High prevalence of *Rickettsia gravesii* sp. nov. in *Amblyomma triguttatum* collected from feral pigs.

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Abstract

A survey of ectoparasites on feral pigs identified two commonly occurring ixodid tick species; *Amblyomma triguttatum triguttatum* and *Ixodes australiensis*. Molecular screening of *A. t. triguttatum* and *I. australiensis* for the presence of *Rickettsia* species detected the presence of rickettsiae belonging to the Spotted Fever Group (SFG) in 78.4% of screened *A. t. triguttatum*. None of the screened *I. australiensis* were positive for rickettsiae. Sequence analysis of the *glt*A and *omp*A loci of positive *Rickettsia* isolates were 100% homologous to the newly described species *Rickettsia gravesii* sp. nov. BWI-1. Serological screening of feral pigs detected antibodies to SFG *Rickettsia* in 50% of serum samples tested. These findings suggest that *A. t. triguttatum* is a potential vector/reservoir for *R. gravesii* sp. nov.

Keywords: *Amblyomma triguttatum*; *Ixodes australiensis*; Spotted Fever Group; *Rickettsia gravesii* sp. nov. BWI-1; molecular; serology; feral pigs.

1. Introduction

Feral pigs, *Sus scrofa* Linnaeus, 1758 are a highly invasive pest species in many parts of the world, including Australia (Choquenot et al., 1996). They are widely recognised as potential vectors of both exotic and endemic disease however few studies have investigated the ectoparsites and tick borne diseases associated with feral pigs, in particular *Rickettsia* species. Ticks are second only to mosquitoes in importance as vectors of human infectious diseases worldwide (Parola and Raoult, 2001), and ticks removed from wild pigs in both southern France and north-eastern Spain have been shown to harbour pathogenic *Rickettsia* species (Ortuno et al., 2006; Sanogo et al., 2003).

Rickettsiae are short, obligate intracellular gram-negative bacteria which require arthropod vectors for their transmission between mammalian hosts, (Fournier and Raoult, 2007). Members of the genus *Rickettsia* may be classified into the spotted fever group, the typhus group, *R. belli* and *R. canadensis* (Renvoisé et al., 2009). Several species of spotted fever group rickettsiae have been documented in Australia including *R. australis*, *R. felis*, *R. honei* and *R. honei* subsp. *marmionii* which are members of the SFG and tick transmitted (Odorico et al., 1998; Schloderer et al., 2006; Unsworth et al., 2007a; b). The occurrence of human cases of spotted fever throughout Western Australia have been reported (Owen et al., 2006a), but no organism has yet been confirmed as the aetiological agent.

A rickettsia of unknown pathogenicity (*Rickettsia gravesii* sp. nov. BWI-1) has recently been isolated from *Ambylomma triguttatum triguttatum* ticks from Western Australia (Owen et al., 2006a; b). Sequence analysis of the rickettsial 16S rRNA, *glt*A, *omp*A, *omp*B and *sca*4 genes demonstrated that *Rickettsia gravesii* sp. nov. BWI-1 is sufficiently divergent to be classified as a novel species (Owen et al., 2006b). As such, this study aimed to investigate the tick species commonly occurring on feral pigs in Western Australia and any *Rickettsia* spp. they may harbour.

2. Materials and Methods

Collection of Ectoparasites

Ectoparasites were removed from feral pigs post mortem and preserved in 70% ethanol containing 5% glycerol prior to identification. The presence or absence of lice and their eggs were recorded from all pigs, however ticks were the predominant ectoparasites collected. Ticks were examined under 1.5-30x magnification using a Wild MZA stereomicroscope and were identified to the species level based on standard morphological features including the presence or absence of eyes, the anal groove position, the coxal and the number of spurs present (Roberts, 1970). Sexing was performed based on scutum size and the presence of a genital pore.

DNA Extraction and PCR

Ticks were diced with a sterile surgical blade to break the exoskeleton prior to DNA extraction using the QIAamp® DNA Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions. Extracted DNA was resuspended in 70µL Elution Buffer and stored at –20°C until required. PCR amplification of the citrate synthase (*glt*A) and outer membrane protein A (*omp*A) genes utilised primers derived from a conserved region of *R. prowazekii* (*glt*A) and *R. rickettsii* (*omp*A) (Regnery et al., 1991).

Amplification of *glt*A was performed using 20µL PCR reactions consisting of 2.5µL of eluted tick DNA, 2.0mM of $MgCl_2$, 0.8mM each of dNTPs, three units of TAQ-1 DNA polymerase and 0.2pM of each primer, with ultrapure water used to adjust the final reaction volume to 20µL. Samples were amplified using a Perkin-Elmer GeneAmp 2400 thermo-cycler with an initial activation cycle of 95°C for five min, 53°C for three min, and 60°C for five min followed by 35 cycles of 92°C for 20 sec, 53°C

for 30 sec and 60°C for two min, with a final extension phase at 72°C for seven min. Each PCR run incorporated a positive control containing *R. australis* (SFG) and *R. typhi* (TG) DNA as well as a negative control.

Amplification of *Omp*A was performed using 25µL PCR reactions consisting of 2.0µL of eluted tick DNA, 0.5mM of $MgCl_2$, 0.1mM each of dNTPs, two units per sample of TAQ-1 DNA polymerase and 0.16pM of each primer, with ultrapure water used to adjust the final reaction volume to 25µL. Samples were amplified using a Perkin-Elmer GeneAmp 2400 thermo-cycler with an initial activation cycle of 95°C for three min, 48°C for three min, and 60°C for five min followed by 35 cycles of 95°C for 20 sec, 48°C for 30 sec and 60°C for two min, with a final extension phase at 72°C for seven min. Each PCR run incorporated a positive control containing *R. honei* (SFG) DNA as well as a negative control. *Rickettsia australis* DNA was not used as a control as it requires its own specific primers for amplification of the *omp*A gene (Fournier et al., 1998; Regnery et al., 1991).

Visualisation and Sequencing of PCR products

All PCR products were electrophoresed at 86V for 50 min in a 1.5% agarose gel containing 20 µg/ml ethidium bromide. Amplification products from 15 tick DNA extracts, five from each sampling area, for both the *glt*A and *omp*A loci were selected for sequencing (a total of 30 isolates). The PCR products were extracted using the QIAquick Gel extraction kit catalogue number 28704, as per the manufacturer's instructions. Purified PCR products were sequenced using the Big Dye version 3.1 terminator kit (Applied Biosystems, USA) and the Applied Biosystems 373 automatic sequencer and were compared to those of previously characterised rickettsiae in GenBank using BLAST analysis (http://www.ncbi.nlm.nih.gov:80/BLAST/).

Serology

Serological testing of feral pig (n=40) and control pig (n=40) sera for rickettsial antibodies using micro-immunofluorescence was performed using the method described by Philip et al. (1978). The control pig sera used was sourced from intensively farmed indoor pigs and collected post mortem at the abattoir. The cut off titre (1:128) was determined by the lowest titre at which zero members of the control group had any reaction to the rickettsial antigens to prevent misreading of false positives in the event of cross reactivity.

Statistical Analysis

Pair-wise analysis of the presence of ticks on feral pigs at time of capture and antibody presence was performed using Fisher's Exact test with Bonferroni's correction.

3. Results

Prevalence of Ectoparasites

Feral pigs were trapped and sampled from the three study areas over a six month period. Ticks were detected on 102 (49.0%) of 208 feral pigs sampled. Pigs in all three sampling areas were prone to tick infestation however there was a marked difference in tick prevalence between areas (Table 1). Ticks were primarily located on pigs in and around the ears, between the front and rear legs and along the belly. Ticks were also infrequently found attached to the facial region of pigs. Two species of ticks were identified based on morphological characterization from feral pigs; *Amblyomma triguttatum triguttatum* and *Ixodes australiensis*. Lice were detected on 199 (95.7%) feral pigs and were identified as *Haematopinus suis*, the common pig louse. Lice were predominantly located on pigs behind the ears and between the front and rear legs.

Molecular Screening of Ticks

Amplification of 88 *A. t. triguttatum* DNA extracts using the *glt*A primers detected 69 (78.4%) samples positive for rickettsial DNA (Table 2). Four negative *A. t. triguttatum* were collected from feral pigs which also had *A. t. triguttatum* collected from them that screened positive for rickettsial DNA. None of the DNA extracts from *I. australiensis* (n = 28) or *H. suis* (n = 7) produced a positive result. All 69 DNA extracts of *A. t. triguttatum* that screened positive for the rickettsial *glt*A gene also screened positive for the rickettsial *omp*A gene. There was no significant difference in the prevalence of *A. t. triguttatum* shown to be infected with rickettsiae between any of the sampling areas.

Sequence profiles were produced from both *glt*A and *omp*A amplification products from 15 randomly selected positive *A. t. triguttatum* DNA extracts (5 from each sampling area) to identify the *Rickettsia* species present. A BLAST search of the GenBank database confirmed all 15 sequences to have 100% homology at both the *glt*A and *omp*A loci for *Rickettsia gravesii* sp. nov. BWI-1 (GenBank accession nos. **DQ269435** and **DQ269437** respectively).

Serology

Screening of feral pig sera (n=40) revealed the presence of anti-SFG rickettsial antibodies ≥1:128 in 50% (20/40) of samples tested. All control sera tested negative for anti-SFG antibodies. There was no significant correlation between tick presence on feral pigs at time of capture and the presence of rickettsial antibodies in their sera.

4. Discussion

Tick species

Several tick species have previously been reported from both domestic and feral pigs in Australia however reports of *A. t. triguttatum* and *I. australiensis* are limited (Masters, 1979; Roberts, 1970). Both *A. t. triguttatum* and *I. australiensis* are three-host ticks with wide distributions throughout Australia and are able to colonise a wide range of hosts (Roberts, 1970). Whilst *A. t. triguttatum* has previously been reported to be a vector of *Coxiella burnetii*, the causative agent of Q fever in humans (Beaman and Marinovitch, 1999; McDiarmid et al., 2000), no diseases have been associated with *I. australiensis* to date (Bengis et al., 2002; Roberts, 1970).

Rickettsial Disease

The current study and recent work has shown *A. t. triguttatum* collected from both humans and wildlife throughout Western Australia to commonly harbour *Rickettsia gravesii* sp. nov. BWI-1 (Owen et al., 2006a). The pathogenic potential of *R. gravesii* sp. nov. BWI-1 is currently unknown however it is closely related to the *R. massiliae* subgroup of SFG rickettsiae (Owen et al., 2006b), which are pathogenic to humans and prevalent in southern and eastern Europe (Brouqui et al., 2007). In this regard it seems pertinent to treat *R. gravesii* sp. nov. BWI-1 with some caution, especially considering the recent recognition of *R. parkeri* as a human pathogen more than 65 years after first being isolated (Paddock et al., 2004).

The prevalence of *R. gravesii* sp. nov. BWI-1 in *A. t. triguttatum* ticks collected from feral pigs in the current study (78.4%) is significantly greater than that of *R. slovaca* detected in *Dermacentor marginatus* ticks from wild pigs by Sanogo et al. (2003) in France (15.7%) and Ortuno et al. (2006) in Spain (17.7%). This high prevalence of *R. gravesii* sp. nov. BWI-1 coupled with the abundance of *A. t. triguttatum* in the environment (Owen et al., 2006a; Pearce and Grove, 1987), may represent an

191 increased health risk associated with occupational and recreational activities which expose people to
192 contact with wildlife and their habitats.
193
194 All three life stages (adult, nymph and larval) of *A. t. triguttatum* recovered from feral pigs were shown
195 to be infected with the *R. gravesii* sp. nov. BWI-1. Additionally, the prevalence of rickettsial
196 antibodies in 50% of feral pigs tested in the present study suggests that the potential for transmission of
197 *R. gravesii* sp. nov. BWI-1 from tick to host and/or vice versa is significant.
198
199 Given the wide host range of *A. t. triguttatum* and the high prevalence of *R. gravesii* sp. nov. BWI-1 in
200 this tick species, there is potential for the transmission of rickettsiae to many different hosts; including
201 humans. This work highlights a need to increase tick awareness, especially for those people who
202 frequent tick infested areas or have contact with feral pigs during the course of their occupational or
203 recreational activities to further enhance the prevention of tick borne disease.
204
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210
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265

Table 1. Distribution of ticks identified from feral pigs across three sampling sites.

Area	No. of pigs examined	No. of pigs with ticks	No. of ticks collected	No. of ticks identified	
				A. t triguttatum	*I. australiensis*
Mundaring	32	18 (56.2%)	131	131 (100%)	0 (0%)
Serpentine	103	63 (61.2%)	349	299 (85.7%)	50 (14.3%)
Dwellingup[†]	73	21 (28.8%)	116	9 (7.8%)	107 (92.2%)
Total	208	102 (49.0%)	596	439 (73.7%)	157 (26.3%)

[†] Significantly fewer ticks present on pigs from Dwellingup than Serpentine ($p<0.001$) or Mundaring ($p<0.01$).

Table 2. Prevalence of rickettsiae in two species of ticks collected from feral pigs across three sites.

Area	Ticks Positive for *Rickettsia* sp. nov. BWI-1	
	A. t. triguttatum	*I. australiensis*
Mundaring	21/27 (77.8%)	n/a
Serpentine	46/59 (72.9%)	0/13 (0%)
Dwellingup	5/5 (100%)	0/15 (0%)
Total	69/88 (78.4%)	0/28 (0%)

Note: Larval stages from 4 pigs from Mundaring and from 5 pigs from Serpentine were pooled (respectively) for PCR screening for *Rickettsia*. No *I. australiensis* were found on feral pigs from Mundaring.