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Original article

An outbreak of scrub typhus in military personnel despite protocols for antibiotic prophylaxis: doxycycline resistance excluded by a quantitative PCR-based susceptibility assay

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Abstract

Scrub typhus is caused by the obligate intracellular bacterium *Orientia tsutsugamushi* and is endemic to many countries in the Asia–Pacific region, including tropical Australia. We describe a recent large outbreak amongst military personnel in north Queensland. A total of 45 clinical cases were identified (36% of all potentially exposed individuals). This occurred despite existing military protocols stipulating the provision of doxycycline prophylaxis. Doxycycline resistance in *O. tsutsugamushi* has been described in South-East Asia, but not Australia. In one case, *O. tsutsugamushi* was cultured from eschar tissue and blood. Using quantitative real-time PCR to determine susceptibility to doxycycline for the outbreak strain, a minimum inhibitory concentration (MIC) of ≤0.04 μg/mL was found, indicating susceptibility to this agent. It seems most probable that failure to adhere to adequate prophylaxis over the duration of the military exercise accounted for the large number of cases encountered rather than doxycycline resistance.

Keywords: Scrub typhus; *Orientia tsutsugamushi*; Doxycycline; Microbial sensitivity testing

1. Introduction

Scrub typhus is caused by the obligate intracellular bacterium *Orientia tsutsugamushi* and is transmitted by the bite of larval trombiculid mites, usually *Leptotrombidium* species (especially *Leptotrombidium deliense* in Australia). The disease remains endemic to many countries of the Asia–Pacific region, with a distribution described as approximating a triangular area of 13,000,000 km^2 including Eastern Russia, Korea, Japan, China, Taiwan, Thailand, southern Asia, tropical Australia and the islands of the south-west Pacific [9]. However, a disease resembling scrub typhus, caused by a bacterium related to *Orientia* has been described from Chile [1] and a new species of *Orientia* (*Orientia chuto*) has recently been isolated from a traveller returning from Dubai [7], some 500 km west of the previously recognised limit of epidemiological distribution. In Australia, the disease is geographically restricted to tropical regions and has historically been most commonly encountered in northern Queensland [12] and more recently the Torres Strait Islands [26,4]. Foci of endemicity are

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also recognised in the Northern Territory [18,2] and the Kimberly region of Western Australia [5,17]. Worldwide, it is predominantly a rural disease, with an estimated 1 million infections annually [19].

Small outbreaks were reported in Queensland, Australia, in 1996, 1997 [14], and 2005 [13], all occurring in soldiers performing military exercises in the Cowley Beach Training Area near Innisfail. Here we describe a fourth outbreak of scrub typhus in Australian army soldiers, which may represent the largest recorded outbreak in Australia for over 50 years.

1.1. Epidemiology of the outbreak

A full company of regular infantry soldiers and support elements deployed into the Cowley Beach training area in late April 2011. There is a requirement for all Australian Defence Force (ADF) members deploying into the Cowley Beach area to take chemoprophylaxis consisting of oral doxycycline 200 mg on entering the training area, 200 mg weekly thereafter, and 200 mg on exit from the area. This regimen is designed to prevent leptospirosis but has also been shown to be effective prophylaxis against scrub typhus [15]. In addition to this, dipping of uniforms in 25% permethrin (Perigen Defence; Bayer) is recommended to protect against mite bites, and soldiers are issued with personal insect repellent for the same purpose.

The first soldiers became unwell approximately two days after extracting from the training area, with the majority falling ill over the subsequent week. Because the soldiers were granted leave on conclusion of the exercise, suspect cases presented in other states within Australia, such as Victoria and New South Wales, in addition to Queensland, which delayed identification of the outbreak. More than 90% of the company were recalled and treated with doxycycline within 24 h, with the remainder captured in the following days. A subsequent dose of 200 mg seven days later was also recommended. A case met the clinical definition if the individual was deployed on the Cowley exercise and presented unwell between 23rd April and 5th May 2011 and displayed at least two of the following symptoms: headache, fever, maculopapular rash or arthralgia. A case met the laboratory definition if there was a ≥4-fold rise in IgG titres to *O. tsutsugamushi* between acute and convalescent serology, or a positive PCR on blood or tissue or a positive culture from clinical specimens.

1.2. Culture of O. tsutsugamushi *from an index case*

O. tsutsugamushi was cultured from one 19-year old soldier meeting the clinical and laboratory case definitions. Polymerase chain reaction (PCR) on whole blood and from a punch biopsy of eschar tissue from his trunk (see Fig. 1) detected *O. tsutsugamushi*–specific DNA [21]. These samples were also inoculated into Vero cell culture and severe combined immunodeficient mice. After culture, the presence of *O. tsutsugamushi* was confirmed by immunofluorescence of the Vero cells (see Fig. 2) and PCR of the mice spleens. He had been provided with doxycycline prophylaxis during the exercise, which he claimed to have taken as directed, and used clothing impregnated with insecticide. Given the development of scrub typhus despite the apparent provision of doxycycline prophylaxis, the possibility of doxycycline-resistant *O. tsutsugamushi* was considered, which has been described in Thailand [22,27] but never in Australia. Subsequent confirmation of many other cases from the same cohort of army personnel from the Cowley beach exercise further raised our concerns given the strong emphasis that the army places upon doxycycline provision. We therefore conducted further experiments to determine if the isolated strain demonstrated reduced susceptibility to doxycycline.

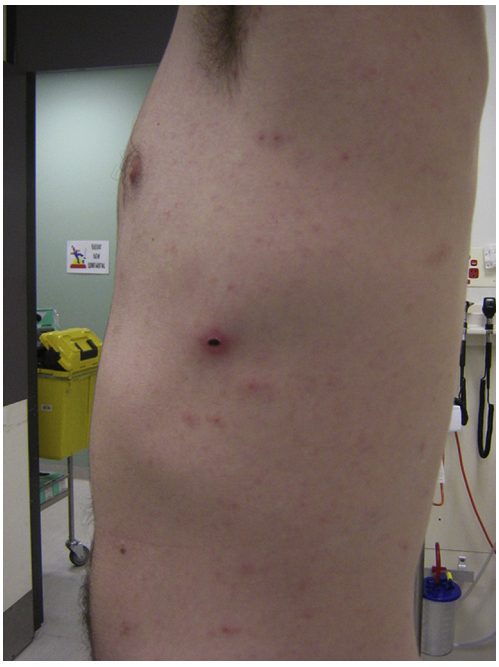

Fig. 1. Diffuse maculopapular rash and prominent eschar in scrub typhus.

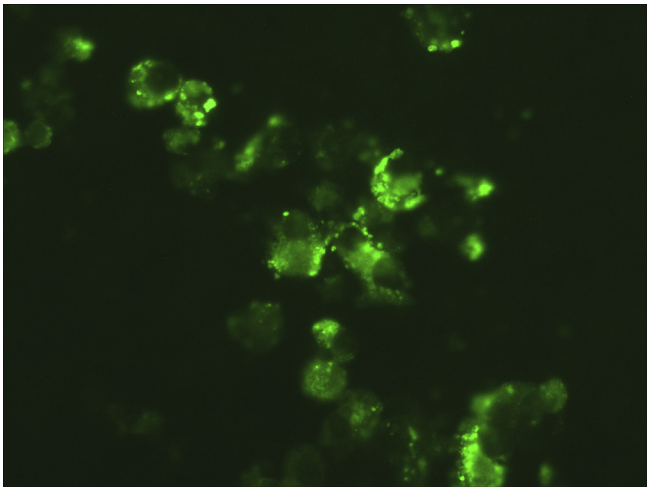

Fig. 2. *O. tsutsugamushi* detected by immunofluorescent (IF) assay following culture in Vero cells and using specific monoclonal antibody; original magnification ×1000. The Australian Rickettsial Reference Laboratory (ARRL) protocol was used for staining and fluorescence microscopy, as previously described [6].

2. Materials and methods

2.1. Susceptibility testing of O. tsutsugamushi

No standardised methods for the antimicrobial susceptibility testing of obligate intracellular bacteria exist, and such testing for *O. tsutsugamushi* has only been infrequently described in the literature. Traditional methods involve the manual counting of bacterial elements within cell culture in varying dilutions of antibiotic [22], but such methods are labour intensive, technically challenging and liable to inaccuracies. More recently, quantitative real time-PCR (qRT-PCR) has been used to determine rates of bacterial replication in the presence of graded dilutions of antibiotic [24,20]. We designed a similar protocol for the testing of the cultured Cowley Beach strain of *O. tsutsugamushi* against dilutions of doxycycline.

Doxycycline hyclate (Vibravenosa; Pfizer, Spain) at a concentration of 20 mg/mL (100 mg per 5 mL vial) was divided into 1 mL aliquots and stored at −80 °C. When required, these were thawed and diluted 1:500 in RPMI 1640 (Sigma−Aldrich; Australia) solution to a final dilution of 40 μg/mL, and then used to create two-fold dilutions ranging from 10 μg/mL to 0.04 μg/mL for susceptibility testing. A monolayer of Vero cells were grown in 24 well tissue culture plates (Interpath Services; Australia) with the addition of 10% fetal calf serum (Sigma−Aldrich; Australia) and 2% L-glutamine (Sigma−Aldrich; Australia). A suspension of the Cowley beach *O. tsutsugamushi* strain (isolated from the index patient) cultured in Vero cells was prepared by scraping of the culture flask and gentle mixing. The bacterial concentration in this suspension was then measured by quantitative real-time polymerase chain reaction (qRT-PCR) before a 1:1000 dilution and inoculation of 0.1 mL into each of the 24 well plates prepared earlier. This corresponded to a bacterial inoculum of 1.2×10^3 cells. The PCR assay used targeted a region of the 16S rDNA gene (*rrs*) that is specific for the *Orientia* genus, as previously described [21]. The PCR was run on the Rotor-Gene 3000 instrument (Corbett Lifesciences; Qiagen) and terminated at 42 cycles and the cycle to threshold value (C_t) determined. Quantification was performed using a standard curve prepared from known concentrations of the PCR amplicon cloned into the plasmid pCR2.1 TOPO [7]. The measured inoculum of *O. tsutsugamushi* was added at a volume of 0.1 mL to all test wells containing dilutions of doxycycline, as well as a growth control well containing no doxycycline. Further control wells containing doxycycline only with no bacteria and a negative control with no doxycycline or bacteria were also included. All test wells were run in duplicate. The plates were incubated at 37 °C in 5% CO_2 for 28 days, with fresh media added every four days to prevent desiccation. Samples of cell culture supernatant from the growth control wells were obtained weekly and tested by qRT-PCR to determine rate of growth. At the end of the experiment, all test wells supernatant were sampled and tested by qRT-PCR. Measured C_t values were plotted against varying concentrations of doxycycline and analysed by linear regression (fixed-effects model) using Stata 12 software (Statacorp LP; Texas, USA).

2.2. Phylogenetic analysis

A 339 bp fragment of the 56-kDa gene was amplified using primers as previously described [8] from the Cowley Beach strain of *O. tsutsugamushi* and the purified product was sequenced (Australian Genome Research Facility, Melbourne; GenBank accession number KT359593). A multiple alignment with the sequences of the 56 kDa gene from 23 other strains was created using MUSCLE [3] and a phylogenetic tree created using the Neighbour-Joining Method implemented by MEGA6 [23].

3. Results

A total of 124 personnel were involved with the Cowley Beach exercise, which lasted from 10th to 21st April 2011. Between 23rd April and 7th May there were a total of 45 clinical cases identified, 27 (60%) of which were confirmed by serological testing and 18 (40%) meeting the clinical definition only. Several clinically suspected cases did not complete paired serological testing for laboratory confirmation. Fourteen cases (31%) required hospitalisation but there were no deaths. The epidemic curve is shown in Fig. 3.

The Cowley beach strain of *O. tsutsugamushi* appeared slow growing in vitro but demonstrated approximately a 1×10^6-fold increase in bacterial load over the 4-week experimental period (see Fig. 4). When tested against different concentrations of doxycycline, low bacterial loads were seen at all antibiotic concentrations, in comparison to a high mean bacterial load (3.5×10^6) in doxycycline-free control wells (see Fig. 5). A linear regression coefficient of 0.282 [95% CI −0.300 to 0.864; $P = 0.34$] suggested no significant trend in bacterial load across all concentrations of doxycycline tested, and the minimum inhibitory concentration (MIC) was ≤0.04 μg/mL.

Sequencing of a fragment of the 56 kDa gene from the Cowley Beach strain showed its closest relative was a strain of *O. tsutsugamushi* isolated from Darnley Island in the Torres Strait [26], with a similarity of 98%. As with the Darnley strain, Cowley Beach is divergent from other sequenced strains. The next closest matches are a cluster of Taiwanese strains, but these only share 90% similarity (see Fig. 6.)

4. Discussion

As this recent outbreak has highlighted, scrub typhus remains a significant problem in endemic areas where exposure to infected mites is likely. Despite the well-established epidemiological link with Cowley beach, and the institutional provision of preventative measures, a very large number of military personnel became infected in this outbreak. Treatment of scrub typhus with tetracyclines (usually oral doxycycline) usually results in rapid resolution of symptoms and is considered first-line therapy. However, cases with

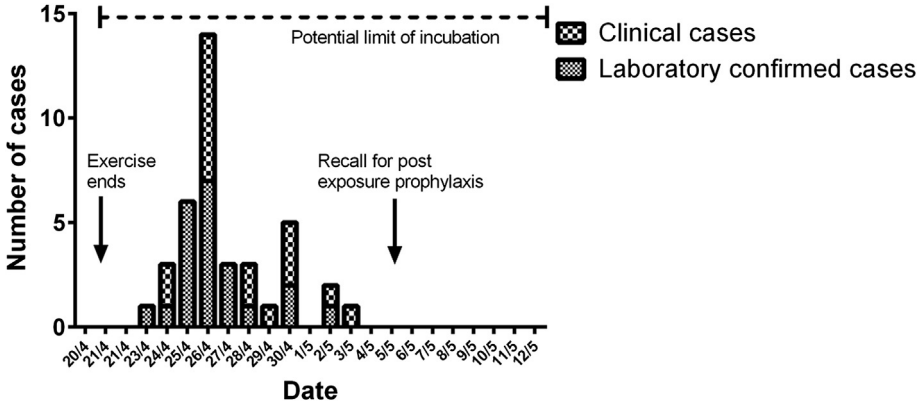

Fig. 3. Epidemic curve of scrub typhus cases following military exercises at Cowley Beach, Queensland, April 2011. Vertical bars show onset date of clinical symptoms, for both laboratory confirmed and clinical cases. Only 39 of 45 cases illustrated; in 6 cases the date of symptom onset could not be ascertained.

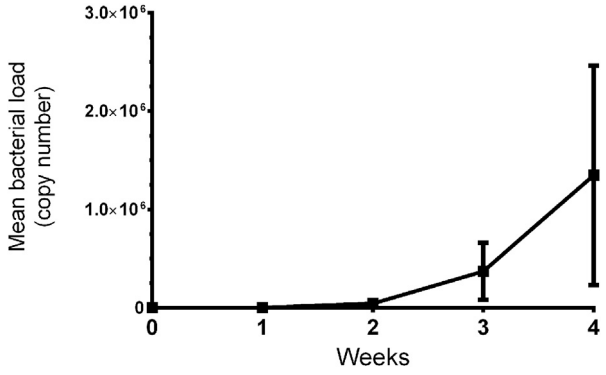

Fig. 4. *O. tsutsugamushi* [Cowley beach] growth curve in Vero cells. An inoculum of 1.2×10^3 cells was used and cultures were incubated at 37 °C in 5% CO_2 for 28 days. Growth control wells were sampled by quantitative PCR weekly and run in duplicate. Data are presented as mean values, +/− standard error of the mean.

delayed response to doxycycline and evidence of in-vitro resistance have been described in Thailand [22,27]. Although there is no standardised susceptibility testing protocols for intracellular pathogens such as *O. tsutsugamushi*, our study would suggest that the outbreak strain remained highly susceptible to doxycycline. Achievable tissue levels of the drug would significantly exceed the putative MIC derived from our in-vitro data. As such, widespread failure of prophylaxis in this context is likely to result from two other factors: lack of adherence to the prescribed regimen or an

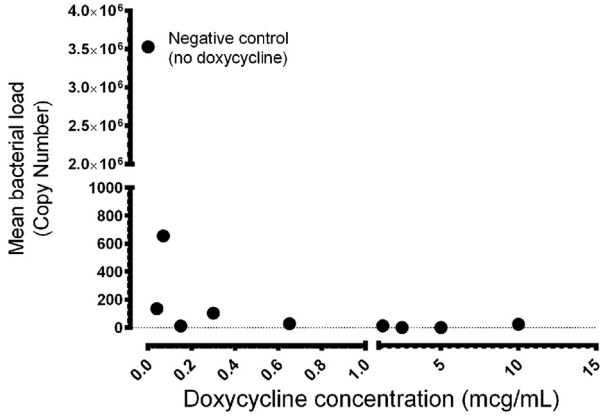

Fig. 5. Quantification of *O. tsutsugamushi* [Cowley beach] against doxycycline concentrations after 28 days growth in Vero cells. Linear regression for values of bacterial load across different doxycycline concentrations suggested no trend (coefficient 0.282, 95% CI −0.300 to 0.864; P = 0.34) to a minimum value of 0.04 μg/mL. Mean copy number for the doxycycline-free growth control wells were 3 logs higher (mean 3.5×10^6).

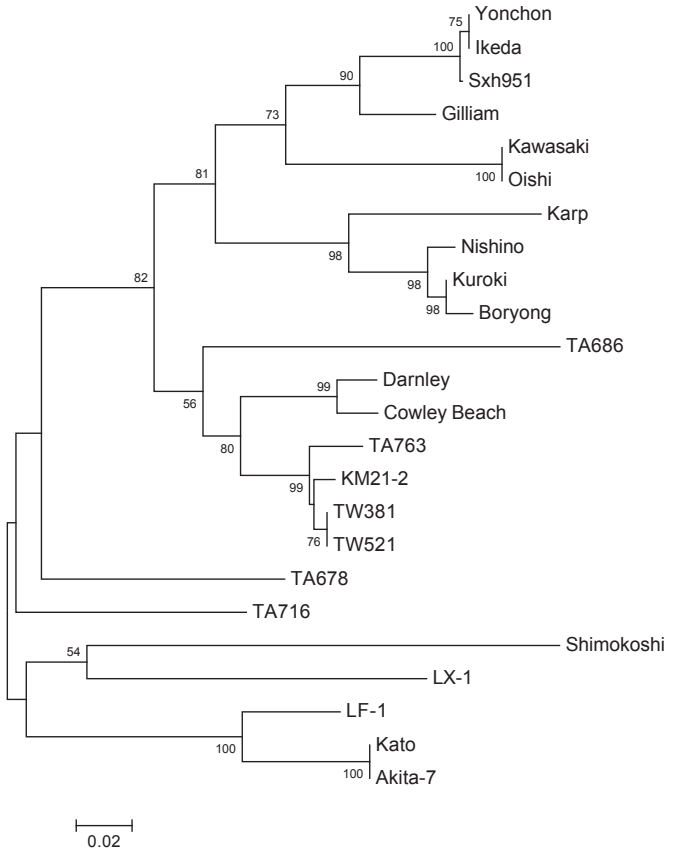

Fig. 6. Phylogenetic tree of *O. tsutsugamushi* strains obtained by Neighbour-Joining analysis of the 56 kDa gene conducted in MEGA6. The optimal tree from 500 bootstrap replicates is shown. The percentage of replicate trees in which the associated strains clustered together is shown next to the branches (only values > 50% are shown). The tree is drawn to scale, with branch lengths representing the number of base substitutions per site.

ineffective dosing schedule that allows 'breakthrough' infection.

We were unable to externally validate the susceptibility testing methods used in this study as there are no established reference strains of *O. tsutsugamushi* with known doxycycline resistance. The ASFC-4 strain from northern Thailand has been considered to clinically respond more slowly to doxycycline, but this may reflect higher density of infection or slower growth rates [10]. Because the soldiers received only one dose of doxycycline at the start of the exposure period, it is possible that no systemic active antibiotic was present later in their 11-day deployment and this allowed infection to occur, even though the *O. tsutsugamushi* strain was sensitive to low levels of doxycycline.

The prophylactic doses of doxycycline chosen by the military are designed primarily for the prevention of leptospirosis, but have been thought to prevent scrub typhus at the same dose [25,15]. However, this has been tested experimentally only to a limited extent and further work may be needed to better define an optimal regimen in Australia. It may be that the current dosing regimen is inadequate to reliably prevent scrub typhus and should be re-evaluated. Data describing doxycycline use was not available for this outbreak, but it is probable that many of the infected individuals failed to take the 'exit' dose of doxycycline at the end of the exercise. This may be the principal reason for prophylaxis failures seen, especially considering that all cases presented after the conclusion of the exercise. This reiterates the importance of maintaining strict adherence to established protocols. While it may be difficult to ensure appropriate administration of chemoprophylaxis in a 'field' environment, the exit dose lends itself well to directly observed therapy prior to redeployment. However, alternative reasons for the failure of prophylaxis should be considered, such as individual variation in serum doxycycline levels.

Azithromycin is an alternative option to treat scrub typhus, and has been shown in-vitro to be more effective than doxycycline, including against strains with reduced susceptibility to doxycycline [22]. In clinical trials, one to three doses of azithromycin had equivalent clinical efficacy to a week of doxycycline [16,11] and is used as first line therapy in pregnancy where doxycycline is contraindicated [28]. It has a much longer half-life than doxycycline, which may make it a useful alternative option for scrub typhus prophylaxis and deserves further study in this context.

Phylogenetic analysis of the 56 kDa gene showed the strain to be most closely related to a strain isolated from Darnley Island in the Torres Strait. *O. tsutsugamushi* strains have never been cultured or typed during previous scrub typhus outbreaks from Cowley beach, so it is unclear whether the strain in this study was the same as the ones responsible for previous outbreaks in this area. Furthermore, given that a cultured isolate was available for only one case, we cannot be certain that all outbreak cases were infected with the same strain.

In conclusion, we describe a large outbreak of scrub typhus in military personnel that occurred despite use of chemoprophylaxis and used a quantitative PCR-based method of doxycycline susceptibility testing for a cultured isolate of *O. tsutsugamushi*. The isolate appeared fully susceptible to doxycycline and the failure of prophylaxis was most likely to have resulted from omitted doses at the conclusion of the military exercise.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

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References

[1] Balcells ME, Rabagliati R, Garcia P, Poggi H, Oddo D, Concha M, et al. Endemic scrub typhus-like illness, Chile. Emerg Infect Dis 2011;17:1659–63.
[2] Currie B, O'Connor L, Dwyer B. A new focus of scrub typhus in tropical Australia. Am J Trop Med Hyg 1993;49:425–9.
[3] Edgar RC. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics 2004;5:113.
[4] Faa AG, McBride WJ, Garstone G, Thompson RE, Holt P. Scrub typhus in the Torres Strait islands of north Queensland, Australia. Emerg Infect Dis 2003;9:480–2.
[5] Graves S, Wang L, Nack Z, Jones S. Rickettsia serosurvey in Kimberley, western Australia. Am J Trop Med Hyg 1999;60:786–9.
[6] Graves SR, Dwyer BW, McColl D, McDade JE. Flinders Island spotted fever: a newly recognised endemic focus of tick typhus in Bass Strait. Part 2. Serological investigations. Med J Aust 1991;154:99–104.
[7] Izzard L, Fuller A, Blacksell SD, Paris DH, Richards AL, Aukkanit N, et al. Isolation of a novel Orientia species (O. chuto sp. nov.) from a patient infected in Dubai. J Clin Microbiol 2010;48:4404–9.
[8] Kawamori F, Akiyama M, Sugieda M, Kanda T, Akahane S, Yamamoto S, et al. Two-step polymerase chain reaction for diagnosis of scrub typhus and identification of antigenic variants of Rickettsia tsutsugamushi. J Vet Med Sci 1993;55:749–55.
[9] Kelly DJ, Fuerst PA, Ching WM, Richards AL. Scrub typhus: the geographic distribution of phenotypic and genotypic variants of Orientia tsutsugamushi. Clin Infect Dis 2009;48(Suppl. 3):S203–30.
[10] Kim MS, Baek JH, Lee JS, Chung MH, Lee SM, Kang JS. High in vitro infectivity of a doxycycline-insensitive strain of Orientia tsutsugamushi. Infect Chemother 2013;45:431–4.
[11] Kim YS, Yun HJ, Shim SK, Koo SH, Kim SY, Kim S. A comparative trial of a single dose of azithromycin versus doxycycline for the treatment of mild scrub typhus. Clin Infect Dis 2004;39:1329–35.
[12] Langan AM, Mathew RY. The establishment of "Mossman", "Coastal" and other previously unclassified fevers of north Queensland as endemic typhus. Med J Aust 1935:145–8.
[13] Likeman RK. Scrub typhus: a recent outbreak among military personnel in North Queensland. ADF Health 2006;7:10–3.
[14] McBride WJ, Taylor CT, Pryor JA, Simpson JD. Scrub typhus in north Queensland. Med J Aust 1999;170:318–20.
[15] Olson JG, Bourgeois AL, Fang RC, Coolbaugh JC, Dennis DT. Prevention of scrub typhus. Prophylactic administration of doxycycline in a randomized double blind trial. Am J Trop Med Hyg 1980;29:989–97.
[16] Phimda K, Hoontrakul S, Suttinont C, Chareonwat S, Losuwanaluk K, Chueasuwanchai S, et al. Doxycycline versus azithromycin for treatment

of leptospirosis and scrub typhus. Antimicrob Agents Chemother 2007; 51:3259–63.
[17] Quinlan ML, Chappell T, Golledge CL. Scrub typhus in Western Australia. Commun Dis Intell 1993;17:570–1.
[18] Ralph A, Raines M, Whelan P, Currie BJ. Scrub typhus in the northern territory: exceeding the boundaries of Litchfield National Park. Commun Dis Intell 2004;28:267–9.
[19] Raoult D. Orientia tsutsugamushi (scrub typhus). In: Mandell GL, Bennett JE, Dolin R, editors. Principles and practice of infectious diseases. Philadelphia, PA: Elsevier; 2010. p. 2529–30.
[20] Rolain JM, Stuhl L, Maurin M, Raoult D. Evaluation of antibiotic susceptibilities of three rickettsial species including Rickettsia felis by a quantitative PCR DNA assay. Antimicrob Agents Chemother 2002;46: 2747–51.
[21] Stenos J, Graves S, Izzard L. Rickettsia. In: Schuller M, Sloots TP, James G, Halliday CL, Carter IWJ, editors. PCR for clinical microbiology – an Australian and International perspective. Heidelberg: Springer; 2010. p. 197–200.
[22] Strickman D, Sheer T, Salata K, Hershey J, Dasch G, Kelly D, et al. In vitro effectiveness of azithromycin against doxycycline-resistant and -susceptible strains of Rickettsia tsutsugamushi, etiologic agent of scrub typhus. Antimicrob Agents Chemother 1995;39:2406–10.
[23] Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 2013; 30:2725–9.
[24] Tantibhedhyangkul W, Angelakis E, Tongyoo N, Newton PN, Moore CE, Phetsouvanh R, et al. Intrinsic fluoroquinolone resistance in Orientia tsutsugamushi. Int J Antimicrob Agents 2010;35:338–41.
[25] Twartz JC, Shirai A, Selvaraju G, Saunders JP, Huxsoll DL, Groves MG. Doxycycline propylaxis for human scrub typhus. J Infect Dis 1982;146: 811–8.
[26] Unsworth NB, Stenos J, Faa AG, Graves SR. Three rickettsioses, Darnley Island, Australia. Emerg Infect Dis 2007;13:1105–7.
[27] Watt G, Chouriyagune C, Ruangweerayud R, Watcharapichat P, Phulsuksombati D, Jongsakul K, et al. Scrub typhus infections poorly responsive to antibiotics in northern Thailand. Lancet 1996;348:86–9.
[28] Watt G, Kantipong P, Jongsakul K, Watcharapichat P, Phulsuksombati D. Azithromycin activities against Orientia tsutsugamushi strains isolated in cases of scrub typhus in Northern Thailand. Antimicrob Agents Chemother 1999;43:2817–8.