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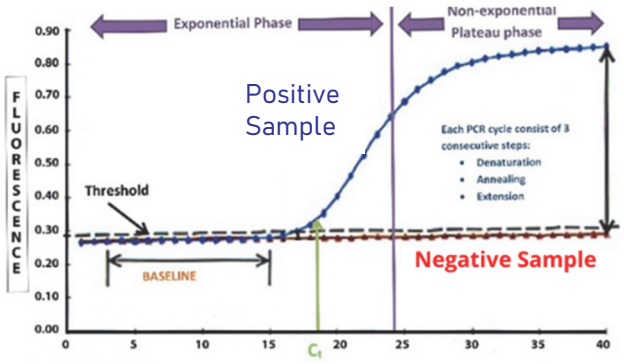
Molecular In My Pocket…

Infectious Diseases

PCR Cycle



Real-time polymerase chain reaction (PCR) is a molecular technique used to detect a pathogen-specific nucleic acid sequence in a sample. Generally, DNA or RNA is first extracted from the sample. However, there are tests in use that do not extract DNA or RNA before performing PCR. If RNA is the target, for example influenza virus, the RNA must first be converted into complementary DNA (cDNA). The DNA then undergoes repeated cycles of heating and cooling to allow for separation into single strands, annealing of oligonucleotide primers and a fluorescent probe(s) to target sequences, and amplification of the target via DNA polymerase. This results in exponential amplification of the target pathogen sequence allowing for detection of small amounts of organism in the original sample. There are a number of probe chemistries, but the two most commonly used probes are either hydrolyzed to release a fluorescent signal separate from a quenching molecule, or the probe binds the target sequence releasing the hairpin structure and separating the fluorescent molecule from the quencher. This results in an increase in fluorescence relative to the amount of target in the sample. When this fluorescence is detected above the baseline (background fluorescence signal), the sample is considered positive for the target.

Quantitative polymerase chain reaction (qPCR): qPCR is a modified real-time PCR technique and is used to simultaneously detect and estimate the copy number (amount) of a specific DNA or RNA sequence in a sample relative to a standard curve. Cycle Threshold (Ct) is defined as the number of cycles required for the fluorescent signal to cross the threshold. The threshold is set to be above the baseline but sufficiently low to be within the exponential growth region of an amplification curve to ensure all reagents are still in excess. The Ct value of each qPCR reaction depends on the initial template amount (copy number) of the target sequence, and it is inversely proportional to the log of this copy number. The Ct values of serially diluted standards of known concentrations can be plotted to generate a standard curve, which produces a linear relationship between Ct and initial amounts of total RNA or DNA, allowing for the determination of the concentration of unknowns based on their Ct values. As a note, most infectious disease molecular assays are qualitative, not quantitative.

Common Tests

Syndromic Panel Testing

Advances in molecular techniques have led to the ability to take a syndromic approach to diagnostics in which a single test is capable of detecting many of the microorganisms most commonly associated with an infection. This is known as multiplex PCR. These assays are highly sensitive and faster than culture-based methods. Common panels include:

- Respiratory infections
- Meningitis/encephalitis
- Gastrointestinal infections
- Sepsis (from positive blood culture)
- Sexually transmitted infections
- Bacterial vaginosis

CT/NG Test

A frequently high-volume molecular assay performed in clinical labs for the detection of *Chlamydia trachomatis* (CT) and *Neisseria gonorrhoae* (NG) nucleic acid in a single test. Molecular detection enables faster and more sensitive results as compared to culture methods.

Resistance gene markers

PCR can also be used to detect the presence of common resistance genes directly from blood culture bottles before traditional bacterial culture-based susceptibilities can be determined. Some include:

- *Enterococcus faecalis/Enterococcus faecium* - *vanA/vanB* genes: vancomycin resistance
- *Staphylococcus aureus* - *mecA* gene: resistance to all penicillinase-stable penicillins and beta-lactams
- *Klebsiella pneumoniae* – bla_{KPC} : beta lactam resistance including penicillins, cephalosporins, monobactams, and carbapenems.
- *Enterobacterales* - bla_{CTX-M}: beta-lactam resistance including penicillins, cephalosporins, and monobactams

Many of these targets have been successfully incorporated into antimicrobial stewardship programs.

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Other Common Molecular Techniques:

Transcription mediated amplification (TMA): Like PCR, TMA involves amplification of a target sequence. However, TMA uses the enzymes RNA polymerase and reverse transcriptase to amplify either target RNA or DNA and generate an amplified RNA product. TMA is an isothermal, single-tube system that enables quick, sensitive, simultaneous detection of multiple pathogenic organisms in a single tube.

Fluorescent *in situ* hybridization (FISH): Fluorescently labeled probe oligonucleotides are used to detect specific regions of DNA/RNA for desired target microbes or resistance genes, with or without amplification. This approach is most commonly used for positive blood cultures speciation and resistance gene detection.

Sanger Sequencing: Sanger sequencing is applied in infectious disease diagnostics for the identification of bacteria and fungi. Primers targeting well conserved regions of the bacterial or fungal ribosome are used to amplify regions that are hypervariable and thus can be used to identify the genus or species, depending on the primer set and organism. For bacteria, this is usually a portion of the 16S gene. For fungi, this may be the 18S and internal transcribed spacer (ITS) regions or part of the 28S ribosome. Commercial and public databases are available for sequence analysis.

Next-generation sequencing (NGS): NGS is a well-established technique in genomics and molecular pathology laboratories but is relatively new for infectious disease applications. There are 2 main categories of NGS approaches in infectious diseases: shotgun metagenomic sequencing and targeted/amplicon sequencing, both displayed in the figure below. Reads of potential pathogens can often be challenging to interpret as they may represent contamination or colonization. Further, bioinformatic pipelines used for metagenomic analyses are highly laboratory specific with only a few commercially available options that have limited publications. At present, only a handful of clinical laboratories offer such testing due to the extensive validation requirements. Data are currently accumulating to determine clinical utility of NGS.

Shotgun NGS: Shotgun metagenomics is the analysis of total RNA and/or DNA in a sample to identify any potential pathogen. Sequences are binned into categories of viral, bacterial, fungal, and parasite to identify potential pathogens, while human nucleic acid is informatically or manually removed using selection techniques. As shown in the figure below, the majority of reads will be human, thus millions of reads must be sequenced to attempt to identify pathogens. Several million reads per sample must be generated for this type of analysis. However, there are currently no guidelines on minimums, quality, or other cutoffs.

Amplicon-based NGS: Amplicon sequencing, also known as targeted sequencing, allows for the detection of a specific group of organisms by using primers (i.e., 16S, ITS), as in Sanger sequencing. This reduces the impact of human DNA interference and allows for a targeted approach (i.e., detection of fungi only). Amplicon-based NGS allows for the interpretation of multiple different species in a sample, unlike Sanger sequencing, which can only identify one species in a sample. Compared to shotgun NGS, amplicon-based NGS has a reduced sequencing cost and decreased human DNA interference, but requires suspicion and selection of the appropriate primers for the group of organisms of interest (i.e., bacterial vs fungal).