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MOLECULAR ASPECTS

The clonal composition of *Mycobacterium tuberculosis* in clinical specimens could be modified by culture

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SUMMARY

Background: The application of molecular tools has revealed that infection by *Mycobacterium tuberculosis* (MTB) is more complex than initially assumed. Genotyping is generally performed on cultures. However, there is no information about bacterial clonal complexity in clinical specimens or whether standard culture procedures can modify this complexity.
Methods: An *in vitro* assay was performed to determine whether culture can modify the clonal complexity of the MTB population in clinical specimens. Pairs of MTB strains (10 pairs) or stain-positive sputa (4 pairs) were mixed in different volumetric proportions. The DNA extracted from the mixtures before and after culture was genotyped using mycobacterial interspersed repetitive unit-variable-number tandem repeat analysis to detect potential changes in the proportion of the mixed strains.
Results: In 6/10 pairs of MTB strains and 2/4 pairs of sputa, marked changes were observed in clonal composition after culture, even in mixtures of strains differing in their drug-susceptibility patterns. In some cases, only one of the mixed strains was detected after culture.
Conclusions: The initial clonal composition in bacteriologically complex clinical specimens could be underestimated if genotyping analysis is performed after culture. Genotyping strategies aimed at analyzing clinical samples must be optimized to reveal the real dimension of clonal complexity in infection by MTB.

1. Introduction

It is traditionally assumed that infection by *Mycobacterium tuberculosis* (MTB) involves a single strain. However, in recent years, the application of genotyping tools[1–4] to the analysis of the bacterial populations in specific clinical cases has revealed that the clonal composition of the bacterial populations involved in some cases of tuberculosis is more complex than initially assumed. Different clonally complex phenomena have been observed, including mixed infections with more than one strain[5–8] and different strains infecting independent anatomical sites[9] or different sites within the lung.[10]

As the concept of clonal complexity has gained ground, it has been suggested that standard procedures in clinical microbiology laboratories could be underestimating such phenomena. Some studies have suggested that several serial isolates per episode should be analyzed to improve detection of clonal heterogeneity.[11–13]

Unfortunately, conventional genotyping tools are not sensitive enough to analyze clinical samples directly, and most studies have applied them only on cultured isolates.[4,14,15] In this study, we used mycobacterial interspersed repetitive unit-variable-number tandem repeat (MIRU-VNTR) genotyping in *in vitro* combinations of MTB strains and respiratory samples to explore whether standard culture procedures could be masking the clonal complexity of the clinical samples.

2. Materials and methods

2.1. In vitro combination assays

2.1.1. Combination of cultured strains of M. tuberculosis

MTB strains were grown in 7H9 broth liquid media (Difco™ Middlebrook, Becton Dickinson, Sparks, Maryland, USA) supplemented with 10% albumin–dextrose–catalase (Becton Dickinson) and 0.05% Tween 80. The bacterial load in each culture was estimated by measuring optical density (OD) values using a CO 8000 Biowave Personal Cell Density Meter (WPA, Biochrom Ltd). Pairs of strains with equivalent OD values were combined in either five different volumetric proportions for the direct approach (90:10,

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10:90, 95:5, 5:95, and 50:50; in a final volume of 1 ml) or three different volumetric proportions for the refined approach (90:10. 10:90 and 50:50). Two hundred microliters of these mixtures was cultured in either Lowenstein–Jensen slants or 7H9 broth liquid medium for 2–3 weeks.

In all the mixtures, the MIRU-types of the individual strains were known before starting the experiment (with allelic variations in at least 4 to 8 loci) to ensure that genotypically different strains were combined. None of the individual strains showed double alleles in any of the loci.

2.1.2. Combination of respiratory samples

Decontaminated stain-positive sputa were selected from our frozen-specimen collection. The MIRU-type of the strains isolated from each sputum specimen was known, because they had previously been genotyped for epidemiological purposes and were all different. The MIRU-types of the strains in each pair showed differences in at least 5 loci.

The sputa were centrifuged at 3500 rpm for 15 min at 4 °C. The pellet was resuspended in 500 μl of phosphate buffer saline, of which 100 μl was inoculated in 7H9 broth liquid medium to test the viability of the bacilli, and the remaining 400 μl was used in the combination assays. Suspended pellets with an equivalent bacterial load estimated by fluorescence microscopy were mixed in pairs. The final volume of each mixture was 800 μl, and 200 μl was cultured in 7H9 broth liquid medium for 2–3 weeks.

2.2. Genotypic analysis of the in vitro mixtures

We performed two types of analysis in order to detect potential changes after culture in the proportion of the combined strains from each pair (Figure 1). Initially, we followed a "direct" approach by applying MIRU-VNTR analysis directly to the mixtures before and after culture in order to obtain qualitative information about the potential changes caused by culture. Next, we followed a "refined" approach, by genotyping multiple independent colonies obtained after plating each mixture before and after culture. This allowed us to obtain more precise quantitative data about the relative proportions of the combined strains in each pair and to define the magnitude of the changes after culture.

2.2.1. Direct approach

MIRU-VNTR analysis was performed directly on 800 μl of the initial mixtures (inoculum) and also on a loopful of the culture obtained after incubation of the inoculum in Lowenstein–Jensen slants for 2–3 weeks (culture). DNA was extracted by boiling the suspensions for 10 min; 5 μl of the extract was used for MIRU-VNTR analysis following standard procedures.[16] We selected only one of the five multiplex polymerase chain reactions (PCRs) in the MIRU15 format to detect potential changes in the strain mixtures. We ensured that the multiplex PCR selected included at least 2 loci at which the strains showed differences. The amplified products were analyzed by capillary electrophoresis[16] and, only in certain cases, by agarose gel electrophoresis. Qualitative changes in the proportion of the strains after culture were evaluated by comparing the amplification signal for each of the combined strains.

2.2.2. Refined approach

The refined approach was performed on a selection of pairs of MTB strains and on all the mixtures of sputa. Serial dilutions were obtained directly from the initial mixtures (inoculum) and from the cultures obtained after 2–3 weeks in 7H9 broth liquid medium

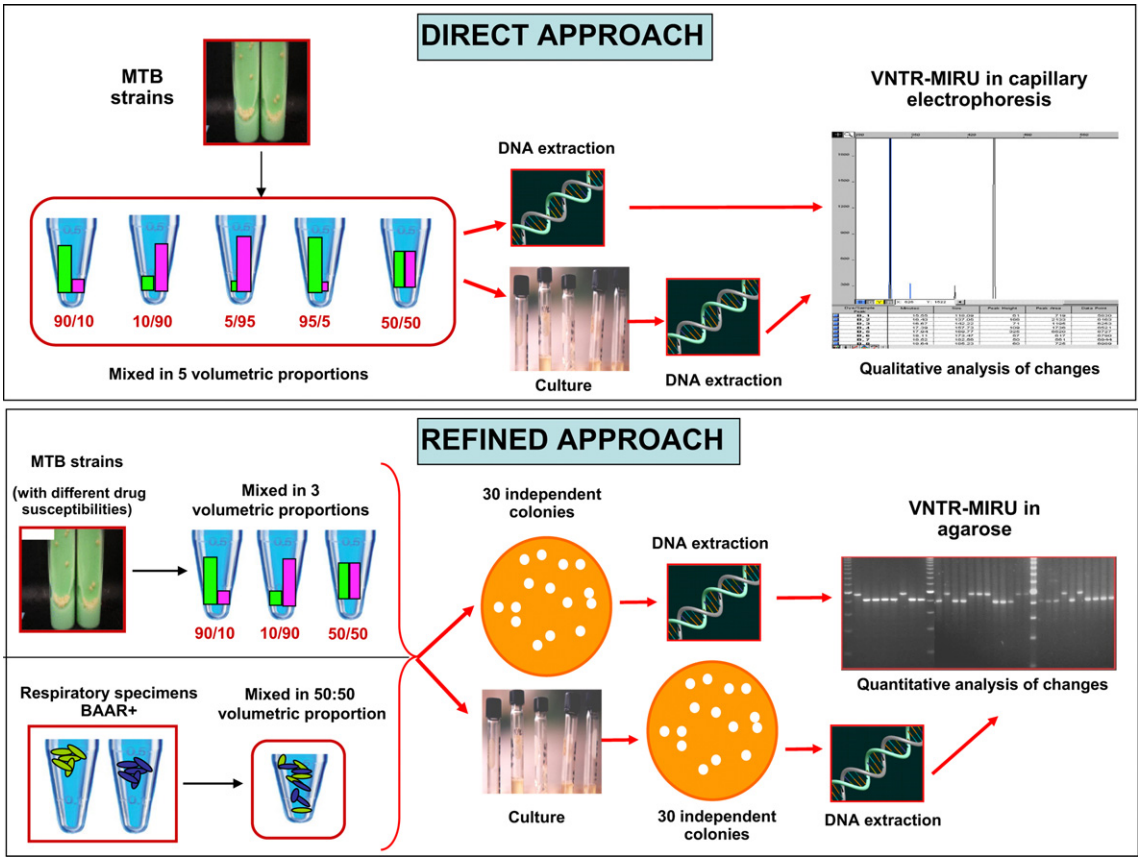

Figure 1. Flow chart describing the direct and refined approaches of analysis.