**Chapter 23: Culture-Independent Diagnostic Techniques**

**Authors:**

**Ms. Fatima Khurshid**  
KAM School of Life Sciences  
[Forman Christian College University]  
[Lahore, Pakistan]  
[fatima.khurshid43@gmail.com]

**ABSTRACT**

This chapter explores the advancements in culture-independent diagnostic techniques in clinical microbiology. These methodologies bypass the need for traditional microbial culture and provide rapid, accurate detection and identification of pathogens. Techniques such as nucleic acid-based assays, proteomics, and high-throughput sequencing have revolutionized diagnostics. Applications range from identifying pathogens in complex polymicrobial infections to detecting antimicrobial resistance genes. The role of these tools in enhancing diagnostic precision, reducing time-to-results, and guiding targeted therapies is discussed, along with limitations and future perspectives.

**Keywords**: culture-independent, molecular diagnostics, sequencing, antimicrobial resistance, microbiology.

**I. INTRODUCTION**

The advent of culture-independent diagnostic techniques (CIDTs) has significantly transformed the field of microbiology and infectious disease diagnostics. Traditional culture-based methods, which have long been considered the gold standard for identifying pathogenic microorganisms, involve the cultivation of bacteria, fungi, viruses, or parasites under laboratory conditions. However, these methods are often time-consuming, labor-intensive, and limited by the inability to grow certain fastidious or non-culturable organisms. CIDTs address these limitations by enabling the direct detection of microbial pathogens from clinical specimens without prior culture.

The development of CIDTs has been driven by advances in molecular biology, genomics, and bioinformatics. Techniques such as polymerase chain reaction (PCR), next-generation sequencing (NGS), and mass spectrometry have revolutionized microbial diagnostics by providing rapid, accurate, and comprehensive pathogen identification. These methods not only enhance diagnostic efficiency but also play a crucial role in epidemiological investigations, antimicrobial stewardship, and infection control practices.

The growing clinical application of CIDTs is particularly evident in the diagnosis of respiratory tract infections, gastrointestinal diseases, bloodstream infections, and central nervous system infections. For example, multiplex PCR panels can simultaneously detect multiple pathogens responsible for community-acquired pneumonia or bacterial meningitis, thereby guiding timely and appropriate antimicrobial therapy. Additionally, metagenomic sequencing approaches facilitate the detection of novel or unexpected pathogens, contributing to the early recognition of emerging infectious diseases.

CIDTs can be broadly classified into nucleic acid-based assays, protein-based assays, and other advanced diagnostic platforms. Nucleic acid-based methods, such as PCR and isothermal amplification techniques, target specific DNA or RNA sequences to identify pathogens with high sensitivity and specificity. Protein-based assays, including enzyme-linked immunosorbent assays (ELISA) and mass spectrometry, detect microbial antigens or host immune responses. Emerging techniques, such as CRISPR-based diagnostics and nanopore sequencing, promise further improvements in diagnostic capabilities.

Despite their advantages, CIDTs also present certain challenges. The absence of culture isolates can impede antimicrobial susceptibility testing (AST), complicating treatment decisions. Moreover, the high sensitivity of molecular assays may lead to the detection of non-viable organisms or commensal flora, potentially causing diagnostic uncertainty. Additionally, the high cost of some molecular assays and the need for specialized equipment and trained personnel can limit accessibility, particularly in resource-limited settings. Therefore, integrating CIDTs with clinical findings and, when necessary, complementary culture-based methods remains essential for accurate diagnosis and patient management.

CIDTs have also facilitated significant advances in the understanding of the human microbiome table 1. Metagenomic and metatranscriptomic studies have provided new insights into microbial community dynamics and their roles in health and disease. This information has implications for diagnosing dysbiosis-related conditions, such as inflammatory bowel disease, and for developing targeted microbiome-based therapies.

**Table 1:** Comparison of Culture-Dependent and Culture-Independent Diagnostic Techniques.

|  |  |  |
| --- | --- | --- |
| **Parameter** | **Culture-Dependent Diagnostics** | **Culture-Independent Diagnostics** |
| Time to Results | Days to weeks | Hours to days |
| Pathogen Identification | Limited to cultivable organisms | Includes non-culturable pathogens |
| Sensitivity | Moderate | High |
| Cost | Low to moderate | Moderate to high |

As the field of diagnostic microbiology continues to evolve, ongoing research and technological innovation promise further enhancements in CIDT performance, accessibility, and affordability. Future developments may include the incorporation of artificial intelligence (AI)-driven data analysis and portable, point-of-care testing devices, ultimately advancing the precision and efficiency of infectious disease diagnostics.

**II. PRINCIPLES OF CULTURE-INDEPENDENT DIAGNOSTICS**

**A. Molecular Diagnostics**

Molecular diagnostics forms the cornerstone of culture-independent diagnostic techniques by targeting the genetic material of microorganisms. These methods rely on the detection of pathogen-specific DNA or RNA sequences, enabling the identification of both culturable and non-culturable pathogens. The primary approaches include:

1. **Polymerase Chain Reaction (PCR) and Its Variants**

PCR is a widely used nucleic acid amplification technique that allows for the detection and identification of microbial DNA or RNA with high specificity and sensitivity. The technique involves three key steps—**denaturation, annealing, and extension**—which are repeated in cycles to exponentially amplify the target genetic sequence.

PCR variants have evolved to enhance diagnostic capabilities, improve sensitivity, and allow real-time quantification. Below are the major PCR types:

**1. Conventional PCR**

* **Principle:** Uses sequence-specific primers to amplify target DNA, producing detectable amplicons.
* **Detection:** PCR products are visualized by gel electrophoresis, usually after post-reaction processing.
* **Application:**
  + Detection of bacterial and viral DNA from clinical specimens.
  + Identification of antibiotic resistance genes.
  + Molecular typing of pathogens for epidemiological investigations.
* **Limitations:**
  + Time-consuming (requires post-PCR gel electrophoresis).
  + Qualitative detection only (presence/absence of DNA, no quantification).
  + Higher contamination risk due to open-tube processing.

**2. Real-Time PCR (qPCR)**

* **Principle:** Unlike conventional PCR, qPCR uses fluorescent dyes or sequence-specific probes to detect DNA amplification in real-time.
* **Detection:** Fluorescent signals increase as DNA is amplified, allowing quantification of the target nucleic acid.
* **Advantages:**
  + Rapid detection (results within hours).
  + High sensitivity and specificity.
  + Enables absolute or relative quantification of pathogens.
  + Lower contamination risk due to closed-tube reactions.
* **Fluorescent Detection Methods:**
  + **SYBR Green Dye:** Binds to double-stranded DNA, producing fluorescence upon binding.
  + **TaqMan Probes:** Fluorophore-labeled probes hybridize to specific sequences, ensuring highly specific detection.
* **Applications:**
  + Diagnosis of bacterial, viral, and fungal infections.
  + Monitoring viral loads in HIV, hepatitis, and COVID-19.
  + Mutation and gene expression analysis.

**3. Multiplex PCR**

* **Principle:** Uses multiple primer sets in a single reaction to amplify different target sequences simultaneously.
* **Advantages:**
  + Detects multiple pathogens in a single test.
  + Reduces assay time and sample volume requirements.
  + Ideal for diagnosing polymicrobial infections.
* **Applications:**
  + Detecting co-infections (e.g., respiratory pathogens like **Influenza A, B, SARS-CoV-2, RSV** in a single test).
  + Simultaneous identification of bacterial and fungal pathogens in sepsis.
  + Antimicrobial resistance gene profiling (e.g., mecA for **MRSA**, vanA for **VRE**).
* **Limitations:**
  + Primer design must prevent cross-reactivity.
  + Sensitivity may be lower for individual targets compared to singleplex PCR.

**4. Reverse Transcription PCR (RT-PCR)**

* **Principle:** Converts RNA into complementary DNA (cDNA) using the enzyme **reverse transcriptase** before amplification via PCR.
* **Purpose:** Essential for detecting RNA viruses and gene expression studies.
* **Advantages:**
  + Enables detection of RNA viruses.
  + Highly sensitive, even for low viral loads.
  + Used in real-time RT-PCR (qRT-PCR) for **quantitative viral load measurement**.
* **Applications:**
  + Diagnosis of RNA viruses (e.g., **SARS-CoV-2, Influenza, HIV, HCV, Dengue**).
  + Gene expression analysis in molecular research.
  + Detection of emerging viral mutations in epidemiological surveillance.
* **Example:**
  + **COVID-19 Diagnosis:** RT-qPCR is the gold standard for detecting SARS-CoV-2 by amplifying viral **N, E, and RdRp genes**.

1. **Nucleic Acid Amplification Tests (NAATs):**
   1. These assays use enzymatic amplification techniques like loop-mediated isothermal amplification (LAMP) and transcription-mediated amplification (TMA) to detect microbial genetic material without complex thermal cycling.

NAATs are molecular diagnostic techniques that amplify pathogen-specific DNA or RNA sequences, enabling highly sensitive and specific detection of infectious agents. Unlike traditional PCR, some NAATs **do not require thermal cycling**, making them faster and more suitable for **point-of-care (POC) testing** and resource-limited settings. These methods use **enzymatic amplification** techniques to achieve rapid results with minimal equipment.

**a. Key Isothermal Amplification Techniques**

Unlike conventional PCR, which requires multiple temperature cycles for denaturation, annealing, and extension, isothermal amplification methods operate at a constant temperature, simplifying instrumentation and reducing processing time.

**1) Loop-Mediated Isothermal Amplification (LAMP)**

* **Principle:**
  + Uses **Bst DNA polymerase** with **four to six primers** to recognize distinct regions of the target DNA.
  + Produces **loop-like structures** that facilitate rapid DNA synthesis at **a single temperature (60–65°C)**.
  + Generates **turbidometric, fluorescent, or colorimetric** signals for detection.
* **Advantages:**  
  ✔ Rapid (results within 30–60 minutes).  
  ✔ High sensitivity (detects low pathogen loads).  
  ✔ Simple instrumentation (water bath or heating block).  
  ✔ Visual readout (color change without electrophoresis).
* **Applications:**
  + Diagnosis of bacterial and viral infections (e.g., **Mycobacterium tuberculosis, SARS-CoV-2, Zika virus, Dengue virus**).
  + Detection of antimicrobial resistance genes.
  + Point-of-care testing in low-resource settings.

**2) Transcription-Mediated Amplification (TMA)**

* **Principle:**
  + Amplifies **RNA targets** using **RNA polymerase and reverse transcriptase**, making it highly efficient for detecting RNA viruses.
  + Uses **isothermal conditions (typically 42–45°C)**, eliminating the need for complex thermocyclers.
  + Produces **millions of RNA copies**, leading to highly sensitive detection.
* **Advantages:**  
  ✔ Faster than PCR (amplification in **15–30 minutes**).  
  ✔ Extremely high sensitivity (detects as few as **10 RNA copies per reaction**).  
  ✔ Efficient for RNA-based pathogen detection.  
  ✔ Automated platforms available (e.g., **Hologic Panther System** for high-throughput testing).
* **Applications:**
  + Detection of **RNA viruses** (e.g., **HIV, Hepatitis B/C, SARS-CoV-2**).
  + Screening for **sexually transmitted infections (STIs)** like **Chlamydia trachomatis and Neisseria gonorrhoeae**.
  + Blood screening for transfusion-transmissible infections.

**Table 2: Comparison of LAMP vs. TMA**

|  |  |  |
| --- | --- | --- |
| Feature | LAMP | TMA |
| Target | DNA or RNA | Primarily RNA |
| Temperature | 60–65°C (isothermal) | 42–45°C (isothermal) |
| Amplification Speed | 30–60 min | 15–30 min |
| Enzymes Used | Bst DNA polymerase | RNA polymerase + reverse transcriptase |
| Detection | Turbidity, fluorescence, color change | Chemiluminescence |
| Best for | Point-of-care settings | High-throughput labs, blood screening |

**Clinical Impact of NAATs**

✔ **Rapid Diagnosis:** Reduces time-to-result compared to culture-based methods.  
✔ **High Sensitivity & Specificity:** Detects infections at early stages, even with low microbial loads.  
✔ **Non-Culture-Based:** Essential for diagnosing **fastidious, slow-growing, or non-culturable pathogens**.  
✔ **Point-of-Care Utility:** LAMP is particularly valuable in **resource-limited settings** where real-time PCR is impractical.

1. **Next-Generation Sequencing (NGS):**

NGS enables comprehensive pathogen detection through whole-genome sequencing, targeted sequencing, or metagenomic approaches. It is particularly useful for identifying novel or unexpected pathogens and for understanding microbial diversity. Next-Generation Sequencing (NGS) is a high-throughput technology that allows for **comprehensive pathogen detection** by sequencing DNA or RNA with unprecedented speed and depth. Unlike traditional sequencing methods (e.g., Sanger sequencing), NGS can generate **millions of sequences simultaneously**, making it a powerful tool for identifying **novel or unexpected pathogens, characterizing microbial communities, and tracking antimicrobial resistance genes**.

**a. Key NGS Approaches in Microbial Diagnostics**

NGS techniques can be applied in various ways, depending on the clinical and research objectives. The three major approaches include:

**1) Whole-Genome Sequencing (WGS)**

* **Principle:**
  + Sequences the **entire genome** of a pathogen from clinical isolates or direct patient samples.
  + Provides a **high-resolution genetic blueprint** of microbial species.
* **Advantages:**  
  ✔ Enables detailed analysis of **virulence factors and antimicrobial resistance genes**.  
  ✔ Facilitates **strain typing** for epidemiological surveillance and outbreak investigations.  
  ✔ Helps identify **mutations linked to drug resistance** (e.g., rifampin resistance in **Mycobacterium tuberculosis**).
* **Applications:**
  + **Hospital-acquired infection (HAI) investigations** (e.g., tracking MRSA outbreaks).
  + **Surveillance of antimicrobial resistance** (AMR) in bacterial pathogens.
  + **Characterizing novel viral strains** (e.g., SARS-CoV-2 variant analysis).

**2) Targeted Sequencing (Amplicon-Based NGS)**

* **Principle:**
  + Selectively amplifies and sequences **specific genomic regions** of interest, such as the **16S rRNA gene** for bacterial identification or resistance genes.
* **Advantages:**  
  ✔ Higher sensitivity for detecting **low-abundance organisms** compared to WGS.  
  ✔ Cost-effective compared to metagenomics.  
  ✔ Useful for identifying **hard-to-culture bacteria** and fungi.
* **Applications:**
  + **Microbiome studies** (e.g., gut and skin microbiota analysis).
  + **Rapid species identification** in sepsis and bloodstream infections.
  + **Detection of antimicrobial resistance markers** in clinical isolates.

**3) Metagenomic Sequencing (mNGS)**

* **Principle:**
  + Sequences **all nucleic acids (DNA/RNA)** in a clinical sample **without prior target selection**, allowing **unbiased detection** of bacteria, viruses, fungi, and parasites.
* **Advantages:**  
  ✔ **Detects novel or unexpected pathogens** in clinical infections.  
  ✔ **Identifies polymicrobial infections** in complex conditions (e.g., pneumonia, sepsis).  
  ✔ Provides insights into **microbial diversity and host-pathogen interactions**.
* **Applications:**
  + **Diagnosis of undiagnosed infections** (e.g., neurological infections, respiratory diseases).
  + **Detection of viral pathogens in immunocompromised patients**.
  + **Outbreak investigations** for emerging infectious diseases.

**Table 3 Comparison of NGS Approaches**

|  |  |  |  |
| --- | --- | --- | --- |
| **Feature** | **Whole-Genome Sequencing (WGS)** | **Targeted Sequencing** | **Metagenomic Sequencing (mNGS)** |
| **Target** | Single pathogen genome | Specific gene regions | All microbial DNA/RNA |
| **Use Case** | Strain typing, AMR detection | Microbiome studies, species ID | Broad pathogen detection |
| **Sensitivity** | High for known pathogens | High for selected targets | Detects unknown & novel microbes |
| **Cost & Complexity** | Moderate | Lower | High |
| **Best For** | Outbreak surveillance, AMR studies | Microbial community analysis | Detecting unknown infections |

**Clinical Impact of NGS in Microbiology**

✔ **Rapid Pathogen Identification:** Overcomes limitations of culture-based methods, particularly for **fastidious or non-culturable pathogens**.  
✔ **Enhanced Outbreak Surveillance:** Enables real-time tracking of microbial evolution and transmission.  
✔ **Comprehensive AMR Detection:** Identifies resistance genes across bacterial populations, aiding in antibiotic stewardship.  
✔ **Personalized Infection Treatment:** Helps clinicians tailor antimicrobial therapy based on genomic insights.

**Challenges and Future Perspectives**

* **High Cost & Data Complexity:** Requires bioinformatics expertise and expensive infrastructure.
* **Turnaround Time:** Can take 24–72 hours, making it less practical for urgent diagnostics.
* **Standardization Issues:** Need for globally accepted protocols for clinical implementation.

Despite these challenges, **NGS is transforming clinical microbiology** by enabling **faster, more precise diagnostics and pathogen surveillance**. Future advancements, such as **nanopore sequencing** and **AI-driven genomic analysis**, will further enhance its applicability in routine clinical practice.

1. **CRISPR-Based Diagnostics:**
   1. Techniques like SHERLOCK and DETECTR utilize CRISPR-Cas proteins for precise nucleic acid detection with high specificity and sensitivity.
2. **Microarray Technology:**
   1. Microarrays detect specific nucleic acid sequences by hybridizing target DNA/RNA to oligonucleotide probes immobilized on a solid surface.

**Clinical Applications:** Molecular diagnostics are extensively applied in the diagnosis of respiratory tract infections (e.g., influenza and SARS-CoV-2), gastrointestinal diseases, bloodstream infections, and central nervous system infections.

**Limitations:**

* High costs and technical expertise requirements.
* Risk of contamination leading to false-positive results.
* Inability to determine antimicrobial susceptibility directly without culture.

**III. TECHNIQUES IN DETAIL**

**A. Nucleic Acid-Based Methods**

Nucleic acid-based methods are fundamental to culture-independent diagnostics, leveraging the detection and amplification of microbial DNA or RNA directly from clinical samples without the need for prior culture. These techniques provide high sensitivity and specificity for pathogen identification and are categorized into the following:

1. **Polymerase Chain Reaction (PCR)**
   * PCR amplifies specific DNA sequences using primers, nucleotides, and DNA polymerase.
   * Variants such as real-time PCR (qPCR) and multiplex PCR allow for the simultaneous detection of multiple pathogens.
2. **Isothermal Amplification Techniques**
   * Techniques like Loop-Mediated Isothermal Amplification (LAMP) and Recombinase Polymerase Amplification (RPA) amplify nucleic acids at a constant temperature, simplifying instrumentation and speeding up the diagnostic process.
3. **Microarray-Based Methods**

Microarrays detect specific genetic sequences through hybridization, enabling high-throughput screening for multiple pathogens simultaneously.Microarray technology is a **high-throughput molecular diagnostic tool** used for detecting multiple microbial genes simultaneously. It enables the identification of pathogens, antimicrobial resistance genes, and virulence factors by hybridizing **nucleic acid probes** to specific target sequences. Microarrays offer a **cost-effective alternative** to sequencing-based methods and are valuable in clinical microbiology for **pathogen detection, genotyping, and epidemiological studies**.

**a. Principle of Microarray Technology**

* **Nucleic acid probes** (short, single-stranded DNA or RNA sequences) are immobilized on a **solid support** (glass slide, silicon chip, or membrane).
* Patient-derived **DNA or RNA** is extracted, labeled with fluorescent or chemiluminescent markers, and hybridized to complementary probes on the microarray.
* **Fluorescence signals** are detected using a scanner, and **pattern analysis** determines the presence or absence of specific pathogens or genetic markers.

**b. Types of Microarray-Based Methods**

Microarrays vary in their design and application, with the most common types being:

**1) Pathogen Detection Microarrays**

* **Detects a broad range of bacterial, viral, fungal, and parasitic pathogens** in a single assay.
* **Example:**
  + **Respiratory Pathogen Microarray:** Simultaneously detects **influenza, SARS-CoV-2, RSV, adenovirus, and bacterial co-infections**.
  + **Gastrointestinal Pathogen Panels:** Identify **Salmonella, Shigella, E. coli, norovirus, and rotavirus**.
* **Advantages:**  
  ✔ **Multiplex capability** – Identifies multiple pathogens in a single test.  
  ✔ **Faster than culture** – Reduces diagnostic time.  
  ✔ **High sensitivity & specificity** – Detects low-level infections.
* **Limitations:**
  + Cannot detect unknown or emerging pathogens (depends on pre-designed probes).
  + Less flexible than sequencing-based approaches.

**2) Antimicrobial Resistance (AMR) Microarrays**

* Detect **resistance genes** and mutations associated with drug resistance.
* **Example:**
  + **Carbapenem-Resistant Enterobacteriaceae (CRE) Panel:** Detects **blaKPC, blaNDM, blaOXA, and blaVIM genes**.
  + **Methicillin-Resistant Staphylococcus aureus (MRSA) Microarray:** Identifies **mecA, mecC, and Panton-Valentine Leukocidin (PVL) genes**.
* **Clinical Impact:**
  + Rapid AMR detection improves **antibiotic stewardship**.
  + Reduces reliance on **phenotypic susceptibility testing**, which can take days.

**3) Virulence Factor Microarrays**

* Identify genes responsible for **toxins, adhesion, and immune evasion**.
* **Example:**
  + **Clostridium difficile Toxin Panel:** Detects **tcdA and tcdB genes** to differentiate toxigenic and non-toxigenic strains.
  + **Shiga Toxin-Producing E. coli (STEC) Microarray:** Detects **stx1, stx2, and eae genes**.
* **Clinical Utility:**  
  ✔ Helps distinguish **highly virulent strains** from commensal or less harmful microbes.  
  ✔ Aids in **outbreak tracking** and epidemiological studies.

**Table 4 Comparison of Microarray vs. Other Molecular Methods**

|  |  |  |  |
| --- | --- | --- | --- |
| **Feature** | **Microarray-Based Methods** | **PCR (qPCR, Multiplex)** | **NGS (Whole-Genome, Metagenomic)** |
| **Detection Range** | Broad, predefined targets | Limited (depends on primers) | Unlimited, can detect novel pathogens |
| **Speed** | 4–8 hours | 2–4 hours | 24–72 hours |
| **Multiplexing Ability** | High (hundreds of targets) | Moderate (up to 10–20 targets) | Very high (entire genomes) |
| **Cost** | Moderate | Lower | High |
| **Best Used For** | Routine diagnostics, AMR panels | Rapid pathogen detection | Epidemiological studies, unknown infections |

**Clinical Applications of Microarray Technology**

✔ **Comprehensive infectious disease panels** – Useful for syndromic testing (e.g., respiratory infections, sepsis).  
✔ **Epidemiological Surveillance** – Tracks emerging resistant strains.  
✔ **Vaccine Development** – Identifies antigenic variations in pathogens.  
✔ **Food Safety & Environmental Testing** – Detects microbial contaminants in food and water supplies.

**Limitations and Future Perspectives**

* **Target-Dependent:** Cannot detect unknown pathogens or emerging genetic variations.
* **Lower Sensitivity for Rare Mutations:** May miss novel drug resistance mechanisms.
* **Requires Specialized Equipment:** Fluorescence scanners and bioinformatics expertise.

**Future Trends**

✔ **Integration with AI & Machine Learning:** Improving probe design and result interpretation.  
✔ **Point-of-Care (POC) Microarrays:** Miniaturized platforms for bedside testing.  
✔ **Combination with NGS:** Hybrid approaches for **enhanced pathogen discovery**.

1. **CRISPR-Cas Systems**
   * CRISPR-based diagnostics, such as SHERLOCK and DETECTR, offer precise and rapid nucleic acid detection through CRISPR-associated enzymes.

**Applications:**

* Respiratory infections (e.g., influenza, SARS-CoV-2)
* Gastrointestinal infections (e.g., norovirus, C. difficile)
* Bloodstream infections (e.g., sepsis pathogens)

**Advantages:**

* High sensitivity and specificity.
* Rapid turnaround time.
* Capability to detect non-culturable and low-abundance pathogens.

**Challenges:**

* High cost and need for specialized equipment.
* Risk of contamination and false-positive results.

**IV. APPLICATIONS OF CIDTS**

Culture-independent diagnostic techniques have a broad range of applications across various medical and public health domains. These applications significantly impact patient care, epidemiology, and infection control strategies.

1. **Clinical Diagnostics:**
   * CIDTs play a crucial role in the diagnosis of infectious diseases, including respiratory infections (e.g., COVID-19, influenza), gastrointestinal infections (e.g., Salmonella, norovirus), bloodstream infections (e.g., sepsis), and central nervous system infections (e.g., meningitis, encephalitis).
2. **Epidemiological Surveillance:**
   * CIDTs are invaluable in monitoring disease outbreaks, tracking pathogen evolution, and detecting emerging infectious diseases. For instance, metagenomic sequencing has been used in real-time surveillance of SARS-CoV-2 variants.
3. **Antimicrobial Stewardship:**
   * Rapid pathogen identification with CIDTs supports timely and appropriate antimicrobial therapy, helping to curb the overuse of antibiotics and the emergence of antimicrobial resistance.
4. **Public Health Interventions:**
   * Health authorities use CIDT data to implement infection control measures, guide vaccination strategies, and manage community outbreaks.
5. **Microbiome Research:**
   * CIDTs facilitate the study of human microbiota, contributing to the understanding of microbiome-related conditions such as inflammatory bowel disease, bacterial vaginosis, and periodontal disease.
6. **Point-of-Care Testing (POCT):**
   * Portable, rapid CIDTs are increasingly used in decentralized settings to improve diagnostic accessibility in primary care clinics, emergency departments, and resource-limited regions.

**III. Metagenomics** Metagenomics involves analyzing the collective genetic material from clinical specimens, offering a culture-independent approach to studying microbial communities. It enables:

* Comprehensive pathogen detection
* Analysis of microbiota and their functional roles
* Identification of antimicrobial resistance genes

Applications include investigating polymicrobial infections, monitoring microbiome changes, and studying emerging pathogens. Limitations include high costs and complex bioinformatics requirements.

Metagenomics is a **culture-independent, high-throughput sequencing approach** that analyzes the collective genetic material of all microorganisms within a clinical sample. Unlike traditional microbiological techniques that rely on culturing, metagenomics enables the **direct detection and characterization of microbial communities**, including **bacteria, viruses, fungi, and parasites**.

**a. Key Features of Metagenomics**

✔ **Comprehensive Pathogen Detection** – Simultaneously identifies multiple microbial species in a sample.  
✔ **Microbiome Analysis** – Studies microbial diversity and function in health and disease.  
✔ **Antimicrobial Resistance Gene Identification** – Detects genetic determinants of resistance, aiding in antibiotic stewardship.

**b. Metagenomic Workflows in Clinical Diagnostics**

1. **Sample Collection & DNA/RNA Extraction**
   * Clinical specimens: Blood, CSF, respiratory secretions, feces, tissue biopsies.
   * DNA/RNA is extracted for downstream analysis.
2. **Library Preparation & Sequencing**
   * DNA is fragmented, adapters are ligated, and sequencing libraries are prepared.
   * High-throughput sequencing is performed (e.g., **Illumina, Oxford Nanopore, PacBio**).
3. **Bioinformatics & Data Analysis**
   * **Taxonomic classification**: Identifies pathogens using reference genome databases.
   * **Functional analysis**: Detects virulence factors, antibiotic resistance genes, and metabolic pathways.
   * **Comparative metagenomics**: Studies microbiome shifts in diseased vs. healthy individuals.

**c. Applications of Metagenomics**

**1) Diagnosis of Polymicrobial Infections**

* Traditional methods struggle with polymicrobial infections due to **bias in culture techniques**.
* **Metagenomic sequencing detects all microbial species**, including fastidious or unculturable pathogens.
* **Example:**
  + Diagnosing bacterial **meningitis** when Gram staining and PCR are inconclusive.
  + Identifying co-infections in pneumonia or wound infections.

**2) Microbiome Analysis in Health & Disease**

* Metagenomics helps characterize the **human microbiome**, revealing **dysbiosis (imbalances)** linked to disease.
* **Example:**
  + **Gut microbiome alterations** in inflammatory bowel disease (IBD).
  + **Oral microbiome profiling** in periodontal disease.
  + **Skin microbiome shifts** in chronic wounds and eczema.

**3) Detection of Emerging & Novel Pathogens**

* Unlike PCR or microarrays, metagenomics does **not require prior knowledge of pathogens**, making it ideal for identifying **novel viruses and bacteria**.
* **Example:**
  + Discovery of **SARS-CoV-2** in early COVID-19 cases.
  + Identification of **Zika virus outbreaks** in South America.
  + Tracking **antimicrobial resistance genes (ARGs)** in hospital-acquired infections.

**4) Surveillance of Antimicrobial Resistance (AMR)**

* Detects known and novel **AMR genes**, even before phenotypic resistance is observed.
* **Example:**
  + Identifying **β-lactamase genes** in multi-drug resistant (MDR) bacteria.
  + Studying AMR patterns in **hospital wastewater and community settings**.

**Table 5 . Comparison of Metagenomics with Other Molecular Methods**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Feature | Metagenomics | PCR (qPCR, Multiplex) | Microarray | NGS (Whole-Genome) |
| Target Organisms | All microbes | Specific (depends on primers) | Predefined targets | Single genome |
| Multiplexing Ability | High | Low to moderate | High | High |
| Detection of Unknowns | Yes | No | No | Yes, but limited to single genomes |
| Antimicrobial Resistance (AMR) Detection | Yes | Only known targets | Predefined genes | Yes |
| Cost | High | Low to moderate | Moderate | Moderate to high |

**e. Limitations of Metagenomics**

* **High Cost**: Expensive sequencing platforms and bioinformatics tools.
* **Complex Bioinformatics**: Requires expertise in data processing and interpretation.
* **Low Sensitivity for Low-Abundance Pathogens**: May need deeper sequencing for rare microbes.
* **Contamination Issues**: DNA from non-pathogenic organisms can complicate interpretation.

**IV. Immunodiagnostic Techniques**

**A. Enzyme-Linked Immunosorbent Assay (ELISA)** ELISA detects pathogen-specific antigens or antibodies in clinical samples. It is widely used due to its simplicity, cost-effectiveness, and scalability.Immunodiagnostic techniques detect and measure **pathogen-specific antigens or antibodies** in clinical samples using **antigen-antibody interactions**. These methods are **highly specific, sensitive, and widely used** for diagnosing infectious diseases, autoimmune disorders, and monitoring immune responses.

**A. Enzyme-Linked Immunosorbent Assay (ELISA)**

**ELISA** is a **high-throughput immunoassay** that detects and quantifies **pathogen-specific antigens or antibodies** using **enzyme-linked detection systems**. It is commonly used for **infectious disease diagnosis, serological testing, and vaccine efficacy studies**.

**1) Principle of ELISA**

✔ **Antibody-Antigen Binding** – A **specific antibody or antigen** is immobilized on a solid surface (microtiter plate).  
✔ **Enzyme-Linked Detection** – A secondary antibody conjugated to an **enzyme (e.g., horseradish peroxidase or alkaline phosphatase)** binds to the antigen-antibody complex.  
✔ **Substrate Reaction** – The enzyme catalyzes a color change in the presence of a **chromogenic or fluorescent substrate**, which is measured using a spectrophotometer.

**2) Types of ELISA**

**a) Direct ELISA**

* Detects **antigen** using a **primary antibody conjugated to an enzyme**.
* **Example**: Detecting **Hepatitis B surface antigen (HBsAg)** in blood samples.
* **Pros:** Quick and simple.
* **Cons:** Lower sensitivity due to the absence of signal amplification.

**b) Indirect ELISA**

* Detects **antibodies** using **an enzyme-labeled secondary antibody** that binds to a primary antibody.
* **Example**: **HIV antibody testing** in blood samples.
* **Pros:** High sensitivity due to signal amplification.
* **Cons:** Potential cross-reactivity with secondary antibodies.

**c) Sandwich ELISA**

* Detects **antigens** using **two specific antibodies** (capture and detection antibodies).
* **Example**: **COVID-19 antigen detection** in nasopharyngeal swabs.
* **Pros:** High specificity due to dual-antibody recognition.
* **Cons:** More expensive due to the requirement for matched antibody pairs.

**d) Competitive ELISA**

* Measures **antigens or antibodies** by competition between labeled and unlabeled antigens/antibodies.
* **Example**: Measuring **testosterone levels** in endocrine studies.
* **Pros:** Useful for detecting small molecules (hormones, drugs).
* **Cons:** Requires precise standardization.

**3) Applications of ELISA**

✔ **Diagnosis of Infectious Diseases**

* **HIV, Hepatitis B/C, Dengue, COVID-19, Syphilis, Lyme disease**.  
  ✔ **Vaccine Development & Serological Studies**
* Monitoring **immune responses post-vaccination**.  
  ✔ **Autoimmune Disease Detection**
* **Antinuclear Antibody (ANA) testing** for lupus and rheumatoid arthritis.  
  ✔ **Toxin & Hormone Detection**
* Detecting **botulinum toxin, thyroid hormones, pregnancy hormones (hCG)**.

**4) Advantages & Limitations of ELISA**

|  |  |  |
| --- | --- | --- |
| **Feature** | **Advantages** | **Limitations** |
| **Sensitivity & Specificity** | High due to antigen-antibody specificity | May have cross-reactivity leading to false positives |
| **Cost & Scalability** | Cost-effective, suitable for high-throughput testing | Requires trained personnel for standardization |
| **Automation** | Easily automated for large-scale diagnostics | Time-consuming compared to rapid immunochromatographic assays |
| **Detection** | Quantitative or qualitative results | Limited to detecting known pathogens with available antibodies |

**5) Future Directions**

✔ **Point-of-Care ELISA** – Miniaturized versions for rapid, bedside testing.  
✔ **Multiplex ELISA** – Detects multiple pathogens in a single assay.  
✔ **Integration with AI & Machine Learning** – For automated interpretation and diagnosis.

**B. Immunofluorescence Assays** These assays use fluorescently labeled antibodies for pathogen detection, offering high specificity and visual confirmation of the target.Immunofluorescence assays (IFAs) are **antigen-antibody-based techniques** that utilize **fluorescently labeled antibodies** for **pathogen detection, disease diagnosis, and biomarker identification**. These assays provide **high specificity, visual confirmation**, and can be used in both **direct and indirect formats** for clinical and research applications.

**1) Principle of Immunofluorescence Assays**

✔ **Antibody-Antigen Binding** – A **fluorescently labeled antibody binds to the target antigen** in a clinical specimen.  
✔ **Fluorescent Tag Excitation** – The fluorophore (e.g., **FITC, TRITC, Alexa Fluor dyes**) absorbs light at a specific wavelength and emits fluorescence.  
✔ **Microscopic Visualization** – The sample is examined under a **fluorescence microscope**, enabling direct visualization of the target antigen.

**2) Types of Immunofluorescence Assays**

**a) Direct Immunofluorescence Assay (DFA)**

* Uses a **fluorescently labeled primary antibody** that directly binds to the antigen in the specimen.
* **Example:**
  + Detection of **Rabies virus antigens** in brain tissue.
  + **Respiratory virus detection** (e.g., Influenza, RSV) in nasopharyngeal samples.
* **Pros:**  
  ✔ Faster and simpler than indirect IFA.  
  ✔ High specificity due to direct binding.
* **Cons:**  
  ✖ Less signal amplification, reducing sensitivity.  
  ✖ Requires high-quality fluorescent-labeled antibodies.

**b) Indirect Immunofluorescence Assay (IFA)**

* Uses an **unlabeled primary antibody** that binds to the antigen, followed by a **fluorescent-labeled secondary antibody** that detects the primary antibody.
* **Example:**
  + **Autoimmune disease testing** (e.g., **antinuclear antibodies (ANA) for lupus**).
  + **Serological diagnosis of viral infections** (e.g., **Epstein-Barr virus (EBV), Syphilis**).
* **Pros:**  
  ✔ Greater sensitivity due to signal amplification.  
  ✔ More versatile – same secondary antibody can detect multiple primary antibodies.
* **Cons:**  
  ✖ Longer procedure with additional steps.  
  ✖ Higher risk of background fluorescence.

**3) Applications of Immunofluorescence Assays**

✔ **Infectious Disease Diagnosis**

* **Bacterial infections**: Identification of **Chlamydia trachomatis, Treponema pallidum (Syphilis)**.
* **Viral infections**: Detection of **SARS-CoV-2, Respiratory Syncytial Virus (RSV), Influenza, Rabies virus**.
* **Parasitic infections**: **Plasmodium spp. (Malaria), Toxoplasma gondii** detection.

✔ **Autoimmune Disease Detection**

* **Antinuclear Antibody (ANA) Test** for **Systemic Lupus Erythematosus (SLE)**.
* **Anti-Glomerular Basement Membrane (Anti-GBM) Test** for **Goodpasture’s syndrome**.

✔ **Cancer Biomarker Identification**

* **Detection of HER2, p53, Ki-67 proteins** in cancer tissues.

✔ **Neurological Disease Diagnosis**

* **Anti-neuronal antibodies** in **autoimmune encephalitis, multiple sclerosis**.

**4) Advantages & Limitations of Immunofluorescence Assays**

|  |  |  |
| --- | --- | --- |
| Feature | Advantages | Limitations |
| Specificity | High due to precise antigen-antibody interactions | Requires well-characterized antibodies |
| Visualization | Direct imaging of pathogens in patient samples | Requires a fluorescence microscope |
| Sensitivity | Indirect IFA allows signal amplification | Background fluorescence may interfere with results |
| Speed | Faster than culture-based methods | More labor-intensive than ELISA |
| Automation | Can be automated for high-throughput testing | Requires skilled personnel for interpretation |

**5) Comparison with Other Immunodiagnostic Techniques**

|  |  |  |  |
| --- | --- | --- | --- |
| Feature | Immunofluorescence (IFA) | ELISA | Western Blot |
| Target Detection | Direct visualization of antigens in tissue/cells | Quantitative antigen/antibody measurement | Protein detection in lysates |
| Sensitivity | High (especially indirect IFA) | Moderate to high | High |
| Speed | Rapid (within hours) | Medium (2–4 hours) | Longer (6–24 hours) |
| Equipment | Requires fluorescence microscope | Requires spectrophotometer | Requires electrophoresis & blotting system |
| Application | Infectious diseases, autoimmune diseases, cancer markers | Serology, biomarker detection | Confirmatory testing for HIV, Lyme disease |

**6) Future Directions in Immunofluorescence**

✔ **Multiplex Fluorescent Assays** – Simultaneous detection of multiple antigens in one sample.  
✔ **Automated Digital Image Analysis** – AI-driven analysis for faster, objective interpretation.  
✔ **Super-Resolution Microscopy** – Improves spatial resolution for detecting subcellular structures.  
✔ **Point-of-Care (POC) Fluorescent Assays** – Handheld fluorescence-based devices for rapid bedside diagnostics.

**C. Lateral Flow Assays (LFA)** LFAs are point-of-care tests providing rapid results, ideal for bedside diagnostics. Common applications include detecting malaria, dengue, and respiratory pathogens.Lateral Flow Assays (LFAs) are **rapid, point-of-care (POC) immunoassays** that detect target molecules (antigens, antibodies, or nucleic acids) in **clinical, environmental, and food samples**. They provide **fast, easy-to-use, and cost-effective** diagnostic solutions, making them ideal for **bedside, field, and home-based testing**.

**1) Principle of Lateral Flow Assays**

LFAs operate on **capillary action**, where a liquid sample moves through a **membrane strip**, interacting with labeled antibodies to generate a visible signal.

✔ **Sample Application** – A patient sample (e.g., blood, saliva, urine) is applied to the sample pad.  
✔ **Capillary Flow** – The liquid moves through the strip via **capillary action**, carrying analytes toward reaction zones.  
✔ **Antigen-Antibody Binding** – If the target analyte (e.g., a pathogen’s antigen or an antibody) is present, it binds to **fluorescent or gold nanoparticle-labeled antibodies**.  
✔ **Signal Detection** – The immune complex reaches the **test and control lines**, forming a **visible band (colored or fluorescent)** to indicate a positive result.

**2) Components of an LFA Strip**

|  |  |
| --- | --- |
| **Component** | **Function** |
| **Sample Pad** | Absorbs and delivers the sample to the conjugate pad. |
| **Conjugate Pad** | Contains antibodies conjugated with gold nanoparticles or fluorescent dyes. |
| **Nitrocellulose Membrane** | Houses the test and control lines where antigen-antibody reactions occur. |
| **Absorbent Pad** | Draws excess liquid to maintain flow. |

**3) Types of Lateral Flow Assays**

**a) Antigen Detection LFAs**

* Detect **pathogen-specific antigens** in patient samples.
* **Examples:**
  + COVID-19 Rapid Antigen Test
  + Malaria Rapid Diagnostic Test (RDT)

**b) Antibody Detection LFAs**

* Detect patient **antibodies** produced in response to infections.
* **Examples:**
  + HIV rapid diagnostic test
  + Dengue IgM/IgG test

**c) Nucleic Acid-Based LFAs**

* Detect **DNA/RNA sequences** using hybridization probes.
* Used as a **readout for isothermal amplification methods** like LAMP (Loop-Mediated Isothermal Amplification).
* **Example:** **Tuberculosis (TB) rapid molecular test**.

**4) Applications of Lateral Flow Assays**

✔ **Infectious Disease Diagnosis**

* **COVID-19, Influenza, Malaria, Dengue, HIV, Hepatitis B/C, Streptococcus, RSV**.  
  ✔ **Pregnancy Testing**
* **hCG-based urine pregnancy tests**.  
  ✔ **Cardiac Biomarker Detection**
* **Troponin I/T rapid tests for myocardial infarction diagnosis**.  
  ✔ **Food and Water Safety**
* **Detection of foodborne pathogens (Salmonella, E. coli, Listeria)**.  
  ✔ **Drug Testing**
* **Urine-based lateral flow tests for detecting narcotics**.

**5) Advantages & Limitations of LFAs**

|  |  |  |
| --- | --- | --- |
| Feature | Advantages | Limitations |
| Speed | Results in 5–30 minutes | May lack sensitivity compared to laboratory-based tests |
| Ease of Use | Simple, no need for specialized equipment | Requires careful handling to avoid false positives/negatives |
| Cost | Affordable for large-scale deployment | Some tests have lower specificity |
| Portability | Can be used at the bedside, in remote areas, or at home | Limited multiplexing capabilities |
| Shelf Stability | Long shelf life, does not require refrigeration | May degrade in humid environments |

**6) Future Trends in LFA Technology**

✔ **Multiplex LFAs** – Detect multiple pathogens in a single test strip (e.g., **simultaneous detection of influenza, RSV, and COVID-19**).  
✔ **Smartphone-Based LFAs** – Use **AI and mobile apps** to enhance test interpretation and data sharing.  
✔ **Fluorescent & Magnetic LFAs** – Improve **sensitivity and accuracy** over traditional colorimetric LFAs.  
✔ **Biosensor-Integrated LFAs** – Combine LFAs with **electrochemical or optical biosensors** for enhanced detection.

**V. Applications in Clinical Microbiology** Culture-independent techniques have transformed diagnostic workflows, offering rapid detection, personalized treatment strategies, and improved infection control measures. Key applications include:

1. Early detection of sepsis and bloodstream infections
2. Diagnosis of respiratory tract infections (e.g., COVID-19, influenza)
3. Identifying antimicrobial resistance patterns

**V. CHALLENGES AND LIMITATIONS**

* **High Cost**: Initial investment in instruments and reagents.
* **Technical Expertise**: Requirement for skilled personnel.
* **Interpretation Complexity**: Challenges in data analysis, particularly for metagenomics.
* **Regulatory and Standardization Issues**: Lack of harmonized guidelines for CIDTs.

**VI. FUTURE PERSPECTIVES**

* **Integration with AI**: Enhancing diagnostic accuracy through machine learning.
* **Point-of-Care Testing (POCT)**: Expansion of portable CIDTs.
* **Global Access**: Reducing disparities in diagnostic availability.

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