Advances in the Diagnosis of Tuberculosis

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19 century → Until now (2007)

<u>still relying on</u> –

direct smear (>10^4 bacilli/)

culture (4-6 wks)

Scope

- Advances in microscopy
- Advances in culture methods
- Advances in IGRAs
- Advances in NAA techniques
- Advances in drug susceptibility testing
- Advances in epidemiologic techniques

Advances in bacilloscopy

Lancet Infect Dis 2006; 6:570–81

- More sensitive (fluorescence microscopy on average 10% more sensitive $p<0.001$)
- Sensitivity greater in low grade positives
- Similar specificity (mean difference 0%;$p=0.21$)
- Takes less time
- Little difference between different flourescent staining techniques
- Limited data in HIV-TB coinfected pts. – available evidence suggests FM may be promising in this population

Sputum processing methods

- Cytocentrifugation
- Bleach method – liquefaction of sputum with sodium hypochlorite and centrifugation
- Treatment of sputum samples with zwitterionic detergent (carboxy-prophylbetaine (CB18)

Lancet Infect Dis 2006; 6:664–74

- Sputum treated with bleach or NaOH and concentrated by centrifugation is more sensitive
- Sputum subjected to overnight sedimentation preceded by treatment with ammonium sulphate or bleach, is, on average, more sensitive, based on a small number of studies
- Specificity for processed smears is similar to that for direct smears
- Insufficient data to indicate whether the gains in sensitivity described above will also apply in patients with HIV infection

Advances in culture methods

Rapid culture methods

- BACTEC system
- MycobactGrowth Indicator Tube(MGIT)
- MB/BacT system
- Septi-check AFB method
- ESP culture system II
- Microscopic observation of broth/slide cultures

BACTEC System

- Radiometric method
- ^{14}C labeled palmitic acid added to liquid 7H12 medium
- Detects M tb by metabolism rather than growth
- $^{14}CO_2$ produced detected by specialized eqpt
- Growth index (GI) measured
- Results available in 7-14days (87-96%)

Ind. J. Tub., 2003, 50,133

MGIT

- Mycobacteria growth indicator tubes
- Capable of analyzing 960 specimen
- Growth detection based on AFB metabolic O_2 utilization
- Results available in 7-14 days
- Cost effective for high load microbiology-labs

MB/BacT system

- Non radiometric continuous monitoring system
- Automated
- Based on colorimetric detection of CO_2
- Slightly longer time than BACTEC system (11.6 days vs 13.7 days)
- Prone to contamination

ESP culture system II

- Based on detection of pressure changes in sealed broth culture bottle by gas production or consumption
- Reliable & less labour intensive
- Used in combination with solid medium not as a stand alone system

Microscopic observation of broth culture

- Rapid detection method
- Relatively inexpensive
- Suitable for endemic countries with high disease burden
- Requires P2 Bio-safety cabinets
- Relatively high technical skill required

MODS assay

- Microscopic observation drug susceptibility assay
- Both for diagnosis and drug resistance
- Based on direct inoculation of the selective 7H9 liquid culture medium in 24-well plates with a sputum specimen subjected to the digestion–decontamination procedure
- Two reagents are used -
  N-acetyl-L-cysteine for digestion
  NaOH for decontamination

- Compared the MODS assay head-to head with two reference methods: automated mycobacterial culture and culture on LJ medium with the proportion method

Variables	MODS	Auto. c/s	LJ media
Sensitivity	97.8	87	84 (p<0.001
Days culture	7	13	26 (p<0.001
Susc. Tests	7	22	68

- Agreement between MODS and the reference standard for susceptibility was >95% for R, H, E and 92% for S

T cell-based IFNγ-release assays

T cell-based IFNγ-release assays

- Region of difference-1: genomic segment of M.tb that is deleted from all strains of BCG vaccine and most environmental mycobacteria
- Two proteins encoded by RD-1:
  - Early secretory antigenic target-6 (ESAT-6)
  - Culture filtrate protein-10 (CFP-10)
- TIGRA based on T-cell response to these antigens
- Types of IGRA - ELISpot (T-SPOT.TB)
ELISA (QuantiFERON-Gold)

ELISpot (T-SPOT.*TB*)

- Peripheral blood mononuclear cells (PMBCs), which includes T cells, are separated from the blood sample by density centrifugation, washed, counted
- Incubated with ESAT-6 and CFP-10 in a 96-well microtitre ELISpot plate for 16 to 20 h
- When sensitized T cells re-encounter these antigens ex vivo, they release a cytokine, IFN γ
- Each such T cell gives rise to a dark spot, which is the “footprint” of an individual M tb specific T cell
- Readout is thus the number of spots that are counted using a magnifying lens or automated reader

ELISA (QuantiFERON-Gold)

- Whole blood from the patient is incubated with ESAT-6 and CFP-10 in a 24-well plate for 24 h
- Sensitized T cells recognize the antigens and secrete IFN γ
- IFN-γ concentration in the supernatant is measured by technique of ELISA

ELISpot (T-SPOT.*TB*)

Study	Study design	No.sub	sens%
Meier T et al, 2005	prospective	72	97
Lalvani A et al, 2001	case control	47	96
Pathan AA et al, 2001	case control	36	92
Ferrara G et al, 2006	prospective	24	83
Lee JY et al, 2006	prospective	87	95
Total		266	

- Gold std : culture-confirmed tuberculosis or clinically highly probable tuberculosis

ELISA (QuantiFERON-Gold)

Study	Study design	No.sub	sens%
Ravn P et al, 2005	prospective	48	85
Mori T et al, 2004	case control	118	89
Kang YAet al, 2005	case control	54	81
Ferrara G et al, 2006	prospective	23	74
Lee JY et al, 2006	prospective	87	70
Total		330	

ELISpot for LTBI

In the absence of a gold standard for LTBI, these studies used degree of exposure to infectious index cases as a surrogate reference standard

CHEST 2007; 131:1898–1906

ELISpot in BCG vaccinated unexposed controls

Study	No.sub	Specificity
Pathan AA et al, 2001	28	100
Lalvani A et al, 2001	40	100
Chapman AL et al, 2002	33	100
Lalvani A et al, 2001	26	100
Total	127	

ELISA in BCG vaccinated unexposed controls

Study	No.sub	Specificity
Mori T et al, 2004	216	98
Kang YA et al, 2005	99	96
Total	315	

IGRAs

- Neither test is confounded by BCG vaccination and therefore more specific than the TST
- Both are more sensitive than TST in active tuberculosis (ELISpot>ELISA)
- ELISpot also performs well in young children with tuberculosis, both LTBI and active tuberculosis

Lancet 2001; 357:2017–2021

293 children – diagnostic sensitivity of the ELISpot was much higher than the TST and was independent of HIV coinfection, malnutrition, and age 3 years, all factors that adversely affect sensitivity of TST

IGRA in HIV-TB coinfection

- ELISpot have high diagnostic sensitivity of 92%

  Aids 2002; 16:2285–2293

- ELISA results were associated with known risk factors for LTBI or h/o TB independent of HIV coinfection

  Respir Res 2006; 7:56

- Although the rates of positive TST results were much reduced in HIV+ vs HIV-subjects, rates of positive ELISpot and ELISA results did not vary significantly by HIV infection status, although there was a trend toward higher rates of positive ELISpot results

  Am J Respir Crit Care Med 2007;175:514–520

Emerging evidence indicates that ELISpot is robust to hematologic malignancy-associated immunosuppression and some types of iatrogenic immunosuppression, including corticosteroids and azathioprine

Reliability of ELISpot and ELISA

- Indeterminate results can be due to -
  - young age (<5 yrs)
  - old age (>80 yrs)
  - immunosuppression
- Indeterminate results 12-21% with ELISA but rare with ELISpot

Lancet 2006; 367:1328–1334

Clin Diagn Lab Immunol 2005; 12:491–496
Thorax 2006;61:616–620
Am J Respir Crit Care Med 2005; 172:631–635
Aids 2005; 19:2038–2041

Direct comparison of ELISpot and ELISA

- Lee JY et al.- head to head comparison of two tests in pts of active TB – higher sens. of ELISpot ($p < 0.05$)

Eur Respir J 2006; 28:24–30

- Two other studies showing similar results (but $p > 0.05$)

Lancet 2006; 367:1328–1334
Clin Microbiol Infect 2006; 12:544–550

Effect of Rx on IGRA

- Magnitude of ELISpot responses declines significantly with Rx in both active TB and LTBI
- But considerable inter-individual variation in the rate of decline of the response
- So cannot be used for treatment monitoring or as a “test of cure”
- However, positive ELISpot results very probably reflect the presence of latent but still-viable bacilli *in vivo*, and this suggests that positive results must be associated with a forward risk of progression to tuberculosis

Future prospects

- Simultaneous measurement of IL-2 and IFN-γ secretion by *M tuberculosis*-specific T cells correlates well with treatment
- Next-generation T-cell based tests measuring dual cytokines promise to provide more clinically useful information

Millington KA, Innes JA, Hackforth S, et al. J Immunol, 2007

Nucleic acid amplification assays

NAA assays

- Enable direct detection of M.tb in clinical specimens
- Amplify M.tb specific nucleic acid sequences using a nucleic acid probe
- Require as few as 10 bacilli in given sample
- Types: AMPLICOR assay
  MTD test
  Real time PCR
  BDProbe Tec MTB test

AMPLICOR assay

- Detects presence of mycobacterial 16S rRNA gene by PCR amplification followed by an ELISA reaction
- Approved by FDA for detection of M.tb in smear + resp. samples (not recd ATT for >7 days or within 12 mth)
- Complete process in about 6.5 hrs
- Automated version – Cobas Amplicor

TMA

- Transcription mediated amplification
- Sample preparation-releases r-RNA
- Reverse transcriptase copies the RNA target
- RNA polymerase mediated amplification-RNA amplicon
- Hybridization protection assay detects RNA amplicon

MTD test

- Mycobacterium tuberculosis direct test
- Isothermal strategy for detection of M.tb rRNA
- Takes 3.5 hrs to yeild results
- E-MTD has FDA approval only for respiratory specimens (AFB +/-)

Real time PCR

- Based on hybridization of amplified nucleic acids with fluorescent-labeled probes spanning DNA regions of interest
- Fluorescent signal increases in direct proportion to the amount of amplified product inside the reaction tube
- Results in 1.5-2 hrs after DNA extraction
- Lower risk of contamination

BDProbeTec Direct TB System

- Semi-automated system
- Based on the strand-displacement amplification (SDA) technique
- Uses the enzymatic replication of target sequences in 16S rRNA gene
- Amplified products are detected with a luminometer
- Many false positive results

Limitations of NAA

- No drug susceptibility information
- Able to detect nucleic acid from both living and dead organisms
- May be falsely positive in whom having recent history of infection and adequate Rx

So assays to detect mRNA under study

Serologic tests

Serologic tests

- Applied mainly for smear & culture negative pulm & EPTB at inaccessible body sites
- ELISA based methods for the detection of mycobacteria antigen in body fluids
- Positive test may perhaps “rule in” a diagnosis, but a negative test cannot “rule out” a diagnosis of tuberculosis
- Used as supportive evidence along with conventional tests

Serologic tests - limitations

- Great individual variability in the number and type of reactive antibodies
- Affected by BCG vaccination, previous infection and environmental NTM exposure
- Persistence of antibodies leads to difficulty in distinguishing between infection and disease
- Low sensitivity in smear negative, HIV co-infection, and disease endemic countries
- Expensive
- Requires trained personnel

Advances in diagnosis of drug resistance

Diagnosis of drug resistance

- Genotypic methods -
  - DNA sequencing
  - Solid phase hybridization techniques
  - Microarrays
  - Real time PCR techniques
- Phenotypic methods -
  - Phage based assays
  - Colorimetric methods
  - The nitrate reductase assay

Genotypic methods

- Search for genetic determinants of resistance rather than resistance phenotypes

- Two basic steps -

  (a) molecular NAA eg. PCR to amplify sections of the M.Tb. Genome known to be altered in resistant strains

  (b) assessing the amplified products for specific mutations correlating with resistance

Genotypic methods - advantages

- Shorter turnaround time
- No need for growth of organism
- Less biohazard risk
- Feasibility for automation
- Possibility for direct application to clinical specimens

Common loci for resistance

Drug	Mutant gene	Freq
Rifampicin	rpoB	~96%
INH	katG / inhA	75-85%
Streptomycin	rpsl	65-75%
Pyrazinamide	pncA	~70%
Ethambutol	embB	~70%

DNA sequencing

- Sequencing DNA of PCR amplified products
- Most widely used – accurate and reliable
- Becoming gold std.
- Both manual and automated procedures
- Used for R, H, S and ciprofloxacin

Clin Microbiol Infect 2003;9:349-359
APMIS 2004;112:838-855

PCR SSCP

- Single strand conformation polymorphism
- Based on the property of ssDNA to fold into a tertiary structure whose shape depends on its sequence
- Single strands of DNA differing by only one or few bases will fold into different conformations with different mobilities on a gel, producing what is called SSCP

Ind. J. Tub.; 2003, 50,197

PCR hybrid complementary DNA

- Performed by mixing amplified DNA from the test organisms and susceptible control strains to obtain hybrid complementary DNA
- Resistant strain – heteroduplex hybrid
- Normal strain – homoduplex hybrid
- Both having different electrophoretic mobility

Solid phase hybridization techniques

- Based on the hybridization of amplified DNA from the cultured strains or clinical specimens to ten probes encompassing the core region of the *rpoB* gene, which is immobilized on a nitrocellulose strip
- Absence of hybridization of the amplified DNA to any of the sensitive sequence specific probes indicates mutations
- Two assays -
  - Line Probe assay – for rpoB gene
  - GenoType MTBDR assay – for katG & rpoB

Tuberculosis (Edinb) 2004;84:311-316

GenoType MTBDR assay

- Hillemann et al. – 99% of strains with mutations in the rpoB gene and 88.4% of strains with mutations in the codon 315 of the katG gene were correctly identified
- Correlation with DNA sequencing was 100% and compared with conventional tests good sensitivity and specificity were also obtained

J Clin Microbiol 2005; 43:3699–3703

Microarrays

- Based on the hybridization of DNA obtained from clinical samples to oligonucleotides immobilized in a solid support, such as miniaturized glass slides
- Technically a solid-phase-type hybridization assay
- Gryadunov et al. - 95% rifampicin resistant and almost 80% isoniazid resistant M. tuberculosis isolates within 12 h in a sample of drug resistant isolates and clinical samples
- Very costly

Clin Microbiol Infect 2005; 11:531–539

Molecular beacons

- Nucleic acid hybridization probes
- Designed to bind to target DNA sequences in regions, such as the rpoB, where resistance mutations are known to occur
- Molecular beacons fluoresce only when bound to their targets, so that a mutation even a single-nucleotide substitution prevents fluorescence
- Can identify drug resistance in sputum samples in less than 3 hours
- Both sensitive (82.7% for H & 97.5% for R) and specific (100% for both H & R)

J Clin Microbiol 2004;42(9):4204– 8
J Clin Microbiol 2001;39(11):4131– 7

Other genotypic methods

- Cleavage fragment length polymorphism (CFLP)
- dideoxy fingerprinting (ddf)
- Hybridization protection assays17
- RNA-RNA duplex basepair mismatch assay

Phenotypic methods

- Assess inhibition of M. tb growth in the presence of antibiotics by detecting earlier signs of growth using various technologies

  eg.- colorimetric methods

  - oxygen consumption
  - early visualization of micro-colonies
  - use of phages

Phage amplification (Pha B assay)

- Based on the ability of viable *M.tb* to support the replication of an infecting mycobacteriophage
- Noninfecting exogenous phages are inactivated by chemical treatment
- Can detect viable M.tb within 48 hrs
- FASTPlaqueTB-MDRi or FASTPlaqueTB-RIF uses the phage amplification technology to determine rifampin resistance
- Albert et al. - sensitivity of 100% and a specificity of 97% to determine rifampin resistance

Int J Tuberc Lung Dis 2004;8(9):1114– 9

Luciferase reporter phage assay

- Firefly luciferase catalyzes the reaction of luciferin with ATP to generate photons efficiently and thereby emit light
- Mycobacteriophages expressing the firefly luciferase gene introduced into viable mycobacteria
- Presence of cellular ATP in viable mycobacteria causes visible light to be emitted when exogenous luciferin is added
- The emitted light is measured by a luminometer or on film (eg, Bronx box)

Luciferase reporter phage assay

- Can determine drug susceptibility in 1 to 4 days
- Other reporter molecule is the green fluorescence protein (GPF) of the jellyfish *Aequorea Victoria* – does not require cofactors due to intrinsic fluorescence nature
- Hazbon et al. compared two detection methods, photographic and luminometric for testing against first line antitubercular drugs
- Sensitivity for H and R resistance – 100%

J Clin Microbiol 2003; 41:4865–4869

The nitrate reductase assay

- Based on the capacity of M. tuberculosis to reduce nitrate to nitrite, which is detected by adding a chemical reagent to the culture medium
- Simple and uses same format and culture media as used in the conventional method
- Recent multicentre study - the test performed very well for H, R, E with accuracy between 96.6 and 98%
- However lower values were obtained for streptomycin

J Clin Microbiol 2005; 43:1612–1616

J Microbiol Methods 2005; 63:145–150

Molecular Epidemiology Techniques

Typing of *M.tuberculosis* Strains

- Commonly Used Methods
  - IS6110 Restriction Fragment Length Polymorphism (RFLP) fingerprinting
  - Spoligotyping
  - Mycobacterial Interspersed Repetitive Units – Variable Number Tandem Repeat (MIRU – VNTR) typing
- Alternative Methods
  - Phage typing
  - Pulsed-field electrophoresis (PFGE)
  - Randomly amplified polymorphic DNA
  - DNA sequencing

Beijing Strain

- First isolated in China in 1995, but now found world wide
- Associated with large outbreaks:
  - -Houston, TX and New York City in US
  - -China, Philippines, Gran Canaria
- Associated with drug resistance, but variable

W Strain

- A subgroup of the Beijing strain
- 1990-93 epidemic
  - 3800 cases
  - 357 cases resistant to INH, RIF, EMB, PZA, SM and often Kanamycin
- IS6110 fingerprinting-18 identical bands

IS6110 – RFLP Analysis

- Currently gold std. for typing
- Restriction endonucleases will cut the ds-DNA at specific recognition sites so fragments of different lengths result
- Gel electrophoresis followed by southern blotting to produce patterns which are Genomic or DNA Fingerprints
- IS6110 is a conserved region of DNA which is unique to bacteria in the M. tuberculosis complex (M. tuberculosis, M. bovis, M. microti, and M. africanum)

RFLP - applications

- Differentiate strains of M. tb
- Monitoring transmission
- Define strain clusters within populations
- Differentiate between reinfection & relapse
- Identify lab cross contaminations
- Understanding molecular evolution at species level

Spoligotyping

- M.tb genome has direct-repeat locus which contains 10 to 50 copies of 36-bp direct repeat sequences
- Separated from one another by spacers that have different sequences
- Spacer sequences between any two specific direct repeats are conserved among strains

Spoligotyping

- Strains differ in terms of the presence or absence of specific spacers, the pattern of spacers in a strain can be used for genotyping (spacer oligonucleotide typing)

- Advantages:

  -Small amounts of DNA are required

  -can be performed on shortly after inoculation

  -both for detection and typing

MIRU

- Mycobacterial interspersed repetitive units
- Based on the variability in the numbers of tandem repeats (40-100 bp elements dispersed in intergenic regions of M. tb genome)
- May replace classical RFLP typing once std protocol is developed