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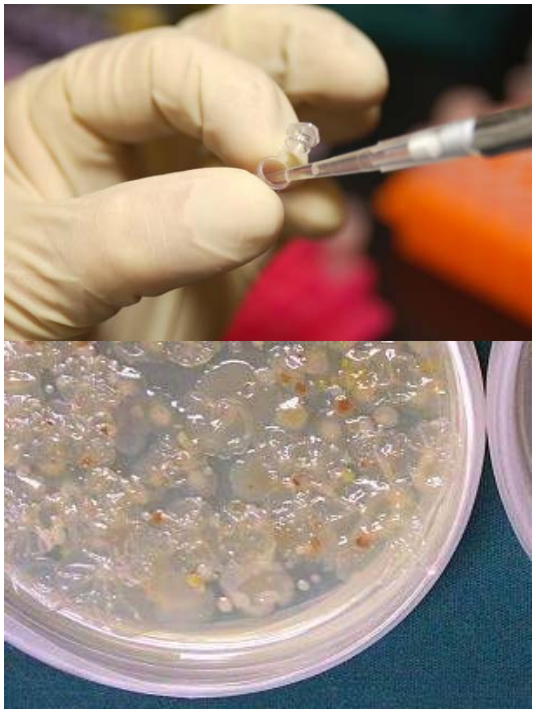

American Society of Cytopathology Core Curriculum in Molecular Biology

Chapter 5:

Applications of Molecular Biology
Diagnosis and Detection of Microorganisms

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Objectives

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- Discuss parameters for specimen collection, preparation, and quality control
- Discuss molecular methods to detect bacterial organisms and resistance to antibiotics
- Describe the use of molecular methods in epidemic epidemiology
- Describe the use of molecular methods in detection of viral diseases
- Discuss molecular methods to detect fungi and parasites

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Molecular Detection and Identification

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- Traditional methods of culture, stains, and biochemical testing are still the major methods used
- But organisms difficult/labor intensive to detect, (for example, *Mycobacterium tuberculosis*), hazardous (*Histoplasma*), or those for which a good laboratory test is lacking such as Hepatitis C or HIV use MP

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Molecular Detection and Identification

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- Used for high-volume tests such as *Streptococcus pyogenes*
- Also, genes that confer resistance to antibiotics such as vancomycin (vanA, etc.) can be detected by MP
- Sequencing of DNA and RNA can be used to find new organisms, subclassify, and predict drug susceptibility

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Types of methods used

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- Same as those to detect human polymorphisms
- PCR (traditional, real-time, and reverse transcriptase as well as DNA sequencing)
- Pulsed-field gel electrophoresis (PFGE)

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Specimen Collection

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- Proper collection and handling to prevent degradation of DNA and especially RNA
- Avoid contamination
- As few as 50 organisms may be detected
- Proper anticoagulant, if used for blood
- Plastic handled swabs for collection (not wood)
- Swab extraction tube system (SETS) for centrifugation of swabs
- Universal Precautions – Appropriate level containment (smallpox – level 4 labs)

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Sample Preparation

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- DNA extraction may need to be modified
- Stronger lysis methods for thick-walled organisms such as fungi, delicate for thin-walled organism such as *Mycoplasma*
- Concentration of organisms considered
- Removal/inhibition of inhibitors of enzyme reactions
- For RNA, removal of all RNases

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Types of Sample Preparation

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- CSF has inhibitors of polymerase – isolate DNA carefully
- Whole blood – remove all hemoglobin and other hemoglobin products - inhibitors of polymerase
- Blood-Isolate white blood cells from RBCs using Ficoll-Hypaque and then lyse for virus DNA
- Sputum – remove acidic polysaccharides (inhibits polymerase)
- Urine – centrifuge and then use DNA isolation methods

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Quality Control

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- Prevent contamination – causes FP in PCR
- Remove inhibitors – otherwise may cause FN
- **Positive control** (shows test is working)
- **Sensitivity control** (positive at lower/upper limits of detection)
- **Reagent blank** – to detect reagent carry-over (should always be negative – no target)
- **Negative template control** – non-target organisms, negates assay if found positive

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Quality Control

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- **Amplification control** – aimed at a housekeeping gene or a human gene that is always present
- If amplified, then negative target is more likely a true negative
- **Internal controls** – monitor steps of process
- Positive result = microorganism is present
- Negative results = microorganisms is not present (within detection limits)

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Eliminating Sources of Error

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- Most FP are reagent carryover contamination
- Some may be from dead or dying organisms in pt. on antibiotics
- Repeating test after 3-6 weeks should be truly negative
- DNA degradation may cause FN
- Prevent by proper collection, sampling, transport media, inhibit DNases and RNases
- Remove inhibitors (hemoglobin, etc.)
- Validate new tests, controls, sensitivity, specificity, reproducibility, personnel

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Bacterial Targets of MP

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- Target sequence must be unique to prevent cross-reactivity between organisms
- Primers or probes meet technical considerations (annealing temps, etc.)
- Many commercially available kits and home-brew PCR protocols
- Home-brew must validate all aspects of test (controls, probes, primers, etc.)

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Molecular Detection of Bacteria

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Methods

- Nucleic-acid based amplification (NASBA)
- Qβ-replicase
- PCR: real-time or quantitative, reverse transcriptase, nested, multiplex

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Molecular Detection of Bacteria

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Detection

- Southern blot
- Gel electrophoresis
- PCR
- Sequencing
- Enzyme immunoassay
- Dot blot hybridization
- Restriction enzyme analysis

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Real-Time or Quantitative PCR

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- Increasingly used – more information than conventional PCR
- Subclinical levels of infection vs. clinical
- Melting-curve analysis by closed tube analysis
- Performed on DNA extracted directly from clinical specimens

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Design of qPCR

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- Unique target gene for primers/probes
- DNA-specific dye SYBR Green instead of probes if no misprimes or primer dimers
- Probe types include fluorescent energy transfer, hydrolysis (Taq Man)
- Gene targets include rRNA and rDNA

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Respiratory Pathogens

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- Traditional methods may be time-consuming and lack sensitivity
- Respiratory infections are an important cause of disease
- Mycobacterium tuberculosis – smear sensitivity = 22-80%, culture = 2-3 weeks, speciation takes several more weeks
- MP tests (AccuProbe, MTD, Amplicor) improves sensitivity and specificity to high 90%s and improves turn-around time to 3.5 – 8 hours.

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Other Respiratory Pathogens

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- *Bordetella pertussis* (whooping cough); culture sensitivity 58% vs. PCR 97%
- *Legionella pneumophila* (Legionnaires' disease) PCR being developed for all species
- *Mycoplasma pneumoniae* 4 weeks for culture; MP correlates best when compared to pt. symptoms and + tests
- Development of multiplex to screen for 4 organisms (*M. pneumonia, L. pneumophila, B. pertussis, and C. pneumoniae*) for respiratory infections

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Streptococcus pneumoniae

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- Traditional methods have not been supplanted by PCR because many people, especially children, are colonized by the organisms and PCR cannot differentiate between infection and colonization.
- The sensitivity and specificity of the test are not high enough to replace traditional methods.

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Urogenital Tract Pathogens

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- http://www.cytologystuff.com/indexmolecular.htm
- *Chlamydia trachomatis*
- *Neisseria gonorrhoeae*
- *Human Papilloma Virus*
- Self Assessment

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Urogenital Tract Pathogens

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- *N. gonorrhoeae* and *C. trachomatis:* 2 most common causes of STD
- MP methods have replaced traditional methods almost exclusively
- Organisms often coexist and checking simultaneously with one test is useful
- Numerous nucleic acid amplification assays are commercially available
- Sensitive, specific, rapid, can be fully automated

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Urogenital Tract Pathogens

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- *Treponema pallidum* (syphilis)
- Serologic testing for antibodies (RPR and VDRL) since cannot be grown in vitro.
- EIA (new enzyme immunoassay) based tests to detect anti-*T. pallidum* antibodies
- RPR and VDRL not specific for syphilis, EIA cannot differentiate between current and past infections but development of PCR has not replaced these tests yet.

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Other Urogenital Pathogens

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- *Haemophilus ducreyi* difficult to grow
- PCR is promising but further development is necessary
- *Mycoplasma* and *Ureaplasma* cause non-gonococcal urethritis
- PCR assays have been developed but not widely used
- Multiplex PCR tests for several urogenital pathogens are being developed to test for more than one organism at a time

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Antimicrobial Agents

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- Inhibitors of microbial growth **–static**, e.g.bacteriostatic
- Agent that kills the organism **–cidal**, e.g. bacteriocidal
- Should be selective against target, have long activity half-life, distribute well in patient, be lethal to organism (rather than –static)

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Development of Resistance

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Several methods by which resistance develops; all involve genetic change in organism

- Destruction of agent
- Elimination of agent
- Altered cell wall
- Alternate metabolic pathways

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Genetic Changes

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- Mutation and selection
- Acquisition of genetic factors from other resistant organisms by plasmids or viruses
- Vancomycin resistance can be transferred from vancomycin-resistant *E. faecalis* to vancomycin-sensitive *S. aureus*
- Its cell wall is built with without the target structure for vancomycin, thus it becomes resistant.

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Molecular Detection of Resistance

- Traditional methods may take at least 48 hours and results may be equivocal
- Minimum inhibitory concentration (MIC) is the least amount of agent needed to inhibit growth of an organism

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MP resistance detection

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Use MP method:

1. MIC is at or near breakpoint of resistance
2. Genes involved in resistance can be directly detected without culture
3. Monitoring spread of resistance in epidemiologic testing
4. MP is the gold standard for new phenotypic assays in development

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Resistance has been detected by MP for several organisms

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- MRSA
- Vancomycin resistant enterococci *(VRE)*
- *M. tuberculosis*
- PCR methods provide rapid, sensitive, and specific results when used to detect resistance

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Molecular Epidemiology

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- **Epidemic** – disease affecting many unrelated individuals at the same time
- **Pandemic** – disease spreading across wide geographic areas
- Causative genetic and environmental factors at a molecular level
- Useful for identifying and typing agents of infection with objective data rather than judgement

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Molecular Epidemiology

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- Need to discover if all organisms in an outbreak are from the same source or from a variety of sources (*E. coli* in spinach outbreak)
- Plasmid analysis, Pulsed Field Gel Electrophoresis (PFGE), RFLP, random amplified polymorphic DNA assay (RAPD), amplified fragment length polymorphism (AFLP), interspersed repetitive elements, internal transcribed spacer elements, spa typing, coa typing, multi locus sequence typing (MLST)

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MSLT – Multilocus Sequence Typing

This test is used in molecular epidemiology to detect various alleles of common housekeeping genes. Variations in these sequences imply that the organisms are not from a common source.

6-7 gene fragments with from 50 to 88 alleles are analyzed and together can determine if two samples are likely to have originated from the same common pool of bacteria. This can be used to control the spread of the disease.

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Criteria for Typing Methods

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1. Typing capacity – target organism able to be typed by method to be used
2. Reproducible – same results when repeated
3. Discrimination – clearly distinguish between unrelated strains
4. Ease of interpretation – easy to read
5. Ease of test performance – easy to do

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Criteria vary among the methods

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- Not any one method is perfect for all criteria
- For some organisms, MP is the exclusive choice
- For others, rapid results can supplement traditional methods
- For remaining organisms, other tests are still being developed

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Viruses

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- Detection of viruses has been aided by MP more than any other type of organism
- Traditional testing to detect antibodies, viral antigens, or virus in culture
- Antibodies are an indirect method of diagnosis
- Antigen detection is only available for some viruses
- Culture is commonest method of detection

  but is time-consuming and may be FN

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MP Methods for Viruses

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- PCR
- RT-PCR
- Quantitative PCR (real-time or qPCR)
- Transcription-mediated amplification (TMA)
- Branched DNA (b-DNA) amplification
- Hybrid capture
- For diagnosis or monitoring

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Human Immunodeficiency Virus

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- Retrovirus that makes a DNA copy of RNA using virally encoded reverse transcriptase
- HIV-1 and HIV-2, rapidly mutates into multiple groups ("clades" or subtypes)
- HIV-1, group M makes up 95% of infections in world (Clades A,B,C,D,F,G,H)
- Diagnosed by detecting antibodies in an EIA and confirming specificity in a Western blot
- Nucleic acid amplification tests are performed after diagnosis to determine viral load, when to start antiviral therapy, and to monitor treatment

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Viral Load Determination

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- Marker for prognosis, to track timing and success of antiretroviral therapy.
- CD4 counts are usually inversely proportional to viral load
- Methods include PCR for integrated DNA, RT-PCR for viral RNA, nucleic acid sequence-based amplification (NASBA), and bDNA.
- Viral load should be measured before therapy, 2-8 weeks after tx. started, and every 3-4 months after to monitor effectiveness.

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Test Performance Features for Viral Load Measurement

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1. Sensitivity – lowest level detected 95%of time
2. Accuracy – ability to detect true value
3. Precision – reproducibility of results
4. Specificity – no FN or FP
5. Linearity – a serial dilution of a standard curve closely approximates a straight line
6. Flexibility – accuracy of measurement of virus regardless of sequence variations

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Genotyping for Resistance Detection in HIV

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- Viral RNA is extracted; PCR used to amplify the whole protease gene and part of the reverse transcriptase gene.
- Genes are analyzed for mutations by sequencing, hybridizing onto microarrays, or reverse hybridizing using line probe assay.
- Significance of any mutations for antiretroviral therapy must be assessed
- Computer analysis using algorithms are used to determine effect on therapy

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Resistance Types

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- Primary resistance mutations – specific for a particular drug, reduce the susceptibility of the virus to that drug, appear shortly after tx. has begun
- Secondary resistance mutations – help the virus replicate in the presence of the drug
- If a resistance genotype is identified, drug tx. should be changed ASAP to avoid secondary mutations in the virus.

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Hepatitis C Virus

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- HCV is an enveloped RNA virus
- Serology used to detect presence of antibodies to HCV
- Western blot used for specificity of genotype
- RT-PCR. Transcription mediated amplification, b-DNA
- Viral load is measured as for HIV
- Pts. with a 2 $\log_{10}$ decrease in HCV after 12 weeks will have a 65% chance of responding; if not this level of decrease, only a 3% chance of responding.

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Summary of MP use for Viruses

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- Use of MP for HIV and HCV is standard
- Increasing in use for other viruses, BK, CMV, EB, HBV, including HPV in cervical samples
- Some other viruses MP only used in special situations, e.g. traditional tests negative but high clinical suspicion
- Outbreak identification - more rapid and sensitive (SARS, West Nile, etc.)
- Multiplex tests for respiratory and urogential samples are very useful.

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Fungi

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- Gene probes have been developed for *Histoplasma, Blastomyces, Coccidiodes*, and *Cryptococcus neoformans*.
- MP is faster and less hazardous to lab personnel than microscopic identification.
- Direct smears and isolation of fungi are still the major methods of detection.

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Parasites

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- Traditional method is morphology in direct specimens.
- Types of parasites detected using MP include *Leishmania, Entamoeba histolytica*, and *Cryptosporidium*

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Summary

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- MP is available in nearly all areas of the laboratory and has shown to be rapid, sensitive, specific, and in some cases an improvement over traditional methodology.
- Rapidity of turn-around time, improved detection, and increased safety for lab personnel are some of the advantages.
- Reference: Molecular Diagnostics, Fundamentals, Methods, and Clinical Applications, Buckingham L and Flaws M, 2007, Chpt. 12

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