*Seek*TB, a Two-Stage Multiplex Real-Time-PCR-Based Method for Differentiation of the *Mycobacterium tuberculosis* Complex

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Tuberculosis (TB) in humans is caused by members of the *Mycobacterium tuberculosis* complex (MTC). The accurate identification of the MTC member causing human infection is important because the treatment of TB caused by some MTC members requires an alteration of the standard drug regimen, it can inform whether transmission is human to human or zoonotic, and it enables accurate epidemiology studies that help improve TB control. In this study, an internally controlled two-stage multiplex real-time PCR-based method, *Seek*TB, was developed for the accurate identification of all members of the MTC. The method was tested against a panel of well-characterized bacterial strains ($n = 180$) and determined to be 100% specific for members of the MTC. Additionally, 125 Mycobacteria Growth Indicator Tube (MGIT)-positive cultures were blindly tested by using *Seek*TB, and the results were compared to those of the GenoType MTBC and TBc ID tests. The *Seek*TB and GenoType MTBC results were 100% concordant, identifying 84 of these isolates as *M. tuberculosis* isolates and 41 as non-MTC isolates. Nine discordant results between the molecular methods and the TBc ID culture confirmation test were observed; however, nucleotide sequencing confirmed the results obtained with GenoType MTBC and *Seek*TB. *Seek*TB is the first-described internally controlled multiplex real-time PCR diagnostic method for the accurate identification of all eight members of the MTC. This method, designed for use on cultured patient samples, is specific, sensitive, and rapid, with a turnaround time to results of approximately 1.5 to 3.5 h, depending on which, if any, member of the MTC is present.

Tuberculosis (TB) remains a major health concern in both developed and developing countries due to the high rates of morbidity and mortality associated with this disease. In 2010, there were an estimated 8.5 to 9.2 million incident cases of TB globally, with an associated 1.5 million deaths, 23% of which were associated with HIV coinfection (28).

The *Mycobacterium tuberculosis* complex (MTC) comprises eight members (*M. tuberculosis*, *M. canettii*, *M. africanum*, *M. bovis*, *M. caprae*, *M. microti*, *M. pinnipedii*, and the attenuated *M. bovis* BCG vaccine strain), ~99% similar on a nucleotide sequence level, that are responsible for causing TB in humans (5).

While *M. tuberculosis* is responsible for the majority of cases of human TB, the accurate identification of other MTC members that cause infection is not routinely performed (21). As a result, the global frequency and distribution of each MTC member remain largely unknown, with some studies suggesting that TB caused by members of the MTC other than *M. tuberculosis* are more prevalent than previously reported (1, 26). *M. africanum*, for example, has been identified as the causative agent of up to 50% of human TB infections in West African countries (15), and *M. canettii* was responsible for ~10% of TB cases in a recent study performed in the Republic of Djibouti (17). Furthermore, *M. bovis* remains an important cause of zoonotic TB worldwide and should not be overlooked in a clinical setting (1, 12). In developing countries, where there is a paucity of data, one study suggested that the prevalence of bovine TB in humans may be as high as 15% (8).

Some MTC members (*M. bovis*, *M. bovis* BCG, and *M. canettii*) are intrinsically resistant to pyrazinamide (PZA), an important first-line anti-TB drug (26). Therefore, the specific identification of these members of the MTC is clinically important for treatment management decisions. This highlights the need for a rapid diagnostic assay that is capable of differentiating the members of the MTC while simultaneously providing information relating to contact and source tracing.

Currently, there is only one commercially available diagnostic assay for the differentiation of the MTC, namely, the Genotype MTBC kit (Hain Lifesciences GmbH, Nehren, Germany). While useful for identifying some members of the MTC, this kit is limited by its inability to accurately identify *M. tuberculosis*, *M. canettii*, *M. africanum*, and *M. pinnipedii* (16). There have also been a number of molecular-based assays for MTC differentiation described in the literature (9, 11, 13, 20, 21). These methods are also limited by an inability to differentiate all members of the MTC, and most of these methods require postamplification processing, which increases method complexity, analysis time, and potential contamination.

Previously, we have reported the design and development of two 4-plex real-time PCR diagnostic assays for the accurate iden-

tification of the MTC, one for the identification of *M. tuberculosis* and *M. canettii* and one for the identification of *M. bovis*, *M. bovis* BCG, and *M. caprae* (22, 23). In this study, additional diagnostic targets were incorporated into these 4-plex assays, resulting in two 5-plex real-time PCR diagnostic assay. By incorporating these targets, we have now devised a diagnostic method and an algorithm which allow for the accurate identification of the MTC and the differentiation of all 8 members, including the capability to differentiate between *M. africanum* West African 1 and *M. africanum* West African 2. This two-stage method, *Seek*TB, can be performed in approximately 3.5 h post-DNA extraction.

MATERIALS AND METHODS

Ethics statement. This study was approved by the research ethics review committee of the University of Zambia School of Medicine, Ridgeway Campus, Lusaka, Zambia. All study participants gave written informed consent in accordance with internationally recognized clinical trial standards.

Bacterial isolates used in this study. The panel of MTC isolates, nontuberculosis mycobacteria (NTM), and other bacterial species used in this study (see Tables S1 and S2 in the supplemental material) was the same panel as that described in a previous study (23), with the addition of 47 MTC isolates provided by the National Reference Center for Mycobacteria, Borstel, Germany (characterized as described previously by Allix-Béguec et al. [2]). All genomic DNA samples used in this study were isolated, quantified, and stored as described previously (22).

PCR primers and hydrolysis probe design. The novel diagnostic targets used in this study were identified by using *in silico* comparative genomic approaches previously described (22, 23). All oligonucleotides used in this study were designed in accordance with previously reported guidelines (10, 24). All primers and probes (see Table S3 in the supplemental material) were designed with similar properties to facilitate multiplexing (23).

Conventional PCR and sequencing. Conventional PCR amplification of the RD713 region (2,798-bp *M. africanum* West African 1) and RD701 (340-bp *M. africanum* West African 2) was performed according to methods described previously by Huard et al. (14). Genomic DNA was also amplified for sequencing to confirm the identity of some clinical isolates. The sequencing primers (see Table S3 in the supplemental material) were used in conventional PCRs as previously described (22, 23). PCR products were purified and sequenced according to methods described previously by Reddington et al. (23).

Real-time PCR. Multiplex real-time PCRs were performed with a LightCycler 480 instrument using the LightCycler 480 Probes Master kit (Roche Diagnostics). The optimized PCR mix for multiplex 1 contained 2× LightCycler 480 Probes Master mix (6.4 mM MgCl$_2$); forward and reverse primers (0.5 μM final concentration); Cyan 500-, carboxy-X-rhodamine (ROX)- and Cy5-labeled probes (0.2 μM final concentration) in addition to 4,4,7,2,4,5,7-hexachloro-6-carboxyfluorescein (HEX)-labeled (0.1 μM final concentration) and 6-carboxyfluorescein (FAM)-labeled (0.4 μM final concentration) probes; dimethyl sulfoxide (4%; Sigma-Aldrich, MO); and template DNA (MTC, 2 μl; internal amplification control [IAC], 2 μl; NTM, 10 μl), adjusted to a final volume of 40 μl with the addition of nuclease-free distilled water (dH$_2$O). The *M. smegmatis* internal control DNA was diluted to contain 100 genome equivalents per 2 μl, and NTM DNA was diluted to contain ~10^4 genome equivalents per 10 μl. For multiplex 2, the optimized PCR mix was the same, with the exception of the FAM-labeled probe, where a 0.2 μM final concentration was sufficient. PCR cycling was performed as previously described (22). To avoid fluorescence leaking from channel to channel, a color compensation file was generated (3), and the noise band was set to 1.2 fluorescence units (multiplex 1) and 1.3 fluorescence units (multiplex 2) in the Cyan 500, FAM, HEX, and ROX channels.

Evaluation of *Seek*TB performance on clinical cultures. Genomic DNA from 125 Mycobacteria Growth Indicator Tube (MGIT)-positive culture samples isolated at the University Teaching Hospital, Lusaka,

Zambia, were tested blindly. Positive MGIT cultures were identified as MTC or non-MTC by using the TBc ID (BD Diagnostics) test, according to the manufacturer's instructions, before DNA extraction. Isolated DNA samples were also tested by using the GenoType MTBC kit (Hain Lifesciences) according to the manufacturer's instructions.

RESULTS

Diagnostics targets. During this study, it was determined that the *wbbl1* assay, outlined previously for the specific detection of *M. tuberculosis-M. canettii* isolates (22), also detected *M. africanum* West African 1. Therefore, an additional assay for the specific detection of *M. africanum* West African 1 was necessary. Region of difference (RD) 713 was previously identified as specific to *M. africanum* West African 1 (7, 19, 27). RDs represent regions of the genome present in *M. tuberculosis* that are deleted in other members of the MTC (4). In members of the MTC with intact RD 713, this region is 4,248 bp, whereas in *M. africanum* West African 1, this region is 2,798 bp (14, 27). An assay targeting a 138-bp region of RD713 (primers RD_713Fw and RD_713Rv and probe RD713 [see Table S3 in the supplemental material]) was designed for the specific detection of *M. africanum* West African 1 and was incorporated into the first multiplex assay. The MTC-specific probe was also redesigned to improve specificity (see Table S3 in the supplemental material).

The second multiplex real-time PCR diagnostic assay was modified from a previously described multiplex assay (23), by incorporating an assay for the specific detection of *M. africanum* West African 2. PCR primers RD701_Fw and RD_701 and probe RD701 were designed to amplify an 81-bp region of RD 701, an RD specific to *M. africanum* West African 2 (7, 19, 27) (see Table S3 in the supplemental material). In members of the MTC with intact RD 701, this region is 2,885 bp, whereas in *M. africanum* West African 2, this region is 340 bp (27).

The incorporation of an additional diagnostic assay to each of the above-described multiplex assays resulted in two 5-plex assays capable of differentiating all 8 members of the MTC and the 2 *M. africanum* clades (see Fig. S1 and S2 in the supplemental material).

Specificities of the diagnostic assays. The specificities of all monoplex assays were evaluated against a panel of 180 MTC, NTM, and other bacteria (see Tables S1 and S2 in the supplemental material). Subsequently, the specificities of the assays were reevaluated in a multiplex format. All assays were 100% specific for the target organisms in both monoplex and multiplex formats.

Using multiplex 1, the RD 713 assay was specific for the 5 *M. africanum* West African 1 isolates tested. The *wbbl1* assay was specific for the 60 *M. tuberculosis*, 8 *M. canettii*, and 5 *M. africanum* West African 1 isolates tested. The MTC assay was specific for the 119 MTC isolates tested. The RD^{canettii1} assay was specific for the detection of the 8 *M. canettii* isolates tested. The IAC (targeting MSMEG_0660) was specific for the detection of *M. smegmatis* DNA.

Using multiplex 2, the *lepA* assay was specific for the detection of the 5 *M. caprae* isolates tested. The RD 701 assay was specific for the 5 *M. africanum* West African 2 isolates tested. The *lpqT* assay was specific for the 14 *M. bovis*, 7 *M. bovis* BCG, and 5 *M. caprae* isolates tested. The diagnostic assay targeting RD 1 did not detect any of the 7 *M. bovis* BCG strains tested but detected all *M. bovis* and *M. caprae* strains tested, allowing for the specific identification of *M. bovis* BCG. The IAC assay was 100% specific.

A typical representation of the amplification curves generated

TABLE 1 Result scenario for each member of the MTC[a]

Multiplex assay	Result with analysis channel and target					Result interpretation[b]
	Cyan 500	FAM	HEX	ROX	Cy5	
1	RD 713	*wbbl1*	MTC *lepA*	RD[canettii1]	IAC MSMEG_0660	
	+	+	+	−	+	*M. africanum* West African 1
	−	+	+	+	+	*M. canettii*
	−	+	+	−	+	*M. tuberculosis*
	−	−	+	−	+	MTC—perform second multiplex
	−	−	−	−	+	Not a member of the MTC
	−	−	−	−	−	Result invalid, test must be repeated
2	*M. caprae lepA*	*lpqT*	RD 1	RD 701	Cy5 (IAC MSMEG_0660)	
	+	+	+	−	+	*M. caprae*
	−	+	+	−	+	*M. bovis*
	−	+	−	−	+	*M. bovis* BCG
	−	−	+	+	+	*M. africanum* West African 2
	−	−	+	−	+	*M. pinnipedii*
	−	−	−	−	+	*M. microti*
	−	−	−	−	−	Result invalid, test must be repeated

[a] +, positive signal observed; −, positive signal not observed.
[b] The interpretations of the results of the multiplex 2 assay take into account the multiplex 1 results.

in each of the analysis channels in the two multiplex assays is shown in Fig. S1 and S2 in the supplemental material.

Sensitivities of the assays. The lower limit of detection (LOD) of the assays was determined by using probit regression analysis (22, 23). LODs of 9.04, 5.88, 0.4, and 5.08 genome equivalents for the MTC-specific (*lepA*), *M. tuberculosis*-*M. canettii*-*M. africanum* West African 1-specific (*wbbl1*), *M. canettii*-specific (RD[canettii1]), and *M. africanum* West African 1-specific (RD713) assays, respectively, were determined.

For multiplex 2, LODs of 5.66, 6.05, 98.28, and 24.9 genome equivalents for the *M. bovis*-*M. bovis* BCG-*M. caprae* (*lpqT*), *M. bovis*-*M. caprae* (RD1), *M. caprae* (*lepA*), and *M. africanum* West African 2 (RD701) assays, respectively, were determined. In both multiplex assays, the IAC at a concentration of 100 cell equivalents was detected in all samples tested.

Interpretation of results. The interpretation of the multiplex assay results was performed as described in Table 1. When isolates are tested, multiplex 1 should always be performed first. If the sample is positive for a member of the MTC but *M. tuberculosis*, *M. canettii*, or *M. africanum* West African 1 is not identified, multiplex 2 should subsequently be performed. Multiplex 2 directly identifies *M. bovis*, *M. bovis* BCG, *M. caprae*, and *M. africanum* West African 2. To identify the remaining members of the MTC, namely, *M. microti* and *M. pinnipedii*, the combined results from both multiplex assays must be taken into account. Assay results can be easily interpreted according to Table 1.

Evaluation of *Seek*TB performance with clinical cultures. MGIT-positive isolates were blindly tested by using two molecular methods, the GenoType MTBC (Hain Lifesciences) line probe assay and *Seek*TB. Both molecular methods demonstrated 100% agreement, identifying 84 isolates as *M. tuberculosis* isolates and 41 isolates as non-MTC isolates. The results of TBc ID (with a reported specificity and sensitivity of 92.4 to 100% and 94.9 to 100%, respectively [18, 25, 29]) and the molecular methods were concordant for 79 MTC isolates and 37 non-MTC isolates. The *ssrA* genes of the 9 discordant samples were sequenced to consolidate results. Nucleotide sequence analysis revealed that the molecular tests were correct for 8 out of the

9 discordant isolates. The remaining isolate returned a mixed sequence, possibly indicating the presence of a mixed culture containing both MTC and non-MTC species.

DISCUSSION

The accurate differentiation of members of the MTC is necessary to (i) perform epidemiological studies, (ii) monitor whether TB transmission is human to human or zoonotic (9, 21), and (iii) administer appropriate anti-TB treatment, due to some members of the complex displaying natural resistance to PZA (1, 26).

The method described in this study, *Seek*TB, is composed of two 5-plex real-time PCR assays which can be performed sequentially. The first multiplex diagnostic assay has the capability of detecting the presence of the MTC isolate while simultaneously differentiating *M. tuberculosis*, *M. canettii*, and *M. africanum* West African 1, taking approximately 1.5 h to complete after DNA extraction. For the majority of TB cases, only the first multiplex assay would have to be performed, as *M. tuberculosis* is thought to be the causative agent of approximately 95% of human infections (6). The second multiplex diagnostic assay would be performed if a member of the MTC was detected in the sample but *M. tuberculosis*, *M. canettii*, or *M. africanum* West African 1 was not identified. The second multiplex assay enables the specific identification of the remaining members of the complex, namely, *M. bovis*, *M. bovis* BCG, *M. caprae*, *M. africanum* West African 2, *M. microti*, and *M. pinnipedii*. The sequential performance of both tests takes approximately 3.5 h after DNA extraction.

The specificity of the diagnostic targets used in this study was initially tested by using a panel of well-defined MTC isolates. These isolates were previously characterized by using methods such as spoligotyping, mycobacterial interspersed repetitive-unit–variable-number tandem-repeat (MIRU-VNTR) analysis, IS*6110*-based typing methods, RD analysis, and biochemical testing in addition to morphological examination. The developed method demonstrated 100% agreement with the previously used methods, correctly identifying all members of the MTC and demonstrating the robustness of the rapid tests developed.

A further evaluation of the *Seek*TB method was performed by blindly testing a panel of uncharacterized positive MGIT cultures and comparing the results with those generated by using the GenoType MTBC test. The results of both molecular methods demonstrated 100% agreement; however, some discordance was observed when the results of these molecular methods were compared to culture results. The concordant molecular method results were indicative of true results in this study, as confirmed by nucleotide sequencing.

While the characterization of the MGIT-positive culture samples resulted in the identification of *M. tuberculosis* only, it should be noted that this is a relatively small number of isolates from one urban geographical region. MGIT-positive culture sample biobanks with a high likelihood of containing a variety of members of the MTC are required to further validate *Seek*TB (and the novel diagnostic targets used). The additional testing of a larger panel of MGIT-positive culture samples from this region and other regions worldwide will be performed in future.

When comparing the two molecular methods tested, the *Seek*TB method offers a number of advantages over the GenoType line probe assay: the developed method is less laborious, requiring less hands-on time; there is no need for the postamplification handling of samples, which significantly reduces the likelihood of introducing contamination; there is an enhanced sensitivity with the real-time-PCR-based *Seek*TB method; and *Seek*TB has the ability to accurately identify *M. tuberculosis*, *M. canettii*, *M. pinnipedii*, and both clades of *M. africanum* in addition to the members of the MTC identifiable by using the GenoType MTBC test.

In conclusion, the diagnostic method developed in this study, *Seek*TB, is the first-described internally controlled, multiplex-PCR-based method capable of rapidly and accurately identifying all members of the MTC. This method has been validated on a large panel of well-characterized MTC isolates and has been successfully used for the culture confirmation of positive African MGIT cultures. Ultimately, *Seek*TB needs to be further tested in the field, both on positive culture samples and directly on clinical samples, such as sputum, to demonstrate its potential and robustness for the specific identification and differentiation of isolates of the MTC.

ACKNOWLEDGMENTS

REFERENCES

1. **Allix-Béguec C, et al.** 2010. Importance of identifying Mycobacterium bovis as a causative agent of human tuberculosis. Eur. Respir. J. **35**:692–694.
2. **Allix-Béguec C, Harmsen D, Weniger T, Supply P, Niemann S.** 2008. Evaluation and strategy for use of MIRU-VNTRplus, a multifunctional database for online analysis of genotyping data and phylogenetic identification of Mycobacterium tuberculosis complex isolates. J. Clin. Microbiol. **46**:2692–2699.
3. **Anonymous.** 2008. LightCycler 480 instrument operator's manual, p 248–256. Roche Diagnostics, Basel, Switzerland.
4. **Behr MA, et al.** 1999. Comparative genomics of BCG vaccines by whole-genome DNA microarray. Science **284**:1520–1523.
5. **Brosch R, et al.** 2002. A new evolutionary scenario for the Mycobacterium tuberculosis complex. Proc. Natl. Acad. Sci. U. S. A. **99**:3684–3689.
6. **Das S, Das SC, Verma R.** 2007. Occurrence of RD9 region and 500 bp fragment among clinical isolates of Mycobacterium tuberculosis and Mycobacterium bovis. Microbiol. Immunol. **51**:231–234.
7. **de Jong BC, Antonio M, Gagneux S.** 2010. *Mycobacterium africanum*—review of an important cause of human tuberculosis in West Africa. PLoS Negl. Trop. Dis. **4**:e744. doi:10.1371/journal.pntd.0000744.
8. **de la Rua-Domenech R.** 2006. Human Mycobacterium bovis infection in the United Kingdom: incidence, risks, control measures and review of the zoonotic aspects of bovine tuberculosis. Tuberculosis **86**:77–109.
9. **Djelouadji Z, Raoult D, Daffé M, Drancourt M.** 2008. A single-step sequencing method for the identification of *Mycobacterium tuberculosis* complex species. PLoS Negl. Trop. Dis. **2**:e253.
10. **Dorak MT.** 7 January 2009, posting date. Real-time PCR. http://www.dorak.info/genetics/realtime.html.
11. **Halse TA, Escuyer VE, Musser KA.** 2011. Evaluation of a single-tube multiplex real-time PCR for differentiation of members of the Mycobacterium tuberculosis complex in clinical specimens. J. Clin. Microbiol. **49**:2562–2567.
12. **Hlavsa MC, et al.** 2008. Human tuberculosis due to Mycobacterium bovis in the United States, 1995-2005. Clin. Infect. Dis. **47**:168–175.
13. **Huard R, de Oliveira Lazzarini L, Butler W, van Soolingen D, Ho J.** 2003. PCR-based method to differentiate the subspecies of the Mycobacterium tuberculosis complex on the basis of genomic deletions. J. Clin. Microbiol. **41**:1637–1650.
14. **Huard RC, et al.** 2006. Novel genetic polymorphisms that further delineate the phylogeny of the Mycobacterium tuberculosis. J. Bacteriol. **188**:4271–4287.
15. **Kallenius G, et al.** 1999. Evolution and clonal traits of Mycobacterium tuberculosis complex in Guinea-Bissau. J. Clin. Microbiol. **37**:3872–3878.
16. **Kjeldsen MK, Bek D, Rasmussen EM, Priemé A, Thomsen VØ.** 2009. Line probe assay for differentiation within Mycobacterium tuberculosis complex. Evaluation on clinical specimens and isolates including Mycobacterium pinnipedii. Scand. J. Infect. Dis. **41**:635–641.
17. **Koeck JL, et al.** 2011. Clinical characteristics of the smooth tubercle bacilli 'Mycobacterium canettii' infection suggest the existence of an environmental reservoir. Clin. Microbiol. Infect. **17**:1013–1019.
18. **Lu P-L, et al.** 2011. Evaluation of the Bactec MGIT 960 system in combination with the MGIT TBc identification test for detection of Mycobacterium tuberculosis complex in respiratory specimens. J. Clin. Microbiol. **49**:2290–2292.
19. **Mostowy S, et al.** 2004. Genomic analysis distinguishes Mycobacterium africanum. J. Clin. Microbiol. **42**:3594–3599.
20. **Parsons L, et al.** 2002. Rapid and simple approach for identification of Mycobacterium tuberculosis complex isolates by PCR-based genomic deletion analysis. J. Clin. Microbiol. **40**:2339–2345.
21. **Pinsky B, Banaei N.** 2008. Multiplex real-time PCR assay for rapid identification of Mycobacterium tuberculosis complex members to the species level. J. Clin. Microbiol. **46**:2241–2246.
22. **Reddington K, et al.** 2011. Novel multiplex real-time PCR diagnostic assay for identification and differentiation of Mycobacterium tuberculosis, Mycobacterium canettii, and Mycobacterium tuberculosis complex strains. J. Clin. Microbiol. **49**:651–657.
23. **Reddington K, et al.** 2011. A novel multiplex real-time PCR for the identification of mycobacteria associated with zoonotic tuberculosis. PLoS One **6**:e23481. doi:10.1371/journal.pone.0023481.
24. **Robertson JM, Walsh-Weller J.** 1998. An introduction to PCR primer design and optimization of amplification reactions, p 121–154. *In* Lincoln PJ, Thompson J (ed), Forensic DNA profiling protocols. Humana Press, Totowa, NJ.
25. **Said HM, Ismail N, Osman A, Velsman C, Hoosen AA.** 2011. Evaluation of TBc identification immunochromatographic assay for rapid identification of Mycobacterium tuberculosis complex in samples from broth cultures. J. Clin. Microbiol. **49**:1939–1942.
26. **Somoskovi A, et al.** 2009. "Mycobacterium canettii" isolated from a human immunodeficiency virus-positive patient: first case recognized in the United States. J. Clin. Microbiol. **47**:255–257.
27. **Vasconcellos S, et al.** 2010. Distinct genotypic profiles of the two major clades of Mycobacterium africanum. BMC Infect. Dis. **10**:80.
28. **WHO.** 2011. Global tuberculosis control 2011. World Health Organization, Geneva, Switzerland. http://www.who.int/tb/publications/global_report/2011/gtbr11_full.pdf.
29. **Yu M-C, et al.** 2011. Evaluation of the Rapid MGIT TBc identification test for culture confirmation of Mycobacterium tuberculosis complex strain detection. J. Clin. Microbiol. **49**:802–807.