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Multilocus Genotyping Identifies Infections by Multiple Strains of *Trichophyton tonsurans*▽

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Acquisition of multiple genetic strains of a single dermatophyte species should not be unexpected in areas of high endemicity, and yet multistrain infections are infrequently reported. This communication details mixed *Trichophyton tonsurans* infections and highlights the need to confirm the presence of multiple strains in a clinical single isolate by use of a multilocus approach.

Clinical and epidemiological investigations, along with population-based evolutionary studies, all benefit from the ability to distinguish pathogen strains. Without the ability to reliably discriminate variants within a species one cannot adequately determine the extent of genetic variation within and between pathogen populations, investigate the origins of phenetic diversity within a species, or gain insight into the relationship between the organism and its host (17). For many fungal pathogens, strain-typing methods have been developed for neutrally evolving areas of the genome, with typing strategies aimed at detecting intraspecific genetic variation in the dermatophytes almost exclusively represented by the ribosomal DNA (rDNA) locus (9, 10, 11, 16, 18, 21).

The rDNA region remains popular for investigations of microbial pathogens. The intergenic spacer is known to exhibit a large degree of variation, and the highly conserved flanking rDNA genes make it relatively easy to target the region. Nevertheless, it is not uncommon for papers describing strain-typing methods using this region to report amplification patterns that are difficult to interpret (11, 12). We similarly reported challenges with initial attempts at characterizing this region from *Trichophyton tonsurans*. However, careful optimization of the PCR amplification procedures ensured a singular, discernible pattern for each strain in the validation set (9). Yet subsequent application of our PCR-restriction fragment length polymorphism (PCR-RFLP)-based strain-typing methods to a large-scale epidemiologic investigation revealed composite RFLP patterns at this locus (2). Notably, examining coding region variations in conjunction with variations in the rDNA locus offered evidence that these patterns were consistent with infections comprised of more than one strain.

Given that clinically derived samples and samples acquired in large-scale studies are rarely plated in a clonal fashion, typing strategies require the ability to discriminate mixed infections (i.e., infections by more than one strain). This is particularly relevant when studies are conducted in areas of high endemicity where more than half of the relevant sample population may be harboring the pathogen and multiple strain types are known to occur. This communication provides details on multistrain *T. tonsurans* infections confirmed from a single clinical isolate by use of a multilocus approach.

MATERIALS AND METHODS

Clinical isolates. The *T. tonsurans* isolates described for this investigation were obtained from a prospective epidemiologic investigation reported on previously by the investigators (2). Scalp cultures were performed for an entire population of day care center attendees, including children identified as clinically symptomatic and those without evidence of infection. Two sterile, soft-headed toothbrushes were massaged over the scalp of each child. One brush was immediately plated onto solid culture medium (Sab-C; Becton Dickinson, Cockeysville, MD) that was maintained at 25°C for 2 to 4 weeks. The second brush was immediately placed into a sterile culture tube containing 5 ml of aqueous medium (yeast nitrogen base without amino acids or ammonium sulfate [Becton Dickinson], keratin [ICN Pharmaceuticals, Aurora, OH], chloramphenicol [Sigma-Aldrich, St. Louis, MO], and cycloheximide [Sigma-Aldrich]). Aqueous cultures were placed in a shaking incubator and maintained at 32°C for 5 days (1).

Fungal material was isolated from all positive cultures, and genomic DNA was isolated using a DNeasy plant Mini kit (QIAGEN Inc., Valencia, CA). Where plated cultures indicated the presence of multiple morphotypes, each colony was subcultured onto an individual Sab-C plate and the genomic DNA was isolated separately.

Molecular strain typing. In total, 14 sequence variations in four gene loci comprised the sequences used for the typing strategy. Within the intergenic spacer of the rDNA locus, a length variant, seven single-nucleotide polymorphisms (SNPs), a small 10-bp insertion, and a small 14-bp deletion were evaluated as described previously (9). In the *ALP1* gene, a 16-bp tandem repeat element present in the 5′ untranslated region (5′UTR) and a single SNP in the 3′UTR were determined using methods previously reported (4).

The two remaining sequence variations incorporated into the strain-typing scheme are located in the coding regions of carboxypeptidase-Y (*CarbY*) and metalloprotease-5 (*MEP5*). These variations were identified through full-length-sequencing efforts directed at the secreted protease genes in *T. tonsurans*. The gene regions were initially amplified and sequenced from 16 strains of *T. tonsurans* by use of oligonucleotide primers directed at the outermost sequences of the coding regions of carboxypeptidase-Y and metalloprotease-5 of the published *T. rubrum* genes (GenBank accession no. AY497024 and AF407189, respectively). The newly generated *T. tonsurans* sequence was subsequently used to design primers for elucidating the entire *T. tonsurans* gene sequence and for designing PCR-RFLP assays for SNP verification. DNA sequencing was performed with DYEnamic ET dye terminator chemistry on a MegaBACE 500 capillary sequencer (GE Healthcare, Piscataway, NJ). Bases were automatically called by use of Sequence Analyzer software (GE Healthcare, Piscataway, NJ) and confirmed by manual inspection with Sequencher DNA analysis software (Gene Codes Corp., Ann Arbor, MI).

The transition (G>A) present in exon 2 of *CarbY* was determined using primers 5′-CAA CGA CCC CAA GAA CGA TCC and 5′-AAT GCC ATT CAC CTC TGA GC to amplify a 1,148-bp fragment directly from genomic DNA. In

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the presence of a G, restriction digestion with BstYI generated two fragments of 858 and 290 bp. When an A was present, BstYI cut the PCR product three times, resulting in a pattern comprised of 570-bp, 290-bp, and 288-bp bands.

One of two sequence variations present in exon 3 of *MEP5* was also included in the typing scheme. The transition C>T was detected after amplification of a 283-bp fragment from genomic DNA by use of primers 5′-CAC GGC CAG GGT GGT AAA GGC and 5′-GGT TGG AAA CTA GAT ATG ATG AGG. Restriction digestion of the PCR product with AciI yielded fragments of 207 and 76 bp in the presence of C, while the amplicon remained uncut in the presence of T. Owing to the nature of this digest, a "reverse" assay was designed to confirm the sequence variation. In the presence of a T, BstBI cut a 251-bp product generated with primers 5′-CTT GCC ATC AGG AGG GGT TTC G and 5′-TCA AAT TTG AGT ACC CTT ATG CTC C into fragments that were 229 bp and 22 bp in length whereas the product remained uncut by BstBI in the presence of C.

Each of the PCRs detailed above was performed in a total volume of 8 μl with RedJumpStart *Taq* polymerase (Sigma-Aldrich) and 3.75% dimethyl sulfoxide according to the manufacturer's recommendations. Annealing temperatures were 58°C for the *CarbY*-derived PCR product and 56°C for the *MEP5* PCR product. Extension times ranged from 20 s to 2 min, depending on the length of the PCR product. Restriction enzymes were acquired from New England Biolabs (Beverly, MA), and digests were incubated for 2 h at the recommended temperatures. PCR-RFLP fragments were separated on either a 2% or a 4% Synergel-agarose gel matrix (Diversified Biotech, Boston, MA). PCRs were performed at least twice, and positive and negative DNAs (i.e., DNAs derived from strains that were sequence verified) as well as a water-only control were included into each genotyping run. All PCR-RFLP assays were highly reproducible and yielded unequivocal restriction patterns on agarose gel electrophoresis with each repeat determination.

Statistics. A chi-square test was used to examine whether disease phenotypes appeared to be influenced by the presence of mixed infections. This test was performed using SPSS version 11.5 software (SPSS, Chicago, IL), with the significance limit set at $\alpha = 0.05$.

Nucleotide sequence accession numbers. The nucleic acid sequence for the *T. tonsurans CarbY* and *MEP5* gene regions have been deposited in the GenBank database under accession numbers EF490685 and EF490686, respectively.

RESULTS

As detailed in our earlier report, a total of 3,541 scalp cultures were collected from 446 children over a period of 2 years (2). A total of 1,048 *T. tonsurans* isolates were recovered from the 3,541 scalp cultures, and all were strain typed for sequence variations in the rDNA and *ALP1* loci. Among these, we observed 892 infection events for 252 children where PCR-RFLP patterns were consistent with a single isolate. Seventy-eight infection events occurred for 57 children where two strain types could be inferred based on the presence of composite restriction patterns in one or more of the genotyping assays. Mixed-strain isolates were recovered, with comparable distribution results, from both solid cultures ($n = 44$) and aqueous cultures ($n = 34$). The mixed infection could be discriminated with colonies that differed in pigmentation by macroscopic morphology for only four of the solid cultures. For the remaining 40 solid and 34 aqueous cultures, the presence of multiple strains was detected only by molecular strain typing.

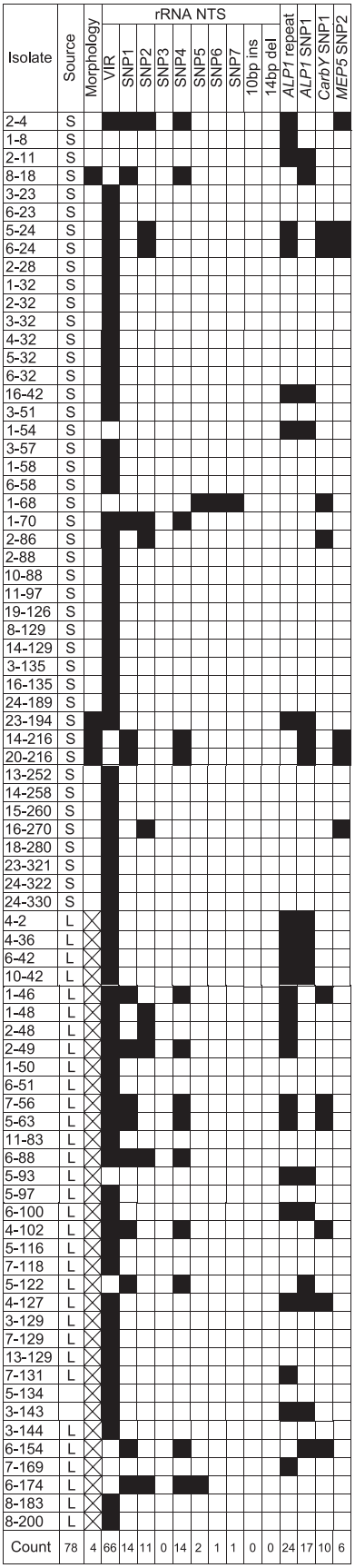
The initial typing strategy comprised 12 sequence variations in two gene loci (rDNA nontranscribed spacer [NTS] and *ALP1*). Within the rDNA locus, the PCR assays designed to detect the length variation displayed patterns consistent with the presence of multiple isolates for 85% ($n = 66$) of the mixed infections. The addition of PCR-RFLP assays designed to identify SNPs residing in the same locus improved detection of mixed infections by seven cases to account for 94% of total mixed infections that were observed (Fig. 1). The tandem-repeat element in the *ALP1* locus identified 31% ($n = 24$) of mixed infections, with 37% ($n = 29$) of mixed infections detected when the additional SNP present in this locus was incorporated (Fig. 1). When these two loci were considered together, length variations alone identified 91% (71) of cases. Knowledge of unique SNPs present within the respective sequences and inclusion of these in the strain-typing strategy was necessary to account for 100% of the mixed infections.

One sequence variation in each of two additional loci were also tested to confirm the ability to detect mixed infections by the presence of composite restriction patterns. These two SNPs were selected from among those that have been identified to date in *T. tonsurans* because (i) they occur with high (~30%) frequency, (ii) they do not appear to be in linkage with the SNPs described as present in the NTS or *ALP1* loci, (iii) they represent SNPs in coding sequences rather than in sequences which are not transcribed (e.g., rDNA NTS) or translated (e.g., *ALP1* UTRs), (iv) they are present in genes that do not contain repetitive elements, and (v) based on our observations, there is no experimental evidence leading us to believe that the genes are duplicated in the genome (i.e., that they exist in multiple copies or have a corresponding pseudogene). Notably, both sequence variations were capable of confirming the presence of mixed infections with *CarbY* and *MEP5*, identifying multiple isolates in 13% and 8% of cases, respectively (Fig. 1).

In the majority of infections where more than one strain type could be identified ($n = 66$), the study participants demonstrated no clinical indicators of disease (i.e., were asymptomatic). The remaining isolates ($n = 12$) derived from children demonstrating signs or symptoms of active disease. When compared with infections caused by a single strain (678 asymptomatic, 214 asymptomatic), mixed infections were no more likely to appear in the presence or absence of clinical disease indicators ($P = 0.084$). Moreover, there was no association between age and the presence of mixed infections (i.e., children at the extremes of age were no more or less likely to harbor multiple isolates).

Among the 57 children demonstrating infection with multiple strains, there were 11 children for whom both isolates appeared more than once, either alone or in combination (Fig. 2a). The isolates appeared exclusively together for only one child (subject 32). For a second child (subject 129), the isolates appeared to occur almost exclusively in combination, with the exception of a single sampling event. For the remaining nine children, the patterns of mixed infections were more consistent with the transient acquisition of isolates and/or the displacement of one isolate by another. Notably, none of these isolates appearing intermittently in these 11 children were low-frequency isolates (i.e., all appeared at a rate of more than 1% in this population). Frequency estimates reflect the number of times the genetic strain type appeared in the population of isolates. Frequencies were arbitrarily assigned as high, moderate, and low when they occurred at rates of >10%, 1% to 10%, and <1%, respectively.

There were 31 children for whom only one isolate identified as part of a multistrain infection reappeared during the period of observation. Representative examples are shown in Fig. 2b. The persistent strains in these individuals were comprised of low ($n = 3$)-, moderate ($n = 8$)-, and high ($n = 19$)-frequency strains. The transient strains that appeared to be acquired or lost were also constituted by low ($n = 2$)-, moderate ($n = 15$)-,



and high (n = 13)-frequency strains. Among the remaining children, there were six for whom neither isolate present in the mixed infection reappeared and nine for whom no determination could be made because the mixed infection was their only infection event.

DISCUSSION

Given the ubiquitous nature of dermatophyte species, it is not uncommon to observe a large fungal burden in populations with heavy concentrations of "at-risk" individuals. In the United States, where *T. tonsurans* is endemic among African American children, infection rates as high as 50% have been observed in large-scale epidemiologic investigations (2). Infection rates for species that are endemic in other geographic regions can be equally impressive. Population surveys conducted in undeveloped and developing countries revealed clinical infection rates that never fell below one in five and often exceeded one in every two of the children sampled (3, 6, 13, 14, 15, 19, 20).

Considering that fomites, in addition to humans, represent a reservoir for the spread of dermatophytes within a population (8), it is not unexpected that individuals in areas of high endemicity have the potential to acquire multiple strain types. Yet multistrain infections are infrequently reported in clinical investigations that have evaluated dermatophytes at the molecular level. Several investigations targeting the rRNA locus report the presence of only one strain contributing to infections of the feet and toenails (10, 16). In one study, serial isolates were described as switching back and forth between two strain types, but in no sample did the strains occur together (10). In contrast, a single study reported that 60% of onychomycosis specimens were comprised of multiple strains (21). However, the discrimination of multistrain infections in the aforementioned study was accomplished by selecting and independently analyzing colonies randomly selected from the plate on which the original specimen was inoculated.

While random selection of colonies may afford the ability to identify the presence of multiple strain types from a single infection, typing a few selected colonies may fail to detect minor strains that are present in low abundance relative to the major strain (17). Using morphotyping as a basis for colony selection does not assure that selection bias is mitigated. We observed far greater numbers of genetic strain types of *T. tonsurans* than of morphotypes; in the present investigation, only 5% of mixed specimens could be discriminated based on macroscopic morphology. Consequently, strain-typing strategies need to be of sufficient specificity to reliably discriminate the presence of multiple strains in routinely processed clinical specimens.

The highly repetitive nature of sequences within the intergenic spacer of the rRNA locus makes this region particularly

FIG. 1. Sequence variations that discriminated multiple isolates in a dual infection according to composite restriction patterns in PCR-RFLP. Shaded boxes indicate composite patterns observed with PCR or PCR-RFLP products. S, solid culture; L, aqueous culture (aqueous culture results are not applicable for morphology).

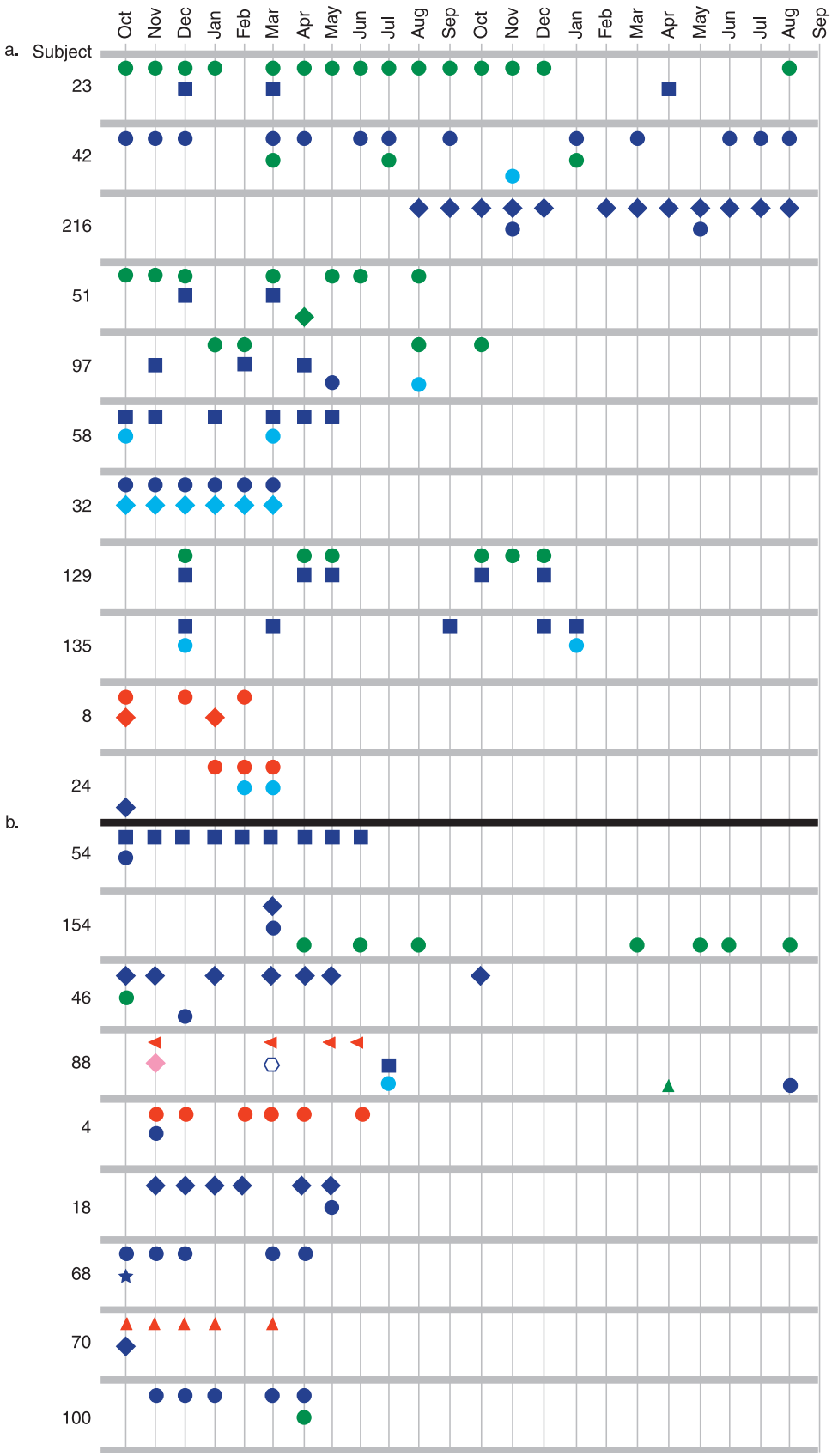


FIG. 2. Examples of mixed infections where both isolates appear more than once (a) and only one isolate recurs on the host (b). Each colored symbol represents a distinct strain type, as detailed previously (2).

susceptible to the generation of PCR artifacts (e.g., as a result of arbitrary priming or misalignment of the template). Consequently, failure to address ambiguous PCR products and/or RFLP patterns that arise during assay development may preclude reliable recognition and discrimination of multistrain infections. In a number of investigations, ambiguous band patterns remained unexplained, leading the authors to propose that (i) the rDNA locus contains cistrons whose intergenic spacer regions differ in composition, (ii) rearrangements are occurring within the intergenic spacer such that primer sites are being duplicated, or (iii) the isolates being typed represent heterokaryons with a different intergenic spacer contributed by each haploid mate (11, 12).

Development of our typing strategy was driven by full-length sequence data of the respective target sequences which allowed us to recognize anomalous RFLP patterns and carefully optimize the assays to eliminate artifacts. Consequently, we did not observe any isolates in our validation set of clonally derived strains that offered evidence of heterokaryosis or a polycistronic rDNA region. Moreover, serial passage of our prototype isolates over 3 years returned the same RFLP pattern on analysis of the daughter strains, arguing against spontaneous rearrangements occurring in the locus (albeit in an in vitro setting) (2).

The presence of multiple strains in the clinical isolates represents the most likely explanation for the composite band patterns observed in this investigation. The RFLP patterns we observed were distinct and reproducible and were constituted by individual patterns that segregated with known genotypes obtained from single strains. Additionally, a large number of strains were represented among the isolates present in these mixed infections, and unique strain types did not exclusively coexist within the population, thus providing additional evidence against a polycistronic nature of the rDNA locus in our North American isolates.

We recognize that evidence exists for heterogeneity in the rDNA region in diploid organisms (5, 7) and that the idea of the polycistronic nature of this locus cannot be excluded without sequence data covering the entire region. Moreover, an argument in support of a polycistronic locus could be made for isolates from individuals such as subject 32, for whom a consistent pattern was observed with each sample. Consequently, a robust typing strategy requires the inclusion of gene loci that are less susceptible to PCR artifact formation. Notably, the secondary locus initially selected for this purpose (*ALP1*) bears a repeat element, thus prompting the addition of two supplementary loci (*CarbY* and *MEP5*) harboring stable single nucleotide sequence variations. If the composite RFLP patterns observed in the intergenic spacer and *ALP1* represented a polycistronic rDNA region or assay artifact, the PCR fragments derived from the *CarbY* and *MEP5* loci would be expected to exhibit only the wild-type or variant sequence but not both; this was, in fact, not the case.

Infections with multiple genetic strains of *T. tonsurans* appear to occur with reasonable frequency in young African American children attending a large urban daycare center. This investigation highlights the requirement that typing strategies be robust enough to detect multiple strain types in a single clinical specimen if the strategy is going to be of utility in large population-based studies. The incorporation of sequence variations from multiple loci, in cases in which full sequence data are available, should allow investigators to unambiguously determine the nature of infections in their population.

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