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SUPPLEMENT ARTICLE

Scrub Typhus: The Geographic Distribution of Phenotypic and Genotypic Variants of *Orientia tsutsugamushi*

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Orientia tsutsugamushi **is the etiological agent of scrub typhus, an acute, mite-borne, febrile illness that occurs in the Asia-Pacific region. Historically, strain characterization used serological analysis and revealed dramatic antigenic diversity. Eyeing a recommendation of potential vaccine candidates for broad protection, we review geographic diversity and serological and DNA prevalences. DNA analysis together with immunological analysis suggest that the prototype Karp strain and closely related strains are the most common throughout the region of endemicity. According to serological analysis, ~50% of isolates are seroreactive to Karp antisera, and approximately one-quarter of isolates are seroreactive to antisera against the prototype Gilliam strain. Molecular methods reveal greater diversity. By molecular methods, strains phylogenetically similar to Karp make up ~40% of all genotyped isolates, followed by the JG genotype group (Japan strains serotypically similar to the Gilliam strain but genetically non-Gilliam; 18% of all genotyped isolates). Three other genotype groups (Kato-related, Kawasaki-like, and TA763-like) each represent ~10% of genotyped isolates. Strains genetically similar to the Gilliam strain make up only 5% of isolates. Strains from these groups should be included in any potential vaccine.**

Scrub typhus, tsutsugamushi disease, or, more accurately, chigger-borne rickettsiosis is an acute, febrile illness among humans that is caused by infection with the bacterium *Orientia tsutsugamushi* following the bite of infected mite vectors [1, 2]. It is endemic to a 13,000,000-km^2 area of the Asia-Pacific rim, extending from Afghanistan to China, Korea, the islands of the southwestern Pacific, and northern Australia (figure 1) [3]. Transmission of the etiological agent to the rodent host or the human incidental host occurs during feeding of the parasitic larval or "chigger" stage of mites primarily of the genus *Leptotrombidium*. Vertical or transovarial transmission appears to be essential to the maintenance of the infection in nature; thus, the mite serves as both the vector and the reservoir (table 1). The vectors can be found in a variety of ecological conditions, from the mountainous regions of northern India to the tropical climates of the Malay Peninsula and Indonesia. Clinical presentation in humans can vary from mild or unapparent disease to fatal disease, with variable mortality rates reported to be in the range 35%–50% in the preantibiotic era [30, 31]. The disease is historically significant; evidence of the disease has been found in writings from as early as 313 A.D. in China and in later descriptions from the early 1800s in Japan [3, 32]. It was not until 1926 that the disease was clearly distinguished from flea-borne or murine typhus, and in 1936, it was described as similar to the mite-borne typhus in Japan [14, 32, 33].

Although well known in the areas of endemicity, this disease did not become more familiar to other areas until World War II. The impact of the disease on the

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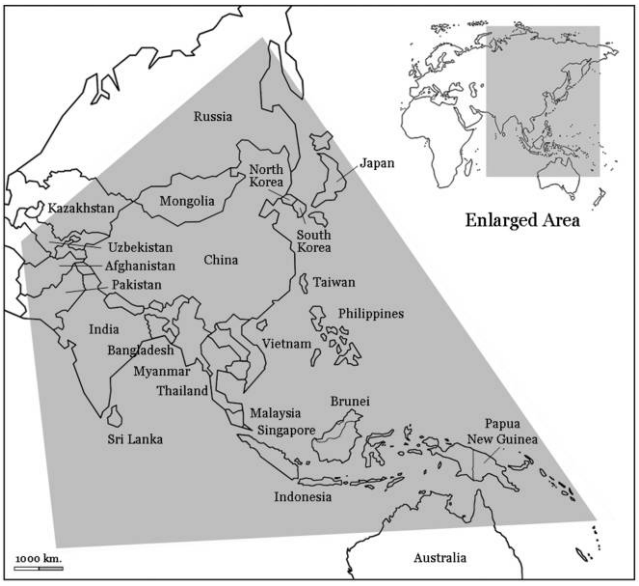

Figure 1. Map of the area where scrub typhus is endemic. The primary vectors for the regions are given in table 1. The prevalent strains of *Orientia tsutsugamushi* in particular geographic regions are listed in table 3.

military peaked during that war, when tens of thousands of cases and hundreds of deaths were reported among Allied and Japanese troops [32, 34]. The dramatic impact of the disease in the Asia-Pacific theater of the war drove the substantial effort during and immediately after the war to prevent, control, and treat the disease [35]. This program, coordinated by the US Typhus Commission, included development of treatments, novel miticides, impregnation of repellent in clothing, environmental control through burning and clearing of troop encampment areas, and one of the first substantial, although ultimately unsuccessful, vaccine trials [32, 36–38]. Unfortunately, the military relevance of this disease was painfully learned again <30 years later during the Vietnam conflict, when it was reported to be second only to malaria as a leading cause of fever among troops in the field [3, 32, 39]. Today, >60 years after the end of World War II, there is still no effective human vaccine against scrub typhus. One important product of the research from the World War II era was the discovery of dramatic antigenic variation among strains of *O. tsutsugamushi*. This variation was shown using several methods available at the time, including cross-neutralization, cross-vaccination, or cross-immunization and complement fixation (CF) [40]. In addition to antigenic variation, research showed great interstrain variability in virulence in humans and rodents that ranged from unapparent disease to disease that was consistently fatal when untreated. To date, there have been >20 antigenically distinct strains reported, including the initially characterized prototypic strains Karp, Gilliam, and Kato (table 2) [32].

Soon after World War II, the introduction of chloramphenicol and, later, the tetracyclines dramatically and effectively eliminated mortality among cases recognized early enough for initiation of treatment [59–61]. Although relapses or repeat infections were common [61], infection was generally responsive to treatment with the new antibiotics, even when antigenically diverse strains were involved. Thus, until recently, the efforts to produce an effective vaccine that began during and immediately after World War II diminished dramatically, primarily because of the existence of effective, rapid-acting antibiotic treatments. The need for greater emphasis on prevention such as would be afforded by an effective vaccine has been prompted by increasing evidence that suggests the presence of antibiotic refractility, the difficulty in diagnosing a disease that mimics several other febrile illnesses, and the increasing popularity of ecotourism in areas of endemicity [32, 58, 62–66]. These developments are presently driving renewed efforts to develop a vaccine, particularly by the US Department of Defense. Modern technologies such as DNA vaccines [67–69], recombinant proteins [70–72], and combination DNA-protein vaccines appear to be promising strategies for disease prevention, as was recently suggested elsewhere [38].

Evident antigenic diversity exists throughout the region of endemicity. However, the paucity of systematic strain collection and information on identification and characterization of strains, especially information on strain prevalences, raises the question of which strains should be selected for inclusion in a vaccine or diagnostic test. Efforts to develop and evaluate a vaccine or serodiagnostic tests would be resource intensive, requiring careful selection of representative strains. The primary purposes of this article are to review historical data on antigenic variation, including characterization of isolates found predom-

Table 1. Putative vectors of human scrub typhus and their geographic foci.

Vector[a] (*Leptotrombidium* species)	Localities
L. deliense	Prevalent in Australia [3, 4], China [3, 5–8], India [3, 9], Malaysia [3, 10], New Guinea [3, 11], Pakistan [1, 3], Philippines [3, 10], and Thailand [3, 12, 13]; present in Sumatra (Indonesia) [3], Myanmar [3], and Pescadores islands (Taiwan) [3]
L. akamushi	Prevalent in Japan [1, 3, 10, 14–16] and Solomon Islands [3, 17, 18]
L. scutellare	Prevalent in Japan [1, 3, 10, 14–16]; present in China [6], Korea [19], Malaysia [3, 20], and Thailand [3, 12]
L. chiangraiensis	Present in Thailand [21, 22]
L. arenicola	Present in Indonesia [3] and Malaysia [1, 3, 23]
L. imphalum	Present in Thailand [3, 21]
L. pallidum	Prevalent in Japan [1, 3, 10, 14–16] and Korea [1, 3, 24, 25]; present in Primorski Krai (Russia) [1, 3, 16]
L. pavlovskyi	Prevalent in Primorski Krai (Russia) [3, 26, 27]
L. fletcheri	Present in Indonesia [1], Malaysia [1, 3, 28, 29], New Guinea [1], and Philippines [1, 3]
L. gaohuensis	Present in Zhejiang Province, China [5]

NOTE. Several other trombiculid mite species are not identified in the table, either because of relative rarity or because they are unproven human vectors [1]—for example, *L. palpale* (Japan, Korea, and Primorski Krai, Russia), *L. tosa* (Japan), *L. fuji* (Japan), *L. orientale* (Japan, Korea, and Primorski Krai, Russia), and *L. intermedium* (China).

[a] Primary chigger vectors of scrub typhus are found in the genus *Leptotrombidium* (*L. deliense* group). The mite life cycle is 2–3 months in warmer climates and ⩾8 months in colder climates; only the parasitic, 6-legged larva or "chigger" stage parasitizes the vertebrate host [3].

inantly in humans, and to summarize genomic information about *Orientia* species that could guide the selection of appropriate strains for diagnostic tests or vaccines [38, 67, 71].

THE NATURE OF STRAIN VARIATION IN *O. TSUTSUGAMUSHI*

Since the preantibiotic era and the years of World War II in the Asia-Pacific theater, investigators have reported that scrub typhus severity and clinical presentation appeared to be strain dependent, varying tremendously from extremely mild or inapparent disease to frequently fatal disease in both humans and laboratory animals [34, 73–75]. The nature of the multiple antigens of *Orientia* strains was examined after various increasingly more sensitive assays and research tools were developed and became available. *Orientia* species are difficult to cultivate and are a risk to laboratory investigators; thus, their analysis now requires use of biosafety-level (BSL-3) facilities [76]. Typically, agent isolation for diagnosis and characterization occurs primarily from specimens of human blood or rodent tissues.

Models for the initial isolation of *Orientia* strains most often involved mice and cell culture but historically have also included rabbits, guinea pigs, gerbils, and hamsters [3, 77]. These methods assumed a model sensitivity sufficient to isolate human-virulent strains for propagation and analysis; however, virulence can vary dramatically both among the *Orientia* strains themselves and among the murine outbred or inbred strains used for isolation [48, 75, 78–83]. Even the age of the mouse host can influence the apparent strain virulence and the capacity to isolate the agent in vivo [84]. Although very limited, there is evidence of strain differences indicated by variation in sensitivity to antibiotics or antibiograms, which is related to reports of antibiotic-refractory scrub typhus [62, 85, 86].

Direct antigenic characterization of *Orientia* strains in infected chiggers or pools of chiggers has been done using 1 of 2 possible chigger-collection methods [1, 12, 23, 28, 87]. In the black-plate collection method, flat, square, black plastic tiles are placed briefly on the ground. Questing, unengorged chiggers that attach to the plates are removed for rickettsial isolation in laboratory mice either by intraperitoneal inoculation or by individual feeding on immobilized mice. Isolation can also be done using cell culture. An alternative collection method is to remove chiggers from the ears of field-trapped rodents and process them for *Orientia* strain isolation and antigenic characterization as described below. The difference between results from the black-plate method of chigger collection and those from the method in which chiggers are removed from trapped rodents involves the potential number of *Orientia* strains identified. Use of the latter method causes possible difficulties in interpretation, because rodent hosts may harbor *Orientia* strains from multiple chigger feedings, and these strains may be picked up by attached chiggers.

Methods of serological characterization include CF, toxin and serum neutralization, direct fluorescent antibody assay (DFA), and indirect fluorescent antibody assay (IFA). Characterizations with DFA or IFA included human and animal sera and were based on either polyclonal or, more recently, monoclonal antibodies often used in conjunction with sodium dodecal sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the bacterium's cellular components. Significantly, strain identification—as well as establishment of strain prevalence for the dif-

Table 2. Prototypical and reference *Orientia* strains as determined by antigenicity.

Strain	Source organism; location; date	Remark(s)	Reference(s)
Karp	Human; New Guinea; 1943	Original prototype strain	[40, 41]
Gilliam	Human; Assam-Burma border; 1943	Original prototype strain	[36, 42]
Kato	Human; Niigata, Japan; 1955	Original prototype strain	[43]
Volner (JHV)	Human; Samar Island, Philippines; 1945	Strain used for vaccine trial	[10, 37, 40]
TA678	*Rattus rattus;* Khao Yai, Khorat Plateau, Thailand; Sep 1963	Strain used in DFA studies by Shirai et al.	[44–46]
TA763 (Fan)	*Rattus rajah;* Chong Mek, Khorat Plateau, Thailand; Nov 1963	Strain used in DFA studies by Shirai et al.; Karp-related strain	[44–46]
TA686	*Tupaia glis;* Khao Yai, Khorat Plateau, Thailand; Sep 1963	Strain used in DFA studies by Shirai et al.; Karp-related strain	[44–46]
TA716 (Chon)	*Menetes berdmorei;* Chong Mek, Khorat Plateau, Thailand; Aug 1963	Strain used in DFA studies by Shirai et al.; Karp-related strain	[44–46]
TH1817	Human; Pak Tong Chai, Khorat Plateau, Thailand; Jul 1965	Strain used in DFA studies by Shirai et al.	[44–46]
Buie	Human; New Guinea; 1943	…	[47]
Seerangee	Human; Malaya; 1934	…	[33, 36, 40]
Kostival	Human; Dobadura, Papua New Guinea; 1943	…	[11, 40]
B-15	Chigger pool; Slavyansky district, Primorski Krai, Russia; 1963	…	[26]
Irie	Human; Miyazaki, Japan; 1971	Low murine virulence	[48, 49]
Hirano	Human; Miyazaki, Japan; 1980	Low murine virulence	[49]
Kuroki	Human; Miyazaki, Japan; 1981	…	[50, 51]
Shimokoshi	Human; Niigata, Japan; 1980	Low murine virulence	[52]
Ikeda	Human; Niigata, Japan; 1979	…	[52]
Yamamoto	Human; Niigata, Japan; 1982	…	[52]
Kawasaki	Human; Miyazaki, Japan; 1981	…	[53]
Saitama	Rodent; Saitama Prefecture, Japan; 1997	…	[54]
Boryong (B119)	Human; Boryong, South Chungcheong Province, South Korea; 1980s	Predominant strain reported in Korea	[55, 56]
Yonchon	Human; Yonchon County, northern South Korea; 1989	…	[57]
Litchfield	Human; Northern Territory, Australia; Aug 1996	…	[58]

NOTE. DFA, direct fluorescent antibody assay.

ferent regions, vectors, and reservoirs by use of these serological techniques—requires comparison with established "prototypes" available at the time of the study. Only as geographically diverse and more-intensive investigations were done have additional prototypes been determined. For example, early CF characterization compared only the Karp, Gilliam, and Kato strains with new isolates [88, 89], whereas IFA and DFA analyses starting in the late 1970s included selected Thai strains that were shown by CF, IFA, and DFA to be antigenically distinct (table 2) [28, 45, 46].

More recently, genetic analysis of the antigen genes of *Orientia* species, focused primarily but not exclusively on the 56-kDa cell-surface antigen gene, has resulted in techniques to differentiate "genotypes" [90–92]. These molecular methods include PCR in conjunction with gel electrophoresis, restriction fragment–length polymorphism (RFLP) mapping, and the sequencing of specific PCR products for direct comparison of products from the same genes of multiple *Orientia* strains. There has also been use of 2D gel electrophoresis that permits the proteomic comparison of drug-susceptible and -nonsusceptible strains [93]. Evidence of divergence in other gene sequences (e.g., genes for 22-kDa proteins and the 47-kDa HtrA protein) is just beginning to be assessed [94, 95]. The entire DNA sequences of 2 strains, the Boryong strain and the Ikeda strain, have been completed [96, 97], and genome sequences of the Karp strain (Naval Medical Research Center, Silver Spring, MD), and the AFPL-12 Thai strain (Centers for Disease Control and Prevention, Atlanta, GA) are under way or near completion [96] (G. A. Dasch and A. L. Richards, unpublished data). As whole-genome sequences of *Orientia* strains are made available for comparison and analysis, the enormous scope of strain variation will likely become even more evident, possibly resulting in increased interest in the species status of strains within the genus [96, 98].

METHODS OF CHARACTERIZING ANTIGENIC VARIATION IN *ORIENTIA* SPECIES

CF. Along with the Weil-Felix test, the CF assay was one of the earliest serological tests used for clinical diagnosis of scrub typhus. Unlike the Weil-Felix test, the CF test was proven useful in characterizing *O. tsutsugamushi* isolates [36, 42, 89, 99, 100]. In fact, CF was used by Shishido [88, 89] to confirm the unique serological identity of the 3 "original" prototypes: the Karp, Gilliam, and Kato strains. Later, Elisberg et al. [101] performed CF testing of serum specimens from guinea pigs inoculated with 77 isolates of *Orientia* species collected from humans, rodents, or chiggers in 5 geographically distinct regions in Thai-

land. Whereas 72 of the isolates could be characterized as Karp, Gilliam, Kato, or a combination, 5 isolates were identified as serologically unique. These novel *Orientia* isolates demonstrated minimal cross-reactivity with the older, established prototypes and were categorized as new strains: TA678, TA686, TA716, TA763, and TH1817 (table 2) [44, 45, 101, 102]. In part because of the extremely tedious and time-consuming nature of the test, as well as the advent of newer, more rapid and sensitive technologies, the CF test is rarely used today in rickettsiology. The amount of complement "fixed"—that is, the amount unavailable to facilitate lysis of hemolysin-sensitized RBCs—is proportional to the mass of the antigen-antibody complex or to the degree of binding of the *Orientia* antigen. Thus, isolates closely related to the prototype strains will bind complement with no resulting lysis except at high serum dilutions, whereas more distantly related isolates will result in easily seen lysis even at high serum antibody concentrations. The test produces a picture of the relative antigenic relatedness of the strains compared [42, 103]. Bengston [42, 100] found that CF testing of a homologous strain—that is, the identical *Orientia* prototype strain is used for both the immunizing antigen and the guinea pig immune serum—fixed complement at much greater dilutions than did testing of a heterologous strain. Specificity was shown by the absence of cross-reaction with non–scrub typhus group antigens. To be used accurately, each component of the CF assay—complement, hemolysin, antigen, antiserum dilutions, sheep RBCs, and so forth—must be carefully standardized.

Cross-neutralization and cross-immunization (cross-vaccination). Although the neutralization test is still used in virus studies, this adaptation is cumbersome, frequently variable [82, 104], and not commonly used today to distinguish between *Orientia* strains. The method requires a susceptible model, such as the mouse for scrub typhus. In the cross-neutralization assay, serum containing specific antibody collected from hyperimmunized animals is incubated with suspensions of *Orientia* organisms and subsequently injected into otherwise susceptible mice [43, 47, 82, 88, 104–108]. Infectivity of the organisms is reduced by the presence of specific neutralizing antibody [79, 82]. *O. tsutsugamushi* strain variation can be observed by comparing neutralization indices between isolates. These are calculated on the basis of the ratio of survivors to nonsurvivors [47, 105]. Similar cross-neutralization can be done in cell culture and by monitoring cytopathic effect [109] or plaque reduction [110] as end points.

In cross-vaccination or cross-immunization, mice or guinea pigs are immunized with either killed *Orientia* organisms or live organisms administered by a nonlethal route of inoculation, usually subcutaneously [40, 79, 111, 112]. Immunized animals are subsequently challenged with dilutions of live homologous or heterologous strains. Typically, protection from the homologous challenge is greater than that from the heterologous challenge. The immunological relatedness of *Orientia* strains can then be characterized by categorization of virulence in the inoculated animals. Challenge with some strains, however, shows no protection. Early studies suggested the promise of vaccine-induced heterologous immunity, at least in the guinea pig model. However, cross-immunity can vary tremendously, and general strain relatedness can be shown but not to the extent demonstrated using the various serological tests [24, 88, 113, 114]. In addition, the relative virulence in challenged animals appears to be highly mouse-strain specific [78, 79, 115].

Strain variation has also been demonstrated using a toxin neutralization assay [79, 116, 117]. With egg yolk sac viability as an end point, the strain-specific toxic effect of *O. tsutsugamushi* can be neutralized by preinoculation incubation with dilutions of homologous sera but less so with heterologous antisera. Similarly, the toxic effect following intravenous administration of the Gilliam strain in mice can be neutralized by preincubation with homologous antisera but not with heterologous antisera [3, 117]. The exact nature of the toxin from the Gilliam strain has not been discovered, and whether it is similar to that from other *Orientia* strains is also unknown. Some inbred mouse strains appear to be resistant to this toxic effect, but unfortunately there has been no follow-up to further characterize the Gilliam strain toxin.

Immunofluorescence and immunoperoxidase assays. Before the molecular studies initiated by the first sequencing of genes from *O. tsutsugamushi* [90–92], most antigenic characterization was done using DFA and IFA [45, 102, 118, 119]. By far the most extensive use of DFA antigen characterization for the identification of regional variation of *Orientia* isolates was by Shirai et al. [17, 46, 87, 120–122] working at the US Army Medical Research Unit, Institute for Medical Research, Kuala Lumpur, Malaysia, from 1978 through 1986. The microdot modification developed there allowed a more complete DFA characterization of isolates from humans [46], rodents [120], and chigger-pools obtained from mice or direct characterization of *Orientia* strains from dissected mites on a single slide [12, 28, 123]. The typical *Orientia* DFA panel included labeled anti-Karp, -Kato, -Gilliam, -TA678, -TA686, -TA716, -TA763, and -TH1817 sera [45]. Because some of the conjugates in the panel were not monospecific, sometimes reacting weakly to heterologous prototype antigens, unlabeled heterologous antisera were commonly mixed with conjugates to yield reagent specificity. It has been suggested that this lack of specificity is caused by certain *Orientia* isolates sharing cross-reacting antigens with Karp, TA716, TA763, and TA686 strains, and such strains are described as "Karp-related" [46, 121]. Although similar to the DFA method, antigenic characterization by IFA has an additional incubation step in which the primary unlabeled rabbit anti-*Orientia* immune serum is "sandwiched" between

the organism and fluorescein isothiocyanate–labeled (goat, etc.) anti-rabbit immunoglobulin. In addition to the cross-reactivity noted above, the IFA and DFA results are somewhat problematic in other ways. These tests are subjective, in that the relative reactivity or brightness of the fluorescing organisms requires observation by a consistent, well-trained eye. Nevertheless, comparative studies with CF testing showed that DFA was more sensitive for characterization of isolates than were earlier techniques [101, 124, 125].

DFA characterization of *Orientia*-infected lines in mite colonies suggested other problems with the method when intergenerational antigenic variation was sometimes observed [23]. For example, 1 filial mite generation might be negative for the Gilliam strain by DFA, whereas the succeeding generation in the mite line might be subsequently reactive. This suggests that the assay may be insufficiently sensitive to detect low numbers of organisms. The newer molecular methods based on PCR should avert such results because of the greater sensitivity of the assay (D.J.K., unpublished data).

In contrast to the DFA, the IFA and indirect immunoperoxidase tests can also be used for serodiagnosis [126, 127]. The IFA test is performed using patient serum specimens to show specific increases in whole globulin or IgG titers in paired sera or elevated single IgM titers typical of active disease. For example, Bozeman and Elisberg [124], using IFA to test serum specimens from Malaysian patients, showed faster increases in titer for homologous infection than for heterologous infection. The indirect immunoperoxidase test is performed similarly; however, the end points are observed using a light microscope [126, 128, 129]. Although the indirect immunofluorescence test was long considered the "gold standard" for serodiagnosis, the lack of standardization of end points and of the antigen strains used in the test has resulted in considerable confusion [127]. For antigenic characterization, the peak serum titer in reactions with a set of the prototype *Orientia* strains is scored as the antigenic type [130].

Monoclonal antibody typing. As noted above, *Orientia* strains can be difficult to characterize or classify using DFA, IFA, or indirect immunoperoxidase assay, because of cross-reactivity [45, 46, 123]. To reduce this problem and to produce more-specific results, some investigators have used monoclonal antibodies developed using prototype *Orientia* strains to characterize human, mite, and other field isolates [50, 53, 55, 131–137]. Monoclonal antibodies specifically derived from and reactive with the prototype strains can be used to classify new isolates by several methods, including DFA, IFA, and ELISA, and also by analyzing the reactivity in electrophoresed immunoblots. Although the specificity associated with monoclonal antibody characterization is theoretically very good, the multiplicity of unique *Orientia* strains is problematic. The development of monoclonal antibodies from each newly identified strain among the ever-increasing number of apparently unique strains for comparison with each previously identified "prototype" may be impractical.

SDS-PAGE immunoblot assay. The reaction of electrophoresed proteins from multiple strains of rickettsiae, isolated from many different sources, with human or animal polyclonal antisera or monoclonal antibodies shows clear strain-dependent differences (D.J.K. and W.M.C., unpublished data) [132, 138]. *O. tsutsugamushi* contains no lipopolysaccharide or outer membrane glycoproteins typical of most gram-negative organisms, and this method yields at least 30 major proteins, including 110-kDa, 70-kDa, 60-kDa, 56-kDa, 47-kDa, and ~22–25-kDa proteins [139–142]. The dramatic antigenic variability of scrub typhus rickettsiae appears to reside primarily but not exclusively in the 110-kDa and 56-kDa proteins [143]. Although the serum samples from most convalescent patients react with ⩾1 of these major proteins, >99% react with a polypeptide in the size range 54–56 kDa, with SDS-PAGE showing distinct antigenic patterns among strains for this antigen [130, 143–147]. The 56-kDa protein has become the most widely studied antigen, especially with use of SDS-PAGE. This antigen is expressed mostly on the cell surface and contains both unique and cross-reactive epitopes. The 56-kDa protein is type specific and the most plentiful cell-surface protein of *O. tsutsugamushi,* consisting of ~520 amino acids, although the protein can vary from ~500 to 540 amino acids in different strains. The protein is usually considered to contain 4 variable domains (figure 2) and to be the primary antigen reflecting variation within the genus [92]. It is not clear what if any role the protein plays in the pathogenesis of *O. tsutsugamushi,* although it is thought that the protein acts as an adherent factor for adsorption of the bacteria to the host cell surface [148, 149] and may be a

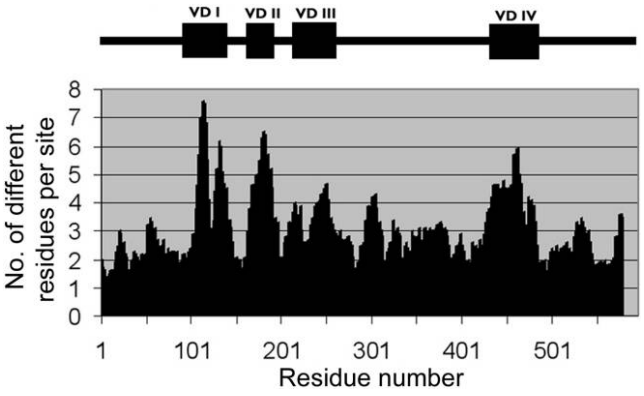

Figure 2. Diversity of amino acids in the alignment of the coding region of the 56-kDa protein of *Orientia tsutsugamushi.* The plot shows the moving mean, for a window of 10–amino acid residues, of the number of different amino acids observed at a position in the amino acid alignment (for >135 complete or nearly complete sequences from *Orientia* strains). The location of the highly variable protein domains VDI–VDIV within the amino acid alignment of the coding region is shown above the graph.

determinant of virulence for individual strains [150]. The characteristic type specificity of the 56-kDa antigen for scrub typhus strains and the lack of high sequence similarity of the 16S ribosomal RNA (rRNA) gene from scrub typhus prototype strains compared with similar sequences for various species of *Rickettsia* (similarity, 91%) provided the basis for the removal of scrub typhus rickettsiae from the genus *Rickettsia* and their placement in the new genus *Orientia* [140, 141].

MOLECULAR CHARACTERIZATION OF ANTIGEN GENES IN *ORIENTIA* SPECIES

Beginning in the early 1990s, a number of studies reported gene sequences for several of the protein antigens of *O. tsutsugamushi* [90–92, 151]. Among these reports were the cloning and molecular characterizations of the gene that encoded the 56-kDa antigen polypeptide from several of the prototype strains, including Karp, Kato, and Gilliam [91, 92]. As mentioned, the 56-kDa protein is a type-specific protein and is not expressed in other bacteria, including other members of the Rickettsiaceae. In fact, a study of the occurrence of the genetic locus encoding this protein among other bacteria, by use of molecular information on the genomes of >1000 other bacteria, indicated that the 56-kDa antigen gene is unique to *O. tsutsugamushi.* Across most of its length, the protein sequence shows no obvious homology or structural similarity to any other protein in the genome database, making it one of the most appealing targets for use in development of a vaccine against *O. tsutsugamushi.* A small portion of the carboxy-terminal end of the protein shows similarity to some outer-membrane proteins from the alphaproteobacteria. This type specificity is unlike that found for some of the other major surface antigens of *Orientia* strains. For instance, the 47-kDa HtrA surface antigen is encoded by a gene within a family of serine proteases that has structural similarity and evolutionary homology with sequences found in almost all major bacterial and eukaryotic (both plant and animal) lineages but not necessarily in all bacterial forms [152, 153].

The sequence of the 56-kDa antigen gene appears to provide the most useful data for studies of genetic differentiation within *Orientia* strains. Other genes may be useful for studies of differentiation within *Orientia* strains, but to date, no other locus has been reported to contain levels of variation even close to those seen for the locus encoding the 56-kDa protein. Thus, no other locus seems to have the same potential of usefulness for fine-structure analysis of strain variation. Specifically, multiple strains have been compared for the small subunit rRNA gene (16S rRNA) and the gene for the groEL protein. *groEL* is a member of the chaperonin gene family, required for proper folding of proteins following translation. The *groEL* gene occurs in most eubacteria and is present in *O. tsutsugamushi.* The gene sequences of the 16S rRNA gene have been widely used for the identification of bacterial species and have been used extensively in bacteria related to *O. tsutsugamushi,* such as *Rickettsia* species [154], in which they vary between species by as much as 0.025. In previous analyses comparing at most 3 strains (Karp, Kato, and Gilliam), the gene showed less divergence between strains in the *Orientia* genus than between those in the *Rickettsia* genus [155, 156], whereas sequence divergence between *Orientia* and *Rickettsia* was 9%–10%. Reliable sequence data for the 16S rRNA gene in *O. tsutsugamushi* have accumulated slowly and are now available for 9 strains. Divergence of this gene within the *Orientia* genus is still found to be less than that within the *Rickettsia* genus, with 8 of the 9 strains showing <1% divergence. The ninth strain, the divergent Shimokoshi strain, shows ~1.5% divergence from all the others. Similar results but with slightly greater levels of variation are seen for the groEL protein [157], sequences of which are available for ~30 isolates. The maximum divergence seen between pairs of *groEL* sequences is ~3.5%. However, the data mainly consist of strains that appear to be phylogenetically close to the Karp prototype strain, and none of the most divergent strains, such as Shimokoshi, have been studied for *groEL* sequences.

This lack of marked divergence for the 16S rRNA and *groEL* genes would seem to imply that all or most *Orientia* strains may represent a single species but that it is a species that also shows a high degree of interstrain variation when assessed using sequences of the locus for the 56-kDa protein. It should be noted that most of the isolates of *Orientia* strains showing the most-divergent sequences for the 56-kDa protein gene, other than Shimokoshi, have not been examined for their 16S rRNA sequences, so the question of species limits within the *Orientia* genus remains open. Gene sequence analysis of the 56-kDa antigen gene (sequence from either the entire gene or from major variable portions of the gene) has now become the major tool for genetic characterization of *Orientia* strains.

PCR analysis of the 56-kDa antigen gene. Soon after publication of the DNA sequence of the 56-kDa antigen gene of the Karp strain of *O. tsutsugamushi* [91], primers derived from that sequence were used to detect and confirm, by using Southern blot analysis, the agent in the blood of infected mice [158] and in individual chiggers [159]. The study of infected mice showed that the PCR method had sensitivity sufficient to detect *O. tsutsugamushi* at blood levels found in typical human infection, 16–160 organisms/mL at 2–14 days after infection, as quantified by murine titration [158, 160]. Unlike the traditional mouse, egg, or cell culture isolation and propagation methods, PCR does not require expensive BL-3 facilities or extended incubation times. The first uses of the method to directly detect and confirm diagnosis soon followed [161–166]. PCR was found to correlate well with traditional culture and mouse inoculation methods in monitoring for the clearance of *O. tsutsugamushi* after antibiotic therapy. DNA could be detected by

the method at up to 8 days after treatment, long after the patient typically becomes afebrile [167]. In a case report, Lee et al. [168] diagnosed scrub typhus by using a nested PCR with primers derived from the 56-kDa antigen gene. Their data suggest that the nested PCR with these derived primers may be more sensitive for the diagnosis of scrub typhus than is standard PCR alone; however, Liu et al. [169], also using primers derived from the sequence of the 56-kDa antigen gene, reported 100% sensitivity for performance of a standard PCR of eschar biopsy specimens from patients with scrub typhus.

Although probably not useful for genetic characterization of strains, the recently developed real-time or quantitative PCR has proven to be more sensitive than PCR for diagnostic purposes, permitting detection of as few as 3–5 copies/μL of blood [170]. The method, which has been applied to the detection of the conserved gene for the 47-kDa HtrA protein, is sensitive (detecting as few as 3–21 copies of *Orientia* sequence per μL of blood), specific in molecular terms (not cross-reacting with non-*Orientia* genetic sequences, such as those from 17 rickettsial strains and 18 other eubacterial strains), and does not risk the problem of contamination that is typically associated with the nested PCR assay. In comparison, in a recent prospective clinical evaluation involving 135 suspect cases of scrub typhus in Korea that used nested PCR with primers derived from the 56-kDa antigen gene, a sensitivity of 82% and a specificity of 100% were reported [171]. In a smaller study, Saisongkorh et al. [172] reported even greater sensitivity (100%); all patients who tested positive for scrub typhus by IFA were also identified by nested PCR. Three patients who had negative results of IFA had positive results of PCR, but disease status for scrub typhus was not determined for these patients. Although they reported similar specificity, Sonthayanon et al. [173] noted a sensitivity of only 45% for real-time PCR with 16S rDNA–derived primers in analysis of blood from 183 patients with serologically confirmed disease in Thailand. PCR assays also allow monitoring of levels of infection in arthropod vectors, mammalian hosts (such as rodents), and nonhuman primates and the comparison of active infection with previous infection (assessed by testing for the presence of antibodies) without the necessity of culturing *O. tsutsugamushi* [72, 159, 174, 175].

The gene sequence for the 56-kDa protein appears to provide the most useful data concerning genomic diversity. However, indirect methods of assessing variation in the gene by PCR have been applied that do not involve determining the sequence directly, but they have limitations. The first studies to make genetic comparisons of strains introduced the use of strain-specific PCR primers, based on the sequences of a number of prototype strains [166, 176]. Samples that yielded the product were classified as belonging to a group identified with a particular prototype strain. However, genetic variation that exists within the PCR product but outside the primer sites would not be detected, nor would simple amplification provide an estimate of the degree of genetic differentiation. In many cases, isolates could be grouped with standard prototype strains [176–178]. For instance, analysis with prototype strain–specific primers based on the gene sequences was capable of classifying strains isolated from patient samples [166, 179]. However, there is a question as to whether samples tested negative because of the absence of *Orientia* DNA or because the product from divergent strains could not be amplified using PCR primers derived from the standard prototype strains.

A further step toward determination of the underlying levels of genetic variability in the 56-kDa antigen gene within populations uses RFLP characterization of the PCR products. In this method, restriction endonucleases are used to treat the products of standard and nested PCR reactions produced using a set of primers that are presumed to amplify all versions of the 56-kDa antigen gene. Variable band patterns can then be compared using electrophoresis. The number of fragments generated for comparison depends on the number and type of restriction enzymes used. Considerable variation in banding patterns was discovered, with attempts made to relate genetic similarity of strains to the antigenic properties of isolates [159, 166, 180–184]. However, some strains showed unique patterns, and assessment of the degree of similarity in many instances was not straightforward [136, 185–189].

Gene sequences of the locus for the 56-kDa protein. The first reports of the gene sequence of this locus indicated that the protein product of the gene could differ between strains [91, 92]. A small number of sequences were compared, and the amount of genetic difference was determined to identify the phylogenetic relationships between strains. The protein was found to be variable in size when different strains were compared, generally having slightly >520 amino acid residues, and was coded by a gene of ~1550 bases [91, 147, 190].

Examination of the available gene sequences for the 56-kDa protein that have been deposited in the sequence databases through mid-2008 indicates that protein sequences encoded by the gene may be as short as 516 amino acids and as long as 541 amino acids. This variation in length is the result of an unusual degree of nucleotide insertion or deletion within the coding frame of the gene that maintains the protein-reading frame without producing premature termination signals. These changes occur only within the variable domain regions of the gene. The ultimate result of this process is the unusually high levels of protein variation and subsequent immunological diversity that exists for the 56-kDa type-specific antigen.

Comparison among prototype strains indicated that the gene had several regions of hypervariability [190]. The 4 primary regions (figure 2), designated variable domains VDI–VDIV [190], roughly correspond to regions of hydrophilic residues

in the protein [92]. When a larger number of sequences are compared, these domains generally correspond to the areas of the protein most subject to sequence divergence at both the nucleotide and protein levels. As the amount of sequence data available for comparison has increased, higher levels of genetic diversity in these variable domain portions of the protein, compared with other parts of the molecule, continue to be observed. This is illustrated in figures 2 and 3; sequence diversity was analyzed among a set of 135 sequences of the gene for the 56-kDa antigen of *Orientia* that included a complete or nearly complete sequence (at least 90% of the coding sequence) of the gene culled from the GenBank database [192]. Figure 2 presents the mean number of different amino acids (with a deletion considered to be an alternative amino acid) for a sliding window of 10 amino acid residues across the aligned length of the protein for 135 sequences. The variable domains, as defined on the first few sequences [192], were observed to remain valid, given the larger data set. The VDI and VDII regions are the most variable in amino acid content. Figure 3 illustrates the data with use of a different measure of variability, the mean gene diversity [191]. The gene diversity within the same 10–amino acid sliding window across the protein in the 135 sequences is illustrated. As is seen in figure 2, the 4 traditional variable domains contain the highest level of diversity within the molecule. The 4 variable domain regions show roughly comparable levels of elevated diversity, compared with the remaining regions of the gene. Both figures 2 and 3, however, show that moderate levels of variability are very widespread within the gene, at levels that would be considered high in most other systems. However, the diversity of forms represented by the variability within VDI–VDIV should be given critical consideration in any detailed plans for vaccine development.

Phylogenetic trees constructed using specific DNA sequences derived from the 56-kDa antigen genes of multiple *Orientia* strains have been obtained by several research groups to show the genetic relationships of various strains [58, 169, 186–203]. Comparison of isolates from Japan, for example, produced phylogenetic trees from sequence comparisons that were used to group the increasing numbers of isolates from humans, rodents, and mites derived from multiple epidemiological investigations within the regions of endemicity [194]. Similar studies have been performed in Taiwan [196], Malaysia [197], India [200], northern Thailand [188], and China [204].

More recently, studies have emphasized the collection of DNA sequence data to characterize interstrain variation. By mid-2008, DNA sequences (size range, ~150 to >2000 bases) from >270 isolates were available for comparison. Approximately one-half of these deposited sequences contain a substantial portion of the coding region (>75% of the ~1560-nucleotide protein-coding region). Recently, however, for

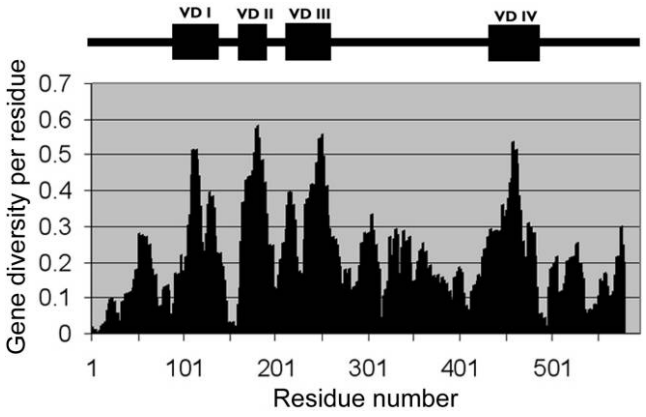

Figure 3. Gene diversity measured at the amino acid level in the alignment of the coding region of the 56-kDa protein of *Orientia tsutsugamushi.* Gene diversity is as defined by Nei and Kumar [191]. The plot shows the moving mean of gene diversity for a window of 10–amino acid residues around a position in the amino acid alignment (for >135 complete or nearly complete sequences from *Orientia* strains). The location of the highly variable protein domains VDI–VDIV within the amino acid alignment of the coding region is shown above the graph.

sequences being deposited in the international sequence databases, there has been a move toward obtaining information for only 350–500 bases of the most variable portion of the gene (P.A.F., unpublished data). Although these smaller fragments are clearly useful for general identification of major antigen types within *O. tsutsugamushi,* whether they are sufficient to completely identify interstrain variation within the gene and within the *Orientia* genus remains to be determined.

Phylogenetic analysis of the gene sequence for the 56-kDa protein was undertaken as part of our investigation. The sequences from the international databases (GenBank, European Molecular Biology Laboratory, and DNA Data Bank of Japan) incorporating >1200 bases of the coding segment of the gene were aligned in 2 ways. Clearly redundant identical sequences were eliminated for the strains obtained by multiple researchers from 118 entries, and these entries were supplemented by 17 unpublished sequences, yielding 135 sequences for comparison. These represent the sequences that are used above for the analysis of diversity and that are presented in figures 2 and 3. For phylogenetic analysis, first, an alignment was obtained using the protein translation of the gene as a primary basis of alignment. Second, the nucleotide sequence was used directly as the basis of an alternative alignment. The 2 alternative alignments were constructed using CLUSTAL W [205], as implemented in the Mega4.0.2 analysis package [206, 207]. The length of the alignment differs from the biological length of the protein or gene because of the inclusion of insertions required to obtain the final alignment. The 2 sets of aligned nucleotide sequences were then analyzed using the neighbor-joining method [208], on the basis of the mean pairwise difference between sequences,

and phylogenetic trees were produced to show the genetic similarities between strains. The phylogenetic tree produced indirectly from an alignment of the protein sequences differed from that produced directly from the nucleotide sequences themselves in only branch length and minor topology, especially among very closely related strains, such as among strains closely related to the prototype Karp strain. The reliability of the placement of branching within the tree was assessed using bootstrapping [209]. Identification of significant subgroups within the phylogenetic tree was performed on the basis of topological analysis and bootstrap values.

Examination of the details of the phylogenetic tree shows that there are several identifiable clades present among the 135 sequences of *Orientia* isolates assessed in our study. A general schematic of the phylogenetic relationships among isolates, as obtained from the analysis of the direct nucleotide alignments, is presented in figure 4. The percentages given after each group represent the frequency of sequence types within each clade that were found in our analysis of 271 reported sequences, most of which were from the DNA databases. This included 135 complete or nearly complete sequences, which were used in our analysis of diversity and phylogenetic relationships, and an additional 136 partial sequences, most of which have been deposited in the public databases. There appear to be at least 9 definable clusters: **Karp-related**, **Saitama**, **Kuroki**, **TA763**, **Gilliam**, **Kawasaki**, **JG**, **Kato**, and a group of divergent strains (clades are in bold type). The **JG** and **Kato** clades appear to include 2 subgroups each, assessed by the degree of sequence divergence. There are also a small number of sequences, not shown in figure 4, that do not fall neatly into any major clade. These sequences may represent either very minor natural groups or may be the result of sequencing error.

The detailed relationship among the 135 isolates is presented in figure 5. The Gilliam prototype strain (and related isolates) is used as the central point to separate strains that fall into a grouping of isolates shown in figure 5 that are related to the Karp prototype strain (including the clades designated **Saitama**, **Kuroki**, and **TA763**) from the more distantly related forms shown in figure 6. Isolates in figure 5 are listed by their GenBank accession number, strain designation, and an indication of their geographic origin. Several studies of *Orientia* isolates from Japan previously identified groupings of strains on the basis of antigenicity and RFLP patterns [54, 193, 194]. Some of the group designations shown in figures 4 and 5 are based on those presented by Tamura et al. [54], following the earlier studies of that research group.

In general, the clade with the largest number of isolates will be referred to as **Karp-related** (figure 5). This clade includes strains very close in sequence to that found for the prototype Karp strain, as well as sequences that show minor divergence from that of Karp but that basically maintain the reading frame

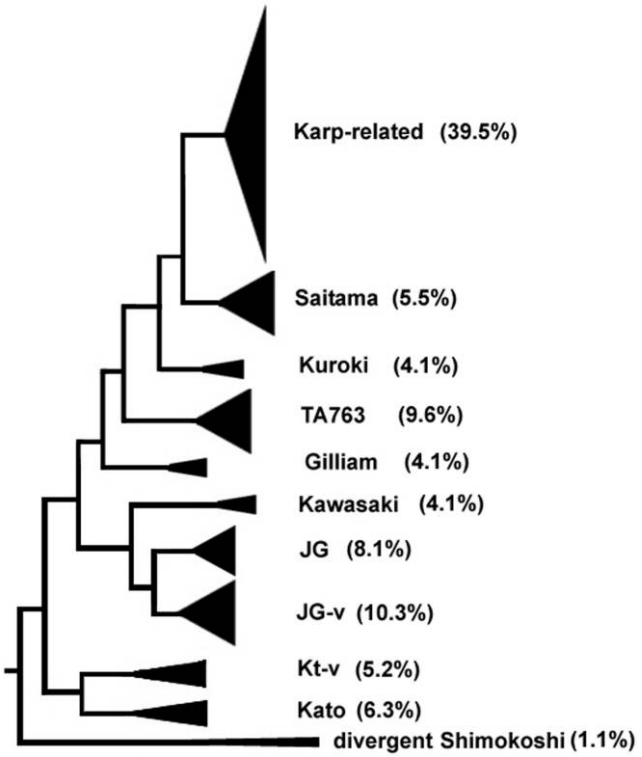

Figure 4. Schematic representation of the phylogenetic relationships among major clades of *Orientia tsutsugamushi* as represented by the DNA sequences of the 56-kDa surface antigen gene. The size of each triangle roughly represents the proportion of all complete or nearly complete sequences from a clade. The percentage in parentheses next to each clade name represents the frequency among 271 sequences, including partial sequences. Detailed phylogenetic relationships are shown in figures 5 and 6.

seen in Karp. Such strains appear to include many of those that have often been referred to as "Karp-like" in studies based on serological similarity. Some isolates that were closely related to the prototype Karp strain were previously designated JP-1 and JP-2 by Tamura et al. [54] on the basis of RFLP differences. Sequence analysis suggests that these 2 subgroups are simply 2 of several subgroups closely related to each other and related to the prototype Karp strain. The other subgroups that make up the **Karp-related** cluster of sequences in figure 5 have not previously received any subgroup designation. Geographically, **Karp-related** strains are found throughout the geographic distribution of *O. tsutsugamushi,* including Japan, Korea, China, and Southeast Asia.

Also closely related to **Karp-related** strains in figure 5 but more distinct in sequence is the group designated **Saitama** by Tamura et al. [54] on the basis of the area of Japan from which many of these isolates were collected. The bootstrap value for the grouping of the **Saitama** clade and the **Karp-related** group is 99%, indicating a very significant evolutionary relationship joining the groups. By incorporation of information on se-

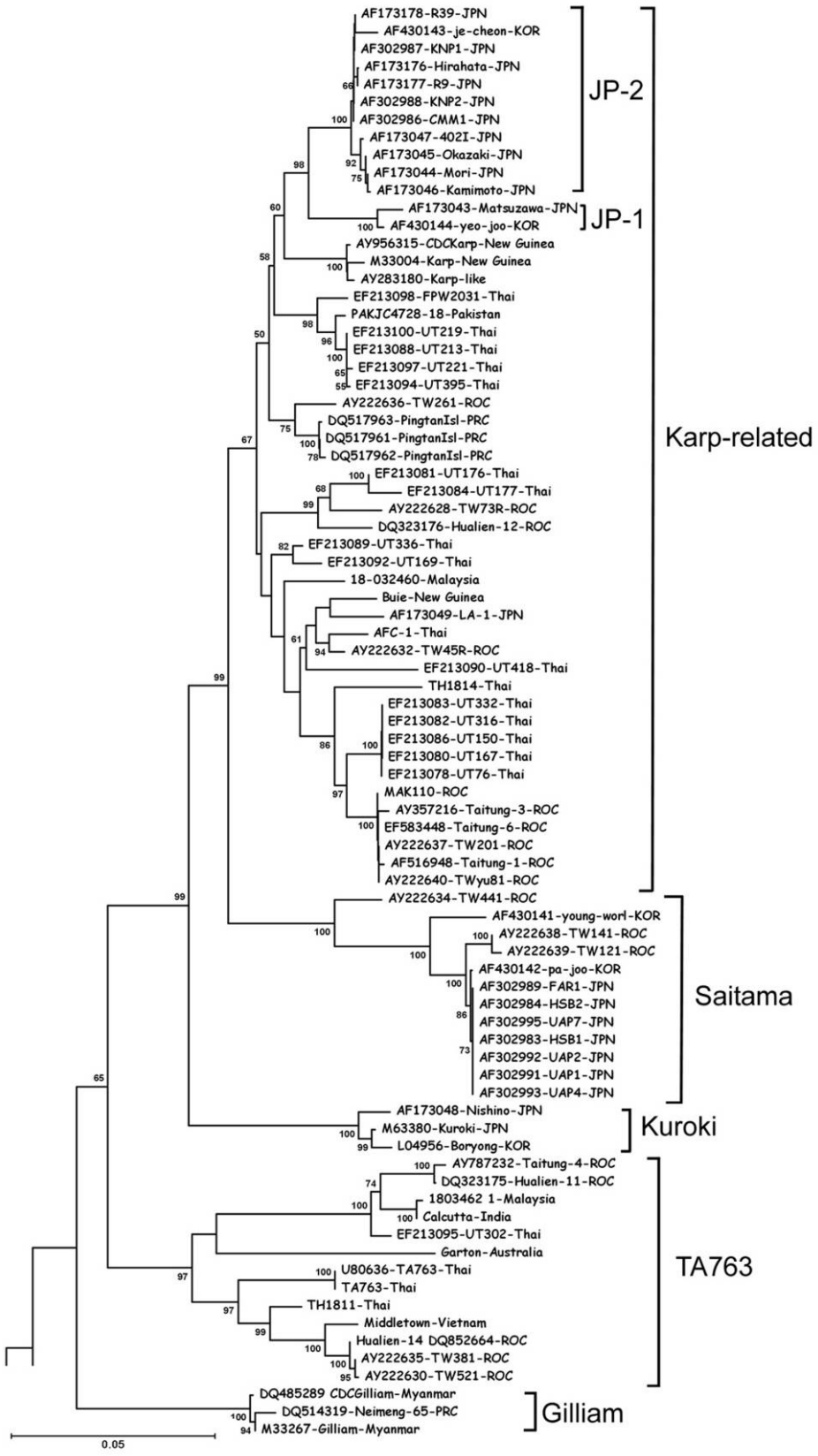

Figure 5. Phylogenetic tree of *Orientia tsutsugamushi* based on the nucleotide sequences of the 56-kDa cell-surface antigen gene. A subset of the phylogenetic tree is made up of the isolates most closely associated with the clade that includes the prototype strain Karp. Isolates are identified by their GenBank accession number, strain designation, and an indication of their geographic origin. The tree was constructed using the neighbor-joining method [170], on the basis of the percentage of nucleotide differences. Major phylogenetic clusters related to antigenic types within the data are indicated. The entire tree given in this figure and in figure 6 is artificially rooted at the point joining the "divergent" clades, including the Shimokoshi prototype isolate, with the remainder of the sequences. The tree includes the prototype strain Gilliam for orientation with figure 6. KOR, Korea; JPN, Japan; PRC, People's Republic of China; ROC, Republic of China.

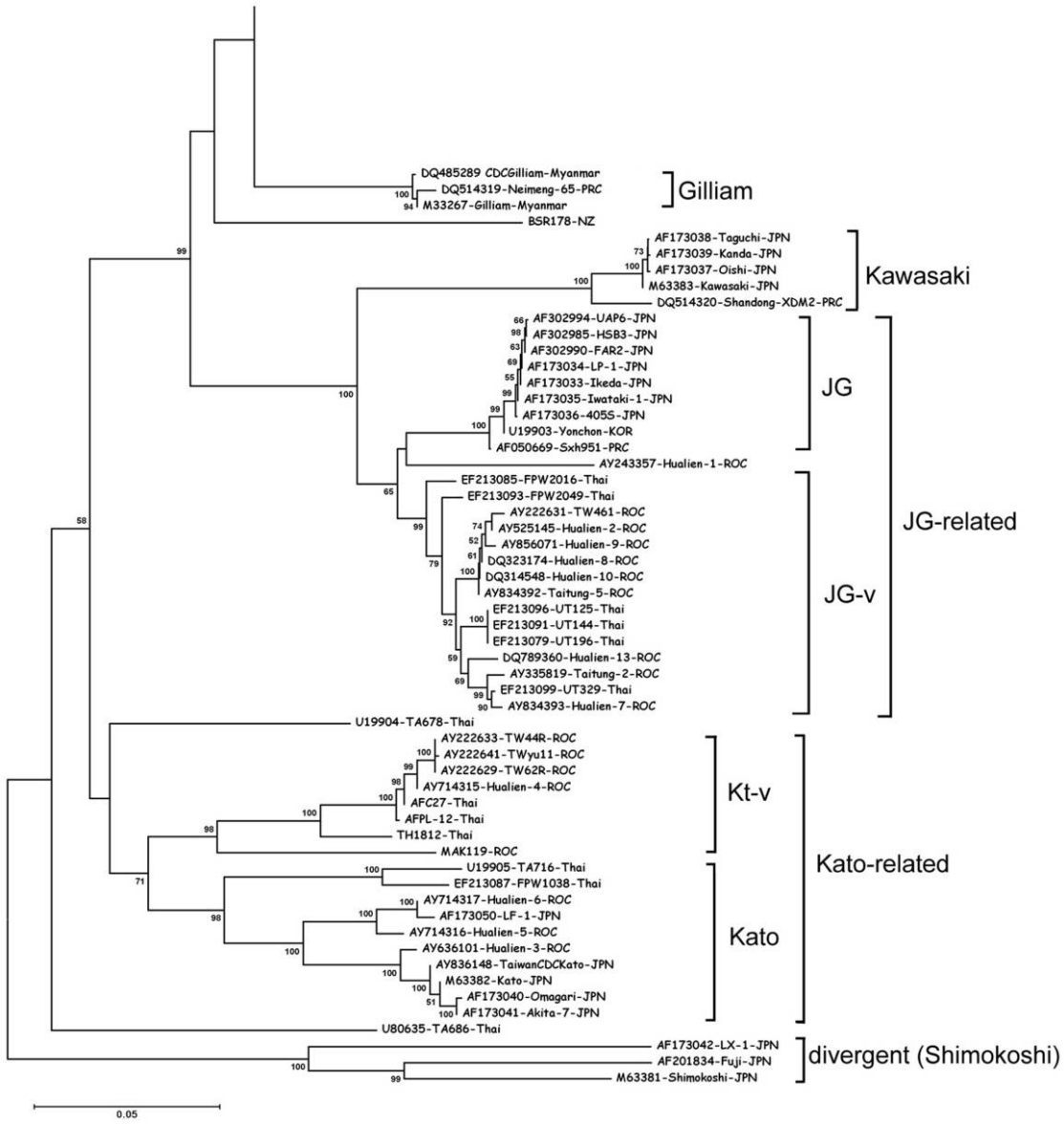

Figure 6. Phylogenetic tree of *Orientia tsutsugamushi* based on the nucleotide sequences of the 56-kDa cell-surface antigen gene. A subset of the phylogenetic tree is made up of isolates associated with the clades with a greater sequence divergence from Karp-related taxa. Isolates are identified by their GenBank accession number, strain designation, and an indication of their geographic origin. The tree was constructed using the neighbor-joining method [170], on the basis of the percentage of nucleotide differences. Major phylogenetic clusters related to antigenic types within the data are indicated. The entire tree given in this figure and in figure 5 is artificially rooted at the point joining the "divergent" clades, including the Shimokoshi prototype isolate, with the remainder of the sequences. The tree includes the prototype strain Gilliam for orientation with figure 5. KOR, Korea; JPN, Japan; NZ, New Zealand; PRC, People's Republic of China; ROC, Republic of China.

quence similarity from isolates for which only partial sequences have been reported, it appears that slightly <40% of sequences can be considered to be in the clade represented by **Karp-related** and **Saitama**. A second group of strains in figure 5 that is further divergent from the **Karp-related** clade is significantly associated (as measured phylogenetically by a bootstrap value of 99%). This small clade, designated **Kuroki**, represents ~5.5% of sequences in the databases. However, on the basis of serological analysis, it may be more prevalent in some regions, especially in Korea, than is represented by the number of sequences available for analysis.

A more variable grouping of strains in figure 5 makes up the clade designated **TA763**, which is named after the prototype strain and which constitutes 10% of all sequences. This clade is clearly related to the cluster of **Karp-related**, **Saitama**, and **Kuroki** clades but with lower support (bootstrap value, 65%). Isolates from the **TA763** clade may not occur in Japan or Korea but are seen in Taiwan and Southeast Asia.

The prototype strain Gilliam is unusual because it is part of a very small clade that is divergent from the clades formed by most other prototypes. Few isolates have been observed that share sequence similarity with the Gilliam prototype strain. The prototype Gilliam isolate was collected in Myanmar (Burma), and a strain with a similar sequence was reported in northern India (Bakshi, unpublished sequence; GenBank accession number DQ286233). There are also a small number of strains with similar sequences that have been found in Inner Mongolia [210]. Strains of the **Gilliam** type make up ~5.5% of *Orientia* sequences in the various data banks.

With regard to the strains detailed in figure 6, another significant grouping of clades is made up of Japanese sequences, designated **Kawasaki** and **JG** by Ohashi et al. [193]. **JG** refers to "Gilliam type in Japan," on the basis of serological cross-reactions [54]. However, analysis of sequences indicates that **JG** is not, in fact, closely related to **Gilliam**, as assessed by sequence similarity. Instead, the **JG** clade clearly appears to be related to **Kawasaki**, and together they make up a separate significant group within *Orientia* species. The sequence types of **Kawasaki**, **JG**, and a subgroup that we designate **JG-v** (JG variant) represent 28% of all sequences. **Kawasaki** type sequences have been found in Japan and China, whereas **JG** and **JG-v** sequences have been observed in Japan, China, and Southeast Asia.

Consideration of figures 4 and 5 reveals that there are other clades that are very divergent from the **Karp-related**, **Saitama**, **Kuroki**, **Gilliam**, **Kawasaki**, and **JG** clades. These include a set of clades that are most closely allied to the prototype Kato strain: **Kato-related**, including **Kato** and **Kt-v** (Kato-variant) in figure 6. Careful examination of figure 6 reveals that the **Kato-related** group is actually quite diverse, both genetically and geographically. This grouping makes up ~11% of all sequences from the databases. Finally, there are a few highly divergent sequences that do not seem to belong to any of the groups that we and others have designated. These include a small but very diverse and very divergent group with sequences somewhat related to that of the prototype Shimokoshi strain. Shimokoshi and other highly divergent isolates make up only ~2% of sequences and may represent sequences from organisms within classification *O. tsutsugamushi* that in the future may be associated with a different species.

There does not seem to be any close relationship between the phylogenetic placement of strains on the tree in figure 5 and their pathogenic potential. On the basis of these molecular analyses, it appears that for a vaccine to be "universally" useful, it must include protein representatives from the **KARP-related**, **Gilliam**, **Kawasaki**, **JG**, and **Kuroki** groups. How to incorporate the more divergent groups is less obvious, although inclusion of **Kato** and **Kt-v** might be considered. The small number of highly divergent strains, such as Shimokoshi, indicates that there will likely always be some proportion of strains that are difficult to incorporate. The Shimokoshi strain has been reported to have low virulence in murine tests [52], so the exclusion of these divergent strains may not be consequential.

Consideration of the geographic origin of the strains for which both nucleotide and calculated amino acid sequences have been obtained and the location of these strains on the tree in figure 5 would suggest that there is only slight evidence of geographic differentiation among strains. Strains from divergent areas (e.g., Japan vs. Thailand) are found in the same phylogenetic grouping, and no phylogenetic grouping appears to be specific to a geographic locality, especially given the nonrandom and patchy nature of sampling represented by strains that have had sequences deposited in the international sequence databases. However, although many of the same prototype forms are observed throughout the range of *O. tsutsugamushi,* local differences in frequency may and probably do occur [178].

GEOGRAPHIC DISTRIBUTION OF ANTIGENIC TYPES

As noted in the introduction, the distribution of scrub typhus is a large, geographically diverse area of the Asia-Pacific rim. Efforts to classify antigenic types within the region appear to be proportional to political and economic stability of countries where it is endemic as much as to the impact of the disease itself. Possibly because other diseases have had a greater impact in many of these countries, the epidemiology of scrub typhus has only relatively recently been investigated. Although serological antigenic characterization data are available for the past ~50 years, genetic characterization data have become available only recently. Antigenic and genetic characterization is important for selection of potential vaccine candidates; however, strain virulence is arguably as important for that selection [121]. Table 3 presents a summary of results from the more extensive epidemiological surveys of prevalence (that used any of the methodologies that we have reviewed) from various geographic areas. Details from these and other less extensive studies are presented below according to geographic region.

Australia. Scrub typhus has been reported throughout the myriad of islands in the Southwest Pacific that includes the Indonesian and Philippine archipelagos and Australia. In Australia, "coastal fever" was recognized as early as 1913 and was recognized as scrub typhus since the 1920s [114, 215, 216]. Distribution is focused on the tropical coastal periphery of northeastern Queensland [217], including the Torres Strait islands off the Cape York Peninsula [201, 218], the tropical region of the Northern Territory, and the adjacent Kimberly region of Western Australia [58, 217, 219]. *Leptotrombidium deliense* has been identified as the primary vector [4]. Fifty-two *Orientia* isolates, collected in the early 1950s by murine inoculation from humans (27 isolates), animals (21), and *L. deliense* pools (4)

Table 3. Selected epidemiological prevalence studies showing the geographic distribution of predominant *Orientia* strains.

Geographic location, isolate sources (no. of isolates)	Method(s)	Predominant strain(s)[a]	Reference(s)
Queensland and Northern Territory, Australia			
Human (27), rodent (21), and mite (4)	DFA	TA716, in North Queensland (94%)	[122]
Pescadores, Taiwan			
Human (17), rodent (17), and mite (15)	DFA	TA716 (100%) and Karp (47%)	[80, 121]
Fujian Province, China			
Human, rodent, and chigger (126 total)	CF, DFA, and IFA	Karp (23%), Gilliam (10%), and TA716 (1%); Karp mix (72%)	[211]
Shandong Province, China			
Human, rodent, and mite (23 total)	IFA, MAb, and RFLP	Gilliam (91%) and Karp (9%)	[212]
Human, rodent, and mite (21 total)	56-kDa Seq	Kawasaki (95%) and Kato (5%)	[213]
Korea			
Human (113)	IFA and MAb	Boryong, primarily in Chungnam Province (78%)	[55]
Akita, Niigata, Kanagawa, Shizuoka, Oita, and Hokkaido prefectures, Japan			
Human (3), mite (89), and rodent (70)	CF	Karp (53%), Gilliam (31%), Kato (11%), and mixed antigenicity (2%)	[15]
Niigata, Kyoto, Tokushima, Akita, Miyazaki, Gifu, and Shizuoka prefectures, Japan			
Human (15), rodent (3), and mite (2)	MAb, RFLP, and 56-kDa Seq	Karp (25%), Gilliam (20%), and Kawasaki (20%)	[194]
Shizuoka Prefecture, Japan			
Human (24), rodent (30), and mite larva (5)	MAb and IFA	Karp (60%), Kawasaki (34%), and Kuroki (7%)	[135]
Niigata, Akita, Kanawagawa, Toyama, Saitama, and Fukushima prefectures, Japan			
Human, rodent, and mite (22 total)	MAb, IFA, and IP	Karp (64%) and Gilliam (36%)	[132]
Primorski Krai, Russia			
Human (1), rodent (1), and mite (1)	IFA, CF, and CN	Gilliam (100%)	[26, 27]
Philippines			
Febrile human serum (23)	CF	Karp (78%), Gilliam (83%), and Seerangayee (61%)	[36]
Rodent (42)	DFA	TA716 (86%), TA686 (81%), and Karp (67%)	[121]
Myanmar (Burma)			
Serum from patients with fever (14)	CF	Gilliam (36%) and Karp-Seerangayee mix (46%)	[36]
Malaysia			
Human (114)	DFA	TA763 (72%), TA716 (70%), and Karp (56%)	[46]
Mites direct[b] (168)	…	Karp (93%)	[20]
Tadzhikistan			
Rodent (4) and mite (4)	CF	Gilliam (100%)	[26]
Thailand			
Human, rodent, and mite (77)	CF	Karp alone (43%); Karp and mix of ⩾2 others (67%)	[101]
Mites direct[b] (146)	DFA	Karp (51%)	[12]
Human (27)	56-kDa Seq	Karp-like (65%), Gilliam-like (22%), and TA716 (4%)	[214]

NOTE. CF, complement fixation; CN, cross-neutralization; DFA, direct fluorescent antibody assay; IFA, indirect fluorescent antibody assay; IP, indirect immunoperoxidase assay; MAb, monoclonal antibody typing; RFLP, restriction fragment–length polymorphism analysis; Seq, protein gene sequencing.

[a] In parentheses is the percentage of isolates that reacted to the specified reagent panels. Primary reactivity is reported.

[b] "Mites direct" indicates that mites are dissected and exudates are stained by DFA.

in North Queensland [113, 114] were characterized by DFA [122]. Of the isolates, 71% were multiply reactive to as many as 4 conjugates. All isolates from humans and chigger pools, 86% of the isolates from rodents, and 94% of isolates overall reacted to TA716, which suggests a high prevalence of this strain in the region sampled. In addition, reactivity to TA763 (42% of isolates), TA686 (33%), and Karp (31%) were reported. With use of PCR and RFLP typing, 47 of these 52 isolates were examined, and it was determined that they made up 10 unique *groEL* types, with restriction patterns distinct from those of isolates from 12 other Asian-Pacific countries [181]. The same 10 unique *groEL* types or a different group of types that differed from the 12 prototypes was seen with the RFLP patterns for the 22-kDa protein gene sequences. The Litchfield strain, the first isolated in the Northern Territory, was recovered in 1996 from the blood of a febrile man who worked in the tropical rain forest of Litchfield Park (table 2) [58]. Although there are few prevalence data from the Northern Territory, at least 6 cases

of scrub typhus dating back to the late 1930s and 1957 were recorded, 9 cases have been reported in Litchfield Park since 1990, and 1 more recent, serologically confirmed case involved a man who had visited the "top end" rain forest area outside the park [220]. The Litchfield strain was characterized by rDNA sequence analysis, which showed that it was distinct from the Karp-like strains from Queensland. Moreover, a comparison of the rDNA sequence of the Litchfield strain with those of various prototype strains suggests that it may represent a distinct type not previously encountered. Collectively, these results suggest the extreme heterogeneity of the Australian isolates, probably associated with their evolution in the geographic isolation of northern Australia. With the exception of 1 of the 2 strains isolated in 2003 and 2004 from patients in Darnley Island that were typed and found to be similar (89.8% homology) to Taiwanese strains TW381 and TW521 [201], little information has appeared concerning variation in the sequences for the 56-kDa antigen gene of Australian isolates. This clearly prevents our ability to assess the degree to which Australia may represent a unique assemblage of *Orientia* strains.

Melanesia, Polynesia, and New Guinea. Scrub typhus was reported during World War II in the Solomon Islands and what is now the Republic of Vanuatu (northern New Hebrides), ~2000 km east and northeast of the Cape York Peninsula of northeastern Australia, in the southeastern-most point of the area where scrub typhus is endemic (figure 1) [18]. Regional seroprevalence studies that used CF to test human serum samples collected in the early 1970s indicated a widespread exposure to the disease in the Solomon Islands (163 [49%] of 335 serum samples with titers $\geq$1:10) and Vanuatu (13 [18%] of 72 serum samples). In the same study, there was no indication of exposure to the east or southeast in the Fiji islands (52 serum samples) or Western Samoa (25 serum samples), nor to the south in New Zealand (58 serum samples). DFA characterization of 7 rodent and 4 *Leptotrombidium akamushi* mite pool–derived *Orientia* isolates from Ndende, Solomon Islands, suggested the predominance of Karp and what the authors called "Karp-like" strains (TA686, TA716, and TA763) [17]. This designation is not equivalent to the "Karp-like" designation used more widely to describe molecular variation for the 56-kDa antigen gene sequences, the convention that we have followed in our analysis of phylogenetic relationships between *Orientia* isolates for which DNA sequences are available (figure 5). The DFA conjugate TA716 was seroreactive with all isolates. There have been no genomic investigations reported that used isolates collected from this region.

Scrub typhus played an important role during World War II in what was then called Dutch New Guinea (Papua) and the proximal islands of the archipelago, including New Britain, Goodenough, and the Schouten Islands, with reported mortality rates of up to 28% [34, 74]. There were several outbreaks among Allied troops near Port Moresby and beyond [11, 221–223]. The highly virulent *O. tsutsugamushi* prototype Karp strain was isolated from the blood of a febrile soldier who had been deployed there [40, 41]. *L. deliense* is the primary vector in the area. One of the earliest accounts detailing the dramatic variation in strain virulence was described among patients exposed in New Guinea [34]. During the last 8 months of 1944, 173 cases were reported at a single hospital. Military personnel from 2 distinct, undisclosed locations who were seen at the hospital were diagnosed on the basis of classic signs and symptoms, including fever, eschar, headache, lymphadenitis, and increases in Weil-Felix OXK titers. Although there were no fatalities among the 135 patients presenting at the military treatment facility from a forward battle area, during the same period on a separate base there were 38 patients who presented and 6 fatalities that occurred. All 6 deaths involved patients who were among the 16 patients from a single unit who received a diagnosis during a 3-week period, yielding a case-fatality rate of 37.5%. The distances between the deployed units were not described, probably because of wartime security concerns. More recently, Kende and Graves [224], using IFA, reported a seroprevalence of 6.5% (6 of 93 serum samples) among healthy volunteers from the Port Moresby area in Papua New Guinea, whereas no seropositive patients were seen among 98 rural volunteers. Molecular information concerning various laboratory stocks derived from the original Karp isolate constitutes our only DNA information about New Guinea (table 3).

Philippines. Although suspected in the Philippines to be an endemic disease as early as 1908, clinical confirmation of scrub typhus did not occur until World War II, when it was reported as a cause of fever and deaths among Japanese and Allied military personnel throughout the archipelago [10, 223, 225]. One isolate collected in that era from a human, the Volner strain, was isolated from the blood of a soldier deployed to the island of Samar in the Guinan region [10]. That isolate was subsequently used to prepare a lyophilized rat lung-spleen vaccine, the first US scrub typhus vaccine ever tested in a field trial (table 2). However, it proved to be unsuccessful [37, 40, 72]. Unlike during World War II, during the Vietnam conflict scrub typhus was of incidental concern in training areas [32, 226]. In 1969–1970, however, there were 6 cases reported among US Air Force personnel training at Clark Air Force Base, located on Luzon, the Philippines [226]. *L. deliense* appears to be the putative vector in the Philippines [10].

Overall, isolation and antigenic characterization of *O. tsutsugamushi* in the Philippines has been limited. Using Gilliam, Karp, and Seerangayee CF antigens, Bengston [36] observed that serum samples from 23 case patients collected in 1945 were either monospecific for Gilliam (22%) or Karp (9%) or were equally seroreactive to all 3 strains (69%) (table 3). Infections occurred on several islands in the archipelago but pri-

marily on Luzon. Kitaoka [227] used the CF test to detect antibodies in feral monkeys, primarily antibodies against the Gilliam strain. Using DFA, Shirai et al. [121] found no prominent single strain but, instead, found a predominance of heterogeneous mixtures of strains (Karp, TA716, TA763, and TA686) in testing 42 isolates from rodents against 8 prototype strains. TA716 was reported to have the greatest frequency (86%), and none of the isolates was reactive to the Gilliam strain. Using the IFA test, Cross and Basaca-Sevilla [228] observed antibodies to Karp, Gilliam, and Kato strains in volunteer populations on Cebu (959 serum samples [8%]), Palawan (2007 [7%]), Capiz (1366 [16%]), and Sorsogon (1744 [6%]).

To the southeast of the Philippines, in western Micronesia, the first diagnosed cases of scrub typhus, occurring from October 2001 through October 2003 in 15 residents of the Republic of Palau, were reported [229]. Seroreactivity of 47.6% was subsequently reported for 212 Palau residents, and a retrospective study that used serum samples collected in the region in 1995 reported seroreactivity of 5.4% [230]. The antigenic diversity of these strains was not reported. Unfortunately, molecular studies are lacking for both the Philippines and Micronesia.

People's Republic of China, Pescadores islands (Republic of China), and Penghu island group. From as early 313 A.D. and throughout China's history, there have been numerous references to "chigger fever" and associated vector mites [5]. Scrub typhus has been reported in several provinces, including the coastal provinces of Zhejiang, Fujian, Guangdong, Guangxi, Jiangsu; Shandong Province, north of the Yangtze River, where scrub typhus was reported more recently; and the inland provinces of Hunan, Sichuan, and Yunnan [169, 6]. The tropical Hainan Island, proximal to Guangdong Province, has reported thousands of cases during the past 50 years. Pingtan Island (People's Republic of China) [204, 211] and the Pescadores islands (Republic of China), situated between Taiwan and Fujian Province (People's Republic of China) are both hyperendemic foci of scrub typhus. Bourgeois et al. [231] reported an outbreak of scrub typhus among Chinese military personnel in the Pescadores from May through November 1975. A serologically confirmed outbreak among 21 Chinese military personnel was reported in the eastern part of the main island of Taiwan in 1970 [232]. *Orientia* isolates were obtained from mites and rodents collected in the area where the soldiers were working. *L. deliense* has been identified as the primary vector in China, because the patient distribution generally corresponds to the distribution of this vector [5]. *Leptotrombidium gaohuensis* is the putative vector in Zhejiang Province, and *Leptotrombidium scutellare* in the Yimong Mountains of Shandong Province, the site of the newly recognized but milder "autumn-winter" disease [169, 6]. Seasonality generally varies with the diverse ecology of the provinces, but typically, cases first appear in May, with numbers peaking in June through July and diminishing in August. In the Pescadores, disease incidence increases from April through November, peaking in June and early July [233], but infecting strains are purported to be of low virulence [234]. On Hainan Island, cases occur throughout the year [5].

Serological characterization of 126 isolates from humans, rodents, and chiggers collected throughout Fujian Province showed at least 4 monospecific antigenic types: Karp (29 isolates), Gilliam (12 isolates), TA716 (1 isolate), and C-41 (10 isolates), an isolate not previously described, as well as cross-reactive combinations of these types (74 isolates) [5, 211]. There was some regional clustering of Gilliam on Pingtan Island (11 [21%] of 52 isolates); Karp in the Fujian Province districts of Guangze (3 [100%] of 3 isolates), Longxi (24 [41%] of 58 isolates), and Jinjiang (1 [13%] of 8 isolates); and TA716 in the Longyan district (1 [100%] of 1 isolate) [211].

In a 1986 DFA analysis of 5 isolates collected from rodents (2 isolates) and *L. deliense* mites (3 isolates) in Fujian and other provinces in 1957–1963 and typed as Gilliam (1 isolate from Zhejiang Province) and TA716 (3 isolates from Fujian Province and 1 isolate from Guangdong) [80]. Overall, 80% were type TA716. Liu et al. [212] typed 23 isolates from humans, rodents, and mites in Shandong Province by using IFA and RFLP analysis. According to serotype analysis, 21 isolates were Gilliam and 2 were Karp. In contrast, by PCR with primers derived from the 56-kDa protein gene, followed by RFLP analysis of the products from the same isolates, 21 isolates were found to be Kawasaki; the remainder were found to be Karp. A subsequent study used DNA extracted from eschars and blood clot samples from 7 seropositive patients with scrub typhus, also from Shandong Province. In an analysis of partial 56-kDa protein gene sequence products, all 7 samples had genotype results indicating that they were related to the Kawasaki prototype strain [169]. These observations point out the problems that exist concerning the relationships between the data collected by different methods and how they correspond to underlying differences between the standard prototype strains that may need to be considered in vaccine development. Other studies that used molecular methods to characterize *Orientia* isolates from humans, rodents, and chiggers in China include studies by Chen et al. [235] in Shangxi Province and by Yang et al. [213, 236] in Shangdong Province. Yang et al. [213] suggest that the Shangdong area shows unusual homogeneity of *Orientia* strains, with almost all being identified as similar to strains in the **Kawasaki** clade. Zhang et al. [210, 237] reported findings from areas of Inner Mongolia and Yunnan provinces. Of 90 rodents trapped in grasslands of Inner Mongolia, 6 tested positive for *Orientia* species (isolation rate, 7%); 3 of the 6 were most similar in genotype to the **Karp** group. By our analysis, 2 of the remaining 3 isolates can be classified as closest

in genotype to the **JG-v** group, and the remaining isolate was closest to the **Kawasaki** clade. The single *Orientia* isolate that was found in samples collected from 20 rodents trapped in Xinjiang Province was typed as being similar to the Karp strain.

Although there were too few isolates from these studies to establish any regional genotypic prevalence data for these 4 provinces, these sequences are predominantly **KARP-related**, with some sequences in the **Kawasaki** and **JG** groups. These results are in line with RFLP data from the same regions and demonstrate the endemicity of *O. tsutsugamushi* in these provinces.

With use of the IFA test and separate Karp, Gilliam, and Kato antigens to characterize 19 seropositive scrub typhus cases from Pescadores, most (68%) of the serum samples from patients were multiantigenically reactive to a combination of either 2 or 3 of the prototypic strains tested; in all these instances, the combination included Karp antigen. In addition, 21% reacted to Karp alone, 5% to Gilliam, and 5% to Kato [231]. In a later DFA characterization of 49 *Orientia* strains isolated in Pescadores from humans (17 isolates), rodents (15), and chigger pools (17), all strains were reactive to TA716 antisera [121]. Almost 47% were typed as being related to TA716 alone, and none reacted to TA678, Kato, or Gilliam reagents. When tested against 8 strains, 53% were multiantigenically reactive. Using PCR and RFLP characterization of 39 isolates from humans in Pescadores, Dasch et al. [182] identified 3 *groEL* types. One unique type proved to be similar to an isolate from the Philippines; 1 set of 8 isolates was grouped with 3 recently found isolates from Thailand but comprised 3 types of 56-kDa proteins that differed from those of the Thai isolates. A group of 30 isolates resembled the Thai prototype strain TH1817 but presented patterns of 2 different 56-kDa protein types [121, 176]. RFLP results and sequences have been reported for 7 isolates from rodents in the Nan Peng Lie islands (3 [43%] were similar to the Karp prototype sequence, 2 [29%] to Kato, and 2 [29%] to Yonchon) [7]. These researchers also tested ground-collected mites (98.5% *L. deliense*) gathered on islands of the Xisa archipelago, Hainan Province, in southern China [8]. RFLP and sequence analysis of *Orientia* DNA derived from mite pools reflected patterns and sequences most closely related to Karp strain (85% of isolates), although the overall prevalence was not determined.

Using IFA monoclonal antibodies and RFLP analysis of the 56-kDa antigen gene for 10 isolates from rodents and *L. deliense* collected in Taiwan, Pescadores, and the nearby Lan-yu island group, Tamura et al. [136] showed that, although related to the Karp and Gilliam prototypes, every isolate was phenotypically and genotypically distinct from previously characterized strains. These studies were extended by Qiang et al. [196], who analyzed sequences of 56-kDa antigen genes in 14 isolates from rodents (12) and mites (2) in Taiwan and found >99% homology among 9 isolates but <96% homology among the remaining 5 isolates, suggesting great diversity in the strains from Taiwan, compared with published strains from other countries. Furthermore, a considerable number of isolates from Taiwan have been deposited in the gene databases but have not appeared in any publications. These sequences make a substantial contribution to our knowledge of strain variability in the Republic of China. In general, examination of the sequences collected from patients, mites, and mammalian hosts in Taiwan suggests that the Taiwanese strains are very diverse. In figure 5, they are found in most of the identified groups, including **KARP-related**, **Saitama**, **TA763**, **JG**, **JG-v**, and **KATO-related**.

A few studies were performed in the Primorye region of the Siberian Maritime Territory (Primorski Krai, Russia), proximal to eastern China. A seroprevalence rate of ~4% was reported by Kulagin et al. [27], who used CF to test 1838 human serum samples collected in 4 districts within the region. *Leptotrombidium pavlovskyi* was reported to be the primary vector in the area. In another study involving Primorski Krai, Tarasevich et al. [26] used CF, cross-neutralization, and IFA analyses to type 1 isolate from a human, 1 from a rodent, and 1 derived from a chigger pool consisting primarily of *L. pavlovskyi.* The 3 isolates were typed as Gilliam by all 3 serological tests. No genetic studies have been reported for isolates from this region.

Japan. Since the typhus epidemics ended after World War II, tsutsugamushi disease has become the most common rickettsiosis in Japan [31, 32]. Typically, outbreaks are associated with the seasonal activity of the vector mites, primarily *L. akamushi, L. scutellare,* and *Leptotrombidium pallidum* [15]. Two forms of the disease have been reported. The recently diminishing but virulent classic summer form, associated primarily with the vector *L. akamushi,* is confined primarily to along the rivers of Niigata, Yamagata, and Akita prefectures in northwestern Honshu [1, 31, 88, 238]. Before the antibiotic era, case-fatality rates for this form of the disease were reported to be as high as 100% for some years, decreasing to ~1% between 1950 and 1980 [239]. A less virulent form, with a steadily increasing incidence since it was first described in 1948, appears in early autumn and again in the spring, is transmitted by *L. pallidum,* and occurs in Chiba, Kagawa, Saitama, and Shizuoka prefectures [1, 32, 54, 240]. Shizuoka Prefecture includes the military training area in the foothills near Mount Fuji, where hundreds of cases have been reported [15, 32, 135, 241]. *L. scutellare* has also been implicated as a vector of this form of the disease [1, 16]. Scrub typhus was also identified in Miyazaki and Kagoshima prefectures, on the southwestern island of Kyushu [49, 53, 242]. In Oita Prefecture, studies that used CF established seroprevalence to *O. tsutsugamushi* among serum samples from humans and rodents [243], and nested PCR demonstrated the presence of *Orientia* organisms in multiple vector mites [244]. The first isolate-positive human case of scrub ty-

phus in Ehime Prefecture, Shikoku Island, was reported in December 1987 [245]. The strain, designated Yamazaki, was serotyped using monoclonal antibodies and found to be a Karp strain.

Using CF and reactions of patient serum samples to Karp and Gilliam strains, Philip [238] demonstrated antigenic heterogeneity in serum samples from 3 family members in Niigata in convalescence from the disease. Shishido [88] used CF to characterize 1 isolate from a human and 12 isolates from rodents in prefectures throughout Honshu Island, as well as Hokkaido. The 3 isolates from rodents in Hokkaido were serotyped as Gilliam, whereas the remaining 10 isolates (77%; 9 from rodents and 1 from a human) were serotyped as Karp. The author also suggested that an unspecified number of isolates from Niigata Prefecture were seroreactive to the Kato strain. Also using CF, Kitaoka et al. [15] serotyped 162 isolates from humans (3 isolates), mites (89), and rodents (70) collected from 1964 through 1966 in Akita, Niigata, Kanagawa, Shizuoka, Oita, and Hokkaido prefectures. The overall results for strain prevalences were 53% Karp, 31% Gilliam, 11% Kato, 2% mixed antigenicity, and 3% untypeable.

In 1980, a new, human-derived strain from Niigata Prefecture, named Shimokoshi, was typed using CF and IFA and found to be distinct from the classic prototypes [52]. Murata et al. [132] developed a panel of monoclonal antibodies highly specific to Karp, Gilliam, or Kato prototype strains. By IFA or indirect immunoperoxidase testing, this panel was used to serotype isolates from humans (15 isolates), rodents (5), and mites (2) that were recovered primarily from central and north-central Honshu. Of the 12 *Orientia* isolates recovered from patients in Niigata Prefecture, 7 (58%) were seroreactive to Karp-specific monoclonal antibodies, and 5 reacted to Gilliam-specific monoclonal antibodies. Akita (1 isolate; Gilliam), Kanagawa (1 isolate; Karp), and Kumamato (1 isolate, Karp) prefectures accounted for 3 of the isolates from humans. The 5 isolates from rodents were obtained in Niigata (1 isolate; Karp), Saitama (1 isolate; Karp), Toyama (1 isolate; Karp), and Fukushima (2 isolates; Gilliam), and the 2 isolates from *L. pallidum* were obtained in Niigata and Saitama (both Karp). All isolates were clearly typed as Karp (54%) or Gilliam (36%). A new type of strain was characterized by PCR sequence analysis in Saitama Prefecture, immediately northwest of Tokyo on Honshu, where human scrub typhus was first reported in 1984 [54]. This is the strain type for the group now referred to as **Saitama**.

Using CF, Misao et al. [242] reported strains in Miyazaki Prefecture that were clearly distinct from the classic prototypes. In addition to multiple Karp, Kato-like, and Gilliam-like isolates [246, 247], several antigenically distinct strains from Miyazaki Prefecture have been isolated and variously typed by DFA, IFA, indirect immunoperoxidase assay, monoclonal antibody testing, and PAGE as Irie, Hirano, Kawasaki, and Kuroki strains [49, 51, 53, 133]. In characterizing the Kawasaki strain from Miyazaki Prefecture as a distinct prototype with the use of monoclonal antibodies, Yamamoto et al. [53] found that 7 of 9 isolates from humans were Karp or Karp-like. They later identified another distinct strain, Kuroki, by SDS-PAGE and analysis of serological reactivity to monoclonal antibodies [50]. In typing multiple isolates from humans, they reported that strains were classified according to primary reactivity as Kawasaki, with very little cross-reactivity with Karp, Gilliam, and Kato antigenic types [50]. Using monoclonal antibodies and IFA, Tange et al. [133] showed that 9 of 9 isolates from humans in Miyazaki Prefecture were Irie or Hirano types. In an intensive investigation in the Gotemba-Oyama district, Shizuoka Prefecture, Kawamori et al. [135] serotyped 59 *Orientia* strains isolated by mouse inoculation. The strains from humans (24), rodents (30), and larval mites (2 from *L. scutellare,* Kawasaki-type; 3 from *L. pallidum,* Karp-type) were collected from 1985 through 1990 from the eastern slope of the Mount Fuji area and were typed using monoclonal antibodies and IFA as Karp (60%), Kawasaki (34%), and Kuroki (7%). In monoclonal antibody serotyping of *Orientia* isolates derived from infected patients in Chiba Prefecture, Kaiho et al. [248] reported that the majority of isolates were related to the Kawasaki strain. Most of the cases were reported in the months of November and December. Using PCR and RFLP, Pham et al. [244] observed primarily Kuroki strains in mite pools collected in neighboring Oita Prefecture, Kyushu. The index case of scrub typhus in neighboring Gifu Prefecture was not identified until 1982 [83]. Of 4 patient isolates that showed dramatic variability in murine virulence, 1 was typed as Kawasaki and 1 as Kuroki by use of IFA with monoclonal antibodies [83, 249]. The most extensive use of molecular methods to characterize strains has been in Japan [54, 165, 166, 176, 185, 186, 190, 194, 195, 250–252]. All methods (PCR primer-specific typing, RFLP, and sequencing) have been used in various studies. Most sequence groups are represented within Japan, with the exception of **Gilliam**, **TA763**, and possibly **JG-v**. Conversely, none of the groups identified in the extensive molecular studies in Japan appear to be restricted to Japan, being shared at least with areas of Korea and China, if not more expansively for the range of *Orientia* isolates.

Korea. Index cases of scrub typhus in Korea were reported among British United Nations troops in 1950 and 1951 during the Korean conflict [19, 32, 24, 253], with initial isolations reported by Fuller and Smadel [254] in 1954. By use of the now-suspect Weil-Felix OXK test, a relatively nonspecific test that detects primarily IgM antibodies [126], antibodies were reportedly found in otherwise healthy Koreans in 1964 [19]. Since the disease was first diagnosed among civilians in 1985 in Chinhae Province, the disease has been reported throughout

Korea as one of the most common febrile illnesses [19, 255–257]. Widespread prevalence is indicated, because almost 35% of almost 10,000 serum samples collected from febrile patients throughout South Korea from 1987 through 1988 tested serologically positive; the incidence has been increasing ever since, with case-fatality rates of up to 37% reported in some localities [19, 178, 255]. Although case reports occur from May through July, up to 90% of reported cases occur from October through November [25, 255]. *L. pallidum* is the putative primary vector in Korea [1, 24, 25]; however, *L. scutellare* mites were found to infest rodents in regions where patients who received a diagnosis had been exposed [137, 257].

Serological antigen characterization, performed using monoclonal antibodies, of mouse-propagated *Orientia* isolates from humans identified the then-new type, the Boryong strain, as the most common (88 [78%] of 113 strains), followed by Karp (14%) and Gilliam (11%) [55, 57, 255]. Both monoclonal antibody serological characterization and phylogenetic tree analysis suggest that the Boryong strain is closely related to the Japanese Kuroki strain [19, 137]. Using nested PCR with primers derived from the 56-kDa antigen gene, Ree et al. [178] characterized isolates from rodents (249 isolates) and mite pools (41 isolates) collected throughout South Korea each autumn from 1997 to 1999. In results similar to those from the study of isolates from humans [55], almost 79% were typed as Boryong and 15% as Karp, most of which were collected from central to north-central South Korea. Altogether, these data suggest that the Boryong strain is predominant in South Korea.

Malaysia. Scrub typhus was first recognized in Malaya (now Malaysia) in 1915 as a disease similar to the Kedani river fever, well known in Japan as mite-borne typhus or tsutsugamushi disease. It was later described in the 1920s among rural plantation workers, including a fatal case [3, 258], and it continues to be a problem [259]. It occurs throughout the year in most states of peninsular (West) Malaysia and in Sabah and Sarawak in East Malaysia (Borneo), with an estimated 500,000 cases annually throughout the country [3]. *L. deliense* is the primary vector in Malaysia [29]; however, infected *Leptotrombidium arenicola* organisms are also present, and infected *Leptotrombidium fletcheri* chiggers have been found at elevations up to 2100 m [1].

Shirai et al. [46] used DFA reagents developed against 8 strains to characterize 114 *Orientia* isolates recovered from patients seen at 3 hospitals in central peninsular Malaysia. Karp and Karp-like strains (TA763 [72%], TA716 [70%], Karp [56%], and TA686 [30%]) were the most frequently reported, making up 86% of isolates. Reactivity to the Gilliam (18%) and Kato (3%) prototype strains was less frequent, and 22% of all isolates were monospecific. Parallel results were reported when field-caught, black-plate–collected *Leptotrombidium* mites from multiple sites within 4 Malaysian states were tested [20]. Almost 4% of mites collected were infected, and 93% of those showed reactivity to Karp strain, 64% to Karp alone, and 96% to the "Karp-related" strains (see "Immunofluorescence and immunoperoxidase assays" section above). There are 2 well-characterized Malaysian mite colonies, *L. arenicola* and *L. fletcheri,* each derived from single, naturally infected mites collected in the 1960s [23, 28]. Typed by DFA, the *L. arenicola* colony contained TA716, TA763, TA686, Karp, and Kato, whereas the *L. fletcheri* colony also contained the Gilliam strain. Although the colonies are somewhat artificial because of years of multiple-passage under laboratory conditions, they represent a snapshot of infected mites in the region. Results of these studies, as well as antigenic characterization of isolates from rodents by DFA [87, 120] or monkey serum samples by IFA [260] from peninsular Malaysia, suggest that Karp and "Karp-related" strains (TA716, TA763, and TA686) predominate in the region.

Thailand. The index human scrub typhus case was reported in 1952, with the first isolation of agents in 1954 [12, 261]. Although there are several proven infected species, the principal vector is *L. deliense* [12, 13], and *Leptotrombidium chiangraiensis* has been recently described as a vector [21].

The climate of Thailand is primarily tropical, and phenotype variation among geographically and ecologically diverse *Orientia* isolates is considerable. Using CF, Elisberg et al. [101] characterized 77 *Orientia* isolates from humans, mites (*L. deliense*), and animals collected in 1962 and 1963 from 4 distinct regions in Thailand. Although unable to show any distinct geographic or ecological relationship among the isolates, they found monospecific reactivity to Karp (43%), Kato (16%), Gilliam (10%), and a mixture of antigenic combinations (25%) but also found 5 weakly reactive or nonreactive strains. These antigenically distinct strains, TA678, TA686, TA716 (Chon strain), TA763 (Fan strain), and TH1817, were also characterized by IFA and were identified as antigenically unique [45]. DFA characterization of 146 isolates from later mite collections (1977–1978) at 6 sites throughout Thailand supported the earlier findings, in that the Karp strain appears to be predominant (60%), as well as "Karp-related" strains TA716 (29%), TA763 (28%), and TA686 (9%) [12]. RFLP and gene characterization of the *groEL* gene for >111 isolates detected 35 different banding patterns, and 31 of these patterns were region specific [183]. Furthermore, Karp-like *groEL* genotypes seemed to be missing for the Thai isolates that were studied [183].

More recently, Manosroi et al. [199] used a nested PCR based on the 56-kDa protein gene and DNA sequencing of a 487–base pair fragment to type patient isolates. DNA was extracted from whole blood samples that were collected from 240 seropositive patients with scrub typhus who presented at 8 medical centers throughout Thailand. The Karp sequence was reported to be the primary sequence in Thailand, accounting for

97% of positive samples, although a low percentage of Kato-like sequences (3%) were reported in southern Thailand. The divergency of the Thai Karp isolates from the Karp type-strain was 3%.

Less-investigated regions of endemicity. As previously described, there are several countries where scrub typhus is proven or suspected to be endemic and areas where limited data have been or are only recently being gathered.

Indonesia is known to be a region where scrub typhus is endemic [262–265], primarily on the basis of the seropositivity for scrub typhus in human serum samples. However, nothing is known about the prevalence of serotypes or sequence types.

Pakistan lies at or near the western fringe of the area of endemicity where *L. deliense* appears to be the primary vector [1]. Using primarily CF and 3 prototype strains, Shirai and Wisseman [102] tested 79 *Orientia* isolates recovered from patients, rodents, and mites in multiple locations throughout Pakistan. Most (90%) were characterized as the Karp strain of variable murine virulence [102]. Although corroborating the high percentage of Karp strains, later DFA characterization of 11 of those isolates identified a similar percentage (82%) of strains characterized as reactive to the TA716 conjugate [80]. To the mountainous north, in neighboring Tadzhikistan, isolates recovered from rodents (4 isolates) and mites (4 isolates) were serotyped using CF and were found to be Gilliam alone [26, 27]. In nearby Uzbekistan, a study involving 108 patients with acute fever showed 6% IgM seropositivity to *Orientia* species with use of a commercial ELISA kit [266]. However, no specific strain characterization was performed.

In India, scrub typhus was recognized as a typhus-like fever in 1917 and in subsequent periodic outbreaks [267]. It is found throughout the country and is reported seasonally from August through October, with *L. deliense* being the primary vector [9]. During World War II, it was a major cause of fever among soldiers deployed along the Assam-India-Burma (Myanmar) border; cases were reported throughout the year in the region, but primarily from October through December, and the mortality rate was 5% [9, 267, 268]. The human Gilliam prototype strain was isolated in 1943 from a soldier in that region. CF antigenic characterization of serum samples collected in 1944 from 15 severe wartime cases typed the isolates as Gilliam (36%), Karp (7%), Seerangayee (7%), or mixed strains (50%) [36]. Mathai et al. [65] reported an outbreak in Tamil Nadu state in southern India that occurred from October 2001 through February 2002. Twenty-eight clinically and serologically confirmed cases and 3 deaths (11%) were recorded. Seventeen patients rapidly defervesced after receiving doxycycline therapy; however, 1 patient died even though that patient received appropriate treatment. It is unclear whether antibiotic resistance was involved in this case, and no isolation, antigenic, or genetic characterization is available from this outbreak. In a more recent outbreak, Bakshi et al. [200] compared real-time PCR with nested PCR by using 66 blood samples collected from febrile patients in Himaschal Pradesh states. Real-time PCR compared favorably with nested PCR; 42 of 48 samples that were reactive in nested PCR were reactive in real-time PCR. Sequence analysis of 10 selected isolates suggested that 8 were most similar to the Kuroki prototype. Although not an epidemiological investigation, the widely disparate origin of patient samples—northern versus southern India—supports the endemicity of scrub typhus in India. Clearly, it remains an ongoing disease risk there.

After not being reported since World War II, scrub typhus suddenly and dramatically reappeared in the Maldives, ~600 km southwest of India, during the summer of 2002 [269]. Investigations revealed 168 cases and 10 deaths, yielding a case-fatality rate of 6%. On Gadhdhoo Island alone, 57 cases and 3 deaths were reported. Although 2 cases were confirmed using PCR, there was no antigenic or genotypic description of the infecting strains. In Sri Lanka, 19 serologically confirmed cases were reported in a 12-month period beginning in November 2002, and one serologically confirmed scrub typhus case, acquired in 2004, was recently reported in a traveler to Sri Lanka [66, 270]. However, these cases were not further characterized antigenically.

DISCUSSION

This review highlights the dramatic degree of phenotypic and genotypic diversity demonstrated by *O. tsutsugamushi* isolates in the vast area of endemicity in the Asia-Pacific region. Recent reports of antibiotic breakthroughs or delayed treatment because of early misdiagnosis have raised concerns among medical professionals. The misdiagnoses often occur in nonindigenous populations or outside regions where scrub typhus is historically endemic, possibly because of increases in ecotourism in what are now relatively politically stable regions. This situation has led to increased interest by international agencies, as well as the US Department of Defense, in better point-of-care or hospital-based rapid diagnostics and effective vaccines. Even easily treatable outbreaks can be problematic in a military operational environment. For example, there have been frequent outbreaks among US Marines and among the Japanese Ground Self-Defence Force near Camp Fuji, Japan; among the Malaysian Army and Police Field Force in Malaysia; and among the Royal Thai Army, in northeast Thailand [32, 241]. As is historically typical of operational exposure in areas of endemicity, the appearance at the local battalion aid station or military treatment facility of large numbers of febrile war fighters with severe fever, headache, rash, and so forth would challenge even the best-prepared medical support [32]. To be effective, diagnostics and vaccines must detect or prevent infection with the most prevalent and/or virulent disease-causing strains.

The importance of antigenic variation to the vaccine effort was underscored early on by Smadel et al. [30, 271–273]. They showed that homologous strain immunity would persist at least 1–3 years. Although severity of the disease was somewhat diminished, immunity to challenge with a heterologous *Orientia* strain was short-lived, as short as 1 month. This suggests that a successful vaccine would have to be multipotent. Even when a mouse model was used, inactivated vaccines or antisera prepared against 1 strain failed to protect against infection with other strains [47]. Likewise, silver leaf monkeys showed no resistance to repeat infection with either homologous or heterologous strains at 14 months [274]. Other factors besides short-lived resistance to heterologous repeat infection are also of concern. Whereas a strain with low human virulence might not be considered for incorporation into a vaccine, because of the reduced risk for those infected, a strain with high virulence and thus a greater risk might be considered even if it had a lower prevalence. For example, the experience in Japan with strains of classic tsutsugamushi disease acquired by farmers working along the rivers of Niigata and neighboring prefectures that proved to be often lethal contrasts dramatically with the experience with the much milder autumn-winter form in the Shizuoka region. In addition to virulence in humans or nonhuman primates, murine virulence is important. Although correlation of human virulence and murine virulence is problematic, the mouse model is still commonly used for calculation of vaccine protection indices, as well as for primary isolation of the organisms from human blood, tissue, and other specimens. Numerous studies have shown that mouse virulence is strain dependent. Indeed, most isolates have been obtained using outbred, selected inbred, or immunosuppressed mice, which, although this method is sensitive, raises the question of the endemicity of *Orientia* strains that are pathogenic to humans but avirulent in mice and thus easily missed [30, 52]. In fact, using nested PCR, Takahashi et al. [275] showed that chigger genotypes did not necessarily correlate to inoculated chigger extract–derived murine isolates.

In the past, antigenic characterization of isolated *Orientia* strains has been performed primarily using CF, a panel of DFA reagents, or IFA testing involving proven monoclonal antibodies directed against existing or generally accepted strains and prototypes, as described above. These reagents have permitted the antigenic classification of isolates and often have identified strains. These reagents are often monospecific, permitting the clear serotyping of most isolates. However, the fact is that many isolates from throughout the area of endemicity are reactive to several conjugates. However useful, these methods can produce confusing results and have not permitted investigators to determine whether cross-reactivity—that is, reactivity to multiple conjugates—is the result of a mixture of antigenic types in the isolation or the result of a single strain displaying variable epitopes. Either of these scenarios is possible when some sort of plaque purification, a slow, labor-intensive task, has not been performed. Moreover, the Karp and so-called Karp-related strains predominate in many studies. Of course, PCR in conjunction with sequence analysis of the products can now address these questions.

Genotyping by PCR and sequence analysis permits a rapid comparison with all previously published sequences of multiple strains through the use of GenBank. Proteonomic comparisons can also be performed. Far fewer exhaustively prepared specific reagents are needed, and the need for BSL-3 facilities, required for most propagative work with live *Orientia* organisms, is obviated. Still, the correlation between serological antigen typing has not been entirely sorted out.

Although we have reviewed several point-prevalence studies, clearly the prevalence data from the vast region of endemicity are sporadic and require far greater refinement through the collection and genotypic testing of samples with use of up-to-date genotyping methods. Nevertheless, some interesting observations can be made here. For example, whereas Karp, Karp-like, and Gilliam-like strains appear to predominate throughout the region of endemicity, many individual studies point to the finding that reactivity to the TA716 conjugate is also common throughout the region (table 3). The percentage of reactivity exceeds 70% in at least 7 of the studies involving isolates from humans, rodents, and mites in multiple regions, including 70% in Malaysia [46], 80% in China [80], 86% in the Philippines [121], 94% in North Queensland, [122], and up to 100% in the Solomon and Pescadores islands [17, 121]. These results are basically consistent with those of genetic studies that have been done in these regions (figure 5). This suggests that the TA716 strain, in combination with the also-prevalent Karp and Karp-related strains, could be incorporated into any multivalent vaccine that may be produced. At any rate, further intensive epidemiological investigation is necessary if we are to ascertain the antigenic variance and strain prevalences of this serious disease.

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