表2 県内区域別の野鼠の *O. tsutsugamushi* に対する IgG 抗体保有状況

県内区域	野鼠種類	検査数	最高抗体価を示した抗原※					最高抗体価複数※※
			Karp 株	Kato 株	Gilliam 株	Kawasaki 株	Kuroki 株	
北東部	アカネズミ	40	7	0	1	3	0	1 (KP=KW=KR)
	ヒメネズミ	5	0	0	0	0	0	0
	クマネズミ	1	1	0	0	0	0	0
	スミスネズミ	1	0	0	0	0	0	0
東部	アカネズミ	31	7	0	1	0	1	0
中部	アカネズミ	29	9	0	0	3	0	1 (KP=KW)
江の川水系	アカネズミ	8	3	0	0	0	0	0
太田川水系	アカネズミ	13	6	0	0	2	0	1 (KP=KW)
西部	アカネズミ	11	7	0	0	1	0	1 (KP=KR)
合計		139	40	0	2	9	1	4

※　最高抗体価を示した抗原の陽性数
※※　複数抗原について最高抗体価が同値であった陽性数.
　　( ) 内は同値であった抗原 (KP：Karp, KW：Kawasaki, KR：Kuroki).

鼠が各1頭, 東部区域では Kuroki 株に対して最高抗体価を示す野鼠が1頭確認された. なお, 複数の抗原に対して最高抗体価を示す野鼠も4頭認められた.

3　*O. tsutsugamushi* DNA 検出状況

捕獲された野鼠164頭の脾臓を材料として PCR 法による遺伝子検出を行ったところ, 11地点, 24頭のアカネズミが遺伝子陽性となった (図1, 表3). 24件のDNA の primer10/11 領域の増幅産物の塩基配列を決定し, 日本 DNA データバンク (DDBJ) に登録されている *O. tsutsugamushi* の標準株等の塩基配列を用いて分子系統樹解析を実施したところ, 24件中22件は Karp (JP-2) 型, 1件は Karp (Saitama) 型, 別の1件は Kawasaki 型のクラスターに属していた (図2). なお, Karp (JP-2) 型の22件の primer10/11 領域は全て配列が100%一致していた.

考　　察

広島県におけるつつが虫病患者の発生は4月から5月にかけて, および10月から12月にかけてが多く二峰性のパターンを示すが, その多くが秋季の県西部の太田川中流域で発生している [3]. これまでの研究からこの地域では, 患者からタテツツガムシ媒介性の Kawasaki 型の *O. tsutsugamushi* の DNA が検出されており [3], またタテツツガムシの生息も確認されている [2]. 今回我々が実施した調査で Kawasaki 型の DNA が検出されたアカネズミもこの地域で捕獲されたものであった. これらを併せ考えると, 広島県内の太田川中流域ではタテツツガムシ媒介性の Kawasaki 型 *O. tsutsugamushi* が分布し, これによるつつが虫病が発生しているものと考えられる.

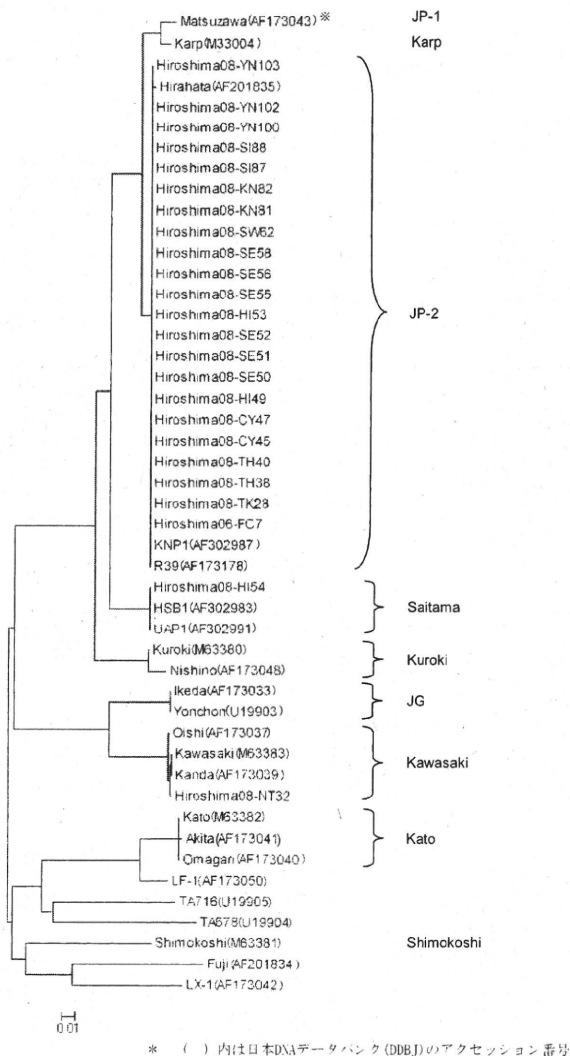一方, 太田川中流域以外の地域では患者発生は散発的

* ( ) 内は日本DNAデータバンク (DDBJ) のアクセッション番号

図2　アカネズミから検出された *O. tsutsugamushi* 56kDa 外膜タンパク遺伝子の系統樹

表3　アカネズミから検出された *O. tsutsugamushi* DNA　とその遺伝子型

県内区域	地点No.	調査地域	検出DNA	遺伝子型
北東部	8	庄原市	Hiroshima08-TK28	Karp (JP-2) 型
東部	11	福山市	Hiroshima08-SI87	Karp (JP-2) 型
	11	福山市	Hiroshima08-SI88	Karp (JP-2) 型
	12	福山市	Hiroshima08-KN81	Karp (JP-2) 型
	12	福山市	Hiroshima08-KN82	Karp (JP-2) 型
	17	尾道市	Hiroshima06-FC7	Karp (JP-2) 型
中部	24	東広島市	Hiroshima08-SW62	Karp (JP-2) 型
	26	広島市	Hiroshima08-YN100	Karp (JP-2) 型
	26	広島市	Hiroshima08-YN102	Karp (JP-2) 型
	26	広島市	Hiroshima08-YN103	Karp (JP-2) 型
江の川水系	30	山県郡北広島町	Hiroshima08-CY45	Karp (JP-2) 型
	30	山県郡北広島町	Hiroshima08-CY47	Karp (JP-2) 型
太田川水系	33	山県郡北広島町	Hiroshima08-TH38	Karp (JP-2) 型
	33	山県郡北広島町	Hiroshima08-TH40	Karp (JP-2) 型
	35	山県郡安芸太田町	Hiroshima08-NT32	Kawasaki 型
西部	39	廿日市市	Hiroshima08-HI49	Karp (JP-2) 型
	39	廿日市市	Hiroshima08-HI53	Karp (JP-2) 型
	39	廿日市市	Hiroshima08-HI54	Karp (Saitama) 型
	41	廿日市市	Hiroshima08-SE50	Karp (JP-2) 型
	41	廿日市市	Hiroshima08-SE51	Karp (JP-2) 型
	41	廿日市市	Hiroshima08-SE52	Karp (JP-2) 型
	41	廿日市市	Hiroshima08-SE55	Karp (JP-2) 型
	41	廿日市市	Hiroshima08-SE56	Karp (JP-2) 型
	41	廿日市市	Hiroshima08-SE58	Karp (JP-2) 型

に春あるいは秋から冬にかけて発生しているが，今回の調査で，県内の広い範囲におよぶ地域でKarp（JP-2）型に感染した野鼠やKarp株に対して高い抗体価を示す野鼠が多く確認されたことから，これらの地域においてはKarp（JP-2）型によるつつが虫病が発生している可能性が考えられる．このKarp（JP-2）型はフトゲツツガムシ媒介性であるが[1]，過去に県内で実施された調査結果から，県内の複数の患者発生地付近ではフトゲツツガムシが確認されていることから[6][7]，県内の広範囲の地域でフトゲツツガムシ媒介性のKarp（JP-2）型が患者の発生に関与している可能性がある．また，Kawasaki株に対して高い抗体価を示すアカネズミは太田川中流域以外でも確認されたことから，Kawasaki型がそれらの地域で患者発生に関与している可能性もある．

なお，今回，廿日市市の1地点の野鼠1頭から検出されたKarp（Saitama）型については，広島県では初めて確認されたものである．他県ではこれまでに埼玉県[8]，岡山県[9]，愛媛県[9]，高知県[9]および徳島県[10]の野鼠から検出されているが，患者からの検出例の報告はない[10]．広島県での本型の確認はその分布を示す新たなデータであり，今後県内の患者発生については，Karp（Saitama）型による可能性にも留意する必要がある．

それら以外にも，Gilliam株あるいはKuroki株に対する高い抗体価を示す野鼠も少数確認されていることか

ら，これらの型の *O. tsutsugamushi* が県内に存在している可能性も考えられる．今後，それらの型を原因とするつつが虫病患者が発生している可能性も視野に入れた検査が必要と考える．

以上，広島県内におけるつつが虫病患者発生の感染環を明らかにするために野鼠類における *O. tsutsugamushi* の侵淫状況の調査を行った結果から，県内では太田川中流域のKawasaki型（タテツツガムシ媒介性）*O. tsutsugamushi* による患者発生以外に，広い範囲でKarp（JP-2）型（フトゲツツガムシ媒介性）による患者発生が起こっていることが強く示唆された．今後は，患者からの *O. tsutsugamushi* の直接検索，また媒介ツツガムシの詳細な調査データの蓄積などを通して，広島県の地域ごとにつつが虫病感染リスクを評価していくことが必要であると考えている．

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病気のはなし

最近のリケッチア症

たかだのぶひろ
髙田伸弘*

サマリー

わが国にみるリケッチア症は寄生性ダニ類が媒介する病種が重要で，微小なツツガムシによるツツガムシ病，また吸血性のマダニによる紅斑熱群が挙げられる．前者は古く知られながら，多様な媒介種が多様な菌型の共生微生物（*Orientia tsutsugamushi*）を保有し，今なお新しい知見は引きも切らない．後者はわが国では 1980 年代後半からようやく実態が知られ，南西日本中心にみられる日本紅斑熱（*Rickettsia japonica*）が重要ながら，北日本ではユーラシア大陸共通性病種の確認も続き，今後とも遺伝解析に伴う多様な知見が得られよう．したがって，検査診断法をさらに改善し，疫学的認識を改めて臨床対応せねばならない．

用語解説

●**共生微生物**…共生（symbiosis）とは，複数種の生物体が相互関係をもちながら同所的に生活する現象をいう．リケッチアを含む細菌類は，例えばダニ類の細胞内に生息しており，そういった共生形態は"細胞内共生"と呼ばれる．宿主と共生微生物とが出会って共生関係になる過程を"伝播"と呼び，卵巣経由で親から子へ共生関係が受け継がれる場合は"垂直伝播"，また環境を介して受け継ぐ場合は"水平伝播"といわれる．一見共生という形態であっても，宿主の性を操作することで自らの伝播をコントロールすることなども知られ，互いに独立した関係でもないことが多いらしく，共生という種間関係は相利共生または寄生（一方の利）といった関係などすべてを含む意味として捉えねばならない．

●**偏性細胞内寄生性**…別の生物の細胞内でのみ増殖可能で，それ自身が単独では増殖できない微生物などの性質をいう．つまり，それは人工培地で生き得ないことを意味する．ただ，リケッチア類は増殖に必要なエネルギー産生系などを備えているのになぜ単独で増殖できないかという理由は，リケッチア類の細胞膜は物質の透過性が高く細胞外では物質の漏出などで生存できないためと考えられている．

●***Orientia tsutsugamushi***…ツツガムシ病原体は，わが国研究者の長い労苦により 1930 年初頭に見いだされ，学説の変遷などを経て菌種名は"*Rickettsia tsutsugamushi*"に落ち着いていた．しかし，その性状分析や遺伝子解析の結果から 1995 年に新属として"*Orientia*"が提唱され，種名も"*O. tsutsugamushi*"と変わって今では広く用いられている．今のところ 1 属 1 種である．

●**寄生性ダニ類**…節足動物のうちクモの仲間は，クモ類とサソリ類，そしてダニ類に分かれる．さらにダニ類は，肉眼的にも大きなマダニ類と日常生活のなかではほぼ見えない微小なコダニ類に分けられる．寄生性とは，皮膚ないし鼻腔粘膜などに口器を刺して一定期間は吸着することを意味するが，ダニ類のうちで寄生できるのはマダニ類（全発育期が吸血性）とツツガムシ類（幼虫期のみ組織液を吸飲），およびヒゼンダニや肺ダニである．このうち，自己の共生微生物（宿主にとっては病原体）の伝播を行うのはマダニ類とツツガムシ類に絞られる．

* 福井大学・シニアフェロー　〒910-1193 福井県永平寺町松岡下合月 23

多様化する病種

　従来のリケッチア症(rickettsiosis)の定義では，紅斑熱群，発疹チフス，ツツガムシ病など真性種のほかQ熱や塹壕熱まで加わり，所属不確定の細菌感染症の集積場と化していた．救いは，その大半の病原種が節足動物媒介性かつ偏性細胞内寄生性という点で共通したことであった．しかし，1980年代後半から新興再興リケッチア症が注目され，遺伝子解析法の急速な進歩に基づく系統樹上でようやく絞り込まれてきた．ただ一方で，遺伝種レベルの細分化に伴い紅斑熱群リケッチアなどは多様化(新種記載の増加)も進むことになった．そういう意味で全体像はなお把握が難しいが，本稿では現時点で国内で焦眉の的になっている病種に絞って概説し，教科書にはない実践的な事柄まで言及したい(表1)．

主な病種の疫学と臨床

1．ツツガムシ病(図1，表1，2)

　ツツガムシ病はわが国で"tsutsugamushi disease"，東南アジアでは多く"scrub typhus"と表記されるが，その病原体は微小なダニのツツガムシの共生微生物であって，親から子へ経卵巣感染で維持される．ムシの種ごとに異なる菌型(血清型)を保有し，国内では古く東北地方日本海側の大河流域でみられたKato型，そして戦後は本州から九州まで分布するKawasaki型など種々の病型が確認され，経緯は複雑である．従来，検査抗原に使われているKarpやGilliamの純粋型は東南アジア由来で国内ではみられない．最近，ほぼ消滅したかと思われていた上記Kato型による感染例が古巣ともいえる★秋田県雄物川中流域で散見されるようになったほか，南西諸島西端の宮古島では東南アジア共通の型の続発も確認されるなど，今なお本病は列島を通じて多様性をみせて尽きるところを知らない．

　ところで，古来から続いていた本症の発生は1960年代に消滅かといわれていたが，1980年頃より再び発生数が増加(年間数百から千例)したということで再興感染症の範疇とされた．しかし，発生数というのは公式届出数に基づくが，その届け出は面倒ということで実際は相当数が不明熱として処理されるようで，たぶん真の発生数は数倍といわれる．統計上でも，古いKato型と新たに知られるようになった多くの型の記録が区分されておらず，厳密な発生消長は知りようがない．ゆえに筆者の本音としては，本病が再興感染症といえるか否か疑問である．統計の価値とは別に，本病は昔から各地に多数実在してきた重要な感染症の一つであるといえる．

　本病を媒介するツツガムシは幼虫期においてのみ脊椎動物に寄生する．その微小さゆえに皮膚上層にしか吸着できないため，吸血ではなく組織液を摂取する．その際にムシの唾液から出た病原体が局所皮膚に潰瘍を形成して増殖し特徴的な"刺し口"が生じる．この病原体が増殖して血流で全身に散布されて発症するまでの日数が潜伏期であり，同病における潜伏期間は★～★日である．治療においては，βラクタム系薬剤は全く無効であるため，テトラサイクリン系薬剤が第一選択となる．なお，1週以上も有効な治療がないと重症化の可能性が高まる点は注意を要する．また，次項の紅斑熱群との鑑別も重要である．

2．新興感染症としての紅斑熱群(図2，3，表1，2)

　マダニ媒介性である紅斑熱群リケッチア(spotted fever group rickettsiae，SFGR)は世界に広く分布し，地域ごとの病原種と病名は多彩である．わが国では，過去の調査不足か，1980年代になって思わぬ新興感染症として日本紅斑熱(Japanese spotted fever)が発掘された．臨床像はツツガムシ病に類似する．認識と検査法普及に伴い，南西諸島も含めて新たな発生地域が見いだされ，年間届出数は南西日本を中心に100例を超えるようになった．最近は紀伊半島の太平洋側森林帯や西九州各地での多発が顕著である．加えてここ数年，欧州や北アジアあるいは東南アジアと共通性のあるマダニ種とその保有SFGRによる症例が知られるようになり，国内の状況も多様なことがわかってきた．

　マダニ類は口器がツツガムシより格段に大きいため，刺し口は皮下毛細血管叢まで届き，刺し口(病原体増殖の揚)が小さくとも病原体が血流に乗って全身に散布されるため発症が早い．病初にみられる手掌や足底の紅斑は特徴的ながら速やかに消退するので注意したい．治療はツツガムシ病

表1 わが国を中心としたリケッチア症の現況

疾患群/疾患名	病原体/主な媒介種	主な発生地域
紅斑熱群（国内で症例確認分） 　日本紅斑熱	*R. japonica*/チマダニ属	南西日本〜極東，東アジア？ （関東や南西諸島でも確認例）
極東紅斑熱（仮称）	*R. heilongjiangensis*/チマダニ属	北アジア〜北日本 （最近，東北地方で確認例）
ヘルベチカ感染症（仮称）	*R. helvetica*/マダニ属	ヨーロッパ，タイ北部，北日本 （近年，北陸地方で確認例）
タムラエ感染症（仮称）	*R. tamurae*/キララマダニ属	東南アジア〜南西日本 （最近，中国地方で確認例）
アジアチカ感染症（仮称）	*R. asiatica*/マダニ属	東アジア〜日本 （近年，中部地方で疑似例）
紅斑熱群（國外の主要種） 　ロッキー山紅斑熱 　シベリアマダニチフス 　ボタン熱 　リケッチア痘	*Rickettsia rickettsii*/カクマダニ属 *R. sibirica*/各種マダニ *R. conorii*/コイタマダニ属 *R. akari*/トゲダニ類	北アメリカ大陸 シベリア，中央アジア，中欧 地中海沿岸〜インド〜アフリカ 北米，ロシア，南アフリカなど
発疹チフス群（世界に散発） 　発疹チフス 　発疹熱	*R. prowazekii*/コロモシラミ *R. tyhpi*/ネズミノミ	世界に散発 世界に散発
恙虫病群（アジア限定） 　ツツガムシ病	*Orientia tsutsugamushi* 　JG ないし JP-2 型/フトゲツツガムシ 　Kawasaki ないし Kuroki 型/タテツツガムシ 　Kato 型/アカツツガムシ 　台湾系の型/デリーツツガムシ	日本，極東〜東南アジア，豪州 （北海道〜九州） （東北中部〜南西諸島） （東北中部の西半部） （最近，宮古島で確認例）

注　従来リケッチア類に含まれていても系統樹上で遠い病原体や限局的分布の菌種は除く．

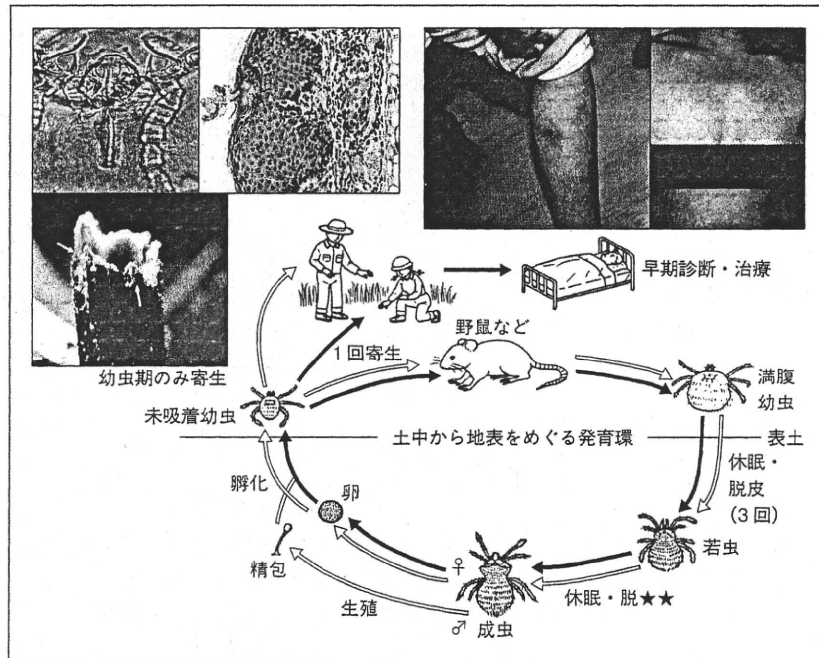
図1 ツツガムシ病の感染環
左上写真は草の先端に集まった幼虫が宿主皮膚に吸着して組織液を摂取する行程．右上写真は刺し口や発疹の状態．

表2　ツツガムシ病と日本紅斑熱の鑑別および対応の比較

事　項	ツツガムシ病	日本紅斑熱（紅斑熱群で概ね共通）
媒介種	フトゲツツガムシ(春と秋) タテツツガムシ(秋〜初冬) アカツツガムシ(夏)	春夏秋(幼若マダニ発生の夏〜秋に多い)
特異症状 　熱型 　発疹 　刺し口 　リンパ節腫脹 　その他	吸着後7〜10日で発症 38〜39℃の弛張熱 主に躯幹(手掌や足底に見ない) 径1cm内外の黒い痂蓋 (頭髪のなかや下着で覆われた部位を含め全身を調べるのが最良) 所属〜全身で＋＋ 比較的徐脈，肝脾腫大	吸着後2〜8日(平均数日)と速い発症 39〜40℃の弛張熱 全身(手掌や足底含む)しばしば出血性 左より小さ目の痂蓋 見られないこと多い 比較的徐脈 ・高サイトカイン血症に伴う全身性炎症反応症候群また播種性血管内凝固症候群(DIC)による重症化や臓器障害の可能性
血液検査 　好中球 　異型リンパ球 　血小板 　CRP 　LDH 　IgM/IgG抗体 　Weil-Felix反応	病初(急性期)と1〜2週後(慢性期)のペア試料が望ましい ＋＋ ＋＋ ↓ ↑ ↑ ・ペア血清につき間接免疫ペルオキシダーゼ染色法または間接蛍光抗体法にて上昇をみる(所管の衛生研究所，国立感染症研究所，ほか民間機関へ依頼)＊ ・上記同様血清につき凝集反応やELISA(試用研究者へ依頼) ・ツツガムシ病でOXK，紅斑熱でOX2かOX19が陽性ながら，低感度で不安定	＋＋ ±〜＋ ↓ ↑ ↑
DNA診断	今では医療機関の検査室でも可能で診断的価値も高い(試料のコンタミに留意) ・生体試料(痂蓋≫血液)につき定番プライマーにてPCR(可能ならシーケンス)	
菌分離	治療前血液につきL929細胞などで継代(検査法としてはやや煩雑)	
治療	テトラサイクリン系の投与 (海外では主にドキシサイクリン)	左の効果低い場合にニューキノロン併用

＊ 実施可能な都道府県立の研究機関は全国の1/5程度に過ぎず，商業ベースで的確な検査が可能な機関も僅少である．国立感染症研究所による行政検査は手続きがやや煩雑ながら可能である．現在進行中の厚労省研究班では，実践的な検査手技やレファレンス体制を都道府県に普及させる試みを急いでいる．

と似るが，重症例ではニューキノロン系薬剤が併用される．

3．その他のリケッチア症

　紅斑熱群はここで挙げた以外にも多くの菌種が世界各地にみられるので，輸入例に遭遇した場合は文献検索ないし専門家への問い合わせで対応したい．一方，古くから知られている発疹チフス群(コロモシラミ媒介の発疹チフスやネズミノミ媒介の発疹熱)は世界の衛生状態の悪い地域で散発するが，わが国では発疹熱を稀にみるだけである．ただ，路上生活者が増加する昨今，発疹チフスの発生などを完全否定はすべきでない．

4．検査診断にみる留意点

　抗体検査の場合，ツツガムシ病は多くの血清型の間で，また紅斑熱群は菌種間で各々交差性をみ

るが，症例ごとに型や菌種を確定するには，国内産の型や菌種の抗原を並べて比較すべきである．ただ，菌体自体を用いた検査法は管理や習熟を要する面もあり，菌体抽出のアルカリ-ポリサッカライド抗原などを用いた凝集反応，またはELISA(enzyme-linked immunosorbent assay)法が簡便な方法として普及が望まれる．PCR(polymerase chain reaction)によるDNA診断法は近年著しく普及し，リケッチア症では特に刺し口の痂蓋(菌の増殖部)から当該遺伝子を検出するという安直とさえみえる方法で100%近い有効性が確認され，診断率が著しく向上した．一方，生きたリケッチアの分離は困難ではあるが，成功した場合には株の樹立により，生菌でしか行うことができない解析が可能となる．なお病理学的検
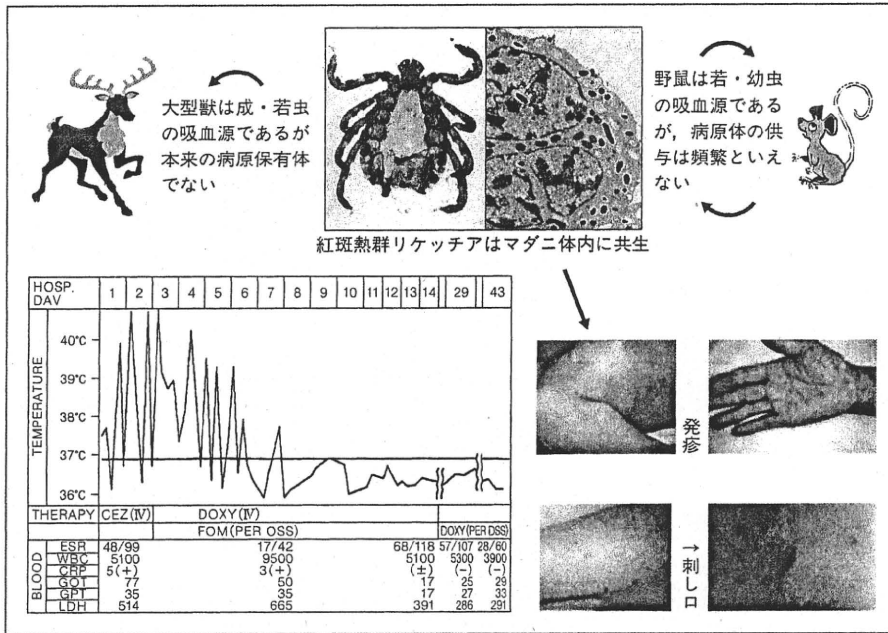
図2　紅斑熱群の感染環
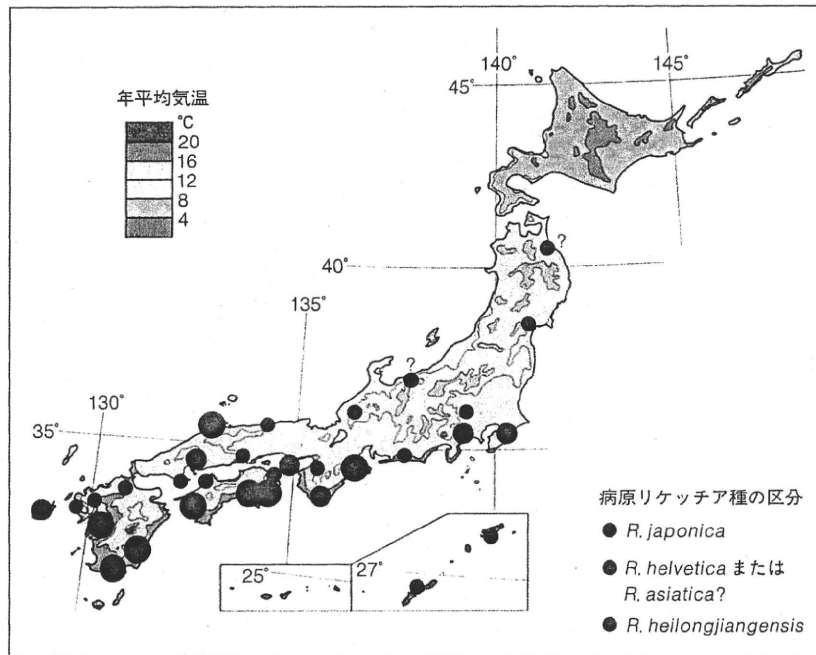上半はマダニと宿主動物での病原体のやりとり．下半は感染後の治癒経過や皮膚所見（発疹，刺し口）．

図3　わが国で確認された紅斑熱群の分布
丸印の大きさは概略の発生頻度を示す．
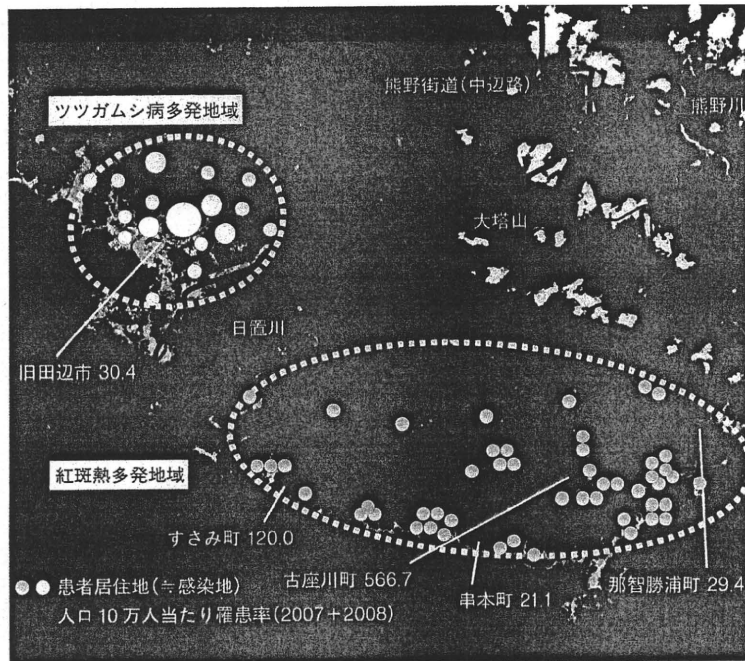
図4　リケッチア症発生地の住み分け
紀伊半島南部の例で，果樹園地帯のツツガムシ病と森林地帯の日本紅斑熱が河川
や山系など環境要因で隔離されている.

索は，診断のためよりむしろ重症例の後追い検査で行われ，単クローン抗体による免疫染色で刺し口や臓器から菌体検出も試みられる.

ところで，リケッチア症は，現行感染症法で4類感染症に分類されるため，それが疑診され，かつ抗体や遺伝子検出ができた場合，最寄りの保健所に届け出る義務がある.ただ，リケッチア症を含むダニ媒介性感染症は届け出指定ながら，特効性のある抗菌薬が正式に保険適用されていないため費用の負担区分が不明確な点は議論がある.

● 疫学的認識の転換(図4)

ダニが介在するリケッチア症は，媒介ダニ種と宿主動物の分布という環境要因に大きく影響される.環境変化と感染拡大の関係を考察すれば以下のとおりである.

①温暖化がいわれるようになってから南方系マダニ種が北上して感染域が拡大したかといえばそうではなく，大半は認識不足で残されていた地域の状況が後追い調査でわかったものと考えるべきで，温暖化の影響はもっと遅れて現れるであろう.

②ただ，シカなどマダニ増殖の背景となる野生動物が近年急増しているのは事実で，それによる感染域の拡大も証明されている.

③ツツガムシ病は果樹園や河岸段丘など開放的な環境(宿主である野鼠が多い)，また紅斑熱は森林環境(宿主となる野獣が多い)に多発傾向があり，しばしばこれらが隣接して住み分ける例すらある.地域医療で留意したい.

では，こういった背景で実際の感染はどう起こるものかを問う場合，まずリケッチア症の感染はすべて「ダニ類の生息する山野」で起こるという漠然とした考え方から改めたい.関係のダニ類はもちろん山野に生息はするが，山沿いの住家裏庭や家庭菜園，散歩道，また大都市内でも公園や河川敷，植生の残る新興住宅地など，住民の身近の草藪にも存在している.また，媒介役となるツツガムシの幼虫，マダニの幼若虫などは肉眼で見えないほど微小であるため，それらが生息する草藪は見分けることは不可能に近い.そういった草藪から屋内に持ち込まれた植物や土壌にも混入する可能性もある.

そのような経路で感染・発症した患者が診察の

際に山野での活動歴を問われたとき，“山野”という言葉の印象から離れた山岳や草原を思い出そうとすると的を射ない．ゆえに，まず住家周辺の環境から問うのが誤解を避けるコツと思われる．このように，ダニ媒介感染症はわが国の“常在感染症”であるという認識で臨み，感染源は身近から検索するのが臨床診断上また疫学対応上でも早道となると思われる．

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Evaluation of *Rickettsia japonica* Pathogenesis and Reservoir Potential in Dogs by Experimental Inoculation and Epidemiologic Survey[∇]

Hisashi Inokuma,* Hironori Matsuda, Leo Sakamoto, Michihito Tagawa, and Kotaro Matsumoto

Department of Clinical Veterinary Medicine, Obihiro University of Agriculture and Veterinary Medicine, Inada, Obihiro, Hokkaido 080-8555, Japan

Rickettsia japonica pathogenesis and reservoir potential in dogs were evaluated by both experimental inoculation and epidemiologic survey. In the experimental inoculation study, dogs 1 and 2 were pretreated with an immunosuppressive dose of cyclosporine 14 days before inoculation and became ill after exposure to *R. japonica*. Dogs exhibited clinical signs, including fever, anorexia, depression, and decreased water consumption, between 36 and 96 h after inoculation, but these signs disappeared spontaneously by 5 days after inoculation. Dogs 3 and 4 were not pretreated with cyclosporine, and no clinical signs were detected in them throughout the 14-day observation period. The control dog was clinically normal and had a normal rectal temperature throughout the study period. We attempted to detect rickettsial DNA from peripheral blood and aspiration samples from kidney and spleen by nested PCR, but all samples examined were negative. The control dog lacked detectable titers to *R. japonica* antigen on day 14, while positive antibodies to *R. japonica* were detected in all four experimentally infected dogs, with titers of 1:160 to 1:80. In the epidemiologic survey, 24 (1.8%) of the 1,363 dogs examined throughout Japan had antibodies against *R. japonica*, with titers of 1:40 or more. However, we observed neither clinical signs at the time of sample collection nor nested PCR results indicative of rickettsial infection in these dogs. In conclusion, dogs in Japan can be exposed to *R. japonica*, and infected dogs with immunosuppressive conditions can temporarily develop clinical symptoms, including fever, anorexia, depression, and decreased water consumption.

Rickettsiae belong to the order *Rickettsiales* and are obligate intracellular, Gram-negative bacteria. Several species cause disease in humans and other animals and are distributed worldwide. This genus comprises the spotted fever group (SFG) rickettsiae and the typhus group (TG) rickettsiae (18). In Japan, *Rickettsia japonica*, classified within the SFG, is the causative agent of Japanese spotted fever (JSF) (15). In 1984, the first JSF patient was reported in Tokushima Prefecture, and since then, most JSF patients have been identified in western Japan (15). Recent epidemiologic studies clarified both the vector and reservoirs of JSF in Japan. *Dermacentor taiwanensis* and *Haemaphysalis flava* are confirmed vectors of *R. japonica* (9). Isolation of *R. japonica* from wild mice indicated that mice are a mammalian reservoir for the pathogen (22). Dogs have also been thought to be a mammalian reservoir for *R. japonica*, as antibodies against *R. japonica* have been detected in canine serum (6, 8). A recent epidemiologic study revealed that antibodies against *R. japonica* were detected in 20 of 1,207 dogs in Japan (21). Dogs are often exposed to a large number of tick species, depending on the distribution of these arthropod vectors in the environment (19). They most likely have an increased risk of tick bites compared to humans, due at least in part to their activity in woodland and bush areas. However, no previous studies have addressed whether dogs that are naturally infected with *R. japonica* readily exhibit detectable clinical signs or whether dogs are important sources of infection. The present study evaluated *R. japonica* pathogenesis and the reservoir potential of dogs through experimental inoculation and an epidemiologic survey.

MATERIALS AND METHODS

Experimental inoculation of *R. japonica* in dogs. Five 6-month-old male beagle dogs weighing between 8 and 11 kg were provided by a commercial breeder. They were housed indoors and maintained in a biosafety level P3 animal care facility, as dictated by the Animal Care and Use Committee regulations of Obihiro University of Agriculture and Veterinary Medicine (permission number 20-86). During a 2-week acclimatization period, we monitored clinical signs, food consumption, and rectal temperatures on a daily basis. During that time 2 milliliters of blood was obtained from the cephalic vein of each dog. Complete blood counts were performed using EDTA-anticoagulated blood. All hematological parameters fell within normal ranges (1). Extracted serum was subjected to an indirect immunofluorescence assay (IFA) to confirm the absence of reactive antibodies to *R. japonica*.

Four dogs were randomly selected for experimental inoculation, and the remaining one was monitored as a control. Two dogs (dogs 1 and 2) in the inoculation group were orally administered cyclosporine (Atopica; Novartis, Basel, Switzerland) with a daily dose of 50 mg/head, beginning at 14 days before inoculation (day −14) and lasting until the final experimental day (day 14), to suppress their immunity. Serum cyclosporine concentrations in dogs 1 and 2 on day 1 were measured by a commercial laboratory and were 35 and 39 ng/ml, respectively. Dogs 3 and 4 of the inoculation group were not treated with any drugs during the experimental period.

After the acclimatization period, four dogs (dogs 1 to 4) were inoculated subcutaneously with 20 ml of L929 cell suspension medium infected with *R. japonica* strain Aoki (day 0). Each inoculum dose contained approximately 2×10^7 infected cells suspended in minimum essential medium (MEM) with 1% fetal bovine serum (FBS). The control dog was inoculated with the same amount of uninfected L929 cells suspended at the same concentration. Clinical signs and food consumption were monitored, and rectal temperatures were recorded twice a day. Blood from the cephalic vein was collected in EDTA tubes every 2 days from day 0 until day 14. Each blood sample was subjected to hematological analysis and PCR. Peripheral blood smears were examined for neutrophil counts

* Corresponding author, Mailing address: Department of Clinical Veterinary Medicine, Obihiro University of Agriculture and Veterinary Medicine, 080-8555 Obihiro, Japan. Phone and fax: 81-155-49-5370. E-mail: inokuma@obihiro.ac.jp.
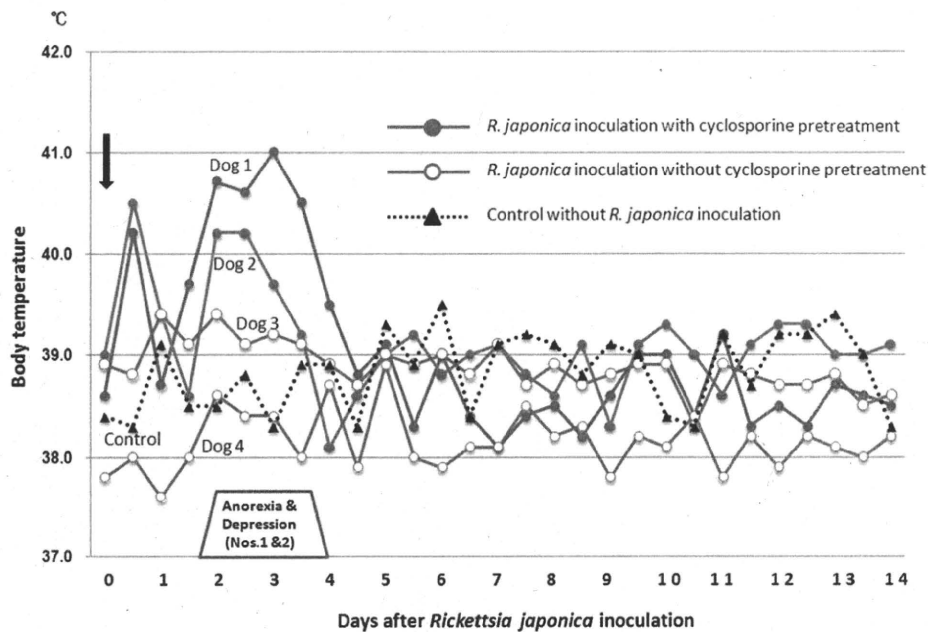
FIG. 1. Changes in body temperature of dogs inoculated with *R. japonica* from the day of inoculation (day 0) to day 14. The body temperature of each dog was measured twice a day (at 8 a.m. and 5 p.m.).

and evidence of platelet aggregation. Sera were collected and IFA was conducted on day 14. Kidney and spleen samples were aspirated for PCR use on day 14.

IFA. Detection of antibodies against *R. japonica* was carried out using IFA as described previously (21). Serum samples were screened at a 1:20 dilution in phosphate-buffered saline (pH 7.2) with 0.5% Tween 20 (PBST). Fluorescein isothiocyanate (FITC)-labeled rabbit anti-canine IgG (Fc) conjugates (Rockland Inc., Gilbertsville, PA) or FITC-labeled rabbit anti-feline IgG (Fc) conjugates (Rockland Inc.) were used as secondary antibodies for the IFA. Reactive antibodies were then detected using a fluorescence light microscope. Samples that reacted with the *R. japonica* antigen at the screening dilution were then titrated to the endpoint. We considered antibody titers 1:40 or above to be positive, as this ratio was used in a previous survey (6).

DNA extraction and PCR. DNA was extracted from EDTA blood samples using a QIAamp DNA minikit (Qiagen GmbH, Hilden, Germany). DNA samples were stored at −20°C in 200 μl of TE (Tris-EDTA) buffer until further analysis. Nested PCR was performed with genus-specific primers for the rickettsial citrate synthase (*gltA*) gene used in our previous study (7); the primer pair RpCS.877p and RpCS.1273r was used for the first amplification. The first round of PCR was carried out in a 25-μl reaction mixture (5 μl of DNA template) under the following settings: 35 cycles of denaturation at 95°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 90 s. The resulting PCR products were then used as a template for the second amplification with primers RpCS.896f and RpCS.1258n. PCR settings for the second round were the same as those for the first round, except that annealing was carried out at 56°C for 30 s. DNA extracted from the *Rickettsia* AT-1 strain was used as a positive control, and distilled water was used for the blank control.

Epidemiologic study. Between January 2006 and June 2008, blood and sera were collected from 1,363 domestic dogs and processed in animal hospitals located within 31 prefectures in Japan (40 dogs in Hokkaido, 40 dogs in Aomori, 60 dogs in Miyagi, 40 dogs in Fukushima, 35 dogs in Tochigi, 50 dogs in Tokyo, 40 dogs in Chiba, 40 dogs in Kanagawa, 40 dogs in Yamanashi, 40 dogs in Shizuoka, 37 dogs in Aichi, 39 dogs in Mie, 40 dogs in Fukui, 40 dogs in Osaka, 40 dogs in Kyoto, 40 dogs in Shiga, 40 dogs in Nara, 40 dogs in Wakayama, 64 dogs in Hyogo, 22 dogs in Tottori, 40 dogs in Shimane, 40 dogs in Hiroshima, 57 dogs in Yamaguchi, 167 dogs in Tokushima, 40 dogs in Kochi, 40 dogs in Fukuoka, 34 dogs in Nagasaki, 40 dogs in Kumamoto, 30 dogs in Oita, 40 dogs in Miyazaki, and 8 dogs in Kagoshima). All animals in the study were routinely active outdoors. Clinical status was determined by the veterinarians treating these animals. Blood samples from each animal were collected in EDTA tubes for DNA extraction, and both blood and sera were stored at −20°C until transfer to the Obihiro University of Agriculture and Veterinary Medicine. DNA was

extracted from each blood sample and evaluated for *Rickettsia* infection using the nested PCR protocol described above. Each serum sample was also examined for the presence of antibodies against *R. japonica* using the IFA method. The samples that were positive for antibodies against *R. japonica* were also examined for antibodies against other domestic *Rickettsia* species, including *Rickettsia helvetica* strain IP-1, *Rickettsia tamurae* strain AT-1, and *Rickettsia asiatica* strain IO-1. The method was exactly the same as that described above.

RESULTS

Changes in the body temperatures and conditions of dogs inoculated with *R. japonica* are shown in Fig. 1. A temperature above 40.0°C was temporally recorded in the afternoon on the day of inoculation in dogs pretreated with cyclosporine (dog 1, 40.2°C; dog 2, 40.5°C). Body temperatures returned to normal by the next morning (day 1), but anorexia and depression appeared in both dogs on the afternoon of day 1. The body temperature of dog 1 increased again to 39.7°C in the afternoon on day 1 and remained higher than 39.5°C until the morning on day 4. Dog 2 also exhibited a temperature of 40.2°C from the morning on day 2 until the morning on day 3. Both dogs exhibited severe anorexia and depression and less water consumption on days 2 and 3; however, their appetites and water consumption improved by the morning of day 4 and had returned to normal by the morning of day 5. Animals that did not receive cyclosporine pretreatment (dogs 3 and 4) exhibited no clinical signs throughout the 14-day observation period. The control dog was also clinically normal and had a normal rectal temperature throughout the study period.

Figure 2 shows changes in white blood cell counts. On day 2, increased white blood cell counts were observed in dogs 1 (27,000/μl) and 2 (18,600/μl). Dog 1 also had an increased white blood cell count (17,300/μl) on day 10. Blood smears revealed that this increased white blood cell count was due to an increase in neutrophils (data not shown). Dog 4 showed a
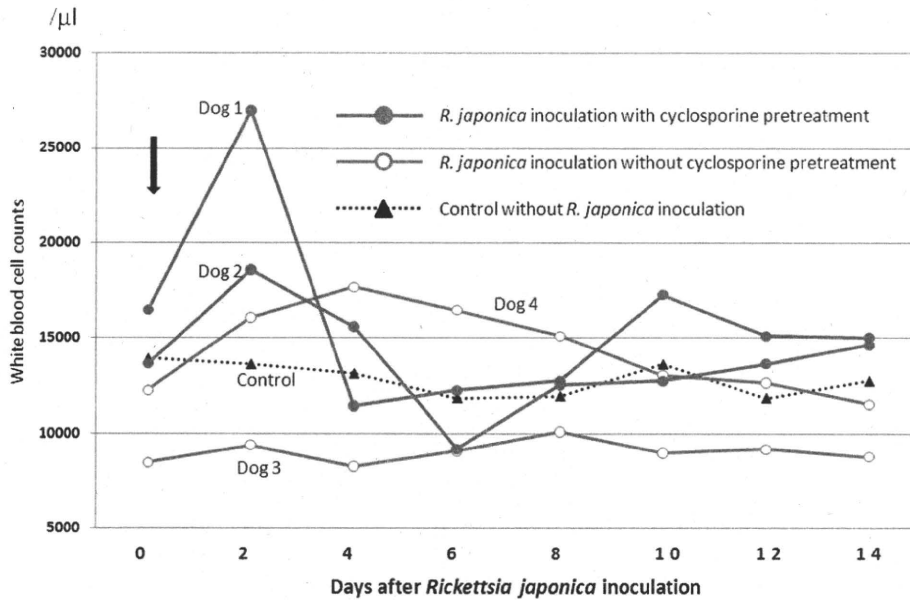
FIG. 2. Changes in white blood cell counts in peripheral blood. Increased counts were observed in both dog 1 (27,000/μl) and dog 2 (18,600/μl) on day 2. Dog 1 also exhibited an increased white blood cell count (17,300/μl) on day 10, Dog 4 showed a slight increase in white blood cell count on day 4 (18,000/μl).

slight increase in white blood cell count on day 4 (18,000/μl). Thrombocytopenia with a platelet count of less than 200,000/μl was not observed in any of the dogs examined (Fig. 3).

We attempted to detect rickettsial DNA from peripheral blood and aspiration samples of kidney and spleen using nested PCR, but all samples were negative.

None of the dogs used in this experiment showed any detectable antibody (titer < 1:20) to *R. japonica* antigen on day 0, just before the inoculation. On the final day of the study (day 14), positive antibodies against *R. japonica* were detected in all four inoculated dogs, with titers being 1:160 (dogs 1, 2, and 3) and 1:80 (dog 4), while the control dog lacked detectable antibody to *R. japonica*.

Among the 1,363 dogs examined, 24 (1.8%) exhibited antibodies against *R. japonica* with titers of 1:40 or more. The profiles of the positive animals are shown in Table 1. Among
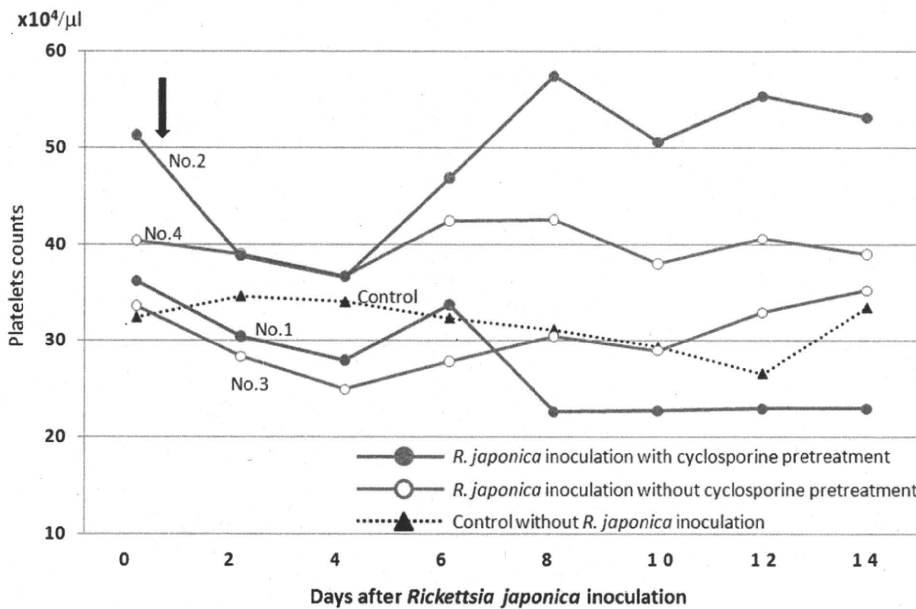

FIG. 3. Changes in platelet counts in peripheral blood. Thrombocytopenia with a platelet count of less than 200,000/μl was not observed in any of the dogs examined.

TABLE 1. Profiles of dogs that reacted with *R. japonica* antigens with titers of 1:40 or more[a]

Location and dog identifier	IFA titer				Prefecture	Age (yr)	Breed	Sex	Present illness
	R. japonica	*R. helvetica*	*R. asiatica*	*Rickettsia tamurae*					
Eastern Japan									
D1	**40**	<40	<40	<40	Miyagi	10	Mix	♀	None
D2	**40**	**40**	<40	**40**	Tochigi	4	G. retriever	♂	None
D3	**40**	**80**	<40	<40	Tochigi	NR	Mix	♀	None
D4	**80**	**80**	**80**	**80**	Tochigi	4	G. retriever	♂	None
D5	**40**	<40	<40	<40	Kanagawa	10	Shiba	♂	None
Western Japan									
D6	**40**	<40	**80**	<40	Wakayama	7	G. retriever	♀	Babesiasis
D7	**40**	<40	<40	<40	Wakayama	2	Mix	♂	None
D8	**40**	<40	<40	<40	Wakayama	10	Mix	♀	None
D9	**40**	<40	<40	<40	Osaka	10	L. retriever	♀	None
D10	**40**	<40	<40	<40	Hyogo	2	Mix	♂	None
D11	**160**	**80**	**40**	**40**	Hiroshima	12	G. retriever	♂	None
D12	**40**	<40	**40**	<40	Hiroshima	10	L. retriever	♂	None
D13	**40**	<40	**40**	<40	Hiroshima	8	Akita	♀	None
D14	**80**	<40	**40**	**40**	Hiroshima	3	Mix	♀	None
D15	**320**	**80**	**160**	**80**	Hiroshima	7	Mix	♂	None
D16	**160**	<40	**80**	<40	Hiroshima	4	Plot hound	♂	None
D17	**40**	<40	<40	<40	Yamaguchi	2	Min. pincher	♀	Kennel cough
D18	**40**	<40	<40	<40	Tokushima	8	G. retriever	♀	Otitis externa
D19	**80**	**40**	<40	<40	Kochi	5	Min. dach.	♀	None
D20	**40**	<40	<40	<40	Nagasaki	NR	NR	NR	NR
D21	**40**	<40	<40	<40	Kumamoto	6	Mix	♀	None
D22	**80**	<40	<40	<40	Miyazaki	NR	NR	NR	NR
D23	**40**	**40**	<40	<40	Miyazaki	NR	NR	NR	NR
D24	**40**	<40	<40	<40	Miyazaki	NR	NR	NR	NR

[a] G. retriever, golden retriever; L. retriever, Labrador retriever; Min. pincher, miniature pincher; Min. dach., miniature dachshund; NR, not recorded. Boldface numbers indicate titers of 40 or greater.

these 24 positive dogs, 5 (20.8%) lived in prefectures in eastern Japan (1 dog, Miyagi; 3 dogs, Tochigi; and 1 dog, Kanagawa Prefecture) and the other 19 (79.2%) were in prefectures in western Japan (3 dogs, Wakayama; 1 dog, Osaka; 1 dog, Hyogo; 6 dogs, Hiroshima; 1 dog, Yamaguchi; 1 dog, Tokushima; 1 dog, Kochi; 1 dog, Nagasaki; 1 dog, Kumamoto; and 3 dogs, Miyazaki). The ages of the positive dogs ranged from 2 to 12 years. The endpoint titers against *R. japonica* of the positive animals ranged from 1:40 to 1:320. Among the 24 dogs seropositive for antibodies against *R. japonica*, 12 dogs also had antibodies against one or more of the other *Rickettsia* antigens. A total of 15 dogs showed the highest titers against *R. japonica*, 1 dog (Tochigi-7) showed the highest titer against *R. helvetica*, and 8 other dogs showed the same titers against two or more antigens. These dogs did not show any clinical signs at the time of sample collection. Nested PCR did not reveal rickettsial infection for any of the 1,363 dogs examined.

DISCUSSION

JSF is the most important spotted fever in Japan. JSF onset in humans occurs 2 to 10 days after a person has worked in the fields and is abrupt. Common symptoms include headache, high fever, and shaking chills. Other major objective signs of JSF in humans include skin eruptions and tick bite eschar (13). Although some domestic and wild animals are suspected to be involved in JSF epidemiology, little is known about JSF reservoir animals. The dog is one potential reservoir animal (14), but the epidemiologic role of dogs with regard to JSF is un-

known, and the pathogenesis of the agent in a canine host has never been examined. Therefore, the present study examined the pathogenesis of *R. japonica* in dogs and the reservoir potential of dogs for *R. japonica* through experimental inoculation and by conducting an epidemiologic survey.

In our inoculation experiment, the two dogs pretreated daily with an immunosuppressive dose of cyclosporine 14 days before inoculation (dogs 1 and 2) became ill after exposure to *R. japonica*. These two dogs exhibited increased body temperatures in the afternoon on the day of inoculation. This could have been a reaction to pathogen inoculation, but it was observed only in dogs 1 and 2. The exact reason for the temporary increase in body temperatures is unknown. Clinical signs in dogs 1 and 2 included fever, anorexia, depression, and decreased water consumption, all of which were observed between 36 and 96 h after inoculation. Increased white blood cell counts, likely as a reaction against the infection, were also recorded on day 2 in dogs 1 and 2. Clinical signs disappeared from these dogs without any treatment. These clinical signs were similar to those observed in canine cases of Rocky Mountain spotted fever (RMSF); however, *R. rickettsii* infection causes more severe disease in dogs. When dogs infected with *R. rickettsii* develop clinical illness, a fever usually develops 2 to 3 days postinfection, while cutaneous lesions consisting of vesicles and/or petechial and ecchymotic hemorrhage develops 4 to 6 days postinfection (10, 12). Other clinical signs of RMSF in affected dogs not receiving any pretreatment include depression, listlessness, anorexia, ocular and nasal discharge, scleral

injection, and increased bronchovesicular lung sounds (2, 4, 5, 17). Skin and vascular lesions were not observed in the present study.

No clinical signs were observed in the *Rickettsia*-inoculated dogs without pretreatment with cyclosporine (dogs 3 and 4). A recent study reported that cyclosporine inhibits lymphocyte activation and expression of cytokine mRNA in dogs (11). These immunosuppressive effects of cyclosporine might affect the susceptibility of dogs to *R. japonica*. Cyclosporine has been widely used to suppress transplant rejection and control pruritus in allergic dermatitis in both humans and dogs. An oral dose of 5 mg/kg of body weight once a day is the most popular dosage administered to atopic dogs (20). For the present experiment, dogs were administered a similar cyclosporine dose of 50 mg per 8 to 11 kg body weight. Thus, a similar situation could occur in dogs with allergic dermatitis that are treated with cyclosporine in areas where *R. japonica* is endemic. Veterinarians should note that *R. japonica* can cause clinical disease in immunosuppressed domestic dogs. It is possible that aged dogs might be more susceptible to the infection of *R. japonica* because of the age-related loss of immune capacity.

Although fever, anorexia, and depression were observed in *R. japonica*-inoculated dogs pretreated with cyclosporine, these symptoms disappeared spontaneously by 5 days after inoculation, and no clinical signs were detected in dogs without cyclosporine pretreatment. These results suggest that *R. japonica* pathogenesis in dogs is much weaker than that of *R. rickettsii* reported previously (3, 17). Notably, the inoculum amount and the infection route differed between the studies; we subcutaneously inoculated each dog with approximately 2×10^7 infected L929 cells suspended in MEM, which was a much greater inoculum than that administered in the previous study of *R. rickettsii*. The latter study intraperitoneally inoculated dogs without any pretreatment with only 3,000 Vero cells infected by *R. rickettsii* suspended in 1 ml of sucrose-phosphate-glutamate buffer, and this caused clinical signs in the dogs (17). Our dogs were inoculated with greater numbers of infected cells, because there are no data available which indicate an optimal amount of pathogen. A quantitative study of experimental inoculation and inoculation route would be useful to this end. It is also possible that inoculation of cell culture-derived rickettsia might differ from that by the tick transmission route with respect to the capacity to cause rickettsemia or disease. The role of tick infestation for rickettsia transmission should be examined in the future study.

A previous study of RMSF observed rickettsemia lasting from 3 to 7 days when dogs were infected by *R. rickettsii* via intraperitoneal injection with a yolk sac suspension or via parasitism of infected *Dermacentor andersoni* ticks (16). This indicates that the dog can serve as a reservoir animal for RMSF. However, as the present study found no rickettsial DNA in peripheral blood or from the aspiration samples from kidney and spleen, dogs may not be suitable reservoir animals for *R. japonica*.

The present study took an epidemiologic approach to evaluate the pathogenesis of *R. japonica* in dogs as well as the reservoir potential of dogs for *R. japonica*. Of the 1,363 dogs examined, 24 (1.8%) had antibodies against *R. japonica* with titers of 1:40 or more. We obtained similar results in a previous study, in that we detected antibodies in 20 of 1,207 dogs ex-

amined (21). These results suggest that dogs in Japan can be exposed to *R. japonica* and produce antibodies against the pathogen under normal conditions. Despite this, none of these dogs from either study had rickettsial DNA in their peripheral blood. As such, we conclude that dogs in Japan have been exposed to this pathogen in the field but do not serve as efficient reservoir animals for *R. japonica*. Moreover, infected dogs, especially those with immunosuppressive conditions, can temporarily develop clinical signs, including fever, anorexia, and depression.

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Short Communication

Molecular Survey of Rickettsial Agents in Feral Raccoons (*Procyon lotor*) in Hokkaido, Japan

Mariko Sashika[1,2], Go Abe[3], Kotaro Matsumoto[1], and Hisashi Inokuma[1]*

[1]Department of Clinical Veterinary Science, Obihiro University of Agriculture and Veterinary Medicine, Obihiro 080-8555; [2]United Graduate School of Veterinary Sciences, Gifu University, Gifu 501-1193; and [3]Wildlife Management Research Center, Hyogo 669-3842, Japan

SUMMARY: Rickettsial infection in feral raccoons (*Procyon lotor*) in Hokkaido, Japan was analyzed by molecular methods. Genus-specific nested polymerase chain reaction (PCR) analysis based on the *Rickettsia* citrate synthase (*gltA*) gene showed that 13 of 699 raccoons (1.9%) examined were positive for *Rickettsia*. Twelve of the 13 partial *gltA* sequence amplicons were successfully analyzed. The nucleotide sequence of one amplicon was identical to both *Rickettsia heilongjiangensis* and *R. japonica*, one was identical to *R. felis*, and the rest to *R. helvetica*. This is the first report on the detection of rickettsial agents in peripheral blood of raccoons.

The raccoon is a medium-sized carnivore native to North America; however, a large number of raccoons have been imported from North America as pets since the 1970s (1). Subsequently, large numbers of raccoons have naturalized in many parts of Japan due to the intentional release or escape of pet raccoons (2). Feral raccoons cause heavy damage to crops and native ecosystems in Japan. Furthermore, raccoons may have introduced nonindigenous pathogens into Japan, such as rabies, raccoon ascarid, and rickettsiosis (3), which have been reported in the United States. Because of these problems, the Hokkaido Government initiated a feral raccoon management program in 1999.

Spotted fever group (SFG) *Rickettsia* is a significant emerging infectious disease whose principal clinical features are fever and rash. In Japan, the first case of rickettsiosis caused by *Rickettsia japonica* was reported in Tokushima Prefecture in 1984 (4). Other SFG *Rickettsia* spp. have recently been detected in Japan, including *Rickettsia helvetica* (5), *R. tamurae* (6), *R. asiatica* (7), and *R. tarasevichiae* (8); however, little epidemiologic data is available (i.e., vectors and reservoir animals). We therefore aimed to characterize rickettsial pathogens by a molecular analysis of peripheral blood samples obtained from feral raccoons in Japan. Another objective of this study was to clarify the epidemiologic role of raccoons for these pathogens in Japan.

A total of 699 raccoons were captured between May and October 2007 and between March and October 2008 as part of raccoon population control programs implemented by the Hokkaido Government and the Ministry of the Environment in west-central Hokkaido, the northernmost of the main islands of Japan. Blood samples were collected from 699 raccoons, and DNA was extracted with a QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany). DNA samples were stored at $-20°C$ in 200 µl of Tris-EDTA (TE) buffer until further use.

Nested PCR was performed with genus-specific primers for the rickettsial citrate synthase (*gltA*) gene (9), and the primer pair RpCS.877p and RpCS.1273r was used for the first amplification. The first round of PCR was carried out in a 25-µl reaction mixture (5 µl of DNA template) under the following conditions: 35 cycles of denaturation at 95°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 90 s. The resulting PCR products were then used as a template for the second amplification with primers RpCS.896f and RpCS.1258n. Cycling conditions for the second round of PCR were the same as the first round, except that annealing was carried out at 56°C for 30 s. DNA extracted from the *Rickettsia* AT-1 strain was used as a positive control, and distilled water was used for the blank control. PCR products were purified with the Qiaquick PCR purification kit (Qiagen) and sequenced as described previously (9). Sequence homology searches of the PCR products were performed with the Basic Local Alignment Search Tool (BLAST; National Center for Biotechnology Information).

According to genus-specific nested PCR analysis, 13 of 699 (1.9%) raccoons were positive for *Rickettsia*. In 12 samples, approximately 322 bp of the *gltA* gene, excluding the primer region, was successfully sequenced. These sequences have been deposited in GenBank under accession numbers HM049647 to HM049658. One amplicon showed 100% nucleotide identity with both *R. japonica* (AY743327) and *R. heilongjiangensis* (AY285776), and another was identical to *R. felis* (U33922). The remaining amplicons were identical to the *gltA* gene of *R. helvetica* (AM418450).

To distinguish between *R. japonica* and *R. heilongjiangensis*, species-specific PCR for the *R. japonica* 17-kDa antigen gene was performed with the primers Rj5 and Rj10 (10). Given the negative result, a conclu-

*Corresponding author: Mailing address: Obihiro University of Agriculture and Veterinary Medicine, Inada, Obihiro 080-8555, Japan. Tel & Fax: +81-155-49-5370, E-mail: inokuma@obihiro.ac.jp

sive determination of whether the amplicons were sequences of *R. japonica* or *R. heilongjiangensis* could not be made. Because sequences of *R. heilongjiangensis* and *R. japonica* are very similar (11), other gene sequences will need to be examined in order to determine which species the samples belong to. The introduction of *R. heilongjiangensis* into Hokkaido from Russia is a possibility given that they are close in proximity and that *R. heilongjiangensis* infection is prevalent in the Russian Far East (12). Further studies will be needed to clarify the relationship between *R. heilongjiangensis* and raccoons.

R. felis was first detected in the United States in 1990 (13) and has since spread throughout the world (14). Although the cat flea, *Ctenocephalides felis*, is currently the only known biological vector of *R. felis* (14), *R. felis* or *R. felis*-like DNA has been detected in several tick species, including those in Japan (15). More epidemiological studies will be needed to confirm the identity of the *R. felis* vector.

Although *R. helvetica* was previously known to exist only in European countries (16), the pathogen has become widespread in Japan, from Hokkaido to the southern island of Kyushu (17). *Ixodes persulcatus* and *Ixodes ovatus* are possible vectors of *R. helvetica* in Japan (5). Feral raccoons in Hokkaido have been infected with *I. ovatus* and *I. persulcatus* (18); therefore, it is logical that *R. helvetica* DNA was detected in peripheral blood of raccoons in Hokkaido.

All rickettsial species detected in the present study, *R. heilongjiangensis* or *R. japonica*, *R. felis*, and *R. helvetica*, are pathogenic to humans (11,14,19). Because raccoons frequently approach areas where humans live, these *Rickettsia* spp. can infect humans via the tick vector introduced by raccoons. More epidemiologic studies are required to confirm the epidemiologic role of raccoons in *Rickettsia* infection.

In conclusion, this is the first report on the detection of SFG *Rickettsia* from peripheral blood of raccoons. Our results indicate that raccoons may be a reservoir for SFG *Rickettsia*.

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Short Communication

Detection of *Anaplasma bovis* DNA in the Peripheral Blood of Domestic Dogs in Japan

Leo Sakamoto[1], Yasuaki Ichikawa[1], Yoshimi Sakata[1], Kotaro Matsumoto, and Hisashi Inokuma*

Department of Clinical Veterinary Science, Obihiro University of Agriculture and Veterinary Medicine, Obihiro 080-8555; and
[1]Merial Japan Ltd., Tokyo 100-0014, Japan

SUMMARY: The prevalence of *Ehrlichia* and *Anaplasma* in 1,427 dogs from 32 Japanese prefectures was evaluated by PCR and DNA nucleotide sequencing. PCR screening demonstrated that 18 dogs (1.3%) were positive for *Anaplasmataceae*. Sequence analysis revealed that 14 of the amplicons were most closely related to *Wolbachia* spp., symbionts of *Dirofilaria immitis*, whereas three were identified as *Anaplasma bovis*. The remaining amplicon could not be sequenced. Almost the entire sequence of 16S rRNA (1,452 bp) from one of the positive specimens was determined, and subsequent phylogenetic analysis confirmed that the detected sequence was that of *A. bovis*. This is the first detection of *A. bovis* DNA fragments in dogs. Species-specific nested PCR showed that 15 (1.1%) of the 1,427 dogs involved in this study were positive for *A. bovis*. The geographical distribution of these dogs ranged from Aomori Prefecture in northern Japan to Kagoshima Prefecture in the south. The relationship between *A. bovis* infection and clinical disease is not yet clearly understood.

Members of the family *Anaplasmataceae* belong to the order *Rickettsiales* and comprise the genera *Ehrlichia*, *Anaplasma*, *Neorickettsia*, *Aegyptianella*, *Wolbachia*, and '*Candidatus* Neoehrlichia', which are obligate intracellular Gram-negative bacteria (1,2). *Anaplasma* and *Ehrlichia* are important emerging tick-borne pathogens in both humans and animals (3). The organisms of greatest clinical importance in dogs include *Ehrlichia canis*, *Anaplasma platys*, and *Anaplasma phagocytophilum*. These bacteria are distributed worldwide, and molecular evidence of these pathogens has been detected in Japan. DNA fragments of *E. canis* were detected in a dog from Kagoshima Prefecture in southern Japan (4), although the only confirmed case of *E. canis* infection occurred in a dog that had been transferred from Indonesia (5). *A. platys* DNA has been detected in dogs in Okinawa and Yamaguchi Prefectures (6–8) and in ticks recovered from Fukushima, Miyazaki, and Kagoshima Prefectures (9). Recently, DNA fragments from *A. phagocytophilum* were detected in deer, cattle, and ticks in Japan (10–13), although not in dogs. There have been no case reports of domestic dogs in Japan infected with these pathogens.

Several new species of *Ehrlichia* and *Anaplasma* have been reported recently in Japan, including *Ehrlichia muris* (14), *Ehrlichia* sp. isolated from *Ixodes ovatus* (15), '*Candidatus* Ehrlichia shimanensis' (11), and *Anaplasma bovis* (11,16). These species may cause infection in dogs, although the pathogenicity of these organisms in these animals is unknown. Positive PCR results for *Ehrlichia* sp. isolated from *I. ovatus* have

been reported in Yamaguchi Prefecture (7), although no epidemiological studies of *Anaplasma* and *Ehrlichia* infection in dogs have been conducted at a national level in Japan. For this reason, we evaluated the prevalence of *Ehrlichia* and *Anaplasma* in dogs from most regions in Japan using PCR and DNA nucleotide sequencing methods. We also undertook the molecular characterization of the *A. bovis* detected in dogs in this study.

Blood samples were collected from 1,427 domestic dogs between January 2006 and June 2008 and processed in animal hospitals from 32 prefectures (Hokkaido, Aomori, Miyagi, Fukushima, Tochigi, Ibaraki, Tokyo, Chiba, Kanagawa, Ishikawa, Yamanashi, Shizuoka, Aichi, Mie, Osaka, Kyoto, Shiga, Nara, Wakayama, Hyogo, Tottori, Shimane, Hiroshima, Yamaguchi, Tokushima, Kochi, Fukuoka, Nagasaki, Kumamoto, Oita, Miyazaki, and Kagoshima). All of the animals involved in this study were routinely active outdoors. The clinical status and epidemiological information, including breed, sex, age, tick infestation, and clinical history, were collected by the veterinarians treating these animals. Blood samples were collected from each animal in EDTA tubes for subsequent DNA extraction, and then stored at $-20°C$ until transfer to Obihiro University of Agriculture and Veterinary Medicine. DNA was extracted from the EDTA blood samples using the QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany). DNA samples were stored at $-20°C$ in 200 μl of TE buffer until further analysis.

Screening PCR was performed using the group-specific primer pair EHR16SD and EHR16SR, which amplifies the 16S rRNA gene of the family *Anaplasmataceae* (17). When a strongly positive band was detected after PCR, the products were purified using the QIAquick PCR purification kit (Qiagen). Direct sequencing of PCR products and analysis of the sequences obtained were performed as described previously (9).

*Corresponding author: Mailing address: Obihiro University of Agriculture and Veterinary Medicine, Inada, Obihiro 080-8555, Hokkaido, Japan. Tel & Fax: +81-155-49-5370, E-mail: inokuma@obihiro.ac.jp

Homology searches based on the sequences of the PCR products were performed using BLAST (National Center for Biotechnology Information).

Additional PCR amplifications using the primer sets fD1 and EHR16SR, or EHR16SD and Rp2, were performed to determine nearly full-length sequences of the 16S rRNA genes of some positive samples (18). Distance matrix calculations and construction of phylogenetic trees were performed using the ClustalW program (version 1.8) in the DNA data bank of Japan (DDBJ; Mishima, Japan [http://www.ddbj.nig.ac.jp/htmls/E-mail/clustalw-e.html]). Distance matrices for the aligned sequences, with all gaps ignored, were calculated using the Kimura two-parameter method, and the neighbor-joining method was used to construct a phylogenetic tree. The stability of this tree was estimated by bootstrap analysis for 100 replications using the same program. The tree figure was generated using the Tree View program (version 1.6.6). The GenBank accession numbers of the 16S rRNA gene sequences used to construct phylogenetic trees and to analyze percent identities were as follows: A. bovis strain South Africa, U03775; A. bovis detected from sika deer in Shimane, Japan, AB211163; A. bovis detected from Haemaphysalis longicornis in Nara, Japan, AB196475; A. bovis detected from cattle in Okinawa, Japan, EU368730-EU368732; A. phagocytophilum strain Webster, U02521; A. phagocytophilum strain CAHU-HGE2, AF093789; A. ovis, AF414870; A. platys, AY077619; A. marginale, AF309867; A. centrale, AF283007; and E. canis, M73221.

Eighteen (1.3%) of the 1,427 samples examined by screening PCR were clearly positive for Anaplasmataceae. The partial 16S rRNA gene-sequences of these positive-PCR products were determined using 345 bp, excluding the primer region. BLAST analysis revealed that 14 samples from Shimane, Kochi, Fukuoka, Oita, Nagasaki, Kumamoto, and Miyazaki were most closely related to Wolbachia spp., whereas three samples from Hiroshima and Fukuoka were closely related to A. bovis. We were unable to determine the sequence of the one remaining positive sample.

As Wolbachia spp. are known to be symbionts of Dirofilaria immitis (heartworm) (19), we speculated that the positive-PCR results might be related to D. immitis infection. A previous study also detected DNA fragments of Wolbachia spp. from dogs in Okinawa, Japan (4). Dogs that were positive for Wolbachia spp. lived in western Japan, which is an endemic area for filarial nematodes. The 14 dogs which tested positive for Wolbachia spp. in our study were also found to be positive for D. immitis infection using an enzyme immunoassay kit (IDEXX canine snap 4D test; IDEXX Laboratories, Westbrook, Maine, USA).

Determination of nearly full-length sequences of the 16S rRNA genes of A. bovis positive samples was attempted using additional PCR. For one sample (Hiroshima-Z27), we successfully sequenced 1,452 bp of the 16S rRNA gene, excluding the primer region. This sequence has been deposited in GenBank under the accession no. HM131217. The sequence demonstrated a nucleotide identity of 99.5% with the 16S rRNA gene of A. bovis detected in South Africa (U03775) and 99.4% with A. bovis from cattle in Okinawa, Japan (EU368731) and from deer in Japan (AB196475). These sequences clustered in the same clade as A. bovis in the 16S rRNA gene-based phylogenetic tree (Fig. 1). This is the first detection of A. bovis DNA fragments in dogs, although such DNA has recently been isolated from ticks, cattle, and deer in Japan (11-13).

A species-specific nested PCR was performed using the primer sets EC9 and EC12A for the first amplification and AB1f and AB1r for the second amplification to determine the percentage of dogs infected with A. bovis. This method has been described previously (11). This nested-PCR evaluation suggested that 15 dogs (1.1%)

83 — A. bovis from cattle in Okinawa (EU368731)
52 — A. bovis from cattle in Okinawa (EU368732)
28 — A. bovis from deer in Shimane (AB211163)
A. bovis from tick NR07 in Nara (AB196475)
89
A. bovis from cattle in Okinawa (EU368730)
100
Anaplasma sp. Hiroshima-dog Z27
99
61 — A. bovis from South Africa (U03775)
100 | A. phagocytophilum CAHU-HGE2 (AF093789)
64 | A. phagocytophilum Webstar (U02521)
A. platys (AY077619)
99 — A. marginale (AF309867)
A. ovis (AF414870)
99
A. centrale (AF283007)
E. canis (M73221)

0.01

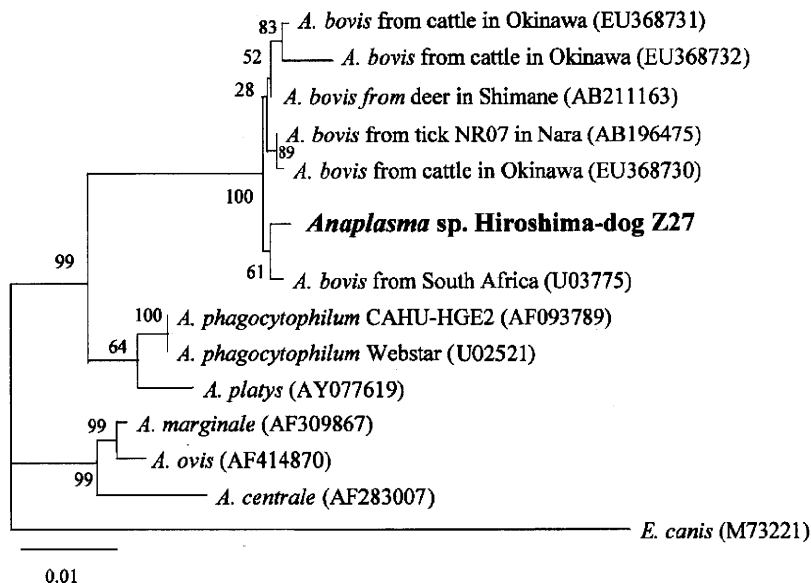Fig. 1. Phylogenetic relationships of various Anaplasma spp. based on nucleotide sequences of the 16S rRNA gene. Scale bar indicates genetic distance (0.01 substitutions/site). The Anaplasma sp. detected in this study is shown in bold type.

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