

## Review

# Application of mass spectrometry to molecular surveillance of hepatitis B and C viral infections

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Detection of genotypes and drug resistance mutations are important molecular tools assisting in clinical management of patients with chronic hepatitis B and C. Together with methods for assessment of genetic heterogeneity and relatedness of viral strains, they form the foundation of molecular surveillance. Currently, all these methods are based mainly on DNA sequencing followed by

phylogenetic analysis. Mass spectrometry (MS) emerged recently as a rapid, cost-effective, reproducible and accurate alternative approach. MS-based molecular assays are highly amenable to automation and provide a suitable platform for routine application to the surveillance of HBV and HCV infections.

## Introduction

Globally, 500 million people are estimated to be chronically infected with HBV or HCV [1,2]. In the US, 3.2 and 2 million people are chronically infected with HCV and HBV, respectively [3–8]. Both viruses cause blood-borne infections and are the major contributors to liver cirrhosis and hepatocellular carcinoma [7,9]. HBV infections can be effectively prevented with vaccination; however, there is no vaccine against HCV. A significant proportion of HCV infections are undetected due, in part, to their asymptomatic nature [10]. Despite a substantial decrease in the incidence over the past two decades, infections with HBV and HCV remain major global public health problems [11].

HBV and HCV are genetically diverse and classified into 10 [12–14] and 6 genotypes [15], respectively. Genetic heterogeneity of both viruses is associated with disease severity, progression to chronic hepatitis and response to therapy [16–18]. Detection of HBV and HCV genotypes and the assessment and monitoring of emerging drug resistance mutations are important molecular tools assisting in the clinical management of patients with hepatitis B and C [19,20]. In public health settings, genetic heterogeneity is used for molecular tracking of HBV and HCV transmissions [21–24].

There are many commercially available assays for identifying HBV and HCV infections [25,26] and for genotyping these viruses [27,28]. However, genetic

analysis of individual HBV and HCV strains is usually accomplished using in-house assays developed and maintained in laboratories involved in molecular surveillance. The majority of these assays are based on phylogenetic analysis of sequences that allows for fine resolution of viral strains and intra-host variants [29]. However, because such assays are costly, time-consuming and require specialized expertise for their conduct and for interpretation of the results generated, their automation would facilitate routine application in clinical and public health laboratories.

Mass spectrometry (MS) was recently developed as a viable alternative to DNA sequencing for the identification and characterization of the genetic diversity of viral strains. The high accuracy and sensitivity, wide dynamic range and high-throughput inherent in MS-based approaches lend them to routine application in molecular surveillance [30].

## MS principles

MS applications are based on measuring the molecular mass of analytes. The approach utilizes direct detection without requiring any detector molecules such as fluorescence or radioactive labels, antibodies or hybridization probes. MS involves three processes: ionization, separation and detection. Ionization may lead to degradation of analytes. To maintain the integrity of nucleic acids, energy-intensive chemical and electrical

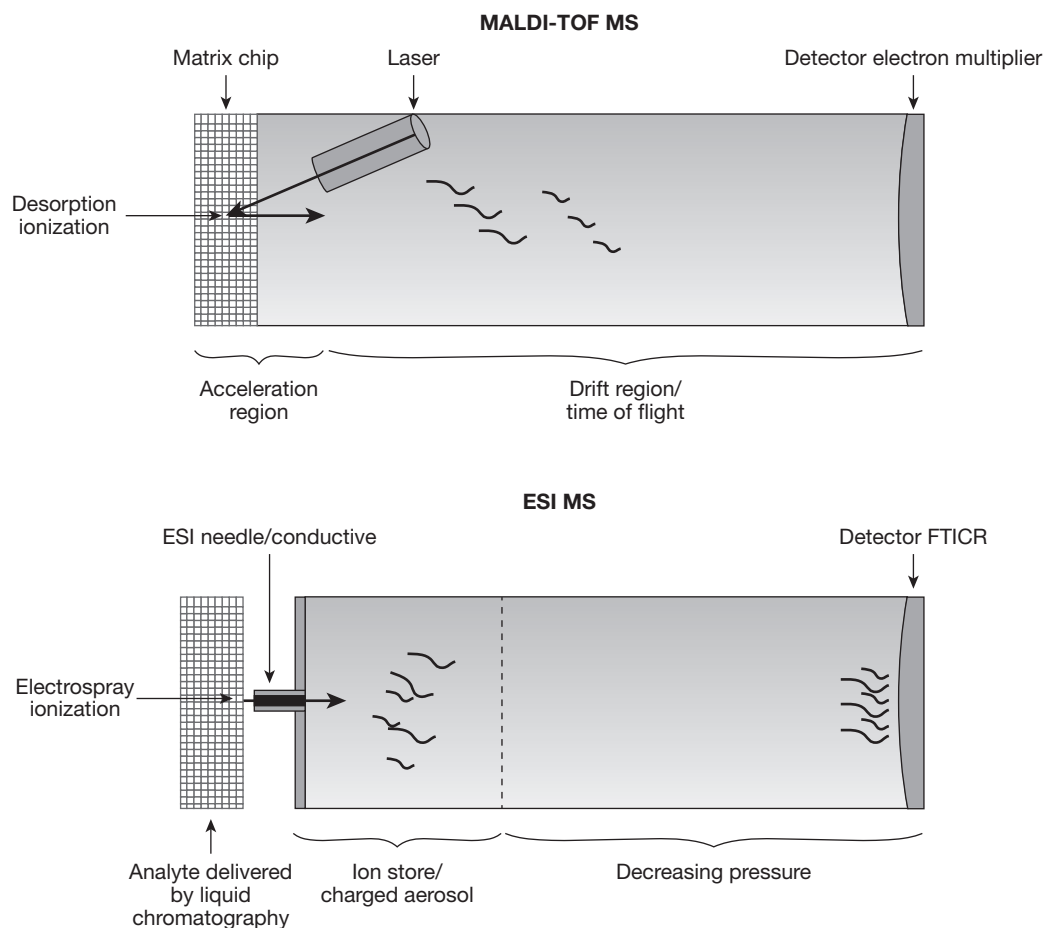
ionization techniques have been replaced with softer matrix-assisted laser desorption/ionization (MALDI) [31] and electrospray ionization (ESI) [32]. Additional technical improvements that reduce depurination and generation of salt adducts together with substitution of DNA with the more ionization-stable RNA [33] have further facilitated the expansion of MS applications to nucleic acid analysis.

MALDI-time-of-flight (TOF) MS (Figure 1A) uses two classes of matrices. ‘Hot’ matrices are higher in energy and more suitable for detection of small RNA molecules, whereas ‘cool’ matrices provide better protection against fragmentation and so are used for larger oligonucleotides. The analyte is imbedded in the matrix and ionized by a 3–4 ns laser pulse. The ions are then accelerated to the equal kinetic energy and sent into a field-free drift vacuum tube where molecules are

separated by their masses to a very high resolution. Detection is achieved by recording the TOF needed for the ionized analyte to reach the detector [34].

In ESI MS (Figure 1B), the analyte is dissolved in organic solvent and injected in a conductive capillary, where it is converted into charged aerosol under high voltage. The aerosol travels through sections of decreasing pressure and forms gas-phase ions of the analyte. ESI is typically coupled with an automated sampler injector and liquid chromatography. The single sample detection rate is several minutes. Detection occurs simultaneously for all generated ions. They are differentiated by their rotational frequency that is transformed into a specific mass reading. Fourier transform ion cyclotron resonance is one of the most accurate and high-resolution techniques of detection [35].

**Figure 1.** Schematic representation of basic configurations of MALDI-TOF MS and ESI



ESI, electrospray ionization; FTICR, Fourier transform ion cyclotron resonance; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MS, mass spectrometry.

## MS platforms for genetic testing

Development of MALDI-TOF and ESI MS enabled numerous applications in molecular diagnostics, ranging from identification of pathogens and disease susceptibility genes [36,37] to DNA sequencing [38] and single nucleotide polymorphism (SNP) discovery [39]. This range was recently broadened to analysis of DNA methylation [40], which was found to be associated with human malignancies [41]. In addition to high precision, rapidity and cost-effectiveness, MS offers ease of multiplexing for the detection of molecular analytes. Several MS applications were developed for the simultaneous detection of bacteria [42] and viruses [43,44].

ESI MS was used in development of the Triangulation Identification Genetic Evaluation of Biological Risks (TIGER) Bioanalyzer (Ibis Biosciences, Abbott Laboratories, Abbott Park, IL, USA) for multiplexed identification of pathogens [45]. MALDI-TOF MS was applied successfully to the development of the MassArray System at Sequenom Inc. (San Diego, CA, USA) [46]. It involves gene-specific PCR followed by primer extension to the site of interest in the presence of all four dideoxynucleotides; masses of the extended primers are detected by MALDI-TOF MS and interpreted automatically into specific SNPs. MassArray iPLEX was designed for SNP analysis and genotyping [47] and was shown to identify minority alleles present in as low as 1:50 ratio in a sample. The high sensitivity and specificity allow for the simultaneous detection of up to 40 SNPs and make this assay suitable for high-throughput genotyping [46]. MassArray iSEQ designed for a high-performance comparative sequencing is based on MALDI-TOF MS of mixtures of short oligonucleotides generated by a base-specific cleavage of target RNA obtained from PCR fragments [48]. This assay yields mass patterns (MSP) specific to the tested PCR fragment. MSPs contain very rich genetic information and can be directly applied to viral genotyping, genetic relatedness analysis [49,50] and detection of new mutations.

## Identification of genotypes

The first MS application to detection of HBV from serum was described 15 years ago [51] and showed the considerable advantages of coupling PCR to MS for molecular testing. However, it is only recently that significant advances in MALDI-TOF MS of nucleic acids provided an opportunity for the development of molecular assays for the detection of viral genotypes suitable for the routine surveillance.

ESI MS has been extensively used for virus detection and screening on a large scale [52]. MALDI-TOF MS seems to be more suitable for a fine genetic

characterization of viral lineages and has been applied to genotyping of HBV and HCV. The MassArray iSEQ platform was implemented for HBV genotyping [50]. In this assay, MSPs experimentally generated from the S gene of tested HBV variants are compared immediately after completion of the MS run with *in silico* simulated MSPs of HBV variants with known genotypes. Genotype is called when similarity of the experimental and simulated MSPs exceeds an established threshold. This iSEQ-based assay is highly reproducible, owing to significant redundancy of generated data that ensures correct genotype calls. The extensive evaluation of the assay against a large panel of HBV strains representing all known genotypes showed complete concordance with results obtained using DNA sequencing and phylogenetic analysis.

Many factors such as viral titre, genotype specificity, reference-panel composition, quality of PCR product and MSP as well as genetic complexity of the intra-host HBV population were examined and found not to have effect on the specificity of genotype calling. The sensitivity of the assay can be largely controlled by maintaining an extensive reference set of sequences with known HBV genotypes and the high quality of PCR products and MSP. However, mixed-genotype infections were found to present a special challenge because the assay detected either the dominant genotype in the sample or produced MSPs with matching scores very close to the lower threshold of the assay. Nevertheless, the design of the assay makes it easily amenable to the detection of any new genotype by simple addition of sequences to the reference panel, allowing for testing of up to approximately 1,000 specimens per day with downstream data analysis completely automated [50].

The MALDI-TOF MS detection is most efficient when applied to complex mixtures of short oligonucleotides. An assay based on a similar principle as MassArray iSEQ, whereby the large polynucleotides are degraded specifically into short oligonucleotides, was designed for HCV genotyping [53]. The HCV 5'-untranslated region (5'-UTR) contains three variable genotype-specific motifs flanked by conserved regions. These motifs were used for restriction-fragment mass-polymorphism (RFMP) analysis. Detection of HCV RNA was based on PCR amplification with primers containing recognition sites for restriction endonucleases. Cleavage with two different sets of the endonucleases generated multiple oligonucleotides, the separation of which using MALDI-TOF MS allowed for the accurate identification of HCV genotypes. The RFMP assay was efficient in detection of HCV genotypes in mixed-genotype infections. It identified genotypes of HCV strains representing only 0.5% of intra-host viral population and reliably determined relative abundance of genotypes [53].

A very efficient primer extension MALDI-TOF MS assay was recently developed for the identification of HCV genotypes and subtypes [54]. In this assay, the PCR fragment derived from 5'-UTR of HCV genome was used as a template for the extension of three oligonucleotide primers designed to detect two sets of genotype-specific SNPs. The products of the primer extension reactions were analysed using MALDI-TOF MS. The assay was shown to be effective in detecting HCV subtypes 1a, 1b, 2a and 3a as well as genotype 4.

### Fine-resolution genetic characterization

HBV and HCV exist as a population of closely related variants or quasispecies in each infected host [55,56]. Some of these variants are resistant to therapeutic treatment. Detection of such variants is important for clinical management of patients on antiviral therapy and monitoring drug resistance of viral strains circulating in different geographic regions. Consensus sequencing of PCR fragments derived from viral genomes may not detect minority variants in complex intra-host viral populations. However, MS-based assays were proven to be simple to perform and sensitive for the detection of minority variants, and as such are suitable for routine molecular characterization of viral strains [57,58].

The MassArray iPLEX platform was applied to the detection of 60 HBV variants containing drug resistance mutations in the reverse transcriptase domain, and precore and basal core promoters [59]. In the assay, PCR fragments amplified from two regions of the HBV genome containing the mutations were used to conduct four multiplex primer extension reactions, each including 12–17 primers. The primer extension products were separated by automated MALDI-TOF MS for the detection of HBV genetic variants. The MS- and sequence-based assays showed 99.9% concordance in detecting mutations. The MS assay detected twice as many minor variants as direct sequencing while achieving close to full automation [59].

The primer extension approaches are suitable for the detection of SNPs. However, viral genetic heterogeneity may affect efficacy of primer binding to annealing sites, thus complicating the assay design and potentially contributing to the decreased sensitivity of the SNP detection. Alternative RFMP-based assays take advantage of the improved assay sensitivity owing to the significant reduction in the number of used primers; however, they have been applied so far to the detection of a limited number of SNPs. The RFMP-based assay was developed for the detection of mutations in the YMDD motif of the HBV reverse transcriptase domain and was shown to be useful for the early identification of HBV breakthrough infections [60]. This assay showed a remarkable ability to detect as few as 100

copies of HBV genome and correctly identify known HBV variants. It could distinguish minority variants in complex intra-host HBV populations and assess relative abundance of the variants [61].

The intra-host HCV genetic heterogeneity was found to be associated with progression to chronic infection and response to antiviral therapy [18]. It is usually assessed using hypervariable region 1 (HVR1) located at the 5'-end of the E2 gene [62]. Such assessment is frequently accomplished by separating HVR1 variants using genetic cloning or end-point limiting dilution [63] followed by sequencing. It can also be achieved by next-generation sequencing [49]. MS significantly reduces assay logistics while maintaining adequate accuracy, thus making MS-based assays for the assessment of HCV genetic heterogeneity suitable for routine molecular surveillance.

Besides evaluation of nucleic acids directly, MS was applied to the detection of HCV HVR1 quasispecies using peptides translated *in vitro* from PCR amplicons [64]. The heterogeneous intra-host HVR1 variants were translated into a mixture of peptides, separation of which by MS allowed for the assessment of the quasispecies complexity. The MS-based assay was used to show weekly changes in HVR1 quasispecies in an HCV-infected chimpanzee. The observed variation in intra-host HCV population was compatible with emergence of neutralizing antibody responses. The assay was found to be more sensitive in detection of quasispecies than the more conventional approach involving separation of HVR1 variants by genetic cloning followed by DNA sequencing [65].

Analysis of the HCV HVR1 quasispecies is used for the identification of transmissions [21–24], which is at the core of outbreak investigations and molecular surveillance for HCV infections. Although sequencing approaches have been confirmed accurate in detection of transmissions, all involve sophisticated sequence analyses and complex data interpretation, thus dramatically limiting the use of these approaches to specialized genetic laboratories. The development of robust and straightforward approaches to the detection of transmission remains an important task for surveillance. Extensive analysis of HVR1 quasispecies using MassArray iSEQ showed that, similar to sequences, MSPs contain genetic information sufficient for accurate discrimination among HCV strains [49], indicating that MS-based assays may serve for the detection of viral transmissions.

### Conclusions

During the past decade, MS has emerged as a rapid, cost-effective and highly reproducible method with numerous applications to microbial identification and detection of mutations. MS- and sequence-based assays perform equally efficiently in identification of HBV and HCV

genotypes, detection of drug resistance mutations and assessment of genetic heterogeneity of intra-host viral populations. MS is highly amenable to automation and, when coupled with computational tools for interpretation of MS data into molecular epidemiological information, may serve in the development of novel genetic assays suitable for routine molecular surveillance.

## Disclosure statement

The authors declare no competing interests.

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