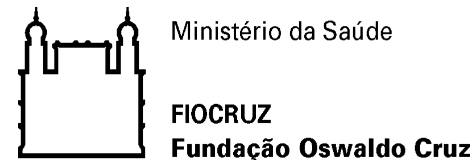

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TATIANA ROZENTAL BURDMAN

RICKETTSIAS *LATO SENSU* EM AMOSTRAS HUMANAS E DE ANIMAIS NO ESTADO DO RIO DE JANEIRO

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Tese apresentada ao curso de Pós-Graduação em Pesquisa Clínica em Doenças Infecciosas do Instituto de Pesquisa Clínica Evandro Chagas para obtenção do grau de Doutor em Ciências, sob a orientação da Prof.ª. Drª. Elba Regina Sampaio de Lemos.

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Orientador: Prof.ª Dr.ª Elba Regina Sampaio de Lemos

Aprovada em: 28/02/2013

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Para Marcinho e Belinha, razões da minha vida.

“The important thing is not to stop questioning. Curiosity has its own reason for existing. One cannot help but be in awe when he contemplates the mysteries of eternity, of life, of the marvelous structure of reality. It is enough if one tries merely to comprehend a little of this mystery every day. Never lose a holy curiosity. ” - Albert Einstein

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RESUMO

A febre maculosa brasileira (FMB), causada por *Rickettsia rickettsii* (uma α-proteobactéria do subgrupo 1) é a rickettsiose mais importante no Brasil, embora existam relatos de casos confirmados e suspeitos de tifo recrudescente, tifo endêmico, ehrlichiose, tifo transmitido pela pulga do gato e rickettsiose variceliforme, além da febre Q, causada por *Coxiella burnetii*, e bartoneloses. *Bartonella* spp. e *C. burnetti*, que pertencem ao subgrupo α-2 proteobactéria e γ-proteobactéria, respectivamente, permanecem sendo estudadas no campo da rickettsiologia. Este estudo foi desenvolvido com o objetivo principal de caracterizar as rickettsias do grupo da febre maculosa (RGFM), *C. burnetii* e *Bartonella* spp. em amostras de pacientes com suspeita de rickettsioses encaminhadas para o Laboratório de Referência Nacional para Rickettsioses, Laboratório de Hantaviroses e Rickettsioses, Instituto Oswaldo Cruz/FIOCRUZ, assim como amostras biológicas de animais domésticos e silvestres, procedentes de diferentes regiões do território fluminense, durante o período de 2007 a 2012. *Rickettsia rickettsii* foi a única RGFM identificada em amostras de pacientes residentes no Estado do Rio de Janeiro, incluindo as amostras procedentes do surto desta zoonose, como doença ocupacional, em um abrigo de animais, onde cinco funcionários evoluíram para o óbito e 114 dos 117 cães apresentaram anticorpos anti-*R. rickettsii*. Adicionalmente, em 2008, um caso fatal de febre maculosa causada por *Rickettsia conorii conorii* foi confirmada, por análise molecular, em um paciente sul-africano em viagem de trabalho ao Brasil. Em relação à febre Q, além do primeiro caso confirmado por análise molecular no Brasil, em 2008, quando um surto em área rural do Município de Itaboraí foi identificado, *C. burnetii* foi identificado como agente causador de uma pneumonia grave associada com osteo-artropatia soronegativa em paciente previamente saudável que adquiriu a infecção em área urbana do Município do Rio de Janeiro. Amostras de carrapatos e de animais silvestres coletados no Município de Piraí foram submetidas à análise molecular e a presença de carrapato da espécie *R. sanguineus* infectado por RGFM foi confirmada. Embora nenhum roedor silvestre tenha apresentado infecção por RGFM, a infecção por *Bartonella* spp. foi identificada em dois exemplares de *Euryoryzomys russatus* e por *C. burnetii* em um exemplar de *Oxymycterus dasythricus* e *Akodon cursor*. Este é o primeiro relato de roedores silvestres infectados naturalmente por *C. burnetii* e *Bartonella* spp. no Brasil. Embora mais pesquisas precisem ser desenvolvidas, os resultados obtidos neste estudo alertam para o risco destas zoonoses no Estado do Rio de Janeiro e também no Brasil, onde somente a FMB é doença de notificação compulsória.

Palavras-chave: Febre maculosa, Bartonelose, Febre Q, Rio de Janeiro, Casos humanos, Animais, Caracterização molecular

Rozental, T. **Rickettsias *lato sensu* in human and animal samples in Rio de Janeiro.** Rio de Janeiro, 2013. xviii 133 f. Thesis [PhD in Clinical Research on Infectious Diseases] – Evandro Chagas Clinical Research Institute.

ABSTRACT

Brazilian spotted fever (BSF) caused by *Rickettsia rickettsii*, an α-proteobacteria of the subgroup 1, is the most important rickettsial disease in Brazil, although there are reports of confirmed and suspected cases of recrudescent typhus, endemic typhus, ehrlichiosis, typhus transmitted by cat fleas, varicelliform rickettsiosis, besides Q fever, caused by *Coxiella burnetii*, and bartonellosis. *Bartonella* and *C. burnetti* belong to the α-2 subgroup proteobacteria and γ-proteobacteria, respectively but remain studied in the field of rickettsiology. This study was conducted with the main objective of characterizing rickettsiae of the spotted fever group (SFGR), *C. burnetii* and *Bartonella* spp. in samples from patients with suspected rickettsiosis sent to the National Reference Laboratory for Rickettsiosis, Laboratory of Hantavirosis and Rickettsiosis, Oswaldo Cruz Institute/FIOCRUZ, as well as biological samples from domestic and wild animals, from different regions of the territory of Rio de Janeiro State, during the period from 2007 to 2012. *Rickettsia rickettsii* was the only SFGR identified in samples from patients in the State of Rio de Janeiro, including samples obtained from the outbreak of zoonotic disease as an occupational disease in an animal shelter, where five workers died and 114 of the 117 dogs studied had antibodies anti-*R. rickettsii*. In addition, in 2008, a fatal case of spotted fever caused by *Rickettsia conorii conorii* was confirmed by molecular analysis in a South African male on a business trip to Brazil. Regarding Q fever, besides the first case confirmed by molecular analysis in 2008, when an outbreak in a rural area of the municipality of Itaboraí was identified, *C. burnetii* was identified as the causative agent of a severe pneumonia associated with seronegative osteo-arthropathy in previously healthy patient who acquired the infection in the urban area of Municipality of Rio de Janeiro. Specimens of wild animals and ticks collected in the municipality of Piraí were tested by molecular analysis and the presence of the infected by SFGR tick species *R. sanguineus* was confirmed. While all small wild rodents were negative for SFGR, *Bartonella* spp. was identified in *Euryoryzomys russatus* (02) and *C. burnetii* in *Oxymycterus dasythricus* (01) and *Akodon cursor* (01). This is the first report of *C. burnetii* and *Bartonella* spp. natural infection in wild rodents in Brazil. Although further investigations are warranted, the results of this study emphasize the risk of these zoonosis in the State of Rio de Janeiro and in Brazil, where only BSF is a notifiable disease.

Keywords: spotted fever, bartonellosis, Q fever, Rio de Janeiro, human cases, animals, molecular characterization

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LISTA DE ABREVIATURAS

BSF	Brazilian spotted fever (Febre Maculosa Brasileira)
ºC	Graus Celsius
DAG	Doença da Arranhadura do Gato
DNA	Ácido desoxirribonucleico
FIOCRUZ	Fundação Oswaldo Cruz
H	Hora
HIV	Vírus da Imunodeficiência Humana
IFI	Imunofluorescência indireta
IgG	Imunoglobulina G
IgM	Imunoglobulina M
IPEC	Instituto de Pesquisa Clínica Evandro Chagas
INC	Instituto Nacional de Cardiologia
kDa	Kilodaltons
kg	kilograma
LHR	Laboratório de Hantaviroses e Rickettsioses
LPS	Lipopolissacarídeo
LRNR	Laboratório de Referência Nacional para Rickettsioses
MG	Miligrama
MS	Ministério da Saúde
PCR	Polimerase Chain Reaction (Reação em Cadeia da Polimerase)
PUBMED	Publicações Médicas (Banco de dados de publicações científicas)
%	Percentual
FMB	Febre maculosa brasileira
RGFM	Rickettsias do grupo da febre maculosa
rRNA	RNA ribosomal
SARA	Síndrome de angústia respiratória do adulto
SUIPA	Sociedade Protetora Internacional de Animais

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1. INTRODUÇÃO

Nas últimas décadas, apesar dos esforços para erradicar as doenças infecciosas, patógenos, na maioria, zoonóticos, até então desconhecidos ou subestimados, emergiram com impacto na saúde humana. O número destas zoonoses (re) emergentes associadas com vetores, que se encontram mundialmente distribuídos, tem crescido como consequência, entre outros fatores, do maior contato humano com animais e artrópodes capazes de transmitir doença, do aumento do interesse da comunidade científica e da maior disponibilidade de novas técnicas diagnósticas.

Rickettsioses são doenças infecciosas causadas por proteobactérias, que, transmitidas por artrópodes reservatórios vetores como ácaros, carrapatos, piolhos e pulgas, se encontram dispersas em diferentes regiões do mundo. Atualmente, com o advento das técnicas moleculares, as espécies, originalmente classificadas na ordem *Rickettsiales*, família *Rickettsiaceae*, foram reorganizadas e distribuídas em: (i) proteobactérias do grupo alfa (subgrupo alfa-1) - gêneros *Rickettsia* e *Ehrlichia;* (ii) proteobactérias do grupo alfa (subgrupo alfa-2) - gênero *Bartonella* e (iii) proteobactéria do grupo gama, na ordem *Legionellales* - a espécie *Coxiella burnetii* (Quadro 1).

Assim, embora o termo rickettsioses atualmente possa ser considerado apenas as infecções causadas pelas espécies do gênero *Rickettsia* ou pelas proteobactérias do grupo alfa, neste estudo, com o objetivo de manter os agentes historicamente descritos no campo da rickettsiologia, como os gêneros *Anaplasma*, *Bartonella*, *Ehrlichia*, *Orientia* e *Rickettsia*, o termo rickettsioses *lato sensu* será utilizado considerando, além da febre maculosa, as doenças causadas por outras proteobactérias, febre Q e bartoneloses.

Transmitidas por artrópodes ectoparasitos (pulgas, piolhos, ácaros e carrapatos), que fazem repasto sanguíneo nos animais que parasitam, mantendo assim um ciclo enzoótico, as rickettsias, considerando o conceito mais amplo, têm sido identificadas como responsáveis por importantes problemas de saúde pública na população humana tanto em países em desenvolvimento quanto na Europa e na América do Norte.

Das rickettsioses descritas no Brasil como a febre maculosa brasileira (FMB), tifo endêmico, tifo epidêmico (forma recrudescente), febre Q e tifo transmitido pela pulga do gato, além dos casos suspeitos de ehrlichioses e rickettsiose variceliforme, a FMB é a rickettsiose de maior importância em nosso território, em especial na região sudeste, onde a maioria dos casos é diagnosticada e notificada. Vale ressaltar que a FMB é a única das doenças neste grupo de notificação compulsória desde 2001.

Quadro 1 - Doenças causadas por Proteobactérias (Rickettsioses *lato sensu*), sua distribuição geográfica, vetores e as espécies relacionadas[a] (Lemos 2009).

Vetor	Espécies	Doenças	Distribuição geográfica
	Rickettsia rickettsii		Hemisfério
	Rickettsia sibirica		Ocidental
Carrapatos	*Rickettsia conorii*		Ásia, Europa
	Rickettsia australis		África, Europa,
	Rickettsia honey		Oriente Médio,
	Rickettsia israeli	Febre	Índia
	Rickettsia japonica	maculosa	Austrália
	Rickettsia africae		Austrália
	R. mongolotimonae		Oriente Médio
	R. slovaca		Japão
	R. parkeri		África
			Europa, África,
	Ehrlichia		Ásia
	chaffeensis		Austrália, Europa
	Ehrlichia sennetsu[b]	Ehrlichiose	Hemisfério
			Ocidental
	Agente da EGH[c]		
			América do Norte
	Coxiella burnetii[d]		
		Febre Q	Japão
			América do Norte
Ácaros	*Rickettsia akari*		
		Febre	
		maculosa	Mundial
Piolhos	*Orientia*	variceliforme	
	tsutsugamushi		
		Tifo do cerrado	Hemisfério
			Ocidental, Ásia
	Rickettsia		
	prowazekii		
Pulgas		Tifo epidêmico	Ásia, Austrália
		(doença de	
	Rickettsia typhi	Brill-Zinsser)	

	Rickettsia felis	Tifo endêmico Tifo transmitido pela pulga do gato	Mundial Mundial Hemisfério Ocidental

[a] Protobactérias do grupo alfa (subgrupo 1) e do grupo gama (*C. burnetti*). As proteobactérias do subgrupo alfa 2, as bartonelas, não foram apresentadas no quadro. Na atualidade o termo rickettsiose se restringe às proteobactérias do subgrupo alfa 1, embora *C. burnetti* e as diferentes espécies de *Bartonella* continuem sendo estudadas no campo da rickettsiologia.

[b] *E. sennetsu*, atualmente *Neorickettsia sennetsu*, o agente da síndrome adenomegálica do Oriente.

[c] EGH = agente da ehrlichiose granulocítica humana, atualmente classificado como *Anaplasma phagocytophilum*.

[d]Febre Q é comumente transmitida pela inalação de partículas aerolisadas contaminadas.

Mais recentemente com a participação do Instituto Nacional de Cardiologia (INC) e do Instituto de Pesquisa Clínica Evandro Chagas (IPEC), o Laboratório de Hantaviroses e Rickettsioses (LHR) têm confirmado casos clínicos de bartoneloses com ênfase nas formas atípicas com endocardite e meningoencefalite.

Quanto à febre Q, a partir da caracterização molecular do agente, *C. burnetii*, em amostras de pacientes e de animais no estado do Rio de Janeiro, diante do aumento crescente de casos, o LHR passou a disponibilizar os testes para o seu diagnóstico, apesar de a mesma não ser considerada doença de notificação compulsória em nosso território.

1.1. FEBRE MACULOSA

1.1.1. Agente Etiológico

As rickettsias são bactérias pleomórficas, gram-negativas, cocóides, cocobacilares, medindo aproximadamente 0,3-0,5 µm de diâmetro por 0,8-2 µm de comprimento. A composição de sua parede celular é semelhante à das bactérias gram-negativas, contendo lipopolissacarídio, peptideoglicano, uma

proteína de 135 kDa (proteína de membrana externa, OmpB), uma lipoproteína de 17 kDa e uma proteína de superfície (OmpA), específica para as rickettsias do grupo da febre maculosa. As rickettsias são inativadas a 56ºC por 30 minutos ou 37ºC por diversas horas e destruídas por formalina, fenol, mertiolato e outros antissépticos, se coram pelos métodos de Giemsa, de Gimenez ou de Machiavello e crescem em culturas de células, ovos embrionados ou em animais de laboratório. Já foram identificadas 25 espécies com 16 associadas com infecção humana, além de mais algumas dezenas sem caracterização definitiva.

Abaixo se encontram enumerados, a partir da análise genética, os quatro grupos de proteobactérias do gênero *Rickettsia*:

1. Grupo ancestral, cuja patogenicidade é desconhecida - *Rickettsia bellii* e *Rickettsia canadensis*;
2. Grupo do tifo - *Rickettsia typhi* e *Rickettsia prowazekii*;
3. Grupo da febre maculosa - composto por mais de 25 espécies, entre elas, *Rickettsia rickettsii* e *Rickettsia parkeri* no continente americano, *Rickettsia conorii* e *Rickettsia africae* na Europa e na África, *Rickettsia japonica* e *Rickettsia israeli* na Ásia e no Oriente Médio (Quadro 1);
4. Grupo transicional - *Rickettsia akari*, *Rickettsia australis* e *Rickettsia felis*.

RICKETTSIAS DO GRUPO DA FEBRE MACULOSA

Rickettsias do grupo da febre maculosa (RGFM) constituem uma diversidade de espécies de rickettsias não patogênicas e patogênicas associadas com doença humana e que se encontram amplamente dispersas no Mundo (Quadro 1)

Rickettsia rickettsii, o protótipo do grupo da febre maculosa, é a espécie mais importante e melhor caracterizada no Brasil. A infecção é adquirida pela picada de carrapatos, cuja espécie *Amblyomma cajennense* é o mais importante reservatório-vetor. Estudos conduzidos sob condições naturais têm sido realizados nas últimas três décadas por diferentes grupos de pesquisa no território brasileiro, confirmando a participação, como vertebrados amplificadores, de caninos, equinos, além de roedores silvestres, marsupiais,

assim como a identificação de carrapatos reservatórios, na manutenção do ciclo enzoótico das rickettsias principalmente nos estados de Minas Gerais, Rio de Janeiro e São Paulo (Lemos et al 1996, Lamos et al 1996a, Horta et al 2009, Ogrzewalska et al 2011, Ogrzewalska et al 2013, Szabó et al 2013).

No Estado do Rio de Janeiro, apesar do ressurgimento de dezenas de casos de febre maculosa nas últimas três décadas em diferentes municípios fluminenses, a ocorrência, em 2005, de um surto com dois óbitos em Itaipava, distrito de Petrópolis, intensificou o interesse por esta zoonose, fato que culminou com aumento na vigilância epidemiológica da FMB no Brasil, a partir de 2005.

Assim, após 30 anos do último relato de casos de FMB no Município do Rio de Janeiro, novos casos fatais foram confirmados em sua área periurbana, onde ocorreram oito casos fatais de síndrome febril e hemorrágica sem esclarecimento etiológico. Em um destes casos fatais foi possível confirmar o diagnóstico de FMB através de técnica molecular (PCR – reação em cadeia da polimerase) (Lemos et al 2002, Lamas et al 2008a).

Sucintamente, em relação aos artrópodes e animais vertebrados, estudos realizados pelo grupo no Estado do Rio de Janeiro têm demonstrado a elevada prevalência sorológica dos cães durante os surtos estudados, confirmando a participação destes animais como sentinelas. Em 2010, após análise molecular de 218 exemplares de artrópodes, procedentes dos municípios do Rio de Janeiro (Jacarepaguá) e de Resende, foi possível identificar exemplares de *Rhipicephalus sanguineus* infectados por *R. rickettsii.* A análise de outros artrópodes como pulgas no Município do Rio de Janeiro mostrou a necessidade de estudos na região administrativa de Jacarepaguá, considerando que 06 exemplares de *Ctenocephalides canis* apresentaram positividade pela análise molecular para rickettsia do grupo da febre maculosa (Lemos 2009, Cunha et al 2009).

1.1.2. Aspectos Epidemiológicos

O vetor mais importante da FMB *é Amblyomma cajennense,* o "carrapato do cavalo", artrópode que pode parasitar tanto animais poiquilotérmicos quanto homotérmicos, embora outras espécies do gênero *Amblyomma,* as espécies

Rhipicephalus (Boophilus) sanguineus, Rhipicephalus (Boophilus) microplus, entre outras, tenham sido identificadas naturalmente infectadas (Pinter et al 2006, Labruna et al 2008, Cunha et al 2009).

A febre maculosa, na maioria das vezes, apresenta-se como casos isolados, mas pode ocorrer na forma de pequenos surtos envolvendo indivíduos de uma mesma família ou de um grupo com atividade em comum. A febre maculosa é mais frequente em pessoas do sexo masculino, em decorrência, provavelmente, do contato com a mata e/ou foco natural da doença, embora todos os indivíduos apresentem suscetibilidade, independentemente de sexo, idade e/ou raça (Lemos et al 2001, Moliterno 2009).

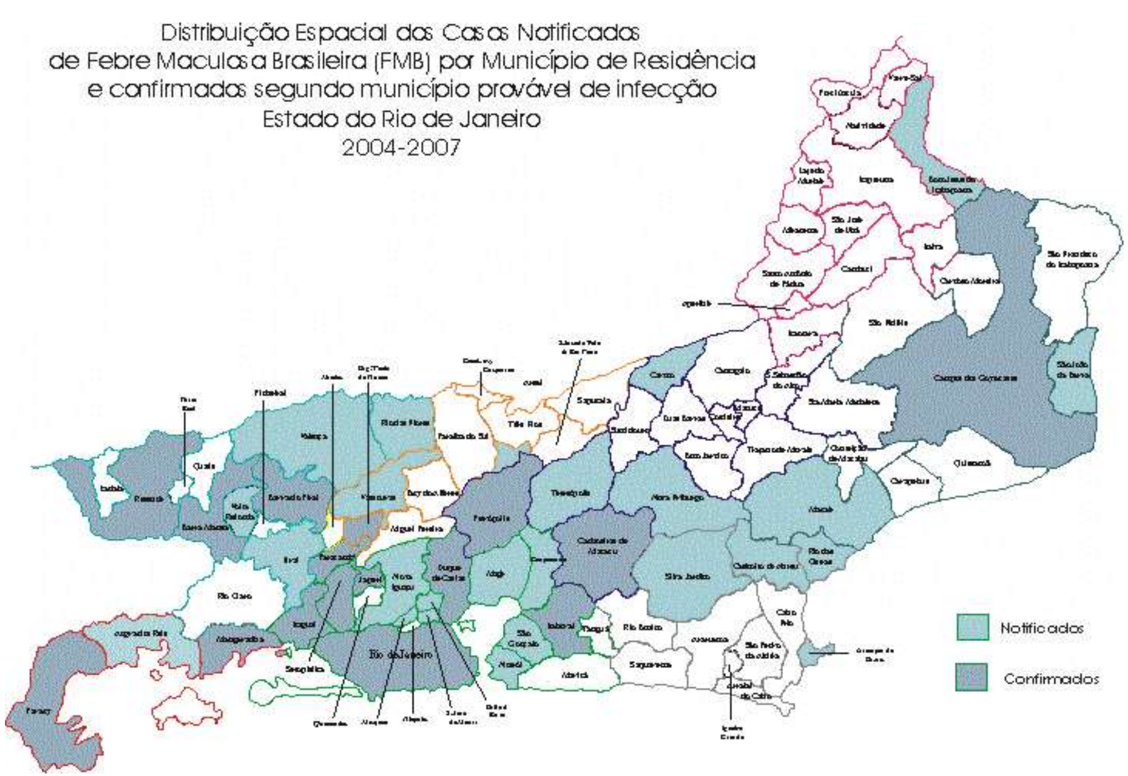
Caracterizada como doença sazonal, a febre maculosa pode ocorrer em todo o período do ano, mas com o maior número de casos no período de maio a outubro, correspondendo a aumento da atividade sazonal dos carrapatos e do concomitante maior contato do homem com esses artrópodes.

No Brasil, desde a notificação obrigatória em 2001 (MS/2001), a FMB, que até recentemente se restringia praticamente aos estados da Região Sudeste, onde casos isolados e surtos têm sido notificados, passou a ser identificada também em outros estados, principalmente nos estados de Goiás, Bahia, Paraná, Rio Grande do Sul e em Santa Catarina. Segundo dados do Ministério da Saúde, a doença acomente mais indivíduos na faixa etária entre 30-49 anos e aqueles do sexo masculino, com relato de exposição ao carrapato em 68% dos casos. A letalidade da FMB variou de acordo com a área geográfica. Enquanto taxas de 20 a 60% foram identificadas nos estados da região sudeste, nenhum óbito foi identificado nos casos confirmados de FMB no estado de Santa Catarina, onde o agente etiológico até o momento não foi identificado (Ministério da Saúde 2011, dados não publicados).

Em uma dissertação de mestrado desenvolvida recentemente no LHR (Moliterno 2009), foi possível identificar, no estado do Rio de Janeiro, que no período de 2004 a 2008, dos 850 casos suspeitos, somente 28 puderam ser confirmados irrefutavelmente. A falta de conhecimento por parte da classe médica e a utilização de testes diagnósticos inadequados para a fase da doença, entre outros fatores, contribuem para este alto indice de casos

compatíveis ou indeterminados. Assim, ainda neste estudo, no território fluminense foi notificado caso de febre maculosa em 14 municípios com uma letalidade de 64%, com ocorrência de dois surtos: um em Itaipava em 2005 e outro em Resende em 2006. A FMB foi mais frequente em indivíduos do sexo masculino (61%) e seis casos foram associados com a medicina do viajante (Moliterno 2009) (Figura 1).

Figura 1. Mapa do Estado do Rio de Janeiro com os municípios com casos notificados e confirmados de febre maculosa brasileira no período de 2004 a 2007 (Secretaria Estadual de Saúde do Estado do Rio de Janeiro).



1.1.3. Aspectos Clínicos e Terapêuticos

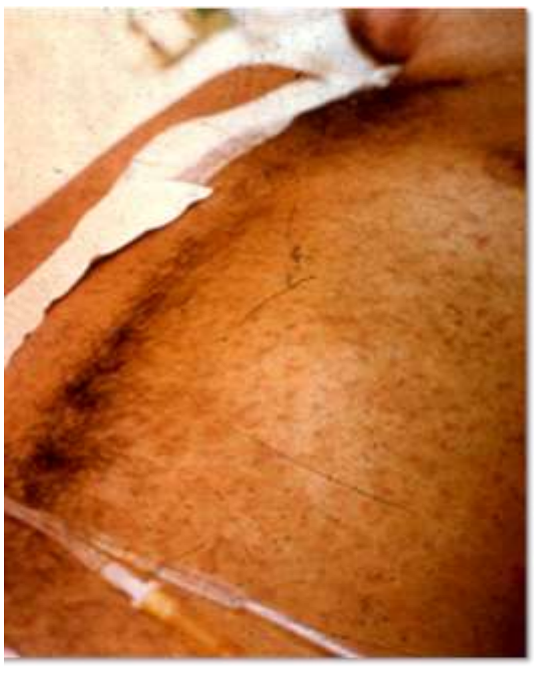
A febre maculosa apresenta um curso clínico variável, desde quadros clássicos a formas atípicas sem exantema. Após um período de incubação médio de 7 dias (2- 14), o paciente desenvolve um quadro febril inespecífico, com mal-estar generalizado, cefaleia, mialgia e artralgia. O exantema geralmente surge entre o terceiro e quinto dia de doença e pode estar ausente em 15 a 20% dos pacientes. O exantema que inicialmente é de colo-

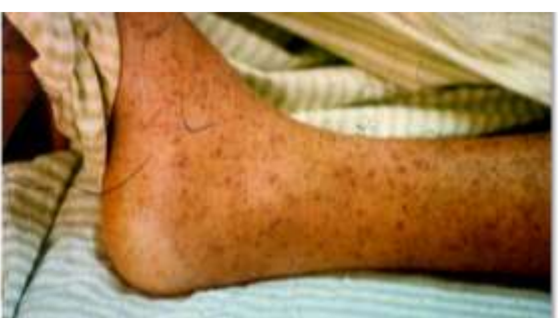
ração rósea, macular, após 1 a 3 dias, torna-se macular e papular, e posteriormente, após 2 a 4 dias, pode evoluir para exantema petequial e, também para necrose e gangrena (Figura 2).

Nos últimos dois anos, uma nova espécie de rickettsia do grupo da febre maculosa, relacionada às espécies *R. africae, R. parkeri* e *R. sibirica*, foi identificada nos estados da Bahia e de São Paulo. Os dois pacientes apresentaram um quadro febril associado com exantema papular, enantema, adenomegalia e cancro de inoculação (Figura 3) (Silva et al 2011, Spolidorio et al 2010).

Embora a suspeita de FMB deva ser realizada com base na tríade clássica - febre, cefaleia e exantema - a ausência de exantema pode ser observada em pacientes idosos e em pacientes submetidos à antibioticoterapia específica precoce. Em pacientes negros, o exantema pode não ser detectado, fato que reforça o conceito de que o exantema não é condição única para suspeita clínica da febre maculosa brasileira.

Figura 2 – Exantema em paciente do Rio de Janeiro com febre maculosa brasileira causada por *Rickettsia rickettsii* (Lemos, 2009)





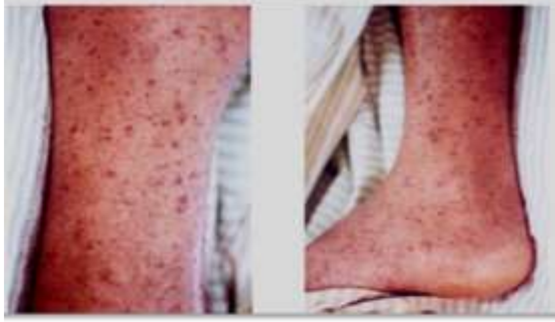
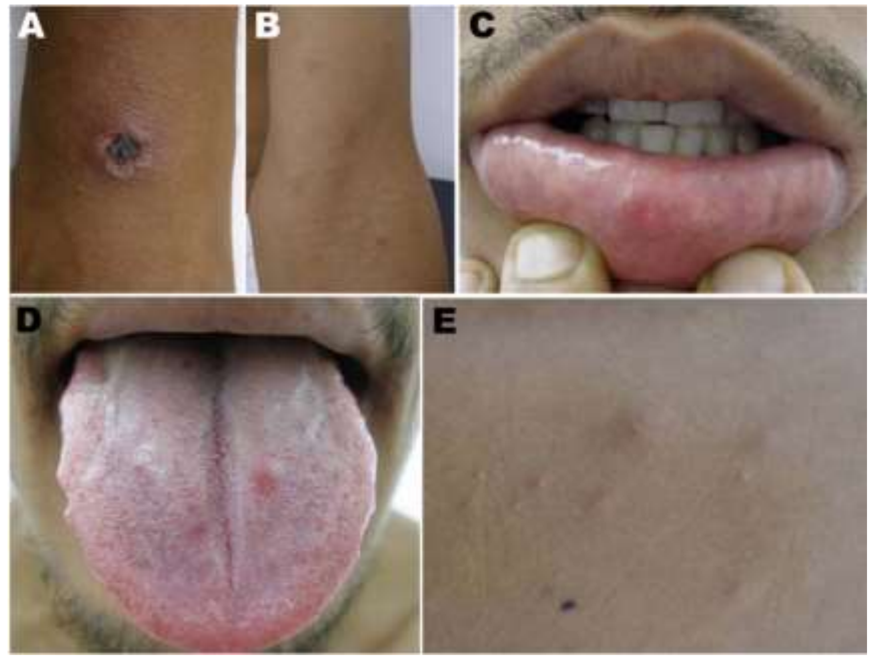


Figura 3 - Exantema papular, enantema, adenomegalia e cancro de inoculação em paciente da Bahia com febre maculosa causada por rickettsia relacionada com as espécies *R parkeri, R sibirica* e *R. africae* (Silva et al 2011).



A evolução para o óbito quase que invariavelmente está relacionada com o retardo no diagnóstico e na antibioticoterapia. Geralmente, a morte do paciente ocorre dentro de 8 a 15 dias após o início do quadro clínico, ainda que casos fulminantes, semelhantes aos casos de meningococcemia, possam ocorrer nos primeiros dias de doença, mesmo em vigência de uma antibioticoterapia correta.

Considerando o polimorfismo das manifestações clínicas, os pacientes podem apresentar quadro gastrointestinal com náusea, vômito, dor abdominal ou diarreia. Alguns indivíduos evoluem com um quadro ictérico. Manifestação pulmonar com tosse, pneumonia intersticial, infiltrado alveolar e derrame pleural, assim como insuficiência respiratória podem ser observadas, em especial nos casos graves, nos quais o paciente pode evoluir para a síndrome de angústia respiratória do adulto (SARA). O paciente pode apresentar também insuficiência renal aguda decorrente de azotemia pré-

renal assim como comprometimento neurológico, como meningite/encefalite e vasculite retiniana, com evolução para coma.

Como o diagnóstico precoce é muito difícil, principalmente durante os primeiros dias de doença, quando pode simular dengue, leptospirose, influenza, histoplasmose, hantavirose, malária, entre outras doenças infecciosas, todos os pacientes com história de contato com carrapatos ou animais deveriam ser tratados empiricamente para febre maculosa, considerando a elevada letalidade diante do retardo do tratamento específico (5º ao 7º dia). Com o surgimento do exantema, a meningococcemia, viroses exantemáticas, dengue, leptospirose, borrelioses (doença de Lyme), ehrlichioses, histoplasmose, sepse com coagulação intravascular disseminada, colagenoses e farmacodermias, entre outras doenças devem ser consideradas no diagnóstico diferencial da FMB (Lemos, 2009).

Quanto ao tratamento, as tetraciclinas e o cloranfenicol são drogas efetivas e consideradas de primeira escolha para as rickettsioses, nas seguintes doses. cloranfenicol (50mg/kg de peso) e tetraciclina (25mg/kg de peso) e o tratamento deve ser mantido por, no mínimo, 7 a 10 dias ou até a ausência de febre por mais de 24h. Terapêutica de suporte para a correção da oligúria, hipotensão, hipocloremia, hiponatremia, hipoalbuminemia, azotemia, edema e coma deve ser instituída nos casos mais graves (Lemos, 2009).

1.1.4. Diagnóstico Laboratorial

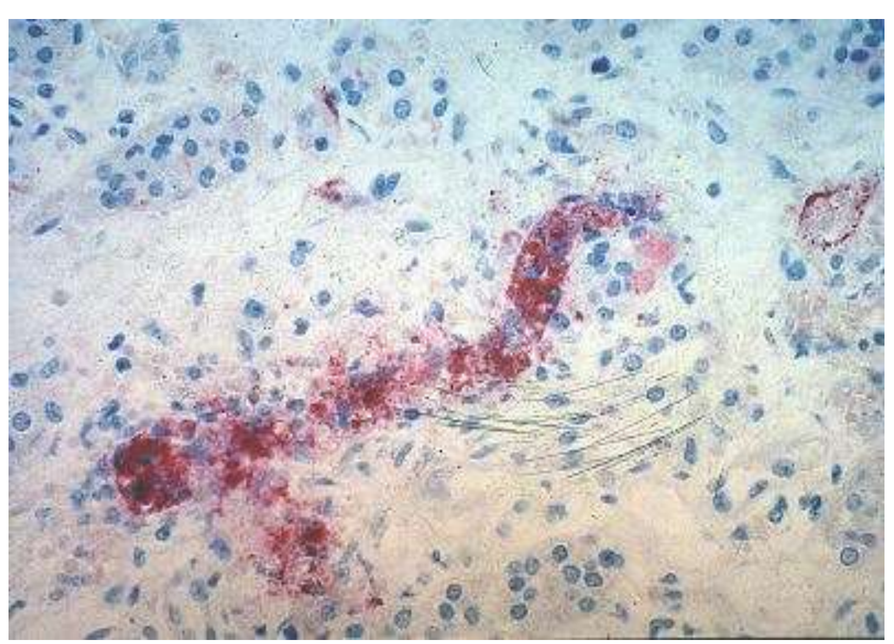
Considerando que os exames laboratoriais utilizados na rotina não auxiliam no diagnóstico - podem ser identificados leucopenia, leucocitose ou contagem normal de leucócitos -, a trombocitopenia, quando presente, deve servir de alerta para o diagnóstico da doença em paciente com história clínica-epidemiológica compatível. O paciente pode evoluir, na ausência de antibioticoterapia específica, para distúrbio de coagulação, azotemia pré-renal e elevação das enzimas hepáticas.

O diagnóstico etiológico é geralmente retrospectivo e realizado a partir de testes sorológicos, pela reação de imunofluorescência indireta, a partir da

análise de duas amostras de soro pareadas e obtidas num prazo ideal entre 2-4 semanas. O aumento de quatro vezes no título de anticorpos da classe imunoglobulina (Ig) M ou G confirma o diagnóstico. A análise molecular a partir da reação em cadeia de polimerase (PCR, *polymerase chain reaction)* se encontra disponível e deve ser realizada na fase inicial da doença, quando anticorpos anti-RGFM ainda não são detectados, especialmente em casos graves e óbitos, quando não é possível a análise de amostras pareadas (Lemos, 2009).

Outras técnicas podem ser utilizadas como a imunohistoquímica (Figura 4) assim como o isolamento rickettsiano, mas que na prática clínica não substituem as técnicas sorológicas e moleculares descritas acima. As condições adequadas de biossegurança devem ser consideradas e por se tratar de microorganismo de classe 3, todas as atividades de isolamento deverão ser realizadas em laboratório de nível 3 de biossegurança (Lemos et al 1996a).

Figura 4 - Técnica de imunohistoquímica (fosfatase alcalina) em fragmento de tecido renal de caso fatal de febre maculosa brasileira em São Paulo (Lemos et al 2001).



1.2. FEBRE Q

1.2.1. Agente etiológico

Coxiella burnetii agente da febre Q, uma zoonose universal, é uma pequena bactéria intracelular obrigatória, pleomórfica cujo tamanho varia de 0,2 a 0,4 µm de largura, com 0,4 a 1 µm de comprimento e que não se cora pela técnica de Gram. Através dos métodos de Giemsa e de Gimenez é possível a sua identificação em amostras clínicas e culturas celulares. Caracterizada como uma proteobactéria, com base na análise da sequência 16S rRNA, foi possível demonstrar que o gênero *Coxiella* pertence à subdivisão gama de Proteobactéria, com os gêneros *Legionella*, *Francisella* e *Rickettsiella* (Figura 5) (Maurin & Raoult 1999, Angelakis & Raoult 2010).

Uma importante característica desta proteobactéria é a capacidade de esporulação, que associada com a alta infectividade determina ampla dispersão. Embora a infecção possa ocorrer em diferentes espécies de animais, como em pequenos roedores silvestres, considerados importante reservatório de *Coxiella,* a infecção humana está geralmente associada com ruminantes em ambiente rurais e com gatos e cães em surtos urbanos. Semelhante ao que é observado com os membros da família *Enterobacteriaceae* (variação liso-rugosa), *C. burnetii* apresenta variações antigênicas relacionadas principalmente com a variação de mutação no lipopolissacarídeo (LPS). Assim, na fase natural encontrada nos animais infectados, artrópodes, ou seres humanos, identificamos a fase I, altamente contagiosa e que corresponde ao LPS liso. Quanto à fase II, obtida somente em laboratórios, após passagens seriadas em culturas, não é infecciosa - corresponde ao LPS rugoso. (Maurin & Raoult 1999, Angelakis & Raoult 2010).

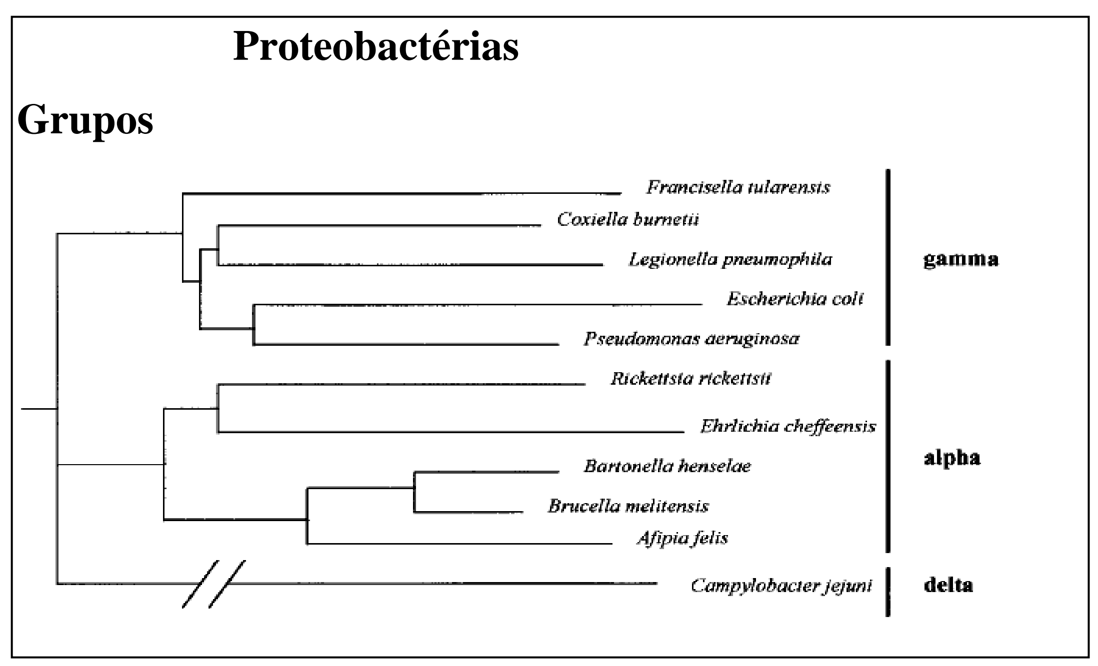
1.2.2. Aspectos Epidemiológicos

O homem contamina-se com aerossóis provenientes de líquido amniótico, placenta e lã; urina, fezes, leite e outras secreções animais também podem conter e disseminar material infectante. Solo, couro, poeira de matadouros, vestimentas de veterinários e fazendeiros podem ser fontes

contínuas dessa infecção. Carrapatos transmitem *C. burnetii* a animais, mas não ao ser humano. Assim, apesar de ser considerada extremamente infecciosa para seres humanos, *C. burnetii* determina infecção assintomática em roedores, marsupiais, pássaros, além dos animais domésticos, principalmente bovinos, caprinos e ovinos.

A febre Q é um importante problema de saúde pública em diversos países, em especial na França, mais recentemente na Holanda, onde, nos últimos três anos, mais de 4.000 casos foram notificados (Van der Hoek et al 2010, Hilbert et al 2011, Roest et al 2011, Sprong et al 2011, Whelan et al 2011). *Coxiella burnetii* é responsável por 5 a 8% dos casos de endocardite infecciosa no sul da França e dados recentes mostram que quadros de febre Q ocorrem em 50 por cada 100.000 habitantes, com a incidência anual da febre Q aguda e endocardite de 2,5/100.000 pessoas e 0,1/100.000 pessoas, respectivamente, com aumento expressivo de casos e surtos nos últimos anos (Frankel et al 2011, Maurin & Raoult 1999).

Figura 5: Árvore filogenética construída pelo método de agrupamento de semelhantes com sequências de genes 16S rRNA na qual é possível verificar a relação filogenética de *C. burnetii* e as outras proteobactérias. (Maurin & Raoult 1999).



Nos Estados Unidos, a febre Q é uma doença de notificação desde 1999 e aproximadamente 80 casos são notificados anualmente com pico de 171 casos em 2007 e com uma incidência média anual de 0,28 /100.000 pessoas (Anderson et al 2009).

No Brasil, onde a doença é praticamente desconhecida e negligenciada, a primeira descrição de febre Q foi em 1953, em São Paulo. Embora existam estudos soro-epidemiológicos que evidenciem a circulação d*e C. burnetii* em população considerada de risco, recentemente casos de febre Q têm sido identificados, incluindo endocardite diagnosticados através de testes sorológicos, moleculares e histopatológicos (Brandão et al 1953, Costa et al 2004, Costa et al 2006, Lamas et al 2013, Ribeiro-Neto et al 1964, Ribeiro Valle et al 1955, Riemann et al 1974, Riemann et al 1975, Siciliano et al 2006, Siciliano et al 2008, Travassos et al 1954).

No Estado do Rio de Janeiro, em uma tese de doutorado desenvolvida no LHR, Lamas e colaboradores através da análise sorológica em pacientes, HIV reativos, atendidos em serviço de saúde na região administrativa de Jacarepaguá, Município do Rio de Janeiro, identificaram quatro pacientes do sexo feminino com anticorpos anti-*C. burnetii* (Lamas et al 2009).

Nos últimos cinco anos, mesmo sem ser considerada doença de notificação compulsória, o LRNR tem disponibilizado testes sorológicos e moleculares para febre Q, e, além do estado do Rio de Janeiro, casos já foram confirmados nos estados de Minas Gerais e Tocantins (ERS Lemos, comunicação pessoal).

1.2.3. Aspectos Clínicos e Terapêuticos

Embora a maioria das infecções provocadas possam ser subclínicas, o quadro inicial pode ser confundido, após um período de incubação de 7 a 28 dias, com influenza. Na fase aguda, manifestações pulmonares associadas com infiltrado intersticial difuso, além da hepatite, geralmente na ausência de ou com discreta icterícia, podem ser observadas (Maurin & Raoult 1999).

Mais raramente alguns pacientes podem evoluir para a forma crônica da doença e a endocardite subaguda pode surgir meses ou anos mais tarde,

com comprometimento principalmente da valva aórtica assim como hepatite granulomatosa com curso mais prolongado. Glomerulonefrite, doença articular e febre de origem indeterminada, dentro de um amplo espectro de manifestação clínica, também têm sido descritas (Angelakis & Raoult 2010).

Considerada também uma doença ocupacional, a infecção por *C. burnetii* pode permanecer assintomática por toda a vida com reativação diante de condições como gravidez, hemodiálise, presença de valvulopatia, prótese valvar, aneurisma aórtico ou ainda imunodeficiência (Fournier et al 1998).

Em relação ao tratamento, não existe consenso quanto à antibioticoterapia e ao período de tratamento, que pode ultrapassar o período de 12 meses, nos casos de endocardite. Cloranfenicol, tetraciclina, quinolonas podem ser utilizadas nas formas benignas. Doxiciclina associada à cloroquina (600mg/dia), por no mínimo 18 meses pode ser utilizada nas endocardites, sendo indicada, na maioria das vezes, a ressecção da valva cardíaca (Baddour et al 2005).

1.2.4. Aspectos Laboratoriais

Considerando a dificuldade do diagnóstico clínico, a confirmação etiológica quase invariavelmente ocorre a partir dos testes sorológicos. O teste de microimunofluorescência indireta é a técnica de referência com a análise dos soros coletados nas fases aguda e convalescente. (Angelakis & Raoult 2010). Outros testes sorológicos como imunofluorescência indireta (IFI), microaglutinação, fixação de complemento, radioimunoensaio, teste de hemólise indireta, e “Western blotting”, entre outros, têm sido realizados, mas apenas os dois primeiros são disponíveis comercialmente (Maurin & Raoult 1999, Fournier et al 1998).

Na febre Q aguda, a soroconversão é geralmente detectada a partir do 7º ao 15º dia do início da doença com títulos de anticorpos IgG anti-fase II ≥ 200 e de IgM anti- fase II ≥ 50. No entanto, o título de corte negativo depende do perfil de cada população. Assim, o ponto de corte utilizado na população francesa não pode ser o mesmo aplicado no Brasil, considerando a quantidade de estimulação antigênica na população estudada. Já na febre

Q crônica, o diagnóstico sorológico é caracterizado pela presença de anticorpos antifase I. Em caso de suspeita de endocardite por *C. burnetii*, a presença de um título de anticorpos de IgG anti-fase I ≥ 800 é considerado altamente preditivo de febre Q (Li et al 2000, Maurin & Raoult 1999).

Embora reações cruzadas tenham sido descritas entre *C. burnetii* e diferentes espécies de *Legionella* e *Bartonella*, a quantificação dos títulos de anticorpos antiantígenos de *C. burnetii* antifase I e antifase II determina maior robustez ao diagnóstico, permitindo assim a determinação etiológica (Maurin & Raoult 1999).

O diagnóstico laboratorial também pode ser realizado a partir de testes moleculares, isolamento e de imunohistoquímica, embora estes dois últimos não sejam as técnicas utilizadas na rotina diagnóstica. A PCR tem se mostrado mais sensível do que técnicas de cultura padrão para o diagnóstico retrospectivo, com amostras congeladas e para o acompanhamento dos pacientes tratados para a febre Q crônica, já que os iniciadores derivados do elemento repetitivo *htpAB* são usados rotineiramente com sucesso no laboratório (Fournier et al 1998). Além da PCR convencional, outras modalidades como nested PCR ou PCR em tempo real em condições de Light-Cycler, SYBR Green ou química TaqMan têm sido utilizadas com sucesso no diagnóstico da febre Q (Klee et al 2006).

Devido à alta sensibilidade e especificidade, o elemento repetitivo IS 1111 é o melhor gene alvo para a detecção de *C. burnetii* em pacientes com febre Q ativa. Recentemente, a sequência completa do genoma de *C. burnetii* se tornou disponível, permitindo uma grande variedade de alvos de DNA (Angelakis & Raoult 2010).

Berri e colaboradores (2000) mostraram a que sensibilidade do *htp*AB foi 100 vezes maior do que a sensibilidade obtida com PCR usando os iniciadores CB1-CB2. A sensibilidade também foi testada em modelos de DNA extraído de esfregaços genitais colhidas dos animais naturalmente infectados (Berri et al 2000)

1.3. BARTONELOSES

1.3.1. Agente Etiológico

Bartonelas são bactérias gram-negativas, intracelulares, parasitas de eritrócitos e de células endoteliais, fastidiosas, anaeróbias eventuais, classificadas na ordem *Rhizobiales*, família *Bartonellaceae*, e que mantém relação filogenética estreita com membros da ordem *Rickettsiales* (Brenner, 1993). As bartonelas podem ser mantidas no laboratório tanto em meio de cultura enriquecido com sangue a 37º C de gás carbônico, assim como em meio celular contendo soro fetal bovino (Magalhães et al 2008).

Até 1993, *Bartonella bacilliformis* era considerada a única bactéria do gênero *Bartonella* causadora de doença humana, a doença de Carrion, restrita às regiões da Colômbia, Equador e Peru. A partir de 1993, com nova classificação taxonômica, com base na análise filogenética, foi possível identificar novas espécies, e atualmente, são reconhecidas mais de 30 espécies e/ou subespécies de *Bartonella*. Mais de doze foram associadas com doença humana, além de *B. bacilliformis: B. quintana* subsp. *arupensis, B. vinsonii* subsp. *berkhoffii, B. henselae, B. elizabethae, B. grahamii, B. washoensis* e, recentemente, *B. koehlerae* (Breener et al 1993, Maureen et al 2012).

1.3.2. Aspectos Epidemiológicos

Considerando que as infecções causadas por *B. bacilliformis*, transmitidas por flebotomíneos, ocorrem restritamente na região andina, e, portanto, nunca foram notificadas no Brasil, neste tópico não será discutida a doença de Carrion ou a verruga peruana.

Este grupo de proteobactérias, com execção de *B. bacilliformis*, se encontra amplamente disperso na natureza e sua manutenção tem sido identificada em diversos mamíferos reservatórios, em especial felinos como o gato doméstico, que servem de fonte de infecção para artrópodes vetores, como a pulga do gato, *Ctenocephalides felis,* por exemplo, uma grande variação no perfil epidemiológico pode ser observada em diferentes regiões do Mundo.

Atualmente mais do que quinze espécies têm sido descritas como agentes etiológicos de diferentes quadros clínicos, sendo que *Bartonella henselae e B. quintana*, são as mais prevalentes entre indivíduos imunocompetentes e imunocomprometidos. Os vetores de *B. quintana* são pulgas, carrapatos e o piolho humano; de *B. henselae*, pulgas e carrapatos. Mais recentemente*, Sarcoptes scabiei* tem sido implicado na transmissão de *Bartonella* spp (Velho et al 2006). Embora muitos vertebrados como roedores, gatos, cães e ungulados, além de animais silvestres, tinham sido identificados como hospedeiros do gênero *Bartonella*, está bem documentado que o gato doméstico é o principal reservatório. Estudos mostram soroprevalências que variam de 14 a 50%. Estes animais podem ter bacteremia persistente e assintomática (Breitschwerdt & Kordick 2000, Hjelm et al 2002, Fabbi et al 2004, Chomel et al 2006, Lamas et al 2010).

Maior atenção deve ser dada à doença da arranhadura do gato (DAG), uma zoonose de distribuição mundial, causada por *Bartonella henselae,* cuja ocorrência tem sido identificada na América do Sul e mais especificamente no Brasil (Lamas et al 2008, Angelakis et al 2010).

Quanto à população humana, estudo sobre a soroprevalência em indivíduos sadios variou de 4 a 15% em alguns trabalhos internacionais, e foi cerca de 13% no Brasil (Costa 2004). Em usuários de drogas injetáveis norte–americanos, a soroprevalência para *Bartonella elizabethae*, epidemiologicamente relacionada a contato com roedores, foi de 33 e 46% em dois diferentes estudos (Comer et al 1996, Comer et al 2001). Em soropositivos para o HIV, a soroprevalência foi de 17% em trabalho norte-americano. Em 2008, Lamas e colaboradores publicaram uma ampla revisão sobre a epidemiologia das bartoneloses com ênfase no Brasil, onde informações mais completas, relacionadas não somente aos crescentes estudos soroepidemiológicos em população humana e de animais, confirmando a elevada dispersão das bartonelas no território brasileiro, podem ser recuperadas (Costa et al 2004, Costa et al 2006, Lamas et al 2008).

Em relação ao gato doméstico, os mais jovens, com idade inferior a um ano constituem a principal fonte de infecção para a população humana

(Loureiro & Hagiwara, 2007, Staggemeier et al 2010). Embora a pulga da espécie *C. felis* seja identificada como o mais importante vetor da doença, muitos aspectos ainda precisam ser esclarecidos considerando que as formas de transmissão precisam ser comprovadas - inoculação bacteriana por conta da arranhadura, mordedura, a própria picada da pulga? - Assim como a possibilidade de transmissão por parte de outros artrópodes como o carrapato (Angelakis et al 2010, Telford & Wormser 2010, Billeter et al 2012).

Em relação à infecção pela espécie *B. quintana*, agente da febre das trincheiras, considerando o número crescente de casos nos últimos dez anos tanto na Europa quanto na África, deve servir de alerta, neste período de grande intercâmbio populacional e de viagens associadas com turismo ou com negócios para a necessidade da inclusão de seu diagnóstico em casos de pacientes com história clínica e epidemiológica compatíveis já que o agente é transmitido de pessoa a pessoa por *Pediculus humanus corporis*.

1.3.3. Aspectos Clínicos e Terapêuticos

Bartonella henselae e *B. quintana* podem causar diferentes quadros clínicos. Em pacientes imunocompetentes podem causar doença da arranhadura do gato, febre das trincheiras e endocardite. Em imunocomprometidos, são causa de angiomatose bacilar, febre com bacteremia persistente e endocardite, que diante do tratamento antiretroviral e maior conhecimento da doença, têm sido raramente identificadas.

Além da doença de Carrion e da verruga peruana, causadas por *B. bacilliformis*, as bartonelas podem determinar os seguintes quadros clínicos (Angelakis et al 2010, Breitschwerdt et al 2011, Graveleau et al 2011, Palumbo et al 2008, Todd et al 2008):

- Alucinação, neuropatia E déficit de visão periférica - *B. koehlerae*
- Angiomatose bacilar: *B. henselae*, *B. quintana*
- Bacteremia e/ou endocardite: *B. henselae*, *B. quintana, B. elizabethae*, *B. vinsonii* subsp. *berkhoffii*, *B. koehlerae*, and *B. alsatica*
- Doença da arranhadura do gato (DAG) e bacteremia crônica: *B. henselae*
- Esplenomegalia: *B. bacilliformis, B. henselae, B. rochalimae*

- Febre das trincheiras: *B. quintana*
- Febre e fadiga: *B. henselae, B. vinsonii* subsp. *berkhoffii*, *B. tamiae*
- Glomerulonefrite - *B. henselae*
- Manifestações pulmonares - *B. henselae*
- Miocardite: *B. vinsonii* subsp. *berkhoffii*, *B. washoensis*
- Osteomielite - *B. henselae*
- Peliose hepática: *B. henselae*
- pseudomalignidade - *B. henselae*
- Púrpura trombocitopênica e anemia hemolítica - *B. henselae*
- Retinite e uveíte: *B. henselae*, *B. grahamii*

A forma mais comum, doença da arranhadura do gato (DAG), é caracterizada pelo surgimento de lesões pápulo-eritematosas, associadas posteriormente com adenomegalia após mordida/arranhadura do gato, num período de incubação médio de sete dias. Embora a infecção possa ser autolimitada dentro de 2 a 6 meses, manifestações gerais inespecíficas, como febre, mal-estar, anorexia, cefaleia e cansaço, podem estar associadas com complicações hepáticas, neurológicas e cardiovasculares, em especial, a endocardite e, neste cenário, o tratamento passa ser essencial (Slhessarenko 1998, Lamas et al 2007, Lamas et al 2008, Pinto et al 2008, Curi et al 2010, Durá-Travé et al 2010, Fournier et al 2010).

Quanto ao tratamento, azitromicina, doxiciclina, eritromicina, e rifampicina são os antimicrobianos mais utilizados no tratamento da DAG e a duração pode variar de 4 a 6 semanas (Rolain et al 2004). Em um estudo randomizado, duplo-cego foi possível, apesar do pequeno número de pacientes incluídos na avaliação de um protocolo de cinco dias de tratamento, verificar a rápida regressão no grupo tratado com azitromicina (Bass et al 1997).

1.3.4. Aspectos Laboratoriais

O diagnóstico das bartoneloses pode ser confirmado por testes sorológicos e moleculares e análise histopatológica. O teste sorológico, mais frequentemente utilizado para a confirmação etiológica, é o teste padrão de referência para o diagnóstico de infecção ativa ou passada, sempre a partir

da análise de amostras pareadas que tenham sido coletadas com um intervalo de 14 dias. Com a sensibilidade de 84% a 95% e especificidade em torno de 74,1%, o teste deve ser utilizado com o ponto de corte de 64 (Agan & Dolan 2002, Rolain et al 2003, Angelakis & Raoult 2010).

O diagnóstico da infecção por *Bartonella* spp. pode ser também realizado através da análise de material histopatológico do linfonodo, da pele, do fígado ou de outros órgãos afetados, com a utilização de testes moleculares, imunohistoquímicos e pelo isolamento bacteriano (Angelakis & Raoult, 2010).

1.4. ANIMAIS E RICKETTSIAS LATO SENSU

O contínuo processo de urbanização e avanço humano sobre as áreas florestais tem provocado mudanças nos habitats naturais, afetando diretamente a estrutura das comunidades de animais e plantas. Estes processos determinaram que diversas espécies se tornassem sinantrópicas tornando-se elos de contato homem *versus* natureza o que pode trazer para o ambiente urbano, doenças infecciosas de ciclos silvestres desconhecidas do homem, ou inserir um ciclo silvestre de doenças originalmente humanas. Deste modo, a emergência ou re-emergência de rickettsioses pode ser facilitada pela transformação dos ambientes naturais.

Os carrapatos são os hospedeiros naturais e vetores para a maioria das rickettsias e para *C. burnetii*. Mais recentemente foram identificados como potenciais reservatórios e transmissores de bartonelas. Animais domésticos, que podem ser fonte de alimento para estes artrópodes, comumente são infectados e desta forma possuem um papel sentinela importante para a infecção humana já que podem aumentar o número de carrapatos infectados no ambiente além de aproximar estes artrópodes das residências e colocá-los em contato com as pessoas.

No Brasil, desde o final do século passado, com o aumento do número de doenças transmitidas por carrapatos, tem se intensificado o número de publicações sobre a participação dos animais domésticos na amplificação do ciclo das rickettsias do grupo da febre maculosa, em especial cães e equinos, assim como carrapatos, seus reservatórios,

coletados tanto de animais domésticos quanto de silvestres. Entretanto, este número crescente de publicações sobre o carrapato faz um contraponto às limitadas informações sobre a participação de vertebrados silvestres na epidemiologia e na dinâmica da infecção pelos mesmos no Brasil. Com mais de 45 publicações na última década, novas espécies de carrapato assim como de novas espécies de rickettsias vêm sendo caracterizadas no território brasileiro como é possível verificar com um levantamento bibliográfico no PUBMED. Embora a capivara (*Hydrochaeris hydrochaeris*) assim como o gambá (*Didelphis* sp.) tenham sido associadas com rickettsias, desde a década de 1940, pouquíssimos estudos têm sido realizados, o que deixa em evidência a necessidade de mais investigação sobre a ecologia da febre maculosa, por exemplo, onde há casos descritos (Magalhães 1953, Lemos 1996, Horta et al 2009). As informações mais comumente publicadas se limitam ao artrópode coletado de vertebrado silvestre como nos artigos recentes de autoria de Szabó et al (2013) e de Ogrzewalska et al (2011, 2013) ou decorrentes de uma avaliação sorológica, com toda a possibilidade de falsa reatividade.

Em relação à identificação de animais vertebrados e invertebrados reservatórios de *C. burnetii* no Brasil, somente nos últimos cinco anos, a partir de estudos conduzidos no LHR, tem sido possível adquirir algum conhecimento sobre a ecologia do agente da febre Q em nosso território. Em uma dissertação de mestrado recentemente desenvolvida no LHR foi possível identificar a presença de infecção por *C. burnetii* em mamíferos domésticos e carrapatos capturados no peridomicílio e em área de vegetação próxima à residência de caso confirmado de febre Q no Município de Itaboraí (Mares-Guia, 2011).

A doença, negligenciada até a grande epidemia que se iniciou em 2007, passou a ser considerada e estudos desenvolvidos na Europa têm demonstrado que, além dos domésticos ruminantes, diversas espécies de animais silvestres, mamíferos de pequeno à grande porte, assim como aves, podem ser considerados reservatórios (Honarmand 2012)

E em relação às bartonelas, muitas informações têm sido publicadas sobre a participação tanto de animais domésticos quanto silvestres, sejam

herbívoros, carnívoros, roedores, morcegos, marsupiais, entre outros (Bai et al 2011, Bown et al 2004, Breitschwerdt & Kordick 2000, Chomel et al 2009, O'Rourke et al 2005).

2. JUSTIFICATIVA

O Rio de Janeiro é o terceiro estado brasileiro em número de casos notificados de FMB. Desde 2005, com a identificação de surtos de FMB em Petrópolis e Resende, seguida pela ocorrência de oito casos fatais, sem confirmação etiológica, e de um caso fatal de FMB na região de Jacarepaguá maior atenção tem sido dada a febre maculosa, embora o número de óbitos continue elevado pelo diagnóstico e tratamento tardios.

A evidência sorológica de rickettsioses, febre Q e bartoneloses em população humana, acrescida da presença de artrópodes positivos pela análise molecular (PCR) para *Rickettsia* spp., alertam para o risco de ocorrência destas zoonoses de alta morbidade e letalidade no território fluminense.

Diante do exposto, levando em consideração a escassez de informações sobre as rickettsioses, em especial quanto a caracterização molecular, este projeto possibilitará, no contexto do Laboratório de Referência Nacional para Rickettsioses, além de um maior conhecimento sobre rickettsias no território fluminense, fornecer subsídios para o adequado planejamento e execução de medidas de prevenção e de controle em áreas periurbanas, em processo contínuo de ocupação humana.

3. OBJETIVOS

3.1. Gerais

Investigar a presença de rickettsias do grupo da febre maculosa, *Bartonella* spp e *Coxiella burnetii* em amostras de pacientes (demanda espontânea do LRNR) encaminhadas ao LHR, durante o período de 2007 a 2012 e de amostras de animais domésticos e silvestres coletadas em diversas regiões do Estado do Rio de Janeiro.

3.2. Específicos

- Analisar por técnicas moleculares as diferentes espécies de rickettsias *lato sensu*, como rickettsias do grupo da febre maculosa, bartonelas e coxiela.
- Verificar a presença de infecção por rickettsias *lato sensu (Rickettsia* sp*p, C. burnetii e Bartonella* spp.) em roedores silvestres
- Investigar a presença de infecção por rickettsias *lato sensu* (*Rickettsia* spp*, C. burnetti,* e *Bartonella* spp.) em animais domésticos
- Identificar a presença de infecção por rickettsias *lato sensu* (*Rickettsia* spp*, C. burnetti* e *Bartonella* spp.) em artrópodes (ácaros, carrapatos, pulgas e piolhos) coletados de vertebrados incluídos no estudo.

4. ARTIGO 1

OCCUPATIONAL EXPOSURE LEADING TO FATAL BSF IN ANIMAL SHELTER EMPLOYEES IN RIO DE JANEIRO, BRAZIL

Manuscrito a ser encaminhado para publicação como LETTER

Occupational exposure leading to fatal BSF in animal shelter employees in Rio de Janeiro, Brazil

To the Editor: Brazilian spotted fever (BSF), caused by *Rickettsia rickettsii*, is the most frequent tickborne disease in Brazil (1, 2, 3). Between 2001 and 2011, over 860 cases were reported and 4 states, Espírito Santo, Minas Gerais, Rio de Janeiro, and São Paulo account for over 80% of cases (Brazilian Ministry of Health, unpublished data). We report a cluster of occupational BSF at an animal shelter in the Municipality of Rio de Janeiro.

The 1st case, a 44-year-old man who worked as veterinary care assistant, became febrile on January 21, 2011. On January 27, he sought medical care at a public health unit for treatment of dengue. He presented fever, myalgia, headache, and asthenia. Support treatment was begun but he developed haemorrhagic phenomena and died 24 hours after hospitalization. No laboratory test was performed during his hospitalization.

On February 3, a 52-year-old man, who worked as stonemason, presented to another public health unit, with 7 day of fever (39,5°C), severe malaise, myalgia, headache, vomiting, asthenia, and general swelling. On basis of clinical findings the diagnosis of dengue was made and support therapy was initiated. The patient remained febrile, thrombocytopenic, and developed pulmonary and renal failure and died 48 hours after. Clinical samples for laboratory analysis were not available.

On March 16, the 3rd employee, a 52-year-old man who worked in the crematorium of animals at the animal shelter, was sought medical attention in public health unit with complaints of headache, fever, chills, myalgia, calf pain, nausea, vomiting and diarrhea. Blood was collected and revealed thrombocytopenia with high level of hepatic enzymes, ureia, and phosphokinase creatinine. He was treated, without improvement, to leptospirosis with 1.500.000 UI of aqueous Crystalline Penicillin G. Two days later he was transferred to a referral hospital where his condition continued to deteriorate and 72 hours later he developed sepsis, and died from multiple system failure. Serum collected on day 4 of the illness was negative for dengue, and leptospirosis. An immunofluorescence assay (IFA) for antibodies against *R. rickettsii* showed IgM/IgG titers of 128/0. Culture for leptospira was negative. The blood sample was positive by polymerase chain reaction (PCR) targeting the *omp*A and *glt*A genes (Eremeeva et al

1994). Sequencing allowed identification of the etiologic agent as *R. rickettsii;* sequences of *ompA* and *gltA* exhibited 100% identity (GenBank accession no. CP003305) and 100% (GenBank accession no. CP003305) respectively.

On April 11, the 4th animal sheter employee, a 32-year-old woman, was taken to an emergency unit with high fever, dehydration, and hypovolemic shock. She had experienced previously 5 days of fever, severe malaise, headache, myalgia, artralgya, and retro-orbital pain. No exanthema was detected. She was transferred to a public hospital with the diagnosis of dengue. Supportive treatment was started but the patient's codition continued to deteriorate and she died of multiple organ failure, 6 days after the onset clinical manifestations. No dengue NS1 antigen was detected in her serum sample. The blood sample was submitted to PCR and confirmmed BSF.

After the confirmation of the BSF cases, an epidemiological investigation was carried out to identify the origin of the outbreak. Control measures were adopted and informations regarding the nature, the transmission route, and the clinical manifestations of BSF were given to employees. In addition, a survey was conducted in 82% of profissionals (115/140) and in 5% (117/2500) of the dogs. Specific-IgG (IFA) was detected in sero from 3 employees and 114 (97%) dogs (titer of 128 to 2048). No dogs presented clinical manifestations.

On April 2012, although control measures and strategies for preventing exposure to tick-borne diseases had been implemented at animal shelter, the 5th employee, a 47 year-old-woman, with influenza-like disease, died within the 1srt day of admission at referral hospital. Both IgM and IgG against *R. rickettsii* at serum samples collected in April 17 were non reactive, but the PCR of autopsy samples (liver, heart, spleen and kidney) collected 24 hours later confirmmed *R. rickettsii* infection.

This cluster demonstrates some key points with regards to BSF:

- None of the patients had exanthema and all died within 5-8 days of illness onset. This rapid course and the lack of prompt initiation anti-ricketttsial therapy have been observed in BSF cases;

- BSF should be a differential diagnosis for febrile patients who have recent exposure to ticks/animals, particularly in area of dengue and leptospirosis transmission

- Although the tick analysis had not been included, due measures to control ticks previouly done at animal shelter, the results and the date of the epidemiological investigation confirm that the origin of the outbreak was the animal shelter;

- PCR is rapide and specific diagnostic technique in the first days of BSF;

- Profissionals who work in animal shelters or kennels should aware of the potential risks for occupational BSF. Recommendations to prevent futher outbreaks of BSF should be periodicaly reviewed.

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5. ARTIGO 2

FATAL SPOTTED FEVER GROUP RICKETTSIOSIS DUE TO *RICKETTSIA CONORII CONORII* MIMICKING A HEMORRHAGIC VIRAL FEVER IN A SOUTH AFRICAN TRAVELER IN BRAZIL.

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Short communication

Fatal spotted fever group rickettsiosis due to *Rickettsia conorii conorii* mimicking a hemorrhagic viral fever in a South African traveler in Brazil

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ABSTRACT

The authors present a fatal case of spotted fever group rickettsiosis (SFGR) caused by *Rickettsia conorii conorii* mimicking a hemorrhagic viral fever in a South African male on a business trip in Brazil. SFGR was confirmed by molecular and immunohistochemical analyses.

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1. Introduction

A wide spectrum of rickettsioses has been recognized in international travelers in the last 2 decades (Freedman et al., 2006; Gautret et al., 2009; Jensenius et al., 2004, 2009; Kun-Hsien et al., 2009; Wilson et al., 2007). Tick-borne spotted fever is increasingly being diagnosed among international travelers, and most cases are acquired in sub-Saharan Africa, mainly in South Africa, where spotted fever group rickettsioses (SFGR) are secondary only to malaria as the most frequent febrile disease in travelers reported to the GeoSentinel Surveillance Network (Jensenius et al., 2009; Kun-Hsien et al., 2009). We report a fatal case of SFGR due to *R. conorii conorii*, mimicking a hemorrhagic viral fever, in a South African man on a business trip to Brazil.

2. The case

On November 27, 2008, a white 53-year-old South African male engineer on a business trip, 2 days after arriving in Brazil, reported headache, fever, chills, sore throat, asthenia, and hematuria. He was medicated with symptomatic drugs, without improvement. Four days later, he was admitted to a private hospital in Rio de Janeiro with worsening of his clinical picture and the appearance of a generalized maculopapular rash, hepatosplenomegaly, and vomiting. There was no eschar. Antimicrobial therapy directed to arenaviruses, community sepsis, and rickettsiosis was initiated, but he developed renal and respiratory failure. Laboratory examination revealed anemia, white blood cells with 50% band forms, and thrombocytopenia (platelet count 69 × 109/L).

Increased transaminases, lactic dehydrogenase, and alkaline phospatase (>1000 U/L) levels were observed. His clinical state deteriorated, and he died with multiple organ failure after 7 days of symptoms. A viral hemorrhagic fever was included in the differential diagnosis because in October 2008, the patient had had a possible contact with a fatal illness associated with a new arenavirus in South Africa (Paweska et al., 2009). Given this possibility, World Health Organization authorities were notified and local health authorities implemented recommendations of the International Sanitary Regulation. Parasitological, bacteriological, and virological analyses were conducted in blood and post-mortem liver biopsy samples. Serological tests for SFGR, dengue, yellow fever, leptospirosis, hantavirus, and arenavirus were all negative. Blood, urine cultures, and blood smears for malaria and other

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parasites were also negative. Blood samples were tested by PCR for arenavirus, hantavirus, and rickettsiae. Segments of rickettsial genes htrA (246 bp), ompA (532 bp), ompB (650 bp), and gltA (381 bp) were amplified (Rozental et al., 2006; Zhu et al., 2005), and the nucleotide sequences of the ompA and gltA amplicons were analyzed: the nucleotide sequences of the gltA amplicon (325 nt) and of the ompA amplicon (491 nt) exhibited 100% sequence similarity to the homologous gltA gene of *R. conorii* (GenBank accession no. HM152564) and outer membrane protein A (OmpA) gene fragment of *R. conorii conorii* (GenBank accession no. GU256251), respectively. Biopsy specimens of the liver were tested by immunohistochemical assay for SFGR using a polyclonal anti-*R. rickettsii* antibody (Rozental et al., 2006) and showed SFGR antigens in perivascular foci inflammation.

3. Discussion

Rickettsiosis is an endemic condition in many areas of the world, and tick-borne spotted fever rickettsioses have repeatedly been associated with febrile disease in travelers in the last 2 decades (Carzola et al., 2008; Font-Creus et al., 1991; Freedman et al., 2006; Gautret et al., 2009; Jensenius et al., 2004, 2009; Kun-Hsien et al., 2009; Rovery and Raoult, 2008; Wilson et al., 2007). Although most cases of international travel-associated rickettsioses acquired in sub-Saharan Africa, particularly in South Africa and neighboring countries, are caused by *R. africae* and *R. conorii* infections with unfavorable outcome have also been described in that area (Carzola et al., 2008; Freedman et al., 2006; Gautret et al., 2009; Wilson et al., 2007).

Although originally SFGR caused by *R. conorii*, the causative agent of the Mediterranean spotted fever, had been recognized as a benign illness, severe cases associated with renal and respiratory failure have been reported (Carzola et al., 2008; Font-Creus et al., 1991; Rovery and Raoult, 2008). This patient had no eschar, and its absence has been described in 14–40% of cases reported (Carzola et al., 2008; Font-Creus et al., 1991; Rovery and Raoult, 2008). In this context, the lack of a tick exposure report, the lack of an eschar, and the late onset of the rash contributed to the lack of any clinical suspicion possibly leading to the delayed introduction of a specific antimicrobial therapy and the fatal outcome. The molecular identification of *R. conorii conorii* in the clinical samples, an exotic rickettsia in Brazil, transmitted to humans by the dog tick *Rhipicephalus sanguineus* mostly in urban settings, confirms that the patient was infected in South Africa, where he had lived and spent more than 6 days before the onset of symptoms (Rovery and Raoult, 2008).

This case emphasizes the need for a high level of suspicion of SFGR since travel-related rickettsioses are not rare events. An alert about the possibility of the occurrence of SFGR in travelers in South Africa should be considered, and advice for tourists before travel should include precautions against tick bites and contact with animals.

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6. ARTIGO 3

RICKETTSIA SPP. INFECTION IN *RHIPICEPHALUS SANGUINEUS* TICKS IN A BRAZILIAN SPOTTED FEVER ENDEMIC RURAL AREA IN RIO DE JANEIRO STATE, BRAZIL.

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Rickettsia spp. infection in *Rhipicephalus sanguineus* ticks in a Brazilian spotted fever endemic rural area in Rio de Janeiro state, Brazil

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INTRODUCTION

Brazilian spotted fever (BSF) is a life-threatening zoonotic tick-borne disease, caused by *Rickettsia rickettsii*, which is transmitted to humans through the bite of infected ticks. BSF is the most prevalent rickettsial disease in Brazil. *Amblyomma cajennense* ticks are considered as being its main vector and reservoir. The immature stage of *A. cajennense* is usually a parasite on humans and is considered to be an eclectic tick, because it feeds on different animal species. *Rhipicephalus sanguineus*, although considered as a vector of *R. rickettsii* in eastern Arizona and Mexico, is not the common vector for BSF [1]. In Brazil, *R. sanguineus* is usually found on dogs from urban and rural environments. BSF is described in several regions of southern Brazil, mainly in the states of Minas Gerais, Rio de Janeiro and São Paulo [2]. In Rio de Janeiro state, since 1970, suspected and confirmed cases of BSF have been detected. Barra do Pirai is a city belonging to the Rio de Janeiro state and 154 km away from the capital. It has been considered to be an endemic area of BSF since 2004, when our group characterized *R. rickettsii* from a human fatal case [3]. In this report, the authors investigate the presence of *Rickettsia* spp. in ticks collected in this area by molecular analysis and show their possible implication as a vector of BSF in this endemic area.

MATERIAL AND METHODS

During the years 2002 and 2003, after notification of a young woman's death from an infectious disease compatible with BSF, a study was carried out. Ticks were collected from vegetation and dogs from 10 different localities in Barra do Piraí. *Rhipicephalus sanguineus* was the most abundant collected species. The ectoparasites were identified through a stereoscopic microscope using Aragão and Fonseca's taxonomic keys for ticks [4]. Weeks after, a male house servant presented with a similar infectious disease, which evolved to death. He was submitted to necropsy and immunohistochemical staining suggested a spotted fever group rickettsiae and sequence analysis showed identity to *R. rickettsii* [3].

DNA was extracted from individual ticks by freezing them in liquid nitrogen and crushing with a sterile micropestle, resuspended with 20 µL of sterile brain heart infusion (BHI) and stored at −20°C until nucleic acid extraction. Total DNA was extracted from the pulverised ticks using QIAamp DNA Mini Kit (QIAGEN™ Hilden, Germany). The DNA was divided into pools organised by locality, species and sex and screened for the presence of *Rickettsia* DNA by PCR using four sets of primers: Rr190.70p/Rr190.602n (OmpA – 532 bp), BG1-21/BG2-20 (OmpB – 650 bp), Tz15/Tz16 (17 kDa – 246 bp) and RpCS.877p/RpCS.1258n (gltA – 381 bp) [5]. If a pool demonstrated an expected PCR product, DNA of each tick specimen that made part of that pool was individually tested.

PCR conditions consisted of an initial DNA denaturation and hot start at 95°C for 5 min, followed by 40 consecutive cycles of 40 s denaturation at 95°C, primer annealing at 55°C for 1 min, extension at 72°C for 1 min 10 sec, and a 7-min extension at 72°C. For each reaction, 8 µL of the DNA template from each individual tick sample were added to 2.5 µL PCR buffer (10× Invitrogen™, Carlshad, CA, USA), 1.2 µL of each primer (20 mM), 1.5 µL $MgCl_2$ (3 mM), 0.25 µL of dNTP mixture (20 mM), 0.25 µL Platinum Taq DNA Polymerase (5 U/µL Invitrogen™) and nuclease free water to a final volume of 25 µL. A total of 5 µL of DNA extracted from *R. rickettsii*-infected *A. cajennense* ticks was used as positive control. PCR products were stained by ethidium bromide and visualised by electrophoresis in 1% agarose gel.

RESULTS

A total of 1233 ticks were collected; 1017 belonged to *R. sanguineus* species, 1 to *A. aureolatum*, and 215 belonged to *Amblyomma* genus. Due to the viability of the samples, only 259 ticks were tested and divided into 52 pools. Thirty-six pools were positive and when individually tested showed expected bands in 85 ticks to OmpB and 17kDa. Eleven ticks showed expected bands to both primers (Table 1).

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Table 1. PCR results for the tick species and stages of development collected from vegetation and dogs in Barra do Piraí, State of Rio de Janeiro, Brazil

Species	Stage	No. of ticks analysed	Positive ticks with		
			OmpB primer only/number analysed (%)	17 kDa primer only/number analysed (%)	Both primers/number analysed (%)
Amblyomma sp.	Nymph	78	02 (2.56)	0 (0.00)	0 (0.00)
Amblyomma cajennense	Male	01	0 (0.00)	0 (0.00)	0 (0.00)
R. sanguineus	Male	47	15 (31.92)	03 (6.4)	03 (6.4)
R. sanguineus	Female	133	43 (32.33)	11 (8.3)	08 (6.02)
TOTAL		259	60 (23.2)	14 (5.41)	11 (4.25)

CONCLUSIONS

Although the DNA sequences from this study have not yet been characterized, the identification of 85 PCR-positive ticks confirms the participation of *R. sanguineus* as a possible vector of BSF in this endemic area and reinforces the importance of domestic dogs as potential infection amplifiers.

It is very important to notice that, in the same area, recently, our group characterized *R. rickettsii* in *R. sanguineus* ticks (Cunha NC, Fonseca AH, Rezende J, Rozenthal T, Favacho ARM, Barreira JD, Massard CL, Lemos ERS).

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7. ARTIGO 4

COXIELLA BURNETII, THE AGENT OF Q FEVER IN BRAZIL: ITS HIDDEN ROLE IN SERONEGATIVE ARTHRITIS AND THE IMPORTANCE OF MOLECULAR DIAGNOSIS BASED ON THE REPETITIVE ELEMENT IS*1111* ASSOCIATED WITH THE TRANSPOSASE GENE.

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Coxiella burnetii, the agent of Q fever in Brazil: its hidden role in seronegative arthritis and the importance of molecular diagnosis based on the repetitive element IS*1111* associated with the transposase gene

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Coxiella burnetii *is the agent of Q fever, an emergent worldwide zoonosis of wide clinical spectrum. Although* C. burnetii *infection is typically associated with acute infection, atypical pneumonia and flu-like symptoms, endocarditis, osteoarticular manifestations and severe disease are possible, especially when the patient has a suppressed immune system; however, these severe complications are typically neglected. This study reports the sequencing of the repetitive element IS*1111 *of the transposase gene of* C. burnetii *from blood and bronchoalveolar lavage (BAL) samples from a patient with severe pneumonia following methotrexate therapy, resulting in the molecular diagnosis of Q fever in a patient who had been diagnosed with active seronegative polyarthritis two years earlier. To the best of our knowledge, this represents the first documented case of the isolation of* C. burnetii *DNA from a BAL sample.*

Key words: Q fever - bronchoalveolar lavage - molecular analysis - seronegative polyarthritis - methotrexate

Q fever is caused by *Coxiella burnetii*, a small obligate intracellular Gram-negative bacterium of the order Legionellales (Stein et al. 1993). *C. burnetii* has a variety of hosts, including many vertebrates and ticks. The transmission of *C. burnetii* in humans typically occurs via the inhalation of contaminated aerosols from fresh or desiccated urine, faeces, milk and birth products; less commonly, transmission occurs through the consumption of raw milk and milk products (Maurin & Raoult 1999, Tissot-Dupont & Raoult 2008).

The incubation period for *C. burnetii* is variable, depending on the infecting dose and the health status of the patient. Primary infection with *C. burnetii* is commonly asymptomatic. In the symptomatic acute disease, flu-like syndrome, pneumonia and hepatitis are considered to be the classic presentations, but rash, pericarditis, myocarditis, aseptic meningitis, encephalitis and osteomyelitis have also been described. Fatalities are rare and are typically associated with other debilitating health conditions. In most patients, the acute disease either has a self-limited course or responds to appropriate therapy (Maurin & Raoult 1999, Tissot-Dupont & Raoult 2008). Following the acute phase of Q fever, a small number of patients present symptoms of Q fever fatigue syndrome (QFS), characterised by headache, joint and muscle pain and fatigue (Ayres et al. 1996, Raoult et al. 2000, Pappas et al. 2003, Arashima et al. 2004, Hickie et al. 2006, Ledina et al. 2007). Chronic Q fever occurs in 1% of infected patients months or years after the initial infection. The most common form of chronic Q fever is endocarditis, which is typically associated with an underlying valvulopathy and immunosuppression. Less-common presentations of chronic Q fever include granulomatous lesions in the bones, joints, liver, lung, testis and soft tissues (Ralph et al. 2007, Tissot-Dupont & Raoult 2008). Studies have shown that *C. burnetii* can be reactivated during pregnancy and in patients with immunosuppression. Studies suggest that *C. burnetii* persists after most instances of acute Q fever, regardless of clinical status and that immunogenic variation in the response to persistent infection can lead to cytokine dysregulation (Penttila et al. 1998, Harris et al. 2000, Pappas et al. 2003, Ledina et al. 2007).

Although impaired T-cell immunity in patients with human immunodeficiency virus (HIV), cancer, lymphoma and pregnancy has been associated with the failure to eradicate *C. burnetii* and progression to the chronic disease, there are no previous reports of an association between Q fever and the use of methotrexate (MTX) for immunosuppressive therapy (Maurin & Raoult 1999, Nausheen & Cunha 2007). Low doses of MTX have been used effectively for various rheumatic and non-rheumatic diseases and opportunistic infections caused by *Histoplasma, Cryptococcus, Nocardia, Mycobacterium*, human herpesvirus, the hepatitis virus and the Epstein-Barr virus in patients receiving MTX therapy (Boerbooms et al. 1995, Feng et al. 2004, Angit & Daly 2009, McLean-Tooke et al. 2009). This article shows that

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Q fever, confirmed by molecular and serological analysis, is a probable complication of immunosuppression resulting from the use of MTX.

On 13 October 2010, a 33-year-old man from Rio de Janeiro, Brazil, was admitted to a private hospital with symptoms of a fever, myalgia and a dry cough. Notably, the patient's medical history showed that he had been diagnosed with seronegative polyarthritis two years earlier and treated with immunosuppressive therapy using MTX 40 days before hospitalisation. Initial tests revealed that the patient had a white blood cell count of $5 \times 10^9 L^{-1}$, with 18% band forms and C-reactive protein (CRP) levels of 310 mg L^{-1} (> 10 mg L^{-1}). The chest radiograph showed consolidation of the lower lobe of the right lung. MTX therapy was suspended and treatment with amoxicillin/clavulanic acid and azithromycin was started. The patient did not respond to antibiotics and five days later, his chest X-ray showed an increase in bilateral pulmonary infiltrates. His condition continued to deteriorate, with progressive hypoxia requiring an elective intubation. In the intensive care unit, additional tests revealed a total leukocyte count of $10.48 \times 10^9 L^{-1}$, a CRP level of 349 mg L^{-1} and a normal transthoracic echocardiogram. The antibiotic regimen was changed to meropenem, amphotericin, ceftriaxone, vancomycin and doxycycline. The blood and bronchoalveolar lavage (BAL) samples were analysed using culture techniques and were negative for the usual bacteria and fungi. Additional laboratory tests - serological and/or molecular diagnostic assays - were also negative for HIV, cytomegalovirus, Human adenovirus, human herpesvirus, *Rickettsia* spp and *Bartonella* spp.

Two serum samples, collected on days 7 and 27 of the illness, were tested for *C. burnetii* using a commercial indirect immunofluorescence assay (IFA) for class-specific IgM and IgG, cut off = 64 (Panbio, Brisbane, AU). Titres of IgM antibodies against *C. burnetii* (phase II) were detected in the serum samples at a titre of 64 and Q fever was confirmed in the second serum sample, with *C. burnetii* serum titres of 512 for phase I and II IgG. The *C. burnetii* DNA sequences were detected by polymerase chain reaction (PCR) performed on the serum and BAL samples collected on day 7 of the illness, using the primers QBT-1 (5'-TATGTATCCACCGTAGCCAGC-3') and QBT-2 (5'-CCCAACAACACCTCCTTATC-3'), which amplify a 687 bp fragment of the repetitive element IS*1111* of a heat shock protein gene (*htp*AB transposase) (Hoover et al. 1992). The PCR was repeated without the positive control and the results were confirmed. The amplicons were purified and the sequencing was performed using an ABI PRISM BigDye Terminator v.3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). The resulting partial sequences were subjected to BLAST analysis and the nucleotide sequence generated from the BAL sample (585 bp) showed 99% sequence identity with the homologous gene fragment of the *htpAB* gene from *C. burnetii* RSA 331, complete genome (GenBank accession JF968204).

A detailed epidemiological history was obtained and exposure to dogs and cats, some of them parturient, was identified during the two years preceding the illness, when the patient moved to a new office located in front of a veterinary clinic. During this time, after a severe flu-like disease, the patient developed seronegative polyarthritis.

The patient recovered and was discharged from the hospital and followed up as an outpatient. Doxycycline was administered to the patient for 14 days and this treatment was extended for an additional week because there was a recurrence of fever. During the outpatient follow-up, the patient developed no signs of Q fever and fully recovered from the osteoarticular manifestations. Subsequently, serum samples were obtained six-nine months after the patient's discharge from the hospital and additional IFA analysis was performed. The serum collected on month 6 was reactive to the phase I and II *C. burnetii* antigen, with IgG titres of 2.048 (phase I and II) and phase II IgM titres of 64. In the serum sample taken on month 9, IFA showed *C. burnetii* phase I and II IgG titres of 512 and the IgM titre was negative.

Although the patient's symptoms had resolved, the IgG antibody titre against the *C. burnetii* phase I and II antigen remained elevated for nine months after successful treatment with doxycycline. Because the most important criterion in monitoring a recurrence of Q fever would be an increase in the IgG titres (4-fold), the patient should be monitored for this possibility every three months.

In our report, the initial etiological diagnosis of a lobar pneumonia in this patient was limited to community-acquired pneumonia, more specifically the pneumonia caused by pneumococcus. Spotted fever and Q fever, among other infectious diseases, were also initially considered because he had a recent history of animal exposure during an international trip to South Africa.

Initially, the patient received amoxicillin/clavulanic acid and azithromycin, but remained febrile and developed hypoxia and acute bilateral pulmonary infiltrates. Therefore, the antibiotic regimen was changed and treatment with broad-spectrum antibiotics in conjunction with doxycycline resulted in a rapid clinical improvement. Studies have shown that patients treated with azithromycin remained febrile for multiple days compared with one day for patients treated with doxycycline (Ralph et al. 2007, Dijkstra et al. 2010).

Although there are reports of opportunist pulmonary infections in patients with defective cellular immunity who were treated with low doses of MTX, these cases are unusual and possibly underdiagnosed; in these cases, as was relevant in this study, MTX therapy was discontinued. We reported a patient with a history of seronegative polyarthritis whose immunosuppressive MTX treatment likely induced the reactivation of *C. burnetii* and led to a severe pneumonia.

Considering that the *C. burnetii* infection was likely acquired in his workplace and the doxycycline therapy resulted in a clinical improvement with complete resolution of the polyarthritis, could this seronegative rheumatologic disease have been diagnosed as QFS before the MTX therapy? There are studies that support this hypothesis (Harris et al. 2000, Raoult et al. 2000, Pappas et al. 2003, Arashima et al. 2004, Hickie et al. 2006, Ledina et al. 2007).

To our knowledge, this paper reports the first documented case of *C. burnetii* present in a BAL sample obtained from a patient after MTX therapy. At present, *C.*

burnetii has only been isolated from urine, semen and bone marrow samples obtained from patients with Q fever (Kruszewska et al. 1996, Maurin & Raoult 1999, Marmion et al. 2005).

Q fever is not a common diagnosis and it may easily be overlooked in immunocompetent or immunodeficient individuals and pregnant women, being misinterpreted as several other infectious diseases. Q fever should be added to the list of diseases known as probable complications of immune suppression and PCR based on the repetitive element IS*1111* of the transposase gene could be used as an important diagnostic method for Q fever.

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8. ARTIGO 5

Q FEVER AS A CAUSE OF FEVER OF UNKNOWN ORIGIN AND THROMBOCYTOSIS: FIRST MOLECULAR EVIDENCE OF *COXIELLA BURNETII* IN BRAZIL.

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Q Fever as a Cause of Fever of Unknown Origin and Thrombocytosis: First Molecular Evidence of *Coxiella burnetii* in Brazil

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Abstract

We report a case of Q fever in a man who presented with fever of 40 days duration associated with thrombocytosis. Serological and molecular analysis (polymerase chain reaction) confirmed infection with *Coxiella burnetii*. A field study was conducted by collecting blood samples from the patient's family and from the animals in the patient's house. The patient's wife and 2 of 13 dogs showed seroreactivity. Our data indicate that *C. burnetii* may be an underrecognized cause of fever in Brazil and emphasize the need for clinicians to consider Q fever in patients with a febrile illness, particularly those with a history of animal contact.

Key Words: Brazil—Fever of unknown origin—Q fever—Serologic and molecular diagnosis—Thrombocytosis.

Q FEVER IS A WORLDWIDE ZOONOSIS caused by *Coxiella burnetii*, a small obligate intracellular Gram-negative bacterium of the order Legionellales. The disease has a broad spectrum of clinical manifestations, ranging from a limited febrile illness, pneumonia, and hepatitis to life-threatening forms such as endocarditis and meningoencephalitis (Tissot-Dupont and Raoult 2008, Cunha et al. 2009). Ticks, farm animals, pets, and many wild animals are possible reservoirs of infection. Transmission to humans is usually via inhalation of contaminated aerosols from urine, feces, milk, and birth products or less commonly by ingestion of unpasteurized milk from infected farm animals. Occupational groups such as veterinarians, farmers, and slaughterhouse workers are at highest risk. Although Q fever is distributed throughout the world, its distribution in Brazil, where it is not a reportable disease, is poorly defined.

Since the first seroepidemiologic study was published in 1953, little additional information has become available and only four cases associated with endocarditis have been confirmed by serology or Gimenez staining of valves (Brandão et al. 1953, Travassos et al. 1954, Ribeiro do Valle et al. 1955, Riemann et al. 1974, Costa et al. 2006, Siciliano et al. 2008, Lamas et al. 2009).

On October 13, 2008, a 47-year-old man from Itaboraí, state of Rio de Janeiro, in southeastern Brazil, was admitted to the Gaffrée Guinle University Hospital/UNIRIO with a fever of >40 days duration, associated with abdominal pain, headache, nausea, fatigue, malaise, and depression. Physical examination was unremarkable, except for abdominal pain on palpation.

Laboratory exams revealed a high erythrocyte sedimentation rate (82 mm in the first hour), leukocytosis (13,100/mm^3) with neutrophilia (74%), and thrombocytosis (611,000/mm^3). Bone marrow aspiration showed slight hyperplasia of the monocyte–macrophage system and granulocytic cells. Echocardiogram and tomographic scans of the abdomen and thorax were normal. Treatment with a fourth-generation cephalosporin (cefepime 4 g/day) was begun, but without improvement.

The patient reported that 3 months before falling ill he had purchased 12 goats (Saneen breed), some of them for personal milk supply and some to sell to the community. The goats

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Partial data of this study were presented as a poster at the XLV Brazilian Society of Tropical Medicine Meeting, Porto Alegre, Rio Grande do Sul, Brazil, March 2009.

were kept together with other animals, 14 dogs and 2 cats. Additionally, the patient had a history of contact with the birth products of three goats that had aborted, 3 weeks before the onset of the symptoms.

Because of a high clinical suspicion of brucellosis, treatment with doxycycline (200 mg/day) and rifampin (600 mg/day) was begun and the fever disappeared in 4 days.

Serological testing for brucellosis, toxoplasmosis, leishmaniasis, cytomegalovirus, hepatitis B and C, syphilis, bartonellosis, ehrlichiosis, rickettsiosis, and histoplasmosis and detection of circulating capsular polysaccharide antigen from *Cryptococcus neoformans* performed on the first available serum sample collected on 40th day after onset of illness were negative. Mycobacteria, leishmania, and fungi cultures were also negative. Two serum samples collected 40 and 70 days after onset of the illness were tested for *C. burnetii* using a commercial indirect immunofluorescence assay for IgG (Panbio). Titers of specific antibodies to phase II antigen of 256 and 1024 were detected in the first and second serum samples, respectively. After DNA extraction using a commercial kit (QIAamp DNA; Qiagen), polymerase chain reaction (PCR) was performed and heat shock proteins genes (htpAB) of *C. burnetii* (687 bp) were amplified from the first serum sample DNA (Hoover et al. 1992). The PCR was repeated without a positive control and the result was confirmed, but DNA sequencing of the amplicon detected was not possible because of insufficient serum samples (Fig. 1). Rifampin was discontinued and the patient was treated with doxycycline for 21 days.

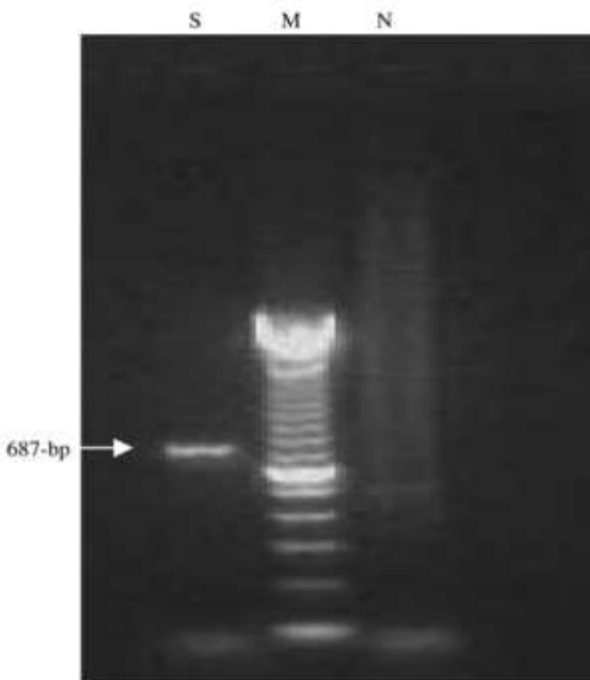

FIG. 1. Agarose gel electrophoresis of *Coxiella burnetii* polymerase chain reaction product amplified from total DNA of serum sample. S, sample from the patient with Q fever; M, molecular size markers (100-bp ladder); N, negative control. The arrow indicates the amplification of a 687-bp htpAB fragment.

In December 2008, a field investigation was carried out in the patient's home and blood samples from his family (wife and daughter) and 13 dogs were collected for analysis. His wife, who handled and fed them goat milk, told the investigators that one of their dogs (a 7-month-old female) had died, and another had aborted puppies, while the patient was in hospital. The wife was seroreactive for *C. burnetti* (titer of anti-phase II IgG of 128) and 2 of the 13 dogs showed indirect immunofluorescence assay (IFA) reactivity (titers of anti-phase II IgG of 64 and 128) but displayed no clinical manifestations. Unfortunately, the goats had been sold to another farmer so biological samples from these animals were not available for analysis.

All the symptoms were resolved and the patient was discharged at 4 weeks after admission. A third sample of the patient's serum collected at 6 months after the onset of illness was analyzed and showed an IgG titer of 128 against phase II antigens of *C. burnetii*.

Although several classical clinical descriptions of Q fever have been recognized in different regions of the world, some atypical and severe forms can be difficult to identify. Fever of unknown origin, clinical pictures mimicking systemic inflammatory disease, or lymphoproliferative disorders have also been described (Tissot-Dupont and Raoult 2008, Cunha et al. 2009). This article reports a case of Q fever presenting as fever of unknown origin and thrombocytosis that recovered after 3 weeks of treatment with doxycycline. Although thrombocytopenia occurs in about 25% of patients in the early phase of the illness, the presence of reactive thrombocytosis has also been described during its later phase (Tissot-Dupont and Raoult 2008, Cunha et al. 2009).

Studies of *C. burnetii* in humans and animals are frequently based on serologic tests, and the prevalence of *C. burnetii* infection varies widely from one country to another (Tissot-Dupont and Raoult 2008). Our findings provide definitive confirmation of Q fever in Brazil, where there are no molecular studies documenting *C. burnetii* infection in humans or animals. Anti-*C. burnetii* antibodies were also detected in the patient's wife and in two dogs, providing further evidence of the circulation of *C. burnetii* in Itaboraí, Rio de Janeiro, Brazil.

Although the PCR results were positive, DNA sequencing of the detected amplicon was not possible because of lack of sufficient biological material in the first serum sample.

Further studies including molecular characterization of *C. burnetii* are necessary to establish the extent and the importance of Q fever in Brazil.

Disclosure Statement

No competing financial interests exist.

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9. ARTIGO 6

MOLECULAR SURVEY OF *RICKETTSIA*, *BARTONELLA* AND *COXIELLA BURNETII* IN WILD SMALL MAMMALS IN RIO DE JANEIRO, BRAZIL.

Manuscrito a ser encaminhado como SHORT REPORT

Running title: *Rickettsia, Bartonella* and *Coxiella* in wild mammals in Brazil

MOLECULAR SURVEY OF RICKETTSIA, BARTONELLA AND COXIELLA BURNETII IN WILD SMALL MAMMALS IN RIO DE JANEIRO, BRAZIL.

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Abstract

This study reports the molecular survey to detection and genetic characterization of *Rickettsia* spp., *Coxiella burnetii* and *Bartonella* spp. in wild small mammals, in Piraí, Rio de Janeiro State, Brazil

Rickettsia spp., *Bartonella* spp., and *Coxiella burnetii* infect a wide range of species of arthropods, domestic and wild vertebrates, including mainly small wild rodent, marsupials, among other, over around the World. In Brazil, since the first studies on *Rickettsia* in wild mammals were published during the 1950's decade, overall publications on these proteobacteria infections have been restricted basically to domestic animals and arthropods (Magalhães, 1953, Lemos et al 1996). During a period of 10 years (2001-2011), over 860 cases of Brazilian spotted fever (BSF), caused by *Rickettsia rickettsii,* were reported (Brazilian Ministry of Health, unpublished data). Q fever, caused by *Coxiella burnetii*, and bartonellosis, more specifically scratch cat disease (SCD), have been also identified in several states though they are not notifiable diseases in Brazil. In Rio de Janeiro State, where has accumulated the third highest number of BSF cases in Brazil, an increasing number of Q fever and SCD cases, associated frequently with goat and cat, respectively, have been identified in different

regions in this state. In this scenario, despite the availability of an increasing number of studies on these arthropod-borne agents in Brazil, few studies have been carried out on the distribution and incidence of these agents among small wild vertebrates. The aim of this study was to investigate the role these vertebrates in the maintenance of *Rickettsia* spp., *C. burnetii* and *Bartonella* spp. in an endemic area for BSF, in Rio de Janeiro, Brazil.

Between 2009 and 2010, a research project on infectious agents in wild small vertebrates was carried out in the Municipality of Piraí (22°37' S, 43°53' W) where wild small were collected with live traps and submitted to necropsy as previously published (Oliveira et al, 2011, 2012; Travassos da Rosa et al 2010). Voucher specimens of animals were deposited in the collection of Laboratory of Biology and Parasitology of Wild Mammals Reservoirs IOC/FIOCRUZ, Rio de Janeiro, RJ, Brazil. A total of 123 small wild animals of 17 different species were captured; *Didelphis aurita* (n = 64; 50,4%), Diphylla eucadata (n=8), *Oligoryzomys nigripes* (n=8), *Marmosops paulensis* (n=6), *Oxymycterus dasythricus* (n=6), *Micoureus paraguayanus* (n=5), *Akodon cursor* (n=4), *Philander frenatus* (n=4), *Sturnira lilium* (n=3), *Carollia perspicillata* (n=3), *Artibeus lituratus* (n=2), *Euryoryzomys russatus* (n=2), *Metachirus nudicaudatus* (n=2), *Nectomys squamipes* (n=2), *Oligoryzomys* sp. (n=2), *Dasypus* sp. (n=1) and *Sphiggurus villosus* (n=1). Taxonomic identification of the rodents was performed by combined morphologic features and molecular analysis using mitocondrial DNA sequencing of Cytocrome B gene (Bonvicino 2001). DNA was extracted from spleen and liver tissues of the animals and tested for the presence of *C. burnetii,* and *Rickettsia* sp. using PCR targeting respectively the repetitive element IS*1111* of a heat shock protein (*htpAB* transposase) gene and the *omp*A and *glt*A genes, as previously described (Eremeeva et al 1994, Rozental et al 2012).

In relation to *Bartonella* spp., the extracted DNA was tested using newly designed primers Bar-gltA-F1-5'-GCTATGTCTGCVTTCTATCAYGA-3' and Bar-gltA-R1-5'-AGAACAGTAAACATTTCNGTHGG-3', targeting a specific fragment of the gene encoding the enzyme citrate synthase (*gltA*). The PCR was incubated at 95°C for 10 min to denature genomic DNA and the thermal cycle reaction programmed for 40 cycles of 30 sec at 95°C, 40 sec at 58°C, and 45 sec at 72°C, with a final extension step of 10 min at 72°C. PCR products were subjected to electrophoresis on a 1.5% agarose

gel and stained with GelRed™ (Biotium, Inc., CA, USA). The 731-bp PCR products were purified using Wizard®SV Gel and PCR Clean-Up System kit (Promega, Corp., Madison, WI, USA).

DNA *C. burnetii* was detected in *Oxymycterus dasythricus, Akodon cursor* and *Bartonella* spp. in *Euryoryzomys russatus*. All small mammals were negative for spotted fever group rickettsiae.

This is the first report of *C. burnetii* and *Bartonella* spp. natural infection in wild rodents in Brazil. The study is particularly relevant because indicate the presence of these zoonotic proteobacteria in wild rodents and alert to potential risk for human health in Brazilian territory where no data has been available regarding the presence of these proteobacteria in wild mammals. Further investigations are warranted in order to characterize the *Bartonella* spp. and verify if it can cause human infection.

Acknowledgments

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Author Disclosure Statement

No competing financial interests exist.

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10. DISCUSSÃO

Com os resultados obtidos nos artigos que compõem a tese é imprescindível reforçar alguns pontos considerados mais importantes que mostram, não somente a complexidade do assunto, mas também a necessidade incontestável de aumentar o nosso conhecimento em relação a este grupo de zoonoses transmitidas por artrópodes, cujo desconhecimento tem continuadamente determinado quadros clínicos graves e fatais, em especial com os casos da FMB.

10.1. A febre maculosa brasileira como doença ocupacional

A FMB pode ocorrer como caso isolado ou como pequenos surtos ("clusters") como verificado, por exemplo, no surto que ocorreu em Itaipava (2005) e Resende (2006) no estado do Rio de Janeiro. Neste novo surto no Município do Rio de Janeiro, onde cinco profissionais da Sociedade Protetora Internacional de Animais (SUIPA) morreram com FMB, serve de alerta também para o perfil de doença ocupacional que esta zoonose, eventualmente, pode apresentar. Assim, veterinários e outros profissionais que trabalham em abrigos de animais, *petshops*, canis, entre outras atividades nas quais há estreito contato com animais e seus ectoparasitas deveriam ser alertados e orientados para a possibilidade da ocorrência desta zoonose fatal, mas geralmente curável, se diagnosticada e tratada precocemente.

Três funcionários que participaram do inquérito sorológico, um deles referia quadro prévio de febre, cefaleia e mialgia. A possibilidade de FMB, com um quadro menos grave, não foi descartada, mas infelizmente, não foi possível realizar nova coleta de sangue do 4º paciente que, apesar de apresentar manifestação clínica, não precisou ser internado.

Quanto à ocorrência do quinto caso de FMB, um ano após os quatro casos de 2011, aponta para a manutenção local da circulação da bactéria nos carrapatos, fato que deve ser fortemente considerado e que, consequentemente, necessita servir também de alerta para que, em caso de doença febril aguda em funcionário da SUIPA e em população residente no

entorno da instituição, a FMB seja incluída no diagnóstico etiológico de imediato, enquanto se investiga a possibilidade de outras doenças como dengue e leptospirose.

Ainda neste contexto de doença ocupacional, a febre Q assim como a bartonelose também devem ser consideradas. Nas duas publicações sobre a febre Q, a exposição a animais - na primeira publicação a partir de ruminantes em área rural do Município de Itaboraí e a segunda em área urbana cujo provável local de infecção foi um *petshop* na zona sul do Município do Rio de Janeiro - sugere a necessidade de sensibilização destes grupos potencialmente expostos a estes agentes.

10.2. Diagnóstico diferencial

Todos os casos de FMB apresentados neste trabalho reforçam a dificuldade do exercício do diagnóstico diferencial com duas endemias em nosso território: dengue e leptospirose. Considerando a complexidade de se pensar em FMB em área de transmissão ativa de dengue, por exemplo, somente com uma adequada sensibilização da classe médica será possível reverter esta elevada taxa de letalidade da FMB que se tem observado em nosso estado. Uma história epidemiológica adequada e a informação de contato com animais e picada de carrapatos, além de viagem, devem servir de alerta para o início de antibioticoterapia específica de forma empírica em um paciente com quadro clínico "influenza-like", compatível com FMB.

No surto da FMB na SUIPA, o contato com os cães deveria também servir de alerta, já que estes animais, assim como os equinos, são considerados animais sentinelas.

Ainda em relação ao surto da SUIPA, é necessário chamar a atenção para o curto período de evolução dos casos para o óbito - 5 a 8 dias e a falta de informação sobre a ocorrência de exantema. Poderia ser uma variante de *R. rickettsi* mais virulenta? Seria a evolução rápida decorrente do uso prévio de sulfa, droga esta associada com pior prognóstico por facilitar o metabolismo bacteriano? Infelizmente, não foi possível obter informações sobre uso de antibioticoterapia nos três primeiros casos, assim como não foi possível verificar a presença de outros fatores que poderiam estar

associados com esta rápida evolução para o óbito como a deficiência de glicose 6-fosfato desidrogenase ou a história de alcoolismo.

10.3. Febre maculosa como doença do viajante

No artigo sobre a febre maculosa fatal causada por *R. conorii* em um paciente sul-africano em 2008 no Brasil, além de confirmar a importância de incluir esta doença do carrapato no diagnóstico diferencial de doenças febris agudas associadas com exantema em paciente com história de viagem, reforça os dados disponíveis na literatura científica, na qual continuadamente se comprova que nos casos febris procedentes dos países da África subsaariana, em especial, a África do Sul, o diagnóstico das rickettsioses deve obrigatoriamente ser considerado, além da malária, da dengue ou da febre tifoide (Freedman et al 2006, Jensenius et al 2004, 2009).

10.4. Os ectoparasitas

Embora as espécies de carrapato do gênero *Amblyomma*, especialmente a espécie *A. cajennense*, sejam consideradas as mais importantes na transmissão da infecção de rickettsias para a população humana, a identificação molecular de *Rickettsia* em exemplar de *R. sanguineus*, espécie que parasita especificamente o cão no Brasil, demonstra a complexidade do ciclo de manutenção das rickettsias na natureza. Curiosamente, *R sanguineus* além de ser a espécie mais importante na transmissão da febre maculosa na Europa e na África, na última década foi identificado como vetor também em território norte-americano (Demma et al 2005). Este achado foi ratificado com outro trabalho desenvolvido com o grupo do LHR e que também foi publicado em 2009 (Cunha et al 2009). Qual a importância da participação desta espécie de carrapato na transmissão humana e o motivo pelo qual não é o vetor no Brasil, são perguntas, entre tantas outras que precisam ser respondidas.

10.5. Febre Q como doença urbana

Nos últimos anos, maior atenção tem sido dada a febre Q no Mundo, em decorrência dos surtos com grande número de pacientes que têm ocorrido no continente europeu, onde mais de 4.000 casos foram confirmados (Van der Hoek et al, 2010, Van der Hoek et al 2011, Van der Hoek 2011a, Hilbert et al 2011, Roest et al 2011). Nestes dois trabalhos apresentados sobre febre Q, é necessário esclarecer que são os dois primeiros registros de casos identificados por técnica molecular no Brasil, com detecção do genoma bacteriano, considerando que os poucos casos confirmados previamente foram com base em evidência sorológica e a partir da análise de uma única amostra de soro (Costa et al 2006, Brandão et al 1953, Lamas et al 2009).

Apesar do crescente número de casos que vem sendo confirmados, especialmente no estado do Rio de Janeiro, além dos estados de Minas Gerais, Tocantins e São Paulo, a febre Q não é considerada uma doença de notificação obrigatória em nosso país, fato que dificulta uma adequada vigilância desta zoonose que deve ser considerada negligenciada.

No terceiro artigo sobre febre Q, cujo local provável de infecção do caso confirmado foi no petshop, está de acordo com os dados de literatura que mostram a ampla diversidade de animais que participam do ciclo de *C. burnetii*, incluindo animais de companhia, com história de parto e aborto recentes (Buhariwalla et al 1999, Cooper et al 2011, Nagaoka et al 1998, Pinsky et al 1991, Porter et al 2011.)

10.6. Animais silvestres, Bartonella, Coxiella burnetii e Rickettsia

A escassez de informações sobre estes agentes em animais silvestres no território brasileiro estimula e reforça a importância desta linha de pesquisa. Complexo e dependente da integração de um grande número de profissionais com perfis de conhecimento diferentes, esta etapa do trabalho exigiu não somente a participação da autora em alguns trabalhos de campo, mas fundamentalmente da colaboração de mastozoólogo, taxonomista, entre outros.

Considerando que somente as amostras dos animais silvestres capturados no Município de Piraí foram incluídos neste artigo, os resultados apresentados nesta pequena nota, nos permite vislumbrar a possibilidade de termos um pouco de conhecimento sobre estes agentes e assim, possibilitar, consequentemente, que diante das informações obtidas, tanto a febre Q quanto as bartoneloses possam ser consideradas doenças de notificação compulsória no Brasil.

A falta de amplificação de RGFM nas amostras dos animais silvestres foi de certa forma um resultado esperado, já que, diferente dos outros dois agentes - *C. burnetii* e *Bartonella* spp. - os vertebrados não são considerados reservatórios de rickettsias, que certamente atuam como amplificadores e fonte alimentar para os carrapatos que atuam como reservatórios e perpetuadores da infecção rickettsiana na natureza.

11. CONCLUSÕES

- ✓ A ocorrência simultânea da febre maculosa brasileira entre membros de mesmo grupo de indivíduos com atividade em comum na SUIPA permite caracterizá-la como doença ocupacional.
- ✓ A falta de diagnóstico e a não instituição imediata de antibioticoterapia específica para febre maculosa influiu diretamente no prognóstico dos pacientes tanto da SUIPA quanto no do paciente sul-africano.
- ✓ O curto período entre o início das manifestações clínicas e o óbito nos pacientes da SUIPA e a falta de exantema sugerem que *R. rickettsii* identificada em nosso estudo represente uma variante mais virulenta. A presença de deficiência de glicose 6-fosfato desidrogenase ou mesmo o uso prévio de sulfa não podem ser descartados como fatores associados.
- ✓ Dois indivíduos, sem história de doença, foram sororreativos (IgG) para febre maculosa no inquérito sorológico realizado na SUIPA. Esta evidência de infecção pregressa sugere a possibilidade de existência de infecções subclínicas ou inaparentes.
- ✓ Uma história epidemiológica de viagem à África e contato com animais identificados neste trabalho corroboram sobre a importância de incluir a febre maculosa no diagnóstico diferencial, principalmente em área de transmissão ativa de dengue e leptospirose, considerando a superposição das manifestações clínicas;
- ✓ Embora *R. sanguineus*, carrapato do cão, não seja o transmissor da febre maculosa para a população humana no Brasil, a identificação de infecção por *Rickettsia* confirma a sua importância na amplificação do ciclo, com a participação do cão, espécie considerada sentinela;
- ✓ A ocorrência de febre Q adquirida em área urbana confirma a necessidade de incluir o seu diagnóstico nos casos clínicos compatíveis com história de contato com material de parto ou de aborto de animais de companhia como cães e gatos.
- ✓ A identificação de *C. burnetii* e *Bartonella* spp. em animais silvestres confirma a complexidade do ciclo destes agentes assim como a necessidade de se obter mais informações sobre o assunto.

12. PERSPECTIVAS

- ✓ Finalizar a análise molecular nas amostras dos animais silvestres das outras áreas do Estado do Rio de Janeiro, nos municípios de Teresópolis, Três Rios e Valença, e posteriormente comparar filogeneticamente as sequências obtidas com as de amostras humanas.
- ✓ Encaminhar para publicação os artigos sobre o surto na SUIPA ("letter") e de animais silvestres no Município de Pirai (nota) para publicação em revista indexada internacional.
- ✓ Preparar mais dois artigos sobre a infecção destas proteobactérias nos animais silvestres no Estado do Rio de Janeiro cujos resultados ainda estão em fase de finalização.
- ✓ Colaborar com o desenvolvimento de outros projetos sobre febre Q e febre maculosa que estão sendo realizados no LHR, com ênfase na região de Itaboraí, Rio de Janeiro.

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14. ANEXOS

ANEXO 1: PARECER DO COMITÊ DE ÉTICA EM PESQUISA/FIOCRUZ

Ministério da Saúde
Fundação Oswaldo Cruz
COMITÊ DE ÉTICA EM PESQUISA-CEP/FIOCRUZ

Rio de Janeiro, 28 de abril de 2011.

Carta: 012/11

De: CEP/FIOCRUZ

Para: - Dra. Elba Regina Sampaio de Lemos e
- Dr. Christian Gabriel Niel

Prezados Senhores,

Estamos encaminhando o parecer do protocolo **559/10** intitulado "**Projeto de Pesquisa Associado às atividades de referência do laboratório de Hantaviroses e Ricketisioses do Instituto Oswaldo Cruz/FIOCRUZ**" com a deliberação de **APROVADO**.

Atenciosamente

Carla Dias Netto
Secretária Geral
CEP/Fiocruz

Comitê de Ética em Pesquisa em Seres Humanos
Fundação Oswaldo Cruz
Avenida Brasil, 4.036 - Sala: 705
Manguinhos - RJ - CEP.: 21.040-3[illegible]
Tels.: (21) 3882-9011 Fax: (21) 2561-48[illegible]
e-mail: etica@fiocruz.br

ANEXO 2. AUTORIZAÇÃO DO IBAMA

Ministério do Meio Ambiente - MMA
Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis - IBAMA
Sistema de Autorização e Informação em Biodiversidade - SISBIO

Licença permanente para coleta de material zoológico

Número: 13373-1	Data da Emissão: 19/11/2007 17:02

Dados do titular

Registro no Ibama: 608054	Nome: PAULO SÉRGIO D ANDREA	CPF: 062.639.198-92
Nome da Instituição : FUNDAÇÃO OSWALDO CRUZ		CNPJ: 33.781.055/0001-35

SISBIO

Observações, ressalvas e condicionantes

1	A participação do(a) pesquisador(a) estrangeiro(a) nas atividades previstas nesta autorização depende de autorização expedida pelo Ministério de Ciência e Tecnologia (CNPq/MCT);
2	A licença permanente não é válida para: a) coleta ou transporte de espécies que constem nas listas oficiais de espécies ameaçadas de extinção; b) manutenção de espécimes de fauna silvestre em cativeiro; c) recebimento ou envio de material biológico ao exterior; e d) realização de pesquisa em unidade de conservação federal ou em caverna. A restrição prevista no item d não se aplica às categorias Reserva Particular do Patrimônio Natural, Área de Relevante Interesse Ecológico e Área de Proteção Ambiental constituídas por terras privadas;
3	O pesquisador titular da licença permanente, quando acompanhado, deverá registrar a expedição de campo no Sisbio e informar o nome e CPF dos membros da sua equipe, bem como dados da expedição, que constarão no comprovante de registro de expedição para eventual apresentação à fiscalização;
4	Esta licença permanente não exime o seu titular da necessidade de obter as anuências previstas em outros instrumentos legais, bem como do consentimento do responsável pela área, pública ou privada, onde será realizada a atividade.
5	Esta licença permanente não poderá ser utilizada para fins comerciais, industriais, esportivos ou para realização de atividades inerentes ao processo de licenciamento ambiental de empreendimentos.
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7	O pesquisador titular da licença permanente será responsável pelos atos dos membros da equipe (quando for o caso)
8	O órgão gestor de unidade de conservação estadual, distrital ou municipal poderá, a despeito da licença permanente e das autorizações concedidas pelo Ibama, estabelecer outras condições para a realização de pesquisa nessas unidades de conservação.
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10	O titular da licença permanente deverá apresentar, anualmente, relatório de atividades a ser enviado por meio do Sisbio no prazo de até 30 dias após o aniversário de emissão da licença permanente.
11	O titular de autorização ou de licença permanente, assim como os membros de sua equipe, quando da violação da legislação vigente, ou quando da inadequação, omissão ou falsa descrição de informações relevantes que subsidiaram a expedição do ato, poderá, mediante decisão motivada, ter a autorização ou licença suspensa ou revogada pelo Ibama e o material biológico coletado apreendido nos termos da legislação brasileira em vigor.
12	A licença permanente será válida enquanto durar o vínculo empregatício do pesquisador com a instituição científica a qual ele estava vinculado por ocasião da solicitação.
13	Este documento não dispensa a obtenção de autorização de acesso ao componente do patrimônio genético ou ao conhecimento tradicional associado nos termos da legislação vigente.
14	As atividades contempladas nesta autorização NÃO abrangem espécies brasileiras constante de listas oficiais (de abrangência nacional, estadual ou municipal) de espécies ameaçadas de extinção, sobreexplotadas ou ameaçadas de sobreexplotação.

Táxons autorizados

#	Nível taxonômico	Táxon(s)
1	ORDEM	Rodentia, Didelphimorphia
2		

Destino do material biológico coletado

#	Nome local destino	Tipo Destino
1	Museu Nacional (UFRJ)	coleção

Este documento (Licença permanente para coleta de material zoológico) foi expedido com base na Instrução Normativa Ibama nº154/2007. . Através do código de autenticação abaixo, qualquer cidadão poderá verificar a autenticidade ou regularidade deste documento, por meio da página do Ibama/Sisbio na internet (www.ibama.gov.br/sisbio).

SISBIO

Código de autenticação: 91827146

Página 1/2

ANEXO 3: COMISSÃO DE ÉTICA NO USO DE ANIMAIS

MINISTÉRIO DA SAÚDE / FUNDAÇÃO OSWALDO CRUZ
VICE-PRESIDÊNCIA DE PESQUISA E DESENVOLVIMENTO TECNOLÓGICO
Comissão de Ética no Uso de Animais
CEUA-FIOCRUZ

CERTIFICADO

Certificamos que o protocolo intitulado :

" Avaliação da circulação de hantavirus entre roedores silvestres no estado do Rio de Janeiro e no estado do Paraná. "

número P-405/07, proposto por Paulo Sérgio D' Andrea, foi licenciado pelo Nº L-049/08.

Sua licença de Nº L-049/08 autoriza o uso anual de :

- 1540 Didelphimorphia *- 3350 Rodentia*

Esse protocolo está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA) e foi APROVADO pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA - FIOCRUZ). Na presente formatação, este projeto está licenciado e tem validade até 9 de junho de 2012.

Rio de Janeiro, 25/09/2008

Dra. Norma Vollmer Labarthe
Coordenadora da CEUA
FIOCRUZ

ANEXO 4: TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

MINISTÉRIO DA SAÚDE
FUNDAÇÃO OSWALDO CRUZ
INSTITUTO OSWALDO CRUZ
DEPARTAMENTO DE VIROLOGIA

LABORATÓRIO DE HANTAVIROSES E RICKETTSIOSES

Laboratório de Referência Nacional para Rickettsioses
Laboratório de Referência Regional para Hantaviroses

Termo de Consentimento Livre e Esclarecido (pequenos animais)

Com o objetivo de avaliar a circulação de Rickettsioses e Hantaviroses no Estado do Rio de Janeiro, eu ____ fui convidado a participar de um estudo que irá avaliar a presença de bactérias e vírus que pode causar doenças em animais domésticos como também no homem. A participação no estudo envolverá uma consulta veterinária e a coleta de cerca de 3 a 5 ml de sangue venoso periférico do meu animal de estimação ____________________, para estudo das infecções por *Bartonella*, *Rickettsia rickettsii*, *Coxiella burnetii*, *Ehrlichia* spp e pelos vírus transmitidos por roedores como hantavírus e arenavírus. Minha participação no estudo é totalmente voluntária, podendo ser interrompida a qualquer momento, e sem nenhuma forma de compensação financeira. Fui informado ainda que receberei os resultados dos exames. Autorizo ainda que amostras de sangue do meu animal de estimação sejam conservadas pelo Laboratório de Hantaviroses e Rickettsioses da Fundação Oswaldo Cruz para estudos futuros, desde que estes venham a ser autorizados pelo Comitê de Ética em Pesquisa da Fundação Oswaldo Cruz.

Caso tenha alguma dúvida ou necessidade de qualquer esclarecimento sobre o estudo, você pode entrar em contato com os pesquisadores relacionados abaixo:

Drª Elba Lemos
Laboratório de Hantaviroses e Rickettsioses
Pavilhão Hélio e Peggy Pereira 1º Pavimento, Sala B116 - Av. Brasil 4350- Rio de Janeiro
Fiocruz- Tel. 2562-1897

Rio de Janeiro, de de 20___.

___________________________________assinatura

1

ANEXO 5: OUTROS PRODUTOS GERADOS DURANTE A TESE

14.1. ARTIGOS EM REVISTAS INDEXADAS

14.1.1. Characterization of *rickettsia rickettsii* in a case of fatal Brazilian spotted fever in the city of Rio de Janeiro, Brazil.

Characterization of *Rickettsia rickettsii* in a Case of Fatal Brazilian Spotted Fever in the City of Rio de Janeiro, Brazil

Cristiane Lamas[1,2], Alessandra Favacho[1], Tatiana Rozental[1], Márcio N. Bóia[2], Andrei H. Kirsten[1], Alexandro Guterres[1], Jairo Barreira[1] and Elba Regina S. de Lemos[1]
[1]*Laboratory of Hantaviruses and Rickettsiosis (Fiocruz);* [2]*Department of tropical Medicine, Fiocruz; Rio de Janeiro, RJ, Brazil*

A lethal case of Brazilian spotted fever (BSF) is presented. Clinical features were initially of gastrointestinal involvement and evolved with progression to septic shock, meningoencephalitis and death on the 6th day of illness. Indirect immunofluorescence assay (IFA) for spotted fever group rickettsia (SFGR) was non-reactive. Diagnosis was confirmed by the polymerase chain reaction (PCR) and the nucleotide sequencing of a fragment of the ompA gene showed 100% homology to *Rickettsia rickettsii*. BSF has not been reported in the city of Rio de Janeiro in the last three decades, and the present description should alert the clinicians to its presence in urban Rio de Janeiro, and to the differential diagnosis with dengue fever, gastroenteritis, leptospirosis and bacterial septic shock, among others.
Key-Words: Brazilian spotted fever, spotted fever group rickettsia, *Rickettsia rickettsii*, lethal case, Rio de Janeiro city, indirect immunofluorescence, polymerase chain reaction, central nervous system involvement.

Brazilian spotted fever (BSF) is a systemic disease caused by *Rickettsia rickettsii*, a bacterium transmitted by the horse tick *Amblyomma cajennense*. It is endemic in the Southeast of Brazil (Rio de Janeiro, São Paulo, Minas Gerais and Espírito Santo states) and affects exposed children and adults [1-4]. Case presentation mimics several conditions which are endemic in the area, such as dengue fever, leptospirosis, gastroenteritis, meningococcal meningits and severe sepsis [1-4]. This paper reports a fatal case of BSF, occurring in July, in the metropolitan area of Rio de Janeiro, with prominent sepsis, rash and neurological and cerebrospinal fluid (CSF) findings.

Case Report

A 48 year-old white male presented, six days prior to hospital admission, with acute onset of high-grade fever, myalgia, headache, nausea, vomiting and diarrhea. He was a heavy smoker, but past medical history was otherwise unremarkable. He sought medical attention and was treated symptomatically. Two days later his symptoms persisted and he developed jaundice: he was given sulfamethoxazole-trimethoprim and was sent home. The following day he sought the Emergency ward because of hematemesis and melena; he was oliguric. He was transferred to an infectious diseases reference hospital with the presumptive diagnosis of leptospirosis. He was a porter in the north area of Rio de Janeiro, and had frequent contact with rodents. He had been bitten by ticks in Campo Grande (west area of Rio Janeiro) two weeks previously, while visiting his brother, who was a horse cart driver. On arrival he was jaundiced, in deep coma with no neck stiffness, systolic blood pressure was 60 mmHg, heart rate =128 bpm, and a generalized purpuric rash was noted. He was intubated, mechanically ventilated, given rapid intravenous fluid, noradrenaline, ceftriaxone, oxacillin and chloramphenicol. Myoclonus was observed, and two hours after admission he presented a generalized tonic-clonic seizure. Initial investigation showed leukocytosis (18,200 cells/mm^3, differential count: 58% polymorphs, 14% band forms), platelet count 90,000/mm^3, hemoglobin = 13.9 g/dL, glucose 129 mg/dL, creatinine 4.4 mg/dL, AST = 314 IU, ALT = 124 IU; blood gas analysis showed metabolic acidosis.

He died six hours after admission; post mortem lumbar puncture was performed. Cerebrospinal fluid (CSF) analysis showed 213 cells per 100 powerfields, with 70% of polymorphs, protein > 1 g/dL, glucose = 11 g/dL. Gram and Indian ink stains were normal. Latex tests for *Cryptococcus neoformans, Haemophilus influenzae, Neisseria meningitidis* and *Streptococcus agalactiae* were negative. CSF culture was negative at 48 hours; blood cultures were negative after seven days incubation.

Indirect immunofluorescence assays (IFA) for spotted fever group rickettsia (SFGR) (Panbio®)-specific immunoglobulin G (IgG) antibodies were performed on serum and CSF.

Polymerase chain reaction (PCR) was performed on whole blood and serum using four previoulsy described oligonucleotide primer pairs as shown in Table 1 and Figure 1 [5,6].

The sequence data of the PCR products were analyzed using the BLAST 2.0 program (National Center for Biotechnology Information) for homology search. The determined sequences were then analyzed for phylogenetic relationships with other sequences registered in the GenBank.

Family members of the index case, who lived where exposure to horse ticks occurred, were recalled for history, physical examination and serology for SFGR two months after the patient's death.

Ticks were captured from grass and garden near the house; they were identified taxonomically, and individually frozen at

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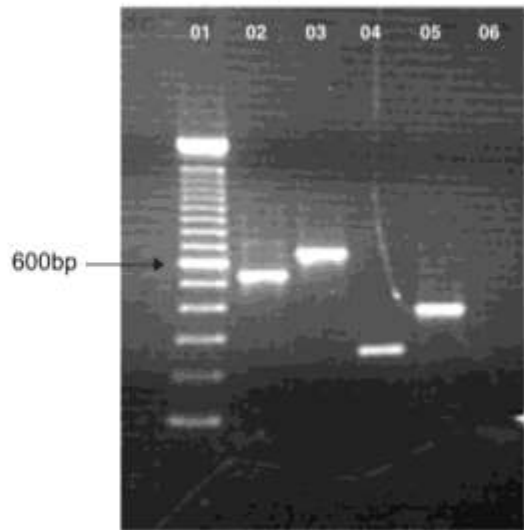
Table 1. Primers used for detection of spotted fever group rickettsia in a PCR assay [5,6]

Primer	Sequence (5'-3')	Amplicon size
Rr190-70	ATGGCGAATATTTCTCCAAAA	532 pb
Rr190-602	AGTGCAGCATTCGCTCCCCCT	
BG1-21	GGCAATTAATATCGCTGACGG	650 pb
BG2-20	GCATCTGCACTAGCACTTTC	
RpCS877	GGGGGCCTGCTCACGGCGG	381 pb
RpCS1258	ATTGCAAAAAGTACAGTGAACA	
TZ15	TTCTCAATTCGGTAAGGGC	246 pb
TZ16	ATATTGACCAGTGCTATTTC	

Figure 1. PCR analysis of whole blood of a fatal BSF case in the city of Rio de Janeiro. Agarose gel electrophoresis, with ethidium bromide, showed the 532 bp fragment amplified with the rompA-specific set of primers (lane 2). Lane 03 - 650 bp fragment amplified with the rompB-specific set of primers. Lane 04 - 246 bp fragment amplified with the 17 kDa-protein-specific set of primers. Lane 05 - 381 bp fragment amplified with the citrate synthase-specific set of primers. Lane 06 - negative control. Lane 01 - ladder 100 bp.



-70°C until processed for DNA extraction. No ticks were collected from horses as they had been applied insecticide.

IFA for SFGR was non-reactive in the patient´s serum and CSF. PCR from whole blood was positive for *R. rickettsii* and sequencing of the product amplified from the ompA gene showed 100% homology to *Rickettsia rickettsii* sequence available at the GenBank.

Eight family members, all of which lived in the same neighbourhood where the patient acquired his illness, were enrolled. Their ages varied between ten and 60 years None presented clinical illness in the preceding two months although all reported frequent tick bites.All had non reactive SFGR IFA.

Twenty-nine ticks (five *Amblyomma cajennense*, two *Riphicephalus sanguineus*, and 22 *Anocentor nitens*) were collected from grass and garden near the house. All ticks showed negative PCR results for SFGR.

Discussion

This previously healthy middle aged man presented clinical features of severe BSF following tick bites in the peak of tick reproduction, when most cases of BSF are reported. Several aspects deserve attention: he had features of a gastroenteritis-like illness and was given sulfonamides, and as previously described, this probably made his outcome worse. He progressed rapidly to acute renal failure, purpuric rash, septic shock and neurological symptoms, manifested as seizure and coma. His spinal fluid analysis showed neutrophilia and low glucose levels. CSF findings in spotted fever group rickettsial disease are diverse: pleocytosis with predominant polymorphs (as in this case), predominance of eosinophils or predominance of lymphocytes. CSF may also be unremarkable [4,7-10]. The predominant cell type in CSF may be related to the timing of CSF analysis; a report on tick-borne encephalitis showed an average of 570 cells with 60% polymorphs in the first three days of illness, and a predominance of mononuclear cells from the fifth day onwards [9].

Another issue relates to jaundice: it is noted more often in BSF than in Rocky Mountain spotted fever, possibly because more adult males (with hepatitis B or hepatitis C co-infection, alcohol use or G6PD deficiency) are present in Brazilian series [3].

Chloramphenicol was started intravenously when he was admitted to hospital because there is no intravenous formulation of doxycycline in Brazil, which would be the best choice in such a severe presentation.

Another important aspect to consider is the geographic location: BSF is no longer a rural endemy, but affects inhabitants of large cities such as Rio de Janeiro and São Paulo, making it important to train doctors to think of this diagnosis, as letality is high (over 40%) with delayed start of antibiotics. BSF is a public notifiable illness since 2001 in Brazil, and the public, including tourist agencies, need to be made aware. No cases had been reported in the city of Rio de Janeiro since 1981, although nearby towns, such as Petrópolis, sought for weekend leisure and tourism, has recently (in 2005) had an outbreak of BSF [11]. The patient's

history of tick bites was clear, but was not elicited until he was seen at a referral hospital.

The last case reported from the city of Rio de Janeiro occurred in 1977; the report [4] accounted for two cases, both involving young males (34 and 44 years old) with cutaneous vasculitis. One of them had meningoencephalitis with abnormal CSF findings. Both were cured with intravenous chloramphenicol.

A complementary search for serological *R. rickettsii* infection in household contacts (all with tick bites) and for molecular evidence of infection in ticks from the area was performed and the results were negative: this is not surprising, because family clusters of BSF occur rarely and the prevalence of rickettsia in ticks, even in endemic areas, is low [1].

Finally, although IFA is the gold standard for diagnosis, molecular diagnosis is crucial in these rapidly fatal cases as IFA may be negative since antibody detection usually takes over ten days [12-15]. The three rickettsial reference laboratories in Brazil, located in the states of Minas Gerais, Rio de Janeiro and São Paulo, are apt to perform molecular biology tests. BSF clinical aspects and the rickettsial reference labs' abilities to make molecular diagnosis ought to be made more widely known, mainly in endemic areas where other infectious diseases as leptospirosis and dengue fever may be misdiagnosed as BSF.

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14.1.2. Eschar-associated spotted fever rickettsiosis, Bahia, Brazil.

Eschar-associated Spotted Fever Rickettsiosis, Bahia, Brazil

Nanci Silva, Marina E. Eremeeva, Tatiana Rozental, Guilherme S. Ribeiro, Christopher D. Paddock, Eduardo Antonio G. Ramos, Alexsandra R.M. Favacho, Mitermayer G. Reis, Gregory A. Dasch, Elba R.S. de Lemos, and Albert I. Ko

In Brazil, Brazilian spotted fever was once considered the only tick-borne rickettsial disease. We report eschar-associated rickettsial disease that occurred after a tick bite. The etiologic agent is most related to *Rickettsia parkeri, R. africae, and R. sibirica* and probably widely distributed from São Paulo to Bahia in the Atlantic Forest.

Brazilian spotted fever (BSF), caused by *Rickettsia rickettsii*, was at one time considered the only tick-borne rickettsial disease in Brazil (*1*). Its transmission in 5 southern states is primarily associated with *Amblyomma cajennense, A. aureolatum*, and *Rhipicephalus sanguineus* ticks; however, many other rickettsiae of unknown pathogenicity are carried by ticks in Brazil (*1,2*). We describe an eschar-associated rickettsiosis in a traveler from the state of Bahia, Brazil; this disease seems to have been caused by the same *Rickettsia* sp. that caused a similar disease in São Paulo in 2009 (*3*).

The Case

In April 2007, a 30-year-old man from Bahia sought care for a 6-day febrile illness that began 9 days after he found a tick attached to his right wrist while hiking and camping in the Chapada Diamantina National Park in Paty Valley (12°48′26″S, 41°19′53″W), a semiarid region in Bahia. Primary signs and symptoms were fever (39–40°C), severe myalgia, and swelling and pain at the site of the tick bite. Two days after onset of illness, the man noticed a scab forming on his right wrist and painful swelling in his right axillary region, followed 2 days later by a generalized rash and painful ulcerative lesions in the mouth. The patient sought medical care, and an outpatient physician prescribed acetaminophen and cefadroxil, which did not reduce symptoms.

On day 6 of his illness, the patient sought care from an infectious disease specialist, who noted a 2.5-cm eschar on the patient's wrist (Figure 1, panel A); disseminated papular rash on his face, trunk, and upper extremities (Figure 1, panel B); and several small erosions on his tongue, buccal mucosa, and lips (Figure 1, panels C, D). The mucosal erosions were painful, and some skin papules formed small pustules (Figure 1, panel E). In the right axilla was a tender, enlarged, 3-cm lymph node. Results of a hemogram and blood biochemistry were unremarkable except for a high level (425 U/L) of lactic dehydrogenase. A rickettsial disease was considered, and the patient was given doxycycline (100 mg 2×/d) for 14 days. The fever and generalized rash resolved within 2 days, and the eschar healed completely within 2 weeks after initiation of therapy.

Acute-phase and convalescent-phase serum samples were evaluated by microimmunofluorescence assay for antibodies to spotted fever group rickettsiae (SFGR) (*4*). Before antimicrobial drug therapy was started, biopsy specimens of the papule and the scab from the eschar were collected, preserved in 10% formol, and evaluated by routine histopathology, immunohistochemical staining, and PCR (*4,5*).

Serum collected on day 6 of the illness was nonreactive with *R. rickettsii* and *R. parkeri* antigens (class-specific immunoglobulin G [Ig] and IgM <32 for both assays, cutoff ≥64). Subsequent testing determined IgG/IgM titers on day 12 to be 128/<32 against *R. parkeri* and 128/32 against *R. rickettsii* antigens and on day 19 to be 128/64 and 512/32, respectively.

Hematoxylin and eosin–stained sections of the papule biopsy specimen demonstrated lymphohistiocytic perivascular inflammatory cell infiltrates in the superficial to middle dermal layers. Immunohistochemical staining for SFGR showed rare antigens in a few small foci of perivascular inflammation.

The sequences for *ompA* (632-bp, GenBank accession no. GQ853063) from the scab and *gltA* (382-bp, GenBank accession no. GQ900666) from the papule specimen each had 100% identity to homologous gene sequences of SFGR detected recently in an eschar specimen from a patient from Peruibe, São Paulo (*3*). The sequences from both organisms were most related to SFGR strain S previously reported from Armenia (*6*) but were not identical to *R. sibirica, R. parkeri*, and *R. africae* (Figure 2). The nucleotide sequence of a 928-bp *sca4* fragment (GenBank accession no.

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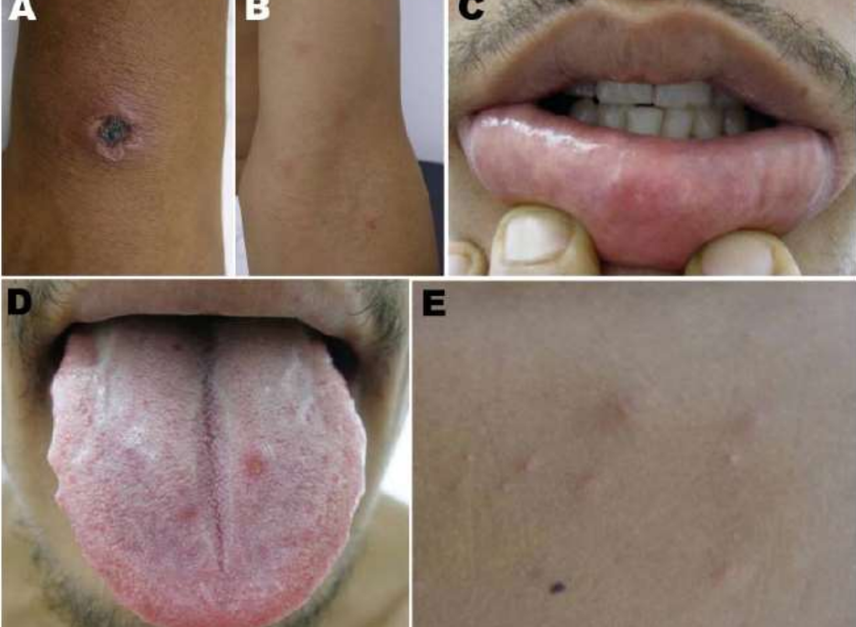


Figure 1. Lesions on day 6 of illness of patient with eschar-associated rickettsial disease, Bahia, Brazil, 2007. A) Eschar on right wrist; B) papular skin rash on left elbow; C) ulcerated lesion on lower lip; D) erosions on tongue mucosa; E) vesicular papular lesions on trunk.

GQ853064) had 99% identity to the homologous fragment of *R. parkeri* (GenBank accession no. AF155059), and the conserved 17-kDa protein gene amplicon (GenBank accession no. GQ853062) was similar to those of many SFGR.

Conclusions

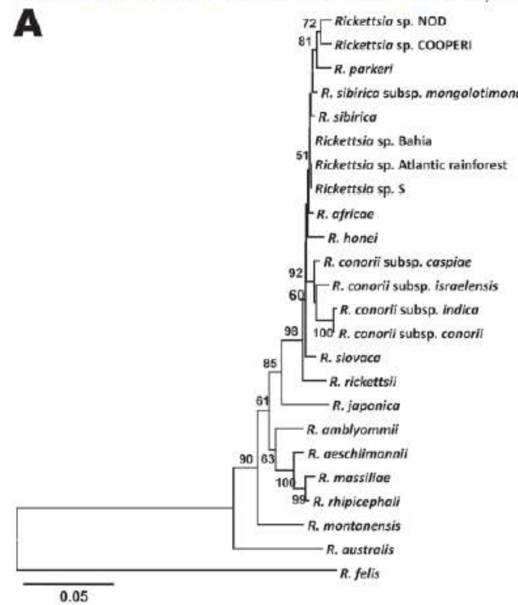
During the past decade, many newly identified tick-borne rickettsiae from South America have been described (*1,2*), including *R. parkeri*, *R. massiliae*, *R. amblyommii*, *R. bellii*, and other *Rickettsia* spp. of unknown pathogenicity. We describe another confirmed case of a novel eschar-associated SFGR disease in Brazil.

Development of an eschar is a characteristic manifestation of rickettsioses caused by *R. parkeri*, 364D *Rickettsia*, and *R. massiliae* (*4,7*). Possible eschar formation in association with Rocky Mountain spotted fever has been reported (*8*), but this manifestation does not seem to be a hallmark of disease caused by *R. rickettsii* or of other rickettsioses in Brazil and South America (*2*). BSF has been most often confirmed solely by serologic testing; however, atypical clinical manifestations, including eschar formation and lymphadenopathy, have been described (*9–12*). Lymphadenopathy and ulcers on the oral mucosa, as found for this patient, have been found in patients with rickettsiosis caused by *R. parkeri* and African tick bite fever (caused by *R. africae*) (*4,13*) but not in the index case-patient from São Paulo (*3*), who seemed to have less severe clinical manifestations than the patient described in this report.

In the scientific literature from Brazil, the earliest reference to an eschar in a suspected case of BSF was in 1932 (*12*). Subsequent eschar-associated cases have been identified in regions where BSF is endemic (e.g., the states of Minas Gerais, Rio de Janeiro, and Espirito Santo) (*9–11*) and in regions where it is not endemic (e.g., states of Santa Catarina, situated along the Argentina border, and Bahia [*14*], where the case reported in this article occurred). Furthermore, clinical descriptions of eschar-associated rickettsioses in Brazil have been reported from BSF-endemic areas with large populations of *A. dubitatum* ticks but no known *A. triste* ticks, which are recognized vectors of *R. parkeri* in southern Brazil (*15*). Although *A. dubitatum*, a human biting tick that is highly prevalent in many BSF-endemic areas (*2*), is a potential candidate for transmission of *R. parkeri* to humans in Brazil, this tick species and its vertebrate hosts, capybaras, have not yet been described in the Paty Valley, Bahia, where the patient acquired the rickettsial infection. Unfortunately, the ticks causing both cases in São Paulo and Bahia were not available for identification.

The taxonomic status of the etiologic agent of this novel rickettsiosis in Brazil cannot be definitively determined until it is isolated. On the basis of the available genetic information presented here and elsewhere (*3*), the pathogen detected in the cutaneous lesion of the patients from Bahia and São Paulo is equally distant from *R. africae*, *R. parkeri*, and *R. sibirica*. Each of these 3 SFGR is

among species long accepted by International Committee of Systematics of Prokaryotes, and this status is consistent with their long evolutionary divergence and differences in their vectors and geographic distributions. Molecular confirmation can and must therefore be used to identify new rickettsial agents because they cannot be identified by clinical case presentations or serologic analyses. Additional efforts will be required to establish the full genetic diversity and range of tick and animal reservoirs of SFGR in Brazil and to determine the prevalence and clinical presentations of different rickettsioses in humans. Clinicians should be alert for tick-borne infectious diseases resulting from ecotourism activities, especially in parks and ecologic reserves in the areas of the Atlantic Forest and other areas of Brazil where many rickettsiae-infected ticks have been identified and most BSF cases have been reported.



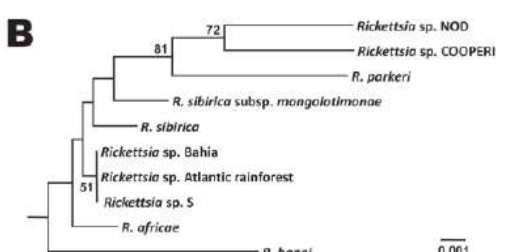


Figure 2. Genetic relationships of the spotted fever group rickettsiae (SFGR) detected in tissue of patient with eschar-associated rickettsial disease, Bahia, Brazil, 2007. Sequence comparison was conducted with MEGA version 4 (www.megasoftware.net). The phylogenetic optimal tree was inferred by using the neighbor-joining method, and distances were evaluated by implementing the Kimura 2-parameter model of substitution (sum of branch length = 0.58588522). In total, 323 nt sites of *gltA* and 401 nt sites of *ompA* were concatenated and evaluated; primer sequences and sites containing gaps and deletions were excluded from the analysis. Statistical reliability of the tree is based on 1,000 bootstrap replicates; only bootstrap values >50 are shown above the branches. The corresponding sequences of reference species and isolates were obtained from the National Center for Biotechnology Information GenBank database. A) Genetic association of *Rickettsia* sp. Bahia and other previously characterized SFGR; B) expanded tree of relationships among new SFGR to *R. africae*, *R. parkeri*, *R. sibirica*, *Rickettsia* sp. S and Atlantic Forest. Scale bars indicate nucleotide substitutions per site.

Addendum

Since submission of this article, recent investigation in Brazil has identified *A. ovale* ticks as potential vectors for the spotted fever group *Rickettsia* sp. described here (*16*).

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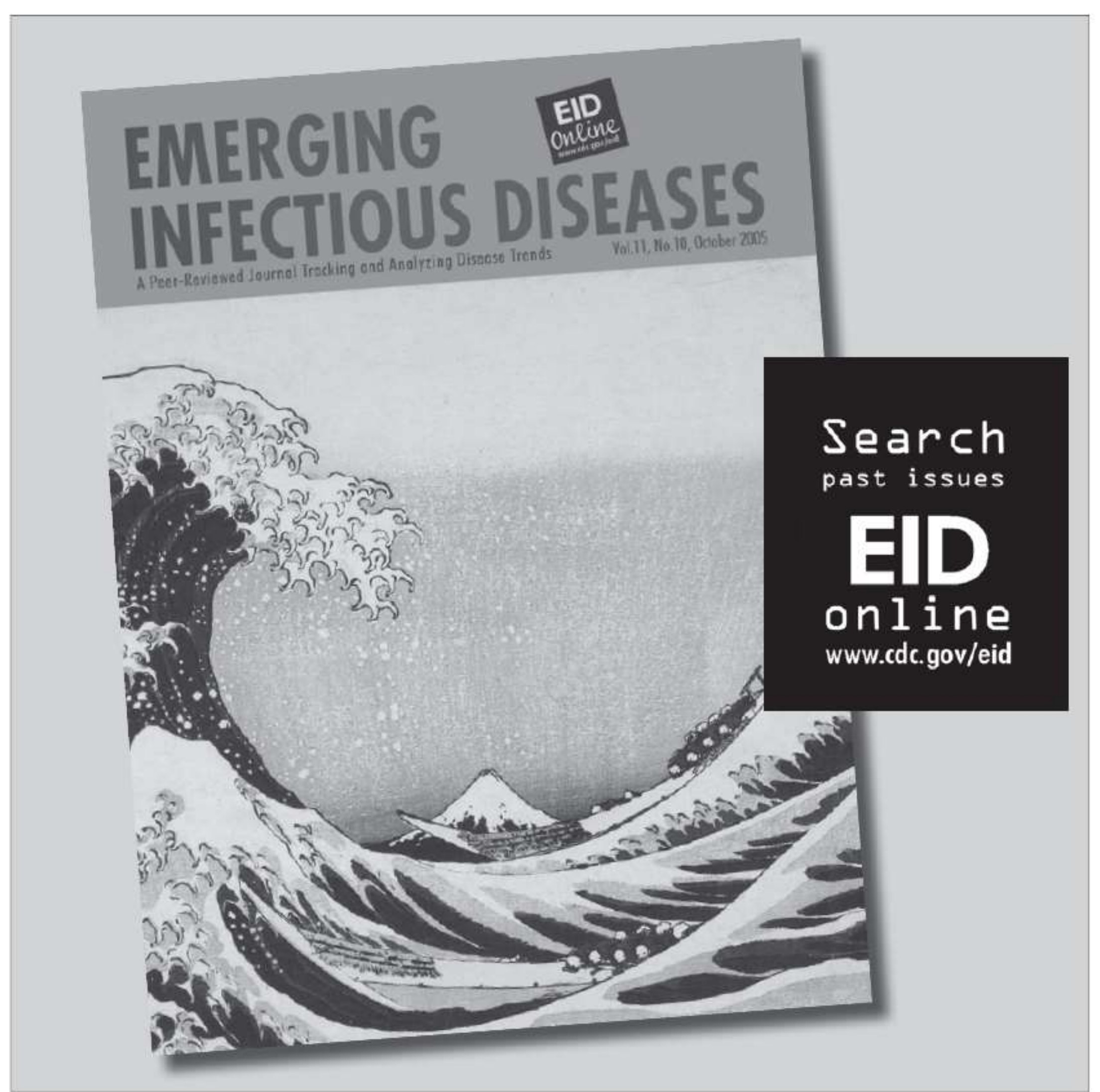

14.1.3. Fatal Brazilian spotless fever caused by *Rickettsia rickettsii* in a dark-skinned patient.

Revista da Sociedade Brasileira de Medicina Tropical

Case Report/Relato de Caso

Fatal Brazilian *spotless* fever caused by *Rickettsia rickettsii* in a dark-skinned patient

Febre maculosa brasileira sem exantema causada por *Rickettsia rickettsii* em um paciente de cor negra

Alexsandra Rodrigues de Mendonça Favacho[1], Tatiana Rozental[1], Simone Berger Calic[2], Maria Aparecida Mota Scofield[3] and Elba Regina Sampaio de Lemos[1]

ABSTRACT

Brazilian spotted fever (BSF) is the most important and frequent rickettsial disease in Brazil. A fatal case of BSF is reposted in a 32-year-old black man, who died of irreversible shock after five days of fever, severe headache and abdominal pain with no rash. Spleen, kidney and heart samples collected at autopsy were positive for *Rickettsia rickettsii* by PCR and sequencing. The authors emphasize the need for a high index of diagnostic suspicion for spotted fever in black patients. Absence of a skin rash should not dissuade clinicians from considering the possibility of BSF and initiating empirical therapy.

Keywords: Fatal Brazilian *spotless* fever. Black patient. PCR.

RESUMO

Febre maculosa brasileira (FMB) é a mais importante e frequente doença *rickettsial* no Brasil. Relatamos um caso fatal de FMB em um homem negro de 32 anos de idade que morreu de choque irreversível após cinco dias de febre, cefaléia intensa, dor abdominal, e sem evidência de exantema. Amostras de baço, rim e coração coletadas na necropsia foram positivas para *Rickettsia rickettsii* por PCR e sequenciamento. Os autores ressaltam a necessidade de um alto índice de suspeita diagnóstica para febre maculosa em pacientes negros. Ausência de exantema não deve dissuadir os clínicos de considerar a possibilidade de FMB e iniciar a terapêutica empírica.

Palavras-chaves: Febre maculosa brasileira sem exantema. Paciente negro. PCR.

INTRODUCTION

Described for the first time in 1929, Brazilian spotted fever (BSF) is caused by *Rickettsia rickettsii* and is considered the most important spotted fever group rickettsiosis (SFGR) in Brazil[1-6]. Since 2001, when national compulsory notification was implemented, the number of notified BSF cases has increased and in the last 10 years, more than 735 confirmed cases have been reported, with a mean mortality of 28% (Brazilian Ministry of Health, 2009). Although the clinical triad of fever, headache and rash is considered the classical description of BSF, which is similar to Rocky Mountain spotted fever (RMSF), this infection has a broader spectrum of manifestations, ranging from asymptomatic and mild disease to the severe form with fatal outcome[3-10]. Differential diagnosis is often difficult and includes SFGR caused by *Rickettsia felis*, dengue, leptospiroses and meningococcemia, among others known diseases in Brazil. The classic red-spotted rash usually develops 3-5 days after the onset of other symptoms on the patient's extremities (ankles, feet, wrists, and hands) and spreads to the body. After six or more days of the illness, the rash can become petechial and can subsequently coalesce to form ecchymoses or gangrene. In Brazil, as well as other places of world, spotted fever cases without rash or with fleeting or atypical skin eruptions (evanescent, localized to a particular region of the body, papulovesicular or ulcerative lesions), have been confirmed. Although in some cases the absence of rash may be due to the prompt institution of therapy with doxycycline/tetracycline or chloramphenicol, in other confirmed cases, in which long delays in the diagnosis and treatment occurred, the characteristic rash does not develop or is not detected, particularly in black patients[8-10].

The serological diagnosis, more specifically indirect immunofluorescence assay (IFA), is the most frequently used method for confirming spotted fever worldwide, but its use is limited during the first 10 days of disease and polymerase chain reaction assay (PCR) should be considered for the diagnosis at this acute onset of illness[5,10].

Herein, the authors report a case of fatal BSF diagnosed at autopsy by molecular methods in a black man with absence of cutaneous lesions.

CASE REPORT

On November 25, 2006, a 32-year-old, previously healthy black man, sought medical attention by a primary care physician with complaints of high fever, headache, nausea, myalgia and asthenia. He was treated with oral amoxicillin, but his symptoms persisted. Two days later, he was admitted to hospital in Itambacuri County, in the State of Minas Gerais, Southeast Brazil, with the presumptive diagnostic of dengue fever. Examination showed fever to 38.8ºC, mild dehydration and hypotension. The laboratory findings showed a hematocrit of 47%, white blood cell count of 4,700/mm³, neutrophils 50%, a platelet count of 60,000/mm³, creatinine of 4.2mg/dl, ureia of 139mg/dl, and total bilirubin level of 6.2mg/dl. After symptomatic

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treatment, the patient developed neurological manifestations, lethargy, mental confusion, agitation and a generalized seizure. Empiric treatment with chloramphenicol was initiated, but the patient's condition worsened and he died from multiple organ failure 24h after admission. Although there was no history of tick bites or exposure to tick-infested habitats, the patient lived in a rural area where two other cases of unexplained deaths in young adults had been notified within the preceding months. An autopsy was performed and serum and tissue samples from various organs were analyzed at the Laboratory of Hantaviroses and Rickettsioses, Oswaldo Cruz Foundation.

Indirect immunofluorescence assays for immunoglobulin M (IgM) and IgG antibodies reactive to *R. rickettsii* (Panbio™, Australia) were performed on serum and were nonreactive. DNA extraction from liver, heart and lung tissue autopsy samples was performed using the QIAamp DNA Blood Mini kit (QIAGEN®, Hilden, Germany), in accordance with the manufacturer's instructions. Polymerase chain reaction (PCR) assay was performed as described previously[5]. Segments of several rickettsial genes, including the 17kDa antigen gene [*htrA*], *ompA*, ompB and citrate synthase [*gltA*] were amplified from liver, heart and lung tissue DNA. The PCR products were purified using QIAquick PCR Purification Kit (QIAGEN) and sequenced using the ABI Prism 377 DNA Sequencer (Applied Biosystems, CA, USA). All sequences were edited with the BioEdit software, identified with the BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and the partial sequence *ompA* from the tissue samples generated in this study (GenBank accession nº FJ959382) showed 99% identity to the corresponding sequence of *R. rickettsii ompA* gene available at the GenBank.

DISCUSSION

The clinical severity of BSF, like RMSF, varies from mild to severe or fatal presentations[1-10]. Nonspecific clinical symptoms, such as fever, headache and muscle pain, occur in the first 10 days after the tick bite of the genus *Amblyomma*, particularly *A. cajennense*. The typical rash usually occurs on days 3 to 5 of the disease. In its early stages, this tick-borne disease can resemble many other infectious diseases, such as dengue and leptospirosis, which are endemic in Brazil, making diagnosis difficult, especially in patients with gastrointestinal and severe systemic manifestations with no presentation of a rash or a known history of tick bites[2-5].

In the case reported here, a diagnosis of BSF was not considered due to the absence of cutaneous lesions. This probably made a correct diagnosis even more difficult. Although the rash may be completely absent or atypical in up to 15-20% of RMSF cases, *spotless* rickettsiosis caused by *R. rickettsii* in black patients has rarely been reported[8,9]. One review of 43 cases of rocky mountain *spotless* or *almost spotless* fever was published in 1992 and the authors demonstrated 61% of these patients had been men and two-thirds of them were black[9].

The only serum sample collected on day 5 of disease was IFA negative for antibodies to spotted fever group rickettsiae. Indirect immunofluorescence assays remains the most commonly used serological technique for diagnosis; however, serological evidence of infection occurs no earlier than 7 to 10 days after the onset of illness[8,10]. Thus, the diagnosis must be based on clinical and epidemiological grounds, because the delay of specific treatment with doxycycline or chloramphenicol increases the risk of severe or fatal outcomes.

In the present case, death occurred five days following the onset of illness, an acute fulminant presentation. Although at least half of all deaths occur within 7 to 9 days of disease, accelerated clinical courses may occur and other variables, such as chronic alcoholism, cardiac problems and glucose-6-phosphate dehydrogenase deficiency (G6PD), associated with absence of early antibiotic treatment, can explain the severity of these cases[10]. None of the conditions previously mentioned were identified, except for delayed treatment, though G6PD deficiency is not routinely performed. PCR is a useful diagnostic method in the early phase of illness when antibodies are undetectable[3,10]. In this case report, *ompA* sequences by PCR amplification detected in tissue fragments of liver, heart and lung were sequenced and *R. rickettsii* was identified as the etiologic agent.

The present case illustrates well the diversity of clinical presentations of BSF and the difficulty of detecting the rash on dark-skinned individuals, emphasizing the importance of the earliest possible antibiotic empiric therapy in severe spotless fever, during the period of May to November, when peak *Amblyomma* reproductive activity is observed.

Finally, although the detection of antibodies against SFGR remains the best recognized and most used laboratory method to confirm BSF, PCR should be considered the gold standard early in the course of disease and is an important diagnostic tool to help identify missed, unconfirmed, or unreported deaths which may be caused by *R. rickettsii* in Brazil.

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14.1.4. Seroprevalence of *Coxiella burnetii* antibodies in human immunodeficiency virus-positive patients in Jacarepaguá, Rio de Janeiro, Brazil.

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Seroprevalence of *Coxiella burnetii* antibodies in human immunodeficiency virus-positive patients in Jacarepaguá, Rio de Janeiro, Brazil

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INTRODUCTION

Coxiella burnetii infection is a worldwide zoonosis; the sporulation capacity and high infectivity of Coxiella explain its ubiquity. Human infection is mainly related to exposure to farm animals; urban outbreaks have implicated cats, dogs and rabbits. There is an association between the chronic form of the infection and immunosuppression, including human immunodeficiency virus (HIV) seropositivity, although the prevalence and severity of illness in this group remains controversial [1]. Recent publications have reinforced the role of pregnancy in perpetuating infection [2,3].

The aim of this study was to describe the risk factors for acquisition of infection of *C. burnetii*, to determine the seroprevalence, and to identify the presence of DNA in blood samples of HIV-positive patients in a semi-rural area in the city of Rio de Janeiro.

METHODS

This was a prospective study of HIV-positive individuals followed up in an AIDS clinic in HMRPS, Jacarepaguá, Rio de Janeiro. Patients were interviewed and had peripheral blood collected after informed consent on their routine consultation day. Active disease and viral loads above 100 000 copies/mL (NASBA) were exclusion criteria. IFI assays using PANBIO slides were performed for detection of IgG antibodies; titres ≥1 : 64 were considered to be reactive. DNA extraction from blood clots was performed using a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. The extract was screened for the presence of *Coxiella* DNA by PCR using one set of primers, QBT-1/QBT-2 (QBT-1, 5′-TATGTATCCACCGTAGCCAGTC-3′; and QBT-2, 5′-CCCAACAACACCTCCTTATTC-3′) (htpAB, 687 bp)[4]. PCR conditions consisted of an initial DNA denaturation and hot start at 95°C for 5 min, followed by 40 consecutive cycles of 30 s of denaturation at 95°C, primer annealing at 60°C for 30 s, extension at 72°C for 1 min, and a 5-min extension at 72°C. Control reactions were always the last to be set up in PCR strips and were the last to be loaded onto gels. Each PCR reaction contained 0.3 μL of Platinum Taq DNA Polymerase (5 units) (Invitrogen, Carlsbad, CA, USA), 10.5 μL of nuclease-free water, 1.25 μL of each primer (1 pmol), and 8 μL of DNA extract in water. PCR products were separated by electrophoresis on 1% agarose gels, and visualized under ultraviolet light with ethidium bromide.

RESULTS

One hundred and twenty-five patients were included, aged 37.1 ± 10.1 years; 64 were females. None used intravenous drugs; 20% inhaled cocaine. Ninety-four of 125 (75.2%) were on antiretroviral therapy. Mean most recent CD4 count was 351 to 500 mm^3. Contact with cats and dogs were reported by 60/125 (48.0%) and 98/125 (78.4%). The frequency of watching and/or helping with animal birth was similar between men and women. Two of 64 women (3.1%) and 10/60 (16.7%) men milked animals, and there was a tendency towards statistical significance in this exposure by the Fisher's correction of chi-square (p 0.06). Exposure to animal hide and wool was infrequent in both sexes. *C. burnetii* phase I antibodies were found in four of 125 (3.2%) samples, and in four of 64 (6.3%) females. Patient 3 had attended a dog birth 3–5 years previously. No DNA amplification was obtained from these patients' clots (Table 1).

Obstetric history showed that patient 1 had four recent pregnancies (childbirths 5, 4 and 3 years previously, and one miscarriage),

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No conflicts of interest declared.

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Table 1. Characteristics of patients with *Coxiella burnetii* antibodies

Age	Sex	*C. burnetii* antibody dilution titres	Cat exposure	Dog exposure	Potential exposure to *C. burnetii*[a]
25	F	1 : 128	0	0	0
28	F	1 : 64	0	1	0
40	F	1 : 64	0	1	1
53	F	1 : 64	1	0	0

0 = no; 1 = yes.
[a]Exposure to wool, hides, animal birth and milking.

patient 2 had one childbirth 7 years previously (miscarriages were not reported), and patient 3 had seven pregnancies (one childbirth 4 years before, six miscarriages, three of which were spontaneous). Patient 4 did not reliably provide her obstetric history, and had no live children.

CONCLUSIONS

Although serological evidence must be analysed carefully, the seroprevalence to *C. burnetii* in Jacarepaguá, Rio de Janeiro of 3.2% suggests its circulation between HIV-positive individuals. This rate is higher than that found in asymptomatic adults (0.9%) in Minas Gerais State, a rural area in south-east Brazil [5]. In this same study, none of 269 ill AIDS patients presented antibodies to *C. burnetii*. In our study, the absence of DNA amplification suggests inactivity of the disease, despite HIV serostatus. This is not surprising, as stable clinical condition was an inclusion criterion. All seroreactive patients were female, and pregnancy may have played a role in perpetuating *Coxiella* antibodies, as the obstetric history was active for most patients. Diagnosis of Q-fever should be considered in cases of acute pneumonia and prolonged fever, especially when there is associated immunosuppression, valvulopathy or pregnancy, and a history of potential exposure to the organism.

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14.1.5. Cat-scratch disease: ocular manifestations and visual outcome.

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ORIGINAL PAPER

Cat-scratch disease: ocular manifestations and visual outcome

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Abstract To describe the intra-ocular manifestations of cat-scratch disease (CSD) found at two uveitis reference centers in Brazil. Retrospective case series study. Review of clinical records of patients diagnosed with CSD in the Uveitis Department of São Geraldo Hospital and the Ophthalmology Department of the Instituto de Pesquisa Clínica Evandro Chagas—FIOCRUZ, from 2001 to 2008. In the 8-year period, 24 patients with the diagnosis of CSD were identified. Twelve patients were male and 12 female. The mean age was 27.04 years (range 7–56). Sixteen patients (66.6%) presented with a history of a cat scratch and all patients reported cat exposure. Visual acuity ranged from counting fingers to 1.0 in the affected eye. Thirteen patients presented with bilateral disease. Sixteen (66.6%) patients complained of systemic symptoms, including fever, lymphadenopathy, liver and spleen enlargement and rash. All patients presented with serum antibodies (IgG) to *Bartonella henselae*. Thirty-seven eyes were affected. The most common findings were small areas of retinal infiltrates which occurred in 11 eyes (29.7%) and angiomatous lesions which occurred in nine eyes (24.3%). Neuroretinitis occurred in only six eyes (16.2%). The most common findings of CSD in our study were retinal infiltrates and angiomatous lesions. CSD patients may present with significant visual loss. Patients may benefit from systemic treatment with antibiotics.

Keywords Cat-scratch disease · Neuroretinitis · Retinitis

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Introduction

Cat-scratch disease (CSD) is a systemic disease caused by the Gram-negative rod *Bartonella henselae*. Ocular manifestation of CSD was primarily reported as a chronic ulcerative conjunctivitis, associated with lymphadenopathy, called Parinaud ocular glandular syndrome. In 1970 Sweeny and Drance described the first case of neuroretinitis associated with CSD [1], with several studies of intra-ocular manifestations of CSD being published since. Intra-ocular changes secondary to *B. henselae* infection include neuroretinitis, subretinal lesions, retinitis, intermediate uveitis, inflammatory masses, and angiomatous lesions [2].

The aim of this study is to report the ocular manifestations and visual outcomes of CSD found at two Brazilian uveitis reference centers.

Patients and methods

The authors reviewed the clinical records of patients diagnosed with CSD at the Uveitis Department of São Geraldo Hospital, Federal University of Minas Gerais and at the Ophthalmology Department of the Instituto de Pesquisa Clínica Evandro Chagas—FIOCRUZ, over a period of 8 years.

Patients were eligible for the study if they presented with intra-ocular changes and serologic evidence of *B. henselae* infection (IgG). Serological analysis was performed by indirect immunofluorescence assay, cut off 1:64, using a commercial kit (*Bartonella henselae* IFA IgG; Bion, Illinois, USA).

All patients underwent complete ophthalmologic examination (visual acuity—Snellen chart, biomicroscopy, tonometry, fundoscopy), fundus photography, fluorescein angiography (FA) and indocyanine green angiography (ICG) where necessary. Patients with positive serology for the human immunodeficiency virus (HIV) were not excluded (Table 1).

This study was approved by the institution's ethics committee.

Results

Twenty-four patients, 12 male and 12 female, presented with ocular changes as a result of CSD. The group had a mean age of 27.04 years, ranging from 7 to 56. Sixteen patients (66.6%) reported a cat scratch and all patients had a history of cat exposure. Systemic findings included fever (eight patients), lymphadenopathy (seven patients), liver and spleen enlargement (one patient), headache (two patients), rash (one patient), and aseptic meningitis (one patient). Thirteen patients (54.1%) presented with a bilateral disease and eleven (45.9%) unilateral (37 eyes). Visual acuities ranged from counting fingers to 20/20 in the affected eyes. Of the 37 affected eyes, 21 (56.8%) presented with visual acuity (VA) worse than 20/200 and 16 (43.2%) better than 20/200. Six eyes (16.2%) presented with mild anterior segment inflammation.

All patients presented IgG-positive for *B. henselae* and seven patients presented IgM-positive. Twenty of the 24 patients (83.3%) were treated with systemic doxycycline, three patients (12.5%) with oral ciprofloxacin and one patient (4.2%) with oral doxycycline and oral steroids. Of the 37 affected eyes, five eyes (13.5%) presented with VA worse than 20/200 after treatment and 32 (86.5%) presented with VA better than 20/200. Of the 24 patients, four (16.6%) were HIV-positive, two of whom showed ocular disease relapse following discontinuation of systemic therapy. One patient was treated for 1 month with ciprofloxacin and the second for 3 months with doxycycline. Both patients were treated after relapses where the CD4+ count reached 250 cells/mm.

Of the 37 affected eyes, six (16.2%) presented with neuroretinitis. Small areas of retinitis occurred in 11 eyes (29.7%) and subretinal lesions occurred in five eyes (13.5%) (Fig. 1). Angiomatous lesions occurred in nine eyes (24.3%) and peripapillary angiomatous lesions occurred in two eyes (5.4%) (Fig. 2a, b). Disc edema was seen in five eyes (13.5%), and two eyes (5.4%) showed optic disc inflammatory mass.

Discussion

Since 1970 when Sweeney and Drance first reported a case of CSD and neuroretinitis, this ocular manifestation has been considered by most authors to be the most common finding in cases of *B. henselae* infection.

Ormerod et al. reviewed 25 published cases of confirmed CSD associated with posterior segment inflammatory disease and found that neuroretinitis associated with macular star was the most common ocular manifestation. In this study the authors describe two cases of multiple retinal arteriolar occlusions, revealing that many other features besides neuroretinitis may occur in CSD [3]. In our study we found that six of 37 affected eyes presented with neuroretinitis. Ormerod and colleagues also described a small number of non-progressive small white intraretinal infiltrates in their two cases. Of the six eyes with neuroretinitis in our study, all eyes presented with associated small areas of retinitis/retinal

Table 1 Demographics of 24 patients with ocular manifestation of cat-scratch disease

Gender	Age (years)	Serology	Cat exposure	Cat scratch	Bilateral or unilateral	Ocular changes	Initial visual acuity	Systemic findings	Treatment	Final visual acuity	HIV status
M	16	IgM/IgG	Yes	Yes	B	Disc edema/serous detachment/retinal infiltrates	CF 30 cm/20/20	Fever/ lymphadenopathy	Doxy	20/20 BE	HIV−
F	9	IgG	Yes	No	U	Neuroretinitis	20/100/20/20	Fever/ lymphadenopathy	Cipro	20/40/20/20	HIV−
M	30	IgG	Yes	No	B	Angiomatous lesions/retinal infiltrates	20/20/CF 20 cm	None	Cipro	20/20/CF 20 cm	HIV+
F	22	IgM/IgG	Yes	Yes	B	Subretinal lesions RE/disc edema/subretinal lesions LE	20/20 BE	Cold 2 weeks	Doxy	20/20 BE	HIV−
F	26	IgG	Yes	Yes	U	Angiomatous lesions	20/30/CF 20 cm	None	Doxy	20/30/20/80	HIV−
F	56	IgM/IgG	Yes	No	B	Disc edema/vitritis	20/60/CF 20 cm	Headache	Doxy	20/30/20/160	HIV−
M	53	IgG	Yes	No	U	Angiomatous lesions RE	CF 50 cm/20/50	None	Doxy	HM/20/25	HIV+
F	7	IgM/IgG	Yes	Yes	B	Neuroretinitis/subretinal lesions	20/20/20/200	Fever/rash/ liver/Spleen	Doxy	20/20 BE	HIV−
M	12	IgM/IgG	Yes	Yes	U	Inflammatory optic disc mass	CF 30 cm/20/20	None	Doxy	20/160/20/20	HIV−
M	18	IgG	Yes	Yes	B	Retinal infiltrates RE/ peripapillary angiomatous lesion LE	20/20/20/800	None	Doxy	20/20/20/120	HIV−
M	27	IgG	Yes	No	U	Retinitis	20/20/20/800	None	Doxy	20/20 BE	HIV−
M	27	IgG	Yes	No	B	Angiomatous lesions BE	20/100 BE	Fever	Doxy	20/20 BE	HIV+
M	24	IgG	Yes	No	B	Angiomatous lesions BE	20/200/20/150	Fever	Doxy	20/80/20/100	HIV+
F	29	IgM/IgG	Yes	Yes	U	Subretinal lesions	20/200/20/20	None	Doxy	20/200/20/20	HIV−
F	14	IgM/IgG	Yes	Yes	U	Angiomatous lesions/ granulomatous lesions	20/20/20/800	Fever/ lymphadenopathy	Doxy	20/20/20/800	HIV−
F	20	IgG	Yes	Yes	B	Neuroretinitis/retinal infiltrates LE	CF 40 cm/20/100	Fever	Cipro	20/20 BE	HIV−
M	40	IgG	Yes	Yes	U	Neuroretinitis	20/70/20/20	Aseptic meningitis	Doxy	20/20 BE	HIV−
F	38	IgG	Yes	Yes	B	Retinal infiltrates BE/macular star LE	20/40/CF 4M	Lymphadenopathy	Doxy	20/20 BE	HIV−
F	42	IgG	Yes	Yes	U	Retinitis	20/100/20/20	Lymphadenopathy	Doxy	20/20 BE	HIV−

Table 1 continued

Gender	Age (years)	Serology	Cat exposure	Cat scratch	Bilateral or unilateral	Ocular changes	Initial visual acuity	Systemic findings	Treatment	Final visual acuity	HIV status
F	25	IgG	Yes	Yes	B	Disc edema RE/retinal infiltrates BE	20/20 BE	Fever/ lymphadenopathy	Doxy	20/20 BE	HIV−
M	18	IgG	Yes	Yes	U	Disc edema RE/retinal hemorrhages	20/30/20/20	Lymphadenopathy	Doxy+Pred	20/20 BE	HIV−
F	25	IgG	Yes	No	U	Neuroretinitis	20/20/CF 4M	None	Doxy	20/20/20/30	HIV−
M	45	IgG	Yes	Yes	B	Neuroretinitis/retinal infiltrates LE	20/100/20/70	Fever	Doxy	20/20 BE	HIV−
M	26	IgG	Yes	Yes	B	Optic disc granuloma RE/ retinal infiltrates LE	LP/20/20	Fever	Doxy	20/200/20/20	HIV−

B, bilateral; BE, both eyes; Cipro, ciprofloxacin; Doxy, doxycyclin; LE, left eye; Pred, prednisolone; RE, right eye; U, unilateral

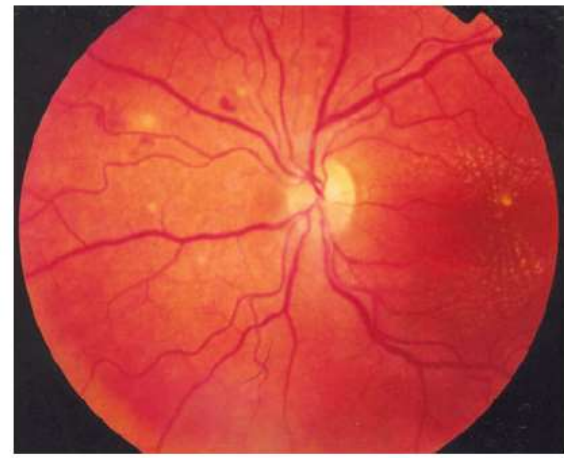

Fig. 1 Fundus photography showing small whitish retinal infiltrates associated with complete macular star

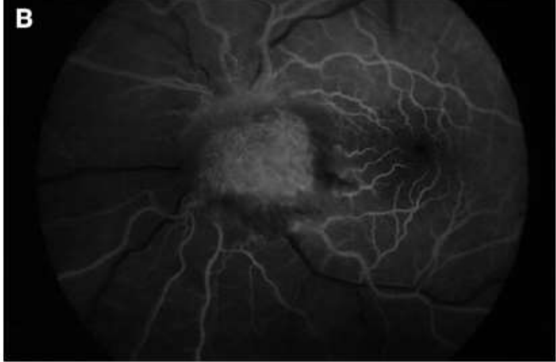

Fig. 2 **a** Fundus photography showing an angiomatous lesion in the optic disk. **b** Fluorescein angiography showing hyperfluorencence of the optic disk corresponding to the angiomatous lesion

infiltrates. This association may be an important clinical feature to help ophthalmologists in the diagnosis of intra-ocular CSD.

Solley et al. published a study of 24 patients (35 eyes) with ocular manifestation of *Bartonella* infection and they found that isolated foci of retinitis or choroiditis were the most common ocular manifestations of CSD. Of the 35 eyes, the retinal or choroidal white lesions were seen in 29 (83%), and 16 (46%) presented with disc edema [4]. In our study we found 16 eyes (43.2%) with small areas of retinitis or subretinal lesions similar to those described by Solley et al. In our study these lesions were the most common finding in CSD.

Less importance has been given to vascular changes in CSD. Our group published two studies showing the abnormal vascular network in CSD associated with HIV infection [5, 6]. Matsuo et al. studied four consecutive patients with CSD and considered granuloma with an abnormal vascular network arising specifically at the optic disc as the hallmark of ocular manifestations of CSD [7]. The relationship between *Bartonella* infection and vasoproliferation has been shown since the isolation of *Bartonella* species in bacillary angiomatosis [8]. Dehio discussed two distinct mechanisms for *Bartonella*-induced vascular proliferation: triggering proliferation and inhibiting apoptosis, and stimulating the production of vascular endothelial growth factor (VEGF) by infected macrophages. VEGF and interleukin-1B, a potentiator of VEGF, are released from macrophage-infected cells in response to *B. henselae* infection [9]. In 2004 Kirb established an in vitro model of *Bartonella*-induced angiogenesis [10]. These studies show that vascular proliferation is one of the most important events in *Bartonella* infection, and our study has shown that these events can occur in the eye.

In the group studied by Solley et al. the authors treated 11 patients out of 24 with oral or intravenous antibiotics, associated or not with steroids, and did not treat 13 patients. The authors reported that there were no differences in final VAs in the two groups. Although there are no prospective studies showing differences between treated and untreated patients, CSD has been considered a self-limited disease. In Japan, Kodama et al. studied 14 patients with CSD: 13 were treated with antibiotics and steroids because of optic nerve involvement; the authors suggested treatment with antibiotics and steroids since a good outcome was achieved and no relapse occurred in their group [11]. Reed et al. described seven cases of consecutive patients treated with oral doxycycline and rifampin. The authors suggested that prompt treatment may shorten the course of the disease [12]. In our study all patients were treated with systemic antibiotics. Of 37 affected eyes, 32 (86.5%) showed a final VA better than 20/200 and only five were worse than 20/200. Two patients showed intra-ocular relapses after the discontinuation of the antibiotics. Although most of the affected eyes evolved with good VA, in some cases this did not occur despite oral treatment. The role of steroids in the treatment of CSD remains unclear; in our study only one patient was treated with oral antibiotics and steroids due to significant macular edema. In this patient the steroids did not seem to affect the patient's recovery.

In conclusion the spectrum of CSD ocular manifestations is very large. Subretinal lesions and small foci of retinitis associated with an abnormal vascular network seem to be the most common ocular finding. Treatment with systemic antibiotics should be considered since significant visual impairment and relapses can occur.

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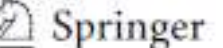

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14.1.6. *Bartonella spp.* infection in HIV positive individuals, their pets and ectoparasites in Rio de Janeiro, Brazil: serological and molecular study.

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Bartonella spp. infection in HIV positive individuals, their pets and ectoparasites in Rio de Janeiro, Brazil: Serological and molecular study

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ABSTRACT

Background: *Bartonella* is the agent of cat-scratch disease, but is also responsible for more severe conditions such as retinitis, meningoencephalitis, endocarditis and bacillary angiomatosis. Its seroprevalence is unknown in Brazil.
Methods: Patients in an AIDS clinic, asymptomatic at the time of the study, were enrolled prospectively. They answered a structured questionnaire and had blood taken for serological and molecular assays. Cat breeder's pets were tested serologically and collected ectoparasites were tested by molecular biology techniques. Blood donors, paired by age and sex, were tested for *Bartonella* IgG antibodies.
Results: 125 HIV positive patients with a median age of 34 were studied; 61 were male and 75% were on HAART. Mean most recent CD4 count was 351–500 cells/mm^3. A high rate of contact with ticks, fleas and lice was observed. *Bartonella* IgG seroreactivity rate was 38.4% in HIV positive individuals and breeding cats was closely associated with infection (OR 3.6, CI 1.1–11.9, $p<0.05$). No difference was found between the sexes. Titers were 1:32 in 39 patients, 1:64 in seven, 1:128 in one and 1:256 in one. In the control group, IgG seroreactivity to *Bartonella* spp. was 34%, and female sex was correlated to seropositivity. Fourteen of 61 (23%) males vs 29/64 (45.3%) females were seroreactive to *Bartonella* (OR 2.8, CI 1.2–6.5, $p<0.01$). Titers were 1:32 in 29 patients, 1:64 in ten and 1:128 in four.
Conclusions: *Bartonella* spp. seroprevalence is high in HIV positive and in blood donors in Rio de Janeiro. This may be of public health relevance.

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1. Introduction

Members of the genus *Bartonella* are small, fastidious Gram negative rod-shaped bacteria that parasitize mammalian erythrocytes and endothelial cells (Slater et al., 1990). At present there are more than 23 recognized species (Brenner et al., 1993) and several have been recovered from a wide range of wild and domestic mammals in the world with a high diversity of geographic distributions and arthropod vectors such as sandflies, the human body louse, the cat flea, and ticks (Lamas et al., 2008). Recognized agents of human disease include *Bartonella bacilliformis*, *Bartonella quintana*, *Bartonella vinsonii* subsp. berkhoffii, *Bartonella henselae*, *Bartonella elizabethae*, *Bartonella grahamii*, *Bartonella washoensis*, *Bartonella koehlerae*, and more recently *Bartonella rochalimae*, *Bartonella alsatica* and *Bartonella tamiae* (Lamas et al., 2008). Contact with animals and vectors seem to be the most important mode of transmission, although recent studies have shown the ability of Bartonellae to survive in stored blood for more than 35 days with the potential for transfusion associated infection (Magalhães et al., 2008).

Bartonellae have pathogenetic characteristics such as red blood cell invasion and lyses, the ability of causing persistent bacteremia and of inducing small vessel endothelial cell proliferation (Brouqui and Raoult, 1996; Anderson, 2001). The pathologic response varies with immune status: in immunocompetent individuals, the response is granulomatous and suppurative, in immunodeficient ones, it is predominantly vasculoproliferative (Resto-Ruiz et al., 2003). Bacillary angiomatosis (BA) and hepatic peliosis (HP), classically associated with the acquired immunodeficiency syndrome (AIDS) (Regnery et al., 1995) are seen less frequently today, possibly because of earlier recognition of HIV serostatus and the lesser number of individuals with CD4 lymphocyte cell counts below 50 cells/mm^3. However, seroepidemiological studies have shown the dispersion of the microorganisms, and clinical syndromes, besides classical cat-scratch disease (CSD), are increasingly described. These include fever of unknown origin (FUO) (Koehler et al., 2003), blood culture negative endocarditis (BCNE) (Houpikian and Raoult, 2005; Fournier et al., 2001; Lamas and Eykyn, 2003; Benslimani et al., 2005; Znazen et al., 2005) and neurological and

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ophthalmological manifestations (Wong et al., 1995; Schwartzman et al., 1995; Curi et al., 2006). The last three carry significant morbidity and potential mortality.

Bartonella spp. infections are common in the general population, with seroreactivity rates varying between 2% and 30% in studies from Europe and North America; prevalence rates are higher in intravenous drug user (IVDU), in homeless and in cat owners (Lamas et al., 2008). In the Southern hemisphere, seroprevalence in adults has varied from 10% to 45% (Costa et al., 2005; Ferres et al., 2006; Buelvas et al., 2008).

Despite a reasonable number of case reports and small case series of *Bartonella* disease in HIV positive individuals (Santos et al., 2000; Gazineo et al., 2001; Plettenberg et al., 2000), there are few seroprevalence studies in this group. Antibodies are present in 10–22.3% in studies from the USA and Europe (Lamas et al., 2008; Blanco Ramos et al., 1999; Wallace et al., 2001) and no studies are available from the Southern hemisphere. Molecular biology studies in South Africa showed a 10% bloodstream infection rate in 188 HIV seropositive outpatients who were chosen randomly for the study, and whose median CD4 count was 395 in the PCR positive individuals and 301 in the PCR negative (Frean et al., 2002). The only clinical information provided in this study was that only one patient possibly had bacillary angiomatosis and was treated for it.

Studies involving BCNE point out to a continuous gradient of *Bartonella* infection, incidence ranging from close to zero in Northern Europe (Werner et al., 2003) to over 28% in Southern France and Northern Africa (Houpikian and Raoult, 2005; Benslimani et al., 2005; Znazen et al., 2005; Hoen et al., 1995).

The aim of our study was to estimate the seroprevalence to *Bartonella* infection in historically more susceptible individuals – HIV positive patients, and in healthy blood donors living in a potentially high exposure area, Jacarepaguá, a forested part of the city of Rio de Janeiro, Brazil, where the world's largest urban forest, part of the Atlantic Rainforest, is located, and where human exposure to vectors and animals is high.

2. Methods

The study took place in the AIDS outpatient clinic of Hospital Municipal Raphael de Paula Souza (HMRPS), Jacarepaguá, Rio de Janeiro, Brazil, between March and July 2005. Ethical approval was obtained from FIOCRUZ, under number 293/05. Patients followed up by one of the authors were consecutively enrolled, if they gave informed signed consent, on the day of their routinely scheduled appointment. A structured and detailed interview was applied. Medical notes were reviewed by the doctor for completeness and accuracy when necessary. Patients were asymptomatic at the time of the study. Exclusion criteria were: pregnancy, systemic active disease (ex tuberculosis, neurotoxoplasmosis), syphilis serology (VDRL titers above 1:8), presence of anti-phospholid antibody syndrome and HIV viral load above 100,000 copies/ml (NASBA) in the last 6 months. The rationale for these criteria was to minimize antibody cross-reactions or false negative results in the case of severe immunosuppression. Patients with mental illness were also excluded. Peripheral blood was then collected (10 ml), clot and serum separated on site, and both were taken to the Rickettsial National Reference Laboratory in FIOCRUZ on the same day, where they were kept at −20 °C until serological and molecular analysis were performed. The control group consisted of the same number of patients, paired by sex and age. Their blood was obtained at HMRPS blood bank, after Ethical Committee approval at the Central Blood Laboratory in Rio de Janeiro (Hemorio), under number CEP HEMORIO 109/07. In Brazil, all blood donors are screened for HIV, hepatitis B and C as well as syphilis and Chagas' disease. Therefore patients in the control group tested negative for these conditions. Ethics Committee approval for animal study was obtained under number CEUA Fiocruz L-0043/07. Additionally, HIV positive patients who were cat breeders were visited in their homes, and their animals studied after informed consent. A veterinary physician examined the animals, collected peripheral blood and their ectoparasites. Ticks and fleas were identified by classical reported enthomological criteria (Aragão and Fonseca, 1961; Walker et al., 2000).

Serological analysis was done using a commercial kit, *B. henselae* IFA IgG (Bion[R], USA); a screening cut-off titer of 1:32 was used. Animal serum was studied using the same kit and cut-off, and commercial anti-dog and anti-cat immunoglobulins from Kirkegaard and Perry, Inc. (Gaithersburg, MD). All serum sample of the HIV positive patients were tested for *Coxiella burnetii* using a commercial IFA kit (Panbio[R], USA). Molecular analysis was performed after DNA extraction from blood clots and tissue samples from ectoparasites, with the use of a commercial kit (QIAamp DNA Mini Kit, Qiagen, Germany). PCR using primers CAT1 [5′-GATTCAATTGGTTTGAA(G/A)GAGGCT-3′] and CAT2 [5′-TCACATCACCAGG(A/G)CGTATTC-3′], targeting the gene htrA was done as previously described (Anderson et al., 1994). Ectoparasites were pooled in 6 pools, randomly.

Data from the interviews were plotted into an EpiInfo database. Using the Statcalc program, qualitative variables were expressed as frequencies and quantitative variables as mean ± standard deviation. The chi-square test and corrections were applied for comparison of frequency of exposure to different variables in patients seroreactive to *Bartonella* spp. The Student *t*-test was applied to compare CD4 counts in patients with and without seropositivity to *Bartonella* spp.

3. Results in the study group

Of the 128 HIV positive patients who were enrolled, 3 were excluded on their following visit due to new illness (tuberculosis in 2, neurotoxoplasmosis in 1). Patient's mean age was 37.1 ± 10.1 years, median 34. Of the 125 patients, 95 (79%) were aged between 22 and 45 years; 61 were male (M) and 64 female (F). None were IVDU, 24/122 (20%) inhaled cocaine, 45/123 (37%) abused alcohol; 24/119 (20.1%) had more than 5 sexual partners in the last 5 years; 19 of 58 (32.7%) were men who had sex with men (MSM); 94/125 (75%) were on highly active antiretroviral therapy (HAART). Diagnosis of HIV seropositivity had been made 3.8 ± 2.6 years previously. Most recent and lowest ever CD4 counts were 351–500 and 101–200 cells/mm^3, respectively. 49 of 120 (41%) patients had incomplete elementary school, 6 (5%) were illiterate. Mean monthly family income varied between US$ 136 and US$ 273. As to co-infections, 6/122 (5%) of patients had hepatitis C (4 M, 2 F) and 2/61 (3.3%) M were hepatitis B antigen positive. Previous HIV related illness had occurred in 88/125 (70.4%) of patients, and there was no sex difference between prevalence of illness. Most frequent past HIV-associated infections were esophageal candidiasis (30%), pulmonary tuberculosis (TB) (24%), nodal TB (24%), miliary TB (22%), Pneumocystis pneumonia (22%), neurotoxoplasmosis (19%), pleural TB (14%) and CMV retinitis (3%). Chronic diarrhea and fever occurred in about 50% of patients, as did herpetic infections. Bacterial sinusitis and pneumonia occurred in about 20%.

Epidemiological data: some form of contact with cats was reported by 60/125 (48%) patients and 20 (16%) had cats as pets; 98 (78.4%) had contact with dogs and 65 (52%) with rats or mice. Exposure to ticks, fleas and lice was referred by 53 (42%), 42 (34%) and 34 (27%) of patients respectively. As to occupation, most women (44/64, 69%) were housewives or worked as house servants and men had occupations in accordance with educational status, mostly as informal street salesmen, office servants, building workers, com-

Table 1
Demographic variables and *Bartonella* spp. serology results in the study group (HIV positive) and control group.

Variables	HIV positive patients (n = 125)	Blood donors (n = 125)
Age in years (mean ± SD, median)	37.1 ± 10.1, 34	37.3 ± 9.4, 35
Sex (male, female)	61; 64	61; 64
Total *Bartonella* IgG seroreactive	48 (38.4%)	43 (34.4%)
IgG *Bartonella* seroreactive males	27/61 (44%)	14/61 (23%)
IgG *Bartonella* seroreactive females	24/64 (37.5%)	29/64 (45.3%)
Total number of individuals with titers of: 1:32	39	29
1:64	7	10
1:128	1	4
1:256	1	0

mercial vehicle drivers and cooks. Of all 125 patients, only 6% had been to university. Most patients lived in the West areas of Rio de Janeiro, and of these 65/89 (73%), lived in Jacarepaguá, where the largest urban forest in the world is located. *Bartonella* spp. seroreactivity rates and other variables are presented in Table 1.

Thirteen of 20 (65%) patients who had cats were seroreactive for *Bartonella* vs 22/65 (34%) of those who did not have contact with them. Breeding cats was closely associated with *Bartonella* spp. infection (OR 3.6, CI 1.1–11.9, $p < 0.05$). Exposure to ticks, fleas and lice, to dogs, rats and mice was not associated with the presence of anti-*Bartonella* antibodies. No difference was found as to CD4 count or sex. Four of 48 (8.3%) had titers equal to or higher than 1:64. Patients with these higher titers bred cats and/or were homeless. Four patients had *C. burnetii* antibodies detected, and 2 of them also were *Bartonella* reactive. Blood clots of the 20 cat breeders were tested for *Bartonella* and none amplified DNA.

Data on the control group is also presented in Table 1. Controls lived in the same area as patients but no other exposure data was available. A difference regarding sex and antibody seroprevalence was found: 14/61 (23%) of males vs 29/64 (45.3%) of females were seroreactive to *Bartonella* spp. (OR 2.8, CI 1.2–6.5, $p < 0.01$).

4. Results in animals

There were 20 cat breeders, 2 of whom were a couple. Eleven homes were visited in August and September 2005, where 22 cats and 29 dogs that lived in the same houses were studied. Fourteen of the 22 visited cats were tested for anti-*Bartonella* antibodies; 5/14 cats (35.7%) were seroreactive. Titers were 1:32 for 2 cats, 1:64 in one, 1:128 in one, and 1:512 in one. None of these cats had ectoparasites collected and they were healthy. They all belonged to male HIV positive patients, none of whom were seroreactive for *Bartonella*. Eight homes of cat breeders were not visited for different reasons: cat died (1), cat was given away after the study interview (1), the home visit would have caused constraint, breaking the consent form premises (4), the patient was taken to a nursing home (1), the patient was not home (1). All settings visited were in the Jacarepaguá areas, but the types of houses visited varied: flats (4), houses with backyards (4), houses without backyards (1) and shacks in slums (2). Cats from all these different scenarios were seroreactive for *Bartonella*. Blood was obtained from 26/29 (90%) dogs. None were reactive for *Bartonella* spp. As to ectoparasites, 37 were collected from 49 dogs and cats; 24 (65%) were *Ctenocephalides canis* ("dog flea") and 13 (35%) *Rhipicephalus sanguineus* ("dog tick"). All were adult except for one nymph and two larvae of *R. sanguineus*. Only one ectoparasite was obtained from a cat (an adult *C. canis*). None of the ectoparasites had *Bartonella* DNA amplified from them.

5. Discussion

This is the first complete study analyzing *Bartonella* seroprevalence in asymptomatic ambulatory HIV positive individuals and in healthy blood donors in Brazil and actually in the Southern Hemisphere; preliminary results were published by our group in abstract form on the HIV positive group (Lamas et al., 2006). A high and comparable rate of seropositivity was found in the HIV positive and in the blood donor groups. The only statistically important association was with breeding cats in the HIV positive group, despite the low socio-economic status of these patients and the high exposure to vectors and vertebrate animals. Despite HIV seropositivity, the inclusion criteria used in the study somehow guaranteed a degree of immunocompetence of these patients. Since 1996 HAART has been made available by government policy to all HIV individuals in Brazil who need it. This has decreased morbidity and mortality in AIDS patients, similarly to what has occurred in other parts of the world. It is interesting to discuss, however, that *Bartonella* infections may have gone undiagnosed in this group of patients, since it was not systematically searched for. Tuberculosis was the most prevalent HIV-associated diagnosis, and previous reports have shown co-infection of *Bartonella* and mycobacteria (Bernit et al., 2003; Rolain et al., 2006; Rovery et al., 2006), especially in diseased lymph nodes, a frequent site of extrapulmonary tuberculosis in adult AIDS patients in Brazil. Treatment for associated conditions with drugs active against *Bartonella* such as rifampin, aminoglycosides, quinolones, macrolides, might have at least partially treated this proteobacterium. Improvement of immune status after initiation of antiretroviral therapy may also have helped controlling *Bartonella* infection.

In the control group, there was a sex difference as to prevalence of *Bartonella* antibodies, as is described in the literature, with a higher proportion of females being *Bartonella* seropositive. This may be explained by differences in cat exposure in HIV positive males, or a higher susceptibility of HIV individuals as a group to infection acquisition.

Pets, especially cats, represent a large reservoir for human infection (Lamas et al., 2008; Skerget et al., 2003). *B. henselae* seroreactivity in humans has a close association with cat ownership and/or cat care. Seroprevalence in cats vary from 14% to 50% in studies, and cats may have persistent and asymptomatic bacteremia (Lamas et al., 2008). In Brazil, a study on seroprevalence of *Bartonella* in 108 stray cats in the city of São Paulo showed presence of antibodies to *Bartonella* spp. in 46% (Slhessarenko et al., 1996). Molecular biology studies show PCR positivity in over 90% of domestic and shelter cats in the state of Rio de Janeiro (Aline Moreira et al., 2009, unpublished data from our lab). Other studies have failed to show such associations, as the one from Catalonia, Spain, where there was 22.3% antibody prevalence to *B. henselae* or *B. quintana* in 340 studied HIV individuals, but the only significant association found was increasing age (Pons et al., 2008). A previous study by Blanco et al. in a different area of Spain, with 52 HIV positive and 85 blood donors, showed a trend in higher seroprevalence for *B. henselae* in the HIV group (17.3% vs 5.9%, p NS), but could not identify risk factors (Blanco et al., 1999). Some studies show controversies on cat ownership and neurological decline in HIV positive patients (Schwartzman et al., 1995; Wallace et al., 2001).

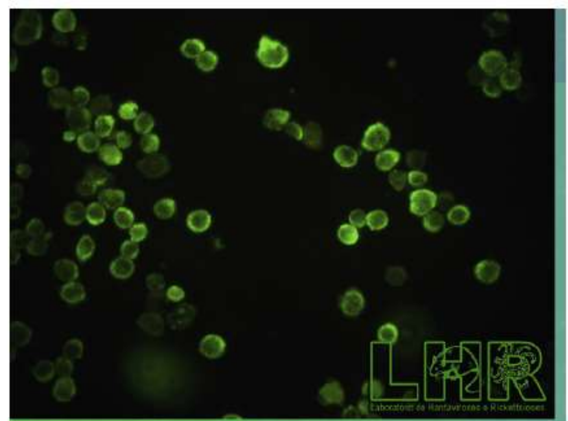

Fig. 1. Reactive indirect immunofluorescence slide for *Bartonella*, 400× magnification. LHR = Laboratorio de Hantavirose e Rickettsioses, Instituto Oswaldo Cruz, Fiocruz, Rio de Janeiro, Brazil.

Dogs may be infected with *B. vinsonii* subsp. berkhoffii (Bvb) (Kordick et al., 1996; Honadel et al., 2001; Breitschwerdt et al., 2007; Henn et al., 2005), *B. henselae*, *B. clarridgeiae*, *B. washoensis*, *B. elizabethae*, *B. quintana* and *B. bovis* (Lamas et al., 2008). The role of dogs as reservoir of *Bartonella* spp. (Honadel et al., 2001; Henn et al., 2005) is less clear than that for cats because domestic dogs are more likely to be accidental hosts, at least in non-tropical regions. However, dogs may be sentinels for human infections because they may become ill (Henn et al., 2005; Breitschwerdt et al., 1999). *Bartonella* seroreactivity in dogs is variable in the literature, depending on geographic differences and on clinical status of the animals. The most often serologically detected *Bartonella* in dogs is Bvb (Kordick et al., 1996; Honadel et al., 2001). In healthy individuals, antibody detection varies from 6.7% to 38% (Lamas et al., 2008; Honadel et al., 2001). In sick dogs, Bvb seroprevalences vary from 10% to over 90% (Lamas et al., 2008). The seroprevalence to *B. henselae* which is reported as 10–27% varies with sickness and health and geographic locale (Lamas et al., 2008). In the present study, all the apparently healthy dogs did not have antibodies detected. This may be because the number of animals tested was small, or because antibodies to Bvb were not sought for or that there was indeed a smaller infection rate in dogs in Brazil. This is suggested by the one published study on seroprevalence in 198 sick dogs in the city of São Paulo, where infection rate was 2% for *B. henselae* and 1.5% for Bvb (Diniz et al., 2007). Unpublished results in Barra do Piraí, a municipality located in a rural area in the state of Rio de Janeiro, show a seroprevalence of anti-*B. henselae* antibodies of 4.8% in 189 healthy domestic dogs (Moreira et al., ongoing PhD thesis at our lab).

A potential limitation of our paper is that *Bartonella* serology presents cross-reactions between the different species, and also between genera such as Coxiella and Chlamydia (La Scola and Raoult, 1996; Maurin et al., 1997; Agan and Dolan, 2002). Despite these limitations, IFA is the gold standard for identification of previous infection (Agan and Dolan, 2002; Regnery et al., 1992). The primary aim in seroepidemiological studies is to show potential exposure of human or animal populations to an infectious agent. Despite limitations, several serosurveys have shown their value in alerting health practitioners and scientists as to the dispersion of pathogens. The likelihood of false negative serological results are high in heavily immunocompromised patients with active *Bartonella* infection, as shown in 7 patients with AIDS and histologically documented bacillary angiomatosis where only one had detectable antibodies to *Bartonella* (Gasquet et al., 1998). This degree of immunosuppression was not present at the time of study enrolment in our patient group, and we do not believe false negative results occurred in our study. Only 2 of the 48 patients reactive to *Bartonella* also showed *Coxiella* antibodies, possibly representing false-positive results. However, one was homeless and the other had a cat.

It is important to point out that our study addresses prevalence in a convenience sample of HIV positive individuals, some of their pets and blood donors in the same rural area in Rio de Janeiro, possibly not reflecting true prevalence in the larger population.

Conclusions *Bartonella* spp. is widely dispersed in HIV positive and in blood donors living in a semi-rural area in the city of Rio de Janeiro. HAART, leading to better immunocompetence, has possibly shifted the spectrum of *Bartonella* syndromes in HIV individuals from BA and HP to CSD, FUO, neuroretinitis and endocarditis. This seems a reasonable assumption since the median CD4 counts of 30, reported in BA and BP in AIDS, are rarely encountered in Brazilian patients today. Cats seem to be the most important reservoir in the setting described. This brings implications in diagnosis and treatment, as well as counselling, of HIV individuals (and those with other causes of immunosuppression), and of patients with other predisposing conditions as valvulopathy, to the potential danger of cat exposure. *Bartonella* infections may be considered opportunistic in HIV positive individuals, and as such, it may be that guidelines addressing this issue should come forward from AIDS societies (Fig. 1).

Conflict of interest

The authors have no conflict of interest or disclosures concerning this paper.

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14.1.7. Cat scratch disease complicated with aseptic meningitis and neuroretinitis.

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Cat Scratch Disease Complicated With Aseptic Meningitis and Neuroretinitis

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Cat scratch disease (CSD) is a self limited condition characterized by fever, lymph node enlargement and less often eye involvement. Central nervous system involvement by *Bartonella henselae* infection is possibly an important cause of morbidity; its role as an agent of aseptic meningitis is unknown. We report a case of a 40 years-old man with CSD accompanied by aseptic meningitis and neuroretinitis. Serum indirect immmunofluorescence (IFI) assays for *B. henselae* were positive and the cerebrospinal fluid (CSF) analysis showed mononuclear pleocytosis and increased level of protein. Serological tests for other etiologies were negative. The patient responded well to antibiotic therapy with oral doxycicline plus rifampin and in the 12th day of hospitalization evolved to total regression of the headache and partial regression of the visual loss. Clinicians should consider CSD as a differential diagnosis when assessing previously healthy patients with aseptic meningitis associated with regional lymphadenopathy and epidemiological history of feline contact.
Key-Words: Cat scratch disease, *Bartonella henselae*, aseptic meningitis.

Central nervous system involvement by *Bartonella henselae* infection is possibly an important cause of morbidity; its role as an agent of aseptic meningitis is unknown. So far only three cases have been described in the literature, two of them in patients with HIV infection [1,2]. We report a case of aseptic meningitis in a previously healthy patient, HIV negative, who also had visual loss due to neuroretinitis, a far more common complication of CSD.

Manifestations of CSD are well described and include self-limited course of fever along with lymph node enlargement linked epidemiologically with a history of intimate contact with cat, mainly scratch or bite. Generally children and young adults are more involved, but cases are seen in any age group [3]. Isolation of the causative agent is very difficult and in the past, therapy was initiated on clinical findings and epidemiological history criteria. Laboratory diagnosis is often retrospective, based mainly in serology and typical findings on histopathological examination of the affected lymph node [4]. Diagnosis of such infections is based on clinical information, histopathology, culture and serology. However, none of these methods alone is sufficiently sensitive or specific. In the past few years polymerase chain reaction (PCR) is emerging as a useful tool for specific and rapid diagnosis [5]. There is no therapeutic consensus on the need for, selection and duration of antimicrobial therapy. *Bartonella spp*. are susceptible to betalactams, rifampin, erythromycin and tetracyclines, and have variable susceptibility to clindamycin, quinolones and co-trimoxazol [6].

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Case Report

S.F.B. a 40 year-old man was referred to the inpatient service of Evandro Chagas Clinical Research Institute (which is dedicated to the research in infectious diseases), with a 15-day history of continuous headache, visual loss, right axilar lymph node enlargement and fever (38.5ºC). This patient referred that seven days before the beginning of symptoms he had been scratched on his right hand by a street cat. Physical examination revealed an enlarged, painful and mobile lymph node in the right axillary region (Figure 1). There was no residual lesion in the region of the scratch. No abdominal mass or signs of meningism were detected. Laboratory findings, including blood cell count, were normal. Serological tests for toxoplasmosis, CMV, syphilis, HIV, Hepatitis B and C and *Sporothrix schenkii* were all negative. Thoracic computed tomographyc scans revealed an enlarged lymph node in left axillary region measuring 3.6 x 3.2 cm with peripheral contrast enhancement. Brain computed tomography scans were normal. Fundus examination of the left eye revealed optic disc edema, perifoveal infiltrates and a stellate macular lesion (Figure 2). Azythromicin 500 mg once daily was started.

On the fifth day of treatment the patient still referred headache and had persistence of fever; a lumbar puncture was done to investigate a meningeal involvement. CSF examination demonstrated a clear liquid containing 25 mononuclear cells per mm^3, glucose level was 53 mg/dL and protein level was 97.4 mg/dL. Based on these findings, antimicrobial treatment was changed to oral doxycicline (100 mg bid) associated with rifampin (300 mg bid).

Serology for *Bartonella spp.* was performed by indirect immunofluorescence assay, using commercial antigen, which revealed a 1:2048 immunoglobulin G (IgG) titer (cut-off 1:64); a second assay was done one week later resulting an IgG titer of 1:1024. Three more serum samples were analyzed and the fourth sample, collected 11 weeks after the first sample, showed IgG titer of 1:256. A sample of citrated whole blood collected

Figure 1. Enlarged lymph node in the right axillary region.

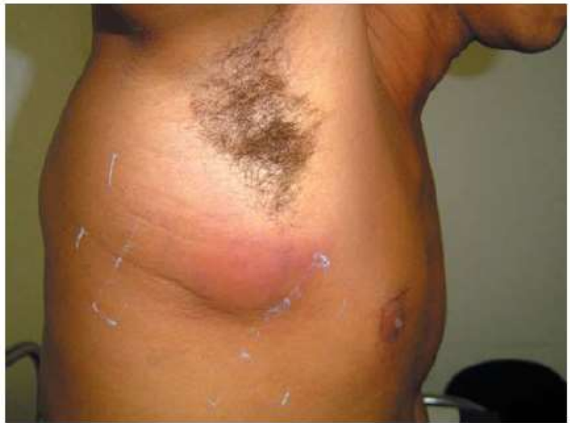
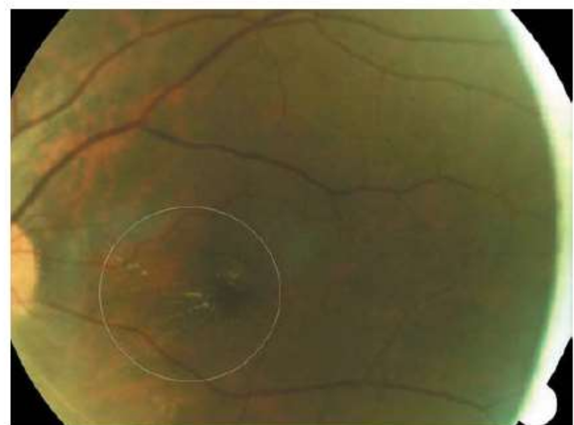


Figure 2. Fundus photography showing hard exudates (circled area) and discrete disc edema.



in the third day of hospitalization was subjected to PCR but was negative for *Bartonella spp*. Lymph node biopsy was not performed because the diagnosis was done based on clinical and epidemiology information and on typical ophthalmic alterations.

On the 12th day of hospitalization there was an outstanding clinical response with total regression of the headache and partial regression of the visual loss. The patient was discharged and advised to follow-up in the Ophthalmology Outpatient Clinic. In the 30th day of treatment there was total recovery of visual acuity and involution of the right axillary lymph node enlargement. Antimicrobial treatment was discontinued then.

Discussion

CSD is perhaps the most common cause of fever and lymphadenitis in children and young adults. In the United States the annual number of cases is estimated to be between 22,000 and 24,000. It has been associated with exposure to cats in about 90% of cases [4,7]. The prevalence of antibodies against *Bartonella henselae* in the population in various studies ranged from 3% in EUA to 61% in Italy, even in those without previous evidence of CSD [8-10]. Epidemiological studies of *Bartonella* infection in Brazil suggests high seroprevalence but the clinical implications are not widely known [11,12].

Typical clinical manifestations usually begin after a cat scratch. A papule then pustule develops seven to 12 days at the inoculation site, followed by enlargement of lymph nodes one to three weeks later, which can persist for few weeks to several months. Low grade fever, malaise, headache are also commonly referred by the patients [13].

Neurological complications of CSD could be considered an emerging condition, being described each day a different pattern of disease [14-16]. The better described manifestation is encephalopathy occurring in 2%-3% of patients. A syndrome of aseptic meningitis is rarely described and we have found three cases in the literature, one reported by Lucey et al. in immunocompetent patient whose CSF analysis revealed 19 leukocytes/mm^3 (100% mononuclear), a protein level of 62 mg/dL, and a glucose level of 76 mg/dL [1]. The other two cases were reported by Wong et al. one in HIV-positive patient [2]. These findings are compatible with the alterations encountered in our patient and probably could be more often than is currently diagnosed.

Ocular involvement occurs in 5% to 10% of patients with CSD, what makes the eye the most commonly affected organ after the lymphatics [17]. The most frequently described manifestation is Parinaud oculoglandular syndrome, which is characterized by eye redness, foreign body sensation and epiphora [18]. In this case we could observe an optic neuropathy with optic disk swelling and the presence of a partial macular star. These alterations are denominated neuroretinitis and CSD is its most common cause. Very often patients also complain of visual loss, generally reversible with antibiotic treatment [19-21].

In relation to laboratory diagnosis, is important to remember that the differentiation among the species of *Bartonella* by serologic test is difficult and the characterization of *Bartonella* by molecular techniques should be also used as a confirmatory diagnostic technique, mainly in disseminated *Bartonella* infections.

Although the PCR has been negative and the diagnosis of this aseptic meningitis has been based on clinical-epidemiological information and serology, we concluded that the patient was probably infected by *B. henselae*. These findings highlight the importance of *Bartonella* infection as a neglected and emergent agent and the need for more clinical studies of this disease in Brazil.

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14.1.8. *Bartonella* native valve endocarditis: the first Brazilian case alive and well.

Bartonella Native Valve Endocarditis: The First Brazilian Case Alive and Well

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Bartonella is an important cause of blood culture-negative endocarditis in recent studies. Seroprevalence studies in the States of Minas Gerais and Rio de Janeiro have shown Bartonella IgG positivity around 14% in healthy adults and 40% in HIV seropositive adults, respectively. A case report of a 46-year-old white male with moderate aortic regurgitation (AR) due to rheumatic heart disease (RHD), admitted due to worsening heart failure, is presented. Clinical features were apyrexia, anemia, polyclonal hypergammaglobulinemia, hematuria and splenomegaly. He was submitted to surgery due to worsening AR. Histopathology of the excised valve showed active bacterial endocarditis and underlying RHD. Routine blood cultures were negative. Indirect immunofluorescence (IFI) assays for _Coxiella burnetii_ were non-reactive. _Bartonella henselae_ IgG titer was 1:4096 prior to antibiotics and 1:512 14 months after treatment. History of close contact with a young cat during the months preceding his admission was elicited.
Key-Words: _Bartonella_ spp., infective endocarditis, blood culture-negative infective endocarditis, rheumatic heart disease, serology, indirect immunofluorescence.

Bartonella endocarditis has been recognized since the first reports from the 1990's [1-8] and its importance in blood culture-negative infective endocarditis (IE) has been reinforced in recent studies [9-14]. Although there are three national rickettsial diseases reference laboratories in Brazil (FIOCRUZ, Rio de Janeiro, Adolpho Lutz, in São Paulo, and FUNEDE, in Minas Gerais), only two probable fatal cases of *Bartonella* endocarditis have been reported so far in our country [14]. There are also case reports of *Bartonella* disease such as bacillary angiomatosis in HIV-positive individuals, disseminated disease in children and cat-scratch disease in Brazil, and there are also groups who work experimentally with *Bartonella* [15-21]. Two seroprevalence studies, one in the State of Minas Gerais [22], and the other in the city of Rio de Janeiro [23], show that the Brazilian population is significantly exposed to *Bartonella*. We present the first case of *Bartonella* endocarditis from Brazil, whose diagnosis was done ante-mortem and who had an extremely favourable outcome.

Case Report

A 46-year-old white male patient was admitted to Instituto Nacional de Cardiologia (INC), Rio de Janeiro, due to worsening heart failure in October 2005. He was born and resided in São Gonçalo, a town situated one hour from Rio de Janeiro. He became ill in 1994, during his job as a security officer. Essential hypertension was diagnosed then, as well as valvular disease. He was followed up in INC outpatient clinic. His first transthoracic echocardiography (TTE) in 1995 showed moderate aortic regurgitation and a calcified aortic valve. He retired a few years later due to heart failure. He was a poor historian, but his present admission was due to progression of fatigue and dyspnea on minimal exertion over the past four months. He denied fever. He was on use of captopril and frusemide. On examination, he was pale, height was 1.82 m, weight 76 Kg; carotid pulses were slow with a rapid descent, heart rate was 88 bpm, bp = 120x70 mmHg, cardiac rhythm was regular, right ventricular ictus was palpable as well as left ventricular ictus, which was located in the anterior axillary line, measuring three digital pulps and was propulsive. Heart sounds were soft, and a left ventricular third sound was heard. A systolic fremitus was palpable in the aortic area. Lungs were clear and abdomen was unremarkable. His lower limbs showed edema and were hyperchromic due to chronic venous insufficiency. First impression was of worsening cardiac function due to progression of his valvulopathy. TTE showed moderate aortic regurgitation, with an average aortic valve gradient of 47 mmHg. Electrocardiogram showed 1st degree atrioventricular (AV) block, 3rd degree right bundle branch block, left ventricular hypertrophy, an enlarged left atrium, ventricular and supraventricular ectopic beats. Hemoglobin was 11.5 g/dL, hematocrit was 25%, and mean corpuscular volume was 74. White cell count and platelets were normal. Creatinine was 2.2 mg/dL, urea was 102 mg/dL, potassium was 5.6 mEq/L and sodium was 134. Albumin was 2.7 g/dL, globulins were elevated at 5.4 (protein electrophoresis performed later showed polyclonal hyperglobulinemia; urinary Bence-Jones protein was absent). Thyroid function tests and B12 vitamin were requested because of a "neuropsychiatric condition", and were normal.

Urinalysis showed hematuria and presence of hemoglobin. HIV and hepatitis B and C serological assays were non-reactive. He was immune to hepatitis A. Ultrasound scan of the kidneys showed preserved cortex and medulla, and kidney length was 10 cm. Despite conventional treatment for heart failure, three weeks after admission he was in New York Heart Association (NYHA) functional class III. Cardiac catheterization done five weeks after admission showed normal

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coronaries. A repeated TTE showed worsening of the aortic gradient (68 mmHg) and more severe aortic regurgitation. He had surgery six weeks after admission. Surgical notes described heavily calcified aortic cusps; he needed temporary pacing due to complete heart block. A metallic St Jude 23 valve was placed in the aortic position. Extracorporeal circulation time was 150 min, clamping time was 120 minutes. He presented mild hypertension post-operatively, but he was discharged from intensive care two days later. Five days after surgery he presented atrial flutter which was reversed with 100 J shock and amiodarone. Moderate left ventricular dysfunction and a small pericardial effusion were seen on a TTE performed two days later. His cardiac function deteriorated and one week later transesophagic echocardiography (TEE) showed severe aortic regurgitation. Histopathology of the excised native aortic valve showed vegetations and was suggestive of active bacterial endocarditis. Aqueous penicillin and gentamicin were started the following day, after collection of three blood culture sets. Insertion of a peripherally inserted central catheter (PICC) was requested. Chest and abdominal tomography scans showed a small pericardial effusion and homogeneous splenomegaly. Blood cultures were negative and were discarded on the 7th day of incubation. Blood was sent off to the Rickettsial Reference Laboratory in FIOCRUZ for *Coxiella* and *Bartonella* serological assays. His antibiotics were changed to ceftriaxone, gentamicin and doxycycline two days after being started. On the 8th day of antibiotics, his hematocrit fell, and hemolysis was considered. Due to severe paraprosthetic leak, he has done another surgery one month after first valve replacement. Surgical notes confirmed paravalvular leak in the coronary and non-coronary cusps, and severe calcification of all valve ring, with injury to the anterior mitral valve leaflet. No signs of endocarditis were seen. Surgical time was 155 minutes, clamping time was 120 minutes. He presented complete AV block and was briefly asystolic in the immediate post-operative intensive care admission, but was well enough to be discharged three days later. He was treated with six weeks of intravenous ceftriaxone, two weeks of gentamicin and six weeks of oral doxycycline. He was discharged home two and a half months after admission, clinically well. He has kept appointments in the outpatient clinic, the last one being on February 2007. When re-called for repeating serology on April 2007, he reported having had close contact with a young cat, which was his wife's, during the year of 2005. He denied being scratched, licked or bitten by the cat, or being bitten by cat fleas. He divorced his wife a few months ago, and found out that the cat had died, for reasons unknown.

Materials and Methods

Routine blood cultures were performed, as well as culture of the excised native aortic valve on chocolate blood agar plates at 37°C with 5% CO_2 in a humid incubator, and the cultures were checked regularly for bacterial growth.

Indirect immunofluorescence assays for *Coxiella burnetii* (Panbio®) and *Bartonella henselae* (Bion®)-specific immunoglobulin G (IgG) and IgM were done in three different serum samples, one dated from December 1st, 2005, the other from December 26, 2005, and the last one from April 4th, 2007.

DNA extraction: total genomic DNA was extracted from serum and paraffin-embedded valvular tissue. QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) was used for DNA preparation, following the manufacturer's instructions. Briefly, two hundred microliter aliquots of serum were homogenized in AL lysis buffer and protease K and incubated overnight (ON) at 56°C. The mixture was added, washed and purified on a spin Qiagen column for 1 min at 14,000 x g in a mini centrifuge (Eppendorf 5245). One hundred microliters of elution buffer was used to resuspend the purified DNA. To obtain DNA from paraffin-embedded valvular tissue, 20 mg of tissue was homogenized in xylene, mixed with a vortex machine and centrifuged. Overnight incubation at 56°C in ATL lysis buffer and protease K was done. After incubation, AL buffer was added, homogenized and incubated for 10 min at 70°C. Separation of DNA was obtained by centrifugation of samples in a Qiagen column for 1 min at 14,000 x g. Extracted DNA was eluted with 100 µL of the elution buffer (Qiagen). All genomic DNA preparations were stored at 4°C until used as a template in PCR assay.

PCR Assays

PCR assay for the 60-kDa heat shock protein was performed as described previously [24,25]. DNAs, prepared from serum and valvular tissue, were used as template for the PCR assays. The extracted DNA was amplified with degenerate primer pair CAT-1 (GATTCAATTGGTTTGAAGGAGGCT) and CAT-2 (TCACATCACCAGGACGTATTC), for amplification of fragment of the 60-kDa heat shock protein (*htrA*), which defines a 414 bp fragment from both *B. henselae* and *B. quintana*. The PCR reaction mixture (total volume of 25 µL) contained 5 µL of the isolated DNA, 2.5 µL of 10-fold PCR buffer, 0.8 µM concentrations of each primer (IDT/PRODIMOL), 200 µM concentrations of each nucleotide, and 0.65 U of *Taq* polymerase (Platinum *Taq*, INVITROGEN). The PCR was accomplished by predenaturing for 5 min at 95°C followed by a total of 40 cycles of 94°C for 1 min, primer annealed at 50°C for 1 min, and extended at 72°C for 1 min in an automated DNA 2,400 thermal cycler (Applied Biossystem). Amplification was completed by holding the reaction mixture at 72°C for 7 min to allow complete extension of the PCR products. Each of the PCR experiments included DNA extracted from either *B. henselae* or *B. quintana* as positive control and water was used as negative control. Ten microliters from each PCR assay was electrophoresed through a 1% agarose gel, stained with ethidium bromide and documented with a gel documented system. The presence of a 414 bp band was considered positive.

Results

Routine blood cultures were negative, as was routine culture of the excised valve. Histopathology of the excised native aortic valve showed vegetations with neutrophilic exudates suggesting bacterial infective endocarditis; there were calcification and fibrosis suggesting underlying rheumatic disease (Figures 1 and 2). Warthin-Starry and PAS stains did not show any microorganism. PCR in serum and paraffin-embedded valvular tissue were negative. Serology IFA IgG and IgM were non-reactive for *C. burnetii*.

Serum titers for *Bartonella henselae* IgG were 1:4096 in the sample collected on December 1st, 2005, and 1:2048 on that of December 26, 2005. Serum collected on April 2007 (14 months after completion of antibiotics) showed a titer of 1:512.

Figure 1. Native aortic valve, hematoxylin-eosin 40x magnified. Vegetation with underlying tissue showing fibrosis and calcification (chronic rheumatic damage).

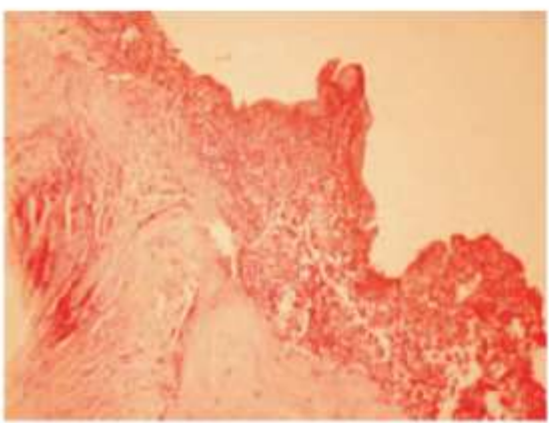
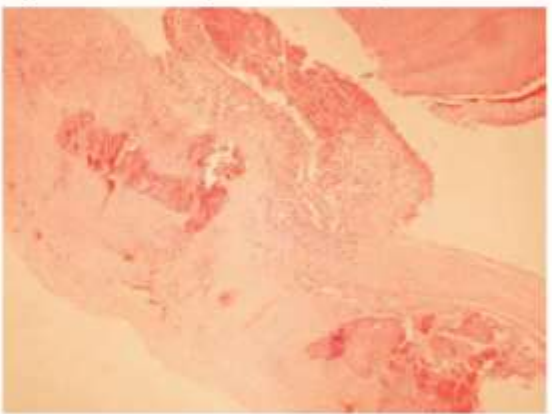


Figure 2. Detail of vegetation with neutrophilic infiltrate.



Discussion

Bartonella disease has a wide spectrum, ranging from asymptomatic to features of fever of unknown origin, uveitis, cat-scratch disease, bacteremia, bacillary angiomatosis and/or peliosis hepatis; the last two manifestations in immunocompromised hosts. Endocarditis has been well described, the first case being published 13 years ago; so far *B. quintana* has been the most frequently identified species, followed by *B. henselae* [26-30]; *B. vinsonii* subsp. *berkhoffi* [30] and *B. elizabethae* [2], which have been infrequently reported. There is a close relationship with acquiring *B. henselae* disease and cat exposure [9,10,26,31-33]. In Brazil, several clinical manifestations of *Bartonella* disease have been reported. Most importantly, seroprevalence studies have shown around 14% *B. quintana* and 13% *B. henselae* IgG antibodies in healthy adults [22] and 40% for *Bartonella sp.* in HIV-positive individuals [23]. In this last study, breeding cats was the only variable associated with *Bartonella* positive serology, despite high exposure to lice, fleas, ticks, dogs, rats and mice in the studied population. Despite the availability of serological studies by reference laboratories in Brazil, only two probable cases of *Bartonella* endocarditis have been reported so far; one involved a native aortic valve and the other a prosthetic aortic valve; both had domestic cats. Diagnosis was based on only one serological sample analysis, no details on associated conditions were mentioned and patients died rapidly (seven and ten days post-admission). This rapid evolution is not usual in *Bartonella* endocarditis [9,10,26,27]; there are several endemic conditions in our country such as Chagas disease and tuberculosis, among other infectious diseases, which may potentially present serological cross-reactions. The present case shows features of *Bartonella* endocarditis, which were similar to what was already published: predisposing valvular condition, absence of fever, exposure of the patient to a young cat at home in the months preceding his progressive heart failure, the need for valvular surgery and a good response to antibiotic regimen containing penicillin, gentamicin and doxycycline [9,10,26,27,32,33]. The diagnosis was made retrospectively by very high IgG titers (1:4096) to *B. henselae* in serum collected prior to antibiotic therapy, and lower levels (1:512) 14 months after completion of specific treatment. There are no antibody kinetic studies on *Bartonella* endocarditis to our knowledge, though a work has been published on cat-scratch disease and HIV-infected individuals, which showed titers declining rapidly over one year [34]. Cross-reactivity to *Coxiella burnetii* was ruled out by specific serology [35]. We can not rule out *B. quintana* cross-reactivity and we are aware of the significant predominance of this organism in countries such as Algeria and Tunisia [30,31]. Also the identification of *B. quintana* in cat fleas in France [36] and in cats [37] and the description of infective endocarditis caused by *B. quintana* in dogs [38] show that *B.quintana* is more widespread than previously thought. PCR was negative on the patient's serum as well as on paraffin-embedded valvular tissue; it has been reported that yield from such tissue is low [10]; besides, the process of decalcifying the valve might have further interfered with DNA extraction.

Bartonella sp. seems to be a prevalent microorganism in Brazil according to two recent seroprevalence studies; rheumatic heart disease is still frequently encountered, and rheumatic valvulopathy is the most important predisposing heart condition to endocarditis in our country. Therefore we assume more cases of *Bartonella* endocarditis are occurring but diagnosis is not being considered by clinicians, possibly because of lack of information regarding the ability of reference labs to perform serology and even molecular biology studies. We suggest investigation of all patients with blood culture-negative endocarditis for *Bartonella* in Brazil.

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14.2. CAPÍTULO NO MANUAL TÉCNICO DO MINISTÉRIO DA SAÚDE

"Diagnóstico Molecular das Rickettsioses com Ênfase na Febre Maculosa Brasileira"

DIAGNÓSTICO MOLECULAR DAS RICKETTSIOSES COM ÊNFASE NA FEBRE MACULOSA BRASILEIRA

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Considerando que os pacientes com suspeita de rickettsiose, mais especificamente, de febre maculosa, devem ser tratados com antibioticoterapia específica imediatamente com base, apenas, na história e nos dados clínicos, neste capítulo, os autores apresentam, com o objetivo de facilitar a compreensão do profissional da saúde, após uma breve revisão introdutória, tópicos sobre por que, como e quando utilizar a técnica molecular disponível, a reação em cadeia da polimerase (PCR) convencional, na rede nacional de vigilância de rickettsioses, com ênfase na febre maculosa brasileira.

1 CONSIDERAÇÕES GERAIS

Rickettsioses são doenças infecciosas causadas por proteobactérias dispersas em diferentes regiões do Mundo onde vetores e reservatórios como ácaros, carrapatos, piolhos e pulgas destes agentes zoonóticos existem. As espécies originalmente classificadas na Ordem Rickettsiales, família Rickettsiaceae, tribo Rickettsia, nos gêneros *Coxiella, Rickettsia* e *Rochalimeae,* foram reorganizadas e distribuídas, a partir da classificação taxonômica, com base na análise molecular. Assim, os gêneros *Rickettsia* e *Ehrlichia* foram classificados como proteobacterias do grupo alfa, no subgrupo alfa 1, as espécies do gênero *Rochalimae,* renomeadas como *Bartonella*, no subgrupo alfa 2 e a espécie *Coxiella burnetii* foi reclassificada no subgrupo gama das proteobactérias, ordem *Legionellales.*

Neste contexto, embora com frequência o termo rickettsioses no Brasil possa fazer referência mais especificamente à febre maculosa, causada pela espécie *Rickettsia rickettsii*, a análise molecular que se encontra disponível na rede nacional de vigilância de rickettsioses poderá ser aplicada para as espécies

historicamente descritas no campo da rickettsiologia como os gêneros *Anaplasma, Bartonella, Erhlichia* e *Rickettsia*,

A emergência e re-emergência de diferentes rickettsias que podem causar impacto na saúde pública em diferentes regiões do Mundo têm sido um tópico de crescente interesse para a pesquisa clínica. Além dos fatores associados com as variações genéticas, as mudanças sócio-ambientais, a dinâmica populacional assim como as pressões ambientais tanto associadas com a população humana como de outros animais, a crescente facilidade das viagens internacionais têm favorecido o surgimento/ressurgimento das rickettsioses no cenário mundial como importantes agravos no contexto da medicina do viajante.

As características inespecíficas no estágio inicial da febre maculosa brasileira podem levar a diferentes suspeitas diagnosticas. Por este motivo, diante das manifestações clinicas, com a presença ou não do exantema, o diagnostico diferencial deve ser feito com leptospirose, dengue, hepatite viral, salmonelose, encefalite, malária, entre outras. O diagnostico diferencial com borreliose/doença de Lyme, um grupo de doenças causadas por espiroquetas do gênero Borrelia, e babesiose,uma doença causada por protozoários do gênero *Babesia* deve ser incluídoem decorrência da semelhança do quadro clínico e epidemiológico.

No caso específico da febre maculosa, a rickettsiose mais importante no Brasil, embora a orientação de tratamento imediato de casos suspeitos, mesmo na ausência de confirmação laboratorial, seja preconizada, a identificação da espécie de rickettsia *lato sensu* causadora da doença humana é clinica e epidemiologicamente relevante e os métodos moleculares para a sua identificação devem ser estabelecidos, já que os métodos convencionais baseados na cultura, detecção de antígenos/anticorpos utilizados no diagnóstico das rickettsioses, embora fundamentais, apresentam uma série de restrições, Neste contexto as técnicas moleculares são, na atualidade, importantes e imprescindíveis instrumentos diagnósticos na rotina dos laboratórios de saúde pública considerando tanto a importância da implementação rápida do controle da infecção quanto as medidas de intervenção tanto individual quanto coletivo.

Embora exista um grande número de métodos moleculares disponíveis – a

grande parte das técnicas moleculares hoje utilizadas apropria-se da PCR ou de suas variações – neste capítulo restringiremos à PCR convencional, por ser a metodologia validada e que se encontra disponível para a rede laboratorial de referência.

Nos últimos cinco anos, a partir da disponibilização da PCR e sequenciamento das rickettsias pela rede de laboratório do Ministério da Saúde foi possível (i) identificar a espécie *Rickettsia rickettsii* em surtos e casos fatais em diferentes áreas do território brasileiro, (ii), identificar nova espécie de rickettsia associada com escara e adenomegalia no estado da Bahia (Silva et al., 2011); (iii) caracterizar molecularmente pela primeira vez no Brasil , em um caso de febre de origem obscura no Rio de Janeiro, o agente da febre Q, *Coxiella burnetii*, cuja ocorrência era baseada em evidência sorológica apenas; (iv) a identifiação de *Bartonella* em amostras de pacientes com endocardite, meningite, entre outras alterações, (v)além (v) da identificação de *R. conorii conorii* em caso fatal de febre hemorrágica em paciente sulafricano em 2008 (de Almeida et al 2010).

2. BREVE HISTÓRICO.

No início da década de 1970, a descoberta das enzimas de restrição, permitiu que genes dos mais variados organismos fossem isolados, clonados e, então, analisados em detalhe. Com o avanço das técnicas de biologia molecular, o estudo destas bactérias intracelulares obrigatórias foi excepcionalmente facilitado. A clonagem molecular forneceu meios pelos quais genes rickettsiais e suas proteínas correspondentes pudessem ser estudadas sem a necessidade de cultivo da rickettsiae como também de sua purificação da célula hospedeira.

A reação em cadeia da polimerase (PCR, do inglês *Polymerase Chain Reaction*) foi a técnica que marcou uma nova era na biologia molecular, possibilitando uma nova forma de isolamento e identificação de genes, sem que houvesse a necessidade de clonagem celular. Através dessa técnica, é possível obter uma grande quantidade de DNA de uma região específica a partir de quantidades extremamente pequenas de um DNA-molde.

A detecção molecular de rickettsias em amostras de sangue, coágulo ou de tecido de biópsia ou necropsia tem sido estabelecida em diferentes regiões do Mundo, onde rickettsioses do grupo da febre maculosa e do grupo do tifo são importante problema de saúde pública. Aplicada no diagnóstico da febre maculosa pela primeira vez em 1989, por Tzianabos e colaboradores, a técnica de PCR possibilita a confirmação diagnóstica na fase inicial da doença, antes da detecção sorológica de anticorpos anti-rickettsia (27).

Antígenos individuais do agente etiológico da febre maculosa (*R. rickettsii*) têm sido clonados e expressos em bactérias e em outros sistemas de expressão. O resultado dos antígenos recombinantes expressos tem sido usado no estudo das respostas imunes obtidas durante infecção com *R. rickettsii*. Estas proteínas, candidatas à vacina em potencial, têm sido identificadas e demonstram produzir proteção em modelos animais (1,14). Da mesma forma, ferramentas moleculares vêm demonstrando ser um passo no entendimento dos mecanismos de patogênese e bioquímicos das rickettsiae. As técnicas moleculares também têm impacto na identificação e caracterização de novas rickettsias patogênicas como também na reorganização de seus grupos.

3. REAÇÃO EM CADEIA DA POLIMERASE

A amplificação de DNA específico através da PCR é um método rápido para detectar infecções causadas por rickettsias. Esta técnica não possui um padrão específico, e a sensibilidade e especificidade diagnóstica pode variar entre os testes (5). Essas particularidades fazem da técnica de PCR uma ferramenta revolucionária na área de diagnostico molecular, pois ela permite que a presença de determinado agente infeccioso possa ser diagnosticada de forma direta, através da identificação dos seus genes, mesmo quando ele está presente em quantidades muito pequenas em determinado organismo hospedeiro. No entanto, o período tardio de obtenção da amostra de sangue associado com o tratamento com doxiciclina, em particular, pode diminuir a sensibilidade do teste (4).

A PCR é realizada em três etapas, repetidas em média, por 30 a 40 ciclos. Um ciclo de PCR envolve três etapas: desnaturação, hibridização e extensão. A fita dupla do DNA alvo é desnaturada através da elevação da temperatura para 92 a 95°C. Na etapa de hibridização, a temperatura é rapidamente reduzida para 35 a 60°C, dependendo essencialmente do tamanho e sequência do iniciador ("primer") utilizado, permitindo a hibridização DNA-DNA de cada iniciador com as sequências complementares que flanqueiam a região alvo. Em seguida, a temperatura é elevada para 72°C para que a enzima DNA polimerase realize a extensão a partir de cada terminal 3' dos *primers*. Esta extensão envolve a adição de nucleotídeos utilizando como molde a sequência alvo, de maneira que uma cópia desta sequência é feita no processo. Este ciclo é repetido por algumas dezenas de vezes. Uma vez que a quantidade de DNA da sequência alvo dobra a cada ciclo, a amplificação segue uma progressão geométrica de maneira que, depois de apenas 20 ciclos, são produzidos mais de um milhão de vezes a quantidade inicial de sequência alvo (24).

Esta escala de amplificação permite, portanto, iniciar com quantidades mínimas de DNA (da ordem de alguns picogramas ou nanogramas) e terminar a reação com grandes quantidades de DNA de uma sequência específica de interesse.

2.1. Reagentes Necessários para a Reação

A reação sempre parte de um DNA molde, extraído convenientemente da amostra, ou de uma amostra de RNA, convertida para cDNA (DNA complementar). O ideal é que o ácido nucléico esteja livre de impurezas (proteínas, lipídeos, outro ácido nucléico, reagentes de extração, etc.) e numa concentração mínima de 5 g/mL, apesar de quantidades bem menores poderem ser utilizadas. Abaixo seguem, em detalhes, os componentes da PCR (24):

2.1.1. **Água:** a qualidade da água do laboratório pode influenciar na reação,

de preferência, use água filtrada em aparelho de ultrafiltragem ou água comercializada livre de DNA e RNA. .

2.1.2. **Tampão**: o tampão da reação de PCR é fornecido juntamente com a Taq polimerase e vem 10x concentrado: faça uma leitura do folheto que acompanha a enzima para conhecer a composição do tampão de reação. O mais importante é observar o quantitativo de Magnésio existente no tampão, elemento fundamental para o funcionamento adequado da Taq.

2.1.3. **Mg ++:** O magnésio é um cofator da Taq polimerase. A concentração deste cofator na reação pode variar, em geral, desde 1,0 até 4,0 mM. Convém otimizar para cada enzima e material biológico, a concentração mais adequada. O Magnésio vem na forma de $MgCL_2$, acompanhando o tubo de enzima, em uma concentração de 50 mM. . É fortemente recomendado também, verificar se o tampão da Taq já contém $MgCl_2$. No caso afirmativo, acrescente somente o volume necessário para obter a concentração final desejada.

2.1.4. **dNTP:** os dNTPs podem vir na forma de um mix, em uma concentração de 20 mM cada. Este estoque deve ser mantido no freezer. Os estoques para uso diário podem ser preparados em volumes pequenos. Mantenha as alíquotas no freezer, use conforme necessidade e, renove sempre estes estoques. Além disso, os dNTPs podem vir separadamente, em concentrações, por exemplo, de 100 mM. Para preparar o mix misture partes iguais de tal forma a reduzir a concentração de cada nucleotídeo para 20 mM. As concentrações finais de dNTP na reação variam de um laboratório para outro.

2.1.5. Iniciadores (“*Primers”*): Antes das etapas experimentais, deve-se

planejar cuidadosamente a sequência e algumas características dos *primers*. Anteriormente concebidos por métodos manuais, hoje existem programas que "desenham" oligonucleotídeos e prevêem seu comportamento nas reações. Além da complementaridade, um ponto importantíssimo da natureza deste reagente é o seu **Ponto de Fusão Médio**, denominado **T_m** (*Temperature of Melting*), que é a temperatura na qual metade dos iniciadores está anelada às fitas de DNA e a outra livre na solução. Para cada par de *primers* que é utilizado, um T_m diferente é determinado. A temperatura média garante que, enquanto metade deles está nas cadeias-mãe, a outra metade está inteiramente disponível para o próximo ciclo. Os dois componentes do par (*Sense* e *Antisense Primers*) devem possuir T_m igual ou muito próximo para evitar alguns problemas na reação. A partir do valor de T_m podemos determinar o **T_a**, que é a temperatura ótima de reação do par de *primers*. Estas temperaturas são afetadas diretamente pela concentração do íon Na^+ na solução e na presença de agentes adstringentes que inibem o anelamento (24).

2.1.6. **DNA molde:** os DNAs deverão ser extraídos das amostras em que se pretende fazer as analises moleculares. .

2.1.7. **DNA polimerase:** Este é o componente mais caro da reação. A mais utilizada ainda é a *Taq (thermus aquaticus)*, numa concentração que varia de 1 a 5U / µl de solução. Normalmente, a maioria das DNA polimerases disponíveis no mercado são fornecidas conjuntamente com uma solução-tampão específica, cuja composição varia de acordo com o fabricante. Basicamente, estas soluções contêm íons diversos que otimizam as condições de reação. Alguns tampões contêm ainda detergentes que inibem a formação de dímeros das cadeias enzimáticas, proteínas e algumas substâncias que agem na denaturação da cadeia molde de DNA,

quebrando as pontes de hidrogênio entre as bases.

2.2. Identificação dos produtos das reações

2.2.1. Eletroforese: Existem dois modelos básicos de eletroforese; baseada em géis de agarose ou em géis de poliacrilamida. As duas substâncias formam tramas de poros de tamanhos variáveis, possibilitando a separação dos fragmentos, que terá sua eficiência dependente da concentração do polímero e da intensidade da voltagem e amperagem aplicada. Em qualquer um dos casos, estas substâncias são dissolvidas numa solução-tampão eletrolítica, obrigatoriamente a mesma que recobrirá o gel na cuba de eletroforese e possibilitará a passagem de corrente elétrica (Tampão de Corrida). Aplica-se uma voltagem entre as extremidades do gel que varia entre 10 e 200 V, e corrente de 50 a 3000 mA.

Para eletroforese de DNA, normalmente utiliza-se o TBE (Tris-Borato EDTA) e o TAE (Tris-Acetato EDTA).

Apesar da sua versatilidade e relativo baixo nível de dificuldade de realização, a eletroforese convencional tem a desvantagem de identificar os fragmentos apenas quanto ao tamanho, e não quanto à sequência.

Podem ser utilizadas concentrações de 0,5 a 3% de agarose no gel. Quanto maior a concentração, maior a sua capacidade de distinguir fragmentos de tamanhos próximos, fator denominado DEFINIÇÃO. Por exemplo, um gel de agarose a 1% pode separar fragmentos com uma diferença de tamanho de 80 pares de bases, enquanto a 2,5% podem-se separar fragmentos com diferença de no mínimo 30 pares de bases (24).

A visualização dos produtos no gel após a corrida se dá pela reação de ligação do DNA com Brometo de Etídio ou Gelred^{tm}. Estes compostos têm a capacidade de inserir-se nas fendas da cadeia de DNA e apresenta fluorescência quando excitado com radiação ultravioleta. Pode-se adicionar o corante (10µg/mL) no gel liquefeito antes da corrida ou levar o bloco a uma solução do corante com a mesma concentração e deixar descansar por alguns minutos (24).

3. PCR E AS PROTEOBACTÉRIAS ESTUDADAS NO CAMPO DA RICKETTSIOLOGIA

Em estudos de infecção por *Anaplasma phagocytophilum*, a PCR foi estimada com sensibilidade variando de 60 a 70% (3), e para o diagnóstico de infecção por *Ehrlichia chaffeensis*, a sensibilidade variou de 52-56% (15) a 87% (8).

Para a febre maculosa, a PCR é provavelmente mais útil na detecção do agente etiológico em amostras de biópsia de pele ou em necropsia de tecidos do que em amostras de sangue coletadas na fase aguda da doença. Isto é explicado devido ao baixo número de rickettsias circulantes no sangue quando na ausência de doença em fase avançada ou infecção fulminante (6). No entanto de forma crescente tem sido possível o diagnóstico molecular a partir de amostras de sangue e de soro na rede laboratorial, confirmando a importância, em especial nos casos graves e fatais, do processamento de qualquer amostra biológica disponível desde que esteja em condições adequadas de armazenamento e transporte.

Um teste da PCR apenas em biópsias de pele não oferece a sensibilidade ideal e um resultado negativo não exclui o diagnóstico, porque isto depende da coleta ter sido realizada no foco da lesão vascular. A confirmação laboratorial da febre maculosa no estágio agudo da doença é aumentada quando a PCR é usada em conjunto com a técnica de imunohistoquímica.

A PCR em amostras de sangue total é mais útil para confirmação de infecções causadas por *A. phagocytophilum*, *E. chaffeensis* e *E. ewingii* devido ao tropismo destes microorganismos por células brancas do sangue. Entretanto, não foi estabelecido qual o período ideal para a coleta da amostra que garanta uma alta sensibilidade para o diagnóstico das ehrlichioses e anaplasmoses. A partir da reação básica de PCR, foram criadas algumas variantes (isto é PCR em tempo real) que podem oferecer vantagens na rapidez de execução e obtenção de resultados, reprodutibilidade, capacidade quantitativa e, baixo risco de contaminação, comparado com a PCR convencional (9).

O gênero *Rickettsia* , com base na análise genômica, é dividido em quatro grupos:

i) **Grupo Ancestral**, composto por *Rickettsia bellii* e *Rickettsia canadensis*; de patogenicidade desconhecida;

ii) **Grupo do Tifo**, composto por *Rickettsia typhi* and *Rickettsia prowazekii*;

iii) **Grupo da Febre Maculosa**, constituído por mais de 25 espécies, entre elas, *Rickettsia rickettsii* e *Rickettsia parkeri* no continente americano, *Rickettsia conorii* e *Rickettsia africae* na Europa e na África, *Rickettsia japonica* e *Rickettsia israeli* na Ásia e no Oriente Médio;

iv) **Grupo Transicional**, constituído por *Rickettsia akari*, *Rickettsia australis* e *Rickettsia felis*.

Até recentemente, a caracterização destas bactérias era baseada apenas em critérios morfológicos, patogênicos, ecológicos e antigênicos. Com a introdução das técnicas de biologia molecular foi possível uma melhor e mais adequada caracterização seja pela análise completa do DNA genômico (12, 16, 19, 28) ou por uma pequena parte do cromossoma bacteriano (7, 17) ou por comparação da sequência de base nucleotídica (18, 20, 22, 26).

Os *primers* mais usados para a detecção de rickettsias em amostras clínicas de pacientes como também de carrapatos são: gene que codifica a proteína 17 kDa; gene da proteína de membrana externa A (*ompA*); gene da proteína de membrana externa B (*ompB*); gene do citrato sintase (*gltA*) e gene D (Tabela) (5, 10, 11, 22, 25).

A proteína de superfície 17 kDa é bem conservada entre as rickettsias do GFM e rickettsias do GT (2). O gene que codifica esta proteína já foi seqüenciado para a maioria das espécies destes dois grupos e vem sendo muito utilizado para a identificação e análise filogenética das rickettsias (27).

Tabela: Oligonucleotídeos utilizados para detecção das proteobactérias no campo da Rickettsiologia.

Gene alvo	Sequência do "primer"	Referência
Gene que codifica proteína 17 kDa [1]	R17-122 (forward), CAGAGTGCTATGGAACAAACAAGG R17-500 (reverse), CTTGCCATTGCCCATCAGGTTG TZ15 (forward), TTCTCAATTCGGTAAGGGC TZ16 (reverse), ATATTGACCAGTGCTATTTC	13, 27
Gene da proteína de membrana externa A (ompA) [1]	Rr190.70p (forward), ATGGCGAATATTTCTCCAAAA Rr190.701n (reverse), GTTCCGTTAATGGCAGCATCT Rr190.70p (forward), ATGGCGAATATTTCTCCAAAA Rr190.602n (reverse), AGTGCAGCATTCGCTCCCCCT	11
Gene da proteína de membrana externa B (ompB) [1]	BG1-21 (forward), GGCAATTAATATCGCTGACGG BG2-20 (reverse), GCATCTGCACTAGCACTTTC	21
Gene Citrato Sintase (gltA) [1]	RpCS.877p (forward), GGGGACCTGCTCACGGCGG RpCS.1258n (reverse), ATTGCAAAAAGTACAGTGAACA	22
Gene D[1]	D1F (foward), ATGAGTAAAGACGGTAACCT D928R (reverse), AAGCTATTGCGTCATCTCCG	10, 25
16S-23S ITS região[2*]	321s (forward) AGATGATGATCCCAAGCCTTCTGG 983as (reverse) TGTTCTYACAACAATGATGATG	31
Gene Citrato sintase (gltA) [2*]	BhCS.781p (forward) GGGGACCAGCTCATGGTGG BhCS.1137n (reverse)AATGCAAAAAGAACAGTAAACA	32
Gene htpAB (IS1111a) [3]	QBT1(forward) TATGTATCCACCGTAGCCAGTC QBT2 (reverse) CCCAACAACACCTCCTTATTC	33
Gene 16S[4*]	ECB(forward) CGTATTACCGCGGCTGCTGGCA ECC (reverse) AGAACGAACGCTGGCGGCAAGCC	34
Gene 16S[4*]	HE1 (forward) CAATTGCTTATAACCTTTTGGTTATAAAT HE3 (reverse) TATAGGTACCGTCATTATCTTCCCTAT	34

1- *Rickettsia*; 2- - *Bartonella; 3- Coxiella burnetii; 4- Ehrlichia*

** Oligonucleotídeos em fase de reavaliação*

Duas proteínas de membrana externa de alto peso molecular, a *OmpB*, uma proteína de superfície específica ao gênero *Rickettsia* (não está presente na *Rickettsia helvetica, R. bellii* e *R. massiliae*) e a *ompA*, uma proteína de superfície presente no GFM (com exceção *Rickettsia helvetica, R. australis, R. bellii* e *R. canadensis*) são imunógenos protetores (14) que reagem com amostras de soro de pacientes em fase de convalescença (1).

Citrato sintase (*gltA*) é um componente de quase todas as células vivas e é uma enzima pertencente ao ciclo do ácido cítrico. A *gltA* está presente no cromossoma de todas as rickettsias.

O gene D, que codifica uma proteína intracitoplasmática – a PS120, está presente na maioria das rickettsiae (10, 25).

4. TESTES MOLECULARES NO DIAGNÓSTICO DAS RICTTSIOSES– POR QUE UTILIZAR?

O diagnóstico laboratorial das rickettsioses até a década de 1980 era fundamentalmente baseado nas técnicas sorológicas, utilizadas para a confirmação de gênero ou grupo etiológico, sem definição de espécie da rickettsia causadora da infecção. Com a disponibilidade do resultado apenas durante o período de convalescença, a sua utilização como método útil para o diagnóstico na fase inicial da doença sempre foi consideravelmente limitado.

Opcionalmente, o cultivo de rickettsias, outra técnica também disponível desde o início do século XX, além de ser laborioso e necessitar, sob o ponto de vista da biossegurança, de um laboratório nível de risco classe 3, é um método restrito a poucos laboratórios que trabalham com pesquisa e/ou saúde pública e que, como os testes sorológicos, tem seu resultado disponível somente quando o paciente se encontra em estado de convalescença.

O diagnóstico molecular em amostra de sangue e/ou de biópsia de pele, assim como o imunohistoquímico em biópsia de pele, tem possibilitado a confirmação etiológica precoce das rickettsioses, nos primeiros dias de manifestação clínica, embora estudos mostrem que a imunohistoquímica em

lesões vasculíticas de pele seja o método mais sensível para a confirmação de febre maculosa na face inicial da doença.

Apesar da PCR ser uma técnica diagnóstica que, além de detectar DNA presente de 5 a 10 rickettsias durante a fase aguda da doença em amostra biológica, possibilita a caracterização da rickettsia a partir do sequenciamento genômico dos produtos obtidos durante o processo de amplificação nucleotídea, o sucesso do diagnóstico molecular depende:

4.1. **tipo de espécimes clínicos**

As amostras biológicas podem ser de sangue total ou coágulo ou fragmentos de tecido preferencialmente mantidos a temperatura de -70oC. Eventualmente, líquor, soro, entre outros materiais biológicos coletados de pacientes, assim como carrapatos ou outros ectoparasitos, sejam procedentes dos pacientes ou coletados durante investigação epidemiológica, podem ser submetidos também à análise molecular, considerando as diversas metodologias de extração de DNA baseada na utilização de fenol-clorofórmio ou mesmo na utilização de outros métodos comercialmente disponíveis.

Amostras de sangue e/ou de fragmento de biópsia de pele coletadas após o uso de antibioticoterapia por mais de 24 horas, ou mesmo a seleção inadequada da área da lesão da pele ou da secção de fragmento de tecido de necropsia, assim como a disponibilidade apenas de material fixado em formol e/ou em parafina, podem reduzir a sensibilidade da técnica de PCR.

4.2. período de coleta dos espécimes clínicos

As amostras devem ser obtidas, preferencialmente, nos primeiros cinco dias de doença, mas impreterivelmente, antes do início do tratamento antimicrobiano específico. As rickettsias são microorganismos intracelulares obrigatórios e o nível de rickettsemia, exceto em casos graves e fulminantes, pode determinar um resultado falso-negativo.

5. TESTES MOLECULARES NO DIAGNÓSTICO DAS RICKETTSIOSES - COMO UTILIZAR?

A PCR desde a sua primeira descrição por Saiki e colaboradores em 1985 tem sido a principal metodologia utilizada na amplificação de ácidos nucléicos de diferentes rickettsias, embora outras técnicas moleculares para a detecção direta do DNA possam ser utilizadas em laboratórios de pesquisa (23).

Considerada uma metodologia sensível, específica e reprodutível em qualquer laboratório que disponha de uma infra-estrutura mínima, é imprescindível que espaços diferentes para os específicos procedimentos laboratoriais sejam disponibilizados, isto é, salas específicas para a extração, amplificação e detecção, para se evitar, assim, contaminações e resultados falso-positivos.

A PCR, conforme descrito previamente, é um processo dependente de um sistema composto de enzima polimerase, iniciadores (primers), deoxinuceotídios trifosfato, tampão Tris-HCl ph 8,4, além de reagentes mínimos como MgCl2 e KCl, cujo desempenho deve ser verificado antes da sua utilização nos diferentes protocolos de amplificação genômica disponíveis. Com a inclusão de controles internos de amplificação é possível controlar os resultados falso-positivo e falso-negativo, aumentando assim a confiabilidade dos resultados.

Embora potencialmente quaisquer amostras biológicas possam ser analisadas pela técnica molecular para a pesquisa de rickettsias, a PCR tem, nos últimos 20 anos, sido aplicada basicamente em amostras de sangue, fragmento de tecido e em carrapatos, eventualmente coletados no paciente ou durante estudos epidemiológicos, considerando, no entanto, as recomendações sobre o manuseio correto, o volume, a fase de coleta de material, assim como o transporte e o acondicionamento do material biológico.

Os espécimes coletados devem ser acondicionados e transportados em frascos estéreis sob refrigeração (4 a 8°C), se encaminhados dentro de um período de 24 horas. Caso o período de transporte ultrapasse às 24 horas, as amostras deverão ser encaminhadas preferencialmente à temperatura de -70 oC ou em gelo seco, podendo ser aceitas amostras conservadas a -20oC.

O volume de sangue é de no mínimo 0,6 ml, podendo ser encaminhado em tubos contendo EDTA ou o coágulo, levando em consideração, no entanto, o risco de contaminação do material durante o seu manuseio para a separação de soro, fato que poderá interferir na amplificação genômica.

Em relação aos fragmentos de tecido, mais especificamente, os fragmentos de biópsia de pele, o material deve conter a lesão vasculítica, pois a sua não inclusão poderá determinar um resultado falso negativo.

6. TESTES MOLECULARES NO DIAGNÓSTICO DAS RICKETTSIOSES, MAIS ESPECIFICAMENTE DA FEBRE MACULOSA - QUANDO UTILIZAR?

Considerando o custo do ensaio, o diagnóstico molecular das rickettsioses deve ser solicitado quando:

a) há necessidade de se definir a espécie de rickettsia circulante durante surtos;

b) ocorrem casos fulminantes, nos quais é impossível o diagnóstico sorológico e as amostras de sangue, biópsia e/ou necropsia estejam disponíveis e adequadamente acondicionas;

c) há possibilidade de circulação de novas espécies de rickettsias;

d) é necessário o diagnóstico rápido para auxiliar na elucidação de quadros clínicos e na vigilância epidemiológica;

e) em caso clínico-epidemiológico compatível com febre maculosa no qual ocorreu, utilizando testes sorológicos, soroconversão menor do que quatro diluições entre as amostras de soro coletadas em um período superior a 14 dias;

f) um teste auxiliar é necessário para o esclarecimento de resultados sorológicos inconclusivos;

g) pacientes apresentam quadro clínico de rickettsioses após história de viagem para exterior, principalmente com o relato de ecoturismo;

7. ENCAMINHAMENTO DAS AMOSTRAS

Considerando que o transporte de material biológico além de cumprir corretamente as normas e legislação, deve levar em consideração, entre outros,

alguns fatores, como o agente biológico objeto de investigação, as diferentes e especificas formas de conservação do material coletado e a distância entre o local de coleta e o laboratório onde as atividades serão realizadas.

De uma forma geral, para que o transporte do material biológico seja realizado dentro dos princípios básicos de biossegurança preconiza-se que:

a) Os recipientes primários para acondicionamento de material coletado sejam higienizáveis impermeáveis, dotados de mecanismos ou dispositivo que impeçam o extravasamento das amostras e confiram total segurança ao seu transporte;

b) Os recipientes sejam corretamente etiquetados de acordo com o seu conteúdo, contendo também dados de identificação do laboratório responsável pelo material;

c) As pessoas responsáveis pelo preparo e transporte do material biológico recebam treinamento que inclua também normas de biossegurança;

d) O material biológico deve ser transportado em veículos oficiais. Não é permitido transportar amostras biológicas no mesmo compartimento em que são transportados os passageiros (Brasil, 2009);

e) O transporte do material deve garantir a qualidade de análise das amostras levando em consideração o tempo e a distância entre o local de coleta de material e o laboratório onde serão executadas as atividades analíticas.

É importante, por fim, que a embalagem esteja corretamente etiquetada com a sinalização de risco, com as informações sobre o destinatário.

8.CONCLUSÃO

Nas últimas décadas com os avanços tecnológicos, diferentes metodologias moleculares vêm sendo aplicadas tanto no diagnóstico genômico direto e precoce das rickettsioses, em especial da febre maculosa, quanto na redefinição taxonômica, como na identificação e caracterização de diferentes espécies e subespécies de rickettsias.

Não obstante dos métodos moleculares para a detecção de rickettsias do grupo da febre maculosa, do grupo do tifo e de ehrlichias não serem utilizados rotineiramente nos laboratórios clínicos, uma série de limitações pode interferir a sua aplicação como instrumento diagnóstico das rickettsioses: 1) a inexistência de um kit comercial aprovado; 2) o custo relativamente elevado da sua implantação em decorrência da necessidade de uma infra-estrutura adequada para a realização da técnica; 3) a utilização de diferentes protocolos desenvolvidos na pesquisa sem uma padronização, fato que pode determinar falsos resultados positivos e negativos; 4) acondicionamento, manuseio e transportes inadequados das amostras e 5) a falta de suspeição clínica pelos profissionais de saúde.

Em relação à falta de suspeição clínica, é importante frisar que a disponibilidade de novas tecnologias diagnósticas, como as metodologias moleculares, não determinará nenhum impacto na sobrevida dos pacientes, se os serviços e os profissionais da saúde não tiverem habilitados para a inclusão do diagnóstico, principalmente da febre maculosa em suas formas mais graves, e também para o imediato tratamento empírico que deve ser instituído a exemplo de outras doenças infecciosas, como meningococcemia e endocardite aguda, antes da confirmação etiológica laboratorial.

Assim, diante do exposto e de uma forma geral, verifica-se que há uma necessidade de sensibilizar, continuadamente, serviços e profissionais para o diagnóstico clínico-epidemiológico das rickettsioses, com o objetivo principal de

reduzir a letalidade e possibilitar, concomitantemente, um maior avanço no conhecimento destas zoonoses, em especial da febre maculosa, no Brasil.

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