Ticks and Tick-borne Diseases xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis

Original article

Isolation of a divergent strain of *Rickettsia japonica* from Dew's Australian bat Argasid ticks (Argas (Carios) dewae) in Victoria, Australia

Leonard Izzard[a], Matthew Chung[b], Julie Dunning Hotopp[b], Gemma Vincent[a], Daniel Paris[c], Stephen Graves[a], John Stenos[a,*]

[a] Australian Rickettsial Reference Laboratory, Geelong, Victoria, Australia
[b] University of Maryland School of Medicine, Baltimore, Maryland, USA
[c] Department of Medicine, Swiss Tropical and Public Health Institute, Basel, Switzerland

ARTICLE INFO

Keywords:
Rickettsia
japonica
Australia
Argas
Carios
Bat

ABSTRACT

A divergent strain of *Rickettsia japonica* was isolated from a Dew's Australian bat argasid tick, *Argas (Carios) dewae*, collected in southern Victoria, Australia and a full-genome analysis along with sequencing of 5 core gene fragments was undertaken. This isolate was designated *Rickettsia japonica* str. argasii (ATCC VR-1665, CSUR R179).

1. Introduction

The importance of bats as reservoir hosts and vectors of emerging infectious pathogens is becoming increasingly recognised. However, it is typically not until these pathogens "spill over" into humans or domestic livestock that public interest and research funding into the pathogen increases (Calisher et al., 2006). In addition, previous and ongoing research has predominantly focused of viral pathogens, whilst the prevalence of bacterial pathogens and their potential impact continues to be largely neglected (Calisher et al., 2006). A number of studies have detected spotted fever group (SFG) rickettsial antibodies in the blood serum of insectivorous bats (Mühldorfer, 2013), and when experimentally infected, *Ornithodoros* spp. ticks were able to transmit *R. rickettsii* to susceptible animals (Parola et al., 2013).

Worldwide, the presence of SFG rickettsiae in argasid ticks is significantly underrepresented compared to their hard bodied counterparts (Brites-Neto et al., 2015) with only a comparatively small number of articles reporting the presence of SFG rickettsiae in *Argas* spp. *Ornithodoros* spp. and *Carios* spp. ticks (Loftis et al., 2005; Cutler et al., 2006; Reeves et al., 2006; Duh et al., 2010; Socolovschi et al., 2012; Milhano et al., 2014; Lafri et al., 2015; Tahir et al., 2016).

To date four characterised and two '*Candidatus*' SFG rickettsial species have been identified in Australia; *R. australis* (Queensland tick typhus) (Andrew et al., 1946), *R. honei* (Flinders Island spotted fever) (Stewart, 1991), *R. felis* (cat flea typhus) (Schloderer et al., 2006), *R. gravesii* (Owen et al., 2006b), '*Candidatus* R. antechini' (Owen et al., 2006a) and '*Candidatus* R. tasmanensis' (Izzard et al., 2009). The primary vectors for 5 of the 6 species are hard ticks, *Ixodidae* spp., the exception being *R. felis*, which is detected in the cat flea, *Ctenocephalides felis*.

2. Materials and methods

2.1. Collection and identification of ticks

Ticks were collected from the roosting boxes of microbats, primarily *Chalinolobus gouldii* and *Vespadelus* spp., during routine monitoring of colonies within Organ Pipes National Park (OPNP) in southern Victoria, Australia (Fig. 1). Ticks were removed from the nesting boxes using plastic specimen collection tubes containing damp gauze cloth to avoid subsequent dehydration. Care was required when handling as the soft ticks were very susceptible to mechanical damage. Ticks were identified using the dichotomous identification key found in the book Australian Ticks by F.H.S. Roberts (1970).

2.2. Rickettsial isolation and culture

The ticks were initially washed in 70% ethanol, followed by a rinse in sterile phosphate buffered saline (PBS), and then homogenized. The homogenate was then filtered using a 0.45 µm sterile syringe filter

* Corresponding author at: Australian Rickettsial Reference Laboratory, The Geelong Hospital, Barwon Health, P.O. Box 281, Geelong, Victoria, 3220, Australia.
E-mail address: len.izzard@gmail.com (J. Stenos).

https://doi.org/10.1016/j.ttbdis.2018.07.007
Received 14 February 2018; Received in revised form 8 July 2018; Accepted 10 July 2018

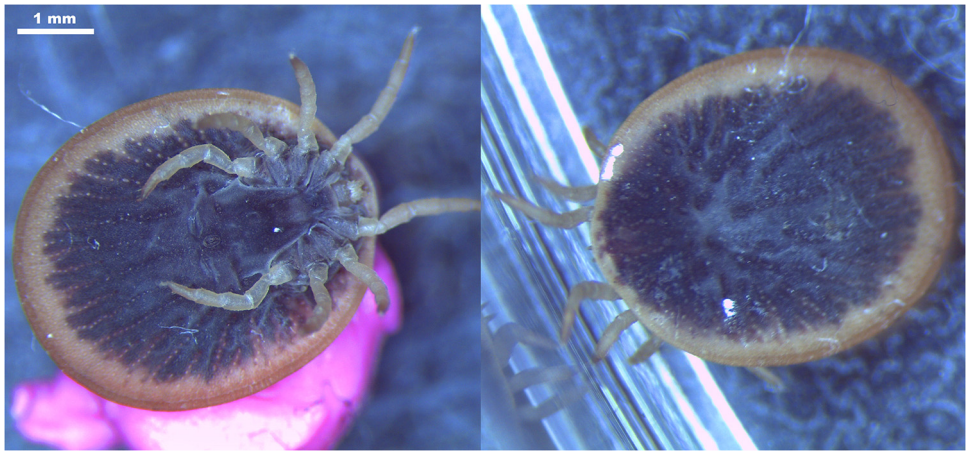

Fig. 1. Representative image of the *Argas (Carios) dewae* ticks collected from the roosting boxes of *Chalinolobus gouldii* and *Vespadelus* spp. bats.

(Millex, USA), to remove the tick debris and any other larger bacteria. The smaller rickettsial cells were small enough to pass through the filter. The filtrate was divided, with half subjected to DNA extraction for initial real time PCR and sequencing of 1098 base pairs of the gltA gene for species determination and the other half added to confluent T25 flask of VERO cells. Cultures were incubated at 34^{0}C, no CO_2, in RPMI 1640supplemented with 25 mM HEPES, 4 mM L-Glutamine and 3% foetal calf serum. After 30 days the cell culture was tested for the presence of a rickettsial agent using real time PCR and the resulting isolate was submitted to the ATCC (VR-1665) and CSUR (CSUR R179).

2.3. DNA extraction from tick homogenates and cultured bacterial samples

2.3.1. Gene segment analysis

DNA from the tick homogenates and isolated agent were extracted using a QIAmp DNA Blood Mini Kit (Qiagen, Germany) using the manufactures instructions. Briefly, 200 µL of sample was combined with 200 µL of buffer AL and incubated at 56 °C for 10 min. Next, 200 µl of glacial ethanol was added and the mix was added to a DNeasy spin column and briefly centrifuged. The column was then washed first with 500 µl of Buffer AW1, followed by 500 µl of Buffer AW2. Finally, the DNA was eluted from the column using 200 µl of AE elution buffer.

2.3.2. Full genome analysis

DNA from the isolate was also prepared for whole-genome sequencing using a modified version of the Gentra PureGene (Qiagen) Gram Negative Bacteria method. Briefly, the isolate was grown in flasks containing confluent Vero cells. The cells were detached, pelleted, and then disrupted by vortexing with 400 µM glass beads. The supernatant was filtered using a 2 µm filter, followed by a 1 µm filter, then incubated with DNAse (Brand) to remove contaminating host DNA. The bacterial agent was washed 3 times by centrifuging at 11,000 x g for 5 min and re-suspending in 300 µM sucrose solution. The DNA was then extracted as per the Gentra PureGene (Qiagen) Gram Negative Bacteria protocol.

2.4. Real time PCR detection of rickettsial agents in tick samples

All tick homogenates were subjected to real time PCR to screen for the presence of *Rickettsia* spp. using a previously described citrate synthase (*gltA*) specific assay (Baird, Lloyd et al. 1992).

Briefly, a 25 µL qPCR mix was prepared containing 2XPlatinum® qPCR SuperMix-UDG Mastermix (Invitrogen, Australia), 200 nM of each primer and probe, 5 mM $MgCl_2$, and extracted DNA. Extracted DNA from cultured *R. australis* was added to one tube as a positive control and water was added to a second tube as a 'no template control' (NTC). The qPCR reaction was performed in a Rotor-Gene 3000 (Corbett Research, Australia), with an initial 3 min 50 °C incubation, followed by a 95 °C for 5 min. After this the temperature was cycled 60 times; first with a 95 °C denaturation step for 20 s, followed by a 60 °C annealing step for 40 s.

2.5. Sequencing and phylogenetic analysis of gene segments

1419, 1098, 514, 4899, and 2876 base pairs of the *rrs, gltA, rOmpA, rOmpB*, and *sca4* genes respectively was amplified using primers that have been previously described (Fournier et al., 2003a, b). Sequencing of the amplicons using BigDye v3.1 technology was performed using a GeneAmp PCR System 2400 thermocycler (Applied Biosystems, USA). The product was then analyzed using an ABI Prism 3730xl DNA Analyzer (Applied Biosystems, USA) at the Australian Genomic Research Facility.

Sequencing traces were assembled in the DNASTAR software package (DNASTAR, Inc. USA) and analysed using BLAST analysis software (NCBI) (Altschul et al., 1990). All sequences have been deposited in GenBank (Table 1). A concatenated tree of the 5 sequences was generated using neighbor-joining and maximum parsimony methods in the MEGA 7 software package.

2.6. Genome sequencing and phylogenetic analysis

The genome of the isolate was sequenced by University of Maryland School of Medicine using Illumina next generation sequencing technology. The complete or draft sequences of 72 *Rickettsia* spp. genomes, including the isolate, were downloaded from NCBI GenBank (Benson et al., 2013) and aligned using the multiple whole-genome aligner, Mugsy v2.1 (Angiuoli and Salzberg, 2011). The core genome alignment, consisting of only the nucleotide base pairs present in all 72 genome sequences, was obtained and concatenated using MOTHUR v1.22 (Schloss et al., 2009). The resulting 502,279 bp alignment was used to generate a sequence identity matrix using BioEdit software (www.mbio.ncsu.edu/BioEdit/bioedit.html) (Fig. 2). In addition, the alignment was used to generate a maximum-likelihood phylogenetic tree using RAxML

Table 1
GenBank accession numbers of *Rickettsia japonica* str. argasii.

Gene	GenBank accession number
rrs	JQ727684.1
gltA	JQ727682.1
rOmpA	JQ727681.1
rOmpB	JQ727680.1
sca4	JQ727683.1