Molecular Detection of *Rickettsia africae* in *Amblyomma variegatum* Collected from Sudan

Ryo Nakao,¹ Yongjin Qiu,² Bashir Salim,^{3,4} Shawgi Mohamed Hassan,³ and Chihiro Sugimoto²

Abstract

Despite the increasing awareness of the importance of emerging vector-borne diseases, human tick-borne diseases, particularly rickettsial infections, are overlooked, especially in the countries such as Sudan with limited resources to perform molecular-based surveys. This study aimed at detection and genetic characterization of *Rickettsia* spp. in ticks collected from Sudan. The samples were first screened for the presence of rickettsial agents by *gltA* real-time PCR and subsequently characterized by *gltA* and *ompA* PCR and size-based multispacer typing. The results demonstrated the wide distribution of *Rickettsia africae* and/or closely related species across Sudan. The results of this report highlight the need for careful consideration of rickettsial infections in patients with nonmalarial febrile illness in this country. Nationwide surveillance on ticks associated with human rickettsial infections in Sudan is warranted.

Key Words: *Rickettsia africae*—Sudan—Real-time PCR—Size-based multispacer typing—African tick-bite fever.

Introduction

THE SPOTTED FEVER GROUP (SFG) rickettsiae are obligate intracellular bacteria transmitted by ticks that caused an emerging disease affecting humans. *Rickettsia africae* is the common SFG rickettsiae as a causative agent of African tick-bite fever (ATBF) in humans. The pathogen transmitted by *Amblyomma* ticks is distributed in sub-Saharan Africa, posing health risks to local communities as well as travelers returning from sub-Saharan Africa (Jensenius et al. 2009). Despite the increasing awareness of the importance of emerging rickettsial diseases, there is a lack of information on the prevalence of SFG rickettsiae, especially in the countries such as Sudan with limited resources to perform molecular-based surveys. The present study therefore aimed at detection and genetic characterization of SFG rickettsiae in ticks collected from Sudan.

Methods

Adult *Amblyomma variegatum*, which were not fully engorged, were collected from indigenous cattle in 2010 in six states in Sudan and South Sudan: Al Gazirah (Abu Ushar

village) (14.55°N, 33.11°E), Al Gadarif (Showak town) (14.38°N, 35.87°E), Central Equatoria (Juba city) (4.85°N, 31.60°E), Sennar (Singa city) (13.15°N, 33.93°E), South Kordofan (Kadugli city) (11.07°N, 29.72°E), and White Nile (Kosti city) (13.17°N, 32.67°E).

A total of 83 ticks (40 males and 43 females) were used in this study, from which genomic DNA was extracted using DNAzol (Invitrogen, Carlsbad, CA) as described previously (Nakao et al. 2011). The samples were first screened for the presence of SFG and typhus group (TG) (SFG/TG) rickettsiae by *gltA* real-time PCR; only real-time PCR-positive samples were subsequently genotyped using *gltA* PCR, *ompA* PCR, and size-based multispacer typing (MST), as described previously (Nakao et al. 2013). Briefly, *gltA* real-time PCR was conducted with primers CS-F (5'-TCGCAAATGTTTAC GGTACTTT-3') and CS-R (5'-TCGTGCATTTCTTTCCA TTGTG-3') and probe CS-P (5'-FAM-TGCAATAGCAAG AACCGTAGGCTGGATG-BHQ1-3') (Stenos et al. 2005) using THUNDERBIRD Probe qPCR mix (Toyobo, Osaka, Japan). The *gltA* PCR and *ompA* PCR were performed, respectively, with primers RpCS.780p (5'-GACCATGAGCA GAATGCTTCT-3'), RpCS.877p (5'-GGGGACCTGCTCA

¹Unit of Risk Analysis and Management, Hokkaido University Research Center for Zoonosis Control, Sapporo, Hokkaido, Japan.

²Division of Collaboration and Education, Hokkaido University Research Center for Zoonosis Control, Sapporo, Hokkaido, Japan.

³Department of Parasitology, Faculty of Veterinary Medicine, University of Khartoum, Khartoum-North, Sudan.

⁴Parasites, Vectors and Vector-borne Diseases, Agricultural Research Council–Onderstepoort Veterinary Institute, Onderstepoort, South Africa.

CGGCGG-3') and RpCS.1273r (5'-CATAACCAGTGTA AAGCTG-3') (Ishikura et al. 2003), and primers Rr.190.70p (5'-ATGGCGAATATTTCTCCAAAA-3') and Rr.190.70In (5'-GTTCCGTTAATGGCAGCATCT-3') (Roux et al. 1996) using high-fidelity KOD-Plus-Neo polymerase (Toyobo). PCR products were then purified with ExoSAP-IT (USB Corporation, Cleveland, OH) and sequenced using the Big-Dye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and an ABI Prism 3130x genetic analyzer according to the manufacturer's instructions.

The sequences obtained were submitted to the DNA Data Bank of Japan (DDBJ) (www.ddbj.nig.ac.jp) under accession numbers LC009786–LC009788. For size-based MST analysis, three intergenic spacers, namely *dkSA-xerC*, *mppA-purC*, and *rpmE-tRNA^{fMet}*, were amplified in a multiplex PCR reaction using fluorescently labeled primers (*dkSAF*, 5'-6-FAM-TCCCATAGGTAATTTAGGTGTTTC-3'; *dkAR*, 5'-TACTACCGCATATCCAATTAAAAA-3'; *mppAF*, 5'-VIC-GCAATTATCGGTCCGAATG-3'; *mppAR*, 5'-TTTCATTTATTTGTCTCAAAATTCA-3'; *rpmEF*, 5'-PET-TTC CGGAAATGTAGTAAATCAATC-3'; *rpmER*, 5'-TCAGG TTATGAGCCTGACGA-3') (Nakao et al. 2013). Multiplex PCR was conducted using the KAPA2G Robust HotStart PCR Kit (Kapabiosystems, Boston, MA), and the amplified fragments were directly analyzed by capillary electrophoresis using the ABI Prism 3130x genetic analyzer and Peak Scanner Software version 1.0 (Applied Biosystems).

Results and Discussion

Out of the 83 samples tested, 25 (11 males and 14 females) were positive for SFG/TG rickettsiae by *gltA* real-time PCR (Table 1). The highest prevalence rate was obtained in samples from South Kordofan State (88.2%), whereas all samples from Al Gazirah State and Central Equatoria State were negative. Subsequent PCR reactions against *gltA* real-time PCR-positive samples amplified *gltA* and *ompA* genes in 15 (60%) and five (20%) samples, respectively. Five samples amplified by *ompA* PCR were subjected to sequencing analysis, giving three different sequence types. One sequence recovered from three samples showed 100% identity with *R. africae* strain ESF-5 (GenBank acc. no. NC_012633). The other two sequences showed the highest similarity with uncultured *Rickettsia* spp. found in ticks from Uganda (Nakao et al. 2013) and Kenya

(Maina et al. 2014); however, the closest rickettsial species excluding environmental samples was *R. africae*, with 4- and 5-bp mismatches in 590-bp amplified region.

By MST analysis, 20 samples yielded amplified products for all three spacers, whereas *rpmE-tRNA^{fMet}* was not amplified in five samples. All 20 samples had a size combination of 225, 195, and 341 bp, respectively, for *dkSA-xerC*, *mppA-purC*, and *rpmE-tRNA^{fMet}*, which is identical to those of three rickettsial species, namely *R. africae*, *R. parkeri*, and *R. rickettsii*. Considering the geographic distributions of these rickettsial agents (Parola et al. 2005, Cazorla et al. 2008), the samples were most likely to harbour *R. africae* and/or closely related species.

This study demonstrated the presence of SFG rickettsiae in four different states of Sudan. Further analysis using the size-based MST method enabled the association of these SFG rickettsiae with *R. africae*. In Sudan, Parola et al. (2001) first reported the presence of *R. africae* in one of 16 *Amblyomma lepidum* samples by PCR. Subsequently, Morita et al. (2004) showed the presence of this pathogen in three different states, namely Al Gazirah, Central Equatoria, and Kassala, through the PCR screening of 22 *Amblyomma* ticks, including 12 *A. variegatum* and 10 *A. lepidum*.

Human tick-borne diseases, particularly *Amblyomma*-borne rickettsial infections, are overlooked. Because it is known that *A. lepidum* and *A. variegatum* are widespread in Sudan and South Sudan (Salih et al. 2004) and that *A. variegatum* is one of the *Amblyomma* ticks occasionally infesting humans (Estrada-Peña and Jongejan 1999), rickettsial diseases associated with *Amblyomma* spp. in humans and animals are expected in these countries. Cattle are suspected to serve as reservoirs of *R. africae* (Kelly et al. 1991). The majority of the inhabitants in the rural areas of Sudan and South Sudan practice animal rearing and agriculture. Animals are kept within the homesteads or in very close premises. The pastoralists, particularly, are in close association with their animals throughout the year. In such situations, ticks feeding on humans and infection with *R. africae* are anticipated. Therefore, nationwide surveillance of ticks associated with human rickettsial infections must be conducted in Sudan.

Some of the real-time PCR-positive samples were negative by conventional PCR, which made it difficult to conduct further genetic characterization of SFG rickettsiae. This drawback of sequencing-based method was successfully

TABLE 1. REAL-TIME PCR, CONVENTIONAL PCR, AND MULTISPACER TYPING RESULTS TO DETECT *RICKETTSIA* SPP. IN ADULT *AMBLYOMMA VARIEGATUM* COLLECTED FROM INDIGENOUS CATTLE IN SUDAN

Origin (state)	Tested	No. of samples:			
		<i>gltA</i> real-time PCR positive	<i>gltA</i> PCR positive ^a	<i>ompA</i> PCR positive ^a	MST positive ^a
Al Gazirah	9 (8/1) ^b	0	0	0	0
Al Gazirah	20 (5/15)	6 (2/4)	4 (1/3)	1 (0/1)	4 (1/3)
Central Equatoria	6 (2/4)	0	0	0	0
Sennar	19 (11/8)	3 (1/2)	1 (0/1)	2 (1/1)	3 (1/2)
South Kordofan	17 (8/9)	15 (8/7)	9 (4/5)	1 (0/1)	12 (7/5)
White Nile	12 (6/6)	1 (0/1)	1 (0/1)	1 (0/1)	1 (0/1)
Total	83 (40/43)	25 (11/14)	15 (5/10)	5 (1/4)	20 (9/11)

^aOnly tested for the samples positive by *gltA* real-time PCR.

^bTotal number of ticks (males/females).

MST, multispacer typing.

circumvented by the introduction of size-based MST analysis, as proposed in the previous study (Nakao et al. 2013). However, the failure of amplification of the spacer *rpmE*-tRNA^{Met} in five rickettsiae-positive samples might imply the necessity of further refinement of this approach, for example, by designing a new primer set and/or including additional spacer region(s). In conclusion, the present study demonstrates the wide distribution of *R. africae* and/or closely related species across Sudan. This fact highlights the need for careful consideration of ATBF in patients with nonmalarial febrile illness in this country.

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Author Disclosure Statement

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References

- Cazorla C, Socolovschi C, Jensenius M, Parola P. Tick-borne diseases: Tick-borne spotted fever rickettsioses in Africa. *Infect Dis Clin North Am* 2008; 22:531–544.
- Estrada-Peña A, Jongejan F. Ticks feeding on humans: A review of records on human-biting Ixodoidea with special reference to pathogen transmission. *Exp Appl Acarol* 1999; 23:685–715.
- Ishikura M, Ando S, Shinagawa Y, Matsuura K, et al. Phylogenetic analysis of spotted fever group rickettsiae based on *gltA*, 17-kDa, and *rOmpA* genes amplified by nested PCR from ticks in Japan. *Microbiol Immunol* 2003; 47:823–832.
- Jensenius M, Fournier PE, Kelly P, Myrvang B, et al. African tick bite fever. *Lancet Infect Dis* 2003; 3:557–564.
- Kelly PJ, Mason PR, Manning T, Slater S. Role of cattle in the epidemiology of tick-bite fever in Zimbabwe. *J Clin Microbiol* 1991; 29:256–259.
- Maina AN, Jiang J, Omulo SA, Cutler SJ, et al. High prevalence of *Rickettsia africae* variants in *Amblyomma variegatum* ticks from domestic mammals in rural western Kenya: Im-

- plications for human health. *Vector Borne Zoonotic Dis* 2014; 14:693–702.
- Morita C, El Hussein AR, Matsuda E, Abdel Gabbar KM, et al. Spotted fever group rickettsiae from ticks captured in Sudan. *Jpn J Infect Dis* 2004; 57:107–109.
- Nakao R, Magona JW, Zhou L, Jongejan F, et al. Multi-locus sequence typing of *Ehrlichia ruminantium* strains from geographically diverse origins and collected in *Amblyomma variegatum* from Uganda. *Parasit Vectors* 2011; 4:137.
- Nakao R, Qiu Y, Igarashi M, Magona JW, et al. High prevalence of spotted fever group rickettsiae in *Amblyomma variegatum* from Uganda and their identification using sizes of intergenic spacers. *Ticks Tick Borne Dis* 2013; 4:506–512.
- Parola P, Inokuma H, Camicas JL, Brouqui P, et al. Detection and identification of spotted fever group Rickettsiae and Ehrlichiae in African ticks. *Emerg Infect Dis* 2001; 7:1014–1017.
- Parola P, Paddock CD, Raoult D. Tick-borne rickettsioses around the world: Emerging diseases challenging old concepts. *Clin Microbiol Rev* 2005; 18:719–756.
- Roux V, Fournier PE, Raoult D. Differentiation of spotted fever group rickettsiae by sequencing and analysis of restriction fragment length polymorphism of PCR-amplified DNA of the gene encoding the protein rOmpA. *J Clin Microbiol* 1996; 34:2058–2065.
- Salih DA, Hassan SM, El Hussein AM, Jongejan F. Preliminary survey of ticks (Acari: Ixodidae) on cattle in northern Sudan. *Onderstepoort J Vet Res* 2004; 71:319–326.
- Stenos J, Graves SR, Unsworth NB. A highly sensitive and specific real-time PCR assay for the detection of spotted fever and typhus group Rickettsiae. *Am J Trop Med Hyg* 2005; 73:1083–1085.

Address correspondence to:
Chihiro Sugimoto
Hokkaido University Research Center for Zoonosis Control
Kita 20, Nishi 10, Kita-ku
Sapporo, Hokkaido 001-0020
Japan
E-mail: sugimoto@czc.hokudai.ac.jp