Concurrent Infection with Murine Typhus and Scrub Typhus in Southern Laos—the Mixed and the Unmixed

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Scrub typhus, murine typhus, and spotted fever group rickettsias all occur in the Lao PDR (Laos) [1,2]. Scrub typhus and murine typhus account for ~16% and 10%, respectively, of acute undifferentiated fever in blood culture–negative adults admitted to hospital in the capital city, Vientiane [1]. However, typhus-like illnesses are significant diagnostic challenges; patients with leptospirosis, dengue, typhoid, and malaria are also common and can present with similar symptoms and signs. Although these pathogens are common and mixed (or concurrent) infections are expected, the laboratory diagnosis of mixed infection is a vexed subject. Reports of mixed infections often use only serological criteria. The problems of antibody persistence and interspecies cross-reaction raise uncertainty as to whether these results represent true mixed infections, sequential infections, or cross-reactions. We report a patient with concurrent scrub typhus and murine typhus, demonstrated by dual PCR positivity, and discuss evidence for identifying mixed infections.

Patient

As part of a study investigating the aetiology of fever among patients with negative malaria tests, we recruited patients at Salavan Provincial Hospital, Salavan Province, southern Laos [3]. A 20-year-old female rice farmer from Naxay Village (15°62’37.06”N; 106°33’42.13”E), Salavan District, whose house was surrounded by vegetable gardens, presented at Salavan Provincial Hospital in July 2009 with 14 days of headache associated with three days of fever, myalgia, and vomiting, having taken five days of oral cephalixin. She was febrile (38.5°C), but physical examination was otherwise normal without rash or eschar. She was suspected to have scrub typhus and was prescribed empirical doxycycline and amoxicillin for seven days and recovered fully. Ethical approval was granted by the Lao National Ethics Committee for Health Research and the Oxford Tropical Research Ethics Committee, United Kingdom, and the patient provided written consent to publication of clinical details.

Subsequently, the patient’s acute serum sample was assayed for immunoglobulin (IgM and IgG) antibody titres against reference O. tsutsugamushi antigens (pooled Karp, Kato, and Gilliam) and R. typhi antigen (Wilmington strain) by indirect immunofluorescent assay [4]. The admission serum had titres of scrub typhus IgM<400 and IgG = 1,600 and murine typhus IgM<400 and IgG<400. Conventional serum was not available. DNA from admission EDTA anticoagulateduffy coat was extracted and used as template for the O. tsutsugamushi 47-kDa-gene-based real-time PCR assay, the R. typhi ompB-gene-based real-time PCR assay, the Rickettsia genus 17-kDa-gene-based real-time PCR assay, and the O. tsutsugamushi groEL-gene-based real-time PCR. Each run contained duplicate low-positive dilutions of linearized pGEM plasmids, ranging from 10² to a single copy/μL, as external controls (Table 1).

The buffy coat was positive for the O. tsutsugamushi 47-kDa and groEL target genes as well as the Rickettsia genus 17-kDa and R. typhi ompB target genes by the diagnostic real-time PCR assays, indicating potential dual positivity for O. tsutsugamushi and Rickettsia spp. The copy numbers determined for both pathogens were within the range normally seen at our laboratory (56/59 and 75/130 copies/μL for the 47-kDa and ompB real-time assays, respectively). That samples were processed in separate pre- and post-PCR work areas, the evidence of multigenic PCR positivity, and that no other dual positive samples were found makes contamination extremely unlikely. Further characterisation was performed (Table 1), including a panel of conventional nested PCR assays targeting the 17-kDa (product size 524 bp), 56-kDa (product size 620 bp), and 47-kDa (product size 785 bp) target genes. All three assays provided positive PCR amplicons and the products were purified and sequenced by Macrogen (Korea). Among the candidates with the same BLAST score results for the 17-kDa PCR ampli- con (367 bp sequence), the geographically closest related strain found was R. typhi strain TH1526 (max. score 640, max. identity 99%, query coverage 97%, E-
value 3e-180), from a patient with murine typhus from Chiang Rai, N. Thailand. The 47-kDa amplicon (744 bp) matched *O. tsutsugamushi* Ikeda strain (max. score 1314, query coverage 100%, E-value 0.0) and the nested 56-kDa amplicon (523 bp) matched *O. tsutsugamushi* T1125175_KH 56-kDa type-specific antigen (max. score = 640, query coverage = 99%, E-value 0.0).

The infecting *O. tsutsugamushi* strain is very similar to the human-pathogenic Cambodian isolate T1125175_KH and the animal-derived (*Rattus ratus*) Thai strain TA763, making this the first Lao scrub typhus patient with a strain similar to another nonhuman vertebrate strain [5,6]. Similarly, human pathogenicity of a Kato-related TA761-like *O. tsutsugamushi* strain originally described from the Indochinese ground squirrel (*Menetes berdmorei*) has been recently reported from Thailand [7].

### Mixed Infections

We present a patient with clear molecular diagnostic evidence of concurrent mixed infection with scrub typhus and murine typhus. Such infections may go unrecognized. Although clinically similar, the diseases have markedly different pathology [8]. Although both pathogens would be expected to respond to doxycycline, *O. tsutsugamushi* generally causes the more severe disease and would not be expected to respond to fluoroquinolones, which have been used for murine typhus [9]. Mixed infection with these two pathogens was demonstrated using PCR and IFA among three patients in Yunnan Province, China [10].

Although culture or molecular detection should be the gold standard for demonstrating mixed infection with very high specificity, this approach will suffer from low sensitivity, as significant proportions of patients with good evidence of mono-infection (with fourfold rises in specific IgM) are PCR negative for both scrub typhus [11] and murine typhus (unpublished data). Moreover, there are cross-reactions between IgM against *O. tsutsugamushi* and *R. typhi* [12] and very few objective data on serological responses in confirmed mixed infections. Western blotting has been used to distinguish serological responses [13]. In Vientiane City, 4% of well adults had IgG antibodies against both scrub typhus and murine typhus [2], suggesting the possibility of previous exposures to both organisms and/or serological cross-reactions.

Mixed *O. tsutsugamushi* and *Leptospira* spp. infections have been reported, but none of these included positive PCR or culture for both pathogens (Table 2). Such infections are especially important as leptospirosis would be expected to respond to penicillins or cephalosporins while scrub typhus would not [14]. Mixed Q fever and scrub typhus infections have been reported in Taiwan but only using serological assays. Mixed infections of *Plasmodium falciparum* with both scrub typhus and murine typhus diagnosed by PCR and/or dynamic serology was documented among febrile pregnant women on the Thai–Burmese border (Table 2). Interpretation would be more intricate if either (or both) pathogen(s) caused chronic infections. This has not been demonstrated for *R. typhi* (although we can find no evidence that it has been expressly looked for), but there have been suggestions that *O. tsutsugamushi* may cause long-term infections [15,16].

We suggest that reports of mixed infections include an explicit discussion of the likely specificity and sensitivity of the diagnostic assays used and the likelihood that the observations represent true concurrent mixed infections (or coinfections), or sequential infections due to persistence of antibody or false positives due to assay cross-reactions (“dual positivity”). A grading system of evidence, analogous to the GRADE guidelines and Infectious Diseases Society of America guidelines [17,18], may be helpful. For example, grade I (culture or molecular detection of both pathogens or direct observation such as in a malaria film), grade II (serological diagnosis with either seroconversion or fourfold antibody responses to both pathogens, without evidence of cross-reactions, or using Western blotting), and grade III (serological diagnosis based on admission serology without exclusion of cross-reactions or antibody persistence or culture, molecular, or admission serological detection). Grades I to III would have decreasing specificity but increasing sensitivity in diagnosing true mixed infections. Seroconversion could also be regarded as grade I evidence if documented with a diagnostic test providing highly specific evidence for seroconversion. The relative importance of sensitivity and specificity will depend on the question being asked and the clinical use of the data. When different grades of evidence are used for different pathogens in a “mixed” infection, we suggest that the grade with the highest number (least specificity) is used.

For patients with grade I evidence, further care is required as molecular methods have different specificities for pathogen diagnosis. Real-time PCR specificity is higher if type-specific genes are used (e.g., 56-kDa and 47-kDa genes for *O.
Table 2. Reports of apparent mixed infections in Asia that included rickettsioses.

| Rickettsial pathogen and diagnosis | Additional pathogen and diagnosis | Number of patients | Country | Reference |
|-----------------------------------|-----------------------------------|--------------------|---------|-----------|
| O. tsutsugamushi | Leptospira spp. | 9/22 (41%) of patients with leptospirosis had evidence for scrub typhus | NE Thailand | Watt et al., 2003 [26] |
| Positive dot blot immunoassay (correlates with IgG titres $=1:1,600$ or IgM titres $=1:400$) | | | | |
| IgM single titre $=1:80$ | MAT 4-fold rise in titre or single titre $=1:320$ | | | |
| O. tsutsugamushi | Leptospira spp. | 1 patient with cholecystitis, pancreatitis, and acute renal failure | Taiwan | Wang et al., 2003 [27] |
| 4-fold rise in specific IgG or IgM titre to $=1:200$ by IFA or a single titre of $=1:400$ | | | | |
| O. tsutsugamushi | Leptospira spp. | 62/540 (12%) of patients with leptospirosis had evidence for scrub typhus | NE Thailand | Suputtamongkol et al., 2004 [28] |
| Admssion IFA IgM titre 1:80 & IgG 1:40 | | | | |
| Leptospira spp. | 1 patient with melioidosis had evidence of leptospirosis and scrub typhus | Taiwan | Lu et al., 2005 [29] |
| Serology technique not stated | | | | |
| Patient 1: PCR positive | Leptospira spp. | 4 patients with leptospirosis had evidence for scrub typhus | Taiwan | Ho et al., 2006 [30] |
| Patient 2: PCR and IgM & IgG positive | Serology technique not stated | | | |
| Patient 3: PCR positive, IgM positive, and 4-fold rise in IgG | Patient 1: single titre 1:1,600 | | | |
| Patient 4: PCR positive, IgG positive, and 4-fold rise in IgG | Patient 2: single titre 1:800 | | | |
| O. tsutsugamushi | Leptospira spp. | 1 patient with acute renal failure and pulmonary haemorrhage | Taiwan | Chen et al., 2007 [31] |
| Admission IFA lgM $=1:80$ plus 4-fold rise in lgG titre on paired sera | MAT seroconversion to 1:400 | | | |
| O. tsutsugamushi | Leptospira spp. | 7/87 (8%) of patients with leptospirosis or scrub typhus had evidence for both pathogens | Taiwan | Lee et al., 2007 [32] |
| IFA 4-fold rise in titre or a single lgM titre $=1:80$ | MAT 4-fold rise in titre or a single titre $=1:320$ | | | |
| O. tsutsugamushi & R. typhi | Leptospira spp. | 11/296 (4%) of patients with leptospirosis had evidence for infection with scrub typhus or murine typhus | NE Thailand | Phimda et al., 2007 [33] |
| IFA 4-fold rise or a single titre of $=1:400$ | Culture or MAT 4-fold rise or a single titre of $=1:1,400$ | | | |
| O. tsutsugamushi | R. typhi | 5/144 (3%) of patients with Q fever or typhus (scrub and murine) had evidence for both infections | Taiwan | Lai et al., 2009 [34] |
| PCR positive and $=4$-fold rise in IgG | PCR positive and $=4$-fold rise in lgG | | | |
| O. tsutsugamushi & R. typhi | Coxiella burnetii | 5/144 (3%) of patients with Q fever or typhus (scrub and murine) had evidence for both infections | Taiwan | Lai et al., 2009 [34] |
| IFA lgM $=1:80$ or 4-fold rise in IgG | IFA anti-phase II IgG $=1:320$ or IgM $=1:80$ or a 4-fold rise in IgG titre | | | |
| O. tsutsugamushi & R. typhi | Plasmodium falciparum | 5/51 (10%) of pregnant women with malaria had evidence for murine typhus or scrub typhus | NW Thailand | McGready et al., 2010 [35] |
| PCR, culture or IFA 4-fold rise in IgM or IgG | Giemsa malaria films | | | |
| O. tsutsugamushi | Leptospira spp. | 5/144 (3%) of patients with Q fever or typhus (scrub and murine) had evidence for both infections | Taiwan | Lai et al., 2009 [34] |
| IFA seroconversion to lgG 1:320 & IgM 1:160 | MAT seroconversion to 1:400 | | | |
| 3/8 (38%) of febrile farmers PCR positive for scrub typhus or murine typhus were PCR positive for both | China | Zhang et al., 2007 [10] |
| O. tsutsugamushi | Plasmodium falciparum | 5/51 (10%) of pregnant women with malaria had evidence for murine typhus or scrub typhus | NW Thailand | McGready et al., 2010 [35] |
| PCR, culture or IFA 4-fold rise in IgM or IgG | Giemsa malaria films | | | |
| O. tsutsugamushi | Leptospira spp. | 1 patient with shock and respiratory failure | Taiwan | Wei et al., 2012 [36] |
| IFA seroconversion to lgG 1:320 & IgM 1:160 | MAT seroconversion to 1:1,600 | | | |
tsutsugamushi) than if genus-specific genes are used (17-kDa genes for Rickettsia spp.), which again are stronger than nonspecific conserved “housekeeping” genes (e.g., gndE and 16S rRNA). Sequencing should be attempted if conventional (nested) PCR products are obtained, as BLAST analysis will provide high-level confidence with confirmation of the amplicon similarity to gene sequences deposited in GenBank and/or genotyping using SNPs will allow for discrimination at a more subtle level. We suggest that where possible mixed infections should be confirmed by culture or detection of specific nucleic acid sequences and that the introduction of a grading system for the strength of evidence for mixed infections should be considered.

Acknowledgments

We thank the staff of the Microbiology Laboratory, Mahosot Hospital, especially the Director, Dr. Rattanaphone Phetsouvanh, and Salavan Provincial Hospital.

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