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Letter to the Editor

Specificity of IS*6110*-Based Amplification Assays for *Mycobacterium tuberculosis* Complex

We have demonstrated homology between DNA from mycobacteria other than tuberculosis strains (MOTT) and a central region of IS*6110* and have urged caution in using diagnostic tests based on this target (6). Hellyer and colleagues (5) have evaluated three IS*6110*-based amplification methods: *Hin*cII strand displacement amplification (SDA), thermophilic SDA, and a previously described PCR (11) and have observed no cross-reaction with 27 nontuberculosis mycobacteria, 26 of which had been used previously in our study (6). They conclude that these data support the use of selected regions of IS*6110* as *Mycobacterium tuberculosis* complex-specific targets.

Hellyer and colleagues suggest that cross-reactivity may be limited to the 181-bp region or alternatively was due to PCR contamination. They describe the use of dUTP/uracil DNA glycosylase to prevent false-positive results, but this strategy has been shown to have a limited impact on false-positive rates in tuberculosis PCR in a recent international collaborative study, in which 8 of 17 laboratories using it recorded false-positive reactions (8).

We have expressed the view that homology is confined to a yet-undefined central region of the insertion sequence (4). We cannot accept the contention that PCR contamination was responsible for our results even though, as the authors suggest, a relatively high concentration of DNA was used for the PCRs. Homology between the 181-bp fragment amplified from H37Rv and genomic DNA from the 26 MOTT was demonstrated by Southern blotting, excluding the possibility of PCR contamination as an explanation of our results. The results obtained in the study of Hellyer et al. may be due to the fact that the target areas used in their PCRs are outside the homologous region, or alternatively they may be due to the small and unquantitated amount of DNA used in their assay, giving false-negative results for nontuberculosis isolates where the homology is lower and consequently a lower predicted level of sensitivity.

The data supporting the contention that IS*6110*-based methods are specific is scanty. Among the authors quoted by Hellyer in support of this idea, Eisenach and colleagues report one positive result for 42 nontuberculosis mycobacteria, a false-positive rate of at least 2.3% (3). Pietrzak and colleagues tested specimens from only 48 patients for whom a diagnosis of tuberculosis was excluded on the basis of culture and history. They state that none of these patients had a positive PCR, but the data is not shown (9). Using the same primers, Querol and colleagues did not test any patients with MOTT (10). The IS*6110* PCR of Eisenach et al. (3) has been shown to have a false-positive rate of 3% with specimens containing MOTT (1). An evaluation of IS*6110*-based methods in nine laboratories from France demonstrated false-positive reactions with an average rate of 7%, although a clear relationship to nontuberculosis isolates or other organisms could not be drawn (2). Those authors conclude that the results of their study suggest "that PCR using IS*6110* as a target for DNA amplification is neither very sensitive nor really specific for the detection of tuberculosis."

The insertion sequence IS*6110* is a member of the IS*3* family. Members of this family are the most widely spread group of bacterial insertion sequences, being found in more than 24 different gram-positive and gram-negative genera (7). To investigate some of the possibilities for cross-reaction, we amplified DNA from a range of organisms, including bacteria likely to be present in oropharyngeal secretions. The PCR products were hybridized with a probe derived from the 181-bp fragment, and discrete bands were demonstrated in PCRs with *Aspergillus*, *Streptococcus pneumoniae*, and *Streptococcus pyogenes* (Fig. 1).

Positivity rates among specimens submitted for clinical diagnosis of tuberculosis are low; consequently, even very low false-positive rates have a considerable impact on the reliability of the test. As IS*3* sequences are widely distributed in organisms found in oropharyngeal secretions and this flora is so heterogeneous, false-positive results at low frequency can be expected. Before IS*6110*-based methods can be recommended, clinical studies of each primer set involving very large numbers of specimens are required. There are now many alternative PCR targets for tuberculosis diagnosis which lack the problems associated with IS*6110*, and we suggest that these methods be employed for clinical diagnosis.

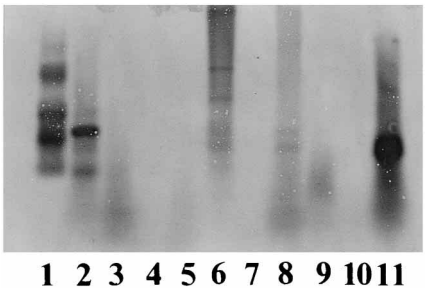

FIG. 1. Southern blot hybridization of nonmycobacterial DNA with IS*6110*. Lanes: 1, *Mycobacterium tuberculosis* H37Rv; 2, *Mycobacterium fortuitum*; 3, *Mycobacterium kansasii*; 4, *Mycobacterium malmoense*; 5, *Mycobacterium xenopi*; 6, *Aspergillus fumigatus*; 7, *Toxoplasma gondii*; 8, *Streptococcus pneumoniae*; 9, *Staphylococcus epidermidis*; 10, *Staphylococcus aureus*; 11, *Streptococcus pyogenes.*

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Authors' Reply

In their original article, Kent and colleagues claimed to demonstrate the existence of homology between an IS*6110*-derived probe and DNA isolated from a variety of nontuberculous mycobacteria (10). The purpose of our study was to determine whether the IS*6110*-based assays used on a routine basis in our laboratory, and which have been adopted by other laboratories around the world, are specific for the *Mycobacterium tuberculosis* complex (8). We performed IS*6110*-based PCR (4, 5, 14) and strand displacement amplification (15) on a panel of nontuberculous mycobacteria supplied by the authors of the earlier paper. Of the 27 strains provided, 22 were purported to possess DNA sequences with homology to IS*6110* (7). We challenged our amplification systems with the equivalent of at least 10^3 genomes of nontuberculous mycobacterial DNA, as determined by titration in a genus-specific PCR assay (3). Meanwhile, the analytical sensitivities of our IS*6110*-based assays were shown to be $\leq$14 copies of the insertion element (equivalent to 1 copy of the *M. tuberculosis* H37Rv genome [1]). None of the 27 strains of nontuberculous mycobacteria yielded a positive result with any of the three IS*6110* assays we employed, while all were positive with the genus-specific assay. These data provide irrefutable support for our assertion that the regions of IS*6110* amplified in our systems are indeed specific for the *M. tuberculosis* complex.

Gillespie and colleagues cite evidence from Southern hybridization analysis to contend that significant homology does exist between a specific region of IS*6110* and DNA from other mycobacteria as well as nonmycobacterial species. However, the figures shown in the original paper and in their response to our article are not convincing. Hybridization of equivalent amounts of genomic DNA with an IS*6110*-derived probe yielded stronger signals with nontuberculous mycobacteria than with an *M. tuberculosis* H37Rv positive control (10). This is most unexpected since this strain is known to possess 14 copies of the IS*6110* element (1). Furthermore, a nested PCR system incorporating a total of 50 cycles of amplification, of which 20 are conducted at low stringency (10, 16), is likely to generate nonspecific products. These will de facto hybridize to a probe containing the PCR primer sequences. To avoid the ambiguity of multiple hybridizing fragments, a probe based upon the intervening sequence between the innermost pair of PCR primers ought to be used. In this manner, strains of the *M. tuberculosis* complex should yield a single hybridizing band of predictable molecular weight.

We have also been criticized for our reliance on decontamination of reaction mixtures with uracil DNA glycosylase (UDG) to prevent false-positive results. Data from our laboratory (8, 9) and the multicenter study by Noordhoek et al. (11) show that UDG is highly effective in reducing the rate of false positives when used in conjunction with appropriate laboratory procedures. These include use of dedicated equipment and physical separation of reagent preparation, sample preparation, amplification, and product detection. The most likely source of false-positive results in previous studies with IS*6110*-based assays is not homologous DNA sequences present in other organisms but either residual or undiagnosed tuberculosis (9, 12, 13) and cross-contamination among specimens during preparation of DNA for amplification (2). UDG has no efficacy against such contamination, which can only be addressed by improved laboratory technique. Culture is the "gold standard" by which molecular assays are frequently judged. However, it is recognized that false-positive cultures are common in some settings. In one study, culture contamination was suspected in 16% of newly diagnosed cases of tuberculosis (6). Individual laboratory results, whether based on conventional microbiology or molecular techniques, cannot therefore be taken in isolation but must be reviewed in the context of the patient's clinical and epidemiological history.

The specificity of our assays having been demonstrated, the most appropriate manner in which to resolve whether IS*6110*-related elements truly exist in nontuberculous mycobacteria and other organisms is by sequence analysis. We emphasize that to avoid the likelihood of contamination, this should be performed on cloned genomic DNA from a panel of different mycobacteria rather than on the products of PCRs.

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