Santa Cruz Biotechnology) in 2% BSA-PBS for 1 h, followed with AlexaFluor 488-conjugated anti-rabbit IgG and DAPI. Images were digitally captured on a Nikon Eclipse TE2000-u microscope coupled to a CCD camera using 200× magnification and processed using Adobe Photoshop.

In actin recruitment assays, HeLa cells grown on glass coverslips in 24 well plates were infected for 1 h at 37°C/5% CO_2 with 50 µl of induced *E. coli* (OD_{600} = 1.0). Cells were washed five times with PBS, fixed for 20 min with 4% PFA in PBS and stained for extracellular *E. coli* using a rabbit anti-*E. coli* antisera followed by AlexaFluor 488-conjugated goat anti-rabbit IgG. Next, cells were permeabilized with 0.1% Triton X-100 and stained for cellular actin using Texas-Red phalloidon. Glass coverslips were mounted onto slides using an antifade reagent, mowiol. Images were captured by confocal fluorescence microscopy on an Olympus DSU spinning disk confocal microscope and back-thinned EM-CCD camera at 1000× magnification and 0.1 µm step size. Z-stack slices were analysed in ImageJ software and single slices assembled in Adobe Photoshop.

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短　報

日本紅斑熱発生地域および近隣の発生が少ない地域における知識および受診行動

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Key words: Japanese spotted fever, recognition

序　文

日本紅斑熱は治療が遅れると重症化することがあるため，早期診断と適切な抗菌薬による早期治療が必要な疾患であり，地域住民や医師への啓発が重要といわれている[1)-3)]．1984年に我が国で初めて症例が報告され[1)]，兵庫県では1988年に淡路島で県内初の患者が確認され，毎年1～4例の患者が発生し，2008年に初めて淡路島外から1例が報告された（兵庫県淡路県民局洲本健康福祉事務所，以下洲本保健所）．淡路島では以前より，洲本保健所が一般市民に対し積極的に啓発活動を行っている[2)]．今回，兵庫県の発生・啓発状況の異なる2地域で日本紅斑熱の知識，情報入手手段と受診行動の実態を調査し，関連を検討した．

方　法

2009年5月～7月の期間に，自記式質問紙票調査（別紙1）による横断研究を行った．まず，淡路島内の日本紅斑熱多発地帯に位置するA校を選び，さらに近隣市（非多発地帯）で進学・就職率等で類似したB校を比較対象に選んだ．A校とB校の所在地を発生地とともに示す（Fig. 1）[4)]．それらの生徒の保護者（A校：299名，B校：469名）を対象に自記式質問票への回答を依頼した．調査項目は回答者の属性，疾患知識，受診行動とした（Fig. 2）．分析方法は学校別に疾患知識および疾患知識と受診行動に関連する要因を解析（Fisher法，P-value 0.05未満を有意とした）した．回答者のうち，医療福祉関係者は解析から除き，率を算出する際は分母から無回答を除いた．

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結果および考察

A校は保護者246名から有効回答が得られ（有効回答率82%），そのうち医療・福祉関係者を除くと187名であった．B校は保護者283名から有効回答があり（有効回答率60%），そのうち医療・福祉関係者を除くと244名であった．性別は両校とも女性が多かった（A校：87%，B校89%）．年齢はA校では年齢の記入のある183名のうち142名（78%）が40代で，平均値44.9歳（標準偏差4.34），中央値は45歳で，B校では235名のうち179名（76%）が40代で，平均値45.1歳（標準偏差6.57）中央値は45歳であった．両校間で，年齢の平均値に有意差はなかった（Welchの検定，p＝0.66）．疾患知識について比較すると（Table 1），2校間で有意差が認められたのは，「病名を知っている」（p-value＝0.029）と「症状を知っている」（p-value＝0.039）であった．知識の有無と受診行動との関連については（Table 2），A校では有意差は認められなかった．B校は，「感染経路を知っている」群（p-value＝0.028）と「症状を知っている」群（p-value＝0.0009）で疾患知識がない群に比べ有意に受診率が高かった．

疾患知識全般について，日本紅斑熱の報告の多い地域のA校のほうが，これまで報告がない地域のB校と比較して，「病名を知っている」と「症状を知っている」率が有意に高かった．これは保健所等による啓蒙活動による[2)]ところが大きいと推察される．疾患知識と受診行動の関係をみると，B校において，症状と感染経路に関する知識との正の関連があった．つつが虫病に関しては松井らがその関連を示したが[5)]，日本紅斑熱に関しては本研究が初めて示した知見である．今回の調査では医療福祉関係者が比較的多いのは，両

Fig. 1 School A and B sites
Prefectures where Japanese spotted fever was reported are in black (2006-2009)[4]

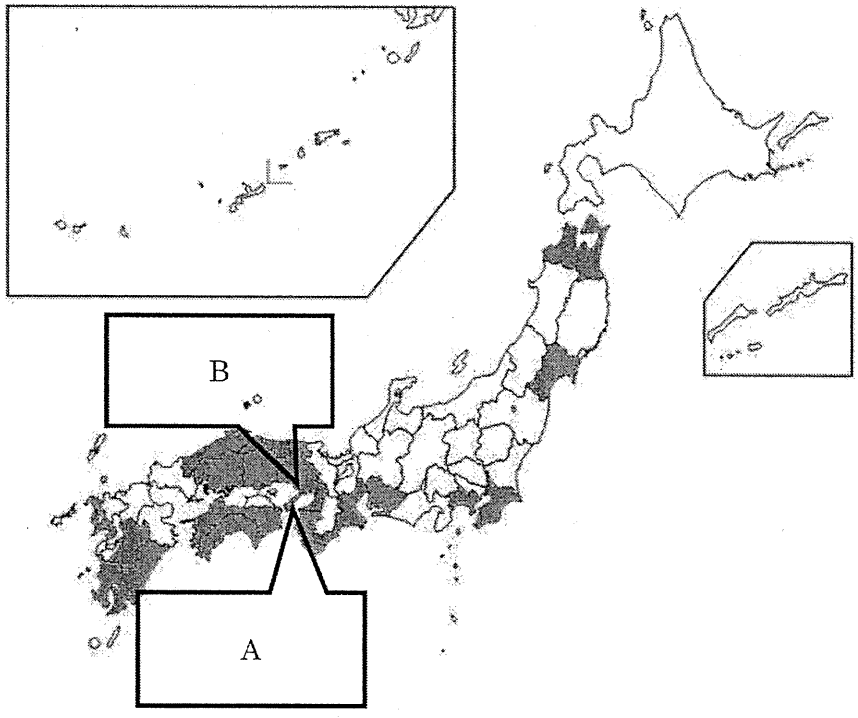

Table 1 Knowledge of Japanese spotted fever (JSF)

	School	Name	Infection route	Symptoms	Infection site	Mortality
JSF	A (n=187)	*58 (31%)* ┐*	13 (7%)	*30 (16%)* ┐*	3 (2%)	14 (8%)
	B (n=244)	*52 (21%)* ┘	12 (5%)	*22 (9%)* ┘	1 (0.4%)	10 (4%)

School A is on Awaji island, where many JSF cases were reported. School B is in Kobe, where few cases were reported.
*$p<0.05$
JSF: name ($p=0.029$), symptoms ($p=0.039$)

Table 2 Knowledge of Japanese spotted fever (JSF)

		With knowledge			Without knowledge				
		consult	Not consult	Consultation	Consult	Not consult	Consultation	Odds	p
A + B	name	77	33	70% (77/110)	227	88	72% (227/315)	0.91	0.77
	infection route	20	5	80% (20/ 25)	280	113	71% (280/393)	1.61	0.49
	symptoms	43	9	82% (43/ 52)	257	109	70% (257/366)	2.03	0.08
	infection site	4	0	100% (4/ 4)	296	118	71% (296/414)	3.60	0.37
	mortality	21	3	88% (21/ 24)	278	115	70% (278/393)	2.90	0.11
A	name	35	23	60% (35/ 58)	93	33	74% (93/126)	0.54	0.097
	infection route	8	5	62% (8/ 13)	119	49	71% (119/168)	0.66	0.67
	symptoms	21	9	70% (21/ 30)	106	45	70% (106/151)	0.99	>0.99
	infection site	3	0	100% (3/ 3)	124	54	70% (124/178)	3.06	0.47
	mortality	11	3	79% (11/ 14)	116	51	69% (116/167)	1.62	0.70
B	name	42	10	81% (42/ 52)	134	55	71% (134/189)	1.72	0.21
	infection route	12	0	100% (12/ 12)	161	64	72% (161/225)	9.99	0.028
	symptoms	22	0	100% (22/ 22)	151	64	70% (151/215)	19.16	0.0009
	infection site	1	0	100% (1/ 1)	172	64	73% (172/236)	1.12	>0.99
	mortality	10	0	100% (10/ 10)	162	64	72% (162/226)	8.34	0.055

School A is on Awaji island, where many JSF cases were reported. School B is in Kobe, where few cases were reported.

平成23年3月20日

Fig. 2 Questionnaire used in this study

別紙1 質問票 (1枚目)

「このアンケートの趣旨に同意して下さるかどうか」に関してお伺いします。

質問1	このアンケートの趣旨に同意してくださいますか？	(同意する ・ 同意しない)

このアンケートの趣旨に同意下さいました方は以降の質問にお答えください。

「ある症状が出た時に受診するかどうか」に関してお伺いします。

質問2	もしあなたが2日～30日前に山野や畑に行き、発熱、発疹(ほっしん)があり、ダニのさし口がある(またはダニにかまれたかもしれない)時、医師の診察を受けますか？	(はい・いいえ)

「つつが虫病」に関してお伺いします。

質問3-(1)	「つつが虫病」という病名を聞いたことがありますか？	(はい・いいえ)
質問3-(2)	質問3-(1)で「はい」と答えた方に質問します。 「つつが虫病」という病名は、どういうところで聞きましたか？ (あてはまるもの、すべてに○をつけて下さい)	1.家族・知人 2.新聞 3.テレビ 4.医学書・医学雑誌 5.医学書以外の本・雑誌 6.保健所 7.講演 8.病院 9.インターネット 10.その他( )
	以降の質問はすべての方にお伺いします。	
質問4	「つつが虫病」は屋外でダニにかまれることにより感染するということを知っていましたか？	(はい・いいえ)
質問5	「つつが虫病」は発熱と発疹を起こす病気ですが、このことを知っていましたか？	(はい・いいえ)
質問6	兵庫県内で「つつが虫病」に感染する可能性がある地域があることを知っていましたか？	(はい・いいえ)
質問7	「つつが虫病」は場合によっては命にかかわる病気であることを知っていましたか？	(はい・いいえ)
質問8	これまでの質問を答えた後で「つつが虫病」について情報を得たいと思いましたか？	(はい・いいえ)
質問9	今後、もしあなたが「つつが虫病」について情報を得るとした場合どのような手段で情報を得たいですか？ (あてはまるもの、すべてに○をつけて下さい)	1.家族・知人 2.新聞 3.テレビ 4.医学書・医学雑誌 5.医学書以外の本・雑誌 6.保健所 7.講演 8.病院 9.インターネット 10.その他( )

「日本紅斑熱(にほんこうはんねつ)」に関してお伺いします。

質問10-(1)	「日本紅斑熱」という病名を聞いたことがありますか？	(はい・いいえ)
質問10-(2)	質問10-(1)で「はい」と答えた方に質問します。 「日本紅斑熱」という病名は、どういうところで聞きましたか？ (あてはまるもの、すべてに○をつけて下さい)	1.家族・知人 2.新聞 3.テレビ 4.医学書・医学雑誌 5.医学書以外の本・雑誌 6.保健所 7.講演 8.病院 9.インターネット 10.その他( )

別紙1 質問票 (2枚目)

	以降の質問はすべての方にお伺いします。	
質問11	「日本紅斑熱」は屋外でダニにかまれることにより感染するということを知っていましたか？	(はい・いいえ)
質問12	「日本紅斑熱」は発熱と発疹を起こす病気ですが、このことを知っていましたか？	(はい・いいえ)
質問13	兵庫県内で「日本紅斑熱」に感染する可能性がある地域があることを知っていましたか？	(はい・いいえ)
質問14	「日本紅斑熱」は場合によっては命にかかわる病気であることを知っていましたか？	(はい・いいえ)
質問15	これまでの質問を答えた後で「日本紅斑熱」について情報を得たいと思いましたか？	(はい・いいえ)
質問16	今後、もしあなたが「日本紅斑熱」について情報を得ようとした場合どのような手段で情報を得たいですか？ (あてはまるもの、すべてに○をつけて下さい)	1.家族・知人 2.新聞 3.テレビ 4.医学書・医学雑誌 5.医学書以外の本・雑誌 6.保健所 7.講演 8.病院 9.インターネット 10.その他( )

「回答いただいているご自身」についてお伺いします。

質問17	年齢	(歳)
質問18	性別	(男 ・ 女)
質問19	学校に通われております生徒様との続柄	1.祖父 2.祖母 3.父親 4.母親 5.兄 6.姉 7.その他( )
質問20	居住されている市	(市)
質問21	御職業	1.農業 2.林業 3.医療・福祉 4.建築業 5.無職 6.その他( )
質問22	仕事中に草木や土に触れる機会はありますか？	(はい・いいえ)
質問23	仕事以外の趣味(キャンプや登山等)や生活(山菜とり等)を目的として山にどの程度入りますか？	1.ほぼ毎日 2.ほぼ毎週 3.ほぼ毎月 4.半年に1回ぐらい 5.年1回ぐらい 6.入らない
質問24	以前あなたまたは周囲の人が「つつが虫病」にかかったことがありますか？	(はい・いいえ)
質問25	以前あなたまたは周囲の人が「日本紅斑熱」にかかったことがありますか？	(はい・いいえ)

以上でアンケートは終了です。ご協力ありがとうございました。

校の医学部進学率がいずれも10%程度と高い（医師は子を医学部に入れる傾向が見られる）ことによる可能性も考えられ，医療関係者は一般市民の調査を目的としたため解析からは除いた．今後同様の調査をする場合は対象の代表性確保が重要である．今回は知識があるかどうかという質問項目のみであったため，よりよい啓発を行うためには，より詳細な知識とリスク認知，およびそれらの受診行動への影響を今後も調査していく必要がある．その際には，各々の知識の正確さなども含めることが肝要と思われた．

なお，この研究の一部は平成21年度厚生労働科学研究費補助金リケッチアを中心としたダニ媒介性細菌感染症の総合的対策に関する研究（主任研究者：岸本壽男）により実施された．調査にご協力下さいました，関係各位に深謝致します．

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Knowledge and Attitude in Medical Behavior in Japanese Spotted Fever in Endemic and Non-endemic Areas

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原　著

つつが虫病および日本紅斑熱について発生頻度が異なる地域での市民医学講座参加者における認知度比較

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Key words: tsutsugamushi disease, Japanese spotted fever, recognition

要　旨

大阪府堺市（講演会A）と兵庫県洲本市（講演会B）で実施された市民向けの医学講座の会場において，つつが虫病と日本紅斑熱について，病名・感染経路・症状についての認知状況および疑わしい症状がでたときの受診行動について自記式のアンケートを実施した．なお，両疾患の感染症発生動向調査における届け出症例数はともに兵庫県の方が大阪府より多い．回収率は，講演会Aは57.9%（113/195），講演会Bは87.2%（61/70）であったが，職種を医療，公衆衛生や，無回答等としたものを除いて講演会A89人，講演会B53人について解析を行った．病名・感染経路・症状の認知度は開催された講演会Bの参加者の方がおおむねよい結果であり，病名（日本紅斑熱），感染経路（つつが虫病），症状（つつが虫病，日本紅斑熱）について，有意差を認めた（$p<0.05$）．しかし，疑わしい症状がでたときの受診行動には，講演会A，Bの参加者において差を認めず，受診につながる情報提供法が今後の課題の一つであると考える．講演会A，Bの参加者をまとめてつつが虫病の病名・感染経路・症状の認知状況と疑わしい症状がでたときの受診行動についてのクロス集計を行ったところ，つつが虫病について感染経路，症状の認知のある群の方が，受診率が有意に高いという結果が得られた（$p<0.05$）．

〔感染症誌　84：48～51，2010〕

序　文

日本における代表的なリケッチア症であるつつが虫病・日本紅斑熱は，重篤な予後をとることもあるが[1)~4)]，比較的まれな疾病ながら，県や地域によっては罹患率が高い感染症であることから，一般市民における認知度に相当の差異があることが予測される．これらの疾病については，これまで一般に患者発生地域を中心に啓発活動が行われてきたが，今後，市民向けの啓発法を考えていく上で，病名，感染経路，症状等の認知状況について調査してみることは有意義であると考えられる．今回は，つつが虫病と日本紅斑熱の感染症発生動向調査における届け出状況が異なる近隣の2府県において開催された市民向けの医学講座の会場において，つつが虫病と日本紅斑熱に関する認知度を調査して結果を比較検討した．

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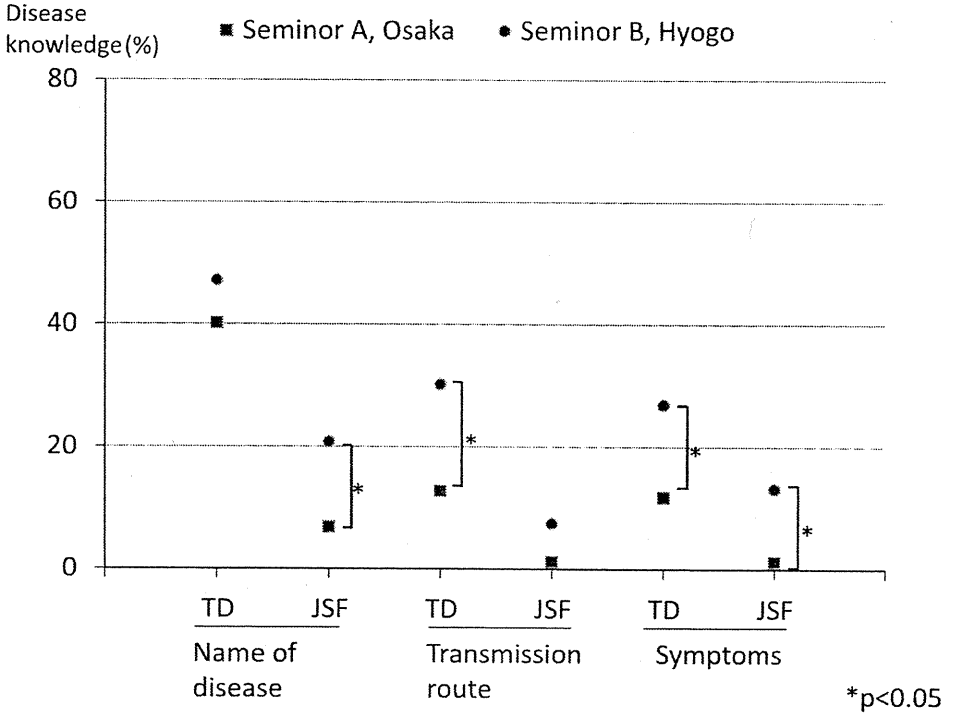
対象と方法

大阪府と兵庫県で実施された市民向けの医学講座の会場において，主催者の了解を得て，自記式アンケートの配布と回収を行った．大阪府堺市で実施された講演会A（以下，A）は，女性団体により平成20年12月2日に実施され，テーマは「現在の感染症のトピックス」について，また兵庫県洲本市で開催された講演会B（以下，B）は，平成21年2月4日（水）に，県民を対象として行われた生活習慣病・がん予防講演会であった．

アンケートでは，つつが虫病と日本紅斑熱それぞれについての病名・感染経路・症状についての認知状況および疑わしい症状がでたときの受診行動等を尋ねた．属性については，職業（「医療・公衆衛生・大学勤務・学生・その他」から選択），および年齢群，性別について尋ねた．居住地についての情報は収集して

Fig. 1 Tsutsugamushi disease (TD) and Japanese Spotted fever (JSF) recognition among Osaka and Hyogo seminar participants



いない.

今回は，医療や公衆衛生以外の職種についての現状評価を行うため，職種を「その他」にチェックをした人のみについて，AとBの回答者間で比較を行った．χ二乗検定は，Epi info ver 3.3.2を用い，有意水準5%で有意であったもののみ本文中にp値を記載した．

成　績

回収率は，Aにおいては，57.9%（113/195），Bにおいては87.2%（61/70）であった．回答者の職業の内訳は，Aは，医療3人，その他89人，無回答21人であり，Bは，医療6人，公衆衛生1人，その他53人，無回答1人であった．以下の集計は，前述のとおり，Aの89人，Bの53人について解析を行った．

解析対象者の属性は，Aについては，男性5人，女性81人，性別不明3名，年代は30歳代24人，40歳代10人，50歳代18人，60歳代34人，70歳代以上3人であった．Bについては，男性6名，女性47名，年代は30歳代1人，40歳代2人，50歳代13人，60歳代24人，70歳代以上13人であった．

「病名を聞いたことがありますか？」という問への回答は，つつが虫病については，Aの回答者では，はい35人・いいえ52人（認知度40.2%：注：割合を算出する場合は，無回答は分母から除いた．以下同じ）および無回答2人，Bの回答者では，はい25人・いいえ28人（認知度47.2%）であった．日本紅斑熱については，Aの回答者では，はい6人・いいえ81人（認知度6.9%）および無回答2人，Bの回答者では，はい11人・いいえ42人(認知度20.8%)であった(p=0.015)（Fig. 1）.

「屋外でダニにかまれることにより感染するということを知っていましたか？」という感染経路に関する問への回答は，つつが虫病については，Aの回答者では，はい11人・いいえ75人（認知度12.8%）および無回答3人，Bの回答者では，はい16人・いいえ37人（認知度30.2%）であった（p=0.012）．日本紅斑熱については，Aの回答者では，はい1人・いいえ84人（認知度1.2%）および無回答4人，講演会Bの回答者では，はい4人・いいえ49人(認知度7.5%)であった（Fig. 1）.

「発熱と発疹を起こす病気ですが，このことを知っていましたか？」という症状に関する問への回答は，つつが虫病については，Aの回答者では，はい10人・いいえ75人（認知度11.8%）および無回答4人，Bの回答者では，はい14人・いいえ38人（認知度26.9%）および無回答1人であった（p=0.024）．日本紅斑熱については，Aの回答者では，はい1人・いいえ83人（認知度1.2%）および無回答5人，Bの回答者では，はい7人・いいえ46人（認知度13.2%）であった（p=0.003）（Fig. 1）.

「2〜30日前に山野や畑に行き，発疹・発熱があり，ダニの刺し口がある（またはダニにかまれたかもしれない）時，医師の診察を受けますか？」という受診態

平成22年1月20日

度についての問への回答は，Aの回答者では，はい39人・いいえ47人・無回答3人であった．無回答の人を除いて集計した潜在的な受診率（以下受診率）は，45.3%であった．一方，Bにおける回答者では，はい23人・いいえ30人で，受診率は，43.4%であった．2つの講演会における回答者の間で，受診率に有意差を認めなかった．

病名・感染経路・症状について，A，Bの回答者ともに一定の認知度が得られたつつが虫病について，病名・感染経路・症状の認知状況と症状出現時の受診態度について無回答は除いてクロス集計を行った．なお，AとBの回答者間で受診率に差がなかったことから，この解析については，A，Bの回答者をまとめて実施した．つつが虫病という病名を知っている群における受診率は52.5%（31/59），知らない群における受診率は，38.5%（30/78），つつが虫病の感染経路を知っている群における受診率は63.0%（17/27），知らない群における受診率は39.6%（44/111），つつが虫病の症状を知っている群における受診率は66.7%（16/24），知らない群における受診率は39.6%（44/111）であった．受診率について有意差が認められたのは，感染経路の認知（$p=0.029$），症状の認知（$p=0.016$）であった．

考　察

今回調査を実施した地域における，感染症法に基づく感染症発生動向調査における届け出症例数は，大阪府は1999年4月～2005年で，つつが虫病4例・日本紅斑熱1例，一方，兵庫県は同期間で，つつが虫病36例・日本紅斑熱28例であった[5]．自治体や医師会による周知活動[6]の成果のためか，両疾患の届け出症例数がともに多い兵庫県において，浸淫地域で開催されたBの回答者の方が病名，感染経路，症状の各認知度についておおむね良い結果を示した．ただし，受診行動についてはAとBの回答者で差がなく，受診行動につながる情報提供が今後の課題の一つであると考えられた．

一方，A，Bをまとめて行った解析によると，つつが虫病の感染経路，症状の認知のある群の方が，疑わしい症状が出た場合の受診率が有意に高いという結果が得られ，感染経路・症状の認知が，受診態度の向上に寄与する可能性が示唆された．

ただし，Bにおいても，日本紅斑熱については，病名・感染経路・症状の認知度については決して認知度が高いとはいえずさらなる情報提供活動が必要である．実際，早期に受診しなかったことによる死亡例が今回調査を行った兵庫県で発生している[4)7)]．また，今回調査を行った大阪府のように，両疾患の届け出症例数が少ない地域においても，感染のリスクはゼロではなく，また浸淫地域への旅行の際などに感染する可能性もあることから，両疾患について，情報を提供する必要性はあると考えられる．

今回の調査は，共に市民向けではあるが医学講座の会場で行われたこと，しかもそれぞれの府県において1回のみの調査機会において実施されたことから，全県下のしかも“一般の”市民における認知度とすることはできない．調査機会を変えて，引き続き検討していきたい．また，回答者の年齢層が異なることも，結果に影響を与えた可能性がある．なお，今回は，回答者の居住地に関する情報を得ていない．同一県内であっても地域において届け出症例数が異なることから今後さらにきめ細かい調査の実施を検討していきたい．

なお，この研究は，平成20年度厚生労働科学研究費補助金（新興・再興感染症研究事業）リケッチア感染症の国内実態調査及び早期診断体制の確立による早期警鐘システムの構築（主任：岸本寿男，分担：岡部信彦）により実施された．

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Tsutsugamushi Disease and Japanese Spotted Fever Recognition Among Medical Seminar Participants for the General Public in Epidemic and Non-epidemic Areas

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Questionnaires on Tsutsugamushi disease (TD) and Japanese spotted fever (JSF) recognition, were distributed at lectures to the general public held in Sakai, Osaka (Lecture A), and Sumoto, Hyogo (Lecture B). Questions included knowledge of transmission routes, symptoms, and seeing physicians after having suspected symptoms. Hyogo had more reported cases of both diseases than Osaka. The response was 57.9% (113/195) to Lecture A, and 87.2% (61/70) to Lecture B. Analysis covered 89 Lecture A and 53 Lecture B respondents after excluding medical and public health specialists and those with unknown occupations. Disease recognition for JSF, knowledge of TD transmission routes, and symptoms of both diseases were better among Lecture B respondents -a statistically significant finding. The two groups saw physicians after having suspected symptoms at roughly the same rate. When these two groups were combined, those with knowledge of transmission routes or symptoms were significantly more likely to see physicians ($p<0.05$).

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NOTE

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Surveillance, recognition, and reporting of Tsutsugamushi disease (scrub typhus) and Japanese spotted fever by general practice clinics in Miyazaki Prefecture, determined by questionnaire survey in 2007

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Abstract In June 2007, a questionnaire survey related to the surveillance, recognition, and reporting of Tsutsugamushi disease (TD) and Japanese spotted fever (JSF) – diseases considered endemic in Miyazaki Prefecture – was distributed to general practice clinics in the prefecture. The response rate was 40.9% (232/567). While 75.5% of the responding clinics knew TD to be a notifiable disease, only 41.8% knew JSF was notifiable. The recognition level of JSF surveillance was lower in the low-incidence areas of JSF within Miyazaki Prefecture. In 2006, 25 cases were clinically suspected as TD by the responding clinics; of the 25 cases, 9 were confirmed and 8 of these were reported to the National Epidemiological Surveillance of Infectious Diseases (NESID). Only 1 of 6 clinically suspected JSF cases from the responding clinics was confirmed in 2006, and it was not reported to NESID. The clinics located in the high-incidence areas for TD tended not to perform laboratory confirmation of the clinically suspected cases of either of the diseases. Considering that NESID requires laboratory confirmation of the reported cases of these diseases, their extent may be underestimated, especially in the high-incidence areas. For clinics in Miyazaki Prefecture, we need to publicize the existence of JSF surveillance and inform clinics about the laboratories available for confirmation of JSF and TD in the prefecture.

Key words Surveillance · Tsutsugamushi disease · Japanese spotted fever

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The National Epidemiological Surveillance of Infectious Diseases (NESID) is a new surveillance system in Japan, created under the Infectious Diseases Control Law enacted in April 1999. Tsutsugamushi disease (TD) and Japanese spotted fever (JSF) were declared notifiable diseases, and reporting of laboratory-confirmed cases became mandatory for physicians. According to the Infectious Disease Weekly Report (http://idsc.nih.go.jp/idwr/index-e.html, from the 14th week in 1999 to the 52nd week in 2006, 3633 TD cases and 378 JSF cases were reported in Japan (population, 127.7 million). Of these cases, 290 and 25 cases of TD and JSF, respectively, were reported from Miyazaki Prefecture (population of 1.1 million). Miyazaki Prefecture has been considered endemic for both diseases. However, recently, the number of notifications for TD and JSF has been decreasing. Because we were not able to determine the true situation, we decided to evaluate the surveillance, recognition, and reporting of TD and JSF from general practice clinics in the prefecture, in collaboration with the Miyazaki Medical Association and the Miyazaki Physicians Association.

In June 2007, questionnaires were sent by reply-paid post cards to 567 clinics registered in the "Himuka Emergency Medical Information Network" as providing an internal medicine service. The chief doctor at each clinic was asked about the surveillance for TD and JSF, and whether the cases diagnosed as TD or JSF in 2006 were confirmed and reported to NESID.

We grouped the data according to clinic location into seven geographical areas: the Northern area (Nobeoka city, Kitagawa town, Takachiho town, Hinokage town, Gokase town), the Hyuga-Irigou area (Hyuga city, Kadokawa town, Misato town, Morotuka village, Shiiba village), the Saito-Koyu area (Saito city, Takanabe town, Shintomi town, Nishimera village, Kijiro town, Kawaminami town, Tuno town), the Nishimoro area (Kobayashi city, Ebino city, Takaharu town, Nojiri town), the Higashimorokata area (Miyazaki city, Kiyotake town, Kunitomi town, Aya town),

the Miyakonojyo-Kitamorokata area (Miyakonojyo city, Mimata town), and the Nichinan-Kushima area (Nichinann city, Kushima city, Kitagou town, Nangou town).

To calculate the reference incidence data for each area, we obtained the number of cases confirmed by the Miyazaki Prefectural Institute for Public Health and Environment (MPI) from April 1991 to March 2007 for TD and from April 1983 to March 2007 for JSF. The confirmation was done by serological test and we calculated the area-specific incidence rate using the suspected place of infection as recalled by the patient.

This was a cross-sectional study. The χ^2 test was done with Epi Info™ ver 3.5.1 (http://www.cdc.gov/epiinfo) ($P < 0.05$).

The questionnaires were returned by 40.9% of the clinics (232/567). The response rates for each area in Miyazaki Prefecture ranged from 28.8% (15/52) to 51.4% (36/70; Table 1). We were unable to classify the location of three clinics.

Of 220 clinics, 166 (75.5%) knew TD to be a notifiable disease. The recognition rates for each area ranged from 57.1%% (8/14) to 100% (14/14). Ninety-four of 225 clinics (41.8%) knew JSF to be a notifiable disease, and the recognition rates for each area varied from 14.3% (2/14) to 53.3% (8/15). The clinics that did not answer this question were not included in the denominator (Table 1).

In 2006, 25 cases were clinically suspected as TD at the responding clinics, but 13 of these cases were not confirmed by any specific test. Of the remaining 12 cases, 9 were laboratory-confirmed (5 cases at MPI and 4 cases at commercial laboratories) and 8 cases were reported to NESID. Three clinically suspected cases were negative for TD by specific tests (laboratory-discarded).

In the same year, six cases were clinically suspected as JSF at the responding clinics: five were not submitted to any specific test for the disease and the remaining one was confirmed by laboratory test, but not reported.

In this study, we did not ask for the criteria used to decide testing in suspected cases.

See the confirmation status by clinic location in Table 2.

From April 1991 to March 2007, the number of test-positive cases of TD confirmed at MPI was 808 for the entire prefecture (Table 1). The numbers of test-positive cases of TD confirmed at MPI, according to the suspected areas of infection of these cases (with incidence rates, per year, per 0.1 million population of each area in 2005, shown in parentheses) were: 0 (0) in the Northern area, 33 (2.2) in Hyuga-Irigou, 15 (0.8) in Saito-Koyu, 248 (18.6) in Nishimoro, 123 (1.8) in Higashimorokata, 217 (6.9) in Miyakonojyo-Kitamorokata, and 158 (11.9) in Nichinan-Kushima. In 14 cases, we could not identify the area of infection.

The area-specific incidence rates of TD were categorized into three groups: low incidence (less than 1.5), middle (from 1.5 to 6.5) and high (more than 6.5). The Northern and Saito-Koyu areas were classified as low incidence, the middle-incidence areas were Hyuga-Irigou and Higashimorokata, and the high-incidence areas covered Nishimoro, Miyakonojyo-Kitamorokata and Nichinan-Kushima.

Table 1. Response rates and recognition levels of requirement for reporting Tsutsugamushi disease and Japanese spotted fever, and incidence of laboratory-confirmed cases in Miyazaki, 2006

Area	No. of clinics	No.of responding clinics (response rate; %)	Recognition of the reporting requirement		Referral incidence	
			Tsutsugamushi disease	Japanese spotted fever	Tsutsugamushi disease	Japanese spotted fever
			No. of clinics that recognized the reporting requirement/no. responding yes or no (%)	No. of clinics that recognized the reporting requirement/no. responding yes or no (%)	No. of cases confirmed at MPI[a] from April 1991 to March 2007 (incidence/year per 0.1 million population)	No. of cases confirmed at MPI[a] from April 1983 to March 2007 (incidence/year per 0.1 million population)
North[b]	70	36 (51.4)	28/36 (77.8)	14/36 (38.9)	0 (0)	1 (0.03)
Hyuga-Irigou[c]	33	15 (45.5)	8/14 (57.1)	2/14 (14.3)	33 (2.2)	0 (0)
Saito-Koyu[d]	52	15 (28.8)	14/14 (100)	4/15 (26.7)	15 (0.8)	1 (0.04)
Nishimoro[e]	44	15 (34.1)	9/15 (60.0)	8/15 (53.3)	248 (18.6)	4 (0.20)
Higashimorokata[f]	228	102 (44.7)	72/98 (73.5)	44/100 (44.0)	123 (1.8)	22 (0.22)
Miyakonojyo-Kitamorokata[g]	100	29 (29.0)	23/27 (85.2)	13/26 (50.0)	217 (6.9)	4 (0.09)
Nichinan-Kushima[h]	40	17 (42.5)	9/13 (69.2)	7/16 (43.8)	158 (11.9)	16 (0.80)
Not classifiable		3	3/3 (–)	2/3 (–)	14 (–)	2 (–)
Total	567	232 (40.9)	166/220 (75.5)	94/225 (41.8)	808 (7.0)	50 (0.43)

[a] MPI, Miyazaki Prefectural Institute for Public Health and Environment
[b] North area: Nobeoka city, Kitagawa town, Takachiho town, Hinokage town and Gokase town
[c] Hyuga-Irigou area: Hyuga city, Kadokawa town, Misato town, Morotuka village and Shiiba village
[d] Saito-Koyu area: Saito city, Takanabe town, Shintomi town, Nishimera village, Kijiro town, Kawaminami town and Tuno town
[e] Nishimoro area: Kobayashi city, Ebino city, Takaharu town and Nojiri town
[f] Higashimorokata area: Miyazaki city, Kiyotake town, Kunitomi town and Aya town
[g] Miyakonojyo-Kitamorokata area: Miyakonojyo city and Mimata town
[h] Nichinan-Kushima area: Nichinann city, Kushima city, Kitagou town and Nangou town

Table 2. Laboratory- confirmation status and reporting of Tsutsugamushi disease and Japanese spotted fever in Miyazaki, 2006

Area	Tsutsugamushi disease suspected				Japanese spotted fever suspected	
	Laboratory-confirmed		Laboratory-discarded	Without laboratory confirmation	Laboratory-confirmed	Without laboratory confirmation
	At MPI	At CoL			At MPI	
North	0	0	1	0	0	0
Hyuga-Irigou	0	1	0	0	0	0
Saito-Koyu	0	0	0	0	0	0
Nishimoro	1	1	0	2	0	0
Higashimorokata	1	1	2	5	1	3
Miyakonojyo-Kitamorokata	3	1	0	5	0	1
Nichinan-Kushima	0	0	0	1	0	1
Total	5	4	3	13	1	5

MPI, Miyazaki Prefectural Institute for Public Health and Environment; CoL, commercial laboratories

From April 1983 to March 2007, the number of cases that tested positive for JSF at MPI was 50 in the entire prefecture. Of these, the 1 JSF case in 1983 was retrospectively confirmed. The number of cases that tested positive for JSF at MPI from April 1983 to March 2007 (with incidence rates, per year, per 0.1 million population of each area in 2005, shown in parentheses) were: 1 (0.03) in Northern area, 0 (0) in Hyuga-Irigou, 1 (0.04) in Saito-Koyu, 4 (0.20) in Nishimoro, 22 (0.22) in Higashimorokata, 4 (0.09) in Miyakonojyo-Kitamorokata, and 16 (0.80) in Nichinan-Kushima. In 2 cases, we could not identify the area.

The area-specific incidence rates of JSF were categorized into three groups: low incidence (less than 0.05), middle (from 0.05 to 0.5), and high (more than 0.5). The low-incidence areas were the Northern area, Hyuga-Irigou, and Saito-Koyu. The Nishimoro, Higashimorokata, and Miyakonojyo-Kitamorokata areas were classified as middle incidence, and the Nichinan-Kushima area was classified as high incidence.

The recognition level of JSF as a notifiable disease (41.8%) was significantly lower than that of TD (75.5%). The recognition rate of JSF as a notifiable disease according to the incidence rates was 30.8% (20/65) in the low-incidence areas, 46.1% (65/141) in the middle-incidence areas, and 43.8% (7/16) in the high-incidence area. The recognition level in the low-incidence area was significantly lower than the total from the other area groups. For TD, no association was found between the area incidence category and the recognition level of reporting TD as a notifiable disease.

The difference between these two diseases in the recognition of the requirement to report them may be explained by three factors: (1) TD had been notifiable since 1950 under the former Communicable Diseases Prevention Law. In contrast, JSF became a notifiable disease only in 1999; (2) JSF is an emerging disease; the first clinical case was reported in 1984;[1] (3) the incidence of JSF is relatively low compared with that of TD in Miyazaki Prefecture.

Using the laboratory diagnosis of TD as the gold standard, the frequency of responding clinics for TD reporting was 88.9%. Of 9 laboratory-confirmed cases identified through the survey, 8 were reported to the Miyazaki Prefecture Health Department. However, the main problem was that of 25 clinically suspected TD cases, laboratory testing was obtained for only 12 cases. The frequency of laboratory testing was worse in high-TD incidence areas (42.9%; 6/14), than in other areas (54.5%; 6/11). Of 6 clinically suspected JSF cases identified in responding clinics, laboratory testing was obtained in only 1 case. Although that test was positive, the case was not reported. Because laboratory confirmation is required by law for both TD and JSF, underreporting of TD and JSF is very likely.

The clinical features of JSF and TD are similar.[2] Epidemiological information on seasonality or endemicity is only partially useful in distinguishing between the two diseases in Japan. Minocycline is basically effective for both diseases, but some cases of JSF were reported to be partially resistant to minocycline monotherapy.[2-4] For those cases, fluoroquinolones were useful. Recently, combination therapy with minocycline and fluoroquinolones has been recommended,[5] especially when the body temperature in patients with JSP is 39°C or more. Distinguishing JSF from TD in the early stages would therefore be clinically useful.

Although MPI is the only laboratory available for JSF confirmation in Miyazaki Prefecture, some commercial laboratories can perform serological testing for TD, using the three standard strains: Kato, Karp, and Gilliam. In addition to these strains, MPI uses two more: Kawasaki and Kuroki. Both are considered the dominant strains in this area[6,7] Therefore, to avoid false-negative TD cases, it is important to re-test the negative cases from the commercial laboratories at MPI, even though cross-reaction of the Kawasaki and Kuroki strains with the three standard ones has been reported .

For the clinics in Miyazaki Prefecture, we need to publicize the existence of JSF, and the facilities available for laboratory confirmation of JSF and TD. This includes the important role played by MPI to confirm these diseases in Miyazaki Prefecture.

The continuing decrease in the number of TD and JSF cases reported to NESID from Miyazaki Prefecture was not evaluated by this study, so we need to follow up the trend and set up another study if necessary. Furthermore, we may need to evaluate TD and JSF surveillance systems in other areas.

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Rickettsia africae Infection in a Japanese Traveller with Many Tick Bites

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African tick bite fever (ATF) is caused by *Rickettsia africae*, and is endemic in sub-Saharan Africa and the eastern Caribbean (1, 2). Cattle ticks of the *Amblyomma* genus act as both reservoirs and vectors (1), and the rate of *R. africae* infection in *A. hebraeum* is 70% in endemic areas (2). Indigenous cases typically occur during agricultural work (1), and 26.9% of adult residents have antibodies to *R. africae* in rural areas of Cameroon (3). Although ATF presents as an acute febrile disease frequently accompanied by headache, myalgia, lymph node swelling, and maculopapular eruption with inoculation eschars, it is basically a self-limited, mild disease (2). Nevertheless, it has also been reported that reactive arthritis may complicate 5% of travel-associated ATF cases, and some patients may develop subacute cranial or peripheral neuropathy (4). In contrast, we report here a case of ATF in a Japanese woman without any general symptoms, in spite of a large number of tick bites.

CASE REPORT

A 61-year-old Japanese woman noticed more than 100 small arthropods on her extremities and trunk the night she returned to Japan (day 1) following an 11-day trip to South Africa; she had stayed in the Kruger National Park for 3 days. Although she removed the ticks as far as possible, 16 brown-grey-, red-, or yellow-coloured ticks, 2×3 mm in size were found on her skin (Fig. 1a, c) when she consulted our hospital on the fourth day (day 4); the remaining ticks were removed (Fig. 1b). On day 19, multiple red papules, which were not itchy, appeared in the different areas affected by tick bite on the 4 extremities and trunk (Fig. 2a, b). The number of papules increased over the following week, but no symptoms, such as fever, myalgia, fatigue, or lymph node swelling, were evident. Although she was given minocycline 200 mg/day, she took it only once. The eruption had developed to pigmentation by day 31. Laboratory examination data were within normal ranges on both days 4 and 20. The ticks were identified as *A. hebraeum* by morphology and by sequencing of the tick *mt-rrs* and tick 12S rRNA gene (5, 6). Using PCR method for rickettsial agents (7), identical sequences with *R. africae* of 17-kDa antigen, *gltA* and *ompA* genes were detected in the body of the ticks and the crust of the eschar on the patient's skin. Because an immunofluorescence assay for *R. africae* was not available in Japan, antibodies to *R. conorii*, which cross-reacts with *R. africae* (8), was tested by immunoperoxidase assay. Positive conversion of antibodies to *R. conorii* were detected on day 20; with IgM titres of 80 and 320, and IgG titres of <40 and 1,280 on days 4 and 20, respectively. Based on these findings, a diagnosis of *R. africae* infection via *A. hebraeum* was made.

Histopathological findings of the eruption on day 4 included perivascular infiltration of mostly lymphocytes and a few

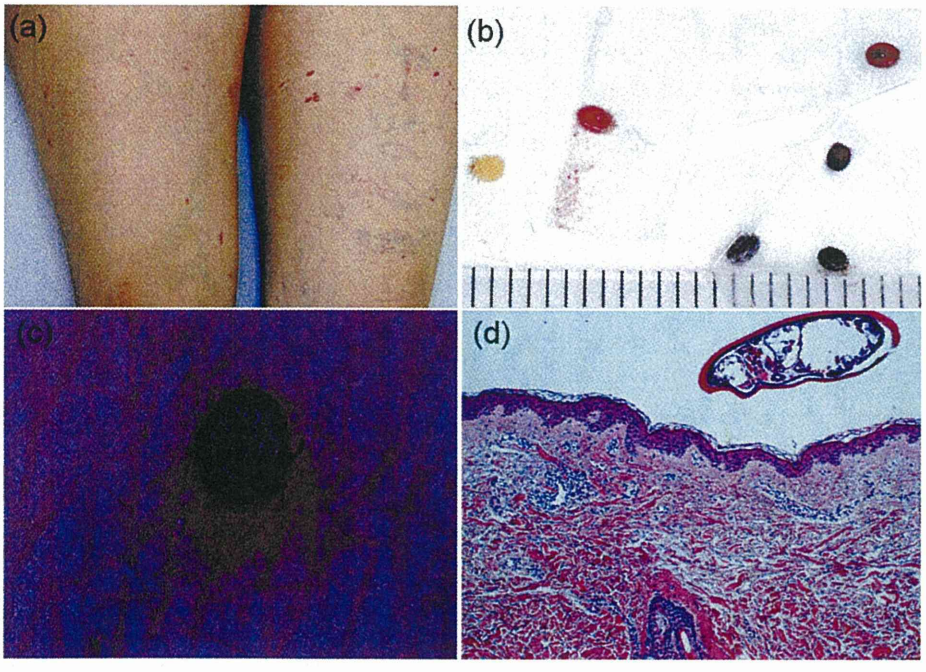

Fig. 1. Clinical findings on day 4, including (a) the appearance of the thighs, (b) some of the removed ticks, (c) a tick on the skin, and (d) histological findings from a section of skin bitten by a tick (haematoxylin and eosin stain, original magnification × 100).

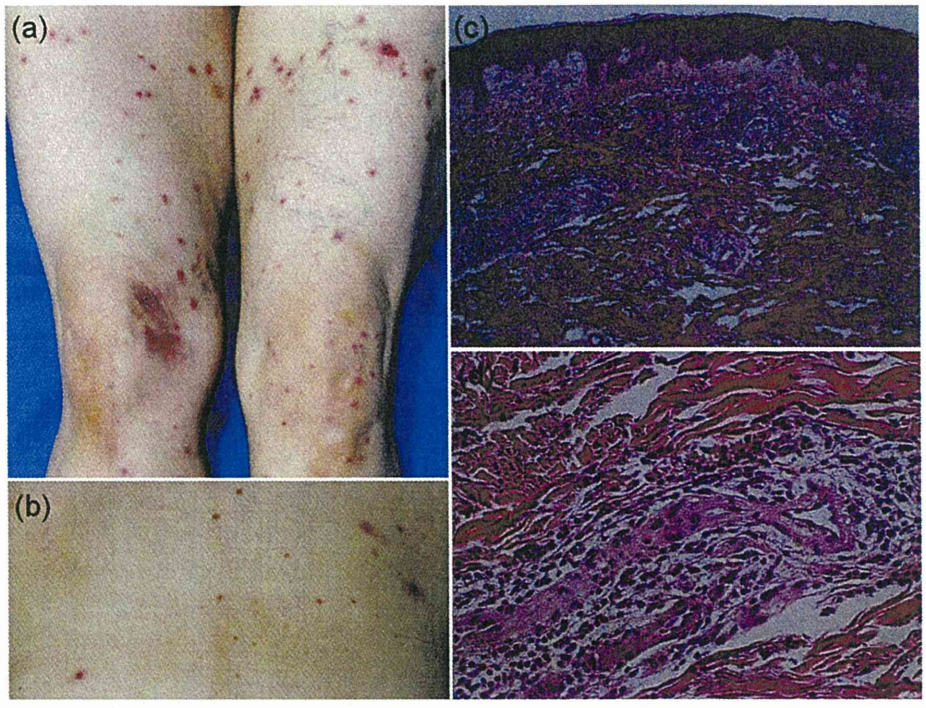

Fig. 2. Clinical findings on day 19, including (a) the appearance of the lower extremities and (b) back, and (c, d) histological findings from the skin eruption (haematoxylin and eosin stain, original magnification (c) × 100 and (d) × 200).

eosinophils in the upper dermis at the site of epidermal tick bite (Fig. 1d). However, when papules appeared on day 19, lymphocyte infiltration was evident not only around the capillaries, but also between collagen bundles, and swelling of capillary endothelial cells was observed through the middle dermis (Fig. 2c, d). These lymphocytes constituted mostly $CD3^+$, $CD4^+$, $CD8^-$ and $CD25^-$ cells.

DISCUSSION

Most patients with ATF exhibit flu-like symptoms. The incubation period from tick bite to the onset of symptoms is usually 5–7 days. In the present case, the tick bites were presumed to have occurred 2–3 days before she noticed the tick on her skin, probably originating in the Kruger National Park and the incubation period to onset of multiple papules was 21–22 days, which is longer than that described in previous reports. In addition, the eruptions in the present patient were not typical of maculopapular eruption usually seen in rickettsiosis, which is more widely distributed.

In rickettsiosis after tick bite, rickettsial pathogens are known to infect endothelial cells, inducing subsequent perivascular infiltration of T cells and macrophages, resulting in vasculitis. Histological findings in ATF eruption are varied; one report showed more abundant neutrophil infiltration than that seen in Mediterranean spotted fever caused by *R. conorii* (9), but another showed only mononuclear cells, but not neutrophils, infiltrating into the dermis (10). In the present case, most infiltrated cells were immunohistochemically identified as helper T lymphocytes.

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ORIGINAL ARTICLE

Histopathological diagnosis of Japanese spotted fever using formalin-fixed, paraffin-embedded skin biopsy specimens
Usefulness of immunohistochemistry and real-time PCR analysis

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Abstract

Japanese spotted fever (JSF) is caused by *Rickettsia japonica*, and lethal cases are reported yearly in southwest Japan. We thus established the method of diagnosing JSF by immunohistochemistry (IHC) and real-time PCR (RT-PCR) using formalin-fixed, paraffin-embedded skin biopsy specimens. Two monoclonal antibodies were used for IHC, and the 17k genus common antigen gene served as the target of RT-PCR. We collected skin biopsy (n = 61) and autopsy (n = 1) specimens from 50 patients clinically suspected of JSF. Immunohistochemically, the rickettsial antigens were localized as coarse dots in the cytoplasm of endothelial cells and macrophages. Thirty-one seropositive cases plus one autopsy case (group A) and nine seronegative cases but with positive IHC and/or RT-PCR (group B) were judged as JSF. Nine cases were regarded as non-JSF disorders based on negative serology, IHC and RT-PCR (group C). Of 50 biopsies (eschar 34, eruptions 10, and scabs 6) from groups A and B, IHC and RT-PCR positivities were 94% (32/34) and 62% (21/34) for eschar, 80% (8/10) and 30% (3/10) for eruptions, and 33% (2/6) and 50% (3/6) for scabs. For IHC, eschar was most suitable, and scabs were insufficient. Unexpectedly, 18 biopsies happened to be fixed in 100% formalin, and this lowered the detection rate by RT-PCR, but IHC was tolerant. Sequence analysis using five skin biopsy specimens confirmed a 114 bp DNA stretch homologous to that reported for the target gene of *R. japonica*. In 26 (84%) of the 31 seropositive patients, the diagnosis was made by IHC and/or RT-PCR earlier than serology.

Keywords: 17k genus common antigen, formalin-fixed paraffin-embedded specimen, immunohistochemistry, Japanese spotted fever, real-time PCR, *Rickettsia japonica*, skin biopsy
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Introduction

There are two rickettsioses endemic in Japan: Japanese spotted fever (JSF) caused by *Rickettsia japonica* (*Rj*) and Tsutsugamushi disease caused by *Orientia tsutsugamushi* (*Ot*). JSF was first reported by Mahara, *et al.* [1,2] in 1984 in Tokushima, Shikoku Island. JSF is thus one of the newcomers to the spotted fever group (SFG) rickettsioses [3]. In Japan, JSF cases must be reported to the health authorities once confirmed [4,5]. Because fatal JSF cases have recently been seen in southwest Japan [6,7], establishment of reliable diagnostic assays is needed.

Serological screening of IgM and IgG antibodies and detection of *Rj* DNA in blood by polymerase chain reaction (PCR) are the main diagnostic tests for JSF [8,9]. Serology requires a minimal twofold increase of antibody titres between the initial and second blood samples. It usually takes a 2-week period; therefore this cannot be used in an emergency situation. Indeed, fatal JSF cases have occurred within 24 h of hospitalization. Empirical treatment was started on clinical suspicion, and serological diagnosis was made retrospectively [6].

We have established diagnostic immunohistochemistry (IHC) using two monoclonal antibodies in formalin-fixed, paraffin-embedded (FFPE) specimens biopsied from eschar and eruptions [10,11]. When tiny samples or superficial

scabs were submitted, false-negative results happened. Therefore, we started detecting *Rj* DNA extracted from FFPE skin biopsies with TaqMan® real-time PCR (RT-PCR). RT-PCR was targeted at the 17k genus common antigen gene to yield short-length products. We utilized FFPE specimens not only to establish histopathological diagnostic tools but also to avoid possible biohazard during handling of the biopsy material.

The aims of the present study are to establish the method for diagnosing JSF with IHC and RT-PCR in FFPE skin biopsies, and to compare these two assays with serology.

Materials and Methods

Clinical specimens

In the period 2004–2010, we collected specimens from 50 patients clinically suspected of having JSF. The clinics and hospitals supplying samples included Mahara Clinic (Anan, Tokushima, *n* = 17), Yamada Red Cross Hospital (Ise, Mie, *n* = 13), Myojin Clinic (Kozagawa, Wakayama, *n* = 9), Uwajima Municipal Hospital (Uwajima, Ehime, *n* = 7), Hyogo Prefectural Awaji Hospital (Sumoto, Hyogo, *n* = 1), Shinano Hospital (Tomi, Nagano, *n* = 1), Yasu Hospital (Yasu, Shiga, *n* = 1) and Notogawa Hospital (Higashiomi, Shiga, *n* = 1).

Biopsy samples were taken from 49 cases, and autopsy material from one. The skin samples (*n* = 61) included eschar (*n* = 42), eruptions (*n* = 12) and scabs (*n* = 7). All but the autopsied tissues, fixed in formalin, were sent to our department within 24–72 h. Normal skin sampled at autopsy served as a negative control. For serological assays, sera were sent to Ohara Research Laboratory, Fukushima, or Prefectural Institutes of Public Health. In total, 89 sera (acute 46 and convalescent 43) were analysed serologically, as described earlier [12]. The cut-off value was set at <×40 for both IgM and IgG. The *Rj* Aoki strain was used as the antigen. Rickettsia was isolated in limited cases in Ohara Research Laboratory, as reported previously [12].

Cultivation of rickettsial strains and preparation of cell blocks

Rickettsiae (Aoki and Katayama strains of *Rj* and Kato, Karp and Gilliam strains of *Ot*) were sent from Ohara Research Laboratory. All strains were passed in cultured L929 cells (fibroblast-like cells of a C3H/An mouse) at a biosafety level 3 containment laboratory in the Department of Microbiology, Wakayama Medical University, Wakayama.

The cells were grown at 32°C in 25 cm^2 plastic cell culture flasks containing Dulbecco's modified Eagle's minimal essential medium (Nissui, Tokyo, Japan) supplemented with 5% fetal calf serum (Hyclone, Logan, UT, USA). The cells harvested 5 to 7 days after inoculation were fixed in 10% formalin overnight. Cell blocks of uninfected and infected L929 cells were prepared by a gelation method using sodium alginate membranes [13]. Cell blocks were also prepared from *R. conorii* (Malish strain)-infected monkey Vero cells (Fuller Laboratories, Fullerton, CA, USA) fixed in 10% formalin.

Monoclonal antibodies and IHC

Mouse IgM monoclonal antibodies, clones S3 and X1, directed to the *Rj* Aoki strain were a gift from Dr Yosaburo Oikawa, Department of Parasitology, Kanazawa Medical University, Kanazawa. Both clones react with the epitope common to SFG rickettsiae, but do not cross-react with *Ot* [14,15].

Sections, 4 μm thick, were prepared from cell blocks and tissue specimens. After inactivating endogenous peroxidase with 0.3% H_2O_2 in methanol for 20 min, sections were heat-retrieved in 10 mM citrate buffer, pH 7.0 for 10 min with a pressure cooker. Incubation with the monoclonals (dilution: 1:100) at room temperature overnight and then amino acid polymers (Simple Stain MAX-PO, Nichirei, Tokyo, Japan) for 30 min at room temperature followed [10,11]. Antigen localization was visualized in 50 mM Tris–HCl buffer, pH 7.6 containing 1 mM 3,3′-diaminobenzidine and 0.006% H_2O_2. Nuclei were counterstained with haematoxylin.

DNA preparation

Five 4-μm-thick FFPE sections were collected in Eppendorf's tubes. After deparaffinization, DNA was extracted using a QIAamp DNA FFPE Tissue kit (#56404; Qiagen, Hilden, Germany). At sample processing, microtome blades were renewed to prevent sample-to-sample contamination.

Real-time PCR

The 17k genus common antigen gene of SFG rickettsia origin was amplified by RT-PCR, according to the previous reports [9,16,17]. Primer pairs for *Rj* consisted of 5′-ATG AAT AAA CAA GGT ACA GGA ACA-3′ (forward: 24mer) and 5′-AAG TAA TGC ACC TAC ACC TAC TC-3′ (reverse: 23mer), generating products of 114 bp length (GenBank D16515). Both primers were 100% homologous to *R. conorii* (GenBank M28480) and *R. rickettsii* (M28479), while three bases (forward) and one base (reverse) were mismatched with *R. typhi* (M28481) and *R. prowazekii* (M28482). Signals were detected with a TaqMan® hybridization probe FAM-GGT GGC GCA TTA CTT GGT TCT CAA TTC GGT AAG GG-TAMRA for *Rj* (Applied Biosystems, Foster City, CA, USA). The number of bases mismatched with the TaqMan® probe (35mer) was one base for *R. conorii*, two bases for *R. rickettsii*, three bases for *R. prowazekii* and four bases for *R. typhi*.

Assays were carried out in 20 μL final volume containing 1.5–3.0 μL of sample DNA, 2× reaction mixture (10 μL, Premix Ex Taq™; TaKaRa Bio, Otsu, Shiga, Japan), 10 pmol primers, and 10 pmol TaqMan® probe. RT-PCR was performed using the DNA Engine Opticom® System (Bio-Rad, Berkeley, CA, USA), with initial holding temperature of 95°C for 30 s, followed by 50 cycles with two-step PCR at 95°C for 5 s and at 60°C for 30 s with fluorescence monitoring on 6-carboxy fluorescein aminohexyl amidite (FAM) channel.

β2-microglobulin (β2m) served as an internal control for effective DNA extraction [18]. Primers designed with Primer3 software (SourceForge, Mountain View, CA, USA) consisted of 5′-TGC TGT CTC CAT GTT TGA TGT ATC T-3′ (forward) and 5′-TCT CTG CTT CCC CAC CTC TAA GT-3′ (reverse) for human/monkey β2m (GenBank NM_004048), and 5′-CAG TGT GAG CCA GGA TAT AG-3′ (forward) and 5′-GAA GCC GAA CAT ACT GAA CTG CTA C-3′ (reverse) for mouse β2m (GenBank NM_009735). The product sizes were 86 bp for human/monkey and 152 bp for mouse.

Sequencing analysis

For sequencing, the SYBR Green method (Qiagen) using the same primer pairs was employed. RT-PCR was performed using Rotor-Gene Q (Qiagen) according to the QIAGEN SYBR-Green PCR Handbook (2009), with initial holding temperature of 95°C for 15 min, followed by 45 cycles with four-step PCR at 95°C for 20 s, at 55°C for 30 s, at 72°C for 30 s and at 57°C for 15 s. The melting curve was checked in the respective reactions. DNA from FFPE sections of L929 cells infected with *Rj* (Katayama strain), Vero cells infected with *R. conorii* and skin biopsies from five cases (eschar: A4, A10, A14 and B1, and scab: B8) were examined. When the plateau was not obtained in the amplification curve in the first run, the second PCR was performed by adding, as a template, 1.5 μL of 1:1000 diluted PCRed aliquot to reaction mixture. After electrophoresis in 1% Agarose gel, the amplified products were extracted with the QIAquick gel extraction kit (Qiagen). Direct sequencing analysis with the dye terminator method [19] was performed in FASMAC Co. (Atsugi, Kanagawa, Japan). The comparison was done using the BLAST program (National Center for Biotechnology Information, Bethesda, MD, USA).

Results

Clinical features of JSF

Table 1 summarizes 31 seropositive cases and one autopsy case (group A) and nine seronegative cases but with positivity of the SFG rickettsial antigen and/or genome (group B). All group B cases exhibited clinical features of JSF, response to antibiotic therapy, and positivities of IHC and/or RT-PCR. Case distribution in groups A and B (n = 41) is shown on Japan's map (Fig. 1). The male to female ratio was 23:18. The mean age was 62.0 years (range, 28–87). Infection occurred during April–December with a peak in September (n = 10). Serologically, *Rj* antibodies of IgM and/or IgG types got elevated in group A, except for case A32 who died acutely. *Rj* was isolated from two cases (A1 and A6) [12].

Nine (18%) of 50 cases were judged as non-JSF disorders (group C, detailed data not shown), based on clinical follow-up features, negative serology and negative IHC/real-time PCR findings in 11 biopsies (eschar 8, eruptions 2 and scab 1). Final clinical diagnoses included tick bite fever of unknown nature (n = 4), Tsutsugamushi disease (Irie/Kawasaki type), streptococcosis, pneumonia, herpes and allergy (n = 1, respectively).

IHC and RT-PCR

Fifty skin biopsy specimens (eschar 34, eruptions 10 and scabs 6) were obtained from group A (n = 38) and group B (n = 12). Rickettsial antigens were immunolocalized as coarse dots in the cytoplasm of endothelial cells and macrophages in the lesions. Both monoclonals consistently gave comparable results. Representative IHC features are demonstrated in Fig. 2. Autopsy tissues (spleen, liver, intestine, salivary gland, kidney and testis) showed positivity of the rickettsial antigens and DNA [11].

Positivity rates of IHC and RT-PCR were 94% (32/34) and 62% (21/34) for eschar, 80% (8/10) and 30% (3/10) for eruptions, and 33% (2/6) and 50% (3/6) for scabs. For IHC, eschar was most suitable, while scabs were insufficient. For scab samples, RT-PCR functioned better than IHC.

Unexpectedly, 18 biopsies sampled in Mahara Clinic happened to be fixed in 100% formalin, and this evidently lowered the detection rate of RT-PCR, whereas IHC was tolerant of such harsh conditions. In samples fixed in 10% formalin, RT-PCR gave positive results in 73% (16/22) for eschar and in 60% (3/5) for eruptions, while for the samples fixed in 100% formalin, the rates were 42% (5/12) for eschar and 0% (0/5) for eruptions (Table 2).

Both IHC and RT-PCR were concordantly positive in 51% (26/51) of 50 skin biopsies plus one autopsy sample. IHC was positive but RT-PCR negative in 33% (17/51). Two (4%) scab samples were IHC negative and RT-PCR positive. In six (12%), both methods gave negative results. On a patient basis, only three (7%) of 41 cases in groups A and B were negative in both methods, and in two of them, only tiny scabs were submitted (cases A27 and A28). In 26 (84%) of 31 seropositive cases, the diagnosis of JSF was made earlier by IHC and/or RT-PCR than by serology.

TABLE 1. Forty-one cases of JSF (31 seropositive, one autopsy and nine seronegative cases)

Patient no	District	Date of onset	Age (year)	Sex	History of tick bite	Rash	Fever >38°C	Rickettsia isolation	IHC	Real-time PCR		Serological assay of *R. japonica* antibodies				Outcome
										Skin biopsy Taq-Man®	Sybr-Green sequencing	Acute		Convalescent		
												IgM	IgG	IgM	IgG	
A1	Tokushima	19 July 2005	87	M	Yes	Yes	Yes	Blood & eschar	+ (Es)	+ (Es) 41.3 Cy		<40	40	640 (2w), 160	640 (2w), 5120	Recovered
A2	Mie	19 September 2007	60	F	Yes	Yes	Yes		+ (Es)	+ (Es) 39.3 Cy		<40	<40	320	1280	Recovered
									+ (Er)	+ (Er) 40.5 Cy						
A3	Mie	26 October 2007	55	M	Yes	Yes	Yes		+ (Sc)	− (Sc)		80	80	320	1280	Recovered
A4	Ehime	27 October 2009	71	F	Yes	Yes	Yes		+ (Es)	+ (Es) 34.5 Cy	+	<40	<40	640	640	Recovered
A5	Tokushima	26 May 2008	79	F	Yes	Yes	Yes		+ (Es)	+ (Es) 37.4 Cy		<40	<40	640	640	Recovered
									− (Er)	− (Er)						
A6	Tokushima	25 July 2004	76	F	Yes	Yes	Yes	Blood	+ (Es)	− (Es)		<40 (2w)	<40 (2w)	640	160	Recovered
									+ (Er)	− (Er)						
A7	Tokushima	24 August 2004	51	M	Yes	Yes	Yes		+ (Es)	− (Es)		80	320			Recovered
A8	Mie	6 October 2006	63	F	Yes	Yes	Yes		+ (Es)	+ (Es) 40.7 Cy		<40	<40	320	640	Recovered
A9	Wakayama	31 May 2010	74	F	Yes	Yes	Yes		+ (Es)	+ (Es) 37.3 Cy		<40	<40	320	640	Recovered
A10	Ehime	7 October 2009	59	F	Yes	Yes	Yes		+ (Es)	+ (Es) 33.8 Cy	+	<40	<40	320	160	Recovered
A11	Ehime	16 June 2010	60	F	Yes	Yes	Yes		+ (Es)	+ (Es) 35.2 Cy		<40	<40	320	160	Recovered
A12	Ehime	3 July 2009	28	M	Yes	Yes	Yes		+ (Es)	+ (Es) 34.6 Cy		<40	<40	180	160	Recovered
A13	Mie	21 September 2007	62	M	Yes	Yes	Yes		+ (Es)	+ (Es) 48.0 Cy		20	80			Recovered
A14	Wakayama	13 August 2009	49	F	Yes	Yes	Yes		+ (Es)	+ (Es) 34.7 Cy	+	<40	<40	160	320	Recovered
A15	Tokushima	23 April 2009	65	F	Yes	Yes	Yes		+ (Es)	+ (Es) 37.1 Cy		<40	<40	160	320	Recovered
A16	Ehime	25 September 2009	60	F	Yes	Yes	Yes		+ (Es)	+ (Es) 33.2 Cy		<40	<40	160	320	Recovered
A17	Ehime	27 April 2009	71	M	Yes	Yes	Yes		+ (Es)	+ (Es) 39.5 Cy		40	<40	80	80	Recovered
A18	Ehime	29 July 2010	60	M	Yes	Yes	Yes		+ (Es)	+ (Es) 35.6 Cy		<40	<40	160	320	Recovered
A19	Tokushima	12 September 2004	65	F	Yes	Yes	Yes		+ (Es)	− (Es)		<40	<40	160	320	Recovered
									+ (Er)	− (Er)						
A20	Tokushima	29 September 2007	68	M	Yes	Yes	Yes		+ (Es)	− (Es)		<40	<40	160 (4w)	80 (4w)	Recovered
A21	Wakayama	24 September 2008	61	F	Yes	No	Yes		+ (Es)	+ (Es) 36.0 Cy		<40	<40	160		Recovered
A22	Tokushima	22 June 2005	52	F	Yes	Yes	Yes		+ (Es)	+ (Es) 41.3 Cy		<40	40	80 (2w)	160 (2w)	Recovered
A23	Mie	7 September 2006	84	M	Yes	Yes	Yes		+ (Er)	− (Er)		<40	<40	80	320	Recovered
									+ (Sc)	+ (Sc) 42.7 Cy						
A24	Tokushima	22 July 2004	77	M	Yes	Yes	Yes		+ (Es)	− (Es)			<40	80	Recovered	
									+ (Er)	− (Er)						
A25	Mie	19 September 2006	70	M	Yes	Yes	Yes		+ (Es)	− (Es)		<40	<40	<40	160	Recovered
A26	Wakayama	4 September 2009	56	M	Yes	Yes	Yes		− (Es)	− (Es)		<40	<40	40	80	Recovered
A27	Mie	4 October 2006	51	M	Yes	Yes	Yes		− (Sc)	− (Sc)		<40	<40	320	640	Recovered
A28	Wakayama	1 October 2007	71	M	Yes	Yes	Yes		− (Sc)	− (Sc)		<40	<40	40	160	Recovered
A29	Mie	2 November 2007	50	F	Yes	Yes	Yes		+ (Es)	+ (Es) 38.7 Cy			+	+	Recovered	
									+ (Er)	+ (Er) 44.7 Cy						
A30	Wakayama	28 July 2010	77	M	Yes	Yes	Yes		+ (Es)	+ (Es) 30.5 Cy			+	+	Recovered	
A31	Wakayama	28 June 2008	56	M	Yes	Yes	Yes		+ (Es)	− (Es)			+	+	Recovered	
A32	Hyogo	19 December 2005	77	M	Yes	Yes	Yes		+ (Au)	+ (Au) 41.5 Cy		<40	<40			Died
B1	Tokushima	23 October 2006	71	F	Yes	Yes	Yes		+ (Es)	+ (Es) 40.0 Cy	+	<40	<40			Recovered
									− (Er)	− (Er)						
B2	Mie	2 October 2006	64	M	Yes	Yes	Yes		− (Es)	− (Es)		<40	<40	<40	<40	Recovered
									+ (Er)	+ (Er) 38.7 Cy						
B3	Wakayama	25 May 2009	65	M	Yes	Yes	No		+ (Es)	+ (Es) 33.6 Cy		<40	<40	<40	<40	Recovered
B4	Mie	2 November 2006	50	M	Yes	Yes	Yes		+ (Es)	− (Es)		<40	<40	<40	<40	Recovered
									+ (Er)	− (Er)						
B5	Tokushima	7 June 2008	38	M	Yes	Yes	Yes		+ (Es)	− (Es)		<40	<40	<40 (2W)	<40 (2W)	Recovered
B6	Tokushima	23 May 2009	54	F	Yes	Yes	Yes		+ (Es)	− (Es)		<40	<40	<40	<40	Recovered
B7	Mie	29 September 2006	29	F	Yes	Yes	Yes		+ (Es)	− (Es)		<40	<40	<40	<40	Recovered
B8	Tokushima	6 May 2007	64	M	Yes	Yes	Yes		− (Sc)	+ (Sc) 43.6 Cy	+	<40	<40	<40	<40	Recovered
B9	Mie	2 August 2007	62	M	Yes	Yes	Yes		− (Sc)	+ (Sc) 40.2 Cy		<40	<40			Recovered

Group A = 31 seropositive cases and one autopsy case (A1–A32), Group B = 9 seronegative cases (B1–B9).
Es, eschar; Er, eruption; Sc, scab; Au, autopsy; Cy, cycles, representing cycle threshold values.