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CONTENT

Rev.	Descrizione modifiche	Data
0	Prima emissione	01/10/2010
1	Cambio ragione sociale	07/01/2013

1. SCOPE

Spoligotyping is a PCR- based method to simultaneously detect and type *Mycobacterium tuberculosis* complex bacteria, exploiting polymorphism in the "Direct Repeat" (DR) region, which is uniquely present in *Mycobacterium tuberculosis* complex bacteria.

2. APPLICATION

Spoligotyping may offer an alternative for typing Southern blotting when rapid results are required. The method is in particular useful to simultaneously detect and type *M.tuberculosis* complex bacteria in clinical samples (suspected nosocomial infections, outbreaks in prisons, etc.). The level of differentiation by spoligotyping is less compared to IS*6110* fingerprinting for strains having five or more IS*6110* copies, but higher for strains with less than five copies. Thus spoligotyping is a preferred method to type *M. bovis* strains, which usually contain only one or two IS*6110* copies. Note that *M. bovis* can be recognized by the absence of reactivity with spacers 39-43.
The mechanism by which spacers and copies of DR are generated, is unknown. With the method described here, the presence or absence in the DR region of 43 spacers of known sequence can be detected by hybridization of PCR-amplified spacer DNA to a set of immobilized oligonucleotides, representing each of the unique spacer DNA sequences. This method will be referred to as s*poligotyping* (from *sp*acer *oligotyping*).

3. DEFINITIONS AND ABBREVIATIONS

PCR*:*	Polymerase Chain Reaction
DR:	Direct Repeat region
IS6110:	Insertion sites in the direct repeat (DR) region of *M. tuberculosis*.
EDTA:	Ethylenediaminetetraacetic acid
ECL:	Enhanced chemiluminescence
SSPE:	Saline-Sodium Phosphate-EDTA
Na_2HPO_4:	Disodium Phosphate
NaCl:	Sodium Chloride
SDS:	Sodium Dodecyl Sulphate
POD:	ß-peroxidase
dNTP:	deoxynucleotide triphosphates

4. RESPONSIBILITIES

Supervision and proper execution of this following method must be supervised by a technically qualified individual and by the coordinator of the specific scientific area.

5. EQUIPMENT AND MATERIALS

5.1 Kit contents

- Two vials containing the positive controls:

    a) *positive control 1.* (*M. tuberculosis* strain H37Rv)
    b) *positive control 2.* (*M. bovis* BCG P3)

Both positive controls are 10 ng/µl. For PCR amplification 2 µl of each positive control should be added to the PCR reaction mixture. The volume in each vial is 25 µl and should therefore be sufficient for approximately 12 PCR reactions.

- Two vials containing the primers for PCR amplification:

a) primer Dra (biotinylated)
b) primer DRb

The primers are shipped lyophilized. To prepare both primers for PCR amplification both primers should be dissolved in 1.0 ml MQ water. Then split in two separate vials of 0.5 ml each and add 0.6 MQ water to each vial. The volume in each vial should be sufficient for approximately 550 PCR reactions.

- One spoligo-membrane.

The membrane is shipped in 20 mM EDTA. Please avoid dehydration at any time, because this will rapidly decrease the quality of the membrane. Store the membrane at + 4℃ for optimal storage condition.

- One manual

- Available spoligtyping products:

Cat. No.	**Description**	**Quantity**
IM9701	Spoligotyping kit	1
IM9702	Spoligotyping kit without controls and primers	1
MN45	Mini-blotter for spoligtyping	1
PC200	Foam cushions	100

5.2 Equipment

- PCR Thermocycler
- Maxi 14 Hybridisation oven with shaking platform
- Miniblotter MN45
- Plate cushions for miniblotter
- X-ray films preferably Hyperfilm ECL
- Incubation box fitting the membrane
- Exposure cassette

Additional Reagents:

- 20 x SSPE
  - 0.2 M Na_2HPO_4 $^*2H_2O$ 35.6 g/l
  - 3.6 M NaCl 210.24 g/l
  - 20 mM EDTA 7.4 g/l
  - pH 7.4

- SDS 10%
  - 10 g SDS / 100 ml demineralised water

- DNA polymerase
  - HotStarTaq

- Streptavidin-POD-conjugate

- ECL detection liquid

6. PROCEDURES

6.1 In vitro amplification of spacer DNA by PCR

Amplification of the spacers is accomplished by using the primers DRa and DRb, which enable to amplify the whole DR region. Only a very small amount of template DNA is required. Typically the PCR is performed on 10 ng purified chromosomal Mycobacterial DNA but, with minor adaptations, DNA extracts from clinical samples or lysed bacteria (from freezer or Löwenstein) can also serve as template. The PCR products are labelled with biotin, because primer DRa is biotinylated.

1) Always include chromosomal DNA of *M. tuberculosis* strain H37Rv and *M. bovis* BCG as positive controls. Use water as a negative control.

2) Prepare the reaction mixture:

- Template DNA (20 ng)	5 µl
- Primer DRa (20 pmol)	4 µl
- Primer DRb (20 pmol)	4 µl
- dNTP-mixture	2 µl
- Concentrated PCR Buffer 10x	5 µl
- Hot Star Taq Polymerase (5 units/µl)	0.2 µl / 0.4 µl
- MQ water (to a final volume of 50 µl)	29.8 µl

Notes:
DNA solutions extracted from sediments have to be amplified using 0.4 µl HotStarTaq; DNA solutions extracted from positive cultures have to be amplified using 0.2 µl.

3) Add one drop of mineral oil to the tubes to prevent evaporation of the PCR-mix during the amplification.

4) Place the tubes in a PCR-apparatus for amplification, and perform the following temperature cycling:

3 min 96℃ (15 min 95℃ HotStarTaq)

1 min 96℃
1 min 55℃ } 30x
30 sec 72℃

5 min 72℃

6.2 Hybridization with PCR product and detection

Hybridization of the biotin-labeled PCR products to the immobilized spacer-oligos that represent spacers of known sequence. The presence of spacers is visualized on film as black squares after incubation with Streptavidin-peroxidase and ECL-detection.

Procedure

1) All buffers should be prewarmed before use. Prepare the following buffers from concentrated stocks, using demineralised water for dilution (quantities for one membrane):

   250 ml 2xSSPE/0.1% SDS, 60ºC,
   250 ml 2xSSPE/0.5% SDS, 60ºC,
   250 ml 2xSSPE/0.5% SDS, 42ºC.
   250 ml 2xSSPE, Room temperature.

   See Annex 1 Preparation of Solutions

2) Add 20 µl of the PCR products to 150 µl 2xSSPE/0.1% SDS at 60.0 ℃

3) Heat-denature the diluted PCR product for 10 min at 99℃ and cool on ice immediately.

4) Wash the membrane for 5 min at 60℃ in 100 ml 2x SSPE/0.1% SDS.

5) Place the membrane and a support cushion into the miniblotter, in such a way that the slots are perpendicular to the line pattern of the applied oligonucleotides.

6) Remove residual fluid from the slots of the miniblotter by aspiration.

7) Fill the slots with the diluted PCR product (avoid air bubbles!) and hybridize for 60 min at 60ºC on a horizontal surface (no shaking!). Avoid contamination of neighbouring slots.

8) Remove the samples from the miniblotter by aspiration and take the membrane from the miniblotter using forceps.

9) Wash the membrane twice in 100 ml 2xSSPE/0.5% SDS for 10 min at 60℃ .

10) Place the membrane in a rolling bottle and allow it to cool down to prevent inactivation of the peroxidase in the next step.

11) Add 3.5 µl streptavidin-peroxidase conjugate (500U/ml) to 10 ml of 2xSSPE/0.5% SDS, and incubate the membrane in this solution for 60 min at 42℃ in a sealed plastic and transparent envelope and place it deeply in a water bath.

12) Wash the membrane twice in 100 ml of 2xSSPE/0.5% SDS for 10 min at 42℃.

13) Rinse the membrane twice with 100 ml of 2xSSPE for 5 min at room temperature.

14) For chemiluminiscent detection of hybridizing DNA, incubate the membrane for 1 min in 10 ml ECL detection liquid.

15) Cover the membrane with a transparent plastic sheet or Saran-wrap and expose a light sensitive film to the membrane for 20 min.

16) If the signal is too weak or too strong the membrane can be used again directly to expose another film for a shorter or longer period.

6.3 Regeneration of the membrane

The hybridized PCR product is dissociated from the membrane in order to regenerate the membrane for the next hybridization. A membrane can be regenerated for at least 15 times.

1) Wash the membrane twice by incubation in 100 ml 1% SDS at 80°C for 30 min.
2) Wash the membrane in 100 ml EDTA pH 8, for 15 min at room temperature.
3) Store the membrane at 4°C until use (sealed in p lastic or wrapped in Saran-wrap, to avoid dehydration of the membrane).

6.4 Interpretation

The spoligotype film is interpreted as follows:

- where the spacer is present mark “n” or “1”
- where the spacer is not present mark “ o” or “0”

A single spoligotype consists of 43 digits binary code and it is matched by specific on-line databases for the identification of the lineage (www.miru-vntrplus.org)

7. RECORDING AND REPORTING

The first step of the spoligotyping method, requiring PCR amplification of many samples, is registered on the Mod. EBP-TM 003/0. After the film development, the analysis is carried out deploying the Mod. EBP-TM 005/0. All the results are registered into the “Quaderno Spoligo” and into the relative databases.

8. RELATED DOCUMENTS

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9. ANNEXES

Annex 1: Preparation of Solutions

Solution 1: 2xSSPE/0.1% SDS (150 ml)

- 150 ml per sample (Max. 7 ml to be collected into a 15-ml Falcon tube)
- 2xSSPE/0.1% SDS at 60℃ 100 ml

Preparation:

20x SSPE	15	ml
10% SDS	1.5	ml
Distilled Water	133.5	ml

Solution 2: 2xSSPE/0.5% SDS (600 ml)

- 250 ml at 60 ℃
- 350 ml at 42 ℃

Preparation:

20x SSPE	60	ml
10% SDS	30	ml
Distilled Water	510	ml

Solution 3: 2xSSPE (250 ml at room temperature)

20x SSPE	25	ml
Distilled Water	225	ml

Solution 4: SDS 1% (200 ml)

10% SDS	20	ml
Distilled Water	180	ml

Solution 5: EDTA pH 8.0 (120 ml)

EDTA 250 mM pH 8	9.6	ml
Distilled Water	110.4	ml