TB Diagnostics: looking back and looking forward

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Disturbing Statistics

- 1/3 of world's population is infected with TB
- 8 Million people develop active TB every year
- TB kills more young women than any other disease
- More than 100,000 children will die from TB this year
- Hundreds of thousands of children will become TB orphans

When Should You Suspect Tuberculosis (TB)?

- TB is a disease caused by *Mycobacterium tuberculosis*. TB disease should be suspected in persons who have the following symptoms:
  - Unexplained weight loss
  - Loss of appetite
  - Night sweats
  - Fever
  - Fatigue
- If TB disease is in the lungs (pulmonary), symptoms may include:
  - Coughing for ³ 3 weeks
  - Hemoptysis (coughing up blood)
  - Chest pain
- If TB disease is in other parts of the body (extrapulmonary), symptoms will depend on the area affected

CHALLENGES IN DIAGNOSIS

DIAGNOSIS of TB

-active disease and latent infection

Microbiological 'Gold Standard'

-low sensitivity in children

-pauci-bacillary

-sputum collection difficult

Symptoms, overlap with HIV

Chest X-ray, appearances overlap

To stop the spread of TB globally, the world needs:

- New TB drugs
  - that will shorten treatment, be effective against susceptible and resistant strains, be compatible with antiretroviral therapies used for HIV/AIDS and that will improve treatment of latent infection
  - will dramatically improve TB treatment and control.

To stop the spread of TB globally, the world needs:

- A new vaccine
  - that is both effective and safe for children, adolescents and adults, including people infected with HIV
  - will decrease TB incidence overall and, along with an effective drug therapy, could eventually control the disease.

To stop the spread of TB globally, the world needs:

- Better TB diagnostics
  - that are rapid, practical and accurate in resource-poor settings
  - are critical to ensuring that people receive proper and timely treatment.

DIAGNOSTIC NEEDS

- *Industrialized countries*
- In developed countries with low incidence of TB, classic symptoms of prolonged cough and fever are not very sensitive predictors of tuberculosis.
- The diagnostic needs for countries in the elimination phase TB control priorities include tools for:
  - A replacement for the tuberculin test (PPD) for the identification of latent infection in high-risk individuals
  - A replacement for chest X-rays and smear for the detection of disease in immigrant and other high risk populations through active case finding
  - A replacement for culture for the detection of patients with early disease, low burden infection or minimal symptoms
  - A replacement for standard molecular fingerprinting for identification of outbreaks and characterization of nosocomial and community transmissions
  - A replacement for cultural and biochemical tests and single-species probes

DIAGNOSTIC NEEDS

- *TB-endemic countries*
- In high TB infection prevalence countries , specific exposure is harder to identify and risk assessment more difficult. Widespread BCG vaccination and HIV co-infection rates may affect the specificity of immunologic tests. Desirable diagnostic tools would include:
- A fast, reliable, simple replacement for smear which would require little or not interpretation. Would function well in HIV-infected individuals and would be specific enough to allow initiation of therapy and sensitive enough to detect sputum smear positive patients
- A more sensitive, faster, simpler and cheaper replacement for culture to supplement sputum smear microscopy at the peripheral level. Sensitive enough to detect smear negative - culture positive patients including HIV patients
- A highly sensitive, simple and cheap screening test for active tuberculosis to eliminate TB symptomatic patients from diagnostic load in overburdened clinics
- A robust, inexpensive drug resistance detection tool to be applied to patients in MDR-TB hot spots in countries offering treatment with second line drugs. Results should be routinely available in 2 weeks for sputum smear positive patients, 4 weeks for sputum smear negative patients
- A simple screening tool for latent infection that is specific for *M. tuberculosis,* does not require a return visit and functions in HIV-infected individuals

TB Diagnostics: Historical Perspective

- Screening test: Past and Present
- Direct Approach
- Indirect Approach

Introduction

- The Institute of Medicine and the CDC recognizes the importance of developing accurate diagnostic tests for TB infection to hasten the process of TB elimination
- A sensitive test would accurately identify people with infection, whether latent or active (maximize true positive results)
- A specific test would accurately identify people who are uninfected (maximize true negative)

Introduction

- In the U.S., the effort to control tuberculosis, its transmission, and ultimately, its eradication has been fought along two important fronts
- The first front is to detect and treat people with infectious tuberculosis
- The second front is to detect high risk asymptomatic people who have latent TB infection and prevent the development of active disease

TST - Historical perspective

- Tuberculin was developed a decade after the discovery of the tubercle bacillus as the cause of TB
- The original preparation (old tuberculin) was obtained from heat sterilized cultures of tubercle bacilli
- Initially touted as therapeutic, which was found to be disappointing, its use eventually led to the discovery of its diagnostic value

TST - Historical perspective

- Old tuberculin was an unrefined product contributing to its lack of sensitivity in the diagnosis of infection with *M. tuberculosis*
- Refinements to the OT preparation led to the development of PPD, still used in present day Mantoux skin testing

TST: False negatives / False positives

False negatives

- Technical factors
  - Application
  - Reading
  - Improper storage of PPD
- Biological factors
  - Poor nutrition
  - Infection
  - Immunosuppressive drugs
  - Malignancy
  - Age
  - Stress

False positives

- Infection with nontuberculous mycobacteria
- BCG vaccination

QuantiFERON®-TB Gold Test

A 100 Year Update for the Diagnosis of Tuberculosis Infection

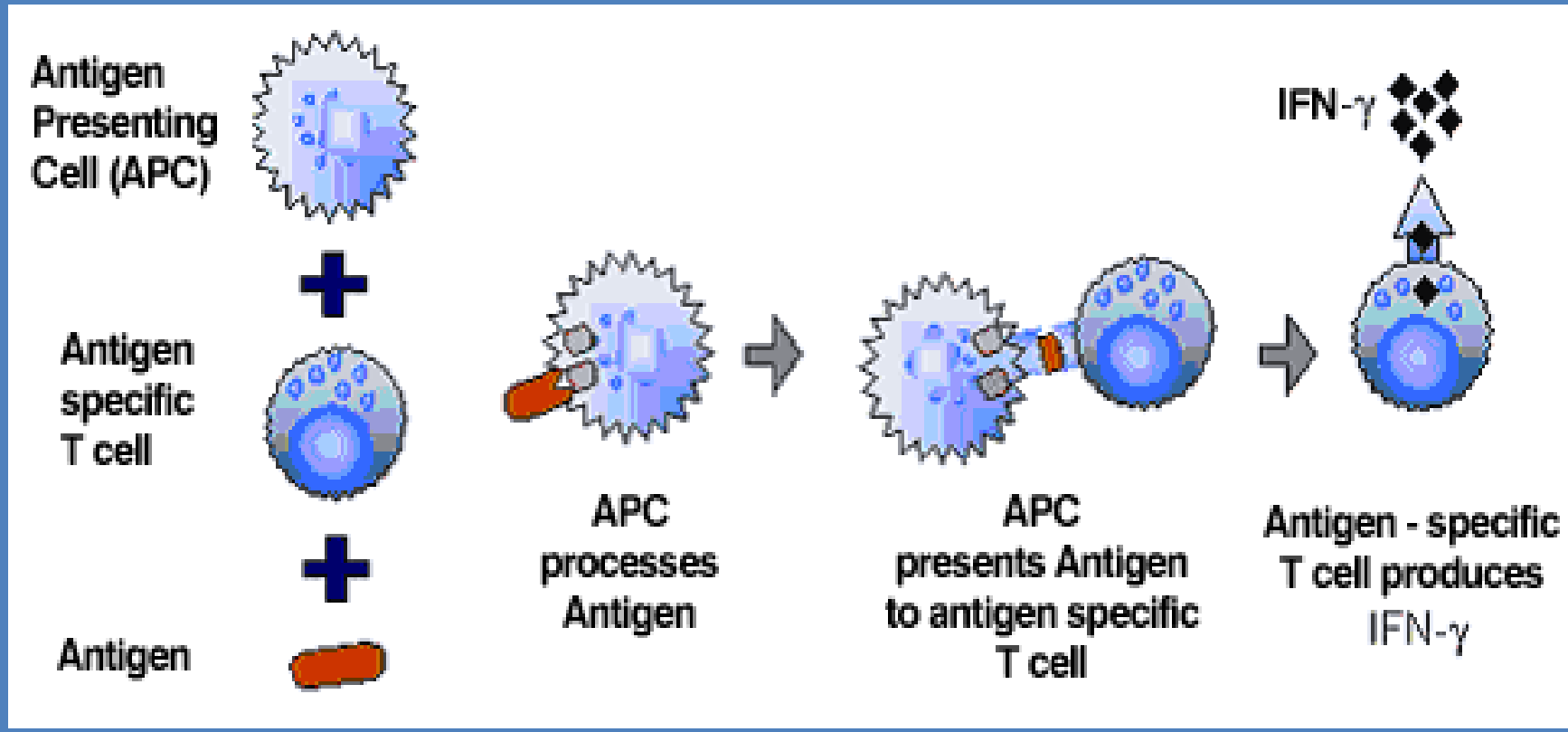
What is Quanti-FERON®-TB Gold

- Blood assay for *M. tuberculosis* > Interferon γ release assay
- *In vitro* test using whole blood specimen for the diagnosis of TB infection, whether latent or active
- Does not distinguish between latent TB infection or TB disease

Quanti-FERON®-TB Gold – Scientific Basis

- Individuals infected with *M. tuberculosis* complex organisms have lymphocytes in their blood that recognize mycobacterial antigens
- This recognition process involves the generation of interferon-γ, a specific cytokine for cell mediated immune response
- The detection and subsequent quantification of IFN-γ is the basis of this test
- The test uses synthetic peptide antigens (ESAT-6, CFP-10) that simulate mycobacterial proteins to generate the immune response

Interferon Gamma Release



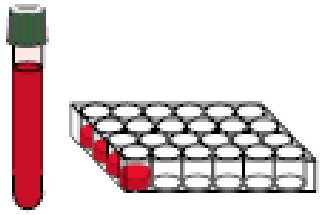
Species Specificity of ESAT-6 and CFP-10

Tuberculosis complex	Antigens	
	ESAT	CFP
M tuberculosis	+	+
M africanum	+	+
M bovis	+	+
BCG substrain		
gothenburg	-	-
moreau	-	-
tice	-	-
tokyo	-	-
danish	-	-
glaxo	-	-
montreal	-	-
pasteur	-	-

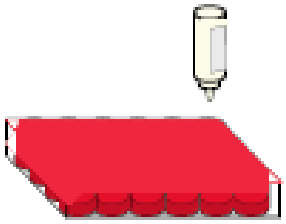
Environmental strains	Antigens	
	ESAT	CFP
M abcessus	-	-
M avium	-	-
M branderi	-	-
M celatum	-	-
M chelonae	-	-
M fortuitum	-	-
M gordonii	-	-
M intracellulare	-	-
M kansasii	+	+
M malmoense	-	-
M marinum	+	+
M oenavense	-	-
M scrofulaceum	-	-
M smegmatis	-	-
M szulgai	+	+
M terrae	-	-
M xenopi	-	-

QFT Assay

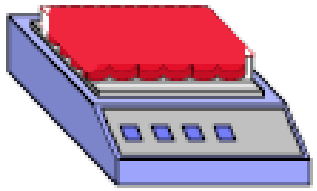
Stage One – Blood Stimulation and Harvesting



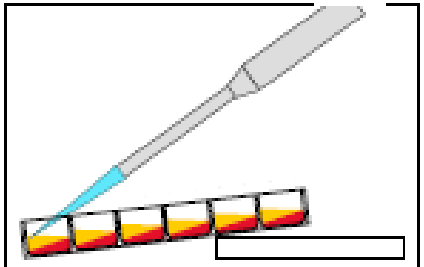
Dispense 1 mL of subject's heparinized whole blood into 4 wells of a 24-well culture plate.



Add 3 drops of the appropriate stimulating antigen to each well.

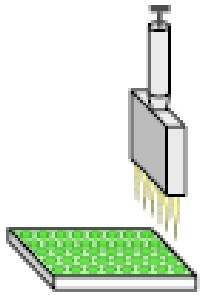


Shake covered plate for 1-2 min. Incubate for 16-24 hrs at 37°C (humidified).

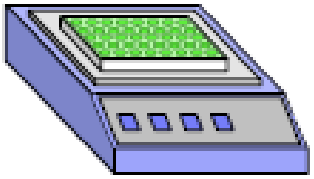


Harvest at least 200 µL plasma from each well. Store in racked microtubes or uncoated microplates.

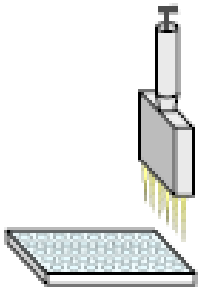
Stage Two – Human IFN-γ ELISA



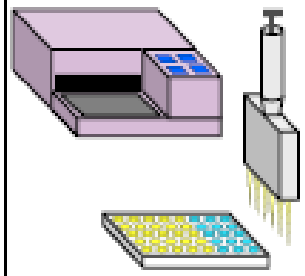
Add 50 µL of conjugate solution to each well. Add 50 µL of plasma or standard.



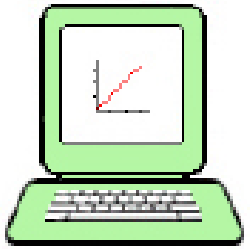
Shake covered plate for 1 min. Incubate for 120 minutes at Room Temperature.



Wash plate ≥ 6 times. Add 100 µL of substrate. Incubate 30 min. at Room Temperature.



Add 50 µL of stop solution. Read absorbance within 5 min at 450nm (620-650nm ref).



Calculate Results using standard analysis programs (QFT-Gold Analysis Software available soon).

Results and Interpretation

RESULT	INTERPRETATION
POSITIVE	ESAT-6 and/or CFP-10 responsiveness detected M. tuberculosis infection likely
NEGATIVE	No ESAT-6 or CFP-10 responsiveness detected M. tuberculosis unlikely
INDETERMINATE	MTB infection status cannot be determined as a result of impaired immunity and/or incorrect performance of the test

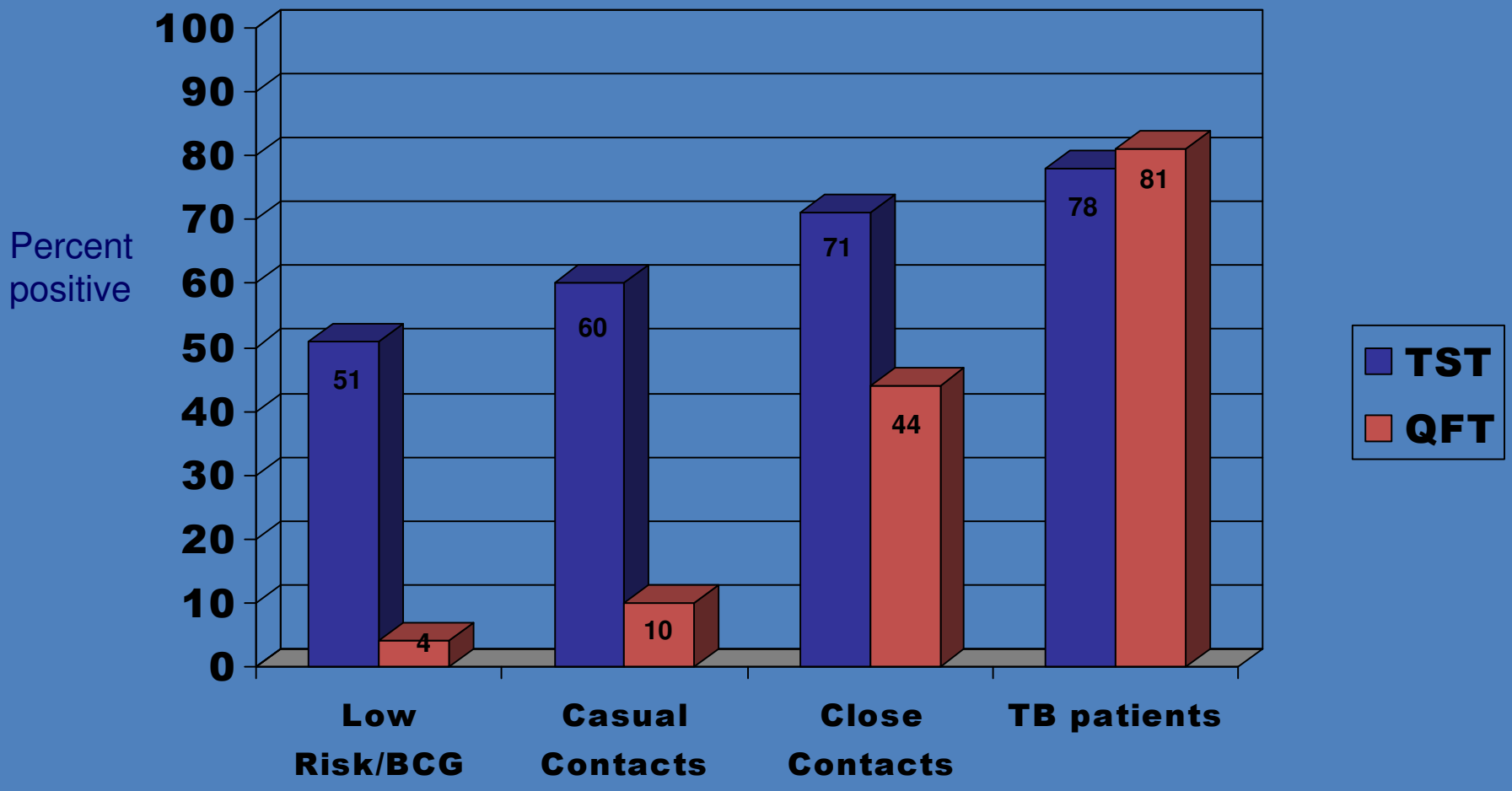
Specificity Estimates

- 216 healthy individuals, no identified risk for TB infection, all BCG (+)
  - Specificity = 98% (213/216 = QFT negative)
    - Mori, et al. AJRCCM 2004;170:59-64
- 532 with no identified risk for TB infection among Navy recruits
  - Specificity = 99.8% (531/532 = QFT negative)
    - CDC; publication in preparation
- 99 healthy individuals with no identified risk for TB infection, all BCG (+)
  - Specificity = 96% (95/99 = QFT negative)
    - Kang, et al. JAMA 2005;293:2756-2761

Sensitivity Estimates

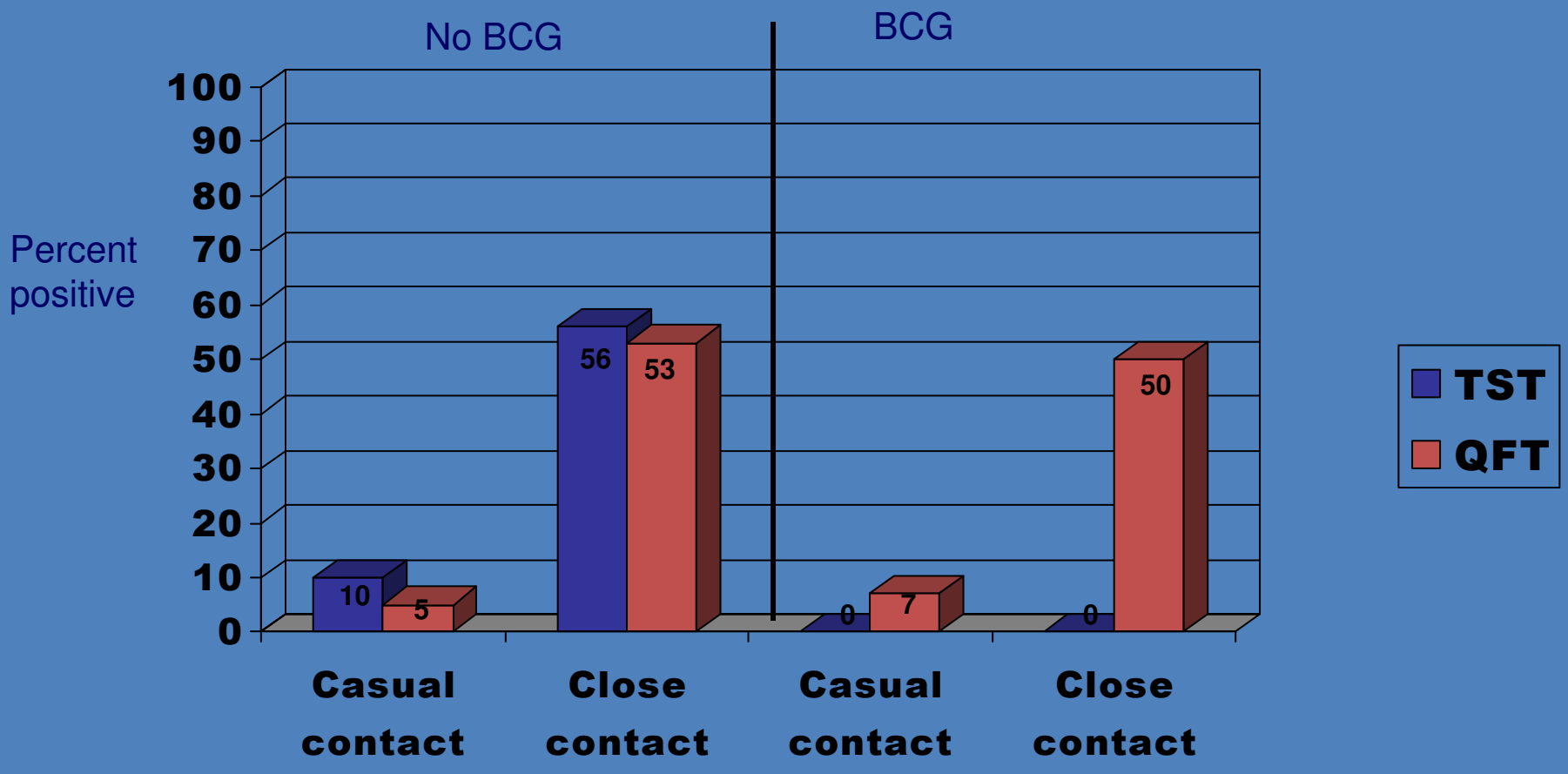
- 118 culture confirmed TB disease, 85% untreated, 15% treated < 7 days
  - 65.8% had positive TST (5mm); Sensitivity 105/118 = 89% for QFT
    - Mori, et al. AJRCCM 2004;170:59-64
- 48 culture confirmed TB disease, 71% untreated
  - Sensitivity 41/48 = 85.4%
    - Ravn, et al. Clin Diag Lab Immunol 2005;12:491-496
- 54 culture confirmed TB disease
  - Sensitivity 44/54 = 81.5%; TST = 77.8%
    - Kang, et al. JAMA 2005;293:2756-2761

Test Agreement, Korea



Increase agreement with increased chance of infection
Kang, 2005

Test Agreement in Contacts, Denmark



Good test agreement between TST & QFT
Brock, 2004

QFT and TST

QFT

- *in vitro* test
- Specific antigens
- No boosting
- 1 patient visit
- Lab variability
- Results possible in 1 day
- Requires phlebotomy
- Includes + control

TST

- *in vivo* test
- Less specific PPD
- Boosting
- 2 patient visits
- Inter-reader variability
- Results in 2-3 days
- No phlebotomy required
- No + control

Direct Approach

- Microscopy
  - Simplest and most rapid procedure
  - Limit of detection: $5x10^3$ bacilli/ml
  - Fluorescent staining: offers the advantage of screening the smear under LPO
  - Results can be influenced by:
    - Type of the specimen
    - Thickness of the smear
    - Extent of decolorization
    - Type of counter-stain
    - Training and experience

Direct Approach

- Culture
  - cornerstone on which definitive diagnosis of TB and mycobacteriosis relies
  - Mycobacterial culture
    - Egg-based solid medium: L J Medium
    - Agar based: Middlebrook 7H10 or 7H11
    - Liquid media
      - Kirchner's
      - Middlebrook 7H9
    - Constraints:
      - slow growth: 4 weeks
      - DST: additional 4 weeks

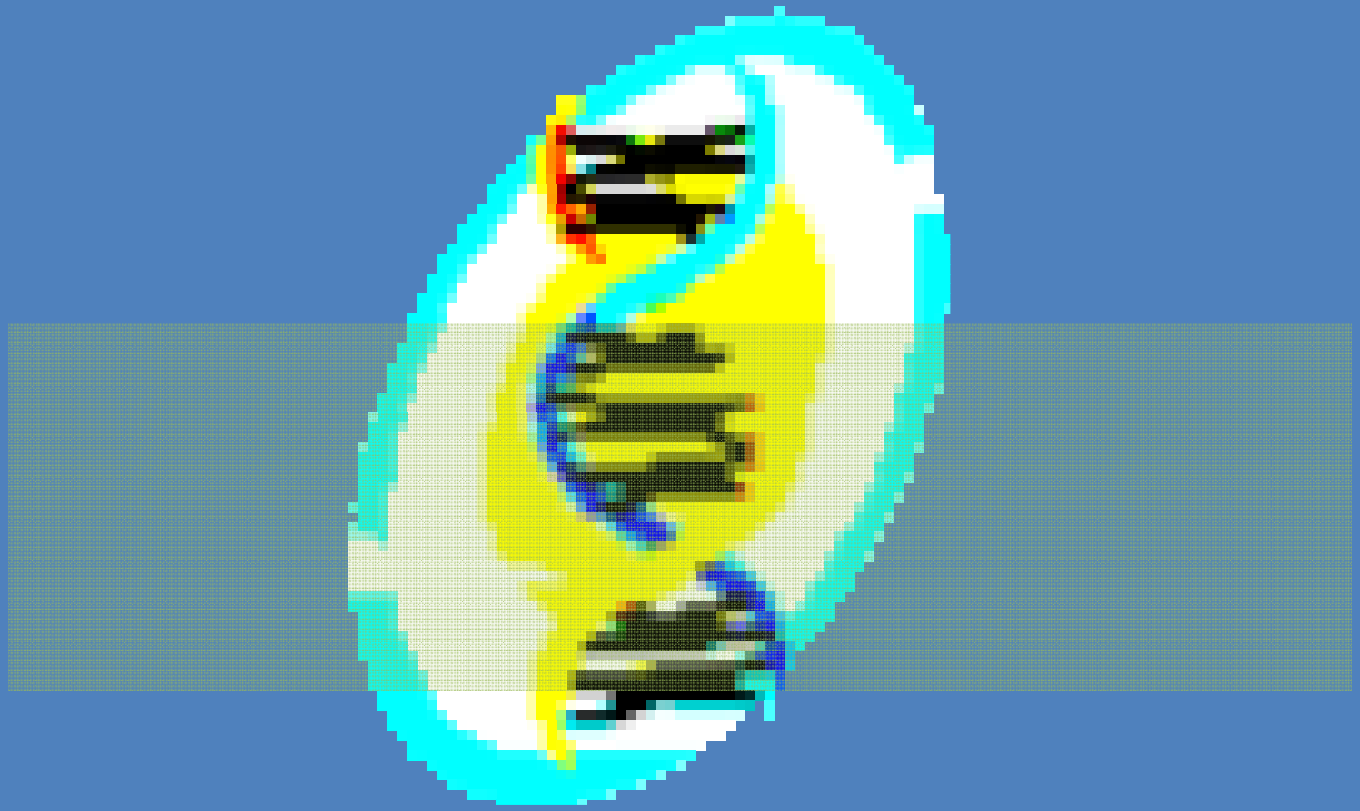
Advances in Culture

- Microcolony detection on Solid
- Septi-check AFB Method
- Radiometric 460
- MGIT 960 Mycobacteria Detection
- MB/Bact System
- ESP Culture System

Detection and Identification of Mycobacteria From Clinical Sample

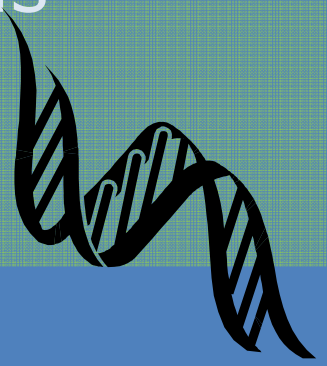
- Genotypic Methods
  - PCR
    - Standard
    - Multiplex
    - Real-time
  - Fingerprinting
  - Sequencing
    - Standard
    - pyrosequencing

The Post-Genomic Era



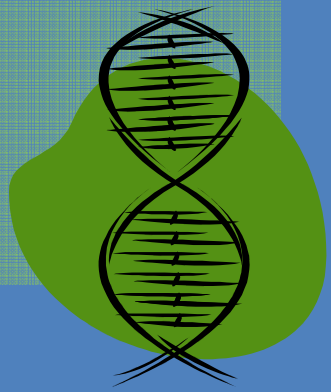
Molecular Testing

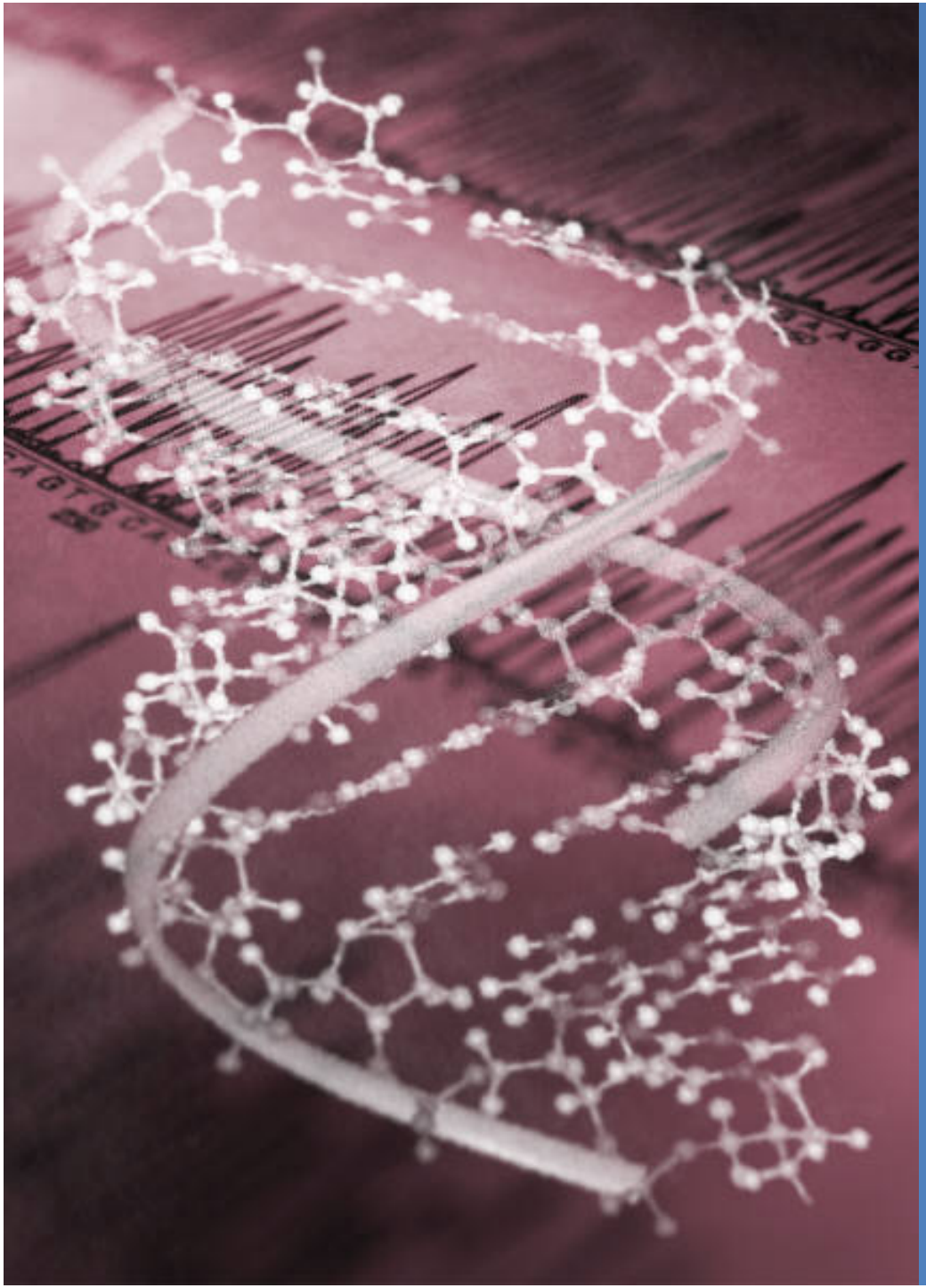
- "diagnostic tool for the new millennium"
- *However…..*
  - difficult questions have arisen regarding the role of such testing in the assessment of clinical infectious diseases.



Molecular Testing

- As more molecular tools flow from bench to bedside..
  - clinicians must acquire a working knowledge of the principles, diagnostic value, and limitations of various assays





PCR:

Basic Principles *and* Overview

Polymerase Chain Reaction

- an enzyme-driven process for amplifying short regions of DNA in vitro
- relies on knowing at least partial sequences of the target DNA a priori and using them to design oligonucleotide primers that hybridize specifically to the target sequences

Basic PCR Principle

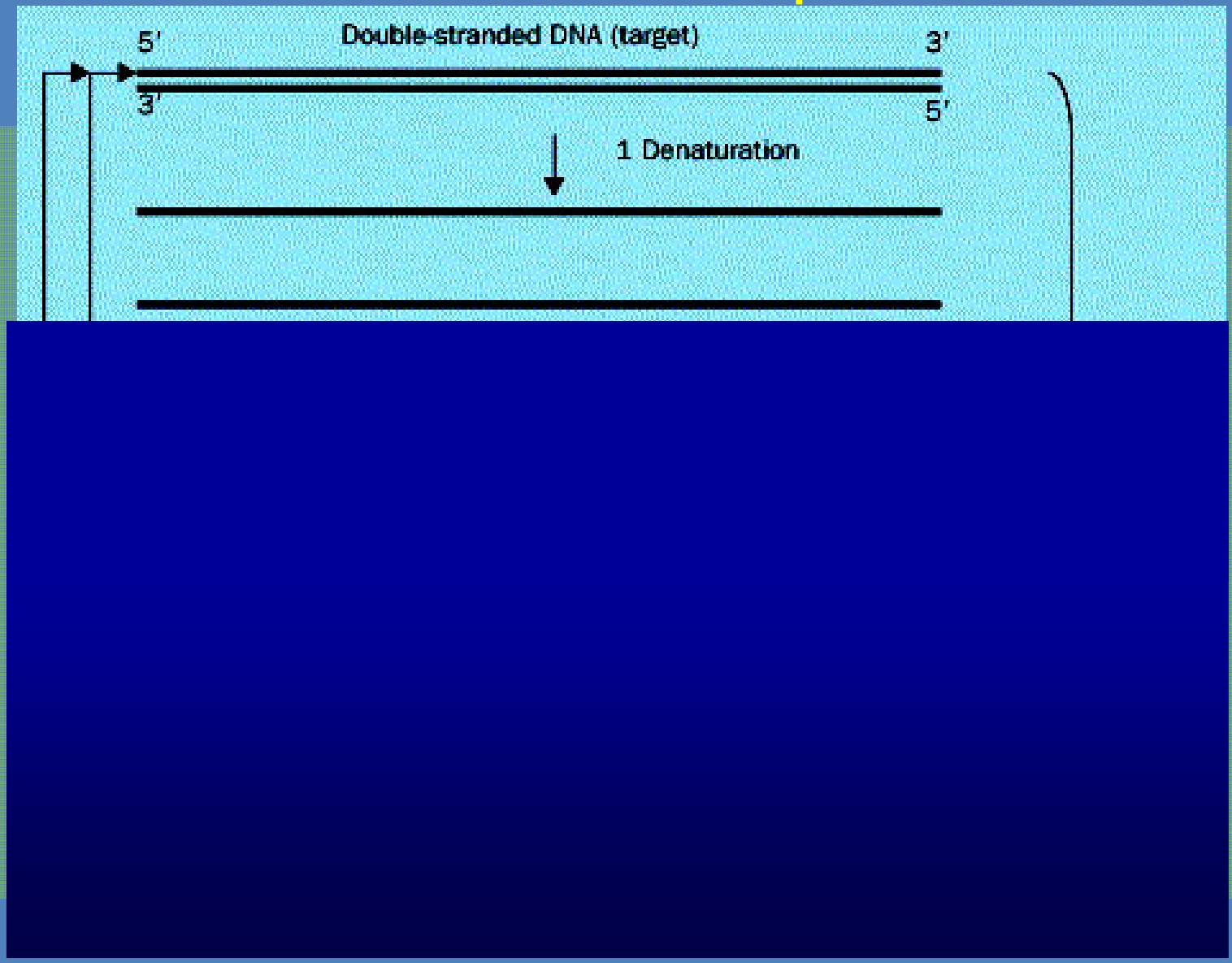
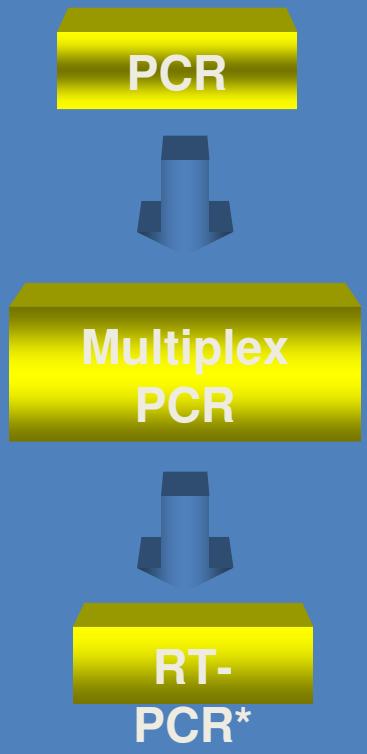


Image from Lancet Infect Dis 2004; 4: 337–48

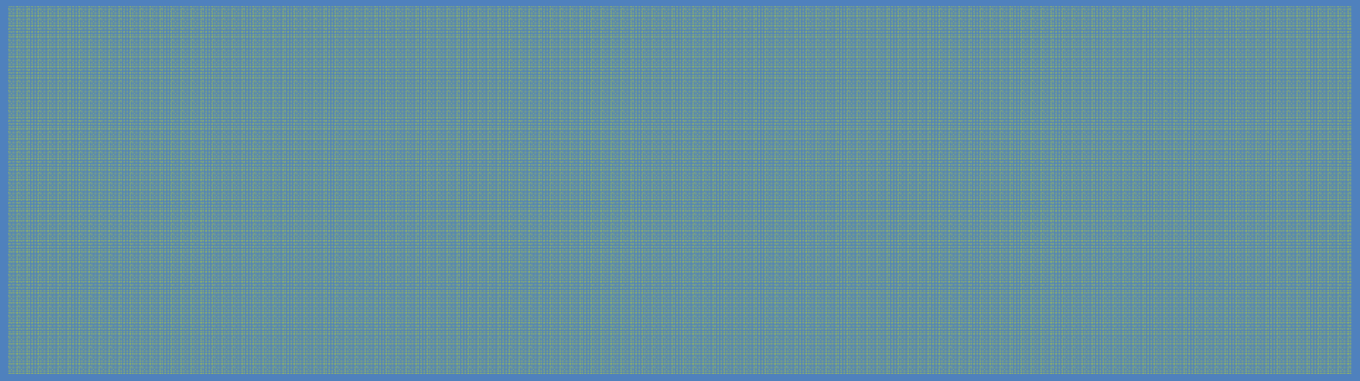
Evolution of PCR



*RT-PCR – Reverse Transcriptase Polymerase Chain Reaction

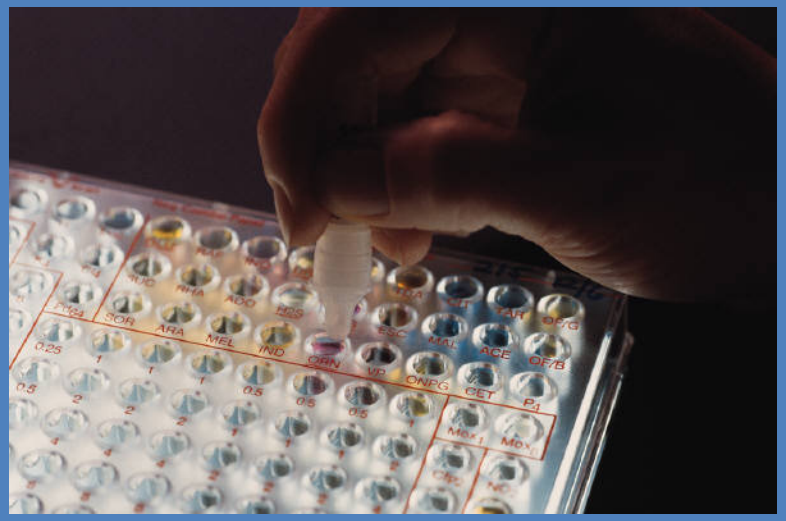
Specific PCR Diagnostics:

Development and Clinical Applications



Conventional Diagnostics *versus* PCR

Pros & Cons



M. tuberculosis PCR

- Only FDA approved as diagnostic adjunct to the conventional smear & culture
- Potential applications
  - Earlier informed decision-making for appropriate use of isolation beds in high prevalence area
  - Regional outbreaks
  - Isolation beds are scarce

Limitations of PCR

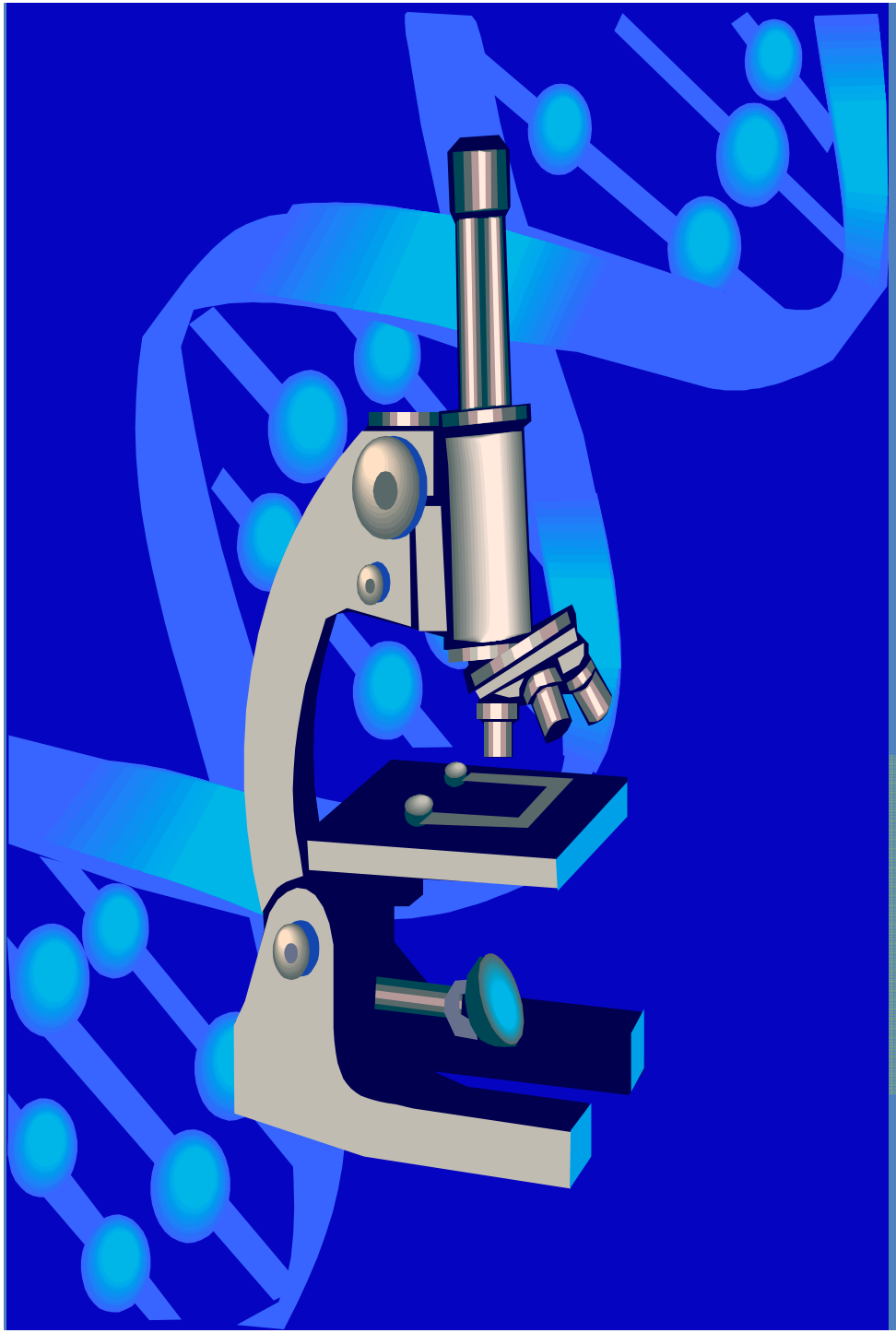
- **False Positives**
  - Background contamination from exogenous sources of DNA
  - "carry over products"
  - Use of universal primers

  Interventions:
    - Use of enzymatic inactivators for "carry over DNA" (i.e. Uracil-n-glycosylase)
    - Meticulous laboratory practice
    - Size-based ultrafiltration devices

Limitations of PCR

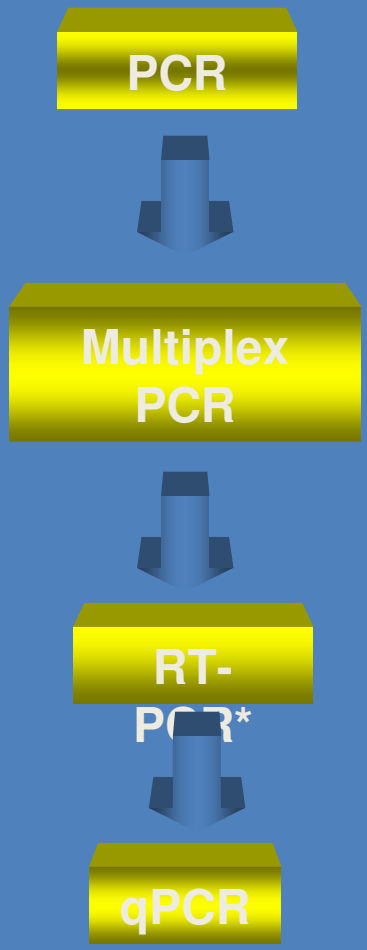
- **False Negatives**
  - Limited small volume for PCR reactions
  - PCR processing
    - Inadequate removal of PCR inhibitors in the sample, such as hemoglobin, blood culture media, urine, and sputum;
    - ineffective release of microbial DNA content from the cells;
    - poor DNA recovery after extraction and purification steps

  Interventions:
    - DNA extraction and Purification from large volume samples prior to amplification
    - Selecting specimen sources or sample fraction
    - Meticulous laboratory practice



Future Directions

Evolution of PCR



*RT-PCR – Reverse Transcriptase Polymerase Chain Reaction

Real Time PCR
in the Diagnosis of Infectious Diseases

Real-time PCR

- Amplification and detection of amplified products are coupled in a single reaction vessel
- Represents a major breakthrough since it eliminates the need for laborious post-amplification processing
- Allows for measurement of product simultaneous with DNA synthesis

Real-time PCR

- Intercalating Dyes
  - Which bind non-specifically to double-stranded DNA generated during amplification
    - Sybr-green 1
- Fluorescent labelled Internal DNA probes
  - Which specifically anneals within the target amplification region
    - Taqman
    - Fluorescence resonance energy transfer
    - Molecular beacon

Basic Principle of the Real Time PCR

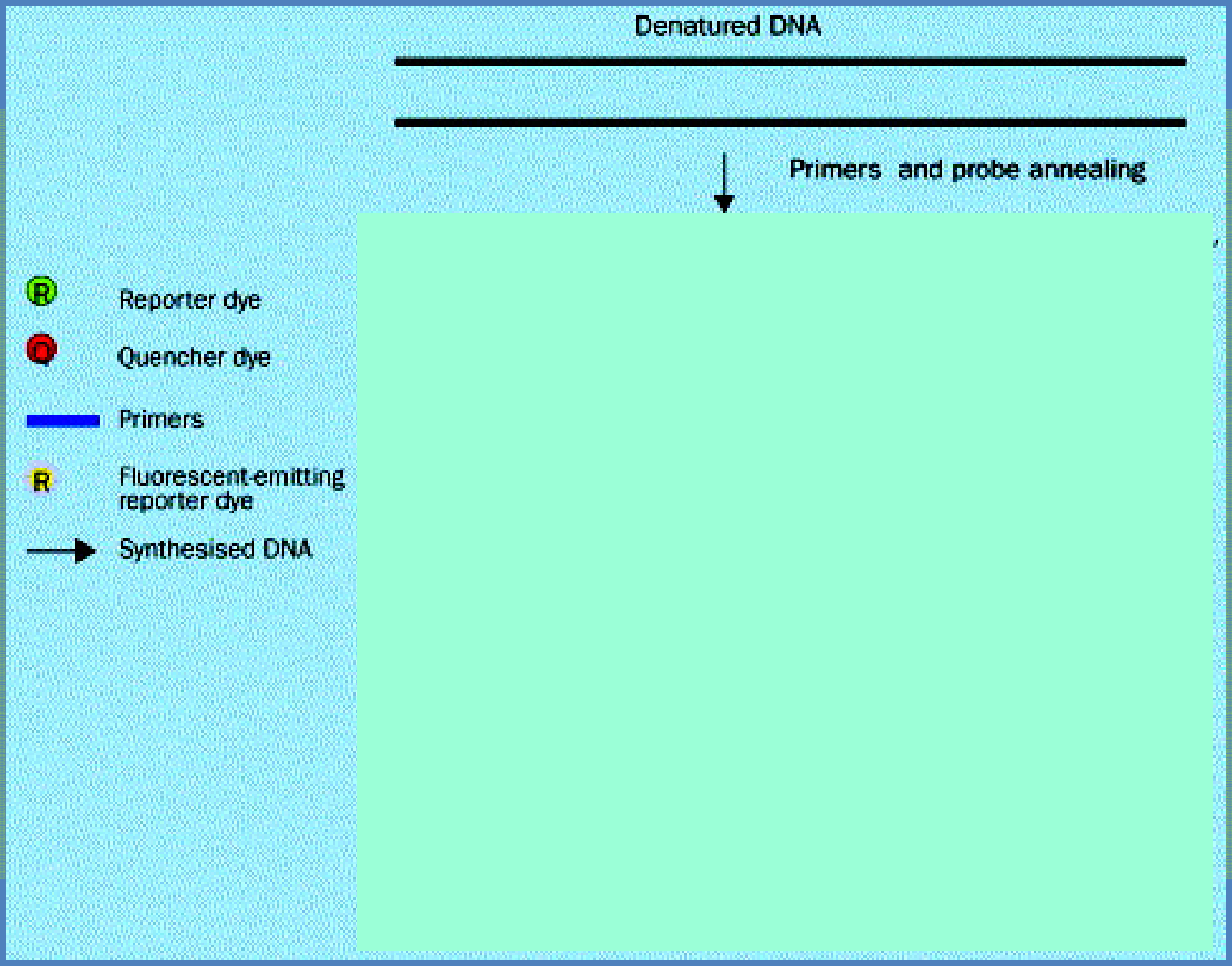


Image from Lancet Infect Dis 2004; 4: 337–48

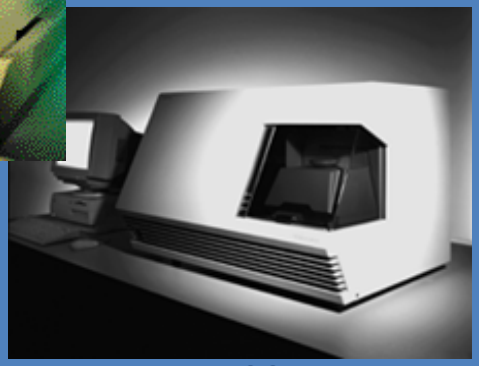
real-time PCR

hardware

iCycler
BioRad

LightCycler
Roche

5700
Applied Biosystems



7700
Applied Biosystems

FluorTracker
Stratagene

FluorImager
Molecular Dynamics

real-time real-time real-time real-time

PCR

amplifies & detects	►	*integrated system*
fluorescent probes	►	*constant monitoring*
fast turn-around	►	*rapid cycling times*
sealed system	►	*low contamination risk*
quantitative	►	*assay design*

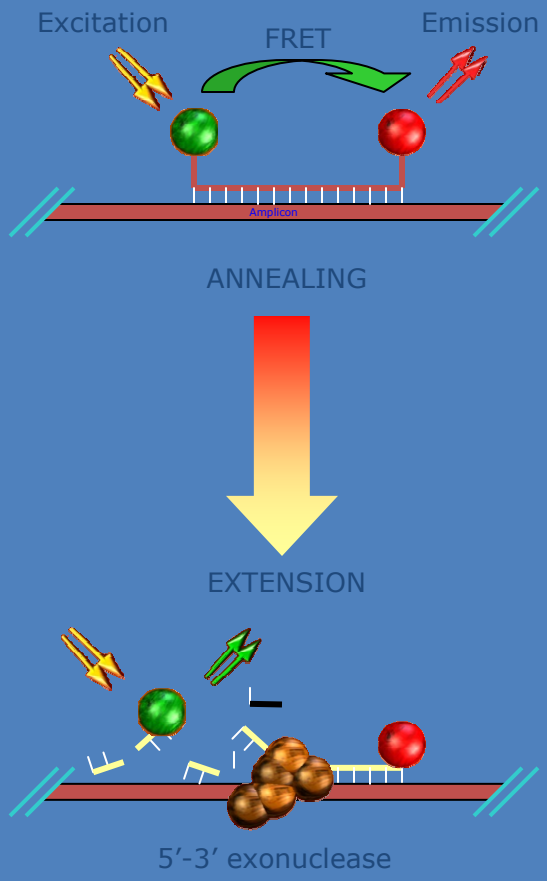
real-time

real-time

real-time

real-time

TaqMan

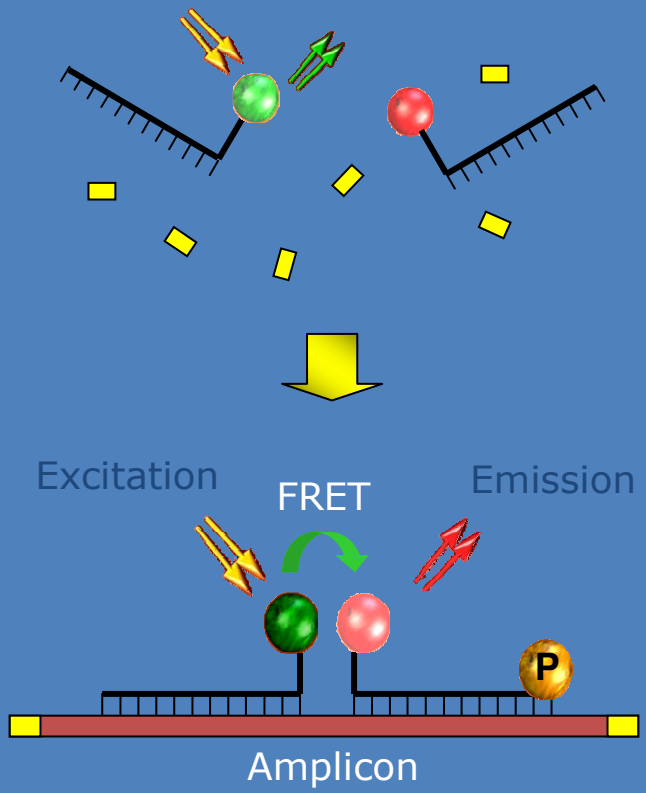


Reporter

Quencher

real-time real-time
LightCycler
real-time
FRET

FRET (Fluorescence Resonance Energy Transfer)
using adjacent hybridization probes



FITC
Red 640
P Phosphate

Table 1 Commercial serological antibody detection tests for the diagnosis of extrapulmonary tuberculosis

Name of test (number of studies in review)	Antigen(s)	Source of antigen	Ig class	Laboratory technique	Manufacturer	Address/URL
Anda-TB* (17)	Antigen 60	Native	IgG, IgA, IgM	ELISA	Anda Biologicals S.A.	Strasbourg, France www.andabiologicals.com
AMRAD ICT TB (1)	38 kDa and four proprietary antigens	Recombinant	IgG	Immunochromatographic test card	ICT Diagnostics	Balgowlah, NSW, Australia
Pathozyme Myco (1)	Lipoarabinomannan (LAM) and 38 kDa	Native LAM and recombinant 38 kDa	IgG	ELISA	Omega Diagnosticshttp://omegadiagnostics.com	Alloa, UK
Pathozyme TB Complex Plus (1)	38 kDa and 16 kDa	Recombinant	IgG	ELISA	Omega Diagnosticshttp://omegadiagnostics.com	Alloa, UK
SEVA TB (1)	31 kDa	Native glycoprotein antigen from culture filtrate of M tuberculosis H37Rv	IgG	ELISA	Jamnalal Bajaj Tropical Disease Research Centre, Mahatma Gandhi Institute of Medical Sciences	Sevagram-442 102 (Wardha) M S (India)

Table 2 Selected characteristics of studies investigating Anda-TB (Anda Biologicals, Strasbourg, France) for the diagnosis of extrapulmonary tuberculosis

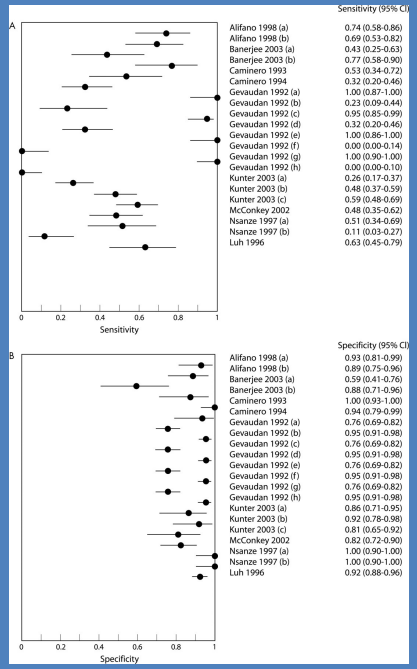
Reference	Data collection	Verification	Reference standard	Disease site	Country	Comparison group	Ig class	No. of participants*	Sensitivity (95% CI)	Specificity (95% CI)
Alifano (1998, a)[41]	Retrospective	Differential	Culture and/ or histology	Multiple	Italy	Mixed disease	IgG	42/44	0.74 (0.58 to 0.86)	0.93 (0.81 to 0.99)
Alifano (1998, b)[41]	Retrospective	Differential	Culture and/ or histology	Multiple	Italy	Mixed disease	IgA	42/44	0.69 (0.53 to 0.82)	0.89 (0.75 to 0.96)
Banerjee (2003, a)[42]	Retrospective	NR	Histology	Lymph node	India	Healthy	IgG	30/32	0.43 (0.25 to 0.63)	0.59 (0.41 to 0.76)
Caminero (1993)[43]	Prospective	Complete	Culture and/ or histology	Pleura	Spain	Pleural TB suspects	IgG	30/48	0.53 (0.34 to 0.72)	1.00 (0.93 to 1.00)
Caminero (1994)[44]	Prospective	Differential	Culture and/ or histology	Multiple	Canary Islands	Non-TB respiratory	IgG	56/31	0.32 (0.20 to 0.46)	0.94 (0.79 to 0.99)
Gevaudan (1992, a)[45]	Retrospective	Differential	Culture and/ or histology	Lymph node	France	Mixed disease	IgG	26/194	1.00 (0.87 to 1.00)	0.76 (0.69 to 0.82)
Gevaudan (1992, b)[45]	Retrospective	Differential	Culture and/ or histology	Lymph node	France	Mixed disease	IgM	26/194	0.23 (0.09 to 0.44)	0.95 (0.91 to 0.98)
Gevaudan (1992, c)[45]	Retrospective	Differential	Culture	Disseminated/ miliary (primary)	France	Mixed disease	IgG	56/194	0.95 (0.85 to 0.99)	0.76 (0.69 to 0.82)
Gevaudan (1992, d)[45]	Retrospective	Differential	Culture	Disseminated/ miliary (primary)	France	Mixed disease	IgM	56/194	0.32 (0.20 to 0.46)	0.95 (0.91 to 0.98)
Gevaudan (1992, e)[45]	Retrospective	Differential	Culture	Disseminated/ miliary (post-primary)	France	Mixed disease	IgG	25/194	1.00 (0.86 to 1.00)	0.76 (0.69 to 0.82)
Gevaudan (1992, f)[45]	Retrospective	Differential	Culture	Disseminated/ miliary (post-primary)	France	Mixed disease	IgM	25/194	0.00 (0.00 to 0.14)	0.95 (0.91 to 0.98)
Gevaudan (1992, g)[45]	Retrospective	Differential	Culture	Genitourinary	France	Mixed disease	IgG	34/194	1.00 (0.90 to 1.00)	0.76 (0.69 to 0.82)
Gevaudan (1992, h)[45]	Retrospective	Differential	Culture	Genitourinary	France	Mixed disease	IgM	34/194	0.00 (0.00 to 0.10)	0.95 (0.91 to 0.98)
Kunter (2003, a)[46]	Prospective	Differential	Culture	Pleura	Turkey	Non-TB respiratory	IgG	88/37	0.26 (0.17 to 0.37)	0.86 (0.71 to 0.96)
Kunter (2003, b)[46]	Prospective	Differential	Culture	Pleura	Turkey	Non-TB respiratory	IgM	88/37	0.48 (0.37 to 0.59)	0.92 (0.78 to 0.98)
Kunter (2003, c)[46]	Prospective	Differential	Culture	Pleura	Turkey	Non-TB respiratory	IgG and IgM	88/37	0.59 (0.48 to 0.70)	0.81 (0.65 to 0.92)
Luh (1996)[47]	Prospective	NR	Culture	Multiple	Taiwan	Mixed disease	IgG	35/224	0.63 (0.45 to 0.79)	0.92 (0.88 to 0.96)

NR, not reported.

Table 3 Selected characteristics of studies investigating commercial tests for the diagnosis of extrapulmonary tuberculosis

Reference	Data collection	Reference standard	Disease site	Country	Comparison group	Name of test*	No. of participants	Sensitivity (95% CI)	Specificity (95% CI)
Banerjee (2003, b)[42]	Retrospective	Histology	Lymph node	India	Healthy	SEVA TB	30/32	0.77 (0.58 to 0.90)	0.88 (0.71 to 0.97)
McConkey (2002)[48]	Prospective	Culture	Meninges	Egypt	Mixed disease	AMRAD ICT	56/74	0.48 (0.35 to 0.62)	0.82 (0.72 to 0.90)
Nsanze (1997, a)[49]	Prospective	Culture and/ or smear	NR	UAE	Mixed disease and healthy	Pathozyme Myco	35/35	0.51 (0.34 to 0.69)	1.00 (0.90 to 1.00)
Nsanze (1997, b)[49]	Prospective	Culture and/ or smear	NR	UAE	Mixed disease and healthy	Pathozyme TB Complex Plus	35/35	0.11 (0.03 to 0.27)	1.00 (0.90 to 1.00)

Figure 2 (A) Sensitivity and (B) specificity estimates of commercial tests for the diagnosis of extrapulmonary tuberculosis (21 studies). Point estimates of sensitivity and specificity from each study are shown as solid circles. The solid lines represent the 95% confidence intervals (CI).



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Conclusions

- Of the recent TB diagnostic advances, including enhanced microscopy, improved serology, alternative liquid culture media, phage replication systems, molecular probes, amplification tests and antigen detection tests, none is ideal or answers the need of all clinical settings.
- More than one type of diagnostic tool will be needed, and extensive clinical trials will be necessary to determine the appropriate use for each test type.
- Diagnostic test development and deployment should be co-ordinated with disease control activities. Improving case detection will have no epidemiological impact in settings where cure rates are low.
- Likewise, drug susceptibility determination will have no utility in settings where strategies to treat resistant isolates are not established.

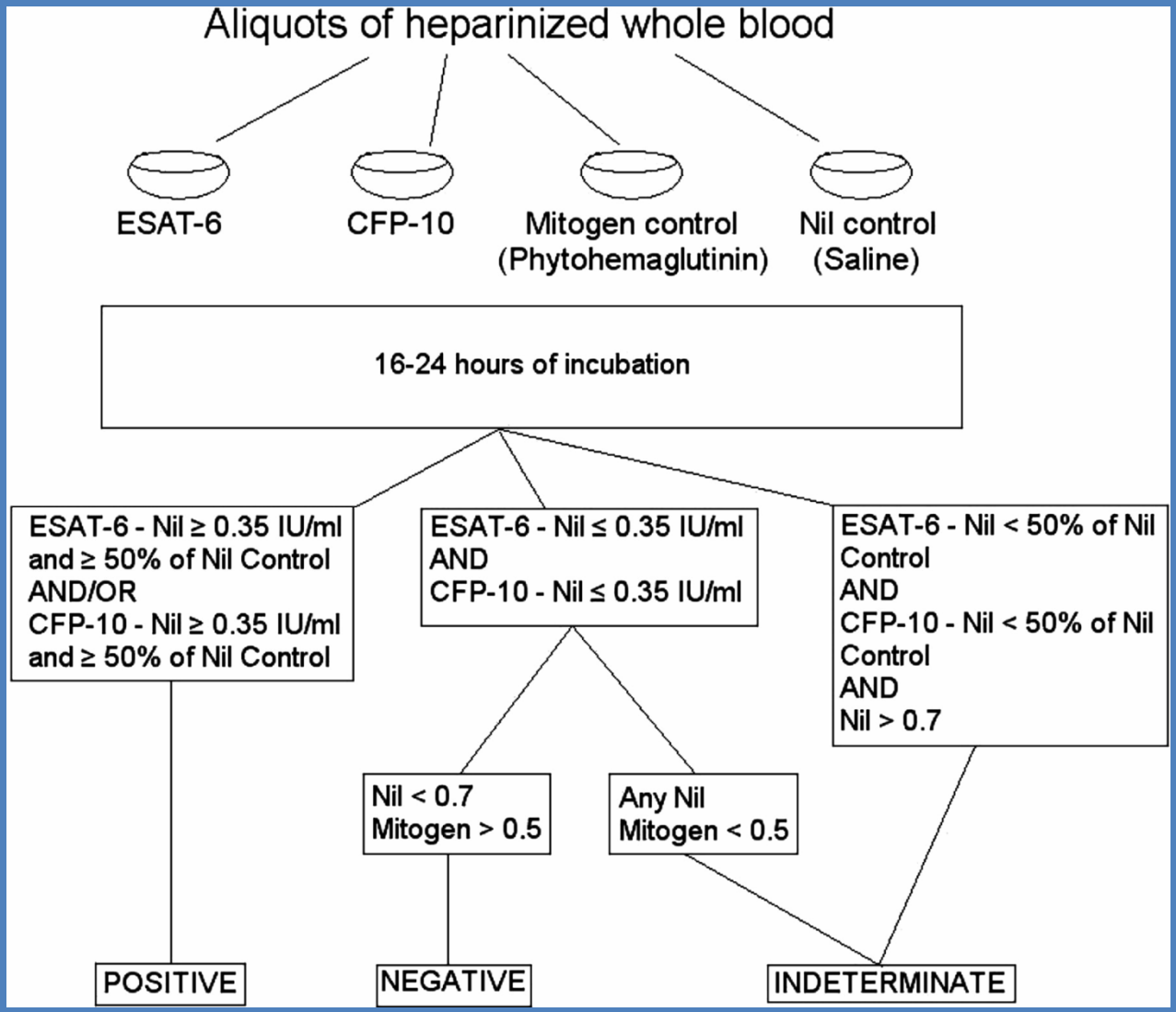
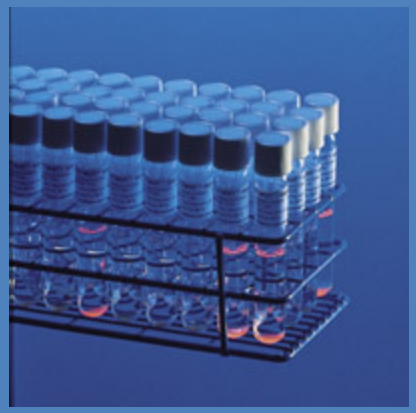


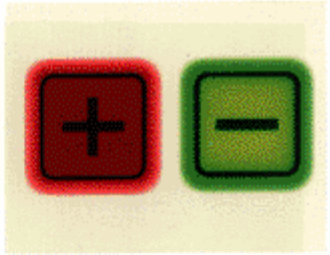
Figure 1. **Procedure and result interpretation of ELISA T-cell–based gamma release essay. ESAT-6 and CFP-10 refer to specific antigens coded by the *esat-6* and *lhp* genes of *Mycobacterium tuberculosis*. For indeterminate results, the test may be repeated if there is evidence of potential technical errors, such as storage at incorrect temperature, pipetting errors, etc.**



Step 1: Select workflow.

Step 2: Scan tube at instrument.

Step 3: Load where indicated by green LED.



Step 4: Remove positives and completed negatives as they occur.