

**National Institute for Infectious Diseases L. Spallanzani**

Identification of antigens and antibodies in body fluids  
*Delia Goletti, MD, PhD*

*Münchenwiler, March 25<sup>th</sup>, 2010*



# Agenda

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- Problems in the diagnosis of TB
- Microbial markers:
  - in sputum
  - In non sputum samples
- Serological tests



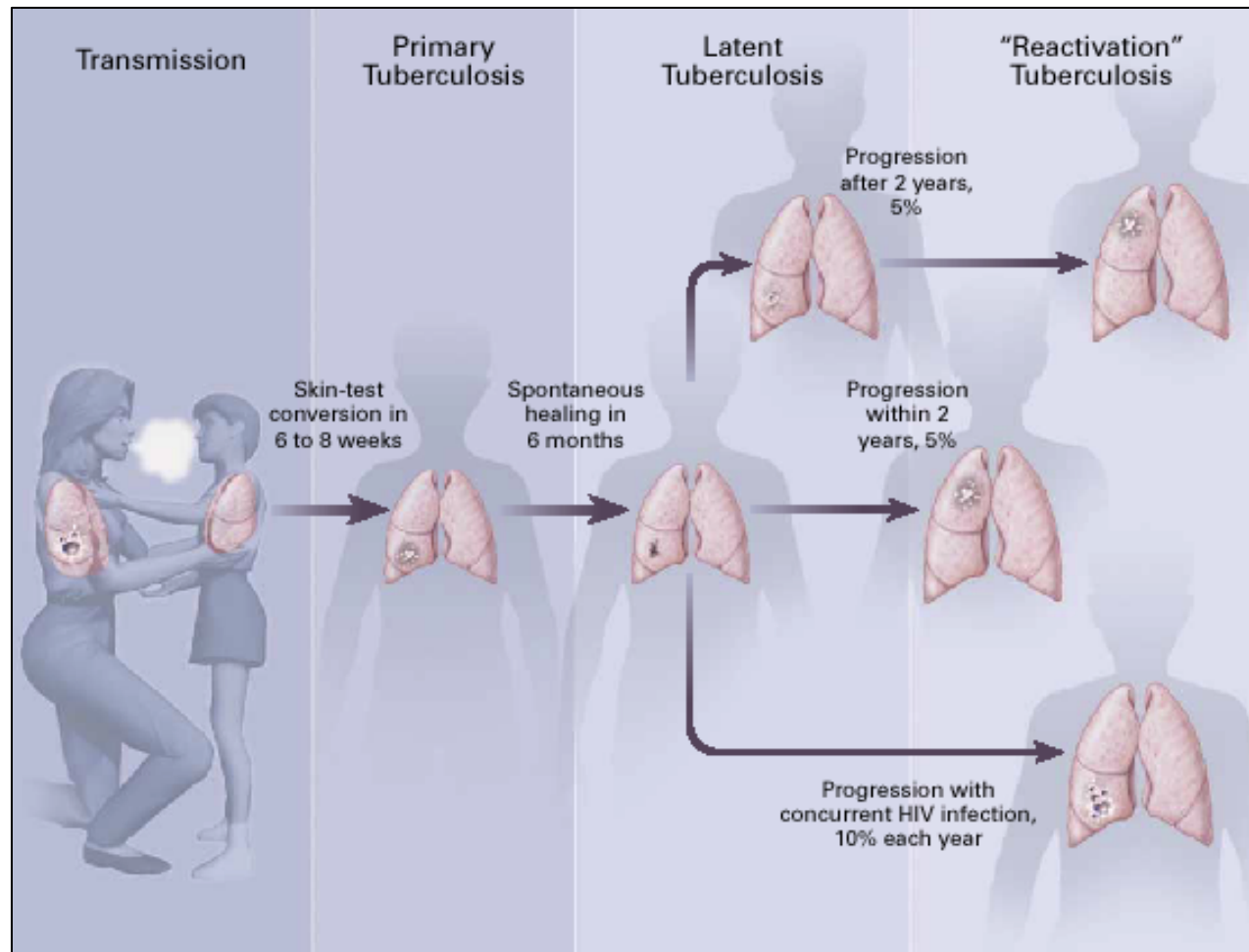
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# Tuberculosis transmission and progression to active disease from latent infection



*Small PM et al, N Engl J Med, 2001*



## Limits of TB Diagnostic Assays

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- **Active disease**
  - Gold standard is isolation of the bacillus by culture:
    - Long time needed (2–6 weeks)
    - Low yield (only 70-85%)
  - Difficult to obtain expectorated sputum



# Agenda

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- Problems in the diagnosis of TB
- Microbial markers in sputum:
  - Ag85
  - mRNA 85B
  - lipid bodies



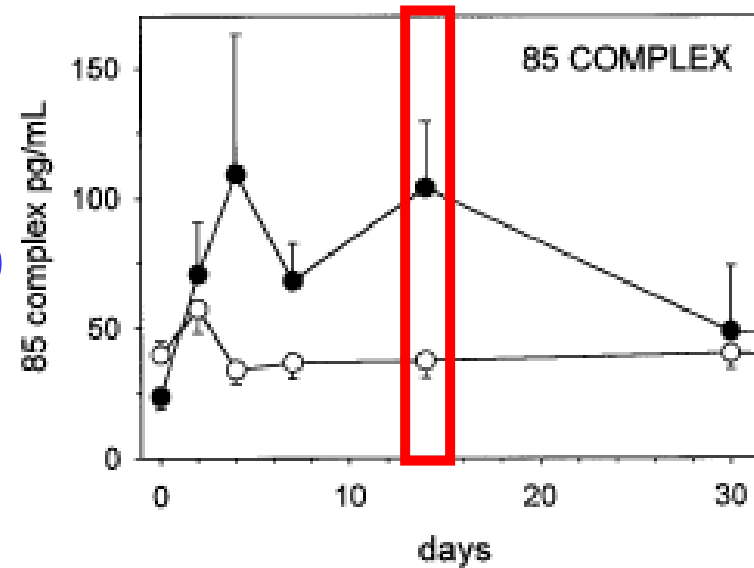
## Microbial markers in sputum

- The antigen 85 complex is critical in cell wall biosynthesis and is induced by isoniazid *in vitro*. Its induction may represent an adaptive transition to a persistent state during therapy
  - *M. tuberculosis* antigen 85 (Wallis RS, JID 1998)
  - *M. tuberculosis* 85B RNA (Desjardin LE, AJRCCM 1999)



## *M. tuberculosis* Antigen 85 in sputum

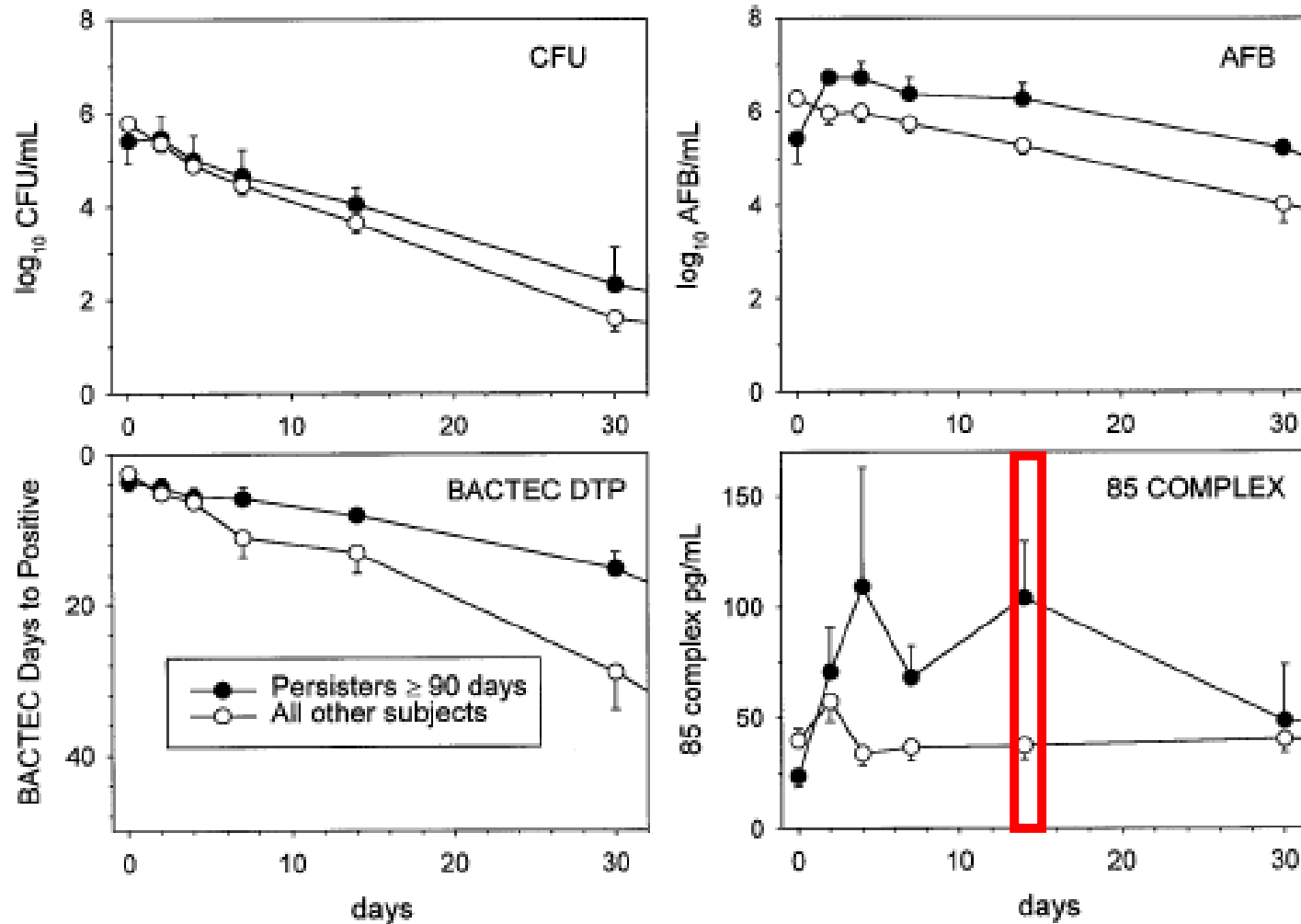
- 42 patients with pulmonary TB
- Antigen 85 expression increased in subjects in whom disease persisted (**persisters**) from days 0 to 14 when the difference between persisters and **nonpersisters** was statistically significant ( $P=0.002$ ).
- Only antigen 85 complex values at day 14 suggested TB persistence at or after day 90 ( $>60$  pg/mL).





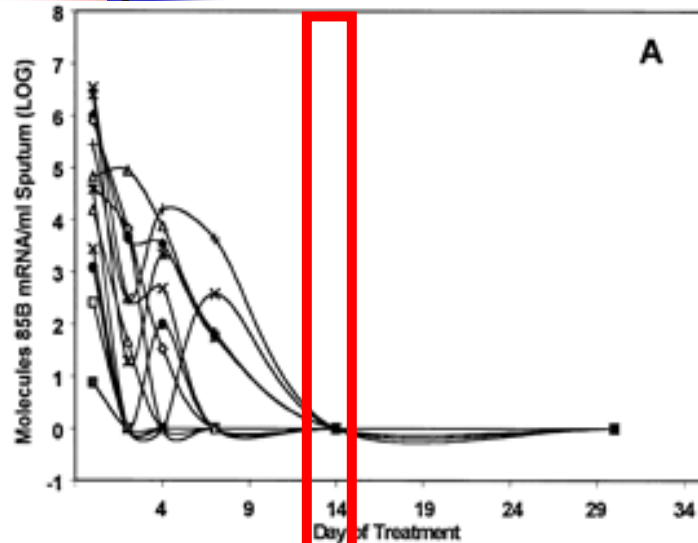


# *M. tuberculosis* Antigen 85 in sputum

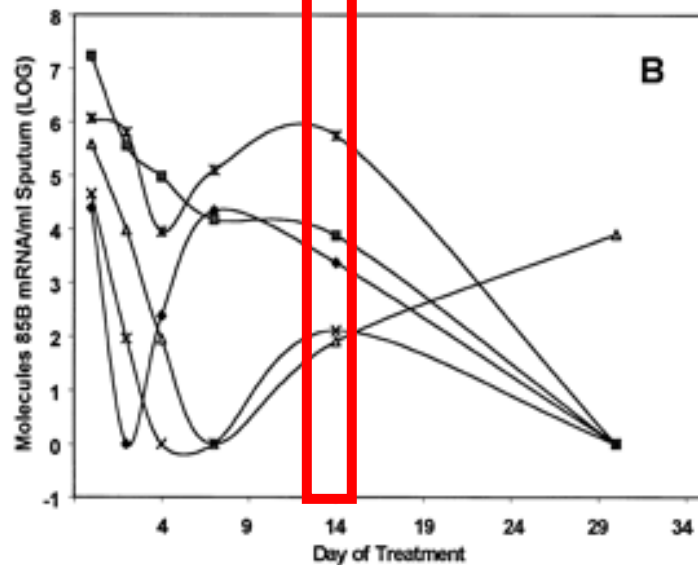




## *M. tuberculosis* 85B mRNA in sputum

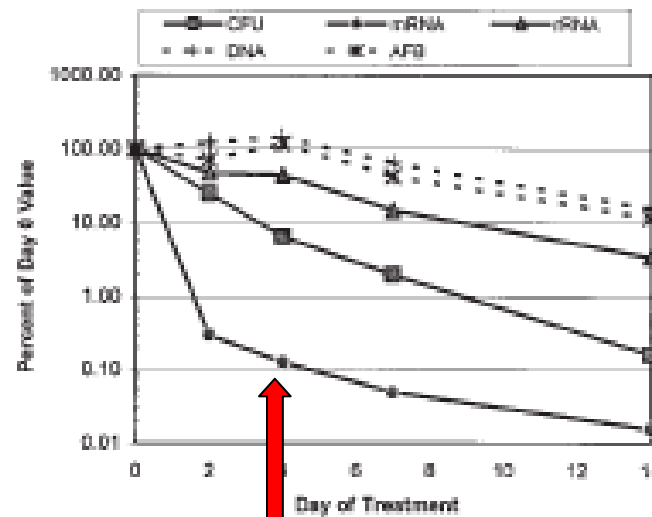


- The 14 patients whose 85B mRNA fell to an undetectable level by day 14 of treatment



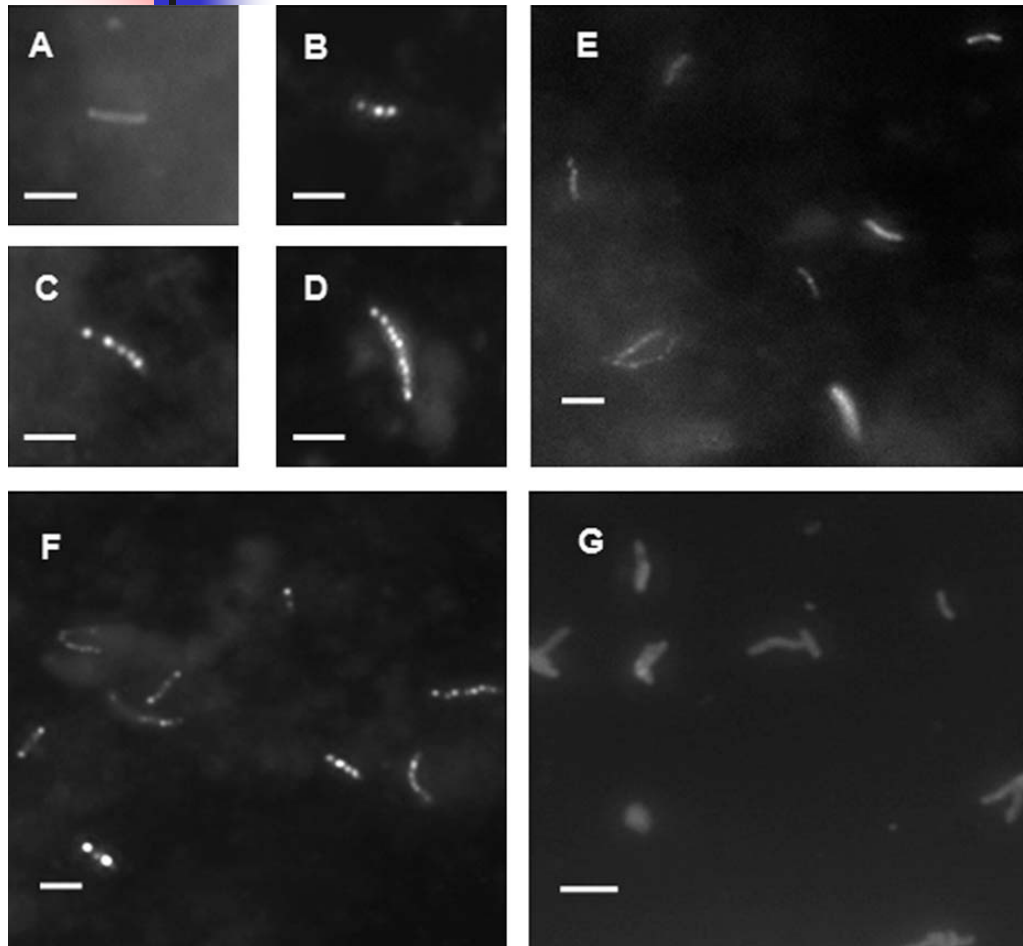
- The 5 patients whose 85B mRNA became negative after 14 days of therapy.

# *M. tuberculosis* 85B mRNA in sputum decay compared to AFB, CFU, DNA and rRNA





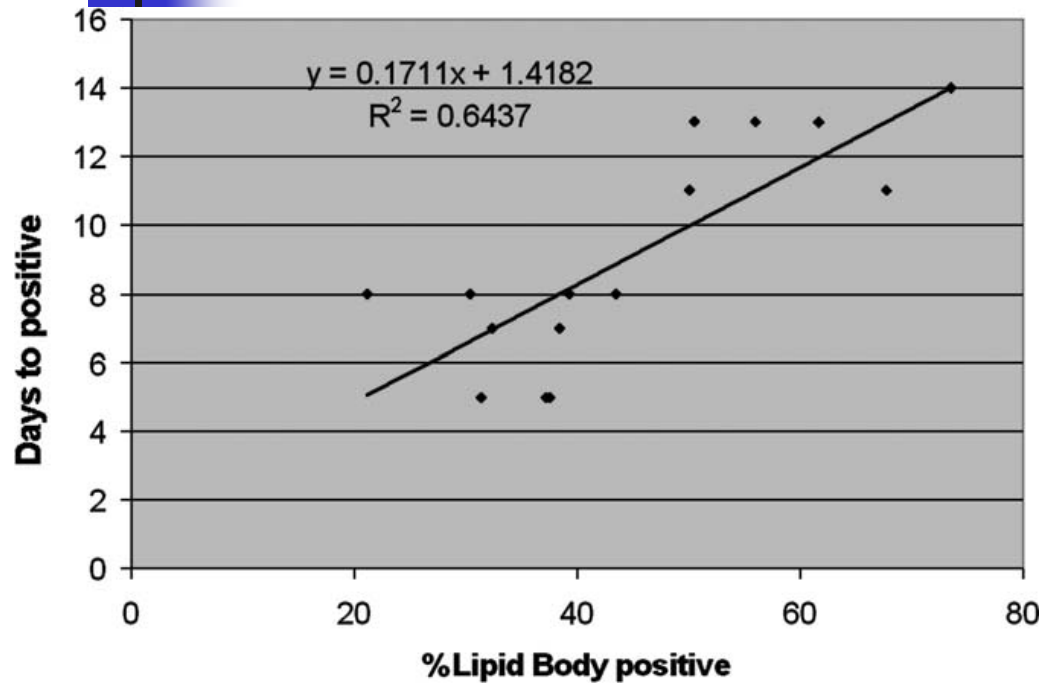
## Lipid Bodies in sputa



- Lipid Bodies in Tuberculous Sputum Samples. Auramine/Nile red-fixed sputum smears and aerobic *M. tuberculosis* growth.
- Variation in lipid bodies per cell:
  - (A) none,
  - (B) three,
  - (C) five, and
  - (D) eight.
  - Samples are shown with (E) low and (F) high proportions of lipid body-positive cells.
  - (G) Aerobically grown mid-log *M. tuberculosis* H37Rv contained negligible lipid bodies.



## Lipid Bodies in sputa



- Time to Positivity in BACTEC Cultures Related to Lipid Body counts determined in the Samples from Which the Cultures Were Prepared



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- Problems in the diagnosis of TB
- Microbial markers in non sputum samples:
  - breath biomarkers
  - urine biomarkers



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## Breath biomarkers of tuberculosis

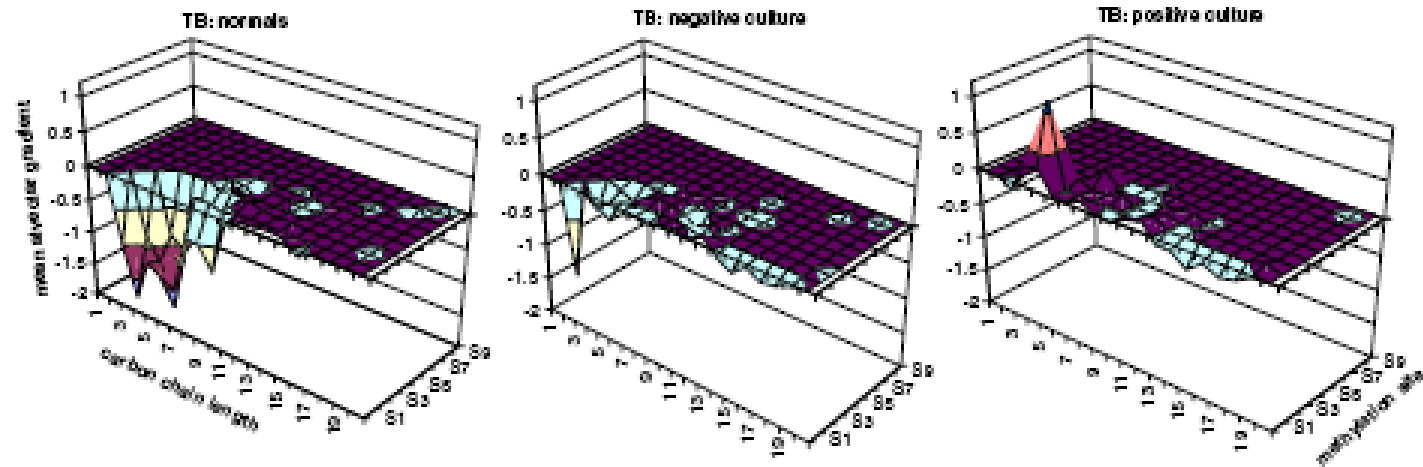
- New research is being done on using electronic noses (E-nose) to identify and characterize volatile organic compounds (VOCs) in sputum and breath from TB patients
- An E-nose is the colloquial name for an instrument made up of chemical sensors (polymer, metallic oxide, bulk acoustic wave or surface acoustic wave sensors) with a pattern recognition system
- The reversible adsorption of VOCs to the sensor surface leads to a change in physical properties (change in resistance and frequency of the sensor) that can be measured





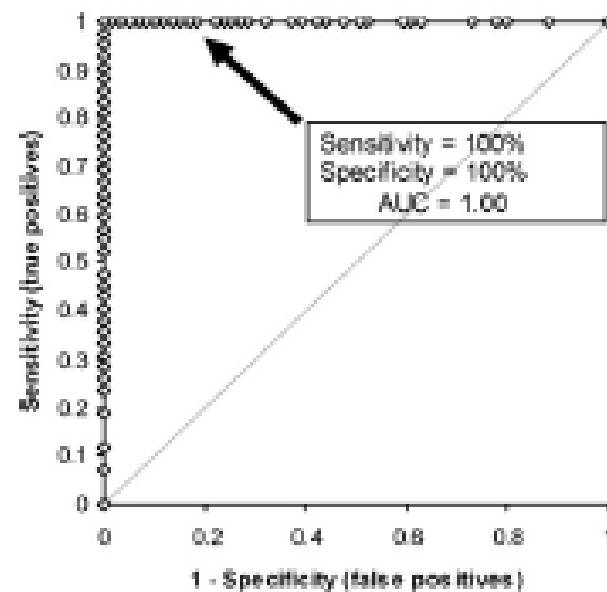
# Breath biomarkers of tuberculosis

## Breath biomarkers of tuberculosis

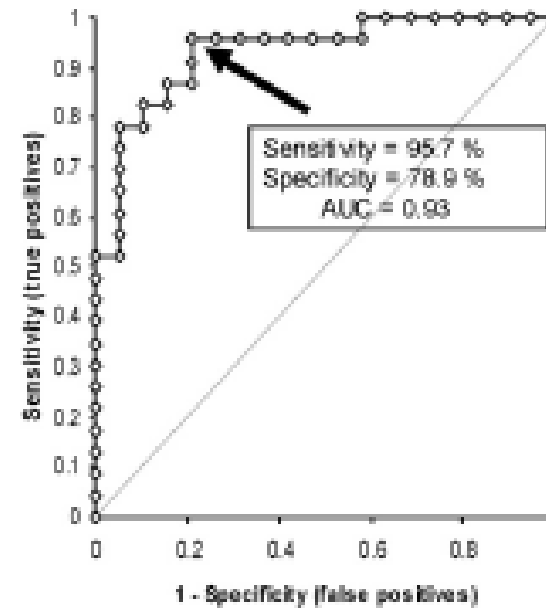




# Accuracy of the breath test



Hospitalized patients  
versus healthy controls



Mycobacterial sputum culture positive  
versus culture negative patients



## Limits of TB Diagnostic Assays

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- Problems in the diagnosis of TB
- Microbial markers in non sputum samples:
  - breath biomarkers
  - urine biomarkers



## General advantages of urine samples

- Noninvasiveness (for the patient)
- Safe specimen acquisition and handling compared to blood and expectorated sputum collection (for Health Care Workers)
- High specimen stability
- Possibility to obtain also from children



## LAM

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- Lipoarabinomannan (LAM) is a major lipopolysaccharide constituent of the cell wall of *M. tuberculosis*
- ELISA-based tests are now available



# LAM detection in urine: HIV-negative vs HIV+ patients (Tanzania)

Table 2: Diagnostic test performance of LAM-ELISA (groups A and B were defined as gold standard positives, Group C as negative controls, other groups with undefined TB status were excluded)

	Sensitivity % (95% CI)	Specificity % (95% CI)	Positive predictive value % (95% CI)	Negative predictive value % (95% CI)	Positive diagnostic likelihood ratio (95% CI)	Negative diagnostic likelihood ratio (95% CI)
<b>Subgroup (n)</b>						
<b>LAM positivity in at least one out of two urine samples</b>						
All (151)	50.7 (38.4-63.0)	87.8 (78.7-94.0)	77.8 (62.9-88.8)	67.9 (58.2-76.7)	4.16 (2.23-7.78)	0.56 (0.44-0.72)
Females (79)	66.7 (47.2-82.7)	83.7 (70.3-92.7)	71.4 (51.3-86.8)	80.4 (66.9-90.2)	4.08 (2.06-8.08)	0.40 (0.24-0.67)
Males (72)	38.5 (23.4-55.4)	93.9 (79.8-99.3)	88.2 (63.6-98.5)	56.4 (42.3-69.7)	6.35 (1.56-25.80)	0.66 (0.50-0.85)
HIV-ve (64)	21.1 (6.1-45.6)	91.1 (78.9-97.5)	50.0 (15.7-84.2)	73.2 (59.7-84.2)	2.37 (0.66-8.50)	0.87 (0.49-1.11)
HIV+ve (87)	62.0 (47.2-75.3)	83.8 (68.0-93.8)	83.8 (68.0-93.8)	62.0 (47.2-75.3)	3.82 (1.78-8.21)	0.45 (0.31-0.66)

-ve = negative | +ve = positive



# LAM detection in urine of HIV+ patients associated with TB disease (South Africa)

Table 1. Sensitivity of tests to diagnose tuberculosis by identification of acid-fast bacilli in sputum, by detecting urinary lipoarabinomannan in unconcentrated and concentrated urine specimens or by using a combination of tests. Data are also stratified by blood CD4 cell counts.




Patient Group	Sputum AFB		LAM (unconcentrated urine)		LAM (concentrated urine)		Sputum AFB + LAM (concentrated urine)	
	Positive (n)	Sensitivity (95% CI)	Positive (n)	Sensitivity (95% CI)	Positive (n)	Sensitivity (95% CI)	Positive (n)	Sensitivity (95% CI)
All patients (n=58)	8	0.14 (0.07-0.25)	19	0.33 (0.22-0.46)	22	0.38 (0.27-0.51)	26	0.45 (0.33-0.58)
CD4 cell count <50 (n=18)	2	0.11 (0.02-0.34)	12	0.67 (0.44-0.84)	12	0.67 (0.44-0.84)	12	0.67 (0.44-0.84)
CD4 cell count 50-100 (n=17)	3	0.18 (0.05-0.42)	6	0.35 (0.17-0.59)	7	0.41 (0.22-0.64)	9	0.53 (0.31-0.74)
CD4 >100 (n=23)	3	0.13 (0.04-0.33)	1	0.04 (0.00-0.23)	3	0.13 (0.04-0.33)	5	0.21 (0.09-0.42)

AFB, acid-fast bacilli; LAM, lipoarabinomannan; TB, tuberculosis.





## LAM in urine: HIV+ vs HIV- (South Africa)

Definite and non TB groups only	All		HIV Negative		HIV Positive		HIV positive CD4 <200 cells/ $\mu$ l	
	Sens. (95%CI)	Spec. (95% CI)	Sens. (95%CI)	Spec. (95% CI)	Sens. (95%CI)	Spec. (95% CI)	Sens. (95%CI)	Spec. (95% CI)
Sputum smear	65 (57, 72)	100 (98, 100)	72* (61, 81)	99 (95, 100)	49* (35, 63)	100 (91, 100)	37** (19, 59)	100 (82, 100)
Urine LAM	13 (8, 19)	99 (97, 100)	6 <sup>#</sup> (3, 14)	99 (95, 100)	21 <sup>#</sup> (11, 35)	100 (91, 100)	37 (19, 59)	95 (84, 99)
Sputum smear and/or urine LAM	68 (60, 75)	99 (97,100)	72 (61, 81)	100 (97,100)	58 (43, 72)	100 (91,100)	53** (32, 73)	100 (83,100)
	PPV (95%CI)	NPV (95% CI)	PPV (95%CI)	NPV (95% CI)	PPV (95%CI)	NPV (95% CI)	PPV (95%CI)	NPV (95% CI)
Sputum smear	100 (95,100)	78 (72,83)	98 (91,100)	83 (76,88)	100 (85,100)	65 (53,78)	100 (65,100)	60 (42,75)
Urine LAM	94 (74,99)	59 (53,64)	83 (44,97)	59 (52,66)	100 (70,100)	55 (43,65)	78 (45,94)	76 (63,86)
Sputum smear and/or urine LAM	99 (94,100)	79 (73,84)	100 (94,100)	83 (76,88)	100 (87,100)	69 (57,80)	100 (72,100)	67 (48,81)
% of sputum smear neg definite TB cases detected by urine LAM	8.6 		0		17.6 		25.4 	



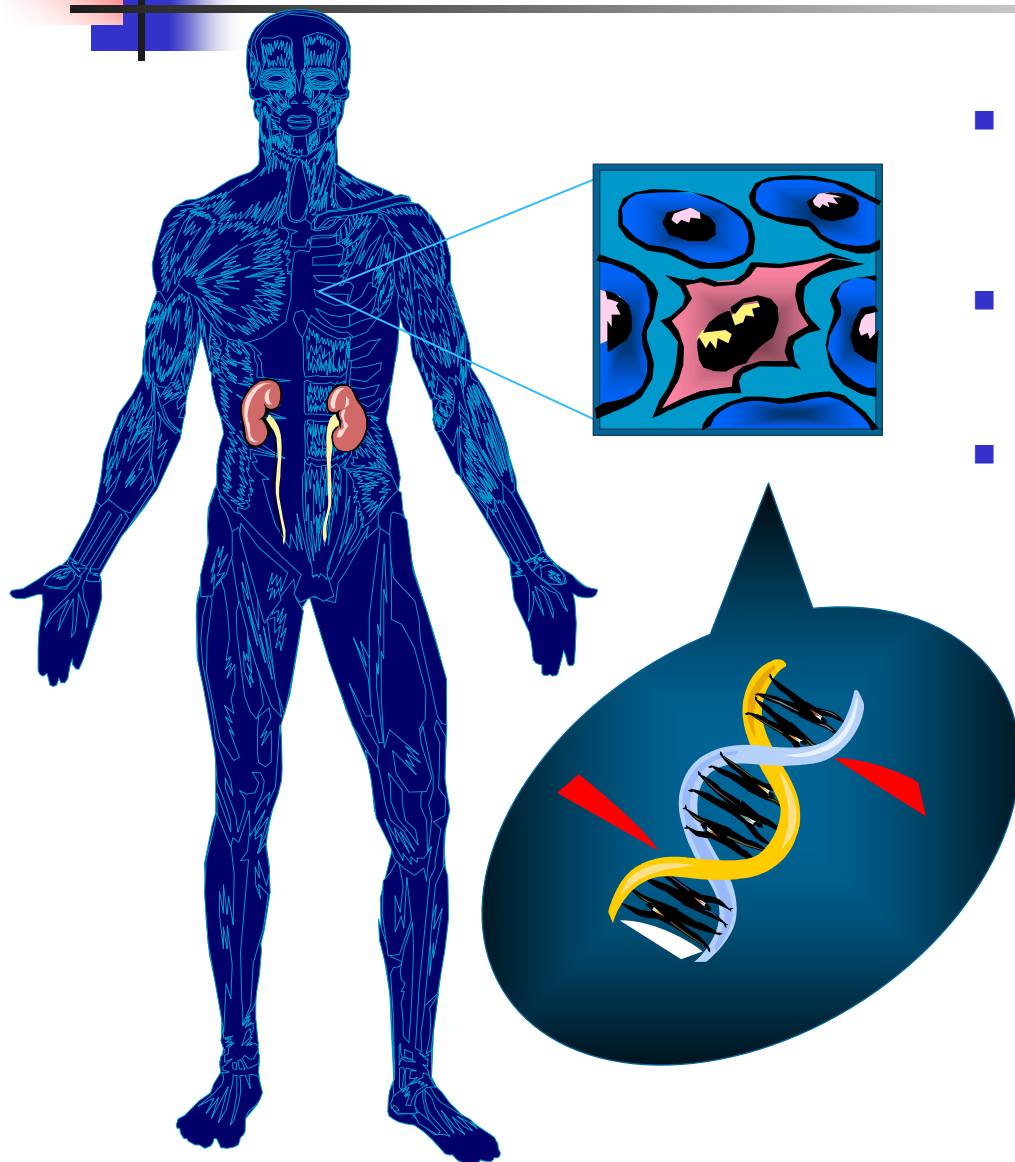
## LAM in urine

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- Higher sensitivity in HIV+, especially with low CD4 T cell counts (hyper-gammaglobulinemia?)
- Good when combined with smear AAR on sputum
- Potential predictor of IRIS (?)



# Tr-DNA: a major advance in diagnostics technology

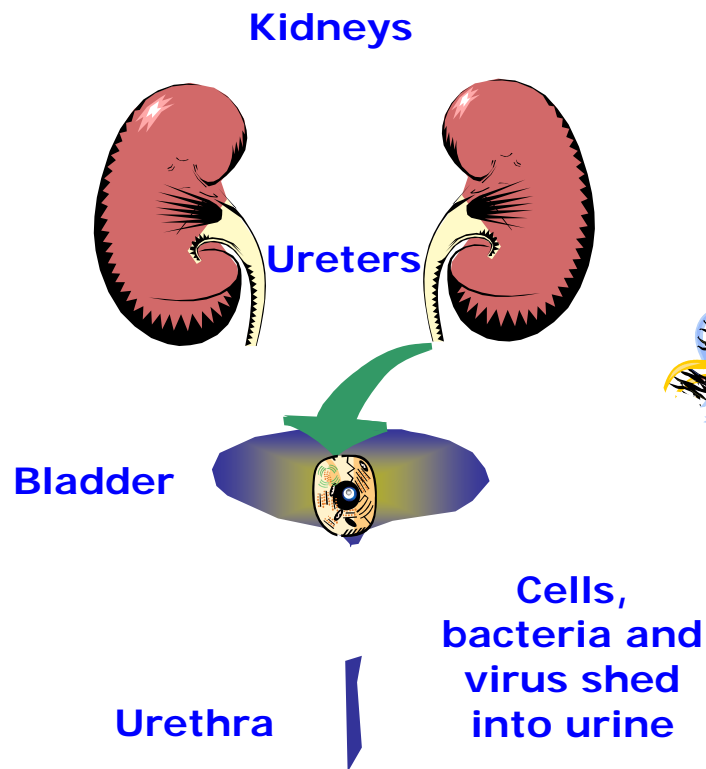


- Nuclear DNA is broken into packets of genetic material
- DNA fragments carried away in blood stream
- Some of this DNA is filtered through kidneys and appears in urine

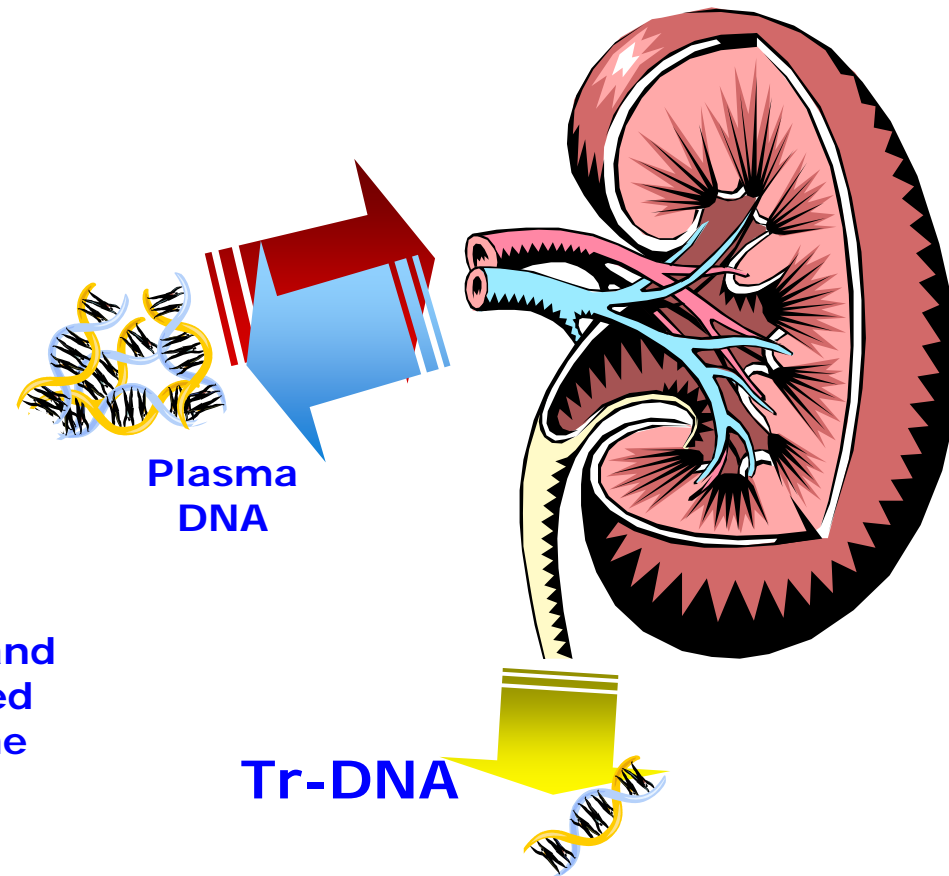
Tr-DNA test analyzes this “transrenal” DNA, searching for genetic signatures from precancerous cells, tumors, a developing fetus, or transplanted organ



# "Urine DNA" vs Transrenal DNA



Often called  
"urine DNA"



**Transrenal-DNA**

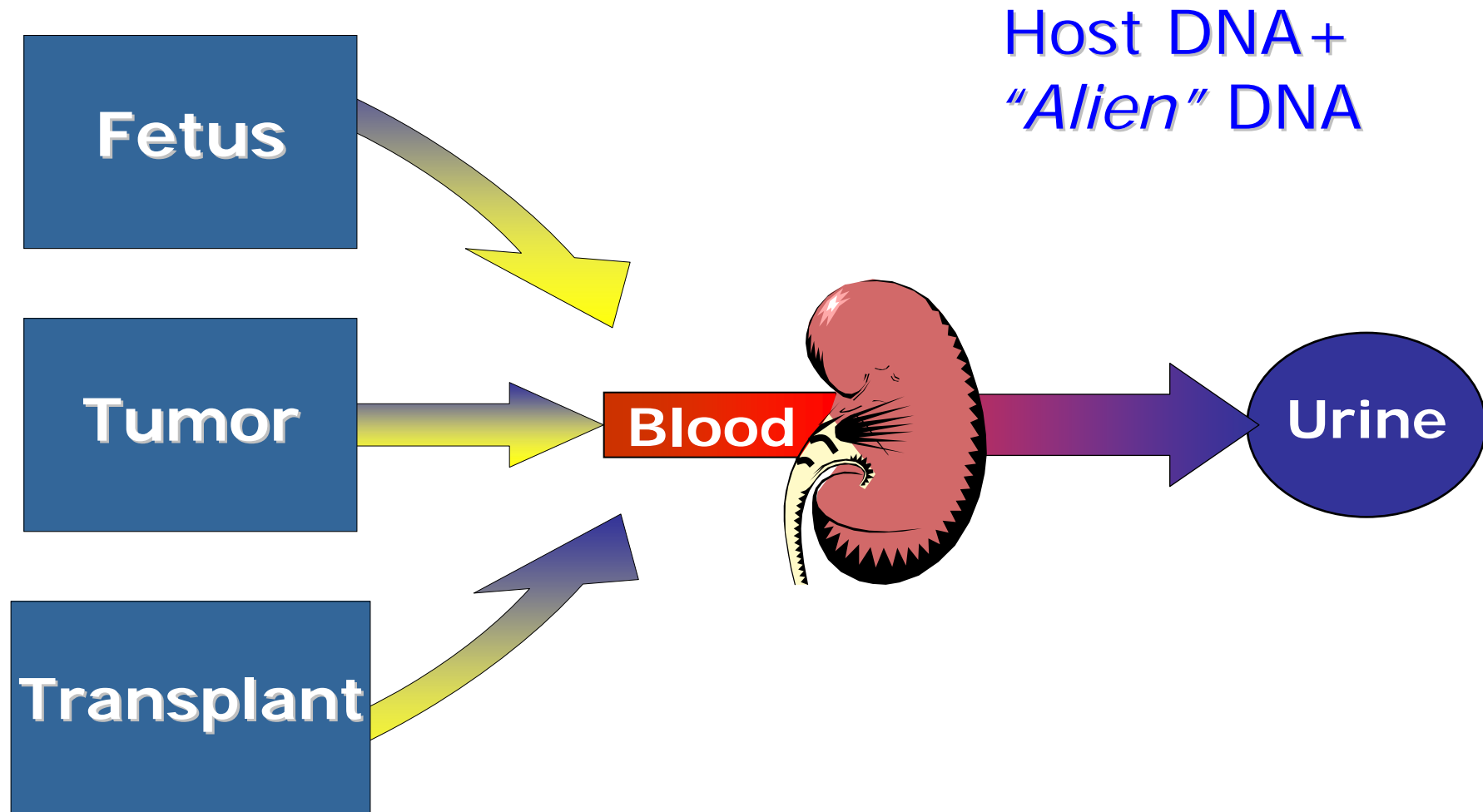


## Characteristics of tr-DNA

- DNA fragment (150-200 bp) that are excreted from the blood stream into the urine (urine supernatants, free DNA)
- Contains genetic information from different cell types throughout the body
- Methods of DNA isolation and analysis are specific



## Origin of the alien DNA in Urine





## Methods

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- Collection of urine specimens (anti-nucleases agents: EDTA)
- Tr-DNA extraction (guanidine and resin)
- Amplification by semi-nested PCR, with target on the IS6110 region (short amplicon size)
- Analysis of PCR products by gel electrophoresis



## Methods


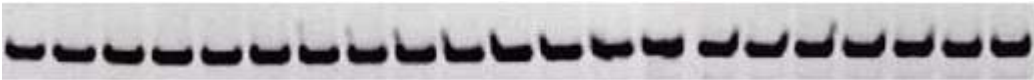
<b>ID</b>	<b>Sequence</b>	<b>Location *</b>	<b>Product, bp</b>
F-785	ACCAGCACCTAACCGGCTGTGG	785	129
R-913	CATCGTGGAAGCGACCCGCCAG	913	
Rn-851	GTAGGCGAACCTGCCCAGGTC	851	67
F-489	GCCCCATCGACCTACTACG	489	330
R-819	TGAGGTCTGCTACCCACAGC	819	
Fn-627	CCCTGAACCGTGAGGGCATCG	627	69
Rn-690	ACAGGCCGAGTTTGGTCATCAGC	690	

- \* Nucleotide numbering is based on the consensus created from the alignment of IS6110 elements nucleotide sequences

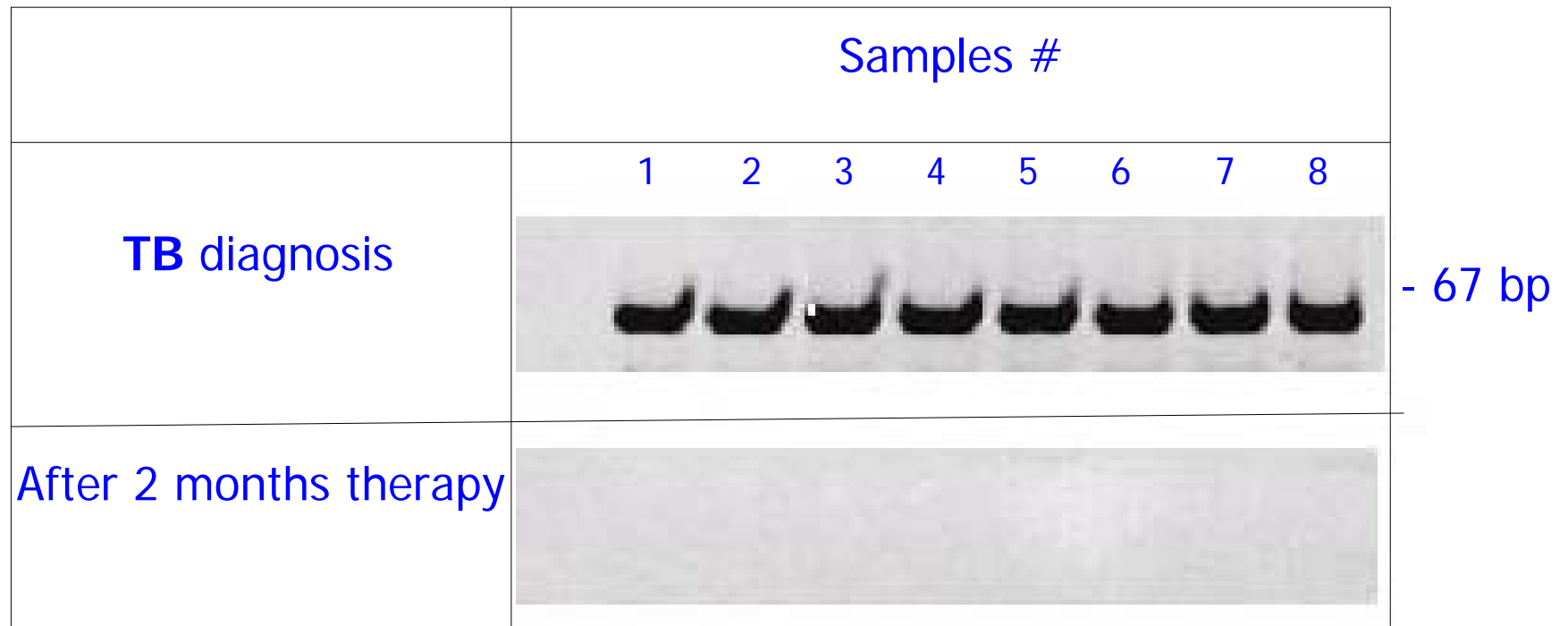


# *M. tuberculosis* Transrenal DNA Is Present in Patients with Active Pulmonary TB Disease



	Samples #
	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Healthy controls	
Active pulmonary TB	 -67 bp

# *M. tuberculosis* Transrenal DNA Is Present During Active Pulmonary TB and Is Lost After an Efficacious Therapy

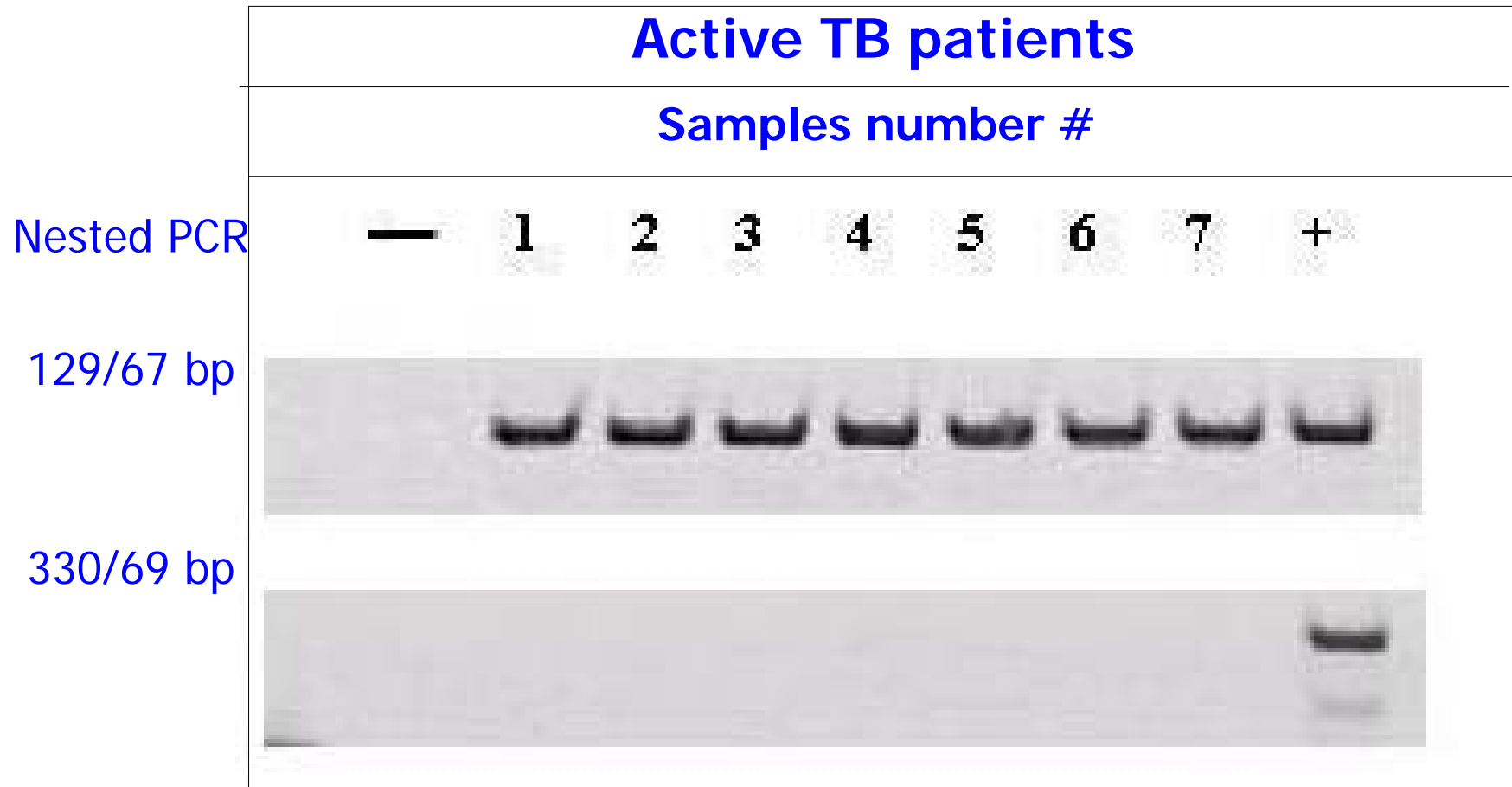




## Characteristics Consistent with tr-DNA

- Present in urine supernatants
- Short DNA fragment (lower than 200 bp)

# Detection of *M. tuberculosis* Transrenal DNA Is Based on the Recovery of Short DNA Fragments





## Remarks

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*M. tuberculosis* specific Tr-DNA was not detected before because:

- DNA isolation techniques were not designed for short DNA fragments
- Larger amplicon size were used
- DNA detection was performed on urine cell sediments



## Conclusions

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*M. tuberculosis* specific Tr-DNA is :

- present in urine of pulmonary TB patients
- lost after an efficacious anti-TB therapy
- detected by using techniques optimized for short DNA fragments
- positive in urine supernatants



# General Advantages of Tr-DNA Technology

- Noninvasiveness (for the patient)
- Safe specimen acquisition and handling compared to blood and expectorated sputum collection (for HCW)
- High specimen stability
- High sensitivity of detection
- Analysis of Tr-DNA compatible with all current methods including PCR and non–amplification-based technologies
- Platform in common with diagnostic test for different diseases



# Agenda

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- Microbial markers:
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  - In non sputum samples
- Serological tests





# Serological tests: antigens evaluated

TABLE 2. Antigens evaluated for serodiagnosis of pulmonary TB

Name(s) of antigen(s) <sup>a</sup>	Protein Rv designation	Reference(s)
<b>Ag85C</b> , 32.5 kDa, FbpC2, MPT-45	0129c	76, 77
<b>38 kDa</b> , Ag 5, PstS1, PhoS, PhoS1	0934	3, 8, 15, 18, 20, 27, 33, 35, 37, 55, 59, 69, 76, 77, 81, 102
<b>Mtb 81/88 kDa</b> , malate synthase, GlcB	1837c	20, 35, 37, 76, 85, 86, 108
<b>DPEP</b> , MPT32; 45/47 kDa, Apa, ModD	1860	19, 26, 76
<b>Ag85B</b>	1886c	23, 67, 77, 103
16 kDa; $\alpha$ -crystallin; 14 kDa, HspX, Acr	2031c	33, 39, 66, 103
27 kDa; MPT51, FbpC1, MPB 51	3803c	10, 69, 85, 108
CFP-10, MTS.A-10, EscB; Lhp, Mtb11	3874	27, 33, 59, 118
ESAT-6	3875	33, 118
Mtb48	3881c	55
DAT		43, 44, 83, 106
TAT		43, 44
SL-I, sulfolipid I		43, 44
Card factor		43, 44, 101

<sup>a</sup> Boldface indicates the name of the antigen in common use.



# Accuracy of the serological tests

TABLE 4. Overall sensitivities, specificities, and likelihood ratios for antigens evaluated for serodiagnosis of pulmonary TB with assays detecting IgG and/or IgA antibodies

Type of compound	Antigen name	Rv designation	No. of studies	Smear status	HIV status	Sensitivity (%) <sup>a</sup>	Specificity (%) <sup>a</sup>	Likelihood ratio positive <sup>a</sup>	Likelihood ratio negative <sup>a</sup>
Recombinant	38 kDa	0934	12	Positive	+/-	47 (39-55)	94 (86-98)	8.22 (3.41-24.85)	0.56 (0.48-0.65)
	Malate synthase	1837c	8	Positive	+/-	73 (58-85)	98 (95-100)	40.78 (14.43-155.7)	0.27 (0.16-0.42)
	MPT51	3803c	5	Positive	-	59 (38-76)	94 (77-99)	10.50 (2.70-69.69)	0.44 (0.26-0.67)
	MPT51	3803c	4	Positive	+	58 (30-82)	97 (84-100)	19.03 (3.73-172.3)	0.44 (0.19-0.73)
	CFP-10	3874	6	Positive	+/-	48 (29-68)	96 (83-99)	12.11 (3.20-64.63)	0.55 (0.35-0.73)
	TbF6 <sup>b</sup>		4	Positive	-	70 (37-90)	93 (69-99)	9.61 (2.23-53.99)	0.33 (0.13-0.66)
	TbF6, DPEP <sup>c</sup>		4	Positive	-	75 (50-91)	95 (86-99)	14.97 (5.43-56.66)	0.26 (0.10-0.53)
Native protein	38 kDa	0934	13	Positive	+/-	49 (37-61)	97 (94-99)	15.73 (8.84-31.55)	0.53 (0.41-0.65)
	38 kDa	0934	7	Negative	-	31 (15-52)	97 (92-99)	9.13 (3.88-24.05)	0.72 (0.51-0.87)
	Ag 85B	1886c	4	Positive	-	53 (20-83)	95 (78-99)	9.36 (2.52-53.81)	0.51 (0.20-0.84)
	Ag 85B	1886c	4	Positive	+	62 (19-92)	97 (89-99)	17.83 (4.04-62.32)	0.39 (0.08-0.84)
	α-Crystallin	2031c	6	Positive	+/-	54 (32-75)	96 (83-99)	13.23 (3.52-66.61)	0.48 (0.28-0.71)
Lipid	DAT		7	Positive	+/-	63 (45-78)	81 (50-96)	3.32 (1.32-13.35)	0.47 (0.30-0.74)
	TAT		4	Positive	+/-	81 (21-99)	44 (24-67)	1.44 (0.42-2.31)	0.42 (0.03-1.71)
	SL-I		4	Positive	+/-	80 (56-93)	59 (8-96)	1.94 (0.89-20.90)	0.34 (0.14-2.22)
	Cord factor		5	Positive	+/-	69 (28-94)	91 (78-97)	7.03 (2.44-20.65)	0.35 (0.06-0.80)

<sup>a</sup> The data represent the posterior means (95% credible intervals).

<sup>b</sup> Polypeptide.

<sup>c</sup> Multiple antigen (additive reactivity).



## Serological tests: malate synthase (Rv1387c)

- Malate synthase (81kda): is present in *M. tuberculosis* culture filtrate, the cell wall and the cytoplasmic subcellular fractions
- It is an enzyme of the glyoxilate pathway used by *M. tuberculosis* during intracellular replication in macrophages and has adapted to function as an adhesin that enhances bacterial adherence to the host cells
- In sputum-smear-positive patients: sensitivity of 73% and a specificity of 98%



## Serological tests: recombinant TbF6

- TbF6t is a single antigen combining 4 distinct antigens (CFP-10, MTB8, MTB48, and 38kDa) as a genetically fused polyprotein
- In sputum-smear-positive patients: sensitivity of 70% and a specificity of 93%



## Serological tests: cord factor

- Cord factor is a major component of *M. tuberculosis* cell wall and it is named for its central role in aggregating mycobacteria into cord structures
- Cord factor contributes to the virulence of *M. tuberculosis* by facilitating cavity formation
- In sputum-smear-positive patients: sensitivity of 69% and a specificity of 91%



## Limits of the serological tests

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- Potential candidate antigens identified; however sensitivity is poor
- Case control studies
- Inadequate number of subjects enrolled



## Conclusions of the serological studies

- Potential candidate antigens for an antibody detection test for pulmonary tuberculosis patients have been identified, although no antigen achieves sufficient sensitivity to replace sputum smear microscopy
- Combinations of select antigens provide higher sensitivities than single antigens
- The use of a case-control design with healthy controls for the majority of studies is a limitation
- Efforts are needed to improve the methodological quality of tuberculosis diagnostic studies

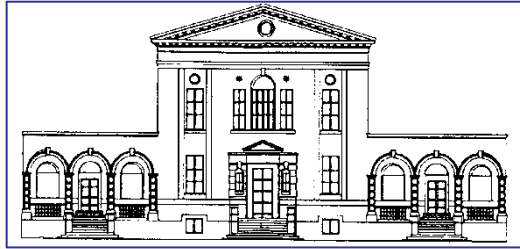


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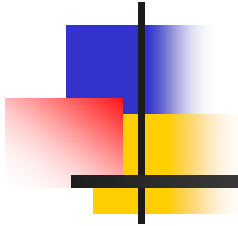
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National Institute for Infectious Diseases L. Spallanzani



**Thank you for this great opportunity!**



## Seven social sins

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- Wealth without work
- Pleasure without conscience
- Knowledge without character
- Commerce without morality
- Science without humanity
- Religion without sacrifice
- Politics without principles

Mahatma Gandhi

