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

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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).

Keywords

Rickettsia; *japonica*; Australia; *Argas*; *Carios*; bat

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

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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., 2006), ‘*Candidatus* *R. antechini*’ (Owen et al., 2006) 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 (Figure 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 (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° C, no CO₂, in RPMI 1640 supplemented 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 $11,000 \times g$ 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 MgCl₂, 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., 2003). 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 3730x1 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) (Figure 2). In addition, the alignment was used to generate a maximum-likelihood phylogenetic tree using RAxML v8.2.0, using a GTRGAMMA model and 100 bootstraps (Stamatakis, 2014), and was viewed using Dendroscope 3 (Huson and Scornavacca, 2012) (Figure 3A).

3. Results

3.1. Tick identification, detection and isolation of rickettsial agents

A rickettsial agent was isolated from Dew's Australian bat argasid ticks, *Argas (Carios) dewae*, a soft tick from the family Argasidae (Figure 1). Rickettsial DNA was detected in 7 (70%) of 10 ticks by using a *gltA*-specific quantitative PCR (qPCR) assay (Stenos et al., 2005). The majority of the ticks collected were female, so no correlation between the sex of the ticks and presence of rickettsia could be determined.

3.2. Gene segment phylogenetic analysis

The 5 gene sequences from the novel isolate were compared to other validated rickettsia species using NCBI BLAST (Altschul et al., 1990). The sequences aligned closest to *R. heilongjiangensis* for the *gltA*, *rOmpB* and *rOmpA*, with sequence identities of 99.5% (1093bp/1098bp), 98.7% (4836bp/4899bp) and 98.2% (505bp/514bp) respectively, while the *rrs* and *sca4* genes aligned closest to *R. japonica*, with sequence identities of 99.9% (1418bp/1419bp) and 99.2% (2854bp/2876bp) respectively. Gene sequence analyses using the neighbor-joining algorithm was undertaken for all five genes, with a concatenated tree shown below (Figure 3B).

Phylogenetic analysis using the original 5 gene fragments, concatenated sequence or whole-genome sequence, all resulted in the isolate consistently aligning within the *R. japonica* and *R. heilongjiangensis* cluster. The criteria defined by Fournier *et. al.* (2003) states that for a rickettsial agent to be considered a new species it must not have more than one gene that exhibits a degree of "nucleotide similarity with the most homologous validated species" of equal to or greater than 99.9%, 99.8%, 99.3%, 99.2% and 98.8% for the *gltA*, *rrs*, *sca4*, *rOmpB* and *rOmpA* genes (Fournier et al., 2003). *Rickettsia japonica* str. *argasii* exhibited percentage identities of 99.9%, 99.5%, 99.2%, 99.1% and 98.1% for the *rrs*, *gltA*, *sca4*, *rOmpB*, and *rOmpA*, so by these criteria, only the *rrs* gene of the isolate was within the percent nucleotide identity threshold, and therefore, satisfies the requirements for classification as a new species.

3.3. Genome phylogenetic analysis

Using the complete or draft genome sequences of 71 *Rickettsia* spp., a identity matrix was calculated for all pairwise comparisons, and the lowest identity was identified within each of the twenty-nine different *Rickettsia* species examined. The isolate showed greatest identity to *R. heilongjiangensis* (99.7%) and *R. japonica* (99.6%) while the pairwise identity between *R. heilongjiangensis* and *R. japonica* was also 99.6%. Therefore, a pairwise identity of >99.7% was chosen as an arbitrary 'species cutoff as this was the level of identity between

the isolate and the validated species *R. heilongjiangensis*. Of the 15 species analyzed that had genome sequences of more than one strain available, only 8 (53%) had strain pairwise comparisons with >99.7% identity in the core genome alignment; *R. amblyommatis* (99.8%), *R. australis* (99.9%), *R. felis* (99.9%), *R. parkeri* (99.9%), *R. prowazekii* (99.9%), *R. rickettsii* (99.9%), *R. slovaca* (99.9%) and *R. typhi* (99.9%). Interestingly, nearly half (7) of the 15 species had strain pairwise comparisons where at least one pair had 99.7% identity: *R. bellii* (99.7%), *R. canadensis* (99.4%), *R. conorii* (99.6%), *R. hoogstraalii* (99.7%), *R. massiliae* (99.5%), *R. rhipicephali* (99.3%), and *R. sibirica* (99.7%). Of these 7 species, three had pairwise similarities between strains lower than the percent identity between the isolate and either *R. heilongjiangensis* (99.7%) or *R. japonica* (99.6%). Of note, they were also lower than the percent identity between *R. heilongjiangensis* and *R. japonica* (99.6%). However, when the species cutoff threshold is dropped to 99.6%, which then incorporates *R. heilongjiangensis*, *R. japonica* and the isolate within the same species, the number of total species whose strains all fall within the new threshold increases to 12 of 15 (80%) (figure 3). Therefore, while the new isolate fulfills Fournier's criteria for classification as a novel species, the additional information provided by genome analysis leads to the view that this isolate is more likely a divergent strain of *R. japonica* as opposed to a novel species. In addition, we believe that *R. heilongjiangensis* most likely also falls under this category and should also potentially be reclassified as a strain of *R. japonica*.

4. Discussion

When comparing individual genes, the concatenated results and whole-genome sequences, *Rickettsia japonica* str. *argasii* consistently clustered with *R. heilongjiangensis* and *R. japonica*, both of which are known to cause disease in humans (Uchida et al., 1992; Mediannikov et al., 2009; Aung et al., 2014). This, combined with the knowledge that several members of the *Argas* spp. are known to aggressively feed on humans (Estrada-Pena and Jongejan, 1999; Otranto et al., 2014; Aubry et al., 2016) suggests that this new rickettsial isolate has the potential to impact human health in the region.

Based on the traditional phylogenetic results, combined with both the novel vector and its remote geographic location compared to *R. heilongjiangensis* and *R. japonica*, we would have normally considered this isolate be a novel species. However, the original phylogenetic methodology compares less than 1% of the genome between *Rickettsia* species, while the addition of full genome sequencing, allowed a comparison with approximately 40% of the genome between species, and it is from these results that it appears that this new isolate is in fact a divergent strain of *R. japonica* and that it is likely that *R. heilongjiangensis* and *R. japonica* are in fact one species. We propose to nominate this rickettsial isolate a novel strain of *R. japonica* and name it *Rickettsia japonica* str. *argasii* after the tick vector from which it was isolated.

As the range of this study was limited to a single location in central Victoria, it would be useful to examine *A. dewae* ticks from roosting boxes in other locations in Victoria, and throughout Australia, for the presence of this rickettsial agent to help elucidate the true geographical range of this species. The pathogenicity of this strain is currently unknown, as it has currently only been isolated from its arthropod vector. However, as mentioned above,

this isolate clustered *R. heilongjiangensis* and *R. japonica*, both of which are known to infect humans. This knowledge combined with the fact that other member of the *Argas* spp. are known to aggressively feed on humans means that the potential exposure risk of this agent should not be disregarded. Pathogenicity studies are required to ascertain its full impact on public health. The likelihood of this agent being present elsewhere is high as the vector of this organism *A. dewae* is known to feed on the primary resident of the nesting boxes tested (Gould's Wattled Bat) (Kaiser and Hoogstraal, 1974), and this species of bat is known to travel great distances, therefore can potentially spreading this agent over great distances.

In addition, testing the blood of bats inhabiting the nesting boxes where the *A. dewae* ticks were found for evidence of SFG rickettsial exposure could also be undertaken and may provide data on whether the bats are being exposed to this agent.

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

Figure 2.
A heat-map of 72 genome sequences showing the level of conservation both within and across different species. The cross (†) symbol indicates a pairwise sequence identity of 99.6% between two sequences. Dendrograms across the top and left of the map show the relationship between different genome sequences.

Figure 3.

(**A**) A phylogenetic tree prepared using the maximum-likelihood method from a 502,279 bp concatenated core genome alignment. Bootstrap values are indicated at each node. Scale bar indicates the nucleotide divergence represented by the branch length. (**B**) A phylogenetic tree prepared using the neighbor-joining method from a concatenated sequence of all five gene segments (*rrs*, *gltA*, *rOmpA*, *rOmpB* and *sca4*) of *Rickettsia japonica* str. *argasii*. Bootstrap values are indicated at each node. Scale bar indicates the nucleotide divergence represented by the branch length.

Table 1.

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

Gene	GenBank accession number
<i>rts</i>	JQ727684.1
<i>gltA</i>	JQ727682.1
rOmpA	JQ727681.1
rOmpB	JQ727680.1
sca4	JQ727683.1