表 2-46　クラミジアの分類と特徴[注]

目 order	科 family	属	種	宿主域	概要
Chlamydiales	*Chlamydiaceae**	*Chlamydia*	*trachomatis*	人	粗でグリコーゲン陽性の封入体。サルファ剤感受性。trachoma（14 の血清型）および lymphogranuloma venereum（4 の血清型）生物型からなる。STD および眼疾患，肺炎の原因菌．完全なゲノム塩基配列が知られている。
			suis	豚	粗でグリコーゲン陽性の封入体。ほとんどがサルファ剤感受性だが，耐性株もある。*C. trachomatis* MOMP と交差抗原性を示す。
			muridarum	マウスおよびハムスター	粗でグリコーゲン陽性の封入体。サルファ剤感受性．MoPn（マウス）および SFPD（ハムスター）の 2 株のみが知られる。MoPn 株の完全なゲノム塩基配列が知られている。*C. trachomatis* と交差抗原性を示す。
		Chlamydophila	*psittaci*	鳥類，哺乳類	ほとんど全ての鳥類に感染し，不顕性感染．幼鳥や時として成鳥に致死性の全身感染．血清型がある。人は偶発宿主。人の *C. psittaci* 感染症は古くからオウム病として知られる。
			abortus	鳥類および哺乳動物	*C. psittaci* に非常に近縁。病原性も類似するが，羊，牛および山羊ならびに馬，ウサギ，モルモット，マウスおよび豚に流産を引き起こす。
			felis	猫	猫に結膜炎および上部気道炎を引き起こす。感染猫の全身の臓器から分離される。血清型はない。人への感染例がある。血清疫学的にもズーノーシスが疑われている。
			caviae	モルモット	封入体結膜炎の起因菌。これまでに分離された菌は全て同一の ompA 遺伝子を有する。
			pneumoniae	人，コアラ，モルモット	*C. psittaci* TWAR として報告された。完全なゲノム塩基配列が知られる。人に呼吸器疾患および循環器疾患を引き起こす。コアラには眼疾患および泌尿生殖器疾患を引き起こす。馬からの分離株は一株で呼吸器から分離された。
			pecorum	哺乳類およびコアラ	多様な病原性を示す。反芻動物では不顕性感染が一般的。コアラでは *C. pneumoniae* と同様に眼疾患および泌尿生殖器疾患を引き起こす。
	Waddliaceae	*Waddlia*	*chondrophila*	牛（？）	牛の流産胎子から未知のリケッチアとして 1986 年に分離。16S rDNA 塩基配列からクラミジア科に分類。
	Parachlamydiaceae	*Parachlamydia*	*acanthamoebae*	原生動物（アメーバ）	*Acanthamoeba* や *Hartmanella* に感染。環境中の水から検出される。疾病との関連性は不明。
		Neochlamydia	*hartmannellae*		
	Simkaniaceae	*Simkania*	*negevensis*	不明	培養細胞への混入微生物として分離。血清疫学的には人の肺炎との関連性がいわれているが，実際には不明。

* グラム陰性。科特異的リポ多糖体エピトープ α Kdo-(2-8)- α Kdo-(2-4)- α Kdo（以前の属特異的抗原エピトープ）をもつ。Chlamyidaceae EB の剛性は 40 kDa 主要外膜蛋白質，親水性システイン・リッチ蛋白質および低分子システイン・リッチリポ蛋白質を含むジスルフィド結合エンベロープ蛋白質による。

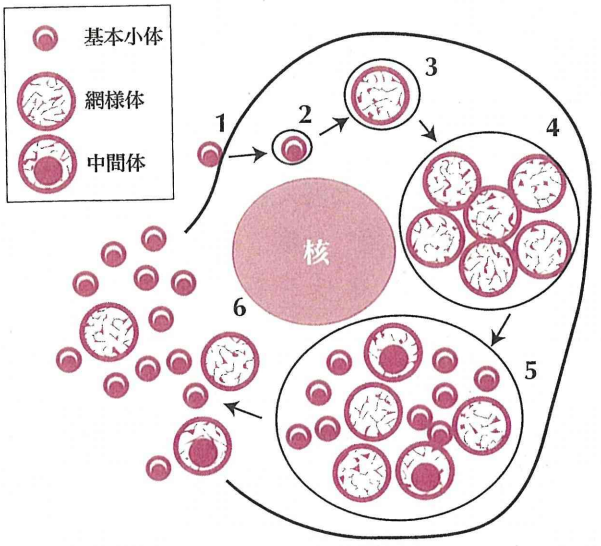


図 2-65　クラミジアの増殖環

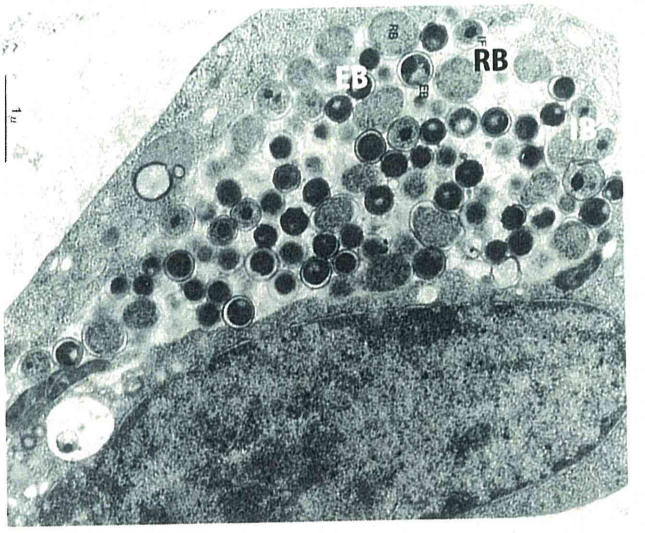


図 2-66　*C. psittaci* の L 細胞における増殖像
細胞質内封入体のクラミジア
EB：基本小体，RB：網様体，IF：中間体
（松本　明博士提供）

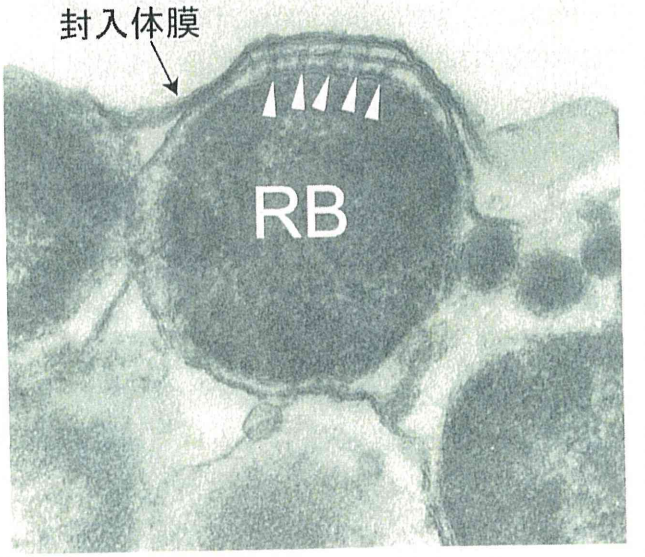


図 2-67　*C. psittaci* 菌体より表出するニードル状の構造（矢頭）
網様体（RB）より表出したニードル状の構造が封入体膜と接触していることが分かる。
（松本　明博士提供）

ラミジア *Chlamydia* 属 2 菌種，クラミドフィラ *Chlamydophila* 属 6 菌種およびワドリア *Waddlia* 属 1 菌種である。

クラミジアは自然界に広く分布し，特に鳥類ではオウム目を含む 18 目 145 種から報告されている。野生のオウム，インコ類におけるクラミジアの保有率は約 5％といわれている。哺乳動物では牛，羊，豚および猫などにクラミジア症がある。

(2) 形　　態

クラミジアは形態学的変化を伴う増殖環を有する。感染性をもち外界への抵抗性がある大きさ 0.2 ～ 0.4 μm の基本小体 elementary body（EB）および感染性はないが高い代謝活性および分裂能を有する網様体 reticulate body（RB）の 2 つの形態をとる（図 2-66 および図 2-67）。細胞壁はグラム陰性菌の膜構造と類似しリポ多糖を含み，外膜および細胞質膜からなる外被膜が DNA およびリボゾームを含む細胞質を包んでいる。基本小体中の DNA は偏在している。感染宿主細胞質内では封入体と呼ばれる膜構造の中に存在している。

(3) 性　　状

クラミジア目の微生物は偏性細胞内寄生性原核生物で，特有の増殖環を有しエネルギー代謝系を欠く。一般細菌にみられるような生物学的性状はない。クラミジアの培養には 6 ～ 7 日発育鶏卵卵黄嚢内接種，L929，HeLa ないし McCoy 細胞などの培養細胞，マウス，モルモットなどの実験動物（腹腔内接種）が用いられる。*C. trachomatis* は血清型が確立されている。他の菌種においては血清型の存在が示唆されているものの確立されていない。ゲノム解析が進んでおり，2011 年初頭の段階で，*C. trachomatis*，*C. pneumoniae* をはじめとして，獣医学領域において特に重要な *C.*

abortus, *C. psittaci*, *C. felis* を含む9菌種において全塩基配列が報告されている。*C. trachomatis*, *C. pneumoniae*, *C. abortus* においては，複数株の解析が報告されている。大きさは1,042～1,230kbpで，ORFは900～1,100個と推定されている。GC含量（モル%）は，クラミジア科のクラミジア属，クラミドフィラ属間で大きな違いはなく39～41であるが，ワドリア科では44，アメーバを宿主とするパラクラミジア科では36%である。増殖に必要な代謝系の多くを宿主細胞に依存しており，ゲノムサイズは他菌種に比べて縮小している。主要な抗原である大きさ約40kDaの主要膜蛋白質 major outer membrane protein, MOMPには型特異性があり，血清型別にも用いられている。またリポ多糖体も外膜に存在し，クラミジア科の共通抗原である。クラミジアにはテトラサイクリン系，ニューキノロン系およびマクロライド系抗生剤が有効である。ペニシリン系抗生剤は分裂増殖を阻止するが，可逆的であり，ペニシリン系抗生剤が無くなると増殖が再び開始されるため，治療に用いてはならない。

a. 細胞内増殖性

クラミジア種間における生活環，細胞内増殖性については大きな違いはない。感染性粒子である基本小体が宿主細胞内に侵入し，封入体内で代謝活性を有する網様体へと変換し活発に2分裂増殖する。網様体は中間体を経て基本小体へと再変換する。この時点で封入体は宿主細胞質を凌駕する程に拡張し，細胞は破壊される。再変換により出現した基本小体は，細胞外へと放出され新しい細胞に感染する（図2-65，図2-66）。菌体を包む封入体膜には，典型的なエンドソームマーカーが検出されず，クラミジア由来の蛋白質（Inc蛋白質群）が検出され，宿主細胞の膜構造より隔離されていることが分かる。封入体膜はライソゾームとの融合などによる細胞内消化からの回避に役立っている。基本小体，網様体ともに菌体表面には注射針様の構造物が認められ，封入体膜と接触している（図2-67）。この構造はⅢ型分泌装置であるとされ，分泌されるエフェクターによりクラミジアは宿主細胞内機能を修飾し，細胞内増殖を可能にしていると考えられている。封入体膜は，ゴルジ体からの分泌小胞と親和性があり，これにより増殖に必要な脂質などの栄養分を宿主細胞から獲得していると考えられている。

（3）感　染　症

a. 鳥類のクラミジア感染症　avian chlamydiosis

オウム病クラミジア *C. psittaci* の宿主域は広い。鳥類ではオウム目を含む18目145種から報告されている。特に，オウム，スズメ，チドリおよびガンカモ目の鳥種が多い。クラミジア感染鳥のほとんどは不顕性感染であり，間欠的に排菌する。感染鳥が排泄する糞便にはクラミジアの感染性粒子である基本小体が多数含まれる。基本小体は乾燥に強く，環境中で感染性を保っている。雛鳥の初感染では一部の感染雛鳥は発症し死亡する。他は保菌鳥となる。保菌鳥は輸送，密飼いなどのストレス，栄養不良などの要因が引き金となり発症する。

発症鳥の症状は鳥種，日齢により異なり，軽症から重症までさまざまであり，時として死亡する。通常，元気消失，食欲減退，鼻腔からの漿液性ないし化膿性鼻漏がある。緑灰色下痢便，粘液便がみられることもある。急性例では症状に気付かないまま死亡することもある。

鳥類間におけるクラミジアの伝搬様式は接触，吸入，経口による水平伝搬であり介卵伝達はない。感染源は病鳥および保菌鳥の排泄物，分泌物，羽毛などの飛沫，汚染された給餌器や飼料，水，病原体を含む排泄物が乾燥した塵などであり，これらのエアロゾルの吸入や，鳥同士のつつきあいなどによる傷口から感染すると考えられている。我が国の鳥類におけるクラミジア保有率は約20%であると考えられる。

生前診断は臨床症状および排泄物からの病原体検出により行う。斃死した場合は臨床症状および剖検所見からオウム病を疑う。いずれも確定診断は病原体の分離ないし検出である。オウム病が疑われた鳥はみだりに剖検するべきではない。病

原体検索およびクラミジア遺伝子検査は生前では糞やクロアカのスワブを材料とする。抗菌薬の治療前に採剤しないと検出は困難であるが，投薬後7～10日間は遺伝子を検出できる場合もある。また，不顕性感染では間欠的に病原体を排出しているので，数週間おきに数回検査をする必要がある。斃死した場合は脾臓および肝臓を材料とする。鳥類用のワクチンはない。飼育環境の衛生および不顕性感染鳥の摘発および治療により拡大，伝播を防ぐ。外部から新しい鳥を導入する場合は数週間の検疫および病原体検査を行うべきである。

鳥類の治療にはドキシサイクリン，クロルテトラサイクリンおよびエンロフロキサシンが用いられる。現在までに耐性菌は見出されていない。鳥種により投薬方法が異なる。罹患鳥には45日間の連続投与が推奨されているが，鳥によっては副作用がみられるため，投与期間中は鳥の健康状態を常にモニタリングし，場合によっては強肝剤やプロビオティックを投与する。

オウム病 psittacosis は，*C. psittaci* が人に感染することによる。インフルエンザ様の呼吸器症状を呈し，致死的な経過をたどることもある。人への感染の大部分は，保菌鳥の排出した菌体を吸入することによる。感染症法では4類感染症に指定されており，我が国では年間20例程の報告があるが，動物園や鳥飼育施設などにおける集団発生事例もある。

b. 猫のクラミジア症 feline chlamydiosis

Chlamydophila felis による結膜炎および上部呼吸器疾患である。時として肺炎に至る。くしゃみ，鼻汁，結膜分泌物および発熱がみられる。SPF猫の感染実験では2週間ほどで治癒する。しかし，自然界では他の細菌の二次感染を引き起こし，長期化すると考えられる。我が国の猫では飼い猫で10～20%，野良猫では約60%から抗体が検出されており，病原体も分離されている。欧州，米国では生および不活化ワクチンが使用されている。我が国でも不活化ワクチンが使用されている。猫ヘルペスウイルスおよび猫カリシウイルス感染症と臨床症状は類似している。獣医師の本菌にたいする抗体保有率は一般人より高率であり，人獣共通感染症である可能性が示唆されている。テトラサイクリン系抗菌薬により治療できる。

c. クラミジア性流行性流産 enzootic abortion of ovine enzootic abortion

Chlamdophila abortus（流行性羊流産菌）により引き起こされる流産で，牛，羊，山羊にみられる。牛では妊娠6～8か月齢で突然流産を引き起こす。流産胎子は，多量の腹腔液貯留，粘膜出血，咽頭・気管の点状出血，肝臓の腫大と灰色の小結節がみられる。我が国でも報告がある。本菌の羊，山羊における流産は，流行性羊流産 enzootic abortion of ewews（ovine enzootic abortion）と呼ばれ，家畜伝染病予防法において届出伝染病に指定されている。妊娠羊に胎盤炎，死流産，また虚弱子の出産を起こす。死流産胎子には浮腫と充出血がみられる。北米，欧州，ニュージーランドなどで発生しており，畜産業における経済的被害が問題となっている。我が国では報告がない。欧米では流産予防のため不活化ワクチンが使用されている。

d. 豚のクラミジア症 swine chlamydiosis

Chlamydophila pecorum による。肺炎，流涙，腸炎，脳炎などを引き起こす。生後2～3週齢の感染子豚は発熱や食欲の減退から発育障害を起こす。心嚢炎や胸膜炎，関節炎や下痢もみられる。北米や欧州で散発的に発生する。我が国でも，死流産や結膜炎が報告されている。

e. 多発性関節炎 bovine polyarthritis

Chlamydophila pecorum による。羊および牛に発熱，跛行，関節炎，結膜炎などを起こす。剖検では関節の漿液性・繊維素性髄膜炎，関節周囲から腱鞘の腫脹，充血や点状出血もみられる。北米や欧州で発生。我が国での報告はない。

f. 散発性牛脳脊髄炎 sporadic bovine encephalomyelitis

Chlamydophila pecorum の感染による。子牛が突然の発熱後，元気，食欲の減退，鼻漏，咳，流涎，脱水，麻痺などを示し，旋回運動，半弓緊張，麻痺を示す疾病である。致死率は30～50%で

ある。脳および脊髄に非化膿性脳炎と軟脳膜炎がみられる。北米や欧州で発生。我が国では輸入牛に1例報告がある。

g. モルモットの結膜炎 chlamydial infection in guinea pigs

Chlamydophila caviae による。4～8週齢の幼若個体に多くみられる。結膜充血から化膿性分泌物を伴う結膜炎までさまざま。我が国での報告はない。*C. trachomatis* 感染の疑われる人の症例より本菌の分離が報告されており，人の性感染症との関連が注目されている。

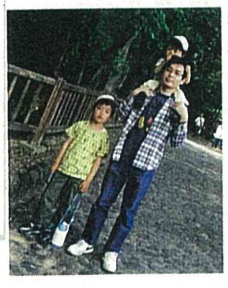
オウム病クラミジア集団発生事例分離株ゲノム配列決定とその意義

大屋賢司[*1・2]　黒田 誠[*3]　関塚剛史[*3]　Garry MEYERS[*4]
岸本寿男[*5]　安藤秀二[*5]　奥田秀子[*2]　福士秀人[*1・2]

要 旨

Chlamydophila psittaci によるオウム病は四類感染症に指定され，年間30例前後の発生が見られる主要な人獣共通感染症である。*C. psittaci* の病原性解析および種鑑別診断系開発を，比較ゲノム解析の視点から行うために，*C. psittaci* 日本分離株の全ゲノム配列決定を試みた。対象とする株は，2001年鳥展示施設における人への集団感染時に分離されMat116株を選択した。Mat116株は，同種他株，近縁他種と似通った遺伝子構造をもつものの，異なっている領域も複数見いだされた。現在は，異なる複数株の配列解読も進行中であり，これらの詳細な比較解析により，本菌の多様な宿主域や病態の理解，鑑別診断系開発に結びつけたい。

[*1] Kenji OHYA（写真・コメント）& Hideto FUKUSHI
岐阜大学応用生物科学部獣医微生物学分野
〒501-1193　岐阜市柳戸1-1
[*2] Kenji OHYA, Hideko OKUDA & Hideto FUKUSHI
岐阜大学大学院連合獣医学研究科応用獣医学
[*3] Makoto KURODA & Tsuyoshi SEKIZUKA
国立感染症研究所病原体ゲノム解析研究センター
[*4] Institute for Genome Sciences, University of Maryland
[*5] Toshio KISHIMOTO & Shuji ANDO
国立感染症研究所ウイルス第一部第五室



学生時代は植物遺伝子工学（！）と感染免疫学，ポスドク時代は赤痢菌の細胞微生物学に従事しました。岐阜大学に赴任して6年，クラミジアのゲノム解読の過程で，やらなければならないことが沢山出てきました。魅力的なテーマは多いのですが，いかんせん人手が（頭も？）足りません。意欲的な大学院生の参入を期待しています。

はじめに

クラミジアは偏性細胞内寄生性を示し，1目4科6属13種からなる（表1）。このうち，人や動物に病原性を示し，医・獣医学上重要と思われるのは *Chlamydia* 属，*Chlamydophila* 属および *Waddlia* 属である。オウム病クラミジア（*Chlamydophila psittaci*）は，人獣共通感染症であるオウム病の原因となり，人においては肺炎などの呼吸器症状を呈す。感染症法では4類感染症に指定されており，年間30例程度が届出されている。我々のグループでは，オウム病クラミジアを始めとした各種クラミジアについて，診断法の開発や実態調査を行っている。しかしながら，オウム病クラミジアについては，公開されているゲノム情報がなく，プロジェクト遂行の障壁となっていた。そこで，*C. psittaci* の鑑別診断法開発や病態解明を，比較ゲノムの視点から行うことを目的として，*C. psittaci* 国内分離株の全ゲノム配列解読を試みた。

オウム病クラミジアのゲノム解析とその意義

クラミジアに関しては，人の伝染性角結膜炎（トラコーマ）や性感染症の原因となる *C. trachomatis* や，*C. psittaci* を始めとして，重要な感染症の原因となるものが多い。しかしながら，臨床上の重要性にも関わらず，「（確立した）遺伝子操作系が存在しない」等の理由により，サルモネラ属菌等他種病原細菌に比べて特に基礎的研究の進展が乏しい。そのような障壁を補填するため，クラミジアに関しては，精力的にゲノム解読が行われており，幾つかの種においては，複数株の配列が公開されている（表2）。*C. psittaci* に関しても2011年になり2つの株（RD1と6BC）の配列決定が報告された。

我々が解析の対象としたのは，2001年鳥展示施設にお

表1　クラミジア目の分類

科	属	種	主な宿主
Chlamydiaceae	*Chlamydia*	*trachomatis*	人
		suis	豚
		muridarum	齧歯類
	Chlamydophila	*psittaci*	鳥類，哺乳類
		abortus	哺乳類（反芻獣）
		felis	猫
		caviae	モルモット
		pneumoniae	人，コアラ
		pecorum	哺乳類，コアラ
Waddliaceae	*Waddlia*	*chondrophila*	反芻獣
Parachlamydiaceae	*Parachlamydia*	*acanthamoebae*	原生動物
	Neochlamydia	*hartmannellae*	原生動物
Simkaniaceae	*Simkania*	*negevensis*	不明

獣医微生物学（南江堂）に掲載されたものを一部改変し作製

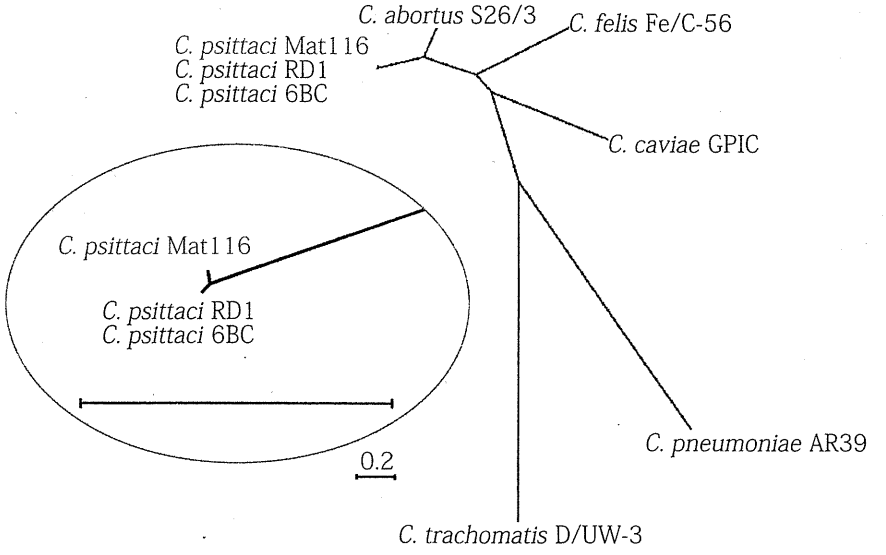
表2　ゲノム配列の解読された代表的なクラミジア種

種名	*C. psittaci*		*C. abortus*	*C. felis*	*C. caviae*	*C. pneumoniae*	*C. trachomatis*
株名	RD1	6BC	S26/3	Fe/C56	GPIC	AR39	D/UW-3
ゲノム（kbp）	1,156	1,172	1,144	1,166	1,173	1,229	1,042
由来疾患	不明	インコ（標準株）	羊 流産	猫 結膜炎	モルモット 結膜炎	人 肺炎	人 STD
GC 含量（%）	38.8	39.1	39.9	39.4	39.2	40.6	41.3
CDS*	959	967	961	1,005	1,009	1,130	894
tRNA	36	38	38	38	38	38	37
rRNA オペロン	1	1	1	1	1	1	2
Pmp** 蛋白質	17	22	18	20	18	21	9
参考文献	1）	2）	3）	4）	5）	6）	7）

* CDS：coding sequence，**Pmp：polymorphic membrane protein

ける人への集団感染時[8]に分離したMat116株を用いた。感染細胞より精製した菌体からゲノムDNAを抽出し，次世代シークエンサーGenome Sequencer 20 system解析に供した。ドラフト配列を既読クラミジアゲノム配列と比較し，コンティグの並びを推定し，サンガー法にてギャップクローズを行い，National Center for Biotechnology Information（NCBI）のシステムを利用しアノテーションを行った。結果得られた*C. psittaci* Mat116株全塩基配列は1.16 Mbp，GC含量は39.1%，遺伝子コード領域（CDS）は999個であった。

Mat116株を始めとした*C. psittaci*ゲノムは，旧*C. psittaci*に属する羊流産クラミジア*C. abortus*と近縁であり，猫クラミジア*C. felis*，モルモットクラミジア*C. caviae*とも近縁であることが示された（図1）。*C. psittaci*株間において配列は似通っているものの，Mat116株は他グループより報告された2株とは多少異なっていることが，系統解析から示された（図1：拡大）。CDS数，および種・株間における病原性や宿主特異性への関与が示唆されている多型膜蛋白質（Pmp）数からもMat116株の同種内における特性や他種クラミジアとの差異を読み取ることができる（表2）。6BC株は1941年にインコより分離され，*C. psittaci*の標準株として実験室内で長く継代されてきた株であること[2]，RD1株はrRNA解析の結果*C. psittaci*とされているものの，その詳細な由来は不明（*C. trachomatis*実験室内保存株ストックより分離された）である[1]。比較的最近，集団発生事例より分離したMat116



各種クラミジアについて，4種類のハウスキーピング遺伝子（*enoA*，*gidA*，*gatA*，*hlfX*）を連結後アライメントし，MEGA ver. 5.02を用いて解析した。系統樹はNJ法にて作製した。遺伝距離は0.2 units barで示す。

図1　4種類のハウスキーピング遺伝子を用いたクラミジア種間の系統解析

株との詳細な比較解析により，本菌の人獣共通感染症としての多様な宿主域や病態に関するヒントが得られるかも知れない。現在は，他種クラミジアとの比較解析の結果同定した，*C. psittaci* 特異的遺伝子を標的とした鑑別診断法を開発中である。また由来や病原性の異なる複数株の配列解読も進行中である。以上のようなアプローチを通じて，オウム病クラミジアの生態の理解，制御に少しでも貢献できればと考えている。

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NOTE *Virology*

Molecular Genetic and Pathogenic Characterization of Psittacid Herpesvirus Type 1 Isolated from a Captive Galah (*Eolophus roseicapillus*) in Japan

Hiroshi KATOH[1)], Souichi YAMADA[1)], Takayuki HAGINO[2)], Kenji OHYA[1,2)], Hiroki SAKAI[3)], Tokuma YANAI[1,3)], Toshiaki MASEGI[1,3)], Tsuyoshi YAMAGUCHI[1,2)]** and Hideto FUKUSHI[1,2)]*

[1)]*Department of Applied Veterinary Sciences, United Graduate School of Veterinary Sciences,* [2)]*Laboratory of Veterinary Microbiology, and* [3)]*Laboratory of Veterinary Pathology, Faculty of Applied Biological Sciences, Gifu University, Gifu 501–1193, Japan*

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ABSTRACT. Psittacid herpesvirus type 1 (PsHV-1) was isolated from a captive galah (*Eolophus roseicapillus*) in Japan that was suspected of having Pacheco's disease (PD), an acute fatal disease in psittacine birds. PsHV-1 has been classified into four genotypes based on the UL16 gene sequence. In the present study, we investigated the genetic and pathogenic characteristics of the isolated virus, FOY-1, compared with a reference strain, RSL-1. The FOY-1 strain was classified into PsHV-1 genotype 2. The FOY-1 strain was found to be less pathogenic to budgerigars than RSL-1, which was classified as genotype 4 in an *in vivo* study. This is the first report regarding the classification of originally isolated PsHV-1 in Japan and its characterization by animal infection experiment.

KEY WORDS: genotype, pathogenicity, psittacid herpesvirus.

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To date, psittacid herpesvirus (PsHV) is the only herpesvirus in psittacine birds, and the virus has been classified as an avian member of the *Alphaherpesvirinae* subfamily by the International Committee on Taxonomy of Viruses [11]. PsHV type 1 (PsHV-1) is the causative agent of Pacheco's disease (PD), an acute fatal disease in psittacine birds [9]. Outbreaks of PD have resulted in massive die-offs of infected birds. Recently, another type of psittacid herpesvirus, PsHV-2, was identified in three African grey parrots (*Psittacus erithacus*) [10]. The sequences of the UL16, whose putative function is capsid assembly, and UL30, whose putative function is DNA polymerase, regions of PsHV-2 both differ from the most closely related PsHV-1 by more than 20%. However, the prevalence and pathogenicity of PsHV-2 is unclear [10, 14].

Based on the UL16 gene sequence, PsHV-1 has been classified into four genotypes. All four of the PsHV-1 genotypes have the potential to cause PD but have distinct biological characteristics [13]. It has been reported that the susceptibility to each PsHV-1 genotype depends on the bird species [13]. For instance, Amazon parrots (*Amazona* spp.) are the most common parrot species diagnosed with PD, and all four genotypes of PsHV-1 have been isolated from these species. PsHV-1s of all four genotypes were also found among birds from the Pacific region such as cockatiels (*Nymphicus hollandicus*) and cockatoos (*Cacatua* spp.). On the other hand, PsHV-1s from African grey parrots with PD have been classified as genotypes 2, 3 and 4, but not genotype 1. In the case of macaws (*Ara* spp.) and conures (*Aratinga* spp. and *Pyrrhura* spp.), genotype 4 has been commonly found and causes mortality, genotype 3 has rarely been found and is less pathogenic and genotypes 1 and 2 have not caused PD at all.

PsHV-1 infections have been reported in several species of psittacine birds in many countries including the U.S.A. [6, 9], U.K. [2], Spain [1] and South Africa [4]; however, genetic and pathogenic information about PsHV-1 is limited. In Japan, avian herpesvirus has been detected in psittacine birds by electron microscopy [15]. However, the virological investigation has been limited. This communication describes the genetic characteristics of PsHV-1 isolated from a captive galah (*Eolophus roseicapillus*) in Japan. In addition, the pathogenicity of this isolate was evaluated by experimental infections using budgerigars (*Melopsittacus undulatus*), a psittacine species that is easier to use than other bird species.

A galah died after exhibiting anorexia and diarrhea in a bird sanctuary in Japan in 2002. Pathological and microbiological examinations were performed in this study to investigate the cause of death. At necropsy, hepatomegaly and splenomegaly were observed (data not shown). Histopathological examination revealed multifocal necrosis in the liver and spleen (data not shown). Intranuclear inclusion bodies were observed in hepatocytes in the liver (Fig. 1) and macrophages in the spleen (data not shown). PCR was carried out in order to detect common pathogens in psittacine birds including herpesvirus [16], beak and feather disease (BFDV) [17] and avian polyomavirus (APV) [7]. DNA was extracted from 50 mg of liver and spleen samples with a SepaGene nucleic acid extraction kit (Sanko Junyaku Co., Tokyo, Japan) according to the manufacturer's instructions. As a result, herpesviral DNA, but no DNA of other pathogenic viruses, was detected in both the liver and spleen (data not shown).

A primary culture of chicken embryo fibroblast (CEF)

* CORRESPONDENCE TO: FUKUSHI, H., Laboratory of Veterinary Microbiology, Faculty of Applied Biological Sciences, Gifu University, 1–1 Yanagido, Gifu 501–1193, Japan.
e-mail: hfukushi@gifu-u.ac.jp

** PRESENT ADDRESS: Laboratory of Veterinary Hygiene, Faculty of Agriculture, Tottori University, Tottori 680–8553, Japan.

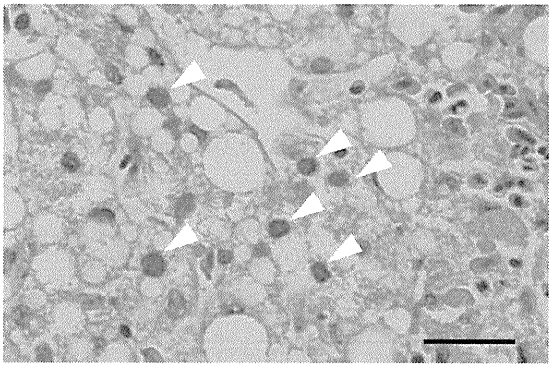

Fig. 1. Histological section of the liver of the galah. Eosinophilic intranuclear inclusion bodies were found in the hepatocytes. The typical inclusion bodies are indicated by arrowheads. Hematoxylin and eosin stain; bar=25 μm.

was used to isolate the herpesvirus. The monolayer was inoculated with 10% (w/v) homogenate of liver or spleen in cell culture medium at 37°C for 1 hr. The inoculated cultures were washed twice with cell culture medium and incubated at 37°C. The cultures were observed for cytopathic effect (CPE) daily. CEF cells inoculated with the liver and spleen samples showed a CPE 2 and 4 days after inoculation, respectively. The CPE was characterized by ballooning and detachment of the cells from the cell culture plate. After three passages, the presence of herpesviral DNA in the culture was confirmed by the PCR used above at 2 days postinoculation. The virus isolated was designated as FOY-1.

The PCR used above was broadly applicable to the detection of the DNA polymerase coding region of herpesviruses in humans and animals [16]. Next, the PCR amplification pattern was examined using five primer sets to investigate further genetic characteristics of FOY-1 as described by Tomaszewski [12]. The locations of primer sets 9F, 9R, 11F, 11R and 23F were UL19 (major capsid protein), UL21 (nucleocapsid protein), UL15 (DNA packaging protein), UL9 (ori binding protein) and UL16, respectively. Amplicons of FOY-1 were produced by using primer sets 9F, 11F and 23F but not 9R and 11R (Fig. 2A). The RSL-1 strain (ATCC VR-915) [3], which is a reference strain for PsHV-1 in this study, was detected with all five primer sets. Tomaszewski *et al.* reported that ten patterns (PsHV-1 variants v1 to v10) were observed using these five primer sets [12]. Although RSL-1 was classified into PsHV-1 variant 1 in this study, the pattern of FOY-1 has never been reported. Since the 23F primer set was able to detect all PsHV-1 variants, FOY-1 was thought to be PsHV-1.

Since PCR amplification patterns of the PsHV-1 have been reported to partly indicate the genotype [13], next, the genotypes of FOY-1 and RSL-1 were identified. A 420-bp fragment of the UL16 gene was generated by PCR with the 23Ff5a/b primer set, which was used for genotyping of PsHV-1 strains in a previous study [10], and sequenced. Partial UL16 gene sequences of FOY-1 and RSL-1 were submitted to DDBJ (Accession numbers: AB510905 and AB510906). A putative 140-amino acid sequence was compared with each of the published PsHV-1 sequences shown in Table 1. The FOY-1 amino acid sequence was closest (99.2% identity) to that of the 1070/93 strain, which is grouped into PsHV-1 genotype 2. The FOY-1 sequence was 79.2% homologous to that of RSL-1.

In a phylogenetic tree based on the UL16 amino acid sequence and constructed by the UPGMA method with the Genetyx-Mac version 15.0.1 computer software (Fig. 2B), the PsHV-1 strains fell into four genotypes, in agreement with the results of Tomaszewski [13]. FOY-1 belongs to genotype 2, whereas RSL-1 belongs to genotype 4. Restriction fragment length polymorphism (RFLP) analysis of the entire viral genomic DNA with restriction endonucleases *Eco*RI, *Pst*I and *Bgl*II was performed to subdivide the genotypes [8]. According to the migration profiles, 12 different restriction patterns have been recognized [8]. FOY-1 was classified as an FFF virus, whereas RSL-1 was classified as an AA2A2 virus (Fig. 2B and 2C). The groups are named on the basis of the cleavage patterns with the restriction endonucleases *Eco*RI, *Pst*I and *Bgl*II. For instance, the first F of FFF and A of AA2A2 mean patterns obtained with *Eco*RI, the second F and A mean *Pst*I, and the third F and A mean *Bgl*II [8].

The pathogenicities of FOY-1 and RSL-1 (isolated from rosellas) were compared using budgerigars as an experimental host. All experimental infection plans were approved by the Committee for Animal Research and Welfare of Gifu University. Twenty-one conventional budgerigars were divided into seven groups and kept in isolators. Before the experiment, we checked whether the birds had been infected with any pathogens, such as herpesvirus, BFDV and APV, by the PCR described above. Three groups of three budgerigars each were orally inoculated with 0.1 m*l* of the FOY-1 strain with 10^6 $TCID_{50}$/bird (Group A), 10^4 $TCID_{50}$/bird (Group B) or 10^2 $TCID_{50}$/bird (Group C), respectively. The other three groups were inoculated with 0.1 m*l* of the RSL-1 strain with 10^6 $TCID_{50}$/bird (Group D), 10^4 $TCID_{50}$/bird (Group E) or 10^2 $TCID_{50}$/bird (Group F), respectively. The virus titers used for inoculation were calculated by a limiting dilution method taking into consideration the CPE as reported in a previous study [5]. The last group was inoculated with 0.1 m*l* of PBS and served as the control (Group G). The birds were observed daily for any clinical signs of disease. At 14 days postinoculation (d.p.i.), the surviving birds were euthanized with an intramuscular injection of ketamine hydrochloride. Liver and spleen samples of all the birds were collected for histopathological examination and herpesviral DNA detection. PCR was carried out using the five primer sets used above to confirm whether the inoculated virus was detected in their tissues [12].

One bird of group A (A-1) showed piloerection at 8 d.p.i., and two birds of group D (D-1 and D-2) showed anorexia and depression at 6 d.p.i (Table 2). No clinical signs were observed in the other birds. At 7 or 8 d.p.i., one bird spontaneously died in each RSL-1-inoculated group. The other

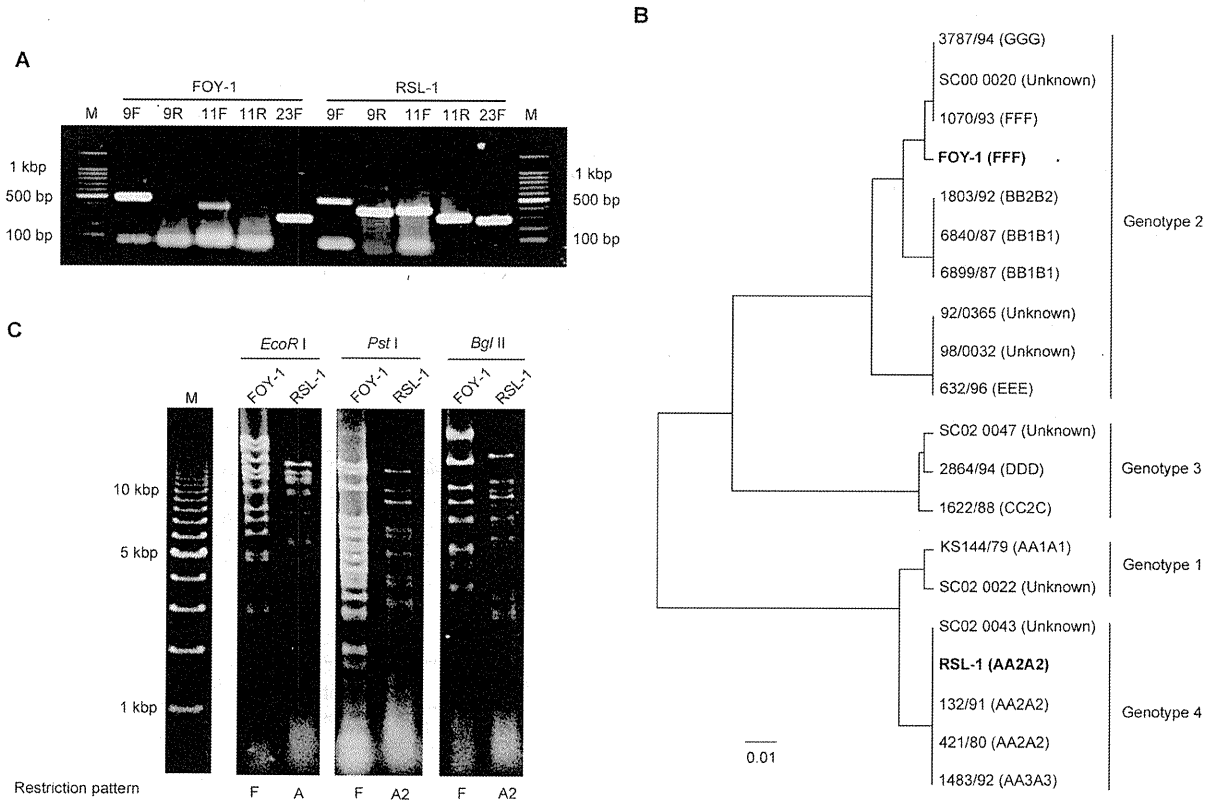

Fig. 2. Genetic characteristic of the PsHV-1 FOY-1 strain. (A) PCR amplification patterns of FOY-1 and RSL-1. Primer names (9F, 9R, 11F, 11R and 23F) according to Tomaszewski *et al.* are shown above each lane [12]. The M lane is a 100-bp DNA Ladder (ToYoBo, Osaka, Japan). (B) Phylogenetic analysis of FOY-1, RSL-1 and other reference strains based on the amino acid sequence of UL16. The tree was obtained using the UPGMA method with the Genetyx-Mac version 15.0.1 computer software. Restriction fragment length polymorphism (RFLP) patterns according to Schroder-Gravenduck *et al.* are shown in parentheses [8]. (C) RFLP patterns of FOY-1 and RSL-1 digestion with *Eco*RI, *Pst*I and *Bgl*II. Restriction patterns according to Schroder-Gravenduck *et al.* are shown under the photographs [8]. The M lane is a 1-kbp DNA Ladder (ToYoBo).

birds in the RSL-1-inoculated groups remained alive until the end of the experiment. On the other hand, all the birds in the FOY-1-inoculated groups remained alive. The overall mortality rate of the RSL-1-inoculated groups, i.e., total number of dead birds in the RSL-1 groups divided by total number of birds in the RSL-1 groups, was 33.3%, whereas the overall mortality rate of the FOY-1 groups was 0%. The histopathological changes in all three dead birds consisted of typical lesions of PD as reported by other researchers [9], such as coagulation necrosis in the liver and eosinophilic intranuclear inclusion bodies in hepatocytes and splenic reticular cells (data not shown). In addition, intranuclear inclusion bodies were found in hepatocytes of the two surviving birds of group D. A histopathological examination found no lesions in the tissues of the other surviving birds. Herpesviral DNA was detected in the liver of all the birds inoculated with RSL-1, whereas the rates of detection of viral DNA in the liver in the three FOY-1-inoculated groups (A, B and C) were 33.3, 0 and 33.3%, respectively.

In conclusion, a genotype 2 strain of PsHV-1 (FOY-1) was isolated from a captive galah that died after exhibiting anorexia and diarrhea and was less pathogenic to budgerigars than RSL-1 of genotype 4. This is the first report regarding the classification of originally isolated PsHV-1 in Japan and its characterization by an animal infection experiment.

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Table 1. PsHV-1 strains used for sequence analysis in this study

Genotype	Virus strain	Host	Restriction pattern	Accession no.
1	KS144/79	Blue-fronted Amazon (*Amazona aestiva*)	AA1A1	AY282614
	SC02–0022	Blue and gold macaw (*Ara ararauna*)		AY421993
2	6840/87	Blue-fronted Amazon (*Amazona aestiva*)	BB1B1	AY282624
	6899/87	African grey parrot (*Psittacus erithacus*)	BB1B1	AY282626
	1803/92	Pooled sample (*Amazons, pionus and conure*)	BB2B2	AY282627
	92/0365	Red-lored Amazon (*Amazona autumnalis*)		AY282631
	98/0032	Unknown		AY282634
	632/96	Green-cheeked Amazon (*Amazona viridigenalis*)	EEE	AY282630
	1070/93	Orange-winged Amazon (*Amazona amazonica*)	FFF	AY282628
	3787/94	White cockatoo (*Cacatua alba*)	GGG	AY282629
	SC00 0020	Yellow-headed Amazon (*Amazona oratrix*)		AY421988
3	1622/88	St. Lucia Amazon (*Amazona versicolor*)	CC2C	AY282635
	2864/94	St. Lucia Amazon (*Amazona versicolor*)	DDD	AY282640
	SC02 0047	Blue-fronted Amazon (*Amazona aestiva*)		AY421999
4	132/91	Yellow-crowned Amazon (*Amazona ochrocephala*)	AA2A2	AY282664
	421/80	African grey parrot (*Psittacus erithacus*)	AA2A2	AY282660
	1483/92	Cuba Amazon (*Amazona leucocephala*)	AA3A3	AY282668
	SC02 043	Blue and gold macaw (*Ara ararauna*)		AY421995

Genotype as presented in Tomaszewski *et al.* [11] and Styles *et al.* [8].

Table 2. Summary of the experimental infection design and results

Group	Virus strain	Dose ($TCID_{50}$/bird)	Bird no.	Clinical symptoms on each day postinoculation													Histopathological lesion	Detection of viral DNA in tissue sample	
				1	2	3	4	5	6	7	8	9	10	11	12	13		Liver	Spleen
A	FOY-1	10^6	1	–	–	–	–	–	–	–	+	–	–	–	–	–	–	+	+
			2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
			3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
B	FOY-1	10^4	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
			2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
			3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
C	FOY-1	10^2	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+
			2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
			3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
D	RSL-1	10^6	1	–	–	–	–	–	+	X							+	+	+
			2	–	–	–	–	–	+	–	–	–	–	–	–	–	+	+	–
			3	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–
E	RSL-1	10^4	1	–	–	–	–	–	–	–	X						+	+	+
			2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
			3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
F	RSL-1	10^2	1	–	–	–	–	–	–	X							+	+	+
			2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
			3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
G	PBS	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
			2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
			3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

A plus sign in the "Clinical symptoms on each day postinoculation" columns indicates that the bird showed clinical symptoms such as piloerection, anorexia, and depression on the indicated day. A minus sign indicates that no clinical symptoms were observed. An "X" indicates that the bird died on the indicated day. A plus sign in the "Histopathological lession" column indicates that typical lesions of PD, such as coagulation necrosis in the liver and eosinophilic intranuclear inclusion bodies, were observed by histological examination. A minus sign indicates that no lesions were observed. A plus sign in the "Detection of viral DNA in tissue sample" columns indicates that the viral DNA was detected in the tissue samples by PCR using the five set primers to confirm the inoculated virus strain. A minus sign indicates that no viral DNAs were detected.

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GENETIC CHARACTERISTICS AND ANTIMICROBIAL RESISTANCE OF *ESCHERICHIA COLI* FROM JAPANESE MACAQUES (*MACACA FUSCATA*) IN RURAL JAPAN

Keiko Ogawa,[1,5] Keiji Yamaguchi,[2] Masatsugu Suzuki,[3] Toshio Tsubota,[4] Kenji Ohya,[3] and Hideto Fukushi[3,6]

[1] School of Veterinary Medicine, Hokkaido University, Kita 18, Nishi 9, Kita-ku, Sapporo, Hokkaido 060-0818, Japan
[2] Hokkaido Institute of Public Health, Kita 19, Nishi 12, Kita-ku, Sapporo, Hokkaido 060-0819, Japan
[3] Faculty of Applied Biological Sciences, Gifu University, Yanagido 1-1, Gifu, Gifu 501-1193, Japan
[4] Graduate School of Veterinary Medicine, Hokkaido University, Kita 18, Nishi 9, Kita-ku, Sapporo, Hokkaido 060-0818, Japan
[5] Current address: United Graduate School of Veterinary Sciences, Gifu University, Yanagido 1-1, Gifu, Gifu 501-1193, Japan
[6] Corresponding author (email: hfukushi@gifu-u.ac.jp)

ABSTRACT: *Escherichia coli* was isolated from wild and captive Japanese macaques (*Macaca fuscata*) to investigate the risk of zoonotic infections and the prevalence of antimicrobial-resistant *Escherichia coli* in the wild macaque population in Shimokita Peninsula, a rural area of Japan. We collected 265 fresh fecal samples from wild macaques and 20 samples from captive macaques in 2005 and 2006 for *E. coli* isolation. The predominant isolates were characterized by serotyping, virulence gene profiling, plasmid profiling, pulsed-field gel electrophoresis (PFGE), and microbial sensitivity tests. In total, 248 *E. coli* strains were isolated from 159 fecal samples from wild macaques, and 42 *E. coli* were isolated from 17 samples from captive macaques. None of the virulence genes *eae*, *stx*, *elt*, and *est* were detected in any of the isolates. The relatedness between wild- and captive-derived isolates was low by serotyping, PFGE, and plasmid profiling. Serotypes O8:H6, O8:H34, O8:H42, O8:HUT, O103:H27, O103:HNM, and OUT:H27 were found in wild macaque feces; serotypes O157:H42 and O119:H21 were recovered from captive macaques. O- and H-serotypes of the 26 isolates were not typed by commercial typing antisera and were named OUT and HUT, respectively. Twenty-eight isolates had no flagellar antigen, and their H-serotypes were named HNM. Similarity of PFGE patterns between wild-derived isolates and captive-derived isolates was <70%. No plasmid profile was shared between wild-derived and captive-derived isolates. The prevalence of antimicrobial-resistant *E. coli* was 6.5% (n=62) in wild macaques, and these isolates were resistant to cephalothin. We conclude that wild Japanese macaques in Shimokita Peninsula were unlikely to act as a reservoir of pathogenic *E. coli* for humans and that antimicrobial-resistant *E. coli* in wild macaques may be derived from humans.

Key words: Antimicrobial resistance, *Escherichia coli*, genotyping, Japanese macaque, *Macaca fuscata*, zoonosis.

INTRODUCTION

Mammals are sentinels for ecosystem health because they sit at or near the top of food chains (Delahay et al., 2009). Nonhuman primates are well suited for zoonosis research because of their genetic and physiologic similarities to humans (Wolfe et al., 1998). However, zoonotic pathogens are sometimes transmitted from humans to nonhuman primates and can cause population declines in threatened species (Leendertz et al., 2006). Few studies have been conducted on zoonotic pathogens of Japanese macaques (*Macaca fuscata*), the only primate indigenous to Japan.

Shimokita Peninsula, Aomori Prefecture, Japan, has the northernmost distribution of Japanese macaques, the northernmost nonhuman primate, in the world. The Shimokita macaque population is isolated and genetically distinct from other macaque populations in Japan (Kawamoto et al., 2008). This population has been conserved as a national natural treasure and is classified as a locally threatened population by Aomori Prefecture (Aomori Prefecture, 2001). However, the population of macaques in Shimokita increased from 187 in the early 1970s to >1,300 in 2005 (Aomori Prefecture, 2008). Seventeen of 29 macaque troops monitored by the local government caused conflicts with the local people by

damaging crops, intimidating people, and invading homes (Aomori Prefecture, 2008). Such direct or indirect contact between wild macaques and humans might lead to pathogen transmission between them.

Escherichia coli is found naturally as intestinal microflora of many species, including macaques and humans. Most *E. coli* strains are commensal, but some are pathogenic to humans. In particular, Shiga toxin-producing *E. coli* (STEC), such as serotype O157:H7, is one of the most important human pathogens in industrialized countries (Beutin, 2006). Although cattle are considered to be the most important reservoir of STEC (Caprioli et al., 2005), wildlife such as deer are associated with STEC (Asakura et al., 1998).

The occurrence of antimicrobial resistance among pathogenic and commensal bacteria is a significant problem affecting medical treatment of infectious diseases. Humans (Ishikawa et al., 2005), food animals (Asai et al., 2005), and wildlife (Gilliver et al., 1999) may act as reservoirs of drug-resistant bacteria through the food chain. Antimicrobial resistance was found to be widespread in enterobacteria from wild rodents in England (Gilliver et al., 1999). However, in Finland, where antimicrobial use is much less common than in England, antimicrobial resistance was found to be almost absent in enterobacteria from wild ungulates and voles (Österblad et al., 2001). The widespread occurrence of antimicrobial resistance in bacteria in wildlife populations may be caused by environmental pollution of antimicrobials or resistant bacteria through human activities such as antimicrobial use in medicine and agriculture.

Our goals for this study were to 1) investigate the risk of transmission of pathogenic *E. coli*, especially STEC, from wild macaques to humans; 2) investigate the transmission of *E. coli* within the wild macaque population; and 3) determine the prevalence of antimicrobial-resistant *E. coli* and estimate the extent of human impact on the wild macaque population.

MATERIALS AND METHODS

Study areas, macaque behavior, and fecal sample collection

Fecal samples were collected in northwestern (41°25′N, 140°50′E) and southwestern (41°8′N, 140°49′E) Shimokita Peninsula, Aomori Prefecture, Japan (Fig. 1), in December 2005 and from October to December 2006. Thirteen troops were surveyed: 12 troops of wild Japanese macaques and a captive troop kept at Wakinosawa Monkey Park (Table 1). Troops that visit farmland seasonally or throughout the year and forage crops were classified as "high damage troops," and those that visit farmland sometimes or seasonally and forage grass plants, grains, or vegetables left around fields were classified as "low damage troops." Habituation levels of the troops were further classified as high (troops that do not run away from humans and pass by humans at close range), intermediate (troops that do not run away but keep away from humans) and low (troops that run away from humans). The local government attached radiotelemetry collars to one or two macaques in each group. Solitary males that did not belong to any troop also were surveyed. Fresh feces (≤1 day old) on the ground were collected during the tracking of troops. Macaque troops were identified from their telemetry information or the location in which they were observed. Fecal samples were stored in sterilized bags at 4 or −20 C until isolation attempts.

Isolation and identification of *E. coli*

One gram of each fecal sample was incubated twice in modified *E. coli* broth (Oxoid, Basingstoke, Hampshire, UK) with novobiocin (20 μg/ml) for 24 hr at 42 C for the recovery of freeze-injured *E. coli* cells (Hara-Kudo et al., 2000). The enrichment culture was selected by the immunomagnetic separation (IMS) method with Dynabeads anti-*E. coli* O157 (Invitrogen, Carlsbad, California, USA). After IMS, the culture was incubated 24 hr at 37 C on CHROMagar O157 TAM plates (CHROMagar, Paris, France) and Tricorol plates (EL-MEX, Tokyo, Japan). Up to four suspected colonies per sample were selected and identified by biochemical tests with triple sugar iron medium (Eiken, Tokyo, Japan); lysine, indole, motility medium (Eiken); VP semisolid medium (Eiken); and Simmon's citrate agar (Eiken). Confirmed *E. coli* isolates were serotyped by agglutination tests with *E. coli* antisera "Seiken" (Denka-Seiken, Tokyo, Japan) following the manufacturer's instructions.

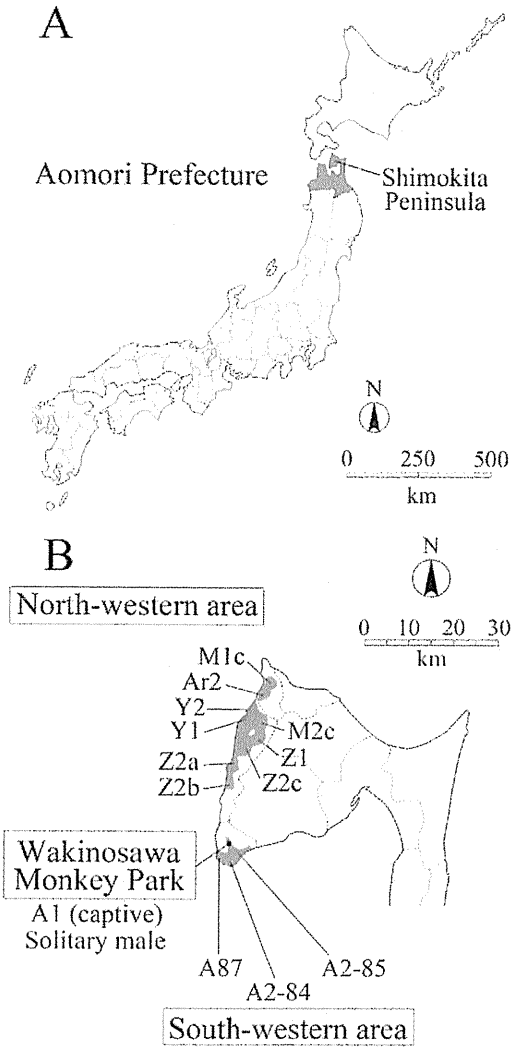

FIGURE 1. Study site and the locations of Japanese macaque (*Macaca fuscata*) troops studied for antibiotic resistant *Escherichia coli* strains. (A) Location of Aomori Prefecture (shaded) and Shimokita Peninsula. (B) Total habitat (shaded) of wild macaque troops surveyed in this study (Aomori Prefecture, 2004; Matsubara, 2006), showing Wakinosawa Monkey Park and approximate locations of each surveyed troop. Habitats of neighboring troops overlapped in marginal regions. Fecal samples of solitary males were collected in Wakinosawa Monkey Park.

TABLE 1. Human-macaque (*Macaca fuscata*) interactions in Aomori Prefecture, Japan (2005–2006).

Area[a]	Troop	Damage level[b]	Habituation level[b]
NW	M1c[c]	H	L
	Ar2[c]	H	I
	Y1[c]	H	I
	Y2[c]	H	I
	M2c[c]	L	L
	Z1	L	L
	Z2a[c]	H	I
	Z2b[c]	H	I
	Z2c	H	L
SW	A2-84[c]	H	H
	A2-85[c]	H	H
	A87[c]	L	H

[a] NW = northwest; SW = southwest.

[b] H = high; L = low; I = intermediate.

[c] One or two individuals in the troop carried radiotelemetry transmitters.

Genotyping of *E. coli*

PCR for the virulence genes for intimin (*eae*), Shiga toxin (*stx*), LT (*elt*), and ST (*est*) was performed on isolates for which serotypes had been determined. DNA extracted with a DNeasy™ Tissue Kit (QIAGEN, Hilden, Germany), or boiled enrichment culture was used as templates. Oligonucleotide primers (Table 2) for PCR were as follows: SK1, SK2 for *eae* (Oswald et al., 2000), Vtcom-u, Vtcom-d for *stx* (Yamasaki et al., 1996), LT_L, LT_R for *elt* (Toma et al., 2003), and AL65, AL125c for *est* (Toma et al., 2003). PCR was performed under the following conditions: initial denaturation for 5 min at 94 C, 30 cycles of 1 min at 94 C, 1 min at 55 C, 1 min at 72 C, and a final extension step of 7 min at 72 C. PCR amplicons were electrophoresed on 2% SeaKem GTG agarose gel (Takara, Otsu, Shiga, Japan) in 1× TAE buffer.

Plasmids were extracted using the methods of Kado and Liu (1981). Extracted plasmid DNA was electrophoresed on 0.8% PFC agarose gel (Bio-Rad Laboratories, Hercules, California, USA) in 1× TBE buffer with a Subcell 192 system (Bio-Rad Laboratories) for 120 min at 100 V.

Pulsed-field gel electrophoresis (PFGE) was performed on dominant isolates. *Escherichia coli* cells in L-broth were suspended in 150 µl of 1× TE buffer (10 mM Tris [pH 8.0] and 1 mM EDTA [pH 8.0]). They were mixed with 150 µl of 1.2% Certified Megabase agarose (Bio-Rad Laboratories) and solidified with a 0.7-mm plug mold (Bio-Rad Laboratories). Plugs were incubated in 2 ml of lysis buffer (0.5 M EDTA [pH 8.0] and lysozyme [3 mg/ml]) for 6–18 hr at 37 C. After lysis, plugs were incubated in 1 ml of proteinase K buffer (0.5 mM EDTA [pH 8.0], proteinase K [1 mg/ml], and 1% [wt/vol] *N*-lauroylsarcosine) for

TABLE 2. Sequences of primers used to detect the virulence genes for *Escherichia coli* from macaques (*Macaca fuscata*) in Aomori Prefecture, Japan (2005–2006).

Primer	Sequence	Target gene	Size (bp)
SK1	5′-CCCGAATTCGGCACAAGCATAAGC-3′	*eae*	881
SK2	5′-CCCGGATCCGTCTCGCCAGTATTCG-3′		
VTcom-u	5′-GAGCGAAATAATTTATATGTG-3′	*stx*	518
VTcom-d	5′-TGATGATGGCAATTCAGTAT-3′		
LT_L	5′-TCTCTATGTGCATACGGAGC-3′	*elt*	322
LT_R	5′-CCATACTGATTGCCGCAAT-3′		
AL65	5′-TTAATAGCACCCGGTACAAGCAGG-3′	*est*	147
AL125c[a]	5′-TTAATAGCACCCGGTACAAGCAGG-3′		

[a] We altered the sequences of the primer from that of AL125 reported by Toma et al. (2003).

12–24 hr at 55 C. Proteinase K was inactivated with 1 ml of Pefabloc solution (10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0], and 2 mM Pefabloc) for 30 min at room temperature. After washing and equilibration, DNA digestion was performed with XbaI (25 U per sample). PFGE was performed on 1% PFC agarose gel (Bio-Rad Laboratories) in 0.5× TBE buffer containing 100 μM thiourea (Liesegang and Tschape, 2002) with CHEF-DR II (Bio-Rad Laboratories). The electrophoretic profile was converted into a TIFF file with Foto/Eclips™ (Fotodyne, Hartland, Wisconsin, USA) and stored in the database with BioNumerics® version 3.0 (AppliedMath, Sint-Martens Latem, Belgium). The similarity among isolates was compared with the unweighted pair-group method with arithmetic mean with a tolerance of 1.5%. The *E. coli* isolates were identified from their PFGE profiles according to Tenover et al. (1995).

Screening for antimicrobial resistance of *E. coli*

Disc diffusion and agar dilution methods (Japanese Society of Antimicrobials for Animals, 2004) were performed to determine microbial sensitivity according to Clinical and Laboratory Standards Institute (CLSI, Wayne, Pennsylvania, USA). Disc diffusion method was performed with BD Sensi-Disc (BD Biosciences, Franklin Lakes, New Jersey, USA). Tested antimicrobials were ampicillin (ABPC, 10 μg), penicillin G (10 units), kanamycin (30 μg), gentamycin (10 μg), streptomycin (SM, 10 μg), erythromycin (15 μg), tetracycline (30 μg), chloramphenicol (30 μg), colistin (10 μg), fosfomycin (50 μg), vancomycin (30 μg), cefazolin (30 μg), cephalothin (CET, 30 μg), cefmetazole (30 μg), cefotiam (30 μg), cefoperazon (75 μg), latamoxef sodium (30 μg), cefotaxime (30 μg), nalidixic acid (30 μg), norfloxacin (10 μg), ofloxacin (5 μg), ciprofloxacin (5 μg), sulfamethoxazole-trimethoprim (23.75/1.25 μg), and sulfamethizole (250 μg). Each isolate was classified as susceptible, intermediate, or resistant, depending on the growth inhibition diameter. Minimum inhibitory concentration of suspected resistant isolates were determined by the agar dilution method with Mueller-Hinton agar (Nissui, Tokyo, Japan) according to the recommendations of CLSI. In brief, suspected resistant isolates were cultured in 1 ml of Mueller-Hinton broth (Difco, Detroit, Michigan, USA) for 2–6 hr at 35 C to match the turbidity of a 0.5 McFarland standard. A 10-fold dilution of cultures was inoculated into Mueller-Hinton agars (Nissui) containing antimicrobials with a microplanter. Resistance to each antimicrobial was determined according to the interpretative breakpoints defined by CLSI (Table 3).

Statistical analysis

The prevalence of antimicrobial resistant *E. coli* isolates from wild macaques was compared with that of captive macaques by Fisher's exact test. Differences were considered significant at $P<0.05$. Statistical analysis was performed using Excel 2007 (Microsoft, Redmond, Washington, USA) with the add-in software Statcel 2 (Yanai, 2004).

RESULTS

Escherichia coli isolates

Escherichia coli was detected in 159 of 265 fecal samples (60%) from wild macaques and in 17 of 20 samples (85%) from captive macaques. In total, 290 strains (248 from wild macaques and 42 from captive macaques) were isolated (Table 4). Six serotypes were identified in the isolates in 2005 and 33 strains of

TABLE 3. Concentrations of antimicrobials and interpretative breakpoints for antimicrobial resistance testing of *Escherichia coli* strains recovered from macaques (*Macaca fuscata*) in Aomori Prefecture, Japan (2005–2006).

Antimicrobial[a]	Concentration[b] (μg/ml)	MIC[c] breakpoint (μg/ml)		
		S	I	R
ABPC	0.0625–512	≤8	16	≥32
CET	0.0625–512	≤8	16	≥32
SM	0.0625–512	≤8	16	≥32

[a] ABPC = ampicillin; CET = cephalothin; SM = streptomycin.

[b] In Mueller-Hinton agar.

[c] MIC = minimum inhibitory concentration.

four serotypes (O8:H34, O8:H42, O8:HUT, and O157:H42) were genotyped by PFGE and plasmid profiles. O- and H-serotypes of the 26 isolates were not typed by the above-mentioned typing antisera and were named OUT and HUT, respectively. Nineteen serotypes were identified in isolates from 2006 and 75 strains of six serotypes (O8:H6, O8:HUT, O103:H27, O103:HNM, OUT:H27, and O119:H21) were genotyped by PFGE and plasmid profiles. Twenty-eight isolates had no flagellar antigen, and their H-serotypes were named HNM. None of the virulence genes examined (*eae*, *est*, *elt*, and *stx*) were detected in the isolates. The isolates were classified into 15 clusters based on the PFGE patterns (Fig. 2). The isolates in each cluster had at least 80% similarity. Seventeen plasmid profiles were observed and named as types a–q (Table 5).

Distribution of serotypes and genetic similarity

Serotype O8 strains were isolated from numerous wild macaque troops in 2005 and 2006 but not from any captive macaques (Fig. 3). Serotype O8 strains consisted of eight clusters (clusters 3 and 8–14; Fig. 2). All isolates of serotype O103:H27 were classified into cluster 2 (Fig. 2), and seven of 12 isolates had identical plasmid profiles (Fig. 2).

The relatedness between *E. coli* isolates from wild macaques and captive macaques was low by serotyping, PFGE, and plasmid profiling. Serotype O157:H42 and O119:H21 strains were isolated from captive macaques in 2005 and 2006, respectively (Fig. 3). Serotype O157:H42 strains had four unique PFGE patterns, and their similarity to wild-derived isolates was <50% (Fig. 2). Serotype O119:H21 strains had a single PFGE pattern, and their similarity to wild-derived isolates was <70% (Fig. 2). No plasmid profile was shared between wild- and captive-derived isolates (Fig. 2).

Antimicrobial resistance

Microbial sensitivity tests were conducted on 33 isolates from 27 fecal samples in 2005 and on 50 isolates from 44 fecal

TABLE 4. Numbers of fecal samples collected and numbers of *Escherichia coli* isolates obtained from Japanese macaques (*Macaca fuscata*) in Aomori Prefecture, Japan (2005–2006).

Area	No. fecal samples			No. *E. coli* isolates		
	2005	2006	Total	2005	2006	Total
Northwestern	56	98	154	57	93	150
Southwestern	35	76	111	12	86	98
Captive	9	11	20	32	10	42
Total	100	185	285	101	189	290

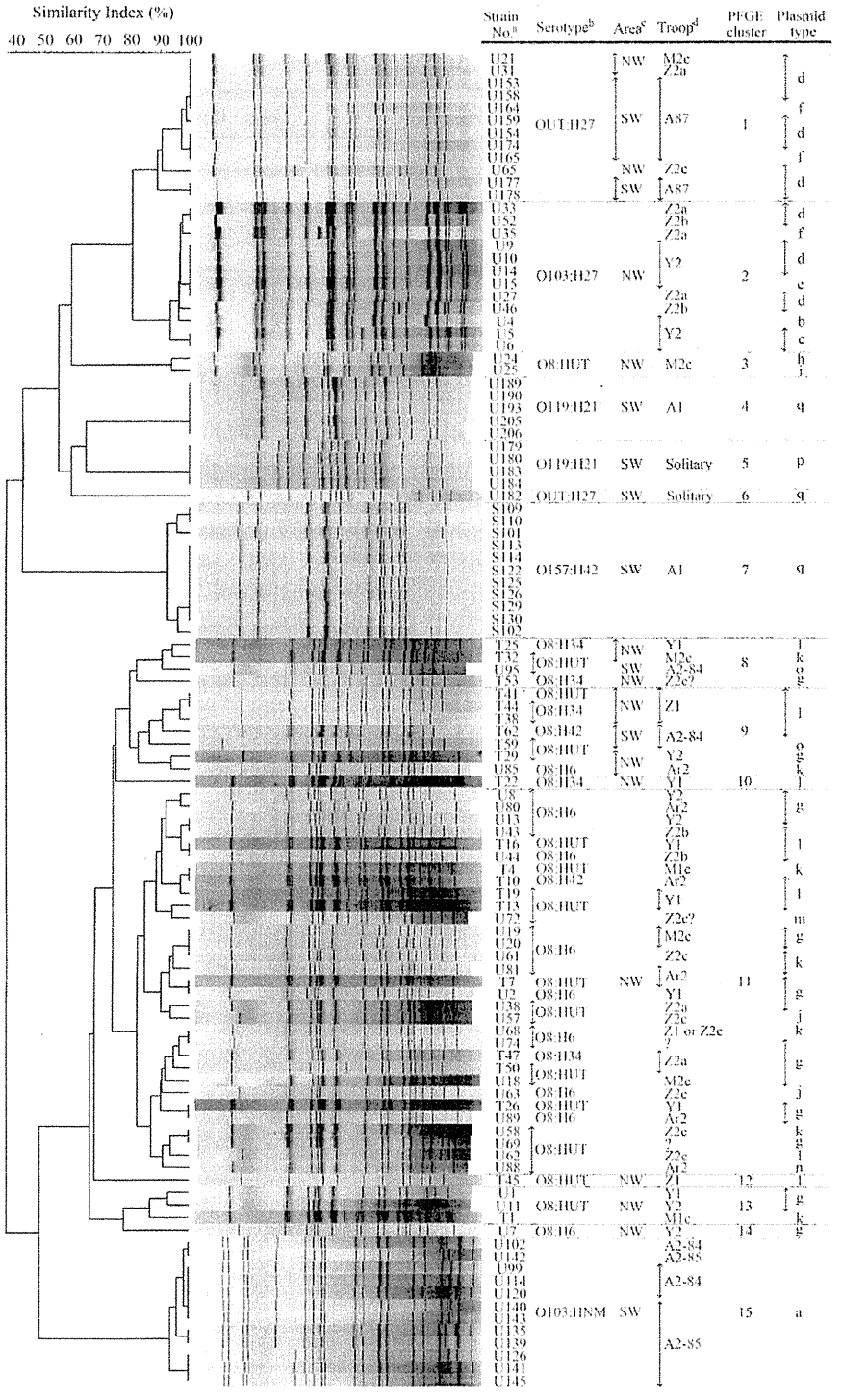

FIGURE 2. Dendrogram of pulsed-field gel electrophoresis patterns of *Escherichia coli* isolates from Japanese macaques (*Macaca fuscata*) sampled in Shimokita Peninsula Aomori Prefecture, Japan (2005–2006). Similarity index scale at top left. (a) Strains beginning with S or T isolated in 2005; strains beginning with U isolated in 2006. (b) UT = untypable; NM = nonmotile. (c) NW = northwest; SW = southwest. (d) Solitary = solitary males not belonging to any troop; Z2c? = likely to be Z2c troop; Z1 or Z2c = troop may have been Z1 or Z2c; ? = unknown troop.

TABLE 5. Plasmid types from *Escherichia coli* isolates from Japanese macaques (*Macaca fuscata*) in Shimokita Peninsula, Aomori Prefecture, Japan (2005–2006).

Plasmid type	Size[a] (kbp)
a	186
b	143, 88, 52
c	173, 52
d	143, 68, 52
e	143, 68
f	143, 88, 68, 52
g	101
h	143, 75, 53
i	143, 75
j	53
k	101, 44
l	101, 53
m	101, 53, 44
n	101, 75, 44
o	101, 75, 53
p	5.3, 3
q	No plasmid

[a] Plasmid sizes were estimated from the migration distances of each band.

samples in 2006. Fifty-eight isolates were resistant to antimicrobials by disc diffusion (17 to CET, 13 to ABPC, 27 to CET and ABPC and one to SM). Minimum inhibitory concentrations to ABPC, CET and SM of these 58 isolates were determined by agar dilution (Japanese Society of Antimicrobials for Animals, 2004). The concentration of each antimicrobial in agar was 0.0625–512 μg/ml. Four isolates (U20, U38, U44, and U61) were resistant to CET. These four isolates came from four troops from the northwestern site in 2006. The prevalence of resistant *E. coli* was 6.5% in wild macaques (n=62) and 0% in captive animals (n=9), but the difference was not significant (Fisher's exact test, P=0.57; n=71).

DISCUSSION

We isolated no pathogenic *E. coli* strains from wild Japanese macaques. Wild macaques are unlikely to act as reservoirs of pathogenic *E. coli*. However, genomic similarity has been observed between enteropathogenic *E. coli* (EPEC) from humans and monkeys, and monkeys were suspected as a reservoir of EPEC in Brazil (Carvalho et al., 2007). Therefore, it is important to continue monitoring for pathogenic *E. coli* among wild macaques, especially where they coexist with humans.

Phenotyping and genotyping revealed that wild macaques of more than one troop carried genetically similar *E. coli* strains. Some troops with similar isolates were in adjacent areas, but some were in separate areas. It is possible that the common strains among wild macaques in our surveyed area are specifically adapted to the internal environment of these animals. The fecal microflora of wild Japanese macaques in snowy areas was different from that of captive macaques, possibly because of the bark-eating habits of the wild macaques (Benno et al., 1987). In our study site, wild macaques mainly fed on dormant buds and bark in winter (Nakayama et al., 1999). Serotype O8, the most frequent serotype in wild macaques, was not isolated from captive macaques, suggesting that serotype O8 strains may be adapted to the internal environment of bark-eating wild macaques.

Another possibility for the similarity of *E. coli* isolates is the transmission of *E. coli*. Some strains, such as serotypes O103:H27, O103:HNM, and OUT:H27, were isolated from wild macaques of particular troops. Those isolates had high genetic similarities in PFGE and plasmid profiles (Fig. 2). Most macaque troops carrying those isolates lived in adjacent areas, suggesting that genetically close strains may be spread in this subset of the Shimokita macaque population. Perhaps other wild animals spread these isolates or the isolates spread environmentally. To examine *E. coli* transmission in the wild macaque population, comprehensive analysis of *E. coli* not only from wild macaques but also from other wild animals and soil in the same area is needed.

Antimicrobial-resistant bacteria among wild animals are thought to be derived from humans or domestic animals through