐色調を帯び，集落中心部に亀裂が生じて噴火口状もしくは亀裂の辺縁がまくれ上がる(図3B)。顕微鏡的に分生子はほとんど産生されず，隔壁を有する細い菌糸と厚膜胞子が観察され，まれにアレウリオ型分生子を形成する株もある(図3C，D)。このような分生子は皮膚糸状菌症原因菌関連菌種の*Chrysosporium* spp.も形成することがある。

宿主内や特殊な培地を用いて35～37℃で培養すると酵母様発育となる。酵母様集落は白色から淡黄色でやや乾燥し，多くのシワをもつ。顕微鏡的には一つの母細胞が細い頚管を介して同時多極的に娘細胞を出芽する多極性出芽が特徴である(図3E，F)。

マルネッフェイ型ペニシリウム症原因菌 *Penicillium marnefferi* [4]

マルネッフェイ型ペニシリウム症は東南アジアに分布する。原因菌は*Penicillium marnefferi*で温度依存性の二形性真菌である。菌糸形の発育速度は中等度で，室温では初め白色から黄色，綿毛状で分生子を産生するにつれて集落表面は青緑色になる。培地中にポートワイン様の紅い色素を急速に拡散する(図4A，B)。

顕微鏡的には分生子柄より分枝した枝は広がり，その先端には4～5個のメツラをつけ，メツラはビン型の複数のフィアライドをつける。分生子は球形から亜球形である(図4C)。

特殊な培地を用いて35℃で培養すると，膜様，灰白色の酵母様集落を形成する。これらの酵母様細胞は1細胞性で，分裂により増殖する。培養物を顕微鏡で観察すると短菌糸の集合体が確認できる(図4D，E)。宿主寄生型もこの短菌糸が変形した酵母様形である。

ブラストミセス症原因菌 *Blastomyces dermatitidis* [4]

ブラストミセス症は，北米大陸の五大湖地方およびアフリカ大陸諸国が流行地域であるが，インドおよび中近東の一部も流行地と思われる。

原因菌は*Blastomyces dermatitidis*である。温度依存性の二形性真菌で，室温で培養すると菌糸形発育し，速度は遅い～中等度，表面は膜様から羊毛状，白色から黄褐色を呈する。顕微鏡的には，短いあるいは比較的長い菌糸様の分生子柄の先端に無色，1細胞性の球形から亜球形の分生子が産生される(図5A～C)。このような分生子は皮膚糸状菌症原因菌関連菌種の*Chrysosporium* spp.も形成することがある。

一方，感染組織内および特殊な培地を用いて35～37℃で培養すると表面

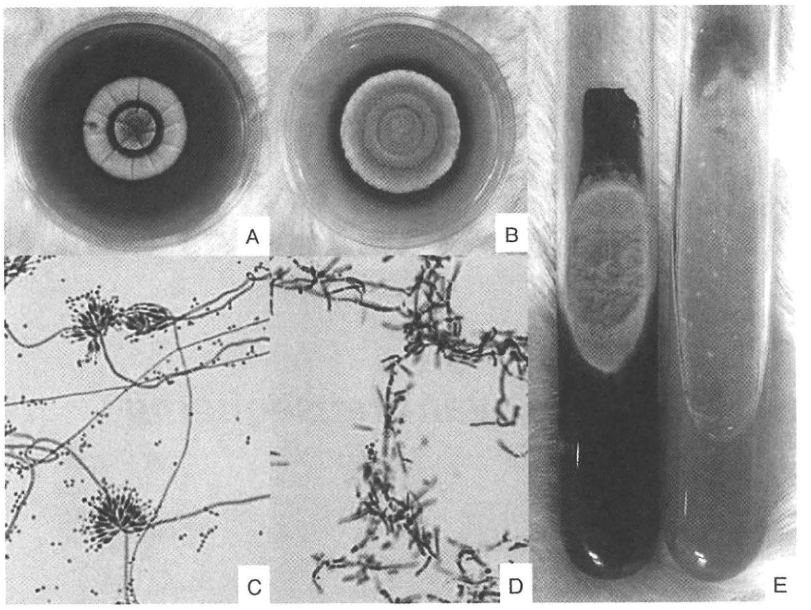

図4 *Penicillium marnefferi*
(A)菌糸形集落，サブロー寒天平板培地，25℃，14日間。
(B)菌糸形集落，ポテト・デキストロース寒天平板培地，25℃，14日間。
(C)フィアライドと分生子。
(D)酵母様集落の顕微鏡所見。短菌糸の集団から構成されている。
(E)左：菌糸形集落，ポテト・デキストロース寒天斜面培地，25℃，14日間。
右：酵母様集落，1％ブドウ糖添加ブレインハートインヒュージョン寒天斜面培地，35℃，7日間。

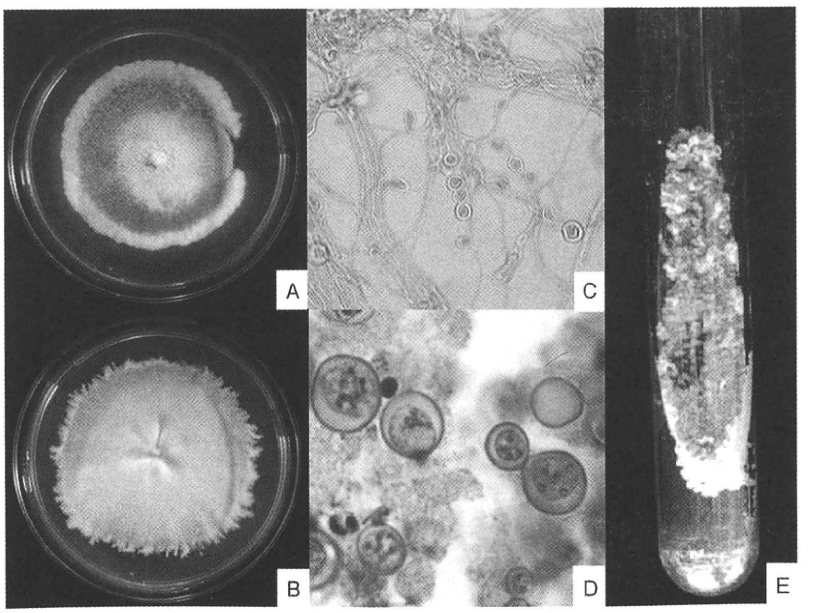

図5 *Blastomyces dermatitidis*
(A)菌糸形集落，サブロー寒天平板培地，25℃，60日間。
(B)菌糸形集落，ポテト・デキストロース寒天平板培地，25℃，60日間。
(C)分生子。
(D)母細胞と娘細胞の接着面が広い酵母様細胞。
（千葉大学名誉教授 宮治 誠先生よりご提供）
(E)酵母様集落，1％ブドウ糖添加ブレインハートインヒュージョン寒天斜面培地，35℃，7日間。

は淡い黄色を帯び，不規則に隆起し，細やかな皺壁を有する酵母様集落となる。温度変化に伴って，菌糸の一部が膨化し，球形の酵母様細胞となり，単出芽により娘細胞を産生し増殖する。母細胞と娘細胞の接着面は*P. brasiliensis*の酵母様形と比較して広いことが特徴である(図5D，E)。

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ドブネズミより分離された *Arthroderma vanbreuseghemii*

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特集　人と動物の共通感染症最前線　7
212-213頁

特集

人と動物の共通感染症最前線 7

Special Issue

ドブネズミより分離された *Arthroderma vanbreuseghemii*

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要　約

人獣共通皮膚糸状菌症原因菌の1種 *Arthroderma vanbreuseghemii* による感染は我が国でも散見される。本菌種はげっ歯類（ドブネズミなど）が保菌し，まず猫がネズミを捕獲することにより感染し，その猫に人が接触して感染すると推測されている。今回，ドブネズミなど33頭の被毛を調べたところ，千葉県で捕獲されたドブネズミ1頭より本菌種が分離され，その遺伝子型は既知の人症例由来株と同一であったので，本菌種の感染にドブネズミの関与が示唆された。

はじめに

皮膚菌糸状症は人と動物の間での接触により感染する人獣共通真菌症で，人が動物由来の皮膚糸状菌に感染すると激しい炎症を起こすことはよく知られている。主な原因菌は *Microsporum canis*，*Trichophyton mentagrophytes*（無性型），*T. verrucosum*，*M. gypseum* などがあげられる。なかでも *T. mentagrophytes* は *M. canis* についで2番目に症例が多い人獣共通皮膚糸状菌症原因菌である。

T. mentagrophytes の有性型は *A. vanbreuseghemii*，*A. benhamiae*，*A. simii* が知られている。このうち *A. vanbreuseghemii* は古くからわが国でも多くの症例が報告されており，人は愛玩動物および実験動物用げっ歯類との接触による感染が報告されている。また，Drouot らによれば海外ではネズミを捕る習性のある猫で本菌種の感染率が高いことから，ネズミが感染に関与していることが示唆されている[1]。しかしながら，わが国では都市型野生動物としてのげっ歯類での本菌種の感染経路ならびに保有率は明らかになっていなかった。そこで，我々は千葉県と東京都で捕獲されたドブネズミ，クマネズミの皮膚糸状菌症原因菌 *A. vanbreuseghemii* および関連菌種の保有率を調べた。

なお2007年に Nenoff らにより，*T. mentagrophytes* の無性型に関する概念が訂正され，世界的には彼らの提唱する有性型と無性型の関係が認められている。その変更点として，かつて，有性型の *A. vanbreuseghemii* の無性型としての *T. mentagrophytes* は好人性の *T. mentagrophytes* var. *interdigitale*，*T. mentagrophytes* var. *nodulare* および *T. mentagrophytes* var. *goetzii* と好獣性の *T. mentagrophytes* var. *granulosum* に分けられていたが，これらを一括して *T. interdigitale* に変更された。かつて好獣性の *A. benhamiae* を有性型とする *T. mentagrophytes* は *T. mentagrophytes* var. *granulosum* と *T. mentagrophytes* var. *erinacei* に分けられていたが，これらは *Trichophyton* sp. と *T. erinacei* がそれぞれ対応することになった。やはり好獣性の *A. simii* を有性型とする無性型は *T. mentagrophytes* var. *quinckeanum* と呼ばれていたが，variety を付けずに *T. mentagrophytes* と名称が変更された。また，かつてこれらの無性型の名称は，多くの場合，variety を付けず，*T. mentagrophytes* もしくは *T. mentagrophytes* complex として扱われてきたが，これらの表現も認められていない。しかし，新規に提唱された *Trichophyton* spp. の名称は混乱を招いていることから，我々は旧来の名称 *T. mentagrophytes* を用いた。

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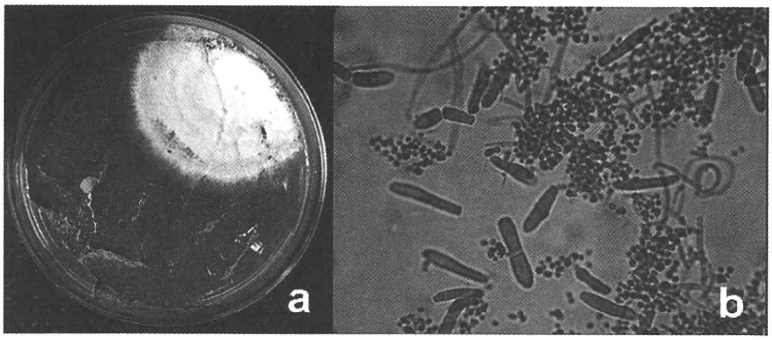
近所にとてもネズミを捕まえるのが上手な三毛猫がいます，時々獲物をもって見せびらかしていますので，ぜひ拝借して調べたいと思うのですが，私の顔が知られていますので写真に撮るだけで我慢です。これだけでも充分変な人と思われていますが…



a：ポテトデキストロース寒天平板培地にて，35℃，14日間培養して生育してきた集落。
b：大分生子，小分生子およびラセン体（ラクトフェノールコトンブルー固定染色，× 200）

写真1

調査方法

2008年8月より2009年6月までに東京都（17頭）もしくは千葉県（16頭）で捕獲されたクマネズミ（*Rattus rattus*）7頭（雄4，雌1，性別不明2），ドブネズミ（*Rattus norvegicus*）26頭（雄9，雌10，性別不明7）計33頭について調べた。いずれのネズミも外見に脱毛，貧毛などの変化を認めなかった。エーテル吸入による軽麻酔下で，市販の歯ブラシで被毛表面を20回こすり，歯ブラシに付着した被毛，落屑をアクチジオンと抗生物質を添加したポテトデキストロース寒天平板培地にて，35℃，14日間培養して，表面がやや褐色を帯びた粉状構造をもつ綿毛状集落を釣菌し（写真1a），形態学的，リボゾームRNA遺伝子ITS領域の配列による分子生物学的同定および分子疫学的解析を行った。

結　果

千葉県で捕獲された雄のドブネズミ1頭より*T. mentagrophytes*（無性型）が分離された。集落は白色綿毛状，培地中に褐色の色素を産生した。少数の大分生子，菌糸に直角に付着する球形の小分生子が多数形成され，ラセン体（コイル状の菌糸）を認めた（写真1b）。

ウレアーゼ活性と毛髪穿孔試験は陽性であった。交配試験による交配型は子嚢果の形成にいたらず，不明であった。

この株のリボゾーム遺伝子ITS領域の配列は*A. vanbreuseghemii*由来配列から構成されるクラスターに属したことから，この株を*A. vanbreuseghemii*と同定した。さらにこの株の遺伝子型は千葉県で確認された人由来株と100％相同（DDBJ Accession No. AB518070）であった。

考　察

愛玩動物由来の*A. vanbreuseghemii*による感染は，わが国も含めて世界各国で多くの症例が報告されており，アウトブレイクに発展した事例も知られている[3]。今回，33個体中の1個体（約3％）と極めて低い保有率ではあったが，ドブネズミから*A. vanbreuseghemii*が分離され，その株の遺伝子型はドブネズミ捕獲地域と同一県内で発症した人症例由来株と同一であった。このことは人および各種飼育動物の*A. vanbreuseghemii*の感染経路に都市型野生動物のげっ歯類が関与していることを示唆するものであり，Drouotらの仮説[1]を支持するものであった。

また，我が国での*A. vanbreuseghemii*人感染例由来の遺伝子型には多型があることが知られている。他の患者由来の本菌種の遺伝子型と一致するげっ歯類由来株を求めて，現在も調査を続けている。

謝　辞　本研究は厚生労働省　新興・再興感染症研究事業「動物由来感染症のリスク分析手法に基づくリスク管理のあり方に関する研究」の一環として行われた。

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END

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Polymerase Chain Reaction Assay and Conventional Isolation of *Salmonella* spp. from Philippine Bats

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ABSTRACT

***Background*:** Salmonellae are important food and waterborne pathogens and the leading causes of the most widespread acute gastrointestinal illnesses around the globe. The organism has been detected in a wide range of host species such as mites, insects, crustaceans, mussels, fish, amphibians, reptiles, birds and mammals including wildlife animals. Salmonellae have been isolated in many species of bats in other countries. In the Philippines, there are 70 species of Philippine bats reported of which nine are considered as endemic. Although human salmonellosis (typhoid, paratyphoid and other *Salmonella*-associated infections) was the primary cause of illnesses and death from the 60 reported foodborne outbreaks (1995 to 2004), no case was ever reported involving Philippine bats. Since transmission of *Salmonella* from wildlife to humans is possible, as advocated by previous reports, the present study endeavored to isolate and molecularly detect *Salmonella* spp. from Philippine bats captured from Aklan, Laguna and Quezon City using conventional isolation method and polymerase chain reaction assay respectively. ***Materials, Methods & Results*:** A total of 96 apparently healthy bats were used in the study. Bats were captured using nylon mobile mist nets of 3 m long and 1.5 m high with 35 mm mesh size. Eleven species of bats were collected and identified following the reported key to the identification of Philippine bats. Majority of the collected species were insectivores under family Vespertilionidae while the largest population of the Philippine bats were frugivores belonging to family Pteropodidae. Necropsy was performed and intestines were collected and subjected to conventional culture method and PCR detection for *Salmonella* spp. Two samples (2.08%) were molecularly detected as positive for *Salmonella* spp. bacterial pathogen. The positive samples were obtained from the intestines of the adult female insectivorous bat species, *Miniopterus australis* and *M. schreibersi*, originating from Pangihan cave of Barangay Pablacion, Malay in Aklan. No *Salmonella* spp. was isolated using the conventional method.

***Discussion*:** The study reports the first detection and molecular evidence of *Salmonella* spp. in Philippine bats by PCR using intestinal samples. In addition, the data strongly indicated that PCR detection appears to be more sensitive over the conventional isolation method. The successful detection was attributed to the ability of PCR to sensitively detect atypical *Salmonella* and non-viable *Salmonella* cells. Results in the present study revealed that the Philippine bats, *Miniopterus australis* and *M. schreibersi*, both adult female insect-eating bats captured in Pangihan cave of Barangay Pablacion, Malay, Aklan harbored *Salmonella* in their intestines. Since salmonellae have been detected in a large variety of environment and host species including insects, these bats may have acquired these microorganisms in water and in their diet. This finding shows that Philippine bats may serve as potential reservoir and carrier of *Salmonella* organisms. The data also strongly indicates that bats may actively contribute in the dissemination of salmonellae into the environment through fecal route. This currently makes Philippine bats as a potential threat to livestock and may pose a serious public health concern, since all serotypes of *Salmonella* are considered to be pathogenic to humans.

Keywords: Bats, *Miniopterus australis*, *Miniopterus schreibersi*, *Salmonella*, PCR.

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INTRODUCTION

Salmonellae are important food and waterborne pathogens of the most widespread acute gastrointestinal illnesses worldwide [1,4,28]. The organism is present in the gastrointestinal tract of warm-blooded and cold-blooded animals and hence, excretion in feces results in contamination of water, food and environment [11]. The organism has been detected in a variety of host species such as mites, insects, crustaceans, mussels, fish, amphibians, reptiles, birds and mammals including wildlife animals [2,10,15,17,21]. Many studies have reported bats as natural hosts of many emerging and re-emerging infectious diseases [19].

Salmonellae have been isolated in many species of bats in other countries [2,15]. In the Philippines, there are 70 species of Philippine bats reported of which nine are endemic as listed in the 2000 IUCN Red List of Threatened Species [16]. Since *Salmonella* is zoonotic in nature and previous study has reported that wildlife may serve as a reservoir for *Salmonella* infections [26], the present study endeavored to isolate and molecularly detect *Salmonella* spp. from Philippine bats captured from Aklan, Laguna and Quezon City using conventional isolation and polymerase chain reaction (PCR) assay respectively.

Two PCR positive samples (2.08%) were obtained from the intestines of the adult female insectivorous bat species, *Miniopterus australis* and *M. schreibersi*, collected from Pangihan cave of Barangay Pablacion, Malay in Aklan. No *Salmonella* spp. was isolated using the conventional method. This finding indicates that Philippine bats are potential carrier of *Salmonella* spp. and may play a significant role in the dissemination of these pathogenic organisms in the environment. Furthermore, the study represents the first detection of *Salmonella* spp. in Philippine bats.

MATERIALS AND METHODS

Collection of bats

A total of 96 apparently healthy bats were used in the study. Forty (40) bats were collected at the Pangihan caves in Barangay Pablacion, Malay and Libertad caves in Barangay Libertad, Nabas in Aklan using nylon mobile mist nets of 3 m long and 1.5 m high with 35 mm mesh size. The mist nets were set up on the entrance and inside the caves. Nylon mist nets of 12 m long and 2 m high with 35 mm mesh size were used to capture twenty four (24) and thirty two (32) bats from the University of the Philippines Los Baños (UPLB) Hortorium in Laguna, and UP Diliman Marine Science Institute (MSI) and Protected Areas and Wildlife Bureau (PAWB) in Quezon City respectively. Seven net nights for one night placed along trails on forest gaps and across the river were set up in Laguna while 14 net nights, seven mist nets for two nights placed near swampy areas in Quezon City.

Species identification of bats

Eleven species of bats were collected and identified following the reported key to identification of Philippine bats [9]. Five species of insect-eating bats and one species of fruit-eating bat were captured from Aklan namely, *Miniopterus australis, M. schreibersi, M. tristis*, *Hipposideros diadema, Myotis macrotarsus* and *Ptenochirus jagori* respectively. Three species of fruit-eating bats, *Ptenochirus jagori, Cynopterus brachyotis* and *Eonycteris spelaea,* were collected from Laguna. Two species of insect-eating bats, *Scotophilus kuhlii* and *Pipistrellus javanicus,* and four species of fruit-eating bats, *Ptenochirus jagori, Cynopterus brachyotis, Rousettus amplexicaudatus* and *Eonycteris spelaea* were captured in Quezon City.

Necropsy of bats and sample collection

After the collection, the body weight of each bat was determined and the dosage for anesthetic was computed using a dose of 0.45 mL of 5% zolazepam-tiletamine[1] per 30 g body weight. The anesthetic was given intramuscularly and the bat was euthanized through intracardiac exsanguination. The body parameter measurements of each carcass were recorded to use for identification purposes.

Each bat was then placed on a necropsy board where the skin over the thorax and abdomen was reflected. The thorax was opened and the internal organs were collected by research collaborators from Japan for other investigative works. In the present study, the peritoneum was incised and the intestine was detached from its mesentery. The entire intestinal tract was cut through the rectum, ligated on both ends and placed on a sterile Petri dish with normal saline solution. The carcass was submitted to the UPLB Museum of Natural History for preservation and storage.

Conventional isolation method

The small intestines were minced and transferred to a pre-labeled tube of nutrient broth[2]. The samples were

incubated at 37°C for 24 h. After incubation, aliquots of the samples were transferred to tetrathionate brilliant green (TBG) broth[3] and incubated under 42°C for 24 h.

All of the enriched samples were streaked on xylose-lysine deoxycholate (XLD) agar[4] plates and incubated at 37°C for 24 h. Expected *Salmonella* colonies appear as pink colonies with black center on XLD media. The suspected colonies were purified, subjected to biochemical tests (triple sugar iron[5], indole[6], Methyl-Red[7], Voges-Proskauer[7], citrate[8], urease[6] and lysine decarboxylase[6] tests) and confirmed using miniaturized identification kit[9].

DNA extraction

The DNA extraction was done based on the National Institute of Molecular Biology and Biotechnology (BIOTECH) protocol. Briefly, each 1.5 mL TBG broth was placed in a 1.5 mL microcentrifuge tube and centrifuged at 8,050 x g for 10 s to remove the minced tissues. The supernatant was transferred to fresh microcentrifuge tube and the debris was discarded. The tube was then centrifuged at 8,050 x g for 5 min to collect the cell pellets. The supernatant was removed and the cell pellets were washed with 150 µL HPLC-grade water. The mixture was mixed using vortex mixer and centrifuged for another 5 min. The supernatant was again discarded and the tube was suspended in 45 µL HPLC-grade water and then mixed. Five microliters of 10% sodium dodecyl sulfate (SDS)[10] was added and mixed. The mixture was boiled for 5 min. After boiling, 0.95 mL of HPLC-grade water was added in the mixture and then mixed. Final dilution of the samples was done using HPLC-grade water.

Polymerase chain reaction assay and analysis

PCR detection of *Salmonella* employed a developed kit[11] that contained a genus-specific primer, *Sal*-05, with the following nucleotide sequence: 5'-GGCAGGGCTCATTT-TTACG-3'. PCR incubation steps were performed using a thermal cycler[12] with the following settings: initial denaturation at 95°C for 5 min, 30 cycles of denaturation at 94°C for 2 min, annealing at 56°C for 2 min and extension at 72°C for 2 min, followed by final extension by polymerase at 72°C for 10 min, and holding temperature at 8°C.

The PCR products along side with 1kb DNA ladder[13] and negative and positive control were resolved using 1% Tris-acetate-EDTA (TAE) agarose gel[13] in an electrophoresis chamber containing 0.5x TAE buffer. The gel was run at 100 V for 30 min until the dye indicator reached the target lane. At the end of each run, the gel was soaked in ethidium bromide solution for 15 min, washed and viewed under an ultraviolet transilluminator machine[14]. The machine was connected to a computer with a software program[15] for the documentation of each run.

RESULTS

The samples obtained from the small intestines of 96 apparently healthy bats were subjected to conventional culture method and PCR detection for *Salmonella* spp. The summary of the species of bats included in the study is shown in Table 1.

A total of 11 species of bats were collected, namely *Miniopterus australis, M. schreibersi, M. tristis, Hipposiderus diadema, Myotis macrotarsus, Ptenochirus jagori, Scotophilus kuhlii, Pipistrellus javanicus, Cynopterus brachyotis, Eonycteris spelaea,* and *Rousettus amplexicaudatus*. These different species are grouped into three families and based on the number of species obtained, the least falls under Rhinolophidae (1/11), followed by Pteropodidae (4/11), while majority were classified under Vespertilionidae (6/11).

Two (2.08%) of the 96 samples subjected to PCR produced the expected 450-bp band for *Salmonella*-positive samples as exhibited in Figure 1. The positive samples came from *Miniopterus australis* and *M. schreibersi*. These chiropterans were both insect-eating adult female bats captured from Pangihan cave of Barangay Pablacion, Malay in Aklan. However, all of the minced small intestine samples of the bats were found negative for *Salmonella* spp. using the conventional isolation method. Suspected colonies from xylose-lysine deoxycholate agar plates were confirmed by biochemical tests (triple sugar iron, indole, methyl-red, Voges-Proskauer, citrate, urease and lysine decarboxylase tests) and further verified by a miniaturized identification kit.

DISCUSSION

A total of 96 apparently healthy bats belonging to three families (Vespertilionidae, Pteropodidae, Rhinolophidae) were collected. Two of the families (Vespertilionidae & Rhinolophidae) were identified as insectivores and one family as frugivores. Based on the number of species obtained, the least falls under Rhinolophidae (1/11), followed by Pteropodidae (4/11) and majority were classified under Vespertilionidae (6/11) (Table 1). However, based on the number of collected

Table 1. Summary of the species of bats tested for *Salmonella* spp. using conventional isolation and PCR methods.

Bat Species (Common name)	Family	Place of Collection			
		A	L	QC	Total
Miniopterus australis (Little bent-winged bat)	Vespertilionidae	21	0	0	21
Miniopterus schreibersi (Common bent-winged bat)	Vespertilionidae	15	0	0	15
Miniopterus tristis (Greater bent-winged bat)	Vespertilionidae	1	0	0	1
Scotophilus kuhlii (Lesser Asian house bat)	Vespertilionidae	0	0	5	5
Pipistrellus javanicus (Javan pipistrelle)	Vespertilionidae	0	0	3	3
Myotis macrotarsus (Philippine large-footed myotis)	Vespertilionidae	1	0	0	1
Ptenochirus jagori (Musky fruit bat)	Pteropodidae	1	17	1	19
Cynopterus brachyotis (Common short-nosed fruit bat)	Pteropodidae	0	4	20	24
Eonycteris spelaea (Common nectar/dawn bat)	Pteropodidae	0	3	1	4
Rousettus amplexicaudatus (Common rousette)	Pteropodidae	0	0	2	2
Hipposideros diadema (Diadem roundleaf bat)	Rhinolophidae	1	0	0	1
Total		40	24	32	96

*A- Aklan L- Laguna QC- Quezon City

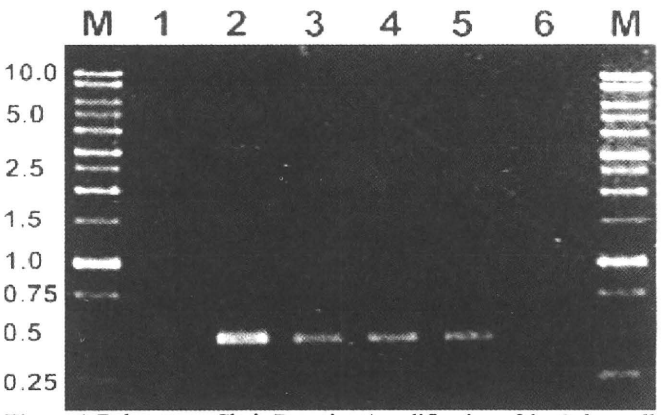

Figure 1. Polymerase Chain Reaction Amplification of the *Salmonella* genus-specific 450-bp PCR products from bat samples. (M) Molecular size marker (1kbp DNA ladder); (1) Negative control (HPLC-grade water); (2) Positive control (*Salmonella* Thyphimurium); 3-4 (Sample 1) & 5-6 (Sample 2) Two *Salmonella* positive samples showing the expected 450-bp amplicon size.

samples, majority were frugivores (Pteropodidae). This can be due to small body size and ability to produce echolocation signals of insectivores which enable them to escape and avoid being trapped in the mist nets [7].

Vespertilionidae is the largest family within the order Chiroptera and is worldwide in distribution. Most species are known to be insectivores and roost in caves. Likewise in the Philippines, previous data have shown that the number of insectivores was almost twice as that of frugivores [7,16].

The present study was able to molecularly detect *Salmonella* species from the small intestines of two (2/96 bats) vespertilionids adult female bats (*Miniopterus australis* and *M. schreibersi*) from Panay Island (Pangihan cave, Aklan). The successful detection was attributed to the ability of PCR to detect atypical *Salmonella* spp. and non-viable *Salmonella* cells which makes it more sensitive and specific in determining the presence of the target *Salmonella* DNA. This result is in agreement with previous studies.

It was previously shown that the detection of *Salmonella* sp. in 391 fecal samples from cattle, pig and poultry in Sweden using commercial PCR-based method (BAX® system) was proven to be satisfactory [6]. In addition, a study using PCR for *Salmonella hilA* gene also successfully amplified the expected 784-bp DNA fragment in all the 33 *Salmonella* strains from 27 serotypes while none from all the non-*Salmonella* strains tested. Furthermore, it was able to detect *S. choleraesuis* subsp. *choleraesuis* serovar Typhimurium in artificially contaminated fecal samples at a concentration of 3×10^2 cfu/mL [20]. Similarly, the use of PCR for specific detection of *Salmonella* spp. in food has been documented. It was reported that PCR targeting specific gene (e.g. *fimA, invA, ssaR)* displayed high degree of diagnostic accuracy, without unspecific amplification or false signals and was found to be faster, less costly and more reliable than the traditional culture methods [5,14,18,25]. Likewise, the BIOTECH *Salmonella* PCR-based detection kit was also establish to be sensitive enough to detect the presence of *Salmonella* in fecal samples, artificially spiked and naturally contaminated animal feeds. Extensive validation trials of the kit were previously performed [24] in collaboration with several government and private testing laboratories on animal feeds and ingredients, and animal organs. Furthermore, the kit has been used on swab samples from ileum and tissue samples from mesenteric lymph nodes of apparently healthy dogs. Atypical *Salmonella* that failed to be detected using the plate culture assay can be positively identified using the kit. Under optimized conditions, the level of PCR assay sensitivity is 10^3 cells [22,23].

Local studies revealed that the combination of TBG enrichment with bacterial lysis method and capillary gel electrophoresis is suitable for a rapid *Salmonella* detection in chicken feces [3]. However, false-negative results can still occur and the bile salt component of TBG broth used as enrichment medium for *Salmonella* spp. may inhibit PCR amplification of DNA [27]. Additionally, other substances that are inhibitory to PCR include large amounts of polysaccharides, phenolic and metabolic compounds in feces [13].

The results of the study strongly indicated that PCR detection appears to be more sensitive over the conventional isolation method. No *Salmonella* spp. isolates were obtained from the small intestines of 96 apparently healthy bats using the conventional method of isolation. The inability to obtain a *Salmonella* spp. culture can be attributed to several factors. It is foremost believed that the short intestinal length and rapid transit time in bats [12] could have prevented the stasis necessary for adherence, colonization and multiplication of bacteria in the distal small intestine. Another consideration is the fact that the pre-enrichment stage which provides nutrition, promotes revival of damaged or stressed cells and multiplication of *Salmonella,* is non-selective, hence it favors the overgrowth of other organisms which may have overwhelmed *Salmonella* species due to its poor competitive nature. Therefore, it is most likely that highly competing non-*Salmonella* organisms may hamper the identification of *Salmonella* on agar plates. Lastly, the atypical appearance of *Salmonella* in the selective media plates may have been overlooked since only those pink colonies with black centers were considered positive on XLD media.

In general, results have shown that there is a significant degree of agreement between the conventional and PCR method, especially for the samples from Laguna and Quezon City. The samples subjected to both methods were found negative for *Salmonella* spp.

CONCLUSION

The study reports the first detection of *Salmonella* spp. in Philippine bats using intestinal samples. Results in the present study revealed that the Philippine bats, *Miniopterus australis* and *M. schreibersi*, both adult female insectivorous bats captured in Pangihan cave

of Barangay Pablacion, Malay, Aklan harbored *Salmonella* in their intestines. Since salmonellae have been detected in a large variety of environment and host species including insects, these bats may have acquired these microorganisms in water and in their diet.

This finding showed that Philippine bats may serve as potential reservoir and carrier of *Salmonella* organisms. The data also strongly indicates that bats may actively contribute in the dissemination of salmonellae into the environment through fecal route. This currently makes Philippine bats as a significant threat to livestock and a serious public health concern, since all serotypes of *Salmonella* are considered to be pathogenic to man [1,8].

Lastly, the results also indicate that majority of the collected species were insectivores under family Vespertilionidae while the largest population of the Philippine bats were frugivores belonging to family Pteropodidae.

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SOURCES AND MANUFACTURERS

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[2] BBL, Maryland, USA
[3] Biotest, Dreieich, Germany
[4] Difco, Maryland, USA
[5] HiMedia, Mumbai, India
[6] Conda, Madrid, India
[7] Hispanlab, S.A., Madrid, Spain
[8] Difco, Michigan, USA
[9] BBL Crystal™ Identification System Enteric/Nonfermenter ID kit, Difco, Maryland, USA
[10] Sigma, St. Louis, USA
[11] *Salmonella* DNA Amplification System™, BIOTECH, Philippines
[12] AB Applied Biosystems™, California, USA
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[14] UVP LLC,California, USA
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伴侶動物に関する研究G

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事　務　連　絡
平成22年5月24日

地方獣医師会会長　各位

社団法人　日本獣医師会
専務理事　大　森　伸　男

犬・猫のカプノサイトファーガ・カニモルサス
感染症に関する衛生情報

このことについて、平成22年5月21日付け事務連絡をもって、厚生労働省健康局結核感染症課から別添写しのとおり通知がありました。

このたびの通知の内容は、犬やネコなどの口腔内に常在するカプノサイトファーガ・カニモルサス細菌を原因とする人の感染症に関するものです。

本病については、我が国においても、これまで重症化した患者の文献報告例が14例あり、近年報告例が増加しているため、このたび、結核感染症課から本病に関するQ＆Aを各都道府県等の衛生主管部局あてに通知したとのことですので、貴会関係者に周知方お願いします。

本件のお問合わせ先
日本獣医師会　事務局事業担当　長野
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資料２）関連各部署への通知文書およびカプノサイトファーガ・カニモルサス感染症に関するＱ＆Ａ
(http://www.mhlw.go.jp/bunya/kenkou/kekkaku-kansenshou18/capnocytophaga.html)

事　務　連　絡
平成２２年５月２１日

社団法人　日本獣医師会　御中

厚生労働省健康局結核感染症課

カプノサイトファーガ・カニモルサス感染症に関するＱ＆Ａについて

今般、別添のとおり、各都道府県、政令市及び特別区衛生主管部（局）長あて通知いたしましたので、ご了知願いますとともに、貴会会員への周知につきましてご配慮の程お願いします。

事　務　連　絡
平成２２年５月２１日

各（都道府県／政令市／特別区）衛生主管部（局）　御中

厚生労働省健康局結核感染症課

カプノサイトファーガ・カニモルサス感染症に関するＱ＆Ａについて

今般、犬猫の咬傷によりカプノサイトファーガ・カニモルサス感染症を発症した事例について国立感染症研究所の発行する病原微生物検出情報（参考参照）に掲載されたこと等を踏まえ、本感染症の情報提供を行い、動物由来感染症に対する予防対策等について理解を深めていただけるよう、別紙のとおりＱ＆Ａを作成しましたのでお知らせします。

貴管内医療機関等関係者への周知につき、ご配慮願います。

なお、本Ｑ＆Ａについては、厚生労働省のウエブサイトにも掲載していることを申し添えます。

（参考）病原微生物検出情報（Vol. 31 p. 109-110: 2010年4月号）
「イヌ・ネコの咬傷感染によるCapnocytophaga canimorsus 敗血症の４症例」
URL：http://idsc.nih.go.jp/iasr/31/362/ki3625.html

カプノサイトファーガ・カニモルサス感染症に関するQ&A

（平成２２年５月２１日）

カプノサイトファーガ・カニモルサス感染症について、現在判明している状況など正しい情報を提供することで、予防対策等について理解を深めていただきたく、厚生労働省において、Q&Aを作成しました。

今後、カプノサイトファーガ・カニモルサス感染症に関する知見の進展等に対応して、逐次、本Q&Aを更新していくこととしています。

＜目次＞

＜カプノサイトファーガ・カニモルサス感染症とは＞

Q１　カプノサイトファーガ・カニモルサス感染症とは何ですか？

A１　カプノサイトファーガ・カニモルサスという細菌を原因とする感染症です。この菌は動物（イヌやネコなど）の口腔内に常在しています。

この病気は、イヌやネコに咬まれたり、ひっ掻かれたりすることで感染・発症します。免疫機能の低下した方において重症化する傾向のある感染症です。なお、動物による咬傷事故等の発生数（注）に対し、報告されている患者数は非常に少ないことから、本病は極めて稀にしか発症しないと考えられます。

注）犬の咬傷事故については、保健所に報告されたものだけでも年間約 6 千件もあり、報告に至らないものを含めるとさらに多く発生していると考えられます。

Q２　どのようにして感染するのですか？

A２　主にイヌやネコなどによる咬傷・掻傷から感染します。

ヒトからヒトへの感染の報告はありません。

Q３．どのような症状になるのですか？

A３　発熱、倦怠感、腹痛、吐き気、頭痛などです。

重症例では、敗血症や髄膜炎を起こし、播種性血管内凝固症候群（DIC）や敗血性ショック、多臓器不全に進行して死に至ることがあります。

なお、重症化した場合、日本では敗血症になった方の約 30％が、髄膜炎になった方の約 5％が亡くなるとされています。

＜予防対策について＞

Q４　感染しないために、どのようなことに注意すればよいですか？

A４　一般的な動物由来感染症予防の対応と変わりありません。日頃から、動物との過度のふれあいは避け、動物と触れあった後は手洗いなどを確実に実行してください。

なお、脾臓摘出者、アルコール中毒、糖尿病などの慢性疾患、免疫異常疾患、悪性腫瘍にかかっている者、高齢者など、免疫機能が低下している方は、重症化しやすいと考えられますので特に注意してください。

Q５　イヌを飼っているのですが、大丈夫ですか？

A５　免疫機能が低下していなくとも、咬傷や掻傷から感染し、発症する事例があるため、日頃から、動物との過度のふれあいは避け、動物と触れあった後は手洗いなどを確実に実行してください。

本病だけでなく、一般的な動物由来感染症予防のためにも、重要です。

＜国内及び海外の発生状況＞

Q６　日本での発生状況はどうなっていますか？

A６　日本においては、これまで重症化した患者の文献報告例が 14 例あります。

その内容をみると、患者の年齢は、40 歳代～90 歳代と中高年齢が多く、糖尿病、肝硬変、全身性自己免疫疾患、悪性腫瘍などの基礎疾患が見られます。感染原因は、イヌの咬傷 6 例、ネコの咬傷・掻傷 6 例、不明 2 例となっています。なお、近年の報告が多いのは、臨床現場で本病が認知されてきたためと思われます。

国内患者の確認報告例（2002～2009年）

発生または報告年	患者（性別・年齢）	感染動物・経路	主な症状	予後
2002	女・90代	猫・咬傷	意識障害	死亡
2004	男・60代	猫・掻傷	敗血症	死亡
2004	男・40代	猫・咬傷	敗血症	回復
2006	女・70代	犬・咬傷	敗血症、DIC、多臓器不全、意識障害	回復
2006	男・60代	不明	敗血症、DIC	死亡
2007	女・70代	犬・咬傷	敗血症、髄膜炎、意識障害	回復
2007	女・59代	猫・掻傷	敗血症、嘔吐	死亡
2008	男・60代	犬・咬傷	敗血症、DIC、黄疸、多臓器不全	死亡
2008	男・50代	犬・咬傷	敗血症、DIC	回復
2008	男・44代	犬・咬傷	敗血症、DIC	回復
2008	男・70代	犬・咬傷	発熱、創部発赤	回復
2008	男・70代	猫・掻傷	敗血症	死亡
2008	男・70代	猫・掻傷	敗血症、DIC	回復
2009	女・50代	不明（犬）	電撃性紫斑、四肢末梢壊死	回復

Q7　諸外国での発生状況はどうなっていますか？

A7　1976年に報告された敗血症・髄膜炎例が、最初の文献報告とされています。その後、現在までに世界中で約250人の患者が報告されています。敗血症の時の死亡率は、日本国内の患者では30%程度とされていますが、オランダでの近年の大規模患者調査では約12%でした。また、感染した場合の発症割合については、オランダの調査では100万人に0．7人、デンマークでは0．6人との報告もあります。

＜専門家の方へ＞

Q8　診断方法はどのようなものがありますか？

A8　患者血液や脳脊髄液、傷口の滲出液を培養して、菌を分離・同定します。培養サンプルからの遺伝子検出（PCR）も可能です。

しかし、医療機関を受診した時に敗血症の状態であることが多く、急激な転帰をたどることや、また、生育が遅い菌であり分離・同定に一定程度の時間を要することから、患者の臨床症状等に応じて早期に適切な治療を開始する必要があります。

なお、血液培養が行える検査施設であれば、分離及びある程度の同定は可能です。

Q9　治療方法はどのようなものがありますか？

A9　カプノサイトファーガ・カニモルサス感染症が疑われる場合には、患者の臨床所見等に応じて早期に抗菌薬等による治療を開始することが重要となります。咬傷に対する抗菌薬としては、ペニシリン系、テトラサイクリン系抗菌薬が一般的に推奨されていますが、*C. canimorsus* にはβラクタマーゼを産生する菌株もあるので、ペニシリン系の抗菌薬を用いる際にはβラクタマーゼ阻害剤との合剤などその影響を受けにくいものを選択するとよいとされています。

（検査に関する相談はQ11をご確認ください。）

Q10　カプノサイトファーガ・カニモルサス感染症と診断した場合に、行政機関への報告は必要ですか？

A10　カプノサイトファーガ・カニモルサス感染症は、感染症法の届出対象疾病ではありませんので、保健所等への届出は不要です。しかし、本菌の調査研究の進展のためにも、国立感染症研究所獣医科学部第一室への情報提供にご協力をお願いします。

Q11　相談窓口を教えてください。

A11　国立感染症研究所獣医科学部第一室（03-5285-1111　内線2622）にお問い合わせください。

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＜Q&A作成に協力をいただいた専門家＞
今岡浩一室長、鈴木道雄主任研究官（国立感染症研究所獣医科学部）

別紙５

研究成果の刊行に関する一覧表レイアウト（参考）

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Prevalence of *Capnocytophaga canimorsus* and *Capnocytophaga cynodegmi* in dogs and cats determined by using a newly established species-specific PCR

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ABSTRACT

Capnocytophaga canimorsus and *Capnocytophaga cynodegmi*, fastidious gram-negative rods, are commensal microbes thriving in the oral cavities of dogs and cats. *C. canimorsus* can sometimes cause fatal systemic infections in humans. In the present study, we established a specific PCR which could identify and distinguish *C. canimorsus* from *C. cynodegmi*. The prevalence of *Capnocytophaga* spp. in dogs and cats was determined using this method. *C. canimorsus* was detected in 74% of dogs and 57% of cats. *C. cynodegmi* was detected in 86% of dogs and 84% of cats. The prevalence of *Capnocytophaga* spp. obtained in this study is somewhat higher than those reported previously where bacterial isolation method was used for identification. This is probably due to the fact that the PCR detection is more sensitive compared to bacterial isolation. Our findings suggest the importance of informing people who belong to high-risk groups as well as health care workers on *C. canimorsus* infection and its potential risk to people particularly to those who are immunocompromised.

1. Introduction

The bacteria of the genus *Capnocytophaga* are capnophilic, fastidious, thin, and facultative anaerobic gram-negative rods displaying gliding motility (Brenner et al., 1989). *Capnocytophaga* spp. are indigenous to the oral cavities of humans, dogs, and cats. Dogs and cats have two species; *C. canimorsus* and *C. cynodegmi*. *C. canimorsus* sometimes causes wound and systemic infections in humans after dog or cats bites (Brenner et al., 1989). *C. cynodegmi* also causes local wound infection, but systemic infection is very rare (Khawari et al., 2005; Sarma and Mohanty, 2001). Detection and identification of *C. canimorsus* have been hampered because of the lack of simple detection systems. Genetic and biochemical similarities between *C. canimorsus* and *C. cynodegmi* make it further difficult to identify these species. Even when highly sensitive molecular methods were applied, sequencing of the product is often required for distinguishing between *C. canimorsus* and *C. cynodegmi* (Gottwein et al., 2006; Janda et al., 2006). In addition, Mally et al. (2009) reported the presence of *C. canimorsus* strains that are hardly distinguished from *C. cynodegmi* by the comparison of the 16S rRNA sequences. Therefore, there is a need for the development of more convenient and specific PCR systems to identify the *Capnocytophaga* spp. (Gaastra and Lipman, 2010). In the present study, a new PCR based detection system is described which discriminates between *C. canimorsus* and *C. cynodegmi*. Using this method the prevalence of both bacteria was determined in the dogs and cats in Japan.

2. Materials and methods

2.1. Bacterial strain

The bacterial strains used in the present study are listed in Table 1. Seven reference strains of *Capnocytophaga* spp. were purchased from the American Type Culture Collection (Manassas, VA, USA) or the RIKEN BioResource Center (Wako, Saitama, Japan). Bacterial species involved in bite infections (*Pasteurella* spp. and *Fusobacterium* spp.) and commensal species of the oral cavity of humans and animals were also obtained from the ATCC and the RIKEN. Other bacterial strains used in a previous study were also included (Kimura et al., 2008). DNA was extracted from bacterial cells by the guanidine thiocyanate lysis and isopropanol precipitation method (SepaGene; Sanko Junyaku, Tokyo, Japan) according to the manufacturer's protocol. Briefly, 100 μl of guanidine thiocyanate solution was added to 100 μl of the specimen, and the phases were then separated by adding 400 μl of sodium acetate and 700 μl of chloroform-agglutination solution, followed by centrifugation at 12,000 × *g* for 15 min. The DNA was precipitated from the upper phase with isopropanol and was resuspended in 27 μl of Tris–HCl buffer (pH 8.0). The concentration of DNA solution was determined by spectrophotometer (NanoDrop 1000, Thermo Fisher Scientific, MA, USA). For the examination of assay sensitivity of specific PCR, the extracted DNA of *C. canimorsus* ATCC35979 or *C. cynodegmi* ATCC49044 were 10-fold serially diluted and used as templates.

2.2. Primers and PCR

As shown in Table 2, two primers, CaL2 and AS2, were newly designed in this study, and used in combination with CaR and CyR primers reported by Kikuchi et al. (2005). PCR detection was performed using puReTaq Ready-To-Go PCR beads (GE Healthcare Bioscience Corp., NJ, USA) under the following conditions: an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 7 min.

2.3. Specimens from dogs and cats

Oral swabs taken from 325 dogs (during 2004–2006) and 115 cats (in 2007) at an animal shelter in Kanagawa

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Table 1
Bacterial strains used in this study and results of primers specificities for *C. canimorsus* and *C. cynodegmi* DNA amplification.

Bacterial species	Strains	Amplification with primers		
		CaL2–AS1	CaL2–CaR	CaL2–CyR
Capnocytophaga canimorsus	ATCC35979	+	+	–
Capnocytophaga cynodegmi	ATCC49044	+	–	+
Capnocytophaga ochracea	JCM12966	–	–	–
Capnocytophaga gingivalis	JCM12953	–	–	–
Capnocytophaga sputigena	ATCC33612	–	–	–
Capnocytophaga granulosa	JCM8566	–	–	–
Capnocytophaga haemolytica	JCM8565	–	–	–
Bacillus anthracis	PAII	–	–	–
Bacillus cereus		–	–	–
Bacillus subtilis	3	–	–	–
Bergeyella zoohelcum	ATCC43767	–	–	–
Brucella abortus[a]	544	–	–	–
Brucella canis[a]	QE13	–	–	–
Brucella melitensis[a]	16M	–	–	–
Brucella suis[a]	1330	–	–	–
Coxiella burnetii	Nine Mile	–	–	–
Escherichia coli	DH5 alpha	–	–	–
Francisella tularensis	LVS	–	–	–
Fusobacterium equinum	JCM11174	–	–	–
Fusobacterium necrophorum	JCM3718	–	–	–
Fusobacterium nucleatum	GTC04469	–	–	–
Fusobacterium varium	JCM3721	–	–	–
Haemophilus influenzae	ATCC10211	–	–	–
Klebsiella pneumoniae	ATCC13883	–	–	–
Leptotrichia buccalis	JCM12969	–	–	–
Leptotrichia trevisanii	ATCC700907	–	–	–
Listeria monocytogenes	ATCC15315	–	–	–
Mycobacterium tuberculosis	ATCC27294	–	–	–
Ochrobactrum anthropi	ATCC49187	–	–	–
Pasteurella aerogenes	ATCC27883	–	–	–
Pasteurella canis	ATCC12947	–	–	–
Pasteurella dagmatis	ATCC51687	–	–	–
Pasteurella gallinarum	ATCC43326	–	–	–
Pasteurella multocida	ATCC43325	–	–	–
Pasteurella multocida	ATCC13361	–	–	–
Staphylococcus aureus	ATCC29247	–	–	–
Streptobacillus moniliformis	ATCC14647	–	–	–
Yersinia enterocolitica	Pal77	–	–	–
Yersinia pestis	Yreka	–	–	–
Yersinia pseudotuberculosis	319	–	–	–

[a] Supplied from National Institute of Animal Health, Tsukuba, Ibaraki, Japan.
[b] Non-specific product (of unexpected length) was observed.

Table 2
Primers used in this study.

Primer name	Sense or antisense	Sequence	Target length	Location at L14637 or L14638[a]
CaL2	Sense (20 bp)	5′-GTAGAGTGCTTCGGCACTTG-3′		71–90 (L14637)
AS1	Antisense (22 bp)	5′-GTGATGCCACCAAACAATACTA-3′	124 bp	194–173 (L14637)
CaR[b]	Antisense (19 bp)	5′-GCCGATGCTTATTCATACA-3′	427 bp	497–479 (L14637)
CyR[b]	Antisense (19 bp)	5′-GCCGATGCTTATTCGTATG-3′	427 bp	495–477 (L14638)

[a] GenBank accession numbers of 16S rRNA gene of *C. canimorsus* (L14637) and *C. cynodegmi* (L14638).
[b] Prepared according to Kikuchi et al. (2005).

Prefecture using sterile cotton-tipped applicators (BD BBL Culture Swab Plus; Nippon Becton Dickinson, Tokyo, Japan) were suspended in heart infusion broth (BD Bioscience, CA, USA) and cultured for 24 h at 35 °C in an aerobic atmosphere of 5% CO_2. Bacterial cells were collected from the culture broth by centrifugation and then resuspended in 200 μl of distilled water followed by heating at 95 °C for 15 min. After centrifuging at 14,000 × *g* for 5 min, the supernatants containing bacterial DNA were collected and used for PCR amplification.

3. Results

3.1. Specificity and sensitivity of PCR

We determined the specificity and sensitivity of the PCR performed with different combinations of primers for discriminatory amplification of the 16S rRNA gene of *C. canimorsus* and *C. cynodegmi*. The CaL2–AS1 primer pair could amplify the target sequences from the DNA derived from both *C. canimorsus* and *C. cynodegmi*. Specific amplification of *C. canimorsus* DNA but not *C. cynodegmi* DNA was achieved by the CaL2–CaR, whereas the DNA fragment of *C. cynodegmi* alone was amplified by the PCR using the CaL2–CyR primer pair (Fig. 1 and Table 1). Amplification of other bacterial DNA, including five species of the genus *Capnocytophaga* isolated from human oral cavity was not observed using the CaL2 forward primer in combination with three reverse primers (Fig. 1 and Table 1). When the CaL2–AS1 primer pair was used for amplification, *Leptotrichia buccalis* gave an unexpected product (Table 1); however, the size of the product was totally different from that of the specific amplicons.

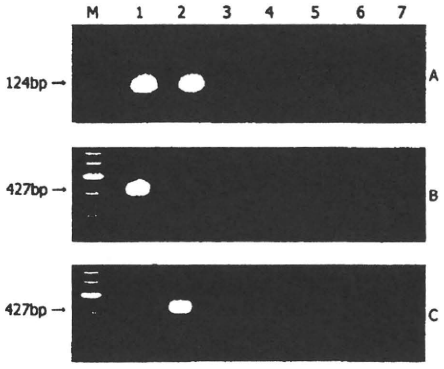

Fig. 1. Specific detection of the 16S rRNA gene of *Capnocytophaga* spp. by PCR using primer sets; CaL2–AS1 (A), CaL2–CaR (B), CaL2–CyR (C). Lane 1: *C. canimorsus*, Lane 2: *C. cynodegmi*, Lane 3: *C. gingivalis*, Lane 4: *C. ochracea*, Lane 5: *C. sputigena*, Lane 6: *C. granulosa*, and Lane 7: *C. haemolytica*.

Table 3
The sensitivity of the PCR with three pairs of primers.

	100 pg	10 pg	1 pg	100 fg	10 fg	1 fg
The amount of DNA of *C. canimorsus*						
CaL2–AS1	+	+	+	–	–	–
CaL2–caR	+	+	+	–	–	–
The amount of DNA of *C. cynodegmi*						
CaL2–AS1	+	+	+	–	–	–
CaL2–cyR	+	+	+	+	–	–

The sensitivity of the PCR with three different pairs of primers was then determined using known amounts of DNA purified from *C. canimorsus* or *C. cynodegmi* culture. It was shown that the sensitivities ranged from 100 fg to 1 pg depending on the primer pair (Table 3).

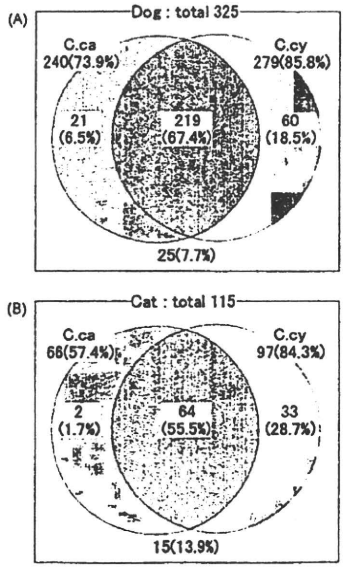

Fig. 2. (A) Prevalence of *Capnocytophaga* spp. in dogs, (B) prevalence of *Capnocytophaga* spp. in cats. C.ca: *C. canimorsus*. C.cy: *C. cynodegmi*. Values are rounded.

3.2. Prevalence of Capnocytophaga spp. in dogs and cats

The findings mentioned above indicated that the PCR using the CaL2 primer together with CaR or CyR allowed us specific amplification of respective species of the *genus Capnocytophaga*. The samples of the dogs and cats were, therefore, examined by PCR with these primers for the presence of specific sequence of *C. canimorsus* and *C. cynodegmi*. As shown in Fig. 2, 240 of 325 (74%) dogs and 66 of 115 (57%) cats tested positive for *C. canimorsus*, while *C. cynodegmi* was detected in 279 of 325 (86%) dogs and 97 of 115 (84%) cats. Both of these species were detected in 219/325 (67%) of dogs and 64/115 (56%) of cats (Fig. 2A and B).

4. Discussion

Capnocytophaga infection is uncommon zoonosis after dog and cat bites. About 200 cases have been reported in the world since 1976 (Gaastra and Lipman, 2010; Macrea et al., 2008). The majority of the disease is caused by *C. canimorsus*. Immunocompromised persons are most vulnerable to the *C. canimorsus* infection. Asplenic, alcoholic, or aged individuals, as well as those who are suffering from chronic diseases, such as diabetes, are considered to be at high-risk (Janda et al., 2006; Lion et al., 1996). The clinical features of *C. canimorsus* infection include sepsis, meningitis, renal insufficiency, and disseminated intravascular coagulation (Lion et al., 1996; Moal et al., 2003). The case fatality rate may exceed 30% (Lion et al., 1996). In Japan, 13 cases of human *C. canimorsus* infection have been reported (Imaoka, 2009; Kikuchi et al., 2005; Ota et al., 2009; Takahashi et al., 2009).

Diagnosis of *C. canimorsus* infection is usually done by isolation of the bacteria followed by biochemical characterization. PCR is also available for identification of *Capnocytophaga* spp. Kikuchi et al. (2005) reported that *C. canimorsus* and *C. cynodegmi* could be differentiated by PCR directed to 16S rRNA gene, whereas in the most recent literature van Dam et al. (2009) reported species-specific real-time PCR. Since we noticed that the primers designed by Kikuchi et al. (2005) amplified the target sequences not only from *C. canimorsus* and *C. cynodegmi* but also from *Capnocytophaga* spp. isolated from human oral cavities, we attempted to improve the original method in this study. By precisely examining the nucleotide sequences deposited in the GenBank, we eventually succeeded to design species-specific primers. Recently the presence of *C. canimorsus* strains which could not be distinguished from *C. cynodegmi* by the sequence analysis of 16S rRNA gene alone was reported by Mally et al. (2009). Since the nucleotide sequence of the CaR primer is shared by the strains identified as *C. canimorsus* by Mally et al. using a phylogenic analysis, the primer designed here for *C. canimorsus* is expected to unambiguously amplify *C. canimorsus* strains.

Because the PCR method established here is rapid and sufficiently sensitive, the method can be applied to identify and distinguish *C. canimorsus* from *C. cynodegmi*. The prevalence of *Capnocytophaga* spp. in dogs and cats was determined using the PCR method. The 16S rRNA gene of *C. canimorsus* and/or *C. cynodegmi* was detected in 92% of dogs and 86% of cats. The prevalence of *C. canimorsus* was higher in dogs compared with that in cats, but *C. cynodegmi* was detected in a similar proportion in both animals. In previous reports, the prevalence of *Capnocytophaga* spp. determined by bacterial isolation was shown to be 36–60% for dogs and 24% for cats (Westwell et al., 1989; Mally et al., 2009). The prevalence determined in this study was higher than that of previous reports, probably because the PCR detection is more sensitive than bacterial isolation.

Although the number of human infections with *C. canimorsus* in Japan remained quite small so far, most septic patients had received intensive care and the mortality rate was around 30% (Imaoka, 2009). Taking into account the fact that around 13 million dogs and 14 million cats are kept as companion animals, and that more than one fifth of 120 million Japanese populations are people over 65 years of ages, the chances of serious infections by *C. canimorsus* are high. It is therefore prudent to disseminate information on possibility of *C. canimorsus* infection through companion animals to people who are at highest risk.

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