6.4 nmol/L and that showed an H243Y mutant type codon. The role of such a polymorphism appears unclear. We found no association between the 3-bp indel in G7 and in vitro dhART susceptibility because mutants were regularly distributed in highly susceptible isolates and in isolates having a diminished susceptibility.

For our samples obtained during 2004–2006, the geometric mean IC_{50} value for dhART was very close to values found in Cameroon during 1997–1998 (mean dhART IC_{50} = 1.11 nmol/L) (*9*), in Senegal in 2001 (mean artemether IC_{50} = 1.3 nmol/L) (*5*), and in Republic of Congo during 2005–2006 (mean dhART IC_{50} = 1.02 nmol/L) (*10*). Ringwald et al. observed a narrower range of IC_{50}s, but their series included only 65 samples (*9*). Previous comparisons between ACs suggested that dhART is 1.7 times more potent than artemether against *P. falciparum* (*9*). Thus, the highest IC_{50} value for artemether observed by Jambou et al. in Senegal (44.7 nmol/L) (*5*) is comparable to the highest IC_{50} value for dhART in our series (31.8 nmol/L). The resistance levels of ACs are still undefined. For artemether, Jambou et al. used a threshold of 30 nmol/L to evaluate the association between the S769N mutation and in vitro susceptibility. The presence of *ATPase6* S769N was not associated with diminished in vitro susceptibility in our series. Conversely, the only S769N mutant that we observed was found in a fully susceptible isolate. Thus, we confirmed that polymorphism exists in this gene in positions 769 and 243, but we did not prove an association between these point mutations and resistance to ACs. Similarly, our results did not support the hypothesis of an association between the 3-bp indel in G7 and resistance to ACs.

ACs, considered the most important class of antimalarial drugs, merit close surveillance for susceptibility. Continued monitoring of the efficacy of their associated partner drugs also appears to be essential.

Acknowledgments

We thank the regular correspondents of the Centre National de Référence du Paludisme. Financial support was provided by the French Ministry of Health (Direction Générale de la Santé). SC received a thesis fellowship from the French Research Ministry.

Sandrine Cojean,*†
Véronique Hubert,*
Jacques Le Bras,*†‡
and Rémy Durand*‡§

*Hôpital Bichat Claude Bernard, Assistance Publique–Hôpitaux de Paris, Paris, France; †Université Paris 5, Paris, France; ‡Hôpital Avicenne, Bobigny, France; and §Université Paris 13, Bobigny, France

References

1. Attaran A, Barnes KI, Curtis C, D'Alessandro U, Fanello C, Galinski MR, et al. WHO, the Global Fund, and medical malpractice in malaria treatment. Lancet. 2004;363:237–40.
2. Mutabingwa TK. Artemisinin-based combination therapies (ACTs): best hope for malaria treatment but inaccessible to the needy! Acta Trop. 2005;95:305–15.
3. Ittarat W, Pickard AL, Rattanasinganchan P, Wilairatana P, Looareesuwan S, Emery K, et al. Recrudescence in artesunate-treated patients with falciparum malaria is dependent on parasite burden not on parasite factors. Am J Trop Med Hyg. 2003;68:147–52.
4. Grandesso F, Hagerman A, Kamara S, Lam E, Checchi F, Balkan S, et al. Low efficacy of the combination artesunate plus amodiaquine for uncomplicated falciparum malaria among children under 5 in Kailahun, Sierra Leone. Trop Med Int Health. 2006;11:1017–21.
5. Jambou R, Legrand E, Niang M, Khim N, Lim P, Volney B, et al. Resistance of *Plasmodium falciparum* field isolates to in-vitro artemether and point mutations of the SERCA-type *PfATPase6*. Lancet. 2005;366:1960–3.
6. Anderson TJC, Nair S, Qin H, Singlam S, Brockman A, Paiphun L, et al. Are transporter genes other than the chloroquine resistance locus (*pfcrt*) and multidrug resistance gene (*pfmdr*) associated with antimalarial drug resistance? Antimicrob Agents Chemother. 2005;49:2180–8.
7. Le Bras J, Andrieu B, Hatin I, Savel J, Coulaud JP. *Plasmodium falciparum:* interpretation of the semi-microtest of in vitro chemosensitivity by H3-hypoxanthine incorporation. Pathol Biol. 1984;32:463–6.
8. Uhlemann AC, Cameron A, Eckstein-Ludwig U, Fischbarg J, Iserovich P, Zuniga FA, et al. A single amino acid residue can determine the sensitivity of SERCAs to artemisinins. Nat Struct Mol Biol. 2005;12:628–9.
9. Ringwald P, Bickii J, Basco LK. In vitro activity of dihydroartemisinin against clinical isolates of *Plasmodium falciparum* in Yaounde, Cameroon. Am J Trop Med Hyg. 1999;61:187–92.
10. Pradines B, Hovette P, Fusai T, Atanda HL, Baret E, Cheval P, et al. Prevalence of in vitro resistance to eleven standard or new antimalarial drugs among *Plasmodium falciparum* isolates from Pointe-Noire, Republic of the Congo. J Clin Microbiol. 2006;44:2404–8.

Address for correspondence: Rémy Durand, Hôpital Avicenne, Laboratoire de Parasitologie Mycologie, 125 rue de Stalingrad, 93009 Bobigny CEDEX, France; email: remy.durand@avc.aphp.fr

Real-time PCR for *Francisella tularensis* Types A and B

To the Editor: *Francisella tularensis*, the etiologic agent of tularemia, is highly infectious and considered a potential bioweapon (*1–3*). Although 4 subspecies of *F. tularensis* are recognized, most cases of tularemia are due to infection by subsp. *tularensis* (type A) or *holarctica* (type B). North America is the only region where both type A and type B cause human disease. Subspecies *novicida* is also found in North America, but it is of reduced virulence. Disease incidence attributable to either type A or type B is

essentially unknown because the traditional method for classification of these subspecies is glycerol fermentation, which requires culture recovery (*4*). *F. tularensis* is fastidious and slow growing, with isolates recovered in a small percentage of cases.

We developed real-time TaqMan PCR assays for classification of *F. tularensis* type A and type B after *F. tularensis* is identified by culture or, in the absence of culture, by a PCR method such as the *F. tularensis* multitarget TaqMan assay (*5*). The type A TaqMan assay targets *pdpD*, which is present in type A, almost entirely absent from type B, and contains a 144-bp insert in *novicida* (*6*,*7*) (F: 5′-GAGACATCAATTAAAAGAAG-CAATACCTT-3′; R: 5′-CCAAGAG-TACTATTTCCGGTTGGT-3′; probe: 5′-AAAATTCTGC"T"CAGCA-GGATTTTGATTTGGTT-3′). The type B assay targets a junction between IS*Ftu2* and a flanking 3′ region (GenBank AY06) (F: 5′-CTTGTACTTTTATTTGGCTACT-GAGAAACT-3′; R: 5′- CTTGCTTG-GTTTGTAAATATAGTGGAA-3′; probe: 5′- ACCTAGTTCAACC"T"-CAAGACTTTTAGTAATGGGAAT-GTCA-3′). In type A and *novicida*, IS*Ftu2* is absent from this position (*8*). Oligonucleotides were designed with Primer Express version 2.0 (Applied Biosystems, Foster City, CA, USA). Probes were synthesized with a 5′ 6-carboxy-fluorescein reporter and an internal quencher (either BHQ1 [type A] or QSY-7 [type B]) at the nucleotide position indicated by the quotation marks.

Assays were optimized by using 1 ng of type A (strain SchuS4) or type B (strain LVS) DNA on the LightCycler 1.2 (Roche Applied Science, Indianapolis, IN, USA). Optimized concentrations (20 μL final volume) were 1× LightCycler Fast Start DNA Master Hybridization Probe mix (Roche), 750 nmol/L primers, 200 nmol/L probe, 5 mmol/L $MgCl_2$ and 0.5 U uracil-DNA glycosylase. PCR conditions were 50°C for 2 min, 95°C for 10 min, 45 cycles of 95°C for 10 s, and 65°C for 30 s, then 45°C for 5 min. Cycle threshold (C_t) values were calculated by using the second derivative maximum method with the y-axis at F1/F3 (LightCycler software version 3.5).

Sensitivity of each assay was assessed by using 10-fold serial dilutions (100,000 to 1 genomic equivalents [GE]) of SchuS4 or LVS DNA. Testing was performed in triplicate, with a reproducible detection limit of 10 GE for both assays. Specificity of each assay was tested with 1 ng of DNA from a panel of 62 *Francisella* isolates (online Appendix Table, available from http://www.cdc.gov/ncidod/EID/vol12no11/06-0629_appT.htm) and 22 non-*Francisella* isolates (*Acinetobacter, Bacillus, Brucella, Corynebacterium, Enterobacter, Enterococcus, Escherichia, Haemophilus, Klebsiella, Legionella, Proteus, Pseudomonas, Serratia, Staphylococcus, Streptococcus,* and *Yersinia* species). Isolates were grown, DNA purified, and quantified as previously described (*5*). Specificity was also evaluated with DNA (2 μL) extracted as previously described from *Francisella*-like tick endosymbionts of *Dermacentor variabilis* and *Francisella*-like soil bacteria (online Appendix) (*9*,*10*). The type A assay recognized all type A isolates with an average C_t value of 17.9 (n = 19). The type B assay detected all type B strains with an average C_t value of 17.1 (n = 21). Neither assay displayed cross-reactivity with *F. tularensis* subsp. *novicida* (n = 7), *F. philomiragia* (n = 15), *Francisella*-like tick endosymbionts (n = 3), *Francisella*-like soil bacteria (n = 7) (Appendix), or non-*Francisella* spp. (n = 22).

To evaluate the ability of the type A and type B TaqMan assays, in conjunction with the multitarget assay, to identify *F. tularensis* and classify subspecies in primary specimens, human, animal, and tick samples were tested (Table) available from DNA was extracted from 200 μL fluid, 25 mg liver, and 10 mg spleen or lung by using the QIAamp DNA MiniKit (Qiagen, Valencia, CA, USA) and 1 μL tested. Multitarget PCR conditions were as described (*5*).

The multitarget and subspecies-specific PCR assays accurately identified and classified *F. tularensis* in all specimens positive by standard diagnostic methods (Table). In addition, the type A and type B assays provided subspecies information for positive specimens in which an isolate was not recovered for glycerol fermentation testing (Table). All specimens negative by standard diagnostic methods tested negative by PCR. These preliminary results suggest that a *F. tularensis* PCR identification method, in combination with the type A and type B assays, provides the capability to identify *F. tularensis* and determine subspecies in the absence of culture.

We describe real-time PCR assays capable of classifying *F. tularensis* type A and type B and distinguishing these subspecies from the less virulent subsp. *novicida*. These assays are designed for use after *F. tularensis* has been identified by culture or by PCR. Supplemental use of these assays will allow laboratories to actively subtype *F. tularensis* isolates and primary specimens, thus providing subspecies information for a higher percentage of tularemia cases. Improved subspecies information will further understanding of the disease incidence and geographic distribution of *F. tularensis* type A and type B in North America.

Acknowledgments

We thank Francis Nano for sharing information regarding the *pdp*D gene; Cheryl Kuske and Susan Barns for sharing DNA from *Francisella*-like bacteria in soil; and Nikos Gurfield, Jean Creek, and Heidi Goethert for providing *Francisella*-like tick endosymbiont DNA samples.

Table. Comparison of standard diagnostic methods with the multitarget *Francisella tularensis* TaqMan assay and type A and type B assays using primary specimens

Specimen	Source	*F. tularensis* identified*	Subspecies identification†	Multitarget *F. tularensis* TaqMan assay‡			Type A assay‡ (C_t value)§	Type B assay‡ (C_t value)
				IS*Ftu2*	*iglC*	*tul4*		
Lymph node aspirate	Human	+		+	+	+	31	–
Bronchial wash	Human	+	A	+	+	+	34	–
Upper lung	Human	+	A	+	+	+	20	–
Lower lung	Human	+	A	+	+	+	26	–
Liver	Human	+	A	+	+	+	29	–
Spleen	Human	+	A	+	+	+	31	–
Pleural fluid	Human	+	B	+	+	+	–	36
Blood	Human	+		+	+	+	–	38
Spleen	Human	–		–	–	–	–	–
Liver	Human	–		–	–	–	–	–
Cerebrospinal fluid	Human	–		–	–	–	–	–
Blood	Human	–		–	–	–	–	–
Liver/spleen	Tamarin	+		+	+	+	28	–
Tissue	Tick¶	+	A	+	+	+	26	–
Tissue	Tick¶	+	A	+	+	+	33	–
Blood	Prairie dog	+	B	+	+	+	–	30
Blood	Prairie dog	+	B	+	+	+	–	27
Spleen	Prairie dog	+	B	+	+	+	–	21
Spleen	Prairie dog	+	B	+	+	+	–	31
Spleen	Prairie dog	–		–	–	–	–	–
Liver	Cat	–		–	–	–	–	–
Liver	Rat	–		–	–	–	–	–
Spleen	Rat	–		–	–	–	–	–
Spleen	Squirrel	–		–	–	–	–	–

**F. tularensis* infection identified by culture, direct fluorescent antibody testing, or serologic testing.
†Subspecies was determined by glycerol fermentation when an isolate was recovered.
‡+ = positive result, $17 \leq C_t \leq 38$; – = negative result, no fluorescence detected after 45 cycles of amplification.
§C_t, cycle threshold.
¶Tick species tested were *Haemaphysalis leporispalustris* and *Dermacentor andersoni.*

Kiersten J. Kugeler,*
Ryan Pappert,* Yan Zhou,*
and Jeannine M. Petersen*

*Centers for Disease Control and Prevention, Fort Collins, Colorado, USA

References

1. Ellis J, Oyston PC, Green M, Titball RW. Tularemia. Clin Microbiol Rev. 2002;15:631–46.
2. Dennis DT, Inglesby TV, Henderson DA, Bartlett JG, Ascher MS, Eitzen E, et al. Tularemia as a biological weapon: medical and public health management. JAMA. 2001;285:2763–73.
3. Sjostedt A. Family XVII. *Francisellaceae*, genus I. *Francisella*. In: Brenner DJ, editor. Bergey's manual of systematic bacteriology. New York: Springer-Verlag; 2005.
4. Olsufjev NG, Meshcheryakova IS. Infraspecific taxonomy of tularemia agent *Francisella tularensis* McCoy et Chapin. J Hyg Epidemiol Microbiol Immunol. 1982;26:291–9.
5. Versage JL, Severin DD, Chu MC, Petersen JM. Development of a multitarget real-time TaqMan PCR assay for enhanced detection of *Francisella tularensis* in complex specimens. J Clin Microbiol. 2003;41:5492–9.
6. Nano FE, Zhang N, Cowley SC, Klose KE, Cheung KK, Roberts MJ, et al. A *Francisella tularensis* pathogenicity island required for intramacrophage growth. J Bacteriol. 2004;186:6430–6.
7. Larsson P, Oyston PC, Chain P, Chu MC, Duffield M, Fuxelius HH, et al. The complete genome sequence of *Francisella tularensis*, the causative agent of tularemia. Nat Genet. 2005;37:153–9.
8. Petersen JM, Schriefer ME, Carter LG, Zhou Y, Sealy T, Bawiec D, et al. Laboratory analysis of tularemia in wild-trapped, commercially traded prairie dogs, Texas, 2002. Emerg Infect Dis. 2004;10:419–25.
9. Barns SM, Grow CC, Okinaka RT, Keim P, Kuske CR. Detection of diverse new *Francisella*-like bacteria in environmental samples. Appl Environ Microbiol. 2005;71:5494–500.
10. Kugeler KJ, Gurfield N, Creek JG, Mahoney KS, Versage JL, Petersen JM. Discrimination between *Francisella tularensis* and *Francisella*-like endosymbionts when screening ticks by PCR. Appl Environ Microbiol. 2005;71:7594–7.

Address for correspondence: Jeannine M. Petersen, Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, Foothills Campus, PO Box 2087, Fort Collins, CO 80522, USA; email: nzp0@cdc.gov