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Evidence of the Presence of IS*1245* and IS*1311* or Closely Related Insertion Elements in Nontuberculous Mycobacteria outside of the *Mycobacterium avium* Complex

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A PCR assay based on the simultaneous detection of IS*1245* and IS*1311* was developed and used to determine the host range of these insertion elements. Specific PCR products were observed in *Mycobacterium malmoense*, *Mycobacterium scrofulaceum,* and *Mycobacterium nonchromogenicum*, indicating that IS*1245* and IS*1311* are not limited to the *Mycobacterium avium* complex.

The 1,414-bp insertion element IS*1245* belongs to the *Staphylococcus aureus* IS*256* family of insertion sequences. It is present in up to 27 copies in *Mycobacterium avium* (8) and was found to be stable during in vivo and in vitro passage (1, 14), making it a popular target for restriction fragment length polymorphism strain typing in recent years (5, 6, 12, 19). The host range was originally demonstrated to be limited to the *M. avium* group (*M. avium* and subspecies *paratuberculosis* and *silvaticum*) by PCR amplification of a 427-bp target sequence within IS*1245*, leading to its use as a species-specific target for diagnostic detection (11).

Recently, Beggs et al. found IS*1245* in strains of *Mycobacterium intracellulare*, demonstrating that the element is present in further species of the *M. avium* complex (MAC) (2).

A closely related insertion element, IS*1311* (85% sequence identity to IS*1245* at the DNA level), has likewise been used as a target for restriction fragment length polymorphism strain typing of *M. avium* (5, 15, 16).

In our laboratory, a PCR assay based on the simultaneous detection of a highly homologous 130-bp portion of IS*1245* and IS*1311* (91% sequence identity) was developed as a diagnostic tool for detection of MAC in clinical specimens and used to determine the host range of these elements.

Mycobacterial strains were received from five collaborating mycobacterial laboratories. Species of nontuberculous mycobacteria (NTM) were identified by 16S ribosomal DNA (rDNA) sequencing (10) and/or 23S rDNA probes (GenoType; Hain Diagnostika, Nehren, Germany). Twelve NTM strains (1 *Mycobacterium shimoidei* strain, 1 *Mycobacterium scrofulaceum* strain, 6 *M. intracellulare* strains, 2 *Mycobacterium malmoense* strains, 1 *Mycobacterium chelonae* strain, and 1 *Mycobacterium szulgai* strain) were identified by a combination of standard biochemical methods (9) and high-performance liquid chromatography (4).

Cells were lysed by incubation at 100°C for 5 min in the presence of 2 M NaOH and 4% Triton. Crude lysates or DNA purified using QIAamp spin columns (Qiagen, Hilden, Germany) were used as PCR templates.

PCR analysis of NTM strains was performed by two independent laboratories (Lab 1 and Lab 2). Amplification was performed on GeneAmp PCR systems 9600 and 2400 (Perkin-Elmer, Weiterstadt, Germany). A 100-μl reaction mixture contained 1 mM $MgCl_2$, 320 μmol of deoxynucleoside triphosphates, 5 U of *AmpliTaq* Gold (GeneAmp; Perkin-Elmer), 100 pmol of each primer, and 5 to 10 μl of lysate with 0.1 to 1 ng of template DNA in 1× PCR Buffer II (Perkin-Elmer). The sense primer N3 (5′ ACTTCCTGCGCAACGTGCT 3′) recognized positions 885 to 903 of IS1245 (GenBank accession no. L33879) and positions 823 to 841 of IS*1311* (GenBank accession no. U16276), and the antisense primer N5 (5′ ATGCCG GCGATGGTGTCG 3′) recognized positions 997 to 1014 of IS*1245* and 935 to 952 of IS*1311*. A 10-min activation step at 95°C was followed by 40 cycles of denaturation for 30 s at 94°C and annealing-polymerization for 30 s at 60°C. PCR products were analyzed on 8% polyacrylamide gels.

Pre- and postamplification work was performed in separate rooms, and negative controls for DNA extraction and purification and PCR were included to avoid false-positive reactions due to carryover of DNA or amplification products. *M. avium* ATCC 35713 genomic DNA served as a positive PCR control.

Specificity of PCR products was demonstrated by restriction with *Hha*I (IS*1245*; restriction sites 832/833 and 925/926) and *Tse*I (IS*1311*; restriction site 858/859) or direct sequencing of both strands using the amplification primers (GATC1500-System; GATC GmbH, Konstanz, Germany).

Extraction and hybridization of genomic mycobacterial DNA was carried out as described previously (19). *M. avium* ATCC 25291 amplification products of the primer pair N3/N5 and P1/2 were labeled (AlkPhos Direct Labeling and Detection kit RPN 3680; Amersham) and used as probes.

The N3/N5 PCR assay was carried out for 54 strains of 17

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TABLE 1. PCR assay for IS*1245* and IS*1311*

Mycobacterium species	No. of positive isolates (no. tested)					
	N3/N5 PCR			P1/P2 PCR		
	Lab 1	Lab 2	Total	Lab 1	Lab 2	Total
NTM						
M. avium	4 (4)	72 (86)	76 (90)	3 (3)	66 (86)	69 (89)
M. avium subsp. *paratuberculosis*	2 (2)		2 (2)	1 (2)		1 (2)
M. intracellulare	7 (13)	15 (56)	22 (69)	3 (7)	4 (56)	7 (63)
M. malmoense	11 (15)		11 (15)	9 (12)		9 (12)
M. scrofulaceum	5 (7)		5 (7)	3 (5)		3 (5)
M. nonchromogenicum	1 (1)		1 (1)	1 (1)		1 (1)
M. celatum	0 (1)		0 (1)			
M. chelonae	0 (2)	0 (1)	0 (3)			
M. fortuitum	0 (1)		0 (1)			
M. gordonae	0 (1)	0 (1)	0 (2)			
M. haemophilum		0 (1)	0 (1)			
M. interjectum	0 (1)		0 (1)			
M. kansasii	0 (1)	1 (1)	1 (2)			
M. marinum	0 (1)	0 (1)	0 (2)			
M. phlei		0 (1)	0 (1)			
M. ratisbonense	0 (1)		0 (1)			
M. szulgai	0 (1)	0 (1)	0 (2)			
M. shimoidei	0 (1)		0 (1)			
M. xenopi	0 (1)	1 (1)	1 (2)			
Total	30 (54)	89 (150)	119 (204)[a]	20 (30)	70 (142)	90 (172)
MTB complex						
M. tuberculosis	1 (38)		1 (38)[a]			

[a] Sensitivity = 58.3%; specificity = 97.4%; positive predictive value = 99.2%; negative predictive value = 30.3% for differentiation of NTM from MTB complex by N3/N5 PCR.

NTM species and 38 strains of *Mycobacterium tuberculosis* complex (MTB complex) by Lab 1 (Table 1). Positive signals were found for the following species: *M. avium* (including the reference strains ATCC 35713 and 25291), *M. avium* ssp. *paratuberculosis*, *M. intracellulare* (including reference strain TMC 146), *M. malmoense*, *M. scrofulaceum* (including reference strain ATCC 35792), and *M. nonchromogenicum*. Strains of 11 further NTM were negative, while one strain of *M. tuberculosis* produced a weak signal (Table 1).

In an independent laboratory (Lab 2), 150 further clinical strains of NTM were tested using N3/N5 PCR. *M. avium*, *M. intracellulare*, *Mycobacterium kansasii*, and *Mycobacterium xenopi* were positive, while six further NTM of different species were negative (Table 1). Thus, the combined results from both laboratories showed a sensitivity of 58.3%, specificity of 97.4%, positive predictive value of 99.2%, and negative predictive value of 30.3% for identification of NTM and differentiation from isolates of MTB complex. For *M. avium* complex, *M. intracellulare*, *M. malmoense*, and *M. scrofulaceum*, the species most frequently causing NTM disease in children with mycobacterial lymphadenitis, the sensitivity increased to 63.4%, but for *M. avium complex* isolates it reached only 84.4%.

Specificity of the N3/N5 amplification products was demonstrated for 19 of 22 non-*M. avium* NTM in Lab 1 by restriction with *Hha*I and *Tse*I or sequence analysis

Characteristic IS*1245* restriction fragments were observed for 3 of 4 strains of *M. intracellulare*, 9 of 10 strains of *M. malmoense*, 4 of 5 strains of *M. scrofulaceum*, and one strain of *M. nonchromogenicum* tested, while IS*1311*-specific fragments were seen for 1 strain of *M. avium* ssp. *paratuberculosis*, 2 of 2 strains of *M. intracellulare*, 2 of 10 strains of *M. malmoense*, and 1 of 5 strains of *M. scrofulaceum*. The amplification product of *M. nonchromogenicum* could not be cleaved by *Tse*I, suggesting a lack of IS*1311*.

Sequence analysis of PCR products for five strains showed sequences homologous to IS*1245* for two strains of *M. malmoense* and superimposed sequences of IS*1311* and IS*1245* for one strain of *M. intracellulare* and two strains of *M. scrofulaceum*. 16S rRNA DNA sequencing reconfirmed the species in each sample.

The amplification product of the strain of *M. tuberculosis* positive by N3/N5 PCR showed 100% homology to IS*1311*. However, a GenBank search (National Center for Biotechnology Information BLAST) did not reveal any homology to published sequences within the *M. tuberculosis* genome.

The presence of IS*1245* was further demonstrated for *M. avium*, *M. avium* ssp. *paratuberculosis*, *M. intracellulare*, *M. malmoense*, *M. scrofulaceum*, and *M. nonchromogenicum* by amplification of a 427-bp internal fragment with the primer pair P1/P2, as described previously (8) (Table 1). All 90 strains positive for PCR with P1/P2 were also positive with N3/N5, except for one strain of *M. avium*.

DNA (1.5 to 3 μg) from six cultured clinical isolates of *M. malmoense* and the reference strain *M. avium* ATCC 25291 was hybridized with the N3/N5 and P1/P2 PCR products derived from *M. avium* ATCC 25291. One isolate of *M. malmoense* showed seven bands using the 427-bp P1/P2 probe and the 130-bp N3/N5 probe, while the other five isolates were negative. The reference strain of *M. avium* showed a two-band

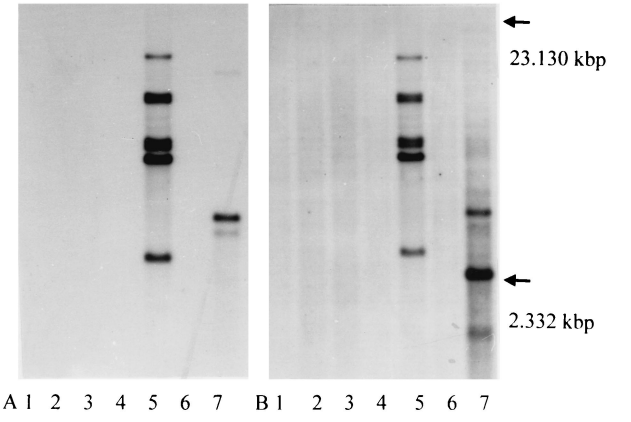

FIG. 1. Hybridization of genomic DNA with P1/P2 (A) and N3/N5 (B). Lanes 1 to 6, clinical isolates *M. malmoense*; lane 7, *M. avium* (ATCC 25291). The arrows indicate the size range based on *Hin*dIII-digested λ-DNA fragments present on the agarose gel.

pattern when hybridized with P1/P2 and a four-band pattern with the N3/N5 probe (Fig 1).

Whittington et al. previously described a number of non-MAC strains that were positive in a PCR for a portion of IS*1311* partially overlapping the target for the primer pair N3/N5 (20). In their study, however, sequence analysis of the amplification products revealed only low homology to IS*1311* (56% homology for *Mycobacterium thermoresistibile* and 58% for *M. tuberculosis*). In contrast, the signals produced by *M. scrofulaceum* in our study were 100% homologous to IS*1245* and IS*1311*, and the product from *M. malmoense* was 100% homologous to IS*1245*.

Since the faint signal produced by N3/N5 PCR from a clinical isolate of *M. tuberculosis* in this study showed 100% homology to IS*1311* and a GenBank search did not reveal any sequence homology of the amplified 130-bp fragment with genomic sequences of *M. tuberculosis*, a mixed infection or cross-contamination with NTM during culture is the most likely explanation (13, 17, 18). However, this hypothesis could not be tested, since the strain was no longer viable. All other strains of *M. tuberculosis* complex were clearly negative by this PCR assay.

While the specificity of the N3/N5 primer pair was confirmed by P1/P2 analysis, we observed a higher number of strains positive in the N3/N5 PCR. This can easily be explained by a higher sensitivity due to the presence of IS*1311*, which would represent a primer target for strains devoid of IS*1245*, or in the case of strains containing IS*1245*, provide a second multicopy target (5, 16).

It remains unclear why hybridization was successful for only one of five strains of *M. malmoense* that were positive in both PCR assays. A possible explanation is that the PCR assay detects elements that are present in only a small subset of cells, below the sensitivity of the hybridization assay. Further experiments based on single colonies will test this hypothesis. While culture contamination of these strains with *M. avium* complex cannot be excluded at this point, this was not indicated by the results of 16S rDNA sequence analysis.

The detection of IS*1245* or a closely related sequence in a clinical strain of *M. malmoense*, as demonstrated by hybridization of genomic DNA, seems to indicate that insertion elements may have spread through horizontal transfer to environmental NTM of differing species. This underscores the need for careful and extensive evaluation of the phylogenetic distribution of these elements among mycobacterial species before interpretation of diagnostic results obtained with insertion elements as genetic markers.

The diagnostic value of the N3/N5 PCR has to be assessed by further studies, since *M. malmoense* and *M. scrofulaceum* represent the second-most-common causes of mycobacterial lymphadenitis in children after MAC, in Europe and the United States, respectively (3, 7, 21). A single PCR, as described in this study, could thus be helpful in differentiating between NTM and tuberculous species in clinical specimens.

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