Detection and Identification of Microorganisms

Chapter 12

Target Microorganisms for Molecular-Based Testing

* Those that are difficult or time-consuming to isolate
	+ e.g., *Mycobacteria*
* Hazardous organisms
	+ e.g., *Histoplasma, Coccidioides*
* Those without reliable testing methods
	+ e.g., *HIV, HCV*
* High-volume tests
	+ e.g., *S. pyogenes*, *N.* *gonorrhoeae, C. trachomatis*

Applications of Molecular-Based Testing in Clinical Microbiology

* Rapid or high-throughput identification of microorganisms
* Detection and analysis of resistance genes
* Genotyping
* Classification
* Discovery of new microorganisms

Specimen Collection

* Preserve viability/nucleic acid integrity of target microorganisms.
* Avoid contamination.
* Maintain appropriate time and site of collection (blood, urine, other).
* Use proper equipment (coagulant, wood, or plastic swab shafts).
* Commercial collection kits are available.
* The Clinical and Laboratory Standards Institute (CLSI) has guidelines for proper specimen handling.

Sample Preparation

* Consider the specimen type (stool, plasma, CSF).
* Consider the number and type of organisms in the sample.
* Inactivate inhibitors (acidic polysaccharides in sputum or polymerase inhibitors in CSF).
* Inactivate RNases.

PCR Detection of Microorganisms: Quality Control

* PCR and other amplification methods are extremely sensitive and very specific. For accurate test interpretation, use proper controls.
	+ Positive control: positive template
	+ Negative control: negative template
	+ Amplification control: omnipresent template unrelated to target
	+ Reagent blank: no template present

PCR Quality Control: Internal Controls

* Homologous extrinsic
	+ Controls for amplification
* Heterologous extrinsic
	+ Controls for extraction and amplification
* Heterologous intrinsic
	+ Human gene control

Sensitivity vs Specificity

* **Sensitivity** and **specificity** are statistical measures of the performance of a test.
* **Sensitivity** (also called the *true positive rate*) measures the proportion of actual positives which are correctly identified as such (e.g. the percentage of sick people who are correctly identified as having the condition).
* **Specificity** measures the proportion of negatives which are correctly identified as such (e.g. the percentage of healthy people who are correctly identified as not having the condition, sometimes called the *true negative rate*).
* When screening for a disease, sensitivity is more important (you can confirm with a more specific test later)
* When confirming a disease, specificity is more important

Quality Control: False Positives

* Contamination: check reagent blank
* Dead or dying organisms: retest 3–6 weeks after antimicrobial therapy
* Detection of less than clinically significant levels

Quality Control: False Negatives

* Improper collection, specimen handling
* Extraction/amplification failure: check internal controls
* Technical difficulties with chemistry or instrumentation: check method and calibrations

**Detection of Bacteria**

Respiratory Diseases

* Respiratory infections are responsible for significant numbers of infections and deaths worldwide.
* These infections are easily spread by inhalation

Bordetella (Whooping Cough)

* ***Bordetella pertussis*** is a Gram-negative, aerobic coccobacillus
* *B. pertussis* is nonmotile. Its virulence factors include pertussis toxin, filamentous hemagglutinin, and tracheal cytotoxin.
* In the US, it killed 5,000 to 10,000 people per year before a vaccine was available. Worldwide in 2000, according to the WHO, around 39 million people were infected annually and about 297,000 died.
* Because of concerns regarding the vaccine, numbers continue to be high
* There are three species that cause most of the pertussis seen in humans:
	+ *B. pertussis* causes the most severe whooping cough in children.
	+ *B. parapertussis* and *B. holmesii* cause a less severe whopping cough.
* In view of its enormous sensitivity and specificity rapid PCR based detection of *B. pertussis* has attracted much attention in recent years. The chromosomal regions that have been used as targets for *B. pertussis* specific PCR include :
	+ the adenylate cyclase toxin (ACT) gene
	+ a region upstream of the porin gene ,
	+ Pertussis toxin (PT) promotor region, and
	+ repeat insertion sequences.
* Among these, repeat insertion sequence IS481 region being present in multiple copies (80–100) in *B. pertussis*, is a target of choice for amplification and detection with greater sensitivity
* However, *B. holmesii* also contains regions homologous to IS481 . Thus PCR targeting IS481 will generate a positive DNA product for both *B. pertussis* and *B. holmesii* as observed.
* As a result it although will provide high sensitivity, it will lack specificity. However, since there are two single nucleotide changes (A/C and C/T variation) in alleles of *B. holmesii* genome; a DNA probe can be diagnostic.
* However, there is another insertion sequence, (IS 1001), that is present in *B. holmesii* but not in *B. pertussis*.
* Thus, testing for both insertion points will give a differential diagnosis.

Tuberculosis

* The presence of acid-fast-bacilli (AFB) on a **sputum smear** or other specimen often indicates TB disease. Acid-fast microscopy is easy and quick, but it does not confirm a diagnosis of TB because some acid-fast-bacilli are not *Mycobacterium tuberculosis*.
* Therefore, a **culture** is done on all initial samples to confirm the diagnosis. (However, a positive culture is not always necessary to begin or continue treatment for TB.) A positive culture for *M. tuberculosis* confirms the diagnosis of TB disease.
* Although sputum smears are the gold standard for diagnosis of tuberculosis, sensitivity in HIV/TB coinfection cases is low, indicating a need for alternative methods.
* Also culture can take as long as 3 weeks.
* Urine is being increasingly evaluated.
* A new method for detecting *Mycobacterium tuberculosis* (MTB) uses combined IMS/ATP assay.
* If the smear is positive, PCR or gene probe tests can distinguish *M. tuberculosis* from other mycobacteria.
* Target probe is the 16S rRNA sequence. Turnaround is reduced to about 2-3 hours.
* But sensitivity is not good enough for clinical specimens
* Urine is being increasingly evaluated.
* A new method for detecting *Mycobacterium tuberculosis* (MTB) uses combined IMS/ATP assay.
* Immunomagnetic separation (IMS) is used to concentrate and recover pathogenic mycobacteria, including MTB . IMS also enables specific target capture and decreases particulate interference in detection assays.
* ATP bioluminescence assays have demonstrated utility in bacteriuria (bacteria in urine) screening , quality control of BCG vaccines, and MTB antibiotic susceptibility testing.
* The determination of ATP using bioluminescence uses the ATP dependency of the light emitting luciferase catalyzed oxidation of luciferin for the measurement of extremely low concentrations of ATP. Luminescence is measured using a luminometer.
* Combining immunocapture with an ATP-based cell viability assay can provide rapid, specific, semiquantitative detection of live cells.
* The method can provide rapid, specific detection of MTB in urine. The method is easy to perform and could be used in settings where the rate of HIV/TB co-infection is high.

Detection of bacterial STDs

* Historically, the diagnosis of sexually transmitted diseases (STDs) has been difficult. The introduction of molecular biology techniques in microbiological diagnosis and their application to non-invasive samples has produced significant advances in the diagnosis of these diseases.
* Overall, detection of *Neisseria gonorrhoeae* by molecular biology techniques provides a presumptive diagnosis and requires confirmation by culture in areas with a low prevalence.
* For *Chlamydia trachomatis* infections, these techniques are considered to be the most sensitive and specific procedures for mass screening studies, as well as for the diagnosis of symptomatic patients.
* Diagnosis of *Mycoplasma genitalium* infection by culture is very slow and consequently molecular techniques are the only procedures that can provide relevant diagnostic information.
* For *Treponema pallidum*, molecular techniques can provide direct benefits in the diagnosis of infection.
* Molecular methods are advisable in *Haemophilus ducrey*i, because of the difficulties of culture and its low sensitivity.

Detection of Viruses

* Because of the difficulty in growing and identifying viruses, it is this field that have benefitted tremendously from the introduction of molecular based methods.

Viruses

* “Classical methods” of detection include antibody detection, antigen detection, and culture.
* Molecular methods of detection include target, probe, and signal amplification.
* Tests are designed for identification of viruses, determination of viral load (number of viruses per mL of fluid), and genotyping by sequence analysis

“Classic” Antibody detection

* IgM and IgG Levels
* IgM is the first antibody produced by the body when it is exposed to a virus. The IgM test is used to screen for early detection of infection and is used to diagnose the disease during onset.
* IgG antibodies develop later and remain present for many years, usually for life, and protect against further infection by the same virus.

HIV

* Molecular testing is important in HIV, not only for detection of the disease, but also continued monitoring of disease treatments.

Detection of HIV

* The first marker that becomes detectable after infection is the HIV RNA, indicated by the green line. This is detectable by current molecular methods at about 11 days from the time of infection or exposure to HIV.
* The second marker that becomes detectable in the laboratory is the HIV p24 antigen, indicated in the purple line. This is detectable by day 16 from exposure.
* And finally, the HIV antibodies that are detectable by current commercial assays occur at about day 22 from the date of infection. So the most widely used serologic tests, which are HIV antibody screening tests, are actually the least sensitive in picking up HIV infection compared to the other 2 markers.
* There are basically 2 windows of HIV infection for detection.
	+ The first is the seroconversion window, which starts from time of infection, indicated by the first arrow on the left-hand side of the timeline, to the time point where antibody becomes detectable. So, this seroconversion window period actually includes the eclipse period and the acute infection period.
	+ The eclipse period is the period at which time that only molecular tests can detect the presence of HIV RNA.
* The acute infection is the period between viral infection detectable by molecular

 tests and a serologic response, which is detectable by serologic assays.

* Now the incidence window is the period from the time of antibody detection first from the infected individual until a specific time point where the serologic assay

 can determine recent infection. So that particular window period is also known as

 the recent infection.

* And, after this assay-specific detection point for recent infection, we see long-standing HIV infection.

Detection of HIV

* The most widely used methods for HIV detection are the ELISA assays, the enzyme-linked immunosorbant assays, which can come in the form of enzyme immunoassay or chemoluminescent immunoassay. They can detect either HIV-1 antibodies, or HIV-2 antibodies, or HIV-1 p24 antigen, or a combination of HIV-1 and -2; and then lastly the fourth generation serologic test is a combination of antibody and p24 antigen.
* And, basically, there are 2 methods, the immunochromatography method, as well as the membrane immunoconcentration method. These rapid test devices are available to detect either HIV-1 antibodies alone, HIV-2 antibodies alone, or a combination of HIV-1 and -2 antibodies.
* The final group of serologic tests are the so-called supplemental tests, also known as confirmatory tests for HIV-1 and -2 antibodies. And there are essentially 2 methods that are commercially available: one is the Western blot and the other is the Immunoblot.
* Early in the epidemic of HIV infection, the first tests that were available for diagnosis or detection of HIV are viral cultures using CD4 cells; these are the human helper T cells that are infected by HIV viruses. And, using these viral cultures, one is able to detect production of viral p24 antigens in the supernatant of cell cultures from CD4 cell lines.
* PCR assays for qualitative and quantitative detection of HIV-1 and -2 are also available. For qualitative PCR assays, one could detect HIV-1 proviral DNA. This is the DNA that is incorporated into the CD4 whole cells, DNA that belong to HIV-1 viral genome. One could also detect a combination of HIV proviral DNA and RNA and also laboratory-developed assays, particularly in certain research investigator laboratories, one could also design a qualitative detection of HIV-2 RNA. For quantitative assays, there are commercially available and FDA- approved assays for quantifying HIV-1 RNA, and then there are laboratory-developed assays for quantifying HIV-2 RNA.
* Two other commercial laboratory tests available utilize transcription-mediated amplification for qualitative detection of HIV-1 RNA, and the branched DNA method, which utilizes signal amplification for quantitation of HIV-1 RNA

Treatment of HIV

* In an HIVinfected individual, the concentration of virus in the bloodstream (viral load (VL)) can be a valuable tool for the clinical management of the infection.
* Broadly, there are three clinical uses for  quantifying HIV in plasma:
	+ diagnosing acute HIV infection
	+ determining prognosis and disease progression
	+ therapeutic monitoring
* Unlike antibody detection, which is confounded by the  trans‐placental transfer of maternal IgG antibodies, VL can also be useful in diagnosing babies born to HIV‐positive mothers.
* Monitoring VL is relevant as a biomarker to monitor the therapeutic efficacy.  Quantifying viral load in plasma enables a clinician to assess the success of treat-ment and detect treatment failure prior to the onset of clinical  symptoms.

Hepatitis

* Viral hepatitis, including hepatitis A, hepatitis B, hepatitis C, hepatitis D and hepatitis E are distinct diseases that affect the liver and have different hepatitis symptoms and treatments.
* Each virus is different and the only commonality between them is that they all cause an inflammation of the liver.
* There is more concern over hepatitis B and C because they are bloodborne pathogens.
* Old methods for detecting hepatitis revolved around antibody and antigen detection. For example the next couple of pages of this powerpoint describe the markers looked for in diagnosing Hepatitis B.
* New methods of hepatitis detection are now available for all of the various types of hepatitis. Using nucleic acid detection has allowed much earlier detection of the viruses and has decreased the window for detection down from 90 days to less than one week after infection.

Antimicrobial Agents

* Inhibit growth (-static); e.g., bacteriostatic, fungistatic
* Kill organisms (-cidal); e.g., bacteriocidal, fungicidal, viricidal
* Antimicrobial agents are classified by
	1. -static/-cidal
	2. Mode of action
	3. Chemical structure

Mechanisms for Development of Resistance to Antimicrobial Agents

* Enzymatic inactivation of agent
* Altered target
* Altered transport of agent in or out
* Acquisition of genetic factors from other resistant organisms

Advantages of Molecular Detection of Resistance to Antimicrobial Agents

* Mutated genes are strong evidence of resistance.
* Rapid detection without culturing
* Direct comparison of multiple isolates in epidemiological investigations

Molecular Epidemiology

* Epidemic: rapidly spreading outbreak of an infectious disease
* Pandemic: a disease that sweeps across wide geographical areas
* Epidemiology: collection and analysis of environmental, microbiological, and clinical data
* Phenotypic analysis measures biological characteristics of organisms.
* Molecular epidemiology is a genotypic analysis targeting genomic or plasmid DNA.
	+ Species-, strain-, or type-specific DNA sequences are the sources of genotype information.

Other Genotypic Methods Used to Type Organisms

* Plasmid fingerprinting with restriction enzymes
* RFLP analysis
* Amplified fragment length polymorphism (AFLP)
* Interspersed repetitive elements
* Ribotyping
* *spa* typing
* Multilocus sequence typing