Antibiotic susceptibility, resistance mechanisms and detection in *Mycobacterium leprae*

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How to test for antibiotic susceptibility in M. leprae?

- *Mycobacterium leprae* does not grow in vitro
- Susceptibility testing in the mouse footpad (Shepard 1960)
  - 20 to 50 mice treated by antibiotics
  - Mice kept and fed for 1 year because of slow growth of *M. leprae*
  - doubling generation time of ~ 10-15 days

  ⇒ rare data on resistance

- Molecular methods developed from 1990 onwards thanks to PCR and genomics

Shepard model of in vivo growth (1960)

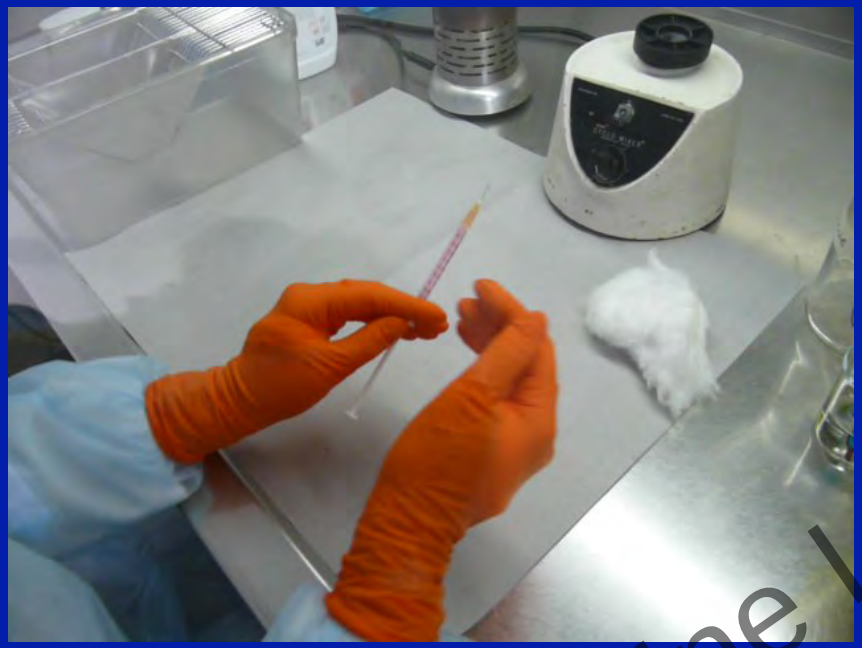
- Inoculation of 5000 bacilli in the left hind footpad of a swiss mouse
- Feed it for one year
- Mince all the tissue of the footpad
- Bacilli will multiply from 5000 (in which 10%at most are alive) to at least 100 000 (visible in microscopy) in several months

Usefulness of the footpad model

- For the diagnosis of drug resistance
  - Phenotype: susceptible or resistant
  - Needs the bacilli alive from the skin specimen
  - Needs groups of mice treated vs. Control untreated
  - Needs to analyse the footpad growth after 7 to 12 months
- To measure the anti- *M. leprae* activity of a drug
  - Groups of mice treated with various doses
  - untreated controls
  - Inoculation with 10-fold serial amount of bacilli

Critical points

- Alive bacilli
- Quantitation and dilutions
- Inoculation of 0.03 ml
- Animal housekeeping
- Treatment administration
  - Food containing drug
  - Gavage
  - injection



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Alive bacilli from skin specimens

- Quick transportation refrigerated
  - 86 biopsies transported in a cold container (4°C) by DHL
  - 55% growth in the mouse footpad
  - No difference between new cases (34) and relapse cases (49)
  - 2 days difference in the time between sampling and inoculation
  - Mean time 3.1 days for growth vs. 4.9 days for no growth ($p < 0.03$)

- You know that there <u>are</u> or <u>are not</u> alive only after 12 months (no macroscopic lesion)
  - Morphological index?
  - New methods to assess viability : RNA measurement, ...

Cambau et al. 2002, Martinez 2009,Lini 2009

Quantitation and dilutions

- Standardized microscopic slides
- Know the surface (D and d) and the volume (v) of the specimen

$$Nombre\ de\ bacilles/ml = \frac{n}{p} \times \frac{S}{s} \times \frac{1ml}{10^{-2}} = \frac{n}{p} \times \frac{\pi D^2/4}{\pi d^2/4} \times \frac{1}{10^{-2}}$$

- Factor of dilution / sensitivity limit (y = number of fields)
- Dilute to inoculate 5 000 bacilli under the volume of 30 µl

Animal housekeeping

- Temperature
- Specific room
- 10 per cage
- Water and food
- Animal housekeeper
- Automate cleaning cages
- Mortality?
- Ethics and laws for animals
- « Infectious » animal housing separate from other animal housing

Treatment administration

- Food containing drug : dapsone
  - Not allowed in some countries
- Oral administration by gavage
  - Animal housekeeper
  - Week ends and holidays
- IV administration
  - mortality

Assessing results

- When to read the controls?
  - 7 months (the hurry microbiologist but might need to inoculate more control mice)
  - 12 months (the patient microbiologist but might loose time)
  - 9 months (in the middle)
- Who will read ?
  - Expertise in dissecting the footpad
    ..\..\..\films leprae OMS\P1030388.MOV
  - Expertise in reading smears (≤ 1 bacilli per field)

Banking reference and other *M. leprae* strains

Reference strains

Nude mice

Rationale and endpoints for molecular detection of drug resistance in *M. leprae*

- Knowledge of mechanisms of drug resistance => define the genotype of resistant strains
- Good correlation between genotype and phenotype of resistance (mouse footpad susceptibility testing) => genotype will predict for phenotype
- Development of molecular techniques that describe the genotype => one genotype obtained from a skin biopsy
- Molecular techniques applicable in expert and in national centers with easy implementation

First trick for PCR efficiency : the bacterial index

Table 1. Efficiencies of in vivo susceptibility testing in the mouse footpad (mouse test) and PCR *rpoB* amplification, according to the number of acid-fast bacilli (AFB) per milliliter in the skin biopsy suspension.

AFB/mL	No. of biopsies tested	Mouse test		*rpoB* amplification	
		Positive	Efficiency, %[a]	Positive	Efficiency, %[a]
10^4 to $<10^5$	3	0	0	1	30
10^5 to $<10^6$	19	8	42	17	89
10^6 to $<10^7$	38	20	53	38	100
$>10^7$	23	18	78	23	100
Total	83	46	55	79	95

[a] Efficiency was defined as [(no. of valid tests/no. of tests run) × 100].

Cambau CID 2002

Genetic modification for acquired resistance in *M. leprae*

= genomic mutation

- Proportion of mutant resistant
  - about 1 for 10^5 to 10^8 bacteria
  - Varies depending on the nature of the drug and its in vivo concentration, i.e. dosage
- Type of genetic modification
  - Point mutation

    TCG => TTG

    Serine => Leucine
  - Deletion

    ACGCCTAGAT ----> AC TAGAT
  - Insertion

    ACGCCTAG ----> ACCTTGCCTA

Knowledge of mechanisms of drug resistance

- Which antileprosy drugs?
  - Dapsone
  - Rifampin
  - Clofazimine
  - Ethionamide
  - Fluoroquinolones (ofloxacin, moxifloxacin)
  - Clarithromycin
  - Minocycline
  - Diarylquinolines (R207910, TMC207)

Region determining **rifampicin resistance** (RDRR) in the subunit B of RNA polymerase encoding gene (*rpoB*)

Insertion

432	433	436	438	441	451	456	458
Gly	Thr	Leu	Gln	Asp	His	Ser	Leu
Ser	Ile	Pro	Val	Asn Tyr	Asp Tyr	Leu Met Phe Trp	Val

*Numbering system of *M. leprae* genome srain TN
RDRR= 432 – 458 = 507-533 in *E. coli*
Position 456 in *M.leprae* = *531* in *E. coli*
Position 456 = previous position 425 in M. leprae

70% of strains tested : **S456L**

Ala411Thr, Arg505Trp need to be validated (Nora Cordona, Cotonou 2012)

Honore 1993, Maeda 1999, Maeda 2001, Cole 2001, Cambau 2002, Zhang 2004, Cambau 2012

Dapsone resistance in *M. leprae*

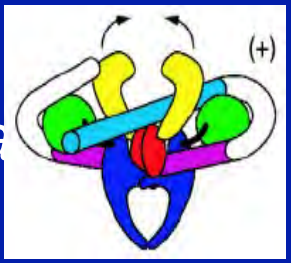
- Diamino-diphenyl-sulfone (DDS)
  - Sulfonamide derivative
  - Competitive inhibitor of dihydropteroate synthase (DHPS)

- Mechanism of dapsone resistance
  - Mutations in *folP1* encoding DHPS
  - at positions 53* and 55*
  - Thr53 => Ile, Arg or Ala
  - Pro55 => Arg, Leu

*Numbering system in *M. leprae*, positions 62 and 64 in *E. coli*

Kai 1999, Gillis, 2000, Cambau 2006

Quinolone resistance in *M. leprae*



- Only one type II topoisomerase in *M. lepra* (no topoisomerase IV)
- Mutation in the genes encoding DNA gyrase
- *gyrA* A91V* is the main mutation described
- gyrA G89C was described
- Confer a 5 to 10-fold lower inhibition of DNA gyrase by ofloxacin (DNA supercoiling experiments)

* Corresponds to 83 in *E. coli* and 90 in *M. tuberculosis*

Kim 2003 ; Maeda 2001, Cambau 1997, Matsuoka 2007 ; Matrat 2008

Knowledge of mechanisms of drug resistance

- Which mechanisms are known?
  - Dapsone : mutation of *folP1* (Williams and Gillis 2000)
  - Rifampin: mutation of *rpoB* (Honoré and Cole 1993)
  - Clofazimine: multiple targets, potassium channel (Cholo 2006, Ren 2009)
  - Fluoroquinolones (ofloxacin, moxifloxacin): mutation of *gyrA* (Cambau 1997) or gyrB (Matrat 2009)
  - Clarithromycin: no resistant strain so far but mutation in *rrl* (23SrDNA) in other mycobacteria (Meier 1994, Wallace 1996)
  - Minocycline: no resistant strain so far but mutation in *rrs* (16SrDNA) in other bacteria
  - Quinolines (R207910): no resistant strain so far but mutation in *atpE* in other mycobacteria (Petrella 2006)

Endpoints in molecular detection of drug resistance in *M. leprae*

2. Good correlation between genotype and phenotype of resistance (mouse footpad susceptibility testing)

=> genotype will predict for phenotype

Concordance of genotype (*rpoB* mutations) and phenotype (mouse footpad susceptibility testing) for rifampin resistance

Results for NRC Paris, France 1989-2006, 123 biopsies

genotype result	Susceptibility result		
	Rif-R	Rif-S	No growth
rpoB mutation	23	1*	1
WT	1**	46	51
Total	24	47	52

*Ser522Cys, 0 /5 mice
**2/7 mice

Concordance = 69/71 = 97%

Updated Cambau 2002 , Honore 1993

Concordance of genotype (*rpoB* mutations) and phenotype (mouse footpad susceptibility testing) for dapsone resistance

Results for NRC Paris, France 1989-2006

Level of résistance	N	Mutation *folP1* at 53 or 55	
		Yes	No
R-100	6	6	0
R-10	4	3	1
R-1	6	1	5
Total strains DDS-R	16	10	6
Strains DDS S	22	0	22

Overall sensitivity = 62.5 % (10/16) and Specificity = 100% (22/22)
Sensitivity for clinical resistance (R-10 or R-100) = 90% (9/10)
Sensitivity for clinical resistance in relapse cases = 100% (7/7)

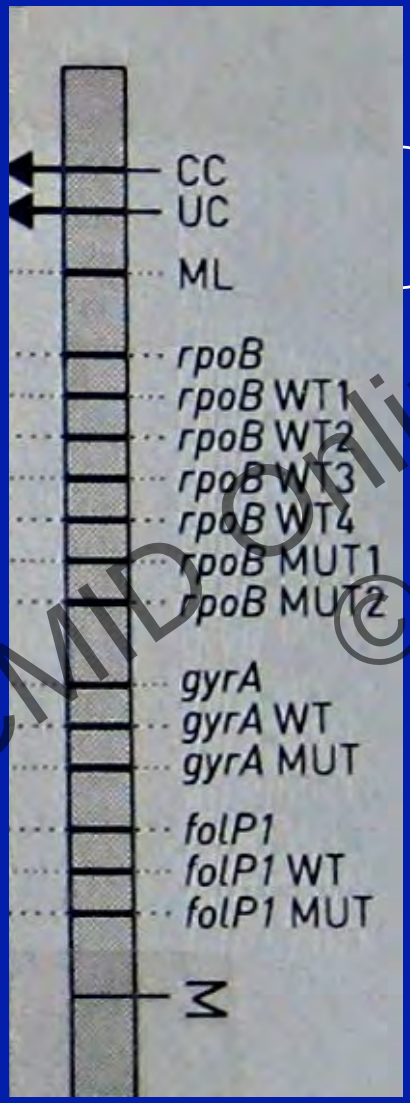
Matsuoka 2007, Cambau 2006

Genotypic methods for detection of drug resistance

- Usually start by gene amplification
- Followed by:
  - DNA fragment analysis without determination of the nucleotide sequence (heteroduplexes): DGGE, DHPLC, SSCP, Touch-Down
  - Determination of the nucleotide sequence: PCR-sequencing, DNA chip, pyrosequencing
  - Hybridization with oligonucleotide probes for mutation and WT sequence: Real time PCR, Reverse hybridation, Microarray, FISH...

Honore 1993, Kim 2003, Saokota 2006 and 2008, Williams 2004, Matsuoka 2008

GenoType LepraeDR DNA strip



Control probes

Presence of *M leprae* DNA

Rifampicin resistance

MUT1 : His451Tyr (His526Tyr)

MUT2 : Ser456Leu (Ser531Leu)

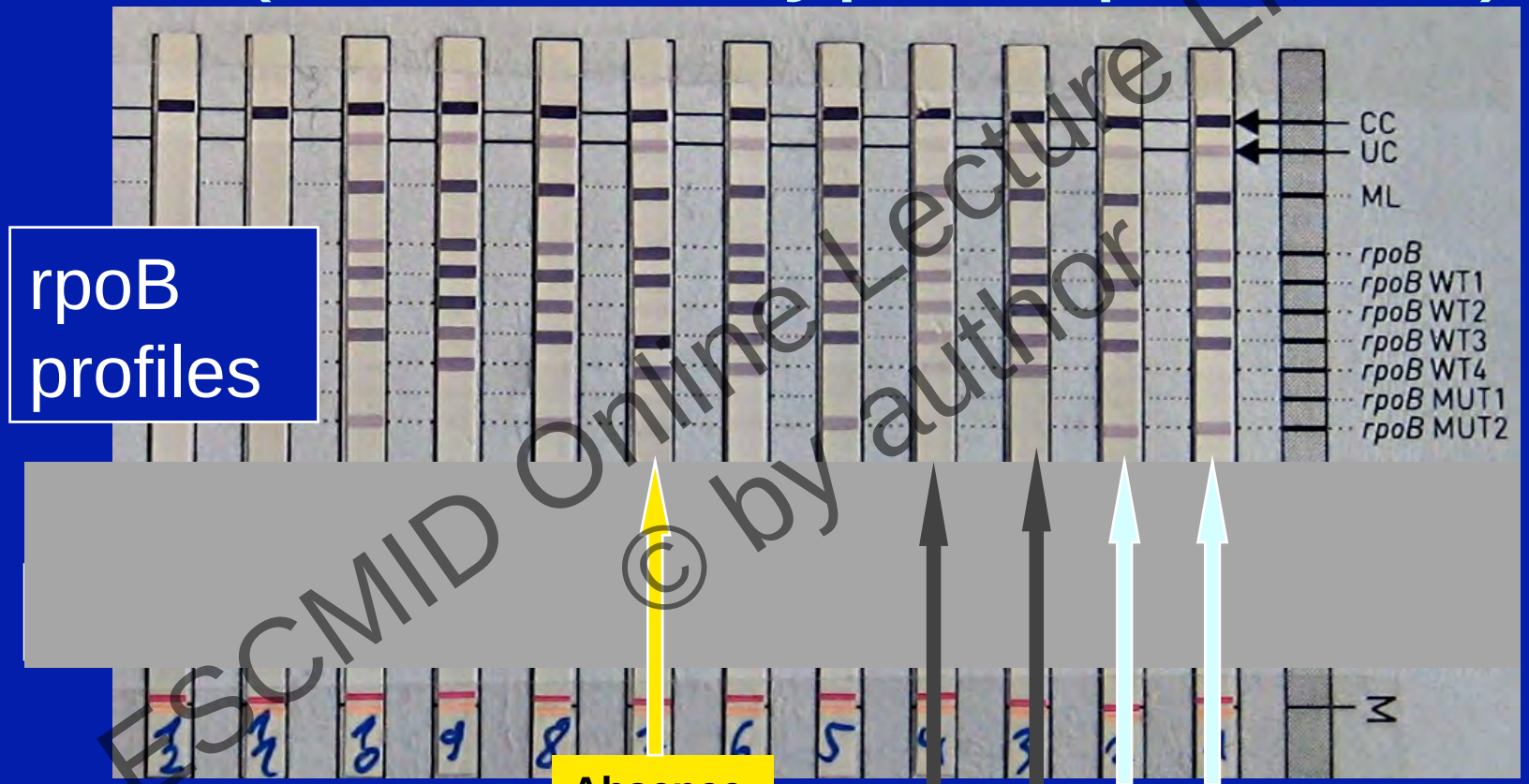
Fluoroquinolone resistance

gyrA MUT : Ala91Val

Dapsone resistance

folP1 MUT : Pro55Leu

Exemples of strips (test GenoType Leprae DR)



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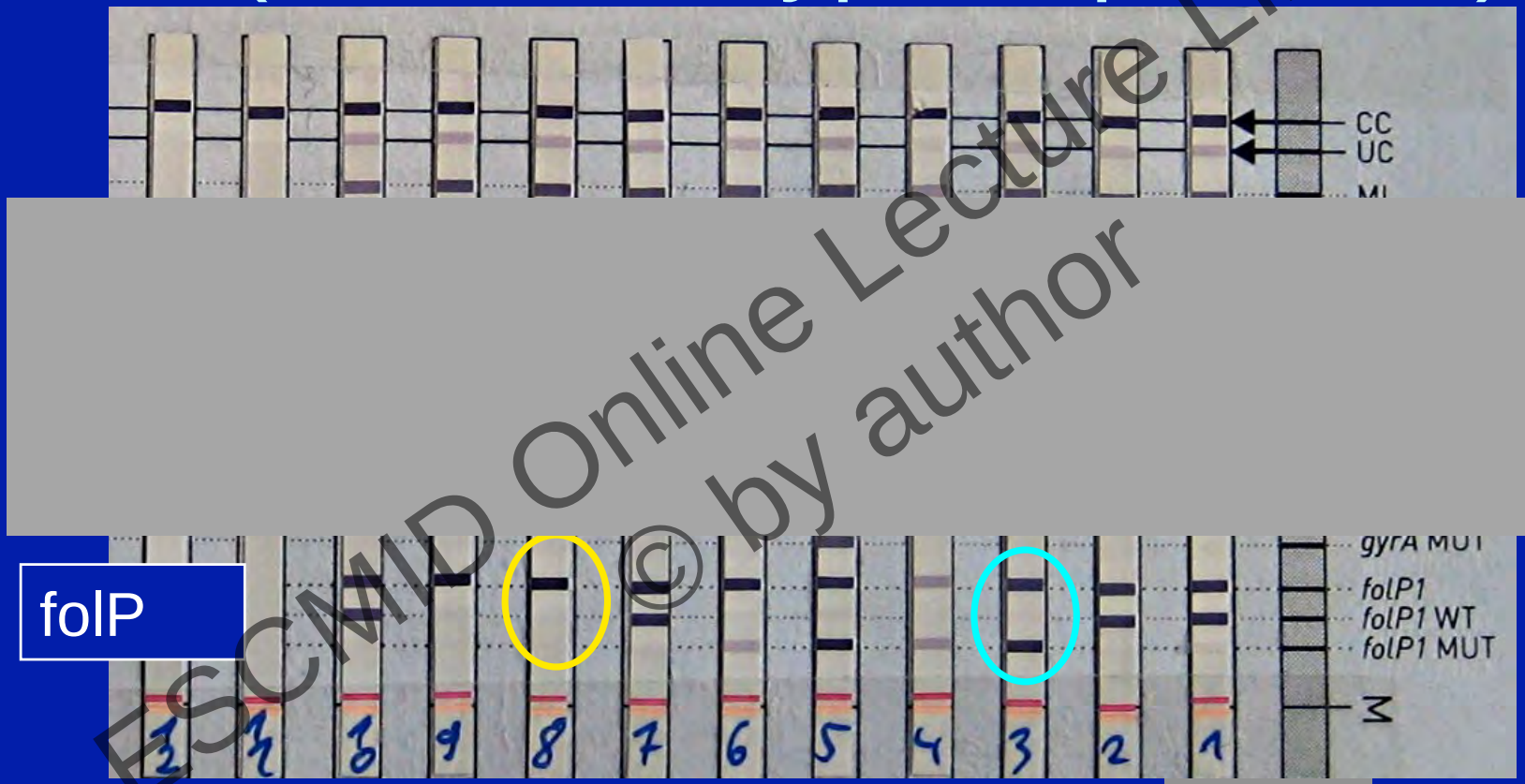


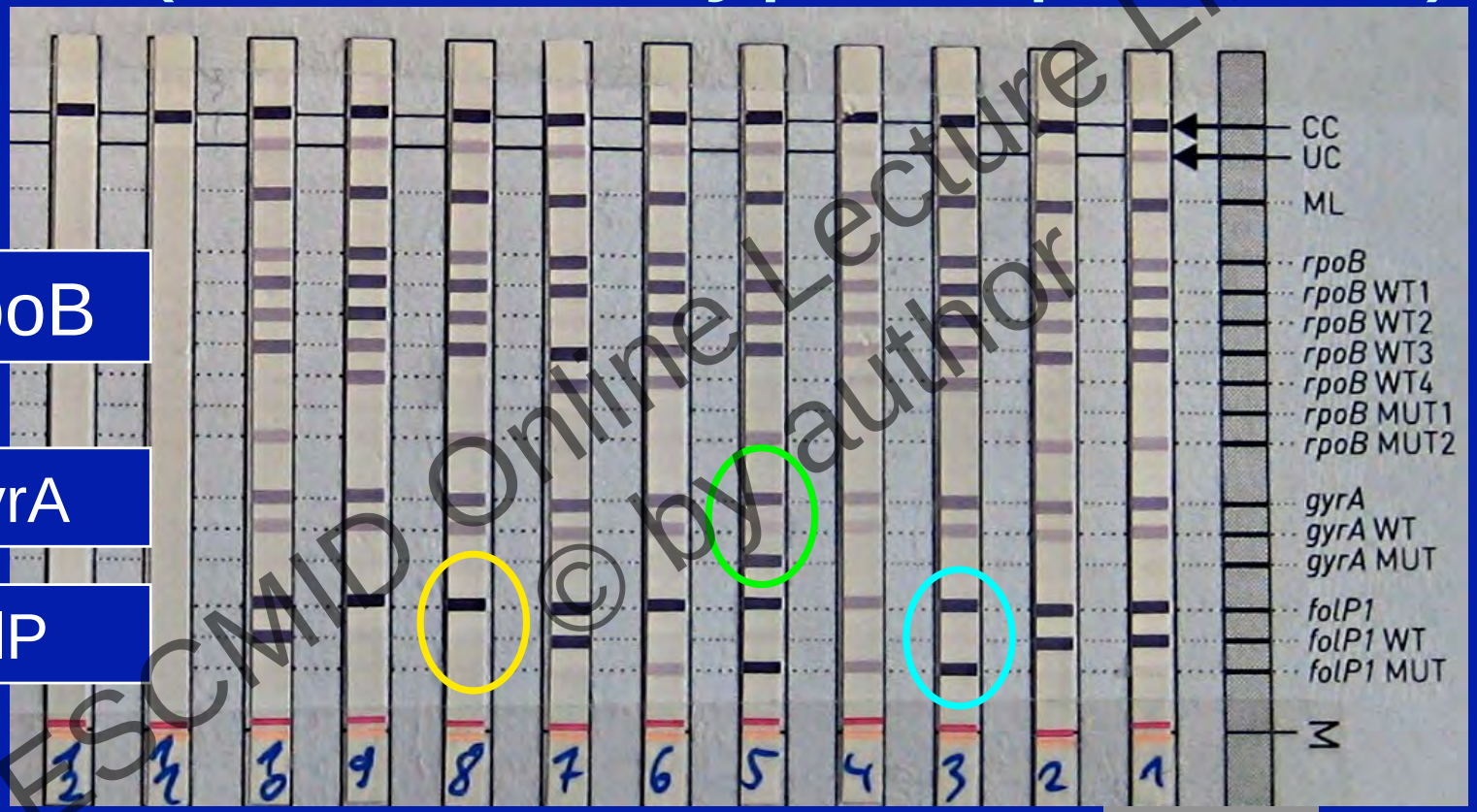
Absence of WTfolP = mutation T53I

folP mutation P55L

WT gyrA

WT folP

Exemples of strips (test GenoType Leprae DR)



rpoB

gyrA

folP

Absence of WTfolP = mutation T53I

A91V Mutation gyrA

folP mutation P55L

WT gyrA

WT folP

Evaluation study: concordance with susceptibility testing

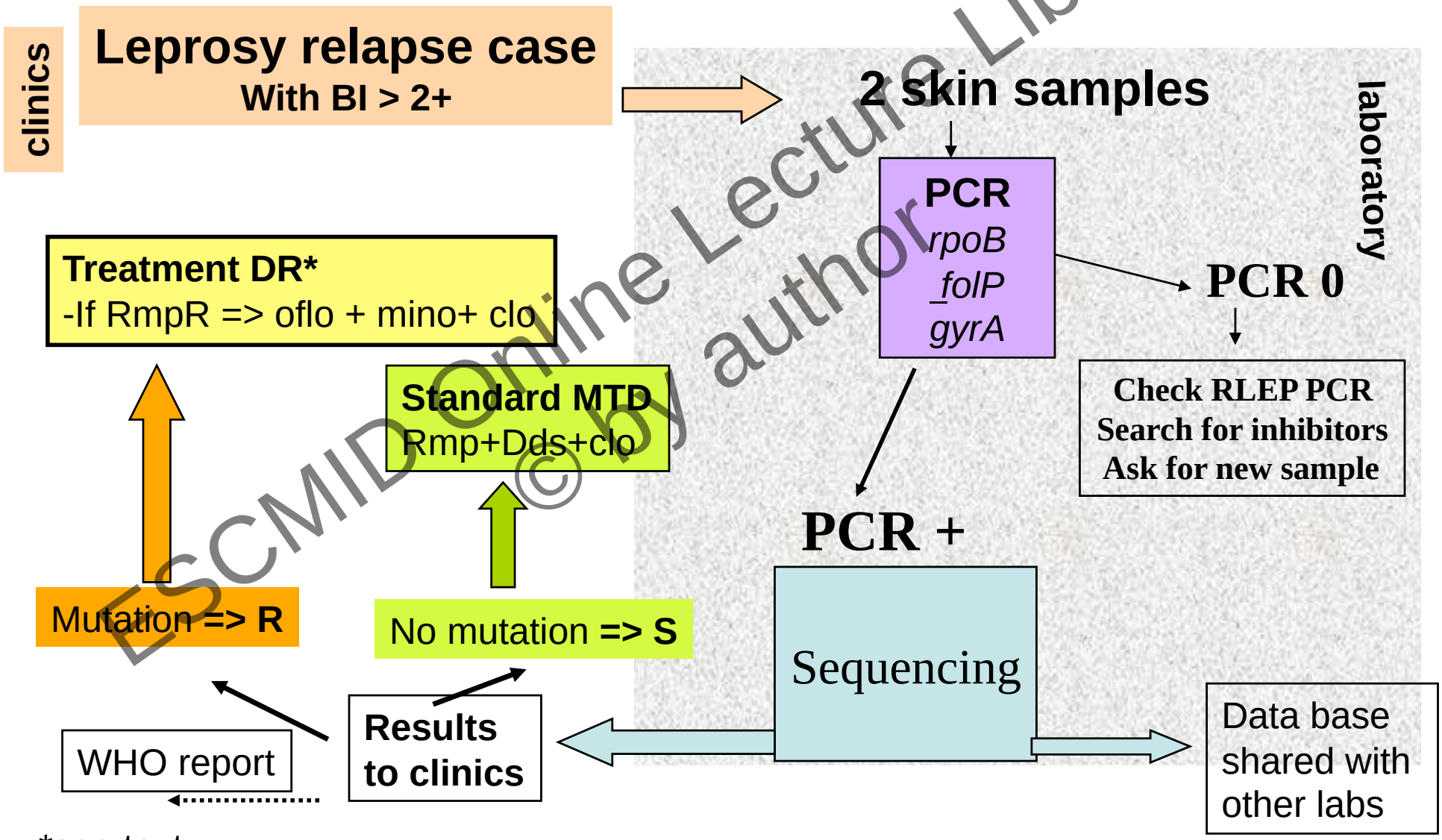
- 84 skin biopsies tested in the mouse footpad
  - For rifampicin: 13 R, 71 S
  - For dapsone: 8 R, 48S
  - For ofloxacin: 1 R, 4 S
- Concordance with resistance in the mouse footpad : 84/84 (100%)

- Concordance with PCR sequencing: 118/120 (98.3%)

False results for 2 strains with a mutation at codon 447: Ser447Cys for one strain and a silent mutation for the second strain: these strains were susceptible in the mouse footpad susceptibility testing

Cambau et al. PlOs NTD 2012

WHO network for surveillance of drug resistance in leprosy



*see text

Results for surveillance of resistance in France 1989-2009

- 199 cases
  - 102 new cases
  - 74 relapses
  - 23 follow up (patients BI+ after treatment)
- 13 rifampin resistant strains
  - 13 relapse cases
  - 11 from west indies, 1 Mali, 1 Cameroun

Resistant cases

Table 3. Characteristics of the 11 patients with rifampin-resistant (assessed by in vivo test in the mouse) leprosy.

Patient no.	Year of biopsy	Age in years	Country	Case	Year of first diagnosis	Previous treatment and duration	Susceptibility to other drugs
1	1989	41	FWI	Relapse	1956	Dds 1956–1960; Slf + Rif 1980–1982	Dds^R, Pth^S
2	1990	58	FWI	Relapse	1959	Dds 1959–1968; Slf 1968–1980; Slf + Dds + Rif + Clf 1980–1987	Dds^R
3	1991	45	FWI	Relapse	1961	Dds 1961–1974; Dds + Rif 1974–1975	Dds^R, Pth^S
4	1992	55	FWI	Relapse	1956	Dds 1956–1973; Rif 1973–1974; Dds + Slf 1975–1991	Dds^R, Pth^S
5	1992	35	Mali	Relapse	1979	Dds 1979–1991; Rif + Ofx 1 month 1992	Dds^R, Pth^R, Ofx^R
6	1992	50	FWI	Relapse	1958	Dds 1958–1977; Rif 1977–1979; Rif + Pth	Dds^R, Pth^R
7	1993	57	Cameroon	Relapse	1960	Dds 1961–1975; Pth + Rif 1975–1992	Dds^S, Pth^S
8	1995	64	FWI	Relapse	1946	Dds 1946–1978; Slf 1979–1982; Rif 1983–1995	Dds^S, Pth^S
9	1995	30	FWI	Relapse	1980	Dds + Rif + Pth 30 months	Dds^S, Pth^S
10	1997	45	FWI	Relapse	1950	Dds for 11 years	Dds^S, Pth^S
11	1999	69	FWI	Relapse	1947	Dds 1947–1972; Dds + Rif 1973–1976	Dds^R

NOTE. Clf, clofazimine; Dds, dapsone; FWI, French West Indies; Ofx, ofloxacin; Pth, prothionamide; R, resistant; Rif, rifampin; S, sensitive; Slf, sulfonamides.

2001: Martinique, relapse, dds, dds+Rmp, DdsS
2002: Martinique, relapse, dds, Rmp, DdsS

Conclusions

- *M leprae* resistance does exist in the world
- Acquisition of resistance is similar to that occured in M. tuberculosis
- Leprosy susceptibility testing is difficult since it required in mouse studiesand Years experiements
- Resistance detection is now mostly done using genotypic detection of mutations known to confer resistance
- However new mutations need to be tested in vitro models (purified targets) or in animal models