Review article

Recent advances in diagnostic testing for viral infections

Selma Souf*

*Corresponding author: College of Science and Technology, Nottingham Trent University, Clifton campus NG11 8NS, Nottingham, UK.
Email: soufselma@gmail.com

Supervisor: Dr Jody Winter, College of Science and Technology, Nottingham Trent University, Clifton campus NG11 8NS, Nottingham, UK.

Viral infectious diseases represent an important portion of global public health concerns with thousands of deaths annually. From serious pandemics and highly contagious infections to common influenza episodes, clinical prognosis often relies on early detection of the infectious agent. Thus, effective identification of viral pathogens is needed to help prevent transmission, set up appropriate therapy, monitor response to treatment and lead to efficient disease management and control. The aim of this review is to outline some of the recent technological advances in viral identification, including polymerase chain reaction, mass spectrometry and next-generation sequencing, and how they are applied in the diagnosis and management of viral infections. These powerful tools combine rapidity and efficiency in detecting viral pathogens and have revolutionized the field of clinical diagnostics. However, a number of drawbacks such as high cost have limited their use in many laboratories, particularly in resource-limited settings. On the contrary, the advent of microfluidic technology has attracted increasing interest from biomedical research groups, and could represent a challenging alternative to diagnose viral infections at lower cost.

Key words: viral infections, laboratory testing, immunoassay, polymerase chain reaction, next-generation sequencing, mass spectrometry

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Introduction

Global pandemics are serious threats to human life. While well-established and characterized viruses such as The human immunodeficiency virus (HIV) and Hepatitis are still killing millions of people, the emerging viruses are also problematic and have caused several serious outbreaks in the recent years. For example, the Severe Acute Respiratory Syndrome-Coronavirus (SARS-CoV) in 2002–2003, Swine Influenza A (H1N1) in 2009 and Ebola Haemorrhagic fever outbreak in 2014 which has caused thousands of deaths in West Africa.

Morbidity and mortality rates are significantly high. Thirty-five million people were infected with HIV in 2013, and 350–400 millions chronic carriers of Hepatitis B virus. According to the World Health Organization (WHO) report of 2014 (WHO, 2014); more than 780 000 people die every year of Hepatitis B and up to 500 000 die of Hepatitis C-related liver diseases. The high prevalence of these diseases has raised the efforts for improving clinical diagnostics.

Effective prevention and clinical management of infectious diseases are intimately linked to early and accurate screening of pathogens, not only by detecting the infectious particles in the organism but also by elucidating the aspects that confer resistance to therapy and immune escape profiles, including mutations and genotype disparity.

Therefore, rapid diagnosis benefits patients in allowing timely therapy to prevent complications; and benefits public health by collecting data for epidemiological studies, to prevent outbreaks and spreading of diseases. In that context, the WHO has established many surveillance programs for disease control such as the global strategy for control and assessment
of HIV drug resistance, the Global Influenza Surveillance and Response System for the control and monitoring of Influenza and the Global Policy on Viral Hepatitis.

At a smaller scale, clinical laboratories are a crucial point for diagnosis of viral diseases by using a range of tools and machineries varying in cost and efficacy.

In a rapidly growing world of technology, the industry is continuously delivering up-to-date instruments but many factors are limiting their implementation in healthcare settings with low income, which unfortunately delays global benefit. This review will describe some of these advanced testing methods, how their specific characteristics have revolutionized the field of laboratory diagnosis and what can be done to overcome their limitations.

### General principles of good laboratory testing

Rigorous and accurate interpretation of laboratory results guarantees effective clinical management of a disease and control of its propagation (Lemon et al., 2007). However, erroneous diagnosis could lead to financial and human loss.

In clinical testing of infectious diseases, it is crucial to determine precisely the presence or absence of the infectious agent or its corresponding antibodies, to prove current or past exposure. Therefore, the ability to say precisely whether the person is infected or not, and to determine the course of the infection has a positive impact on the therapeutic strategy.

The usefulness and reliability of laboratory results depend on the performance and operational parameters of the intended test. The performance parameters directly relate to the results by estimating their **Accuracy**, **Precision**, **Sensitivity**, and **Specificity** (Lalkhen and McCluskey, 2008). While these are statistical values (percentages), they have different explanations and involve comparison with the reference method or ‘gold standard’ for the desired test (Guzman et al., 2010).

**Accuracy** describes how close the obtained results are to those obtained with the reference method and it is expressed as a percentage of correct results. **Precision** refers to the reliable reproduction of one test on the same sample, and obtaining similar results.

These two parameters must be regularly monitored using local quality control (QC) and quality assurance (QA) procedures in order to maintain reliability of the test. In perfect conditions, an ideal test would have 100% accuracy and 100% precision; however, external factors and methodological differences can cause small variations.

**Sensitivity** (also called the true positive rate) is the percentage of patients with confirmed infection (by the ‘gold standard’ method) who will have positive results. It is usually measured by the lower limit of detection of the analyte producing a positive result.

**Specificity** (also called the true negative rate) is a qualitative assessment, showing the capability of the test to distinguish target from non-target analyte. This measure is expressed as the percentage of infection-free patients who will have a negative result. The closer the values are to the reference, the higher the sensitivity and specificity of the test.

On the contrary, operational parameters concern simplicity and ease in performing the test such as the turnaround time (TAT). TAT is a key performance indicator defined as the interval time between sample registration to result reporting. Sample preparation and any other pre-analytical steps are within this interval. Assay completion in less than 60 min is ideal so manufacturers aim to construct diagnosis instruments allowing shorter TAT, which is particularly beneficial for point-of-care settings (Hawkins, 2007).

The WHO has established ASSURED criteria (Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free and Deliverable to end users) (Wu and Zaman, 2012) for diagnostics in resources-limited point-of care settings (Blacksell, 2012). The aim is to provide better management of the disease, such as immediate delivery of the results and rapid record of the disease status, to improve clinical decision-making.

### Traditional laboratory methods for the diagnosis of viral infections

For long time, clinical laboratories have relied on a diverse range of techniques to diagnose viral infections.

In developed countries, electron microscope (EM) has for long time been considered an efficient tool for direct detection of viruses through visualization and counting of the viral particles in body fluids, stools or histopathologic samples. The identification is based on morphological characteristics specific to each virus family and requires a certain amount of viral particles (up to 106 particles/ml). However, specimen preparation that must be performed beforehand (Goldsmith and Miller, 2009) may reduce the virus concentration which makes the analysis harder. In addition, electron microscopy requires substantial technical skills and expertise; the presence of unusual or look-alike structures such as cellular debris and organelles may confuse the electron microscopist with their virus-like shapes (Fauquet et al., 2005).

The combination of EM with culture-based methods has shown great contribution in the diagnosis of viral infections, along with serology testing for detection of antibodies targeted against the virus. These conventional methods are still fundamental practices in many hospital laboratories.

**Cell culture** is one of the most popular methods for isolating viruses using cell lines. These latter vary according to the targeted viruses (for example; rhesus monkey kidney cells are used for isolation of Influenza A virus).

Evidence of virus growth is seen through the cytopathic effect (CPE) exhibiting specific characteristics and alterations of
the cells (Robbins, Enders and Weller, 1950). The virus definitive identification is then performed using Immunofluorescence (IF) staining. Nevertheless, virus isolation using cell culture is not ideal in case of viruses not amenable to growth in cell lines (norovirus, hepatitis virus) or producing CPE (Papafragkou et al., 2013).

In addition, small volumes of the sample may not allow inoculation of many cell types, and thus compromise the results. For example, the standard inoculation of a cell culture medium with cerebrospinal fluid (CSF) sample requires a minimum of 0.2 ml; however, much more is needed to inoculate a combination of different cell types, which could be invasive to the patient (McIntyre, 2007, Hemattana et al., 2016).

The time required for isolating viruses by cell culture is very long (weeks), limiting the usefulness of this technique when rapid diagnosis is needed. Cell culture needs highly skilled and experienced personnel for accurate interpretation of the CPE and adequate facilities for handling mammalian cell lines or highly pathogenic viruses.

In the shell vial technique, one of the rapid culture methods, inoculated cells are subjected to centrifugation, incubation and then IF staining with monoclonal antibodies, specific to the range of viruses suspected to cause the infection, such as respiratory viruses, Herpes simplex virus or Varicella zoster virus. The virus-induced antigens are detected 2–4 days later.

Rapid culture has limited benefits as it does not target a wide range of viruses and has low sensitivity.

Complement fixation test (CFT) is one of the oldest methods in the history of clinical virology (Casals and Palacios, 1941). The complement reacts only with antigen–antibody complex in a non-specific manner. Thus, in the presence of the complex, the complement is not free to interact with sensitized sheep red blood cells (RBCs) used as an indicator, and which remain unlysed. The test is said to be ‘positive’.

CFT is supposedly easy to perform, convenient and requires inexpensive material. However, it is labour intensive and lacks sensitivity. In-house standardization through titration of the reactants and preparation of controls is crucial for obtaining effective testing.

Haemagglutination inhibition test is generally used for detecting arboviruses, influenza and parainfluenza virus subtypes and provides relative quantitation of the virus particles. The principle relies on the capacity of haemagglutinin (HA); a viral protein present in the envelope, to bind to erythrocytes (RBC) and to form a lattice pattern termed ‘agglutination’. In the assay, serial dilutions of the sample serum are added to a fixed amount of viral HA and agglutinable RBCs. If Influenza antibodies are present in the serum, the agglutination process is prevented. The corresponding dilution rate at which complete haemagglutination is observed and considered.

Variants of the agglutination assay are used for the diagnosis of wider range of viral diseases other than influenza (Grandien et al., 1987; Sandeep et al., 2002). However, the test is time consuming, and demanding, particularly in terms of QC.

This list of conventional techniques is not exhaustive, and only the most commonly used were cited. The following sections will discuss more recent developments in the field, which replaced the conventional practices and allowed screening (qualitative test), surveillance (quantitative test) and confirmation of diagnosis.

### Recent methods in the diagnosis of viral infections

As stated before, this review will discuss only some of the main developments in diagnostic technologies that have ushered the new era of clinical virology, and lay out their key advantages and limitations that will be summarized in Table 1.

### Immunoassay-based tests

Antibodies produced immediately after invasion of a foreign substance can inform on primary infection, reinfection or a reactivation state. Therefore, measuring the level of immunoglobulins (Ig) is a widely considered approach for the diagnosis of viral infections.

Automated immunoassay-based methods are among the most frequently used for testing, and are effective because of the high specificity and binding affinity between antigen and antibody. Therefore, the principle of the test relies in the formation of an immuno-complex between antibody present in the patient sample and synthetic antigen present in the reagent or vice versa, to generate a measurable signal.

Immunoassays use labels conjugated to synthetic antibodies or antigens which are linked to a solid phase, and used to capture corresponding antigens or antibodies present in sera samples. These labels could be radioactive isotopes, enzymes that cause change in colour or light-generating substances. Consequently, this principle has generated several methodologies for the testing.

Radio-immunoassay (RIA) is probably the initiating method (1960s); it uses radioisotopes (such as Iodine 125) to label antigen or antibody. The amount of substance to analyse is determined by the amount of the generated radioactivity. RIA is a highly sensitive method but the main drawback is the handling and disposal of hazardous radioactive substances.

The enzymatic labelling alternative using alkaline phosphatase or horseradish peroxidase as markers is, however, the most widely used and was long considered a reference method (Engvall and Perlmann, 1972; Voller, Bidwell and Bartlett, 1976). These enzymes induce emission of signals or change in colour respectively, and allow the amount of analyte of interest to be measured. This enzyme-linked immunoassay (ELISA) has numerous variants, including ELISA, and they differ in the enzyme used and the signal detection principle.
Table 1. Summary of the main viral diagnostic methods

| Diagnostic technique | Principle | Strengths | Weaknesses | Variants | References |
|----------------------|-----------|-----------|------------|----------|------------|
| Immunoassay          | Formation of antigen–antibody through recognition and binding | High sensitivity, High specificity, High speed throughput, Quick TAT (20 min or less), Different types of tags, labels, Automated method | Must rely on QC assurance, High risk of interferences, High cost | RIA, EIA (FPIA, MEIA, CLIA) | Gupta et al. (2015), Mixson-Hayden et al. (2015) |
| NAAT                 | Amplification and detection of sequences from the viral genome (DNA or RNA) | High sensitivity, High specificity, Multiplexed platforms, Genotyping, Determination of the viral load, New compact and portable formats | Longer run time, Requires specific primers for the targets | RT-PCR, qPCR, NASBA, TMA | Garcia-Arroyo et al. (2016), Reijans et al. (2008), Renois et al. (2010), Afshar and Moliaie (2012), Mercier-Delarue et al. (2014), Wu et al. (2014) |
| NGS                  | Polymerization of DNA template by incorporation of labelled dNTPs, and terminate the extension | High sensitivity, High specificity, Identification of novel genomic sequences, Genotyping, Accurate detection of mutations and drug resistant mutations | High cost, Needs bioinformatics skills for data analysis, Delay in use for routine clinical diagnostics | Pyrosequencing Fluorescently labelled dNTP Detection of released hydrogen ion (H+) | Capobianchi et al. (2013), Bartolini et al. (2015), Lowe et al. (2016), Liu et al. (2013), Van den Hoecke et al. (2015), Rothenberg et al. (2011) |
| MS                   | Ionization of the sample, then separation and detection of the particles according to their mass-to-charge ratio (m/z) | High sensitivity, Versatility, Cost-effectiveness, High workload | Expensive equipment, Limited database library | MALDI-TOF MS, ESI MS, Often combined with other methods: PCR-MS | Lévéque et al. (2014), Mengelle et al. (2013), Qian et al. (2014) |

CLIA, chemiluminescent immunoassay; dNTP, deoxyribonucleotide triphosphate; EIA, enzyme immunoassay; ESI, electrospray ionization; FPIA, fluorescence polarization immunoassay; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MEIA, micro-particle enzyme immunoassay; MS, mass spectrometry; NGS, next-generation sequencing; NAAT, nucleic acid amplification test; NASBA, nucleic acid sequence-based amplification; qPCR, quantitative polymerase chain reaction; RIA, radio-immunoassay; RT-PCR, real-time polymerase chain reaction; TMA, transcription-mediated amplification.

The main variants of EIA are as follows:

- **Fluorescence polarization immunoassay (FPIA)**: uses fluorescent label and polarized light.
- **Micro-particle enzyme immunoassay (MEIA)** (Tassopoulos et al., 1997): widely used and relies on alkaline phosphatase enzyme and a corresponding fluorescent substrate.
- **Chemiluminescent immunoassay (CLIA)**, which uses chemiluminescent or light-emitting labels. Companies like ROCHE or Abbott are exploiting this method, and high-volume laboratories are gradually replacing MEIA technology with CLIA for its high-speed throughput and ease of measurement.

In clinical practice, serological studies of Hepatitis B rely on immunoassay as a key tool for detection of Hepatitis B virus (HBV) markers. The up-to-date versions of immunoassay methods are able to detect the lowest levels of the main marker HBsAg, undetectable by conventional methods, particularly in sera samples of asymptomatic patients. The latest screening standard detects as little as 0.05 IU/ml, the equivalent of 0.2 ng/ml viral antigen (Deguchi et al., 2004) and high sensitivity (>99%) was observed at different stages of the disease or even in patients showing seroclearance.

A newly developed CLIA allowed denaturation of the HBV particle, and used monoclonal antibodies against inner
and outer structural epitopes in order to obtain higher assay sensitivity (Shinkai et al., 2013) and sufficient information on the HBV genotypes.

Automated immunoassay techniques for virus detection overcome some of the limitations encountered with the conventional tests, particularly the delay to response.

**Limitations**

Despite the popularity of immunoassay in clinical testing, erroneous results may occur for many reasons, which confer inconsistencies to the testing.

- Immunoassays are more prone to interferences than any other assay, which leads to false-positive or false-negative results. In most cases, interferences are due to the presence of agents with structural similarities to the reagents (Miller, 2004), and fluctuate according to the concentration of the interfering substance and the analyte. Endogenous antibodies (autoantibodies, hetero-antibodies or human anti-animal antibodies) are commonly the main culprit in these interferences (Emerson and Lai, 2013). Their binding to capture antibodies and detection antibodies in the absence of antigen (analyte) mainly leads to false-positive result (Berth and Willaert, 2016).
- Accurate QC guarantees reliable results and assumes that the assay is performed well. Therefore, poor QC measures lead to low accuracy and precision. In immunoassays, QC is assured by regular calibration and control of the reagents, using standardized solutions delivered by the manufacturers.
- The high cost of reagents and equipment is another drawback in immunoassays, particularly for resource-limited settings, considering that the most accurate and sensitive methods are automated.

**Amplification-based assays**

Developed by Mullis and Faloona (1987), nucleic acid amplification by polymerase chain reaction (PCR) has revolutionized the field of molecular diagnosis. The basic PCR assay relies on extraction and purification of the nucleic acid, then exponential amplification of the target sequence, using a thermostable polymerase enzyme and specific primers. The resulting amplicons are then identified using a fluorescence-based detection system, and the result is reported in international units IU/ml.

Soon after its invention, modifications in PCR were tested and patented, with the aim of improving the assay capabilities. The term nucleic acid amplification tests (NAAT) was applied to this range of new variants.

NAAT are very popular in the diagnosis and management of viral infections (HBV, HCV, HIV, Influenza viruses, ...) because they allow determination of the viral load. In other terms, quantitation of the viral nucleic acid by amplifying the target sequence thousands-fold. In most cases, they are now considered a reference, or ‘gold standard’ method for diagnostic practices such as screening donated blood for transfusion-transmitted viruses (CMV, HIV, HCV, ...) (Jackson, 1990).

The most widely used variants of conventional amplification are real-time PCR (quantitative PCR) and reverse transcription-PCR (RT-PCR). Both are nowadays becoming benchmarks in assessing the viral load, and while the first method quantifies DNA throughout the reactions in real time (Ntziora et al., 2013); the second performs RT of the mRNA (RNA messenger) and amplifies the resulting cDNA (complementary DNA). It also quantifies RNA. The combination of both techniques increases sensitivity in detecting viruses, particularly influenza viruses. The WHO recently approved a newly developed reverse transcriptase-PCR assay after the first death from MERS-CoV (Middle East Respiratory Syndrome-Coronavirus) infection reported in 2012 (Abd El Wahed et al., 2013).

Other amplification-based tests such as nucleic acid sequence-based amplification (NASBA) and transcription-mediated amplification (TMA) are suited for detection of RNA viruses by amplification of the mRNA instead of conversion to cDNA (Mercier-Delarue et al., 2014, Wu et al., 2014).

Among the latest enhancements in PCR systems, extraction, amplification and detection steps have been combined within one unit. As an example, BioFire Film Array technology applies two sequential amplifications and can detect a panel of respiratory viruses with high sensitivity in 1 h and within a single instrument (Pierce et al., 2012).

On the contrary, microfluidic technology has also benefited PCR-based systems; and allowed further decrease in the detection time (15 min), with high sensitivity and cost-effectiveness. In the 2009 epidemic, detection of Influenza A virus H1N1 greatly relied on a chip-type real-time PCR instead of the tube-based principle. The portable format of such systems is very convenient during epidemics and outbreaks (Song et al., 2012).

Multiplexed NAAT were designed to detect multiple viruses or subtypes in a single run. Their detection platforms can comprise up to 20 viruses using diversified panels (Mahony et al., 2007), for example, concurrent detection of HAV, HBV and HCV infections, as well as co-infections (Park et al., 2012).

In Hepatitis B, PCR is considered the gold standard method for assessing the HBV DNA level with high accuracy, and the assays are standardized according to the WHO International Standard for Hepatitis B virus (NIBSC), such as the reference Roche Cobas HBV test. The test was the first FDA approved assay, with a detection limit of 10.2 IU/ml. The combined system with Cobas AmpliPrep has an automated sample extraction process and highly sensitive detection for all HBV genotypes (Pyne et al., 2012).

In HIV patients taking antiretroviral treatment (ART), ultrasensitive assays permit quantitation of low copy numbers
of HIV subtypes (<50 copies/ml) and long terminal repeats in latent reservoirs and from cell lysates omitting the nucleic acid extraction step (Vandergeeten et al., 2014). Such assays could be valuable tools in large cohort studies or pandemic situations.

**Limitations**

The limitations of PCR are an important parameter to consider, despite the cost-effectiveness and reliability in the diagnosis of viral infections. The risk of contamination is very high while handling, especially during the sample preparation step, in addition, real-time PCR has a longer run-time (2–5 h) by comparison to other techniques.

In the case of influenza, many PCR-based assays were designed to detect only a particular subtype of the virus responsible for important pandemics (Hall et al., 2009), and the need for designing specific primers for the target requires handling by experienced operators, capable of detecting errors, particularly that PCR is prone to false-positive results.

The high mutation rate of some viruses could trigger mutation within PCR primer regions of the viral genome, which would lead the virus to escape the detection by this assay.

**Next-generation sequencing**

Next-generation sequencing (NGS) is one of the greatest achievements of the modern era. Beyond genome sequencing from known organisms, it allowed discovery of novel viruses responsible for unknown human diseases (Palacios et al., 2008), and tracking of outbreaks and pandemics such as influenza (Baillie et al., 2012) to understand their emergence and transmission profiles (Leung et al., 2014; Isakov et al., 2015).

The journey began in the 1970s with the works of Sanger and Barrell, followed by Maxam and Gilbert, who first initiated the principle of sequencing oligonucleotides via enzymatic polymerization, using radiolabelled primers. In their experimental protocols, they relied on using dideoxy-nucleotides to terminate the extension of the polymer, hence the name: chain termination or dideoxynucleotide method (Sanger, Nicklen and Coulson, 1977).

Since then, the principle has remained virtually the same, but improvements and automation have dramatically increased the speed and accuracy in delivering maximum volume of data comparing to dideoxynucleotide sequencing (Shedure and Ji, 2008). Technically, NGS is inclusive of three main steps: sample preparation, sequencing and data analysis. Systems available in the market differ mostly in their sequencing or reading techniques. Efficient and accurate clinical diagnosis of viral infections using NGS is increasingly aiming to provide accurate longer read-length in a shortest time and at a lower cost. Bioinformatic platforms are key components of the sequencing process. They allow interpretation of the sequencing output through computational analysis (Naccache et al., 2014), and then convert it into useful information on species, genotypes and the occurrence of mutations conferring virulence or resistance to antivirals.

Pyrosequencing is currently the variant of choice within NGS systems. Based on the detection of pyrophosphate (PPi) after incorporation of a nucleotide in a DNA polymerization process, it uses luciferase to catalyse light-generating processes, and the collected light is then recorded. Although Illumina is considered the most frequently used pyrosequencing platform, 454 FLX; a subsidiary company of Roche, was the first high-throughput analyser in the market and was used to determine human papillomavirus (HPV) types (Barzon et al., 2011), subtypes and variants, present in cervical specimens. Other variants detect hydrogen ions that are released throughout nucleotide incorporation reaction (Rothberg et al., 2011).

Generating high volumes of sequence data has allowed the compilation of viral nucleotide databases and acquisition of de novo sequences to understand the genetic variability of viruses (Szpara, Parsons and Enquist, 2010). HIV is by far the most sequenced because of the global priority of AIDS as a serious endemic, and because of the high mutation rate of the virus.

So far, only gene sequencing methods have been successful in genotyping tricky HBV, unlike conventional PCR or serological assays. Therefore, sequencing has allowed better clinical management of HBV infection and related complications (Margeridon-Thermet et al., 2009).

In data analysis, recent technical approaches have included adjustment of the software reading platforms for simultaneous detection of genotypes and mutants of clinical importance (Germer et al., 2013), and partial sequencing of the HBV S gene with high sensitivity (98.64%), considering that portion as the site of most drug-resistance mutations (Wang et al., 2013). Nevertheless, implementation of NGS in clinical settings is increasing, particularly for detecting low-abundance drug-resistance patterns such as in HIV and HCV (Palmer et al., 2005; Verbinnen et al., 2010). Nucleotide sequencing of HCV sub-genomic regions is now the method of choice for genotyping.

**Limitations**

The principal requirements for NGS are initially access to a sequencer, and considerable skills in bioinformatics and expertise in data analysis, plus adequate handling systems for storage of generated data. Adding to that, despite the outstanding results delivered by prototypes in trials, many are still at the research level, and not yet approved for use in routine clinical practice (Jiangjin et al., 2015).

NGS is undeniably a key technology in specialized clinical laboratories, but its implementation is still a challenge in many countries, where not only their resource-limited settings cannot afford a sequence analyser, sample and library preparation, but the vast majority of the population cannot afford the cost of the test.
Mass spectrometry (MS) is nowadays a benchmark of laboratory qualitative and quantitative investigation, particularly in bacteriology (Sauer and Kliem, 2010).

The principle of MS relies on converting the sample into charged particles (ions) by ionization process. These ions are separated according to their mass-to-charge ratio (m/z) and analysed by a detector. The result obtained is compared to a reference database (library), existing within the system and delivered as an interpretive spectrum.

In clinical laboratories, matrix-assisted laser desorption ionization (MALDI) and electrospray (ES) are the most used ionization methods because they allow processing of considerable amounts of analyte (Emonet et al., 2010). These approaches have extensively been evaluated experimentally and provided excellent results, either used alone or combined with other molecular methods, such as PCR, in order to enhance sensitivity. The combination (RT-PCR/ESI-MS) was able to detect viral pathogens usually undetected by regular testing methods, and provided rapid and detailed data (types and subtypes) within a short time (Lévêque et al., 2014).

Detection of genomic variations or mutations in Influenza A using MALDI-TOF has been a key tool in the management of outbreaks (Chen et al., 2011). MS application to the structural investigation of biomolecules showed the efficiency of MALDI-TOF-MS coupled to antibody magnetic nanoparticles in detecting influenza viruses (Chou et al., 2011; Yea et al., 2011), through concordant results with the gold standard PCR-based method. This blend of two powerful manu neries (PCR-MS) can detect drug resistance to antiviral therapy as well as the presence of multiple viruses within the same sample and diagnose for co-infections, when assays are multiplexed.

The increasing interest in MALDI-TOF MS in clinical virology has led to research advances in the diagnosis of Hepatitis B. The method was in fact not only able to detect HBV at relatively low viral load levels (100 HBV DNA copies/ml) (Hong et al., 2004) but it also allowed detection of up to 60 HBV variants and genotypes at a cost lower than $10/sample for all the variants with high throughput (Luan et al., 2009). The same method was applied for genotyping and successfully detected the eight HBV genotypes accurately (Ganova-Raeva et al., 2010), and also minor HCV genotypes occurring at very low level (Kim et al., 2005).

Mass spectrometric-based methods are versatile, sensitive, rapid and cost-effective, and do not require interpretation software for data analysis. The automated machinery necessitates easy sample preparation and fewer operators. The analysis capacity can reach up to 960 specimens/day, which makes it suitable for routine diagnosis in high-volume laboratories and large-scale studies. Tests can also be performed efficiently on archived specimen.

Limitations

The main limitation of MS is the high cost, particularly in high pandemic areas, which are usually the poorest; not all laboratories can afford a mass analyser for their activities. The second major drawback is within the reference library. The identification is limited by known data from well-identified organisms only; therefore, rare mutations cannot be detected if they do not exist within the reading platform, but there is hope that MS database libraries will rapidly expand.

Advantages of the recent methods

- High sensitivity and specificity assays approaching 100%.
- Possibility to combine different methods in one assay enhances detection capability and accuracy (PCR-MS, microfluidic chip technology-based PCR, ...).
- Automation of the assays reduces the number of operators and manual workload.
- Small sample volumes are needed, so the assays can still be performed in particular cases (CSF, new-borns, etc.)
- Some assays such as MS remain efficient after several freezing–thawing of the samples (archived samples).
- Rapid TAT: minimum time required from sample collection to results reporting is 30 min, and microarray tools can deliver results within seconds.
- Low detection limit: 10–100 copies/ml by PCR.
- Multiplex reactions: detection of a wide range of pathogens in a single run is time saving.
- Detection of rare drug-resistance patterns.
- NGS generates many sequencing data per run and sequences long-reads.
- Low reaction cost when using microfluidic chips, and use in POC settings.
- Lower risk of contamination by processing single tube within one unit.

The latest technologies and resource-limited settings

The methods described above have shown outstanding performance in saving thousands of lives in developed countries. However, accurate diagnosis is still a challenge in resource-limited settings because of the difficulty in acquiring these equipment and technical expertise. While expensive tools such as PCR-based systems or mass analysers are only available in reference laboratories or in military facilities, point-of-care or near-patients clinics that serve more than 80% of the poor population struggle with the testing devices available. As an example, the WHO has established guidelines for monitoring ART efficacy in HIV-1 infected patients in developing countries, by suggesting only CD4+ cell count and ELISA-based assays as alternatives to the expensive tests (WHO, 2006a). Ultrasensitive p24 (Patton et al., 2008) and the ExaVir assay (Kokkayil et al., 2014), measure the level of p24.
antigen and the activity of reverse transcriptase, respectively, to replace viral load monitoring. Unfortunately, their lack of sensitivity has induced failure of treatment and emergence of resistant strains (Vekemans, John and Colebunders, 2007).

Hepatitis is another expensive burden to manage in poor countries, where accurate assessment of the epidemiological profile is hard to establish. In the case of HBV, most POC facilities rely on screening for HBsAg solely using rapid diagnosis tests. These low-cost devices do not allow determination of the course of the disease to inform whether to initiate an antiviral treatment or not.

**Automation, microfluidics and future prospects**

Providing high-level epidemiological monitoring of viral diseases is undeniably a global public health ambition, and despite rapid progress in the development of diagnostic methods in recent years, improvements are needed for better cost, size (Loman et al., 2012; Frey et al., 2014) and TAT (Xu et al., 2013).

After the Ebola Haemorrhagic fever in 2014, the US governmental Department of Energy has developed an innovative rapid and portable test to detect specifically Ebola virus within seconds. The test aims to target other RNA and exotic viruses such as Dengue and West Nile for effective management of viral outbreaks. Other examples include fully NAAT systems and small devices to sequence single molecule DNA using nanopore technology (Eisenstein, 2012).

Microfluidic technology is regarded with optimism for the management of infectious diseases by allowing timely therapy. It provides a key potential solution for remote areas and near-patients facilities by avoiding turnaround trips of the patients between the clinic and the laboratory. Their use is also beneficial where time is crucial, or when physical spaces do not allow setting up of conventional methods.

Lab-on-a-chip (LOC) is a very small device that integrates laboratory processes within a few square centimetres. It uses very small volume of samples to perform immediate reactions within the chip or in a portable device. The reactions vary from nucleic acid amplification and detection, to cell count and immunoassays; therefore, microfluidic diagnostics compete with large instruments in performing laboratory tests at a lower cost, to benefit low-income settings and remote areas.

A wide range of LOC devices were approved by the FDA in the diagnosis of viral infections such as Influenza (Cao et al., 2012), HIV (Alyassim et al., 2009) and HBV (Zhi et al., 2014) and further development is in progress. As examples, Daktari Diagnostics use affinity chromatography for CD4+T-cell count as an alternative to flow cytometry to monitor HIV in developing countries (Cheng et al., 2007). The bench top analyser GeneXpert made by Cepheid has an integrated sample preparation and PCR system for molecular diagnosis of influenza and other bacterial infections in a light portable format.

Improvement of QC programs, QA and standardization of assays, kits and reagents are important to fulfil requirements for accuracy. The Centre for Disease Control and Prevention (CDC) is continuously implementing laboratory-testing guidelines particularly for HIV, before further tests will be approved (CDC, 2014).

The WHO and non-profit organizations such as the Foundation for Innovative New Diagnostics (FIND) aim to maximize efforts to implement surveillance programs and control of communicable diseases (Loman et al., 2012), set up new policies and improve diagnostic services in low resource settings.

**Conclusion**

The recently developed viral diagnostic methods are reshaping the field of clinical microbiology, and could contribute to reducing the prevalence of serious infectious diseases. However, the technical capabilities alone are insufficient if not supported by health promotion strategies to increase awareness about the importance of early detection and regular screening of persons at high risk.

Finally, good quality diagnosis has a cost that only developed countries can afford in routine practice so far, and this is delaying the implementation of new methods in the developing world and the endemic areas. However, there is hope that efforts will continue towards developing new good quality tests affordable in low-income countries, which would substantially strengthen disease control strategies for their populations.

**Author biography**

Selma obtained an MSc with Honours in Biomedical sciences from Nottingham Trent University. She works as a biomedical scientist at Institut Pasteur, within the Immunoassay division, and has particular interest in laboratory diagnosis issues in low-income settings and how they can be solved at lower cost. She also has interest in structural biology and microbiology.

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