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**HOW DOES MOLECULAR
BIOLOGY CHANGE
IDENTIFICATION AND
ANTIBIOTIC SUSCEPTIBILITY
TESTING OF BACTERIA**

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CONVENTIONAL, NON-MOLECULAR METHODS

- **are appropriate for identification of species which readily grow and multiply in vitro to determine their**
 - **microscopic morphology**
 - **Gram-stain**
 - **colony characteristics**
 - **motility**
 - **sporulation**
 - **anabolic and catabolic activities**

UNCULTIVABLE BACTERIA

How to identify uncultivable pathogens?

UNCULTIVABLE BACTERIA

R. KOCH postulates

**Isolation and identification
of bacteria in pure culture
in an artificial medium**

Modified by Falkow, 1988

AIMS OF CULTIVATION

- **testing for viability**
- **isolation of pure culture**
- **identification by colony morphology and biochemical reactions**
- **antibiotic sensitivity testing**
- **production of antigens**

CULTIVATION

When the cultivation of bacteria, which is the *sine qua non* of microbiological practice become difficult or impossible, these essential functions become unachievable.

UNCULTIVABLE BACTERIA

Mycobacterium leprae and *Treponema pallidum* - two of the oldest human pathogens are still uncultivable on artificial media.

As well:

Bartonella henselae (Relman, 1990);
Tropheryma whippelii (Relman, 1992)
Whipple's disease

UNCULTIVABLE BACTERIA

Factors responsible for uncultivability

UNCULTIVABLE BACTERIA

Question:

- **Does bacterial uncultivability represent an intrinsic property of bacteria or does it reflect the deficiency of our knowledge? (Bhattacharya, 2002).**
- **Probably the answer is a combination of both of the above factors.**

UNCULTIVABLE BACTERIA

Why *M. leprae* does not multiply under cell free conditions?

Intracellular ATP content in cells of *M. leprae* increased consistently in the medium containing adenosine after 4-6 weeks of cultivation and decreased thereafter. This was a result of the characteristic property of *M. leprae* cell wall (Nakamura, 2001).

UNCULTIVABLE BACTERIA

Implications of bacterial uncultivability:

- **theoretical**
- **practical**

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THEORETICAL

**Limitation of Koch's postulates
stimulated their modification - as
molecular postulates (Falkow,
1988)**

UNCULTIVABLE BACTERIA

PRACTICAL

- growth in cell lines, Mc Coy, - *C. trachomatis* (Fredlund et al. 2004; APMIS 112:771)
- growth in embryonated eggs - *Rickettsia* and *Chlamydia* in the yolk sac
- growth in laboratory animals
 - guinea pigs and mice for the cultivation of *Rickettsia*
 - food pad of mice for *M. leprae*
 - rabbit tests for the maintenance of *T. pallidum*.
- development of novel methods of cultivation e.g. co-cultivation

UNCULTIVABLE BACTERIA

Search for hidden pathogens in diseases thought to be non infective:

- Sarcoidosis**
- Inflammatory bowel disorders - Crohn disease**
- Rheumatoid arthritis**
- Systemic lupus erythematosus**
- Wegener granulomatosis**
- Tropical sprue**
- Kawasaki disease (Friedericks, Relmar, 1998)**

Non culture based methods

- **direct microscopy**
- **immunological methods (antigen antibody reactions)**
- **chromatographic methods**

Molecular identification

- **growth and cell multiplication are not obligatory (non-culture diagnosis)**

Methods

- **hybridisation of DNA with specific probes**
- **polymerase chain reaction products with specific primers (oligonucleotides)**
- **sequencing of DNA from PCR**

Criteria which should be fulfilled both by conventional and molecular identification procedures:

- **highest possible sensitivity and specificity**
- **rapid, non-laborious**
- **simple**
- **cheap**

Molecular methods

Molecular biology has opened a new frontier for the genotypic identification and characterization of fastidious microorganisms e.g. *Mycobacterium* and *Legionella*.

Growth in vitro is no longer necessary for microbial identification (Greer et al., 1991).

Nucleic acid amplification techniques:

- 1. Target amplification systems: PCR (polymerase chain reaction), TMA (transcription mediated amplification) or strand displacement (SD)**
- 2. Probe amplification systems: Q β replicase (Q β R) or thermostable DNA ligase (LCR)**
- 3. Signal amplification- the signal generated from each probe molecule is increased by using compound probes or branched probe technology (TABLE 1)**

TABLE 1 Nucleic acid amplification methods

Amplification method	Amplification category	Manufacturer or license holder	Enzyme(s) used	Temp requirement	Nucleic acid target
PCR	Target	Roche Molecular System, Inc., Branchburg, N.J.	<i>Taq</i> DNA polymerase	Thermal cycler	DNA (RNA)
TMA	Target	Gen-Probe, Inc., San Diego, Calif.	RT, RNA <i>pol</i> , RNase H	Isothermal	RNA and DNA
NASBA	Target	Organon-Teknika Corp., Durham, N.C.	RT, RNA <i>pol</i> , RNase H	Isothermal	RNA and DNA
SDA	Target	Becton-Dickinson & Co., Rutherford, N.J.	Restrictive endonucleonase, DNA polymerase	Isothermal	DNA
Q β R	Probe	Vysis, Inc., Naperville, Ill.	Q β replicase	Isothermal	DNA and RNA
LCR	Probe	Abbott Laboratories, Abbott Park, Ill.	DNA ligase	Thermal cycler	DNA
bDNA	Signal	Chiron Corp., Emeryville, Calif.	None	Isothermal	DNA and RNA

Murray, 1999, Manual of Clinical Microbiology

Criteria for introducing molecular techniques into the diagnostic laboratory:

- 1. Which organisms need a test better than those offered by traditional methods?**
- 2. Do the available molecular tests fulfill the required criteria of high sensitivity and specificity, speed, simplicity and clinical relevance?**
- 3. Which clinical specimens should be tested?**

Molecular techniques are needed

- **for the detection of organisms that cannot be grown in vitro or**
- **for which current culture techniques are too insensitive**
- **too costly or**
- **too time consuming**

Molecular Methods

Problems

1. Contamination of negative specimens by either template carryover or cross contamination from positive controls prepared in parallel.

EX: cross reactivity between *T. gondii* and *Nocardia asteroides* may lead to false positive results in some systems, and misdiagnosis of a cerebral *Nocardia* infection has been reported (Mc Hugh et al., 1995). Thus, clinical validation is indispensable.

Molecular Methods

Problems cont.

- 2. False negative results:
Inhibitors of enzymes used in amplification tests may be present. Such inhibitors can be detected by spiking a small amount of nucleic acid target into the specimen (Monteiro et al., 1997)**

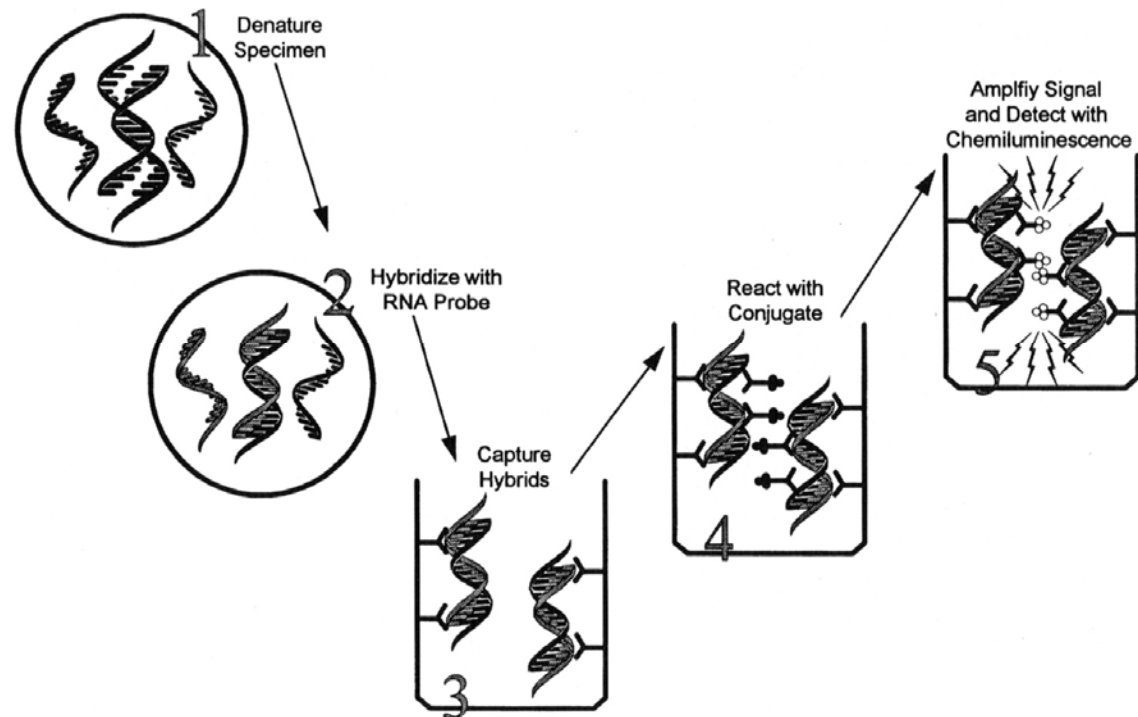
Molecular Methods

Problems cont.

- 3. Disadvantage: molecular methods reveals only what you are looking for - the physician knows which pathogen is causing the disease.**
- 4. The expense - costs of reagents, equipment, space needed to separate preamplification from postamplification procedures have hindered introduction of molecular methods in many clinical laboratories.**

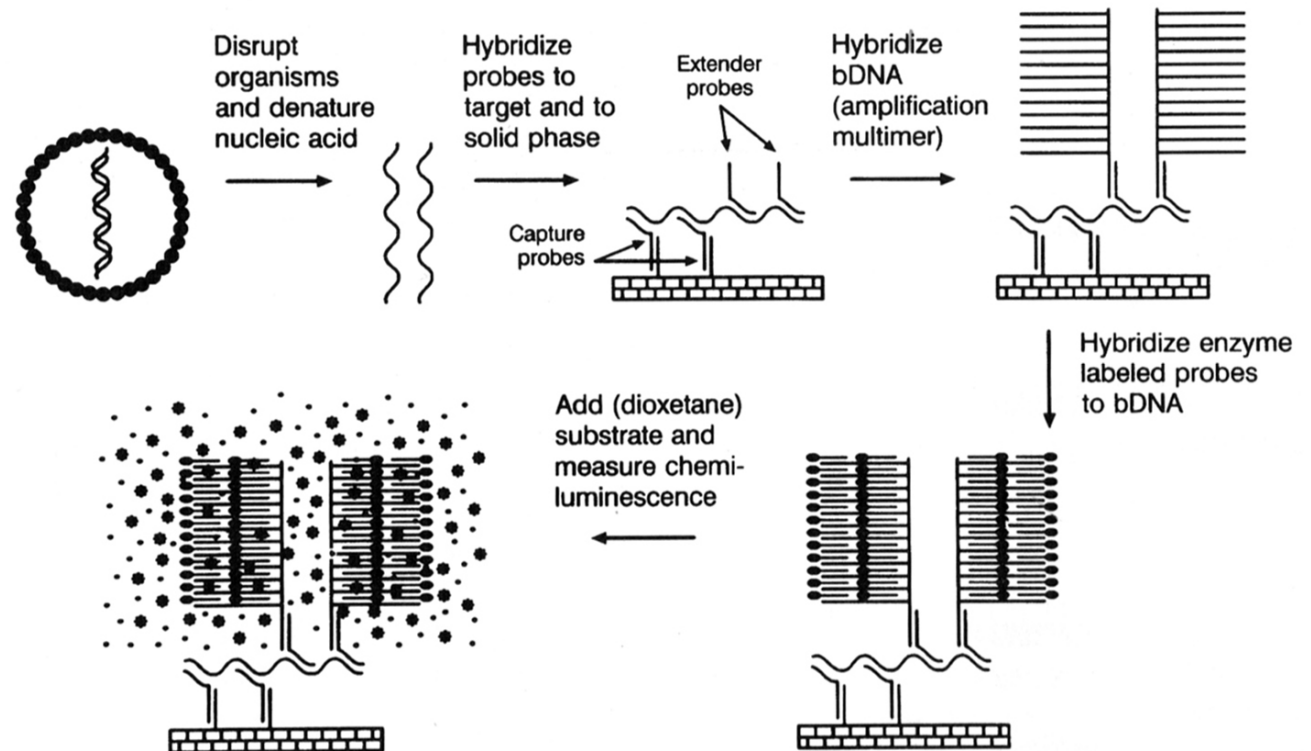
Molecular methods

Digene hybrid capture system



Murray, 1999, Manual of Clinical Microbiology

bDNA based signal amplification



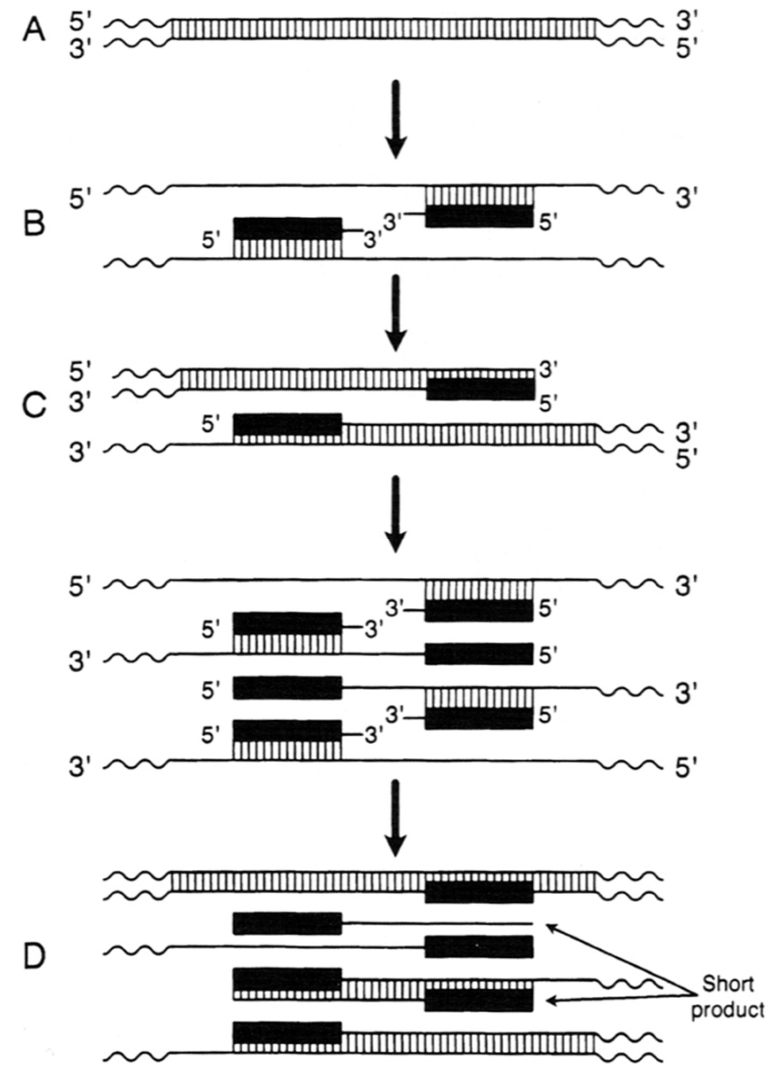
Murray, 1999, Manual of Clinical Microbiology

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Murray, 1999, Manual of Clinical Microbiology

APPLICATIONS OF MOLECULAR DIAGNOSIS

**Unculturable, slow growing microorganisms:
The inability to culture and analyze the principal etiologic agent of non A non B hepatitis (HCV) limited medical advances in this area. Using molecular methods, investigators were able to isolate HCV nucleic acid (Choo et al., 1989). Analysis and cloning of the HCV genome have provided the viral antigens necessary for the development of specific serologic tests.**

Nocardia

- many medically significant species which are difficult to distinguish by conventional techniques
- molecular methods such as PCR restriction enzyme analysis and 16S ribosomal DNA (rDNA) sequencing
- DNA Microarray for simultaneous identification and characterization without prior amplification of target DNA or pre-identification of the pathogen (Cleven et al. 2006, J Clin Microb, 44:2389)

Unusual bacteria

Analyses of small subunit (16S) bacterial rRNA gene has expended the understanding of phylogenetic relationships among members of the bacterial kingdom (WOESE, 1987).

16S rRNA sequencing is applied for the identification of novel pathogens prior to the availability of in vitro culture methods. In 1990, a patient infected with HIV 1 died of an overwhelming infection with an acid fast organism - now known as *Mycobacterium genavense* which at the time, could not be propagated in vitro (Hirschel et al, 1990)

***Tropheryma whippelii* - causative agent of Whipple's disease (Relman, 1992; Ramzan, 1997).**

Subtyping of microorganisms

Important implications for prognosis and therapy.

Diversity between cagA positive and cagA negative H. pylori strains. Infection with cagA positive strain increases the risk for development of gastric ulcer and cancer (Covacci et al., 1993; Simala-Grant et al. 2004; APMIS 112:886).

Group A RSV infection has a greater severity than group B among hospitalized infants.

HPV (human papilloma virus) is a common cause of dysplasia, intraepithelial neoplasia and carcinoma in the female genital tract. Certain types 16, 18- are associated with a high risk of progressive neoplasia.

Different HCV genotypes have distinct profiles of pathogenicity, infectivity and response to antiviral therapy.

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Monitoring the disease by organism quantitation

HIV load, HBV and HCV

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Detection of antimicrobial drug resistance

CONVENTIONAL, NON-MOLECULAR METHODS

Slow, difficult or non-culturable organisms are inappropriate for identification by conventional, non-molecular techniques.

- *Chlamydia trachomatis*
- *Chlamydia pneumoniae*
- *Mycobacteria*
- *Legionella* spp.

1. Resistance in *Mycobacterium*

Multi-resistant strains (MRS) (resistant to rifampicin and isoniazid) isolated in hospitals in the USA, South East Asia and parts of Europe. Rapid identification has become a major public problem

Mycobacteria

- **direct detection of Mycobacteria in clinical specimens by molecular methods**
- **detection of mutations causing drug resistance**

Reasons for using genetic tests to detect resistance genes

2. Detection of resistance genes or mutations that result in resistance in organisms directly in clinical specimens to guide therapy early in the course of a patient's disease long before cultures are positive.

Reasons for using genetic tests to detect resistance genes

- 3. Genetic methods are more accurate than antibiograms for following the epidemiologic spread of a particular resistance gene in a hospital or community setting**
- 4. Gold standard for the detection of resistance when evaluating the accuracy of new susceptibility testing methods.**

Detection of antimicrobial drug resistance

Depends on knowing the genotypic basis of the resistance (Sundsfjord et al. 2004; APMIS 112:815). For organisms with unknown resistance mechanisms, conventional susceptibility testing remains essential (Tang and Persing, 1999)

Enterococci

(Eigner et al. 2005, J Clin Microb, 43:2920)

- simultaneous identification of vancomycin-resistant *Enterococcus* (VRE) species: *E. faecium*, *E. faecalis*, *E. gallinarum*, *E. caselliflavus*, *E. flavescens* and their glycopeptide resistant genotypes (*vanA*, *vanB*, *vanC1*, *vanC2/3*)
- DNA-probes are immobilized on nitrocellulose strips (Geno Type assay), results in 4,5 hours

Detection of antimicrobial drug resistance

Enterococci cont.

- **VITEK-2 system for automated rapid Identification (ID) takes 3 hours.**
- **Antimicrobial susceptibility testing (AST) in 9 hours.**

Identification

Vibrio spp.

A 90-plex PCR assay was combined with a long oligonucleotide DNA microarray. Identifies regions specific for species, serogroup, biotype, antimicrobial resistance and pathogenicity markers for *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, and *V. mimicus*.

(Vora et al. 2005, PNAS, 102:19109)

Identification

Chlamydia trachomatis (Fredlund et al. 2004, APMIS, 112:771)

- Conventional diagnostics: cell culture: McCoy cells develop intracytoplasmatic inclusions which are identified by fluorescein-conjugated antibodies - laborious.
- Instead - nucleic acid amplification tests (NAATs). Major target sequences for NAATs are located on the cryptic plasmid (alternative: *omp1* gene on the chromosome)

mec A containing *S. aureus*, almost all strains which possess the gene expresses the resistance to penicillin. This is not the case for coagulase negative staphylococci, in which resistance can be induced by propagation of isolates in progressively higher concentrations of oxacillin.

1. Oxa resistant *S. aureus* MIC between 2 and 8 µg/ml may contain the *mecA* gene determinant or may produce high amounts of enzyme that slowly hydrolyse oxacillin.

Applications:

- 1. Aminoglycoside resistance genes - in Gram negatives there is a large number of different types of aminoglycoside resistance genes; in Gram positive organisms the situation is more uniform**
- 2. Resistance to beta-lactam drugs**

**Oxacillin resistance in
Staphylococci- *mec A* is a
problem, especially with
coagulase negative strains-
differentiation of border line
isolates**

**Beta-lactam resistance in
pneumococci**

**Beta-lactamases in Gram negative organisms:
TEM-, SHV-, OXA-, CARB- and ROB-
Bla TEM-1 gene was detected in
Enterobacteriaceae, *Haemophilus*, and
Neisseria gonorrhoeae. (>150 TEM, >50
SHV, >30 CTX-M;
www.lahey.org/studies)**

**Isoelectric focusing is used as a screening test
for the identification of novel beta-
lactamase genes, but DNA sequencing has
become the gold standard for analyzing
novel beta-lactamase genes.**

3. Chloramphenicol resistance

Chloramphenicol acetyl transferase (CAT) is produced by Gram positives and Gram negatives

4. Glycopeptide resistance

5. Macrolide, lincosamide and streptogramin resistance

6. Mupirocin resistance

7. Quinolone resistance

