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リスザルにおける *Pasteurella multocida* 莢膜型 F 型による甚急性パスツレラ症の 1 例

森口真理子，伊東隆臣，加藤行男，岡谷友三アレシャンドレ，宇根有美

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特集

人と動物の共通感染症最前線　10

リスザルにおけるPasteurella multocida莢膜型F型による甚急性パスツレラ症の1例

森口真理子[*1]　伊東隆臣[*2]　加藤行男[*3]　岡谷友三アレシャンドレ[*3]　宇根有美[*1]

要　約

約10頭のリスザルを飼育する施設で，若齢の個体が左大腿部に咬傷を受け，翌日に死亡した。死亡個体の咬傷部位および実質臓器からは *Pasteurella multocida* 莢膜型F型が分離された。また，同様の莢膜型を示す *P. multocida* が同居リスザルの口腔内からも分離された。莢膜型F型は家禽コレラ事例から稀に分離される莢膜型で，今回，初めてサルへの高病原性が確認された。

はじめに

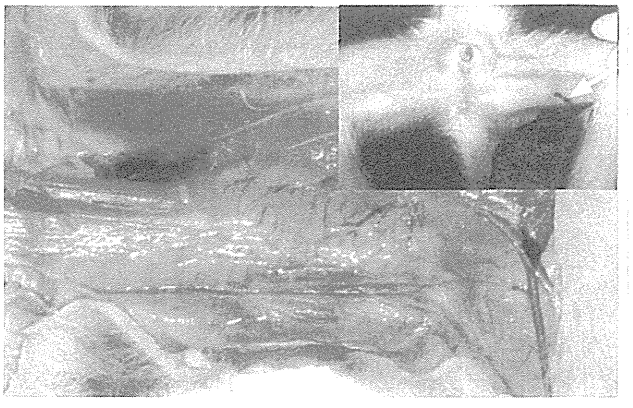
パスツレラ症は，*Pasteurella* 属菌を原因菌とする日和見感染的要素の強い感染症で，その代表的菌種である *P. multocida* は牛の出血性敗血症や家禽コレラ[3]などを引き起こす。また，犬や猫を健康保菌者とし，これらの動物の咬傷や引っ掻き傷が原因となり人にパスツレラ症を起こす。サル類では，気嚢炎のヒヒ[1, 2]や敗血症のリスザル[2]から *P. multocida* が分離された症例報告があるのみで，サル類に対する *P. multocida* の病原性について不明な点が多い。

発生状況

約10頭のリスザルを飼育する施設での発生である。新規導入個体と以前より飼育している個体を同居させた。その翌日の昼頃に突然，1歳10か月の雄ザルが跛行し始めた。第2病日，状態悪化し，白血球数減少（1400/μL）と電解質異常がみられたため，輸液・投薬したが死亡したので，病理学的および微生物学的に検索した。また，本施設では，本例死亡の3か月前から下顎部腫脹を呈する個体が観察され，その数が4頭に達しており，うち1頭が死亡した。さらに，複数回の口腔スワブの細菌検査で全頭から *P. multocida* 莢膜型F型が分離された。

結　果

剖検時，左側大腿部全体が高度に腫脹および発赤し，大腿筋が硬直していた。大腿部内側（腫脹部中央部）に，サルの歯型と見做される弧を描くように分布する小型の裂傷3か所が認められた。同部には顕著な蜂窩織炎があり，皮膚の水腫性肥厚，混濁する滲出液の滲出，半膜様筋の変性・壊死が観察された（図1）。脾腫は軽度で，濾胞・脾材不明瞭であった。肝臓は赤褐色を呈し，一部脆弱であった。また，左大腿部皮下織水腫部スワブ塗抹標本に多数の単～

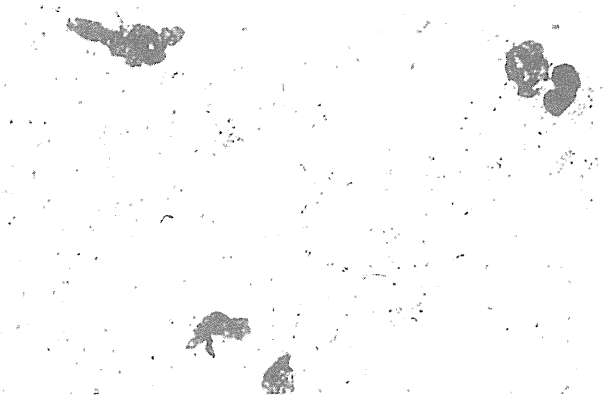


広範な筋変性，皮下水腫および血管怒張がみられた。
挿入図：左大腿部の咬傷部（矢印）。

図1　左大腿部の咬傷部皮下組織

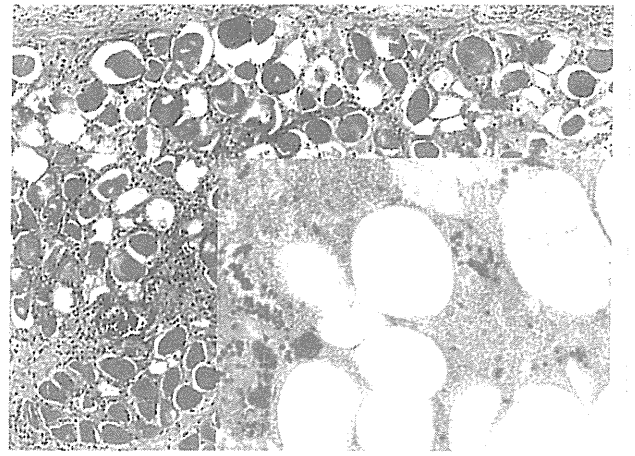
[*1] Mariko MORIGUCHI（写真左・コメント）& Yumi UNE（写真右）：麻布大学獣医学部病理学研究室　〒252-5201 神奈川県相模原市中央区淵野辺 1-17-71
[*2] Takaomi ITO：大阪ウォーターフロント開発株式会社海遊館
[*3] Yukio KATO & Alexandre T. OKATANI：麻布大学獣医学部公衆衛生第二研究室

検索に至らぬ点も多々ありましたが，貴重な症例に遭遇し，多くのことを学ぶことができました。今回の発表に当たり，ご指導いただきました宇根先生，加藤先生，岡谷先生をはじめ，ご協力いただいた工藤先生および病理学研究室の皆様にこの場を借りて感謝申し上げます。



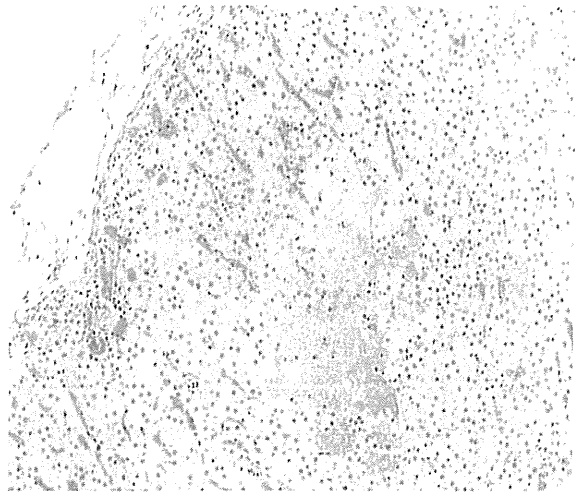
多数の単～短連鎖した球桿菌と少数の好中球が存在。
ギムザ染色。

図２　左大腿部皮下スワブ



高度の筋壊死と軽度の細胞浸潤を伴う細菌増殖。HE 染色。
挿入図：左大腿部筋肉。トルイジンブルー染色。

図３　左大腿部筋肉



HE 染色。

図４　副腎皮質にみられた細菌塊

短連鎖した球桿菌と少数の好中球がみられた（図 2）。組織学的に左大腿部蜂窩織炎部にはおびただしい細菌が観察され（図 3），同様の細菌は副腎，リンパ節にもみられた（図 4）。蜂窩織炎部，肝臓と脾臓から *P. multocida* 莢膜型 F が分離・培養された。

まとめ

以上の所見より，本例は左大腿部創傷から侵入した *P. multocida* による敗血症性ショックで死亡したものと推察した。パスツレラ症の病態は血清型，特に莢膜型に関連するとされ，草食獣の出血敗血症型は，発症後数時間から 2 日で急死する高致死率の病型で，莢膜型 B と E が分離される。本例の病態はこれと一致した。また，莢膜型 F 型は，家禽コレラで分離されるマイナーな莢膜型で，今回の検索により，初めてサルへの高病原性が確認された。

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人と動物の共通感染症最前線　10

愛玩用輸入小型哺乳動物における *Salmonella* の分離

三村有里恵[*1]　菅沼琴世[*1]　畑中律敏[*1]　岡谷友三アレシャンドレ[*1]　宇根有美[*2]　田向健一[*3]　加藤行男[*1]

要　約

2003年から2012年に愛玩用として輸入された直後の小型哺乳動物30種701頭およびペットとして飼育されているフクロモモンガ65頭から*Salmonella*の分離を行った。*Salmonella*は，輸入小型哺乳動物11種77頭（11.1％）から分離され，その血清型には人の胃腸炎患者から高頻度に分離される血清型が含まれ，多剤耐性を示す株もあり，輸入小型哺乳動物は*Salmonella*のキャリアーとして重要な役割を果たしているものと思われた。一方，飼育フクロモモンガの*Salmonella*の分離率（4.6％）は，輸入フクロモモンガの分離率（52.3％）より有意に低く，飼育中にその分離率が低下するものと思われた。

はじめに

近年のペットブームにより，様々な動物が輸入され，愛玩用として飼育されている。2005年9月に「感染症法」の改正が行われたことにより，全ての輸入哺乳動物に対して衛生証明書の添付が義務付けられた。しかし，この法律では一部の感染症のみが規制対象であり，*Salmonella*感染症を含むその他多くの感染症については対象外であるため，これら感染症の病原体保有状況は不明である。そこで本研究では，愛玩用として輸入された輸入小型哺乳動物および国内で飼育されている飼育フクロモモンガにおける*Salmonella*の保有状況を調査した。

調査方法

供試材料として2003年から2012年にかけて愛玩用として輸入された直後の小型哺乳動物30種701頭および国内でペットとして飼育されているフクロモモンガ65頭の腸管内容物あるいは糞便を用いた。*Salmonella*は，定法に従い分離・同定を行った。分離された*Salmonella*は，血清型別を行うとともに，11種類（アンピシリン，セファロチン，セファゾリン，シプロフロキサシン，クラブラン酸アモキシシリン，ゲンタマイシン，カナマイシン，ナリジクス酸，ストレプトマイシン，スルファメトキサゾール・トリメトプリム，テトラサイクリン）の薬剤を用いてディスク拡散法により，薬剤感受性試験を行った。

調査成績

愛玩用として輸入された小型哺乳動物のうち11種77検体（11.1％）より*Salmonella*が分離された。ネズミ目では9種38検体(6.0％)，カンガルー目では32検体(53.3％)，ハリネズミ目では7検体（70.0％）から*Salmonella*が分離された。分離された*Salmonella*の血清型は*S.* Enteritidis，*S.* Typhimurium，*S.* Litchfield，*S.* Newportなど人の胃腸炎患者から高頻度に分離される血清型を含む計22血清型に型別された（表1）。薬剤感受性試験の結果，耐性を示したのはフクロモモンガとミミナガハリネズミから分離された株で，全て多剤耐性であった（表2）。

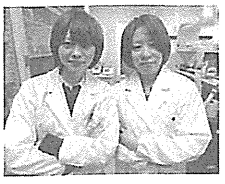
飼育フクロモモンガ65検体中3検体（4.6％）より*Salmonella*が分離された（表3）。飼育フクロモモンガからの*Salmonella*の分離率は，表1に示した輸入フクロモモンガからの分離率（53.3％）と比べ有意に低かった。

以上より，輸入小型哺乳動物は*Salmonella*のキャリ

[*1] Yurie MIMURA（写真左，コメント），Kotoyo SUGANUMA（写真右），Noritoshi HATANAKA，Yukio KATO & Alexandre T. OKATANI：麻布大学獣医学部獣医学科公衆衛生学第二研究室　〒229-8501 相模原市淵野辺 1-17-71

[*2] Yumi UNE：麻布大学獣医学部獣医学科病理学研究室

[*3] Kenichi TAMUKAI：田園調布動物病院　〒145-0071 東京都大田区田園調布 2-1-3



諸先生方，ならびに公衆衛生学第二研究室の皆様のお力添えにより何とか形にすることができました。ご指導，ご協力下さった皆様に，深く感謝申し上げます。

表1　輸入小型哺乳動物における *Salmonella* の分離状況

動物目	検体数	陽性検体数（%）	血清型
ネズミ目 (6科28種)	631	38（ 6.0）	Enteritidis, Bredeney, Bovismorbificans, Derby, Stanley, Welterverden, Anatam, UT*
カンガルー目 (フクロモモンガ)	60	32（53.3）	Bardo, Litchfield, Telelkebir, Paratyphi B, Poona, Stanley, Newport, Typhimurium, Starleyville, Agona, Brazzavile, Virchow, Handen, UT*
ハリネズミ目 (ミミナガハリネズミ)	10	7（70.0）	Kentuckey
合計	701	77（11.0）	22血清型

UT*：市販血清で型別不能

表2　輸入小型哺乳動物から分離された *Salmonella* の薬剤耐性

動物種	検体数	耐性株保有検体数（%）	血清型（検体数）	薬剤耐性
フクロモモンガ	60	7（11.7）	*S.* Paratyphi B（4）	AMPC, SM
			S. Poona（3）	AMPC, ST, TC
ミミナガハリネズミ	10	7（70.0）	*S.* Kentuckey（5）	CPFX, GM, NA, SM, ST, TC
			S. Kentuckey（2）	CPFX, GM, KM, NA, TC

表3　飼育フクロモモンガにおける *Salmonella* の分離状況

動物目	検体数	陽性検体数（%）	血清型
飼育フクロモモンガ	65	3（4.6）	*S.* Agona, UT*

UT*：市販血清で型別不能

アーとして重要な役割を果たし，人への感染源となる可能性があり，今後もその保有状況を監視するとともに，これらの動物の取り扱いには十分注意する必要があるものと思われた。一方，飼育フクロモモンガの *Salmonella* の分離率（4.6%）は，輸入フクロモモンガの分離率（52.3%）より有意に低く，飼育中にその分離率が低下するものと思われた。

サル類における重症例を含む美麗食道虫症の集団発生

山中寛子，東由季子，若林　伸，平　健介，堀　浩，宇根有美

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特集

人と動物の共通感染症最前線　10

サル類における重症例を含む美麗食道虫症の集団発生

山中寛子[*1]　東由季子[*2]　若林　伸[*1]　平　健介[*3]　堀　浩[*2]　宇根有美[*1]

要　約

集団飼育されているエリマキキツネザル数頭に流涎が認められ，うち1頭が嚥下障害により死亡し，同施設のリスザル群においても流涎が観察された。死亡個体の舌および食道に多数の線虫寄生が認められ，その形態から美麗食道虫と同定された。本寄生虫は，反芻類等を終宿主として食道に寄生し，サルでは古くから舌に感染する線虫として知られており，ともに通常は機能障害を起こさない。世界各地で50数例の人体寄生例が報告されている。本例は，美麗食道虫の濃厚感染により舌を含む口腔粘膜の顕著な過形成，角化亢進により嚥下障害を生じ，低栄養状態となり死亡したサルの初めての事例である。

はじめに

美麗食道虫 *Gongylonema pulchrum* は，羊，牛などの反芻類や豚を含む動物の食道に寄生する線虫で，1850年にLeidyらによってアメリカ合衆国で初めて人体寄生が報告された[1]。甲虫類やゴキブリを中間宿主とし，人への感染は偶発的に中間宿主を摂食することによる。人体寄生例は現在までに50数例の報告があり，アメリカ合衆国，旧ソビエト連邦，中国，ブルガリアなど世界中で発生している。今回サルで致死例を含む美麗食道虫の集団発生例を経験したので報告する。

発生状況

集団飼育されているエリマキキツネザル（*Varecia variegate* spp.）5頭のうち1頭が高度の流涎および舌苔形成を示して死亡した。同居の4頭にも流涎が認められ，そのうちの1頭が嚥下障害を生じ，飢餓状態で4か月後に死亡した。また，同施設のリスザル（*Saimiri sciureus*）群においても流涎が複数個体に観察された。

結　果

死亡したエリマキキツネザルの舌は全体的に顕著に腫大し，表面全体に凹凸があり，高度の舌苔形成を伴い黒色を帯びていた。舌のみならず頬部および咽頭部粘膜も肥厚し，表面粗造で，舌尖～舌根，口角周囲の口粘膜一面に細線維状の線虫の寄生が認められた。食道においても全長にわたり，粘膜および外膜にも同様の線虫が寄生していた（図1）。組織学的には，舌を含めた口腔粘膜上皮の顕著な角化亢進，過形成と上皮内線虫寄生と虫卵が観察され，種々の程度に炎症細胞が浸潤していた（図2）。病変部より採取した線虫の形態から美麗食道虫と同定された。なお，胃には寄生虫はみられなかった。リスザルでは，肉眼的に舌に著変はみられなかったが，組織学的には舌粘膜内にキツネザルと同様の線虫の感染を認めた。また，同施設の他の種類のサルの舌表面を掻把し，鏡検した結果，流涎のみられたサルはもとより，臨床症状の明らかでないサルにおいても美麗食道虫の虫卵が検出された。その後，駆虫薬投与で臨床症状（流涎）は消失し，プレパレントピリオド3か月後の舌スワブ，糞便検査でも虫卵は検出されなかった。

[*1] Hiroko YAMANAKA（写真・コメント），Sin WAKABAYASHI & Yumi UNE：麻布大学獣医学部病理学研究室　〒229-8501　神奈川県相模原市淵野辺1-17-71

[*2] Yukiko HIGASHI & Hiroshi HORI：那須ワールドモンキーパーク　〒325-0001 栃木県那須郡那須町高久甲6146

[*3] Kensuke TAIRA：麻布大学獣医学部寄生虫学研究室

自分の行った発表が雑誌に掲載されることが未だに信じられませんが，本症例を通して貴重な経験をさせて頂き，自分自身の成長に繋げることができました。終始ご指導頂きました宇根先生を始め，研究に協力して下さった平先生，那須ワールドモンキーパークおよび病理学研究室の皆様に，深く感謝しております。

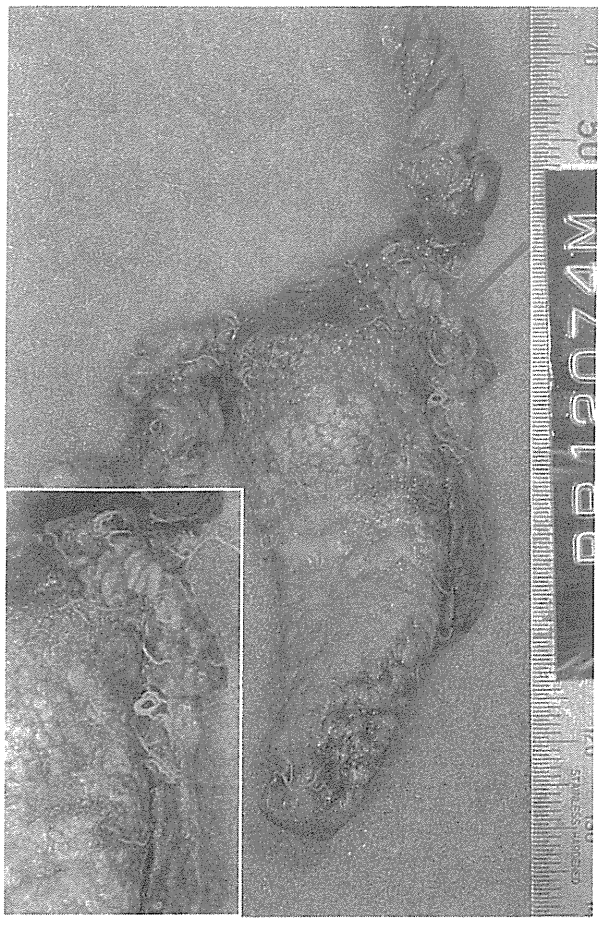

図1　ホルマリン固定後の舌全体
舌は全体に腫大・硬化し，舌苔の形成が高度である。線虫の寄生（矢印）が認められた。
挿入図：舌根部の拡大。多数の美麗食道虫が見られた。

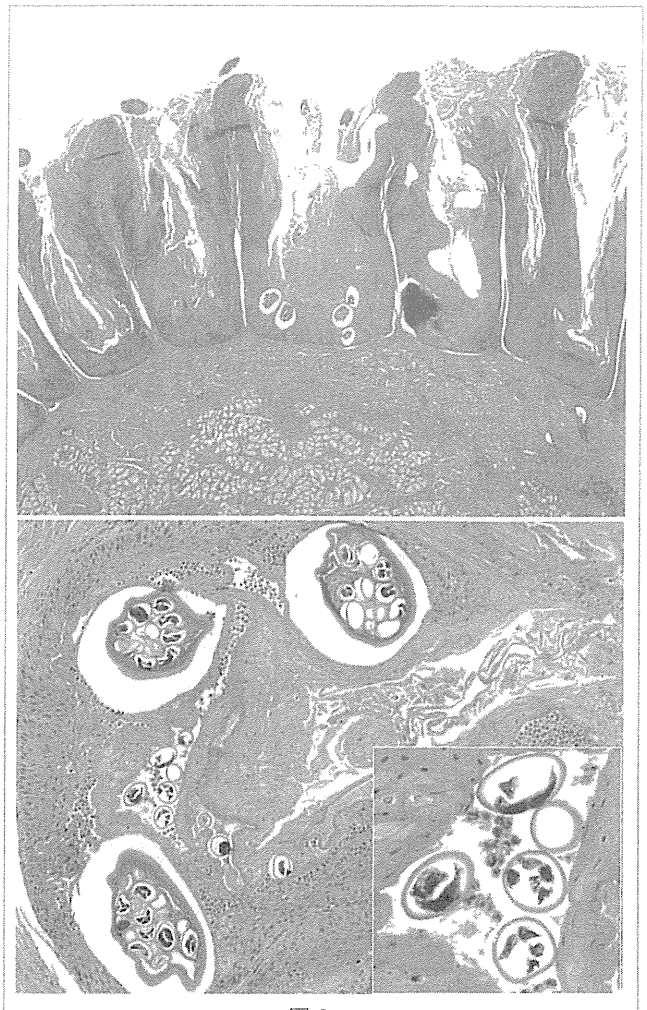

図2
上：エリマキキツネザルの舌粘膜，HE 染色。粘膜上皮の過形成と角化亢進による粘膜の顕著な肥厚。
下：エリマキキツネザルの舌粘膜内の成虫と虫卵，HE 染色。粘膜内に線虫寄生と虫卵が観察された。挿入図：虫卵拡大図。

まとめ

以上のことから，サル類における美麗食道虫症の集団発生と診断した。死亡例では，美麗食道虫の濃厚感染により舌を含む口腔粘膜に広範かつ高度の過形成性反応が生じたため，嚥下障害を招来し，死に至ったと推察した。通常，美麗食道虫は動物に感染した場合，食道粘膜内に止まり，臨床徴候の発現や機能障害を起こすことはない。しかし，人では口内炎，舌麻痺などの症状が報告されており，今回の事例と酷似していた。重篤化したサル類の美麗食道虫症は稀で，さらに死亡例の報告はない。よって，宿主の免疫状態や種による感受性を含めて，重篤化の機構を解析することで，人体寄生における病原性の検討や治療に貢献できるものと考える。最後に，偶発的要素が多いとはいえ，美麗食道虫は，人獣共通寄生虫病であることから，中間宿主の駆除および終宿主への駆虫の励行により感染環を断つことが重要である。

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DISEASE IN WILDLIFE OR EXOTIC SPECIES

Outbreak of Yersiniosis in Egyptian Rousette Bats (*Rousettus aegyptiacus*) Caused by *Yersinia pseudotuberculosis* Serotype 4b

S. Nakamura*, S. Settai*, H. Hayashidani†, T. Urabe‡, S. Namai‡ and Y. Une*

** Laboratory of Veterinary Pathology, School of Veterinary Medicine, Azabu University, Fuchinobe 1-17-71, Chuo-ku, Sagamihara, Kanagawa 252-5201, † Institute of Symbiotic Science and Technology, Tokyo University of Agriculture and Technology and ‡ Tobu Zoo, Japan*

Summary

This report describes an outbreak of yersiniosis in Egyptian rousette bats (*Rousettus aegyptiacus*) caused by *Yersinia pseudotuberculosis* serotype 4b. Twelve of 61 bats died between November and December 2008 or in May 2009. The bats often displayed multiple yellow–white nodules in the spleen and liver. Microscopically, these consisted of focal necrosis accompanied by inflammatory cell infiltration and colonies of gram-negative bacilli. The bacterial colonies were identified immunohistochemically as *Y. pseudotuberculosis* O4 and *Y. pseudotuberculosis* serotype 4b was identified by bacteriological examination. Polymerase chain reaction demonstrated that the isolate harboured the virulence genes *virF*, *inv* and *ypmA*. YPMa is as a superantigenic toxin that is associated with acute systemic infection in man and may contribute to the virulence of *Y. pseudotuberculosis* in bats.

Keywords: Egyptian rousette bat; superantigen; *Yersinia pseudotuberculosis*; zoonosis

Yersinia pseudotuberculosis is an important causal agent of zoonosis with global distribution (Fukushima *et al.*, 2001). Infection with *Y. pseudotuberculosis* is typically acquired orally via the ingestion of food and water contaminated with the faeces of carriers such as free-living rodents and birds (Schiemann, 1989; Han *et al.*, 2003). Yersiniosis due to *Y. pseudotuberculosis* is clinically manifested as enteritis, mesenteric lymphadenitis and occasionally septicaemia, and occurs in man and a wide variety of animals (Mair, 1973). Yersiniosis has caused significant mass mortality in zoological parks amongst a variety of animals (Allchurch, 2003). *Y. pseudotuberculosis* has been classified into serotypes O1–O15 based on expression of the Oantigen, and seven pathogenic serotypes are recognized (O1–O6 and O10; Nagano *et al.*, 1997). A new pathogenic serotype (O7) was isolated from a Bolivian squirrel monkey (*Saimiri boliviensis*) (Nakamura *et al.*, 2009). The pathogenicity of *Y. pseudotuberculosis* is associated with several virulence factors that are encoded on a 70 kb virulence plasmid (pYV) and include *Yersinia* adhesin A (YadA) and *Yersinia* outer membrane proteins (Yops) (Cornelis *et al.*, 1998). Additionally, a chromosomal high-pathogenicity island (HPI) encodes an iron-uptake system characterized by the siderophore yersiniabactin (Schubert *et al.*, 2004), the superantigenic toxin *Y. pseudotuberculosis*-derived mitogen (YPM) (Abe *et al.*, 1997) and invasin, which allows efficient entry into mammalian cells (Grassl *et al.*, 2003) and plays an important role in systemic infection. YPM-producing strains can be separated into three clusters based on the production of the YPMa, YPMb or YPMc variants of YPM (Carnoy *et al.*, 2002). Among these strains, those that produce YPMa display superantigenic activity and high pathogenicity (Carnoy *et al.*, 2000).

There are few reports of yersiniosis in zoo animals and little data on the serotypes and virulence

Correspondence to: Y. Une (e-mail: une@azabu-u.ac.jp).

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characteristics of *Y. pseudotuberculosis* isolated from these species.

Between November 28th and December 5th 2008, nine of 61 Egyptian rousette bats housed in a zoological park in the Kanto region of Japan died consecutively without obvious clinical signs. Additionally, three bats died suddenly over 6 days in May 2009. Complete necropsy examinations were performed on the 12 bats and tissues from the last animal (number 12) were subjected to histopathological examination. Samples from this bat were fixed in 10% neutral buffered formalin and embedded in paraffin wax. Sections were stained with hematoxylin and eosin (HE) and Gram's stain. Immunohistochemistry (IHC) was performed using a commercially available set of rabbit antisera specific for the *Y. pseudotuberculosis* serotypes O1, O2, O3, O4, O5 and O6 (Denka-Seiken Co., Tokyo, Japan) and a set of antisera specific for the *Yersinia enterocolitica* serotypes O1–2, O3, O5, O8 and O9 (Denka-Seiken). Bacteriological examination of the liver, spleen and lung was performed for bat 12 as described by Iwata *et al.* (2008). Additionally, polymerase chain reaction (PCR) examination of the bacterial isolates for the detection of the virulence genes *virF*, *inv*, *ypm* (*ypmA*, *ypmB* and *ypmC*) and *irp2* was performed using six sets of primers as described by Iwata *et al.* (2008). The *virF*, *inv* and *irp2* genes were used as markers for the presence of pYV, invasin and HPI, respectively (Table 1).

At necropsy examination, all of the bats displayed enlargement of the spleen and liver, which contained multiple yellow–white nodules (Fig. 1), and fine white, nodules were observed in the lung. No irregularities were seen in the intestine or lymph nodes, including the mesenteric lymph nodes. Histopathologically, the nodules observed in the liver and spleen consisted of focal necrosis accompanied by inflammatory cell infiltration, including neutrophils and macrophages, with numerous bacterial colonies (Fig. 2). The bacterial colonies were composed of gram-negative bacilli. Intracapillary bacterial colonies were detected in the lung and kidney. Additionally, small bacterial colonies associated with small foci of necrosis were seen in the femoral bone marrow. There were no microscopical changes in the intestine or mesenteric lymph nodes. The bacterial colonies were immunolabelled for *Y. pseudotuberculosis* O4 only (Fig. 3). On bacteriological examination only *Y. pseudotuberculosis* serotype 4b was isolated and PCR analysis demonstrated that the isolate had *virF*, *inv* and *ypmA* genes, but did not possess *ypmB*, *ypmC* or *irp2* (Fig. 4).

Similar gross lesions have been reported in animals infected with *Y. pseudotuberculosis* (Hubbert, 1972; Baskin *et al.*, 1977), and on the basis of the microbiological and immunohistochemical findings, the deaths of these bats were interpreted to relate to sepsis associated with *Y. pseudotuberculosis* serotype 4b infection.

PCR analyses revealed that the *Y. pseudotuberculosis* isolate possessed *virF*, *inv* and *ypmA* genes. The *virF* gene is a marker for the presence of the virulence plasmid pYV, which encodes several critical pathogenic factors, including YadA, which is involved in adhesion to host cells and autoagglutination, and 11 secreted Yop proteins, which play important roles in avoiding the host immune response by affecting the function of phagocytes (Cornelis *et al.*, 1998). Invasin is involved in adhesion to host intestinal epithelial cells (Grassl *et al.*, 2003), while YPMa encoded by the *ypmA* gene functions as a superantigenic toxin. YPMa is considered to be the virulence factor associated with a variety of the clinical signs observed in human patients

Table 1
Primers for PCR detection of virulence genes from *Y. pseudotuberculosis*

Virulence factor	*Target gene*	*Sequence (5′–3′)*	*Annealing temperature (°C)*	*Size of product (base pairs)*
pYV	*virF*	TCATGGCAGAACAGCAGTCAG ACTCATCTTACCATTAAGAAG	55	590
Invasin	*inv*	TAAGGGTACTATCGCGGCGGA CGTGAAATTAACCGTCACACT	55	295
YPMa	*ypmA*	CACTTTTCTCTGGAGTAGCG GATGTTTCAGAGCTATTGTT	55	350
YPMb	*ypmB*	TTTCTGTCATTACTGACATTA CCTCTTTCCATCCATCTCTTA	52	453
YPMc	*ypmA* and *ypmC**	ACACTTTTCTCTGGAGTAGCG ACAGGACATTTCGTCA	49	418
HPI	*irp2*	AAGGATTCGCTGTTACCGGAC TCGTCGGGCAGCGTTTCTTCT	55	280

*Since *ypmC*-specific primers have not been described, both *ypmA* and *ypmC* were detected for evaluation of *ypmC*. An isolate can be presumed to be positive for *ypmC* if it is negative for *ypmA* and positive for *ypmA* and *ypmC*.

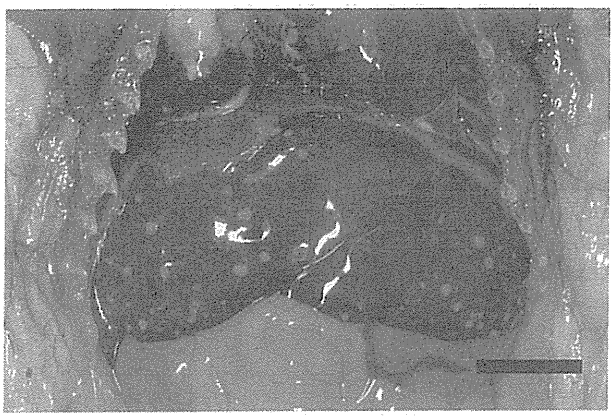

Fig. 1. Multiple yellow–white nodules in the liver. Bar, 1 cm.

including fever, scarlatiniform rash, diarrhea, vomiting and arthritis (Uchiyama *et al.*, 1993). YPMa-harbouring *Y. pseudotuberculosis* is considered to be highly virulent and capable of inducing the above clinical signs in addition to sepsis, while other *Y. pseudotuberculosis* strains have low virulence and result in signs confined to gastroenteritis (Fukushima *et al.*, 2001). HPI-positive serotypes do not typically harbour YPMa and are considered to have lower virulence than YPMa-positive strains (Fukushima *et al.*, 2001). As pointed out above, pathogenic *Yersinia* spp. have numerous virulence factors, but the relationship between these virulence factors and pathological findings have not been investigated extensively.

Y. pseudotuberculosis is able to survive for long periods of time in soil and water, even in cool environments, and the contamination of food and water can be a potential source of infection (Schiemann, 1989; Han *et al.*, 2003). Additionally, wild rodents and birds are considered to be carriers of *Y. pseudotuberculosis* (Mair, 1973; Fukushima *et al.*, 1988). In non-

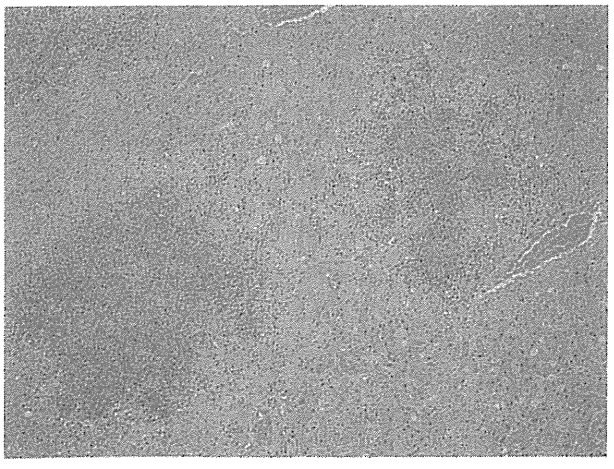

Fig. 2. Multifocal necrosis with numerous bacterial colonies in the liver. HE. ×100.

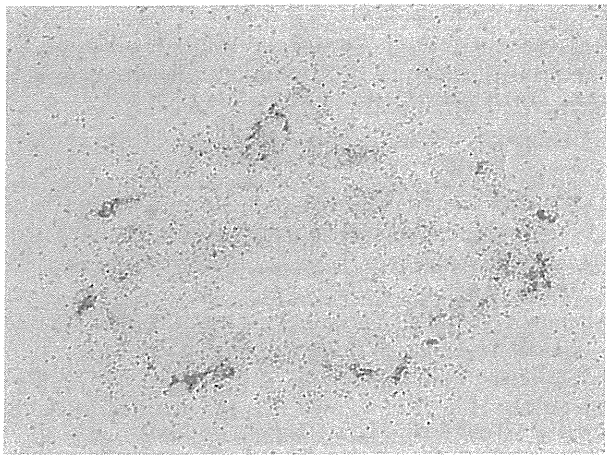

Fig. 3. Bacterial colonies in the liver immunolabelled for *Y. pseudotuberculosis* O4. IHC. ×200.

human primates, large quantities of *Yersinia* spp. are shed in faeces and can spread rapidly directly or indirectly to other animals (Baggs *et al.*, 1976). For these reasons, the possibility of faeces-mediated transmission among chiropteran species cannot be ruled out. Furthermore, it is possible that asymptomatic liver, spleen or lung carriage for long periods followed by stress may have triggered the development of yersiniosis.

To our knowledge, this is only the third report of yersiniosis in chiropteran species (Childs-Sanford *et al.*, 2009; Muhldorfer *et al.*, 2010). However, detailed investigations of the causative *Yersinia* strains by pathological and bacteriological examinations were not performed in the two

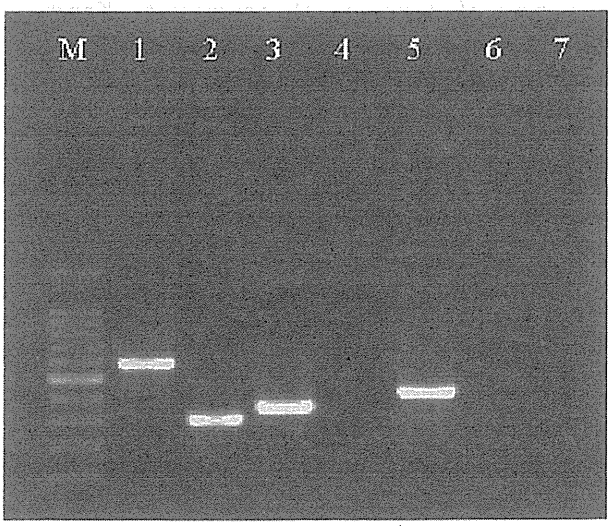

Fig. 4. PCR for detection of virulence genes of the *Y. pseudotuberculosis* 4b isolate. Lane: M, molecular weight markers (100 base pair ladder); 1, *virF*; 2, *inv*; 3, *ypmA*; 4, *ypmB*; 5, *ypmA* and *ypmC*; 6, *irp2*; 7, negative control.

previous reports. From the public health point of view and to help resolve the mechanisms of infection by bacterial pathogens, additional studies of yersiniosis are needed in animals.

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Novel Bat-borne Hantavirus, Vietnam

To the Editor: Compelling evidence of genetically distinct hantaviruses (family *Bunyaviridae*) in multiple species of shrews and moles (order Soricomorpha, families Soricidae and Talpidae) across 4 continents (*1–7*) suggests that soricomorphs, rather than rodents (order Rodentia, families *Muridae* and *Cricetidae*), might be the primordial hosts (*6,7*). Recently, the host range of hantaviruses has been further expanded by the discovery that insectivorous bats (order Chiroptera) also serve as reservoirs (*8,9*). Conjecturing that Mouyassué virus in the banana pipistrelle (*Neoromicia nanus*) in Côte d'Ivoire (*8*) and Magboi virus (MGBV) in the hairy split-faced bat (*Nycteris hispida*) in Sierra Leone (*9*) represent a much broader geographic distribution of bat-borne hantaviruses, we analyzed tissues from bats captured in Mongolia and Vietnam.

Total RNA was extracted from 51 lung tissues, collected in RNAlater Stabilization Reagent (QIAGEN, Valencia, CA, USA), from insectivorous bats, representing 7 genera and 12 species, captured in Mongolia and Vietnam. cDNA was then prepared by using PrimeScript II 1st strand cDNA Synthesis Kit (Takara Bio, Otsu, Shiga, Japan) for reverse transcription PCR (RT-PCR), and using oligonucleotide primers previously designed for amplification of soricid- and talpid-borne hantaviruses (*1–7*).

A novel hantavirus, designated Xuan Son virus (XSV), was detected in 1 of 5 Pomona roundleaf bats (*Hipposideros pomona*) by using a hemi-nested large (L)–segment primer set (outer: HNL-2111F, 5′-CARTCWACWGTIGGIGCIAGTGG-3′, and HAN-L-R1, 5′-AACCADTCWGTYCCRTCATC-3′; inner: HNL-2111F and HAN-L-R2, 5′-GCRTCRTCWGARTGRTGDGCAA-3′) and a nested small (S)–segment primer set (outer: OS-M55F, 5′-TAGTAGTAGACTCC-3′, and XSV-S6R, 5′-AGITCIGGRTCCATRTCRTCICC-3′; inner: Cro-2F, 5′-AGYCCIGTIATGRGWGTIRTYGG-3′, and JJUVS-1233R, 5′-TCACCMAGRTGRAAGTGRTCIAC-3. The bat was captured during July 2012 in Xuan Son National Park, a nature reserve in Thanh Sơn District, Phu Tho Province, ≈100 km west of Hanoi (21°07′26.75′′N, 104°57′29.98′′E).

For confirmation, RNA extraction and RT-PCR were performed independently in a laboratory in which hantaviruses had never been handled. After initial detection, the L-segment sequence was extended by using another primer set (PHL-173F: 5′-GATWAAGCATGAYTGGTCTGA-3′; and TNL-5084R: 5′-GATCCTGAARTACAATGTGCTGG-3′). To calculate the number of virus copies in tissues by real-time RT-PCR, we used a virus-specific primer set (XSV-F: 5′-GTTGCACAGCTTGGTATTGG-3′; and XSV-R: 5′-TTAGCACCCAAACCTCCAAG-3′) and probe (XSV-Probe: 5′-ACAGCTCCTGGCATGGTAAATTCTCC-3′).

Pairwise alignment and comparison (with ClustalW, www.clustal.org) of a 4,582-nt (1,527 aa) region of the RNA-dependent RNA polymerase–encoding L segment indicated sequence similarities of 71.4%–71.5% and 75.9%–78.7% at the nucleotide and amino acid levels, respectively, between XSV and Mouyassué virus and MGBV. Sequence analysis of a 499-nt (166 aa) region of the nucleocapsid-encoding S segment showed that XSV differed by 42.8%–58.3% from representative hantaviruses harbored by rodents and most soricomorphs. XSV sequences were identical in lung, liver, kidney, and spleen; and the highest number of virus copies (7.6×10^1) was in lung tissue, determined by real-time RT-PCR. No additional hantavirus-infected Pomona roundleaf bats were found by RT-PCR that used XSV-specific primers.

Phylogenetic analyses was performed with maximum-likelihood and Bayesian methods, and we used the GTR+I+Γ model of evolution, as selected by the hierarchical likelihood-ratio test in MrModel-test version 2.3 and jModelTest version 0.1 (*10*), partitioned by codon position. Results indicated 4 distinct phylogroups, with XSV sharing a common ancestry with MGBV (Figure). Similar topologies, supported by high bootstrap (>70%) and posterior node (>0.70) probabilities, were consistently derived when various algorithms and different taxa and combinations of taxa were used. Moreover, as we reported previously, the incongruence between some hantaviruses and their reservoir hosts might be indicative of host-switching events (*5–7*).

The striking sequence divergence of XSV presented considerable challenges for designing suitable primers for RT-PCR and sequencing. Also, sequencing efforts were constrained by the limited availability of tissues and concurrent virus isolation attempts. Consequently, we were unable to obtain the full-length sequence of XSV. Similarly, the inability to detect hantavirus RNA in tissues from other species of bats in this study might be attributed to several factors, including the highly focal nature of hantavirus infection, small sample sizes of bats of any given species, primer mismatches, and suboptimal cycling conditions.

Bats of the genus *Hipposideros,* family Hipposideridae, are among the most speciose insectivorous bats; ≈70 species are distributed across Africa, Europe, Asia, and Australia. Pomona roundleaf bats are frequently found in or near limestone or sandstone caves. Their colony sizes vary from few to many hundreds of individuals. The vast geographic distribution of the Pomona roundleaf bat throughout

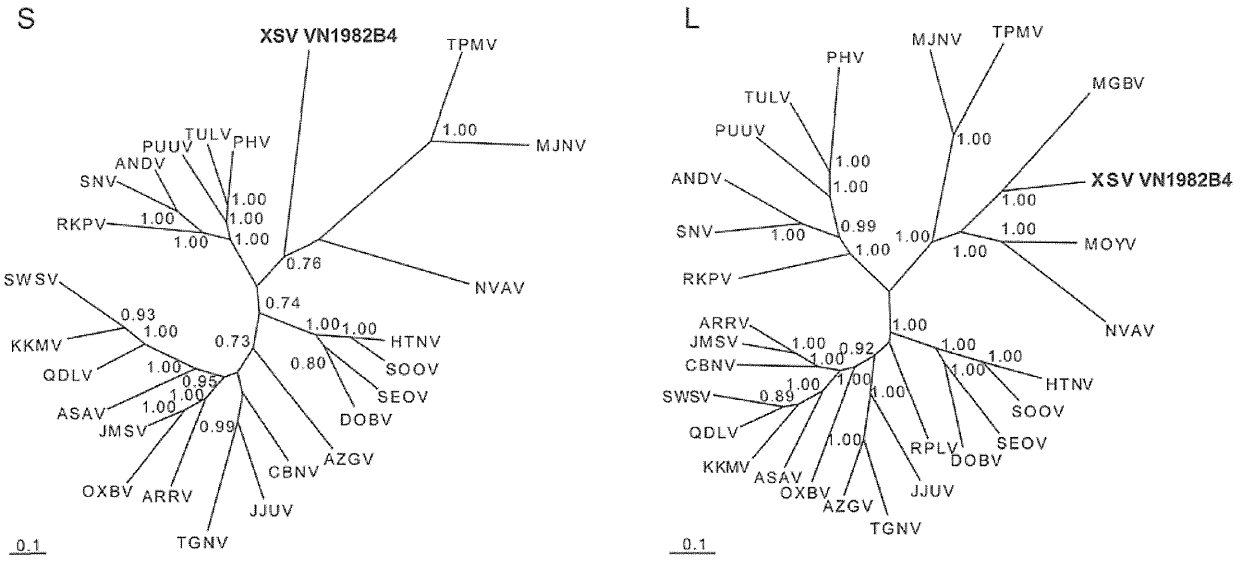

Figure. Phylogenetic trees, based on 499-nt and 4,582-nt regions of the small (S) and large (L) genomic segments, respectively, of Xuan Son virus (XSV VN1982B4) (GenBank accession nos. S: KC688335, L: JX912953), generated by the maximum-likelihood and Bayesian Markov chain Monte Carlo estimation methods, under the GTR+I+Γ model of evolution. Because tree topologies were similar when RAxML and MrBayes were used, the tree generated by MrBayes was displayed. The phylogenetic position of XSV is shown in relation to chiropteran-borne hantaviruses, Mouyassué virus ([MOYV] JQ287716) from the banana pipistrelle and Magboi virus ([MGBV] JN037851) from the hairy slit-faced bat. The taxonomic identity of the XSV-infected Pomona roundleaf bat was confirmed by mitochondrial DNA analysis (GenBank accession no. JX912954). The numbers at each node are Bayesian posterior probabilities (>0.7), and the scale bars indicate nucleotide substitutions per site. **Boldface** indicates the Xuan Son virus detected in Pomona roundleaf bat, Vietnam. Representative soricomorph-borne hantaviruses include Thottapalayam virus ([TPMV] AY526097, EU001330) from the Asian house shrew; Imjin virus ([MJNV] EF641804, EF641806) from the Ussuri white-toothed shrew; Jeju virus ([JJUV] HQ663933, HQ663935) from the Asian lesser white-toothed shrew; Tanganya virus ([TGNV] EF050455, EF050454) from the Therese's shrew; Azagny virus ([AZGV] JF276226, JF276228) from the West African pygmy shrew; Cao Bang virus ([CBNV] EF543524, EF543525) from the Chinese mole shrew; Ash River virus ([ARRV] EF650086, EF619961) from the masked shrew; Jemez Springs virus ([JMSV] FJ593499, FJ593501) from the dusky shrew; Seewis virus ([SWSV] EF636024, EF636026) from the Eurasian common shrew; Kenkeme virus ([KKMV] GQ306148, GQ306150) from the flat-skulled shrew; Qiandao Lake virus ([QDLV] GU566023, GU566021) from the stripe-backed shrew; Camp Ripley virus ([RPLV] EF540771) from the northern short-tailed shrew; Asama virus ([ASAV] EU929072, EU929078) from the Japanese shrew mole; Oxbow virus ([OXBV] FJ539166, FJ593497) from the American shrew mole; Rockport virus ([RKPV] HM015223, HM015221) from the eastern mole; and Nova virus ([NVAV] FJ539168, FJ593498) from the European common mole. Also shown are representative rodent-borne hantaviruses, including Hantaan virus ([HTNV] NC_005218, NC_005222), Soochong virus ([SOOV] AY675349, DQ562292), Dobrava-Belgrade virus ([DOBV] NC_005233, NC_005235), Seoul virus ([SEOV] NC_005236, NC_005238), Tula virus ([TULV] NC_005227, NC_005226), Puumala virus ([PUUV] NC_005224, NC_005225), Prospect Hill virus ([PHV] Z49098, EF646763), Andes virus ([ANDV] NC_003466, NC_003468), and Sin Nombre virus ([SNV] NC_005216, NC_005217).

Vietnam and in Bangladesh, Cambodia, China, India, Laos, Malaysia, Myanmar, Nepal, and Thailand, provides opportunities to ascertain the genetic diversity and phylogeography of XSV and XSV-related hantaviruses. In this regard, although hantavirus RNA was not detected in archival tissues from bats of ≈20 genera, including several other *Hipposideros* species (*8*,*9*), many more genetically divergent hantavirus species are probably harbored by insectivorous bats. Not all orphan viruses warrant intensive study at the time of their discovery. However, insights into the ecology and transmission dynamics of newfound bat-borne hantaviruses might prepare us to more rapidly diagnose future outbreaks caused by emerging hantaviruses.

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Satoru Arai,
Son Truong Nguyen,
Bazartseren Boldgiv,
Dai Fukui, Kazuko Araki,

Can Ngoc Dang, Satoshi D. Ohdachi, Nghia Xuan Nguyen, Tien Duc Pham, Bazartseren Boldbaatar, Hiroshi Satoh, Yasuhiro Yoshikawa, Shigeru Morikawa, Keiko Tanaka-Taya, Richard Yanagihara, and Kazunori Oishi

Author affiliations: National Institute of Infectious Diseases, Tokyo, Japan (S. Arai, K. Araki, H. Satoh, S. Morikawa, K. Tanaka-Taya, K. Oishi); Institute of Ecology and Biological Resources, Hanoi, Vietnam (S.T. Nguyen, C.N. Dang, N.X. Nguyen, T.D. Pham); National University of Mongolia, Ulaanbaatar, Mongolia (B. Boldgiv); National Institute of Biological Resources, Seoul, South Korea (D. Fukui); Hokkaido University, Sapporo, Japan (S.D. Ohdachi); Institute of Veterinary Medicine, Ulaanbaatar (B. Boldbaatar); Chiba Institute of Science, Chiba, Japan (Y. Yoshikawa); and University of Hawaii at Manoa, Honolulu, Hawaii, USA (R. Yanagihara)

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Address for correspondence: Satoru Arai, Infectious Disease Surveillance Center, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku, Tokyo 162-8640, Japan; email: arais@nih.go.jp

Possible Cause of Liver Failure in Patient with Dengue Shock Syndrome

To the Editor: We report a rare hepatic ultrasonograph finding for a patient with liver failure associated with dengue virus (DENV) infection. This finding might shed light on the pathogenesis of liver involvement in this disease.

In March 2006, a 10-year-old previously healthy boy was hospitalized for a 3-day history of fever, headache, and nausea/vomiting. Fever subsided on the day of admission, but the patient was in shock (blood pressure 80/40 mm Hg) and had gastrointestinal bleeding and hematuria. Physical examination showed an obese, confused patient with generalized petechiae and hepatomegaly. The initial diagnosis was dengue shock syndrome (DSS). The patient was intubated and received intravenous fluid infusion, packed red blood cells, ceftriaxone, sodium bicarbonate, and ranitidine before being transferred to King Chulalongkorn Memorial Hospital in Bangkok. The patient's blood pressure increased to 130/90 mm Hg after the initial fluid resuscitation (28 mL/kg free flow), and systolic pressure remained at ≈130 mm Hg until transfer.

Laboratory examinations found 14,930 leukocytes/mm^3, hemoglobin 16.4 g/dL, hematocrit 48.2%, platelet 18,000/mm^3, blood urea nitrogen 33 mg/dL, creatinine 1 mg/dL, sodium 128 mEq/L, potassium 6.2 mEq/L, chloride 91 mEq/L, total CO_2 5 mEq/L, total bilirubin 6.9 mg/dL, direct bilirubin 3.9 mg/dL, aspartate transaminase 3,507 IU/L, alanine transaminase 2,775 IU/L, prothrombin time 43 seconds (international normalized ratio 3.4), and partial thromboplastin time 93.5 s (control 28.7 s). Blood and urine cultures showed negative results. Serum was positive for IgM against DENV. Unfortunately, we did not investigate other viral causes of liver failure.

DSS with liver failure was diagnosed and treated with intravenous fluid, sodium bicarbonate, omeprazole, fresh frozen plasma, platelet transfusion, vitamin K, and recombinant factor VIIa concentrate (NovoSeven; Novo Nordisk, Bagsvaerd, Denmark). Despite stable blood pressure over the next 6 days, liver enzymes continued to rise with progressive jaundice (online Technical Appendix, wwwnc.cdc.gov/EID/article/19/7/12-1820-Techapp1.pdf). Hepatic ultrasonograph on the second

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SHORT COMMUNICATION

Molecular Phylogenetic Analysis of *Orientia tsutsugamushi* Based on the *groES* and *groEL* Genes

Satoru Arai,[1] Kenji Tabara,[2] Norishige Yamamoto,[3] Hiromi Fujita,[4] Asao Itagaki,[2] Marina Kon,[3] Hiroshi Satoh,[1] Kazuko Araki,[1] Keiko Tanaka-Taya,[1] Nobuhiro Takada,[5] Yasuhiro Yoshikawa,[6] Chiaki Ishihara,[7] Nobuhiko Okabe,[1,8] and Kazunori Oishi[1]

Abstract

DNA sequences encoding the GroES and GroEL proteins of *Orientia tsutsugamushi* were amplified by the PCR and sequenced. Pairwise alignment of full-length *groES* and *groEL* gene sequences indicated high sequence similarity (90.4–100% and 90.3–100%) in *O. tsutsugamushi*, suggesting that these genes are good candidates for the molecular diagnosis and phylogenetic analysis of scrub typhus. Comparisons of the 56-kD type-specific antigen (TSA) protein gene and the *groES* and *groEL* genes showed that genotypes based on the 56-kD TSA gene were not related to a cluster containing the *groES* and *groEL* genes in a dendrogram, suggesting that a gene rearrangement may be associated with homologous recombination in mites.

Key Words: *Orientia tsutsugamushi*—*groES* and *groEL* genes—Phylogeny—Scrub typhus—Japan.

Introduction

The bacterium *Orientia tsutsugamushi* belongs to the family Rickettsiaceae and is transmitted by trombiculid mites such as *Leptotrombidium pallidum*, *Leptotrombidium akamushi*, and *Leptotrombidium scutellare*. It is the causative agent of scrub typhus, an emerging zoonosis, spreading throughout central, southern, and eastern Asia, and Oceania (Kelly et al. 2009). In Korea, 17,451 scrub typhus cases, *i.e.*, 7.2 cases per 100,000 individuals, were reported from 2001 to 2005 (Bang et al. 2008). In Japan, 313–791 cases have been reported annually since 1999, when nationwide patient surveillance began. Less than ten fatal cases of scrub typhus have been reported in Japan each year, but the public health impact of scrub typhus has remained in endemic areas. The number of people that participate in outdoor activities in forests, mountains, and similar areas is increasing in Japan, which increases the risk of transmission. Recently, several research groups conducted field surveys to determine the prevalence of *O. tsutsugamushi* among wild rodents and mites (Ogawa and Ono 2008). The serological diversity (Tamura et al. 1984) and genotypic diversity (Tamura et al. 2001) of *O. tsutsugamushi* have also been examined. These studies suggest that many genotypes/serotypes of *O. tsutsugamushi* are distributed throughout Japan and that the risk of infection with scrub typhus remains high.

Phylogenetic and phylogeographic analyses of *O. tsutsugamushi* have typically been based on the gene encoding the 56-kD type-specific antigen (TSA) protein thus far. This protein is expressed on the outer membrane surface and is the primary immunogen in human infections that is responsible for eliciting neutralizing antibodies. In general, a 56-kD TSA-based indirect fluorescence antibody technique (IFAT) is used for the diagnosis and classification of serotypes. There is a high diversity of 56-kD TSA genotypes among *O. tsutsugamushi*, and consequently type-specific PCR based on the 56-kD TSA gene is conducted to identify different genotypes. However, genes encoding major immune antigens that are modulated by the immune response of the host, such as the 56-kD TSA gene, are usually not suitable for phylogenetic and phylogeographic analyses. Housekeeping genes, such as heat shock protein genes or the 16S ribosomal RNA (rRNA) gene,

[1]Infectious Disease Surveillance Center, National Institute of Infectious Diseases, Tokyo, Japan.
[2]The Shimane Prefectural Institute of Public Health and Environmental Science, Matsue, Shimane, Japan.
[3]The Saitama Prefectural Institute of Public Health, Saitama, Japan.
[4]Mahara Institute of Medical Acarology, Anan, Tokushima, Japan.
[5]University of Fukui, Eiheiji-cho, Fukui, Japan.
[6]Chiba Institute of Science, Choshi, Chiba, Japan.
[7]Rakuno Gakuen University, Ebetsu, Hokkaido, Japan.
[8]Kawasaki City Institute for Public Health, Kawasaki, Kanagawa, Japan.

are feasible candidates for such analyses because they are unaffected by the host immune response. The *groEL* gene, which encodes the 60-kD heat shock protein GroEL, is more diverse than the 16S rRNA gene. The sequences of the *groES* and *groEL* genes might be reconstructed more accurately with gene tree reconstructions, similar to 16S rRNA-based phylogeny. Thus, a comparison of the 56-kD TSA gene and the *groEL* or *groES* genes would help understand the molecular epidemiology of *O. tsutsugamushi.*

This study compared the entire *groES* and *groEL* gene sequences of six reference samples, *i.e.*, the Kaisei, Sato, Kato, Kuroki, Matsuzawa, and Shimokoshi strains, and 15 field strains of *O. tsutsugamushi.* Phylogenetic comparisons between the 56-kD TSA gene and the *groES* or *groEL* genes suggest that gene rearrangement may be associated with homologous recombination in mites.

Materials and Methods

Genomic DNA was extracted using a PureLink genomic DNA mini kit (Invitrogen, San Diego, CA) from six *O. tsutsugamushi* reference strains isolated from patients in Japan, *i.e.*, Kaisei, Sato, Kato, Kuroki, Matsuzawa, and Shimokoshi, and 15 field isolates from rodents in Japan, *i.e.*, UAP1, UAP2, UAP4, UAP6, UAP7, FAR1, FAR2, HSB1, HSB2, HSB3, KNP1, KNP2, CMM1, O2, and O3 from infected L929 cells (Fujita et al. 2000, Tamura et al. 2001). DNA samples from two wild rodents in Japan (SH216 and 05-5) and two patients in Shimane, Japan (TD-7 and TD-17) were used for amplification (Tabara et al. 2012). Additionally, three DNA samples, SH205, SH234, and SH245, from rodents that were captured in Japan were also used for amplification. The gene encoding the *O. tsutsugamushi* 56-kD TSA protein was amplified from DNA samples by PCR using the primer set described by Furuya et al. (1993). To identify long sequences, 1722–1880 bp fragments were obtained using the primers Ot56-M329F (5′-CTAAGGTTAAYTGTARCRAGGTGC-3′) and Ot56-Rev1512R (5′-TCCACATACACACCTTCAGCAGCA-3′). In addition to the 56-kD TSA gene, the *groES* and *groEL* genes were also amplified. PCR was performed using oligonucleotide primers, which were newly designed based on the Ikeda strain and other *O. tsutsugamushi* strains. The primers groEL-14F (5′-TTGTACATRGCGATCAATGTCGT-3′) and groEL-1667R (5′-TAGAAATCCATTCCGCCCATAC-3′) were used for the first-round reaction, and the primers groEL-14F and groEL-772R (5′-GAGCTTCTCCGTCTA CATCATCAG-3′) were used for the second-round reactions to detect the *groEL* gene. In addition, the primers groESL-M594F (5′-GGCTATARGCAATGATATAGCTAAACCAG-3′) and groEL-2258R (5′-GCGAACTATTAGTGCTAG CAACTAC-3′) were newly designed to identify the *groESL* gene operon of *O. tsutsugamushi.* First- and second-round PCR were performed using 20-μL reaction mixtures containing 250 μL of deoxyribonucleotide triphosphates

FIG. 1. Phylogenetic trees generated using the Bayesian Markov chain Monte Carlo estimation methods (MCMC) with the GTR+I+Γ model of evolution, which was estimated from the data based on the alignment of the coding regions of the 271- to 285-nucleotide *groES* (ES), 1651- to 1668-nucleotide *groEL* (EL), and 420- to 1481-nucleotide 56-kD type-specific antigen (TSA) (56K) genes of the following *Orientia tsutsugamushi* strains: Kaisei (56-kD TSA, JX235719; groESL, JX188391) from patient, Sato (56-kD TSA, JX235718; groESL, JX188392) from patient, Kato (56-kD TSA, M63382; groESL, JX188393) from patient, Kuroki (56-kD TSA, M63380; groESL, JX188394) from patient, Matsuzawa (56-kD TSA, AF173043; groESL, KC688324) from patient, Shimokoshi (56-kD TSA, M63381; groESL, JX188395) from patient, UAP1 (56-kD TSA, AF302991; groESL, JX188396) from *Apodemus speciosus*, UAP2 (56-kD TSA, AF302992; groESL, KC688329) from *A. speciosus*, UAP4 (56-kD TSA, AF302993; groESL, JX188397) from *A. speciosus*, USP6 (56-kD TSA, AF302994; groESL, KC688325) from *A. speciosus*, UAP7 (56-kD TSA, AF302995; groESL, JX188398) from *A. speciosus*, FAR1 (56-kD TSA, AF302989; groESL, JX188399) from *A. speciosus*, FAR2 (56-kD TSA, AF302990; groESL, KC688326) from *A. speciosus*, HSB1 (56-kD TSA, AF302983; groESL, JX188400) from *A. speciosus*, HSB2 (56-kD TSA, AF302984; groESL, JX188401) from *A. speciosus*, HSB3 (56-kD TSA, AF302985; groESL, KC688327) from *A. speciosus*, CMM1 (56-kD TSA, AF302986; groESL, KC688328) from *A. speciosus*, KNP1 (56-kD TSA, AF302987; groESL, KC688320), KNP2 (56-kD TSA, AF302988; groESL, KC688321), O2 (56-kD TSA, KC688322; groESL, KC688330) from *A. speciosus*, O3 (56-kD TSA, KC688323; groESL, KC688331) from *Apodemus argenteus*, SH205 (56-kD TSA, KC693730; groESL, KC688332) from *A. speciosus*, SH234 (56-kD TSA, KC693731; groESL, KC688333) from *A. speciosus*, SH245 (56-kD TSA, KC693732; groESL, KC688334) from *A. speciosus*, TD-7 (56-kD TSA, JX188388; groESL, JX235722) from patient, TD-17 (56-kD TSA, JX188387; groESL, JX235720) from patient, 05-5 (56-kD TSA, JX188390; groESL, JX235721) from rodent, and SH216 (56-kD TSA, JX188389; groESL, JX188402) from *Mus musculus*. The phylogenetic positions of these strains are also shown relative to the following representative *O. tsutsugamushi* strains: Gilliam (groESL, AY191585) from patient, Taiwan (56-kD TSA, DQ485289) from patient, Ikeda (56-kD TSA, AF173033; groESL, NC010793) from patient, Younchon (56-kD TSA, U19903) from patient, Karp (56-kD TSA, M33004; groESL, M31887) from patient, Kawasaki (56-kD TSA, M63383; groESL, AY191587) from patient, Boryong (56-kD TSA, AM494475; groESL, NC009488) from patient, Nishino (56-kD TSA, AF173048) from patient, Young-worl (56-kD TSA, AF43141; groESL, AY191588) from patient, 402I (56-kD TSA, AF173047) from patient, Darnley (56-kD TSA, AY860955) from patient, FPW1038 (56-kD TSA, EF213087; groESL, EF551288) from patient, FPW2016 (56-kD TSA, EF213085; groESL, EF551289) from patient, Fuji (56-kD TSA, AF210834) from *Leptotrombidium fuji*, IHS I (56-kD TSA, DQ530440) from patient, Hwasung (groESL, QY191589) from patient, LF-1 (56-kD TSA, AF173050) from *Leptotrombidium fletcheri*, LX-1 (56-kD TSA, AF173042) from *Leptotrombidium* mite, TA678 (56-kD TSA, U19904) from *Rattus rattus*, TA716 (56-kD TSA, U19905) from *Menetes berdmorei*, TSA763 (56-kD TSA, RTU80636) from *Rattus rajah*, TW52-1 (56-kD TSA, AY222630) from *Rattus norvegicus*, UT302 (56-kD TSA, EF213095) from patient, and UT418 (56-kD TSA, EF213087; groESL, EF551310) from patient, with *Rickettsia helvetica* (DQ442911), *Rickettsia prowazekii* (Y15783), and *Rickettsia typhi* (AF462073) as outgroups. Genotypes based on the 56-kD TSA gene are shown on the right of 56K. The numbers at each node are the posterior node probabilities (>0.70) based on 150,000 trees. Two replicate MCMC runs consisting of six chains of 10 million generations were sampled every 100 generations with a burn-in of 25,000 (25%). The scale bar indicates the nucleotide substitutions per site. The country of origin is shown in parentheses.

ES
Rickettsia prowazekii
Rickettsia typhi
Rickettsia helvetica
SH234
KNP2
KNP1
CMM1
Matsuzawa
Karp
Boryong
TD-17
05-5
Shimokoshi
Kuroki
Sato
Kato
SH216
O3
SH245
SH205
O2
HSB3
FAR2
UAP6
UAP2
Kawasaki
TD-7
Ikeda
HSB2
HSB1
FAR1
UAP7
UAP4
UAP1
Kaisei
0.1
EL
Rickettsia helvetica
Rickettsia typhi
Rickettsia prowazekii
Sato
05-5
TD-17
Matsuzawa
CMM1
KNP1
KNP2
SH234
Kaisei
TD-7
Ikeda
HSB3
UAP1
UAP4
UAP7
FAR1
HSB1
HSB2
UAP2
UAP6
FAR2
O2
Karp
UT418
Kato
SH205
SH245
FPW2016
FPW1038
Shimokoshi
SH216
Young-worl
O3
Gilliam
Kawasaki
Kuroki
Boryong
Hwasung
0.1
56K
LX-1(JAPAN)
LX-1
Shimokoshi(JAPAN)
Shimokoshi
Fuji(JAPAN)
Fuji
Kato(JAPAN)
LF-1(JAPAN)
Kato
TA716(THAILAND)
FPW1038(THAILAND)
TA716
TA678(THAILAND)
TA678
TA763(THAILAND)
TA763
TW521(THAILAND)
Darnley (AUSTRALIA)
UT302(THAILAND)
Sato(JAPAN)
CMM1(JAPAN)
KNP1(JAPAN)
KNP2(JAPAN)
SH234(JAPAN)
TD-17(JAPAN)
05-5(JAPAN)
Matsuzawa(JAPAN)
Karp(NEW GUINEA)
402I(JAPAN)
O2(JAPAN)
O3(JAPAN)
UT418(THAILAND)
Karp-related
Young-worl(SOUTH KOREA)
SH205(JAPAN)
SH245(JAPAN)
UAP1(JAPAN)
UAP4(JAPAN)
UAP7(JAPAN)
FAR1(JAPAN)
HSB1(JAPAN)
HSB2(JAPAN)
UAP2(JAPAN)
Saitama-related
Nishino(JAPAN)
Kuroki(JAPAN)
Boryong(SOUTH KOREA)
Boryong/Kuroki
Taiwan(TAIWAN)
IHS_I(INDIA)
Kawasaki(JAPAN)
FPW2016(THAILAND)
SH216(JAPAN)
Kaisei(JAPAN)
Younchon(SOUTH KOREA)
Ikeda(JAPAN)
TD-7(JAPAN)
UAP6(JAPAN)
FAR2(JAPAN)
HSB3(JAPAN)
Gilliam/Kawasaki
0.1

(dNTPs), 1 U of Expand Long template DNA polymerase (Roche Applied Science, Penzberg, Upper Bavaria, Germany), and 0.25 μM of each primer.

Initial denaturation was conducted at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 40 s, annealing at 55°C for 30 s, and extension at 72°C for 2 min using a GeneAmp PCR 9700 thermal cycler (Perkin-Elmer, Waltham, MA). PCR products were separated with MobiSpin S-400 spin columns (MoBiTec, Goettingen, Germany), and amplicons were sequenced directly using an ABI Prism 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequences were processed using Genetyx version 9.1 (Genetyx Corporation, Tokyo, Japan) and aligned with CLUSTALW and CLUSTALW2 (Thompson et al. 1994) using publicly available *O. tsutsugamushi* sequences. The phylogenetic analysis was conducted using Bayesian interference trees, which were generated with Bayesian Metropolis Hastings Markov Chain Monte Carlo (MCMC) tree-sampling methods implemented in MrBayes (Ronquist and Huelsenbeck 2003) using a GTR+I+Γ model of evolution, *i.e.*, the hierarchical likelihood-ratio test (hLRT) in MrModeltest2.3 (http://www.abc.se/~nylander/mrmodeltest2/mrmodeltest2.html) (Posada and Crandall 1998) with partitioning based on codon positions.

Results

Sequence and phylogenetic analysis of the groESL *gene*

The 271- to 285-bp and 1651- to 1668-bp *groES* and *groEL* genes of *O. tsutsugamushi* encoded predicted GroES and GroEL proteins with 90–95 and 550–555 amino acids, respectively. The interspecies variation among strains in the *groES* and *groEL* genes was negligible (0–5.6% at the nucleotide level and 0–4.0% at the amino acid level), with the exception of the Shimokoshi strain. Analysis of the *groESL* gene operon, which measures approximately 2.8-kbp and includes the *groES* and *groEL* genes of *O. tsutsugamushi*, revealed four known conserved motifs (two ribosomal binding sites, GGAGGA and AGGAG; one −35 region, TTGAAA; and one −10 region, TATGA) (Stover et al. 1990). A potential *E. coli* sigma 32 heat shock promoter (TTCACACTTGAA) was also found 5′ upstream of the *groES* gene. This study confirmed the overall high sequence similarity of the *groESL* gene operon among strains.

The partially sequenced 56-kD TSA gene, measuring 420–1481 nucleotides in length, was amplified from the genomic DNA of the Sato, CMM1, KNP1, KNP2, SH234, 05-5, and TD-17, and Kaisei, UAP6, FAR2, HSB3, TD-7, and SH216 strains, which showed that the sequences were very similar to those of the Karp-related-type and Gilliam/Kawasaki-type *O. tsutsugamushi*, respectively (Fig. 1, 56K). Exhaustive phylogenetic analyses based on the nucleotide and deduced amino acid sequences of the *groES* and *groEL* genes, which were generated using Bayesian MCMC estimation methods, indicated that both genes were very similar in this species. Interestingly, the two phylogenetic trees were not entirely identical, although they strongly resembled each other. The *groES* and *groEL* genes were highly conserved among species, and the differences between strains were less than 9.7%. Thus, highly conserved genes such as *groES* or *groEL* genes could be suitable targets for the molecular diagnosis of scrub typhus.

Discussion

The present study identified the *groESL* gene operon of *O. tsutsugamushi* that includes the *groES* and *groEL* genes. The shared identities among strains were 90.4–100% and 90.3–100% for the *groES* and *groEL* genes, respectively. Recently, the *groEL* gene was used to identify *Streptococcus*, *Enterococcus*, *Staphylococcus*, *Bartonella*, *Mycobacterium*, and *Ehrlichia*. The present study demonstrated that the sequences of the heat shock protein genes *groES* and *groEL* are highly conserved among *O. tsutsugamushi* strains. Phylogenetic analyses indicated that the 56-kD TSA gene strongly resembled, but was not identical to, the *groEL* and *groES* genes, and these gene sequences are distinctly different from those in *Rickettsia*.

There are reports that indicate that the 56-kD TSA gene is polymorphic among species (Kollars et al. 2003), but gene-based molecular techniques have not been applied to investigate the relationships of the 56-kD TSA gene. Interestingly, phylogenetic analyses revealed no correlation between the 56-kD TSA gene and geographic location of *O. tsutsugamushi* strains and vector *Leptotrombidium*, although the gene is highly polymorphic among species (Fig. 1).

Phylogenetic analysis of the *groES*, *groEL*, and TSA genes detected no common clustering in the dendrograms of each phylogenetic tree (Fig. 1). Two and three clusters were detected in the *groES* and *groEL* gene-based dendrograms, respectively. The clustered strains did not belong to the same genotype according to analysis of the 56-kD TSA gene. A recent report identified genetic recombination between *O. tsutsugamushi* and its vector mite (Sonthayanon et al. 2010), which presaged the mismatches between the 56-kD TSA, *groES*, and *groEL* genes. Thus, the genomic DNA of *O. tsutsugamushi* is undoubtedly rearranged in different strains. More importantly, these findings show that the evolutionary history and transmission dynamics of *O. tsutsugamushi* are far richer and more complex than originally imagined. Given the sympatric and synchronistic coexistence of several *O. tsutsugamushi* strains and different species of vector mites in the field over evolutionary time, it seems plausible that ongoing exchanges within *O. tsutsugamushi* continue to drive its evolution. Phylogenetic analyses based on the *groESL* gene using more isolates will unveil further interesting evolutionary processes that have occurred in *O. tsutsugamushi*.

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Author Disclosure Statement

No competing financial interests exist.

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