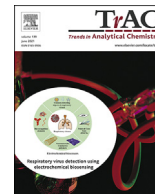




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A new approach to identifying pathogens, with particular regard to viruses, based on capillary electrophoresis and other analytical techniques



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ABSTRACT

Fast determination, identification and characterization of pathogens is a significant challenge in many fields, from industry to medicine. Standard approaches (e.g., culture media and biochemical tests) are known to be very time-consuming and labor-intensive. Conversely, screening techniques demand a quick and low-cost grouping of microbial isolates, and current analysis call for broad reports of pathogens, involving the application of molecular, microscopy, and electromigration techniques, DNA fingerprinting and also MALDI-TOF methods. The present COVID-19 pandemic is a crisis that affects rich and poor countries alike. Detection of SARS-CoV-2 in patient samples is a critical tool for monitoring disease spread, guiding therapeutic decisions and devising social distancing protocols. The goal of this review is to present an innovative methodology based on preparative separation of pathogens by electromigration techniques in combination with simultaneous analysis of the proteome, lipidome, and genome using laser desorption/ionization analysis.

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1. Introduction

Microorganisms have always been extremely important for human life and they are known for both positive and negative reasons. They are particularly associated with food sciences [1], genetic engineering, medicine [2], biotechnology as well as other areas of life sciences [3]. They are used for their unique features which enable production of various therapeutic compounds [2], and food and food-related products [1], as well as decomposition of such components as lignocellulosic biomass for second-generation ethanol [3]. On the other hand, the genetic features and biochemical abilities of microorganisms make them dangerous for industry (e.g. food spoilage) as well as for human health. Approximately 1400 pathogens can cause human diseases [4], with pathogenic bacterial strains and viruses posing a particular threat.

The first group of pathogens is currently a real problem. This is mainly due to the growing number of strains resistant to commercially available antibiotics widely used in therapies. Mass production of these substances in recent years and the lack of discipline in their use in many sectors (e.g. in medicine, animal husbandry and agriculture) have contributed to microbes developing a number of defense mechanisms [5]. Bacteria show two types of antibiotic resistance. The first one is congenital resistance, characteristic of the species, and the second one is acquired resistance, characteristic of a particular strain. Congenital (innate) resistance may be connected with a lack of characteristic binding sites (e.g. porin deficiency) or presence of specific structures that prevent the drug from entering the cell (e.g. a layer of exopolysaccharide or alginate polysaccharide). This type of resistance is not the result of implementing specific therapeutic agents and usually does not involve medical consequences. More dangerous is the acquired resistance of bacteria which were originally sensitive to the drug. In addition, the latter type of resistance can be divided into primary and secondary resistance. Primary resistance is related to spontaneous mutation that can occur without contact with the

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drug and cannot be passed on to other bacteria species. In contrast, secondary (induced) resistance occurs as a result of pathogen coming into contact with antibiotics; genes with information about the drug resistance mechanism can be transferred to other bacterial cells. Moreover, the growing problem is that microorganisms may exhibit multiple resistance mechanisms at the same time. Therefore, depending on the range of resistance, they can be classified into three groups: extensively drug-resistant (XDR), sensitive to up to two groups of antibiotics; multidrug-resistant (MDR), resistant to three or more groups of antibiotics; and drug-resistant (PDR), resistant to all groups of antibiotics. Currently, antibiotic resistance of bacteria is a global problem. It is estimated that the drug-resistant strains cause about 2.8 million infections and approximately 25,000 deaths per year only in the United States [5]. In Europe antibiotic-resistant pathogens cause about 25,000 deaths a year as well. It is believed that already in 2050 resistant strains will cause more deaths than cancers [6].

Next to the pathogenic drug-resistant bacteria, viruses are another group of pathogens highly dangerous to people. Viruses can be defined as infectious agents, which propagate themselves via multiplication in parasitized living cells [7]. The form of virus existence and replication is at the same time virtue and disadvantage. Their multiplication cannot occur without appropriate host cells. However, after the integration viruses become a part of the cell and therefore their complete destruction cannot be achieved without cell death. Another problem which stems from the intracellular existence of the viruses is that it is hard or even impossible to produce effective medicine to control them [8]. Moreover, the submicroscopic size of the viruses (the size of the majority of animal virus's ranges from 30 to 300 nm) enables very fast spread of viral units [7]. It is even more relevant for viruses whose target cells are exposed to environmental influence. Microbial agents such as bacteria, fungi or viruses can move around the environment by air via air pollutants. Recent reports suggest that atmospheric particulate matter (PM) may increase the effectiveness of pathogen spread because it creates a microenvironment suitable for their persistence. These particles can act as carriers for microorganisms and, what is more, they can serve as nutrition for the latter and make them more toxic. Inhalation transports particles (especially these smaller than 2.5 μm) deep into the lungs. This allows the pathogens to develop within the respiratory tract and cause infections [9].

The respiratory tract infections caused by viruses, such as the influenza virus, are one of the most frequent infections in the world. Annually influenza virus causes infections in 5–15% of the population [10]. Coronaviruses (CoVs) also cause respiratory tract infections. There are seven known human coronaviruses: HCoV-229E, HCoV-OC43, HCoV-NL63, HCoV-HKU1, SARS-CoV, MERS-CoV and SARS-CoV-2. In 2003, SARS was the cause of the rapidly spreading respiratory disease with a mortality rate of nearly 10%. In 2012, MERS-CoV caused severe acute respiratory infection with a fatality rate close to 40% [11]. Currently in 2020, the world is struggling with another epidemic caused by a virus from this group, SARS-CoV-2. Studies from many countries indicate the existence of positive correlations between high levels of air pollution (PM_{2.5}, PM₁₀, nitrogen dioxide, and ozone) and SARS-CoV-2 infections [9].

The situation with SARS-CoV and SARS-CoV-2 has revealed that fast and relevant detection of the infection is one of the most effective ways to prevent its distribution and reduce the ravages caused by the epidemic [12]. Rapid identification of bacterial and viral infections is a strategic stage in the fight for the patient's life because only a thorough understanding of the source of the disease allows implementation of a proper therapy. Unfortunately, despite their high accuracy, the methods currently used for this purpose (based on molecular biology) are expensive, time-consuming, and

can be performed only in well-equipped laboratories with a high biosafety level (BSL 2 or 3) and qualified staff, access to which is limited in many regions of the world [13]. The accurate identification of pathogenic microorganisms is essential for scientists involved in many areas of applied research and industry, from clinical microbiology to food production. There are a lot of criteria for the classification of the numerous methods used in identification and characterization of microorganisms; however, generally they can be divided into direct and indirect techniques. Direct methods include conventional isolation of pure cultures of microorganisms and their various phenotypic analyses, while indirect methods are culture independent and include microscopic techniques which are the powerful tools in the identification of microbes by visualization of the characteristic structures. It also makes it possible to detect microbes in a viable but not culturable state.

Identification of pathogens based on the traditional methods – which includes determining their morphology, physiology, chemistry and biochemical characterization – is estimated to take at least 2–5 days. In addition, most phenotypic methods used in microbiological laboratories are time and material consuming. Importantly, phenotypic methods are not always suitable to identify microorganisms at the species level, or much more often at the strain level. One of the strategies to reduce time needed for microbial identification is the use of molecular biology techniques, which may also be supplemented with numerous molecular fingerprinting techniques [13]. Each of these methods has their strengths and weaknesses, and the most recent research approach involves the use of a compilation of multivariate techniques. Such implementation and application of hyphenated techniques seems to have a great potential for future development. In order to obtain the most precise identification, classification and characterization of pathogens, it is extremely important to choose appropriate techniques, as well as have a thorough understanding of the mechanisms of their action (Fig. 1).

Taking the above into consideration, today's world of science faces an enormous challenge: how to develop new pathogen identification methods which are fast, cheap and easy to use, as well as accurate and repeatable.

2. Analytical challenges and strategies in determination of pathogens

Due to the constantly growing number of infections caused by pathogenic microorganisms, the development of fast and cheap methods of microbial identification that are also effective and unambiguous is an extremely important aspect and the challenge of modern science. Reliable and efficient identification of pathogens is a key tool enabling implementation of appropriate therapies, and thus saving lives. Unfortunately, conventional techniques used in diagnostic laboratories to identify pathogens based on biochemical tests that differentiate bacteria phenotypically are time consuming, work intensive and often inadequate to differentiate between phenotypically similar species [13].

2.1. Molecular biology approach

The rapid development of molecular biology has enabled the use of a more precise diagnostic tool, which is polymerase chain reaction (PCR), followed by sequencing of the conserved fragment of the 16S rRNA gene. This method was developed in the early 1980s. It is based on in-situ DNA replication that allows exponential amplification of target DNA in the presence of synthetic oligonucleotide primers and thermostable DNA polymerase [14]. A new version of the PCR technique is real time-PCR (RT-PCR), which

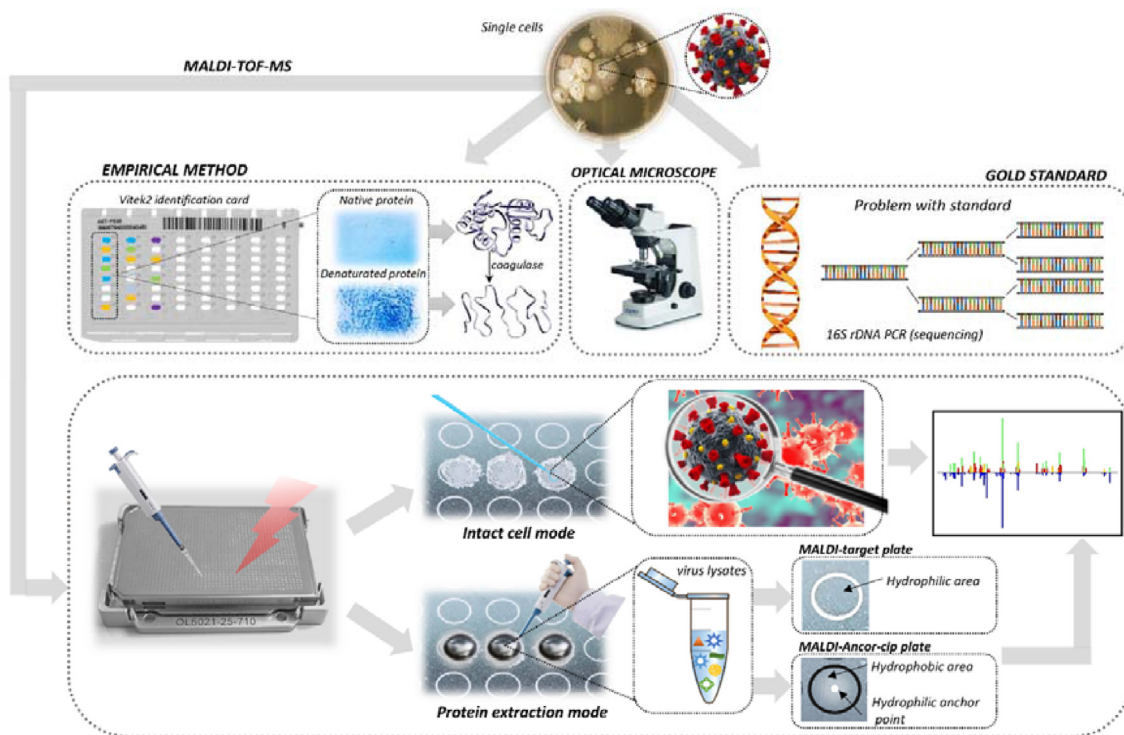


Fig. 1. Pathogen identification approaches with particular regard to viruses.

allows tracking the obtained amplicons in real time after each cycle of the amplification reaction. The application of RT-PCR reduces the risk of sample contamination and shortens the analysis time from about 5 h in the case of classic PCR to about 40 min [13]. The sensitivity and efficiency of PCR techniques may depend not only on the presence of enriching components in the culture medium but also on DNA extraction solution, temperature and cyclic conditions as well as the concentration of primers or matrices [14]. Products obtained from the PCR reaction are usually subjected to sequencing – a technology that allows determining the order of nucleotide bases in a DNA molecule. Nowadays, 16S rRNA sequencing technique is considered as a “gold standard” in microorganism identification [13].

Molecular biology methods are characterized by high sensitivity, accuracy, discriminatory power and reproducibility. However, next to the numerous advantages there are many limitations. The main problems include a long analysis time of up to 3 days. This is mainly related to the fact that such research is usually carried out by specialized external institutions with qualified staff, access to commercial databases and dedicated equipment [14]. In addition, the costs of analysis are so high that this technology cannot be used in routine diagnostics. At the same time, the problem is the limited number of gene sequences available in databases and the lack of strict criteria for interpreting the results [15]. Analysis of the 16S rRNA gene also does not allow differentiation of many closely related species, particularly if they overlap in more than 98% of the sequence. Such a phenomenon was observed, among others, for the *Burkholderia* and *Staphylococcus* species [16].

Another group of microbes that are dangerous to human life are viruses. The structure of the viruses is relatively simple in comparison to other living forms. Viral particles mainly consist of genetic material and proteins. Genetic material can be represented by RNA ((+) single-stranded, (–) single-stranded and double-stranded) or DNA (linear double- or single-stranded and circular double-stranded). Viral proteins are divided into two main groups:

structural and non-structural proteins. Structural proteins form the viral unit and comprise the proteins of the capsid, which protect nucleic acids; the envelope proteins, which take part in the viral-in-cell fusion; matrix proteins, coating the inner leaf of the viral envelope and taking part in envelope assembly. Viral envelope also consists of a lipid bilayer derived from the host cell membrane. The non-structural viral proteins, such as RNA/DNA polymerases and proteases, are coded by the viral genome, but are absent in virus particles [17]. Such a simple structure enables identification of viruses through two main ways, by determining the genetic material or by profiling proteins. For this reason, as in the case of bacteria, viruses are mainly identified through PCR analysis.

In the laboratory practice, quantitative PCR analysis is usually used for virus identification. The detection of amplified viral nucleic acids in quantitative PCR is performed with specific fluorescent probes comprising a small nucleotide sequence attached to the fluorescent indicator. The technique is highly sensitive but suffers from numerous drawbacks. Firstly, PCR analysis requires isolation of genetic material from the investigated microorganism with very high quality, as any impurities derived from the clinical sample (RNase or DNase, EDTA, phenol, urea, denatured albumin, prosthetic groups of hemoglobin, polysaccharides, etc.) can inhibit Taq polymerase and lead to false-negative results [18]. It is even more essential for the RNA-containing viruses, where identification begins with transformation of RNA to DNA by reverse transcription. It was shown that high concentration of viral RNA in the sample can inhibit reverse-transcription-PCR [19]. Even though it was possible to overcome the problem by simple sample dilution, this factor may not be taken into account by laboratory staff, especially in routine analysis. This could be the reason for the false-negative results of the SARS-CoV-2 in the patients with clinical symptoms of COVID-19 as it was shown by Li et al. [20]. The authors also suggest that the presence of a coinfection as well as improper specimen collection and transportation might have burdened the analysis. The statistics gathered in Beijing Haidian Section of Peking University Third

Hospital from 21 to 31 January 2020 shows that approximately 20% of tests were false negative [20]. The second important issue in PCR analysis is the selection of appropriate primers for the reaction. DNA polymerases can only add new nucleotides, which are called primers, to a DNA fragment that already exists. What is usually used as primers are the highly conserved regions of DNA. The design of appropriate primers is a painstaking work that often requires full genome sequencing with subsequent data processing, which may take several months [21]. Moreover, viruses – particularly the RNA-containing ones – can easily mutate, which may lead to changes in the matrix or even in conserved genome areas. Changes in the matrix sequence lead to mismatches with probe sequence and therefore to false-negative results in quantitative PCR [22], which often requires continuous probe adjustment [23]. The changes in conserved regions can reduce the template-primer binding affinity that contributes to low efficiency of the reaction [21]. Additionally, the high adaptive properties of viruses are a reason for their great genetic diversity, which may burden the analysis due to possible cross-reactivity among viruses with a close genome sequence. The sensitivity of the PCR analysis also makes it very vulnerable to cross-contamination, which can lead to false-positive results [21]. Sample contamination may occur at each stage of the investigation: during sample collection and transportation, at the stages of genetic material isolation, and post-PCR isolation by aerosol of a purified genome or PCR products. Finally, some virus infections may exhibit very similar symptoms (e.g. influenza and SARS), so for correct identification of the infectious agent, several parallel tests for each possible virus should be done.

PCR analysis can thus be considered as a labor-extensive and high-cost technique, which restricts its usefulness. For instance, identification of HIV infection with a PCR test is done only for newborns, as in these cases the alternative method detecting particular antibodies cannot be used due to the presence of maternal antibodies in an infant up to 18 months after birth. However, as with other RNA viruses, the quantitative PCR analysis for HIV produces many false-positive and false-negative results because of high genetic variability of the analyte and difficulties in designing good primers.

2.2. Immunochemistry techniques

Viral protein detection is the basis of such immunoassay tests as ELISA, immunoblotting, and immunofluorescence assay. It is based on the interaction of specific antibodies with virus proteins [24]. Antibodies are proteins produced by higher life forms as the response to the presence of foreign material (antigen), which can be viruses or their parts (e.g. proteins). A distinctive feature of an antibody is a specific binding to a particular antigen, which enables the former to identify the presence of antigen in the sample. The virtue of immunoassay analysis is that the specimen can be checked without any pretreatment, which makes it possible to perform such tests automatically and continuously. However, immunoassay analysis suffers from cross-reactivity, leading to false-positive results. Cross-reactivity is a possibility of antibody binding to molecules with similar epitopes (binding sites). The genetic modifications of epitopes, often observed in viruses, are the reason for false-negative results [25]. Although serological tests are considered easier to perform than PCR, in general they are less specific and their usage may be limited by the fact that antibodies appear later during the disease course. Moreover, the production of antibodies is a complex process, which among others involves immunization of the animals at the initial stage of assay, which excludes quick response to changes in the viral proteome. Additionally, high concentration of bilirubin or hemoglobin in the sample can also interfere with the analysis, as their absorbance

interferes with the optical detection system of some assays [25]. Finally, the immunoassay antibody needs to be prepared individually for each antigen (proteins in particular) [24].

2.3. LDI approaches

What has proven to be an alternative to molecular biology techniques is matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), a relatively new but very promising method of microorganism identification. The use of soft ionization makes it possible to record the characteristic molecular profiles of a microorganism (the so-called “fingerprint”), which can be compared with the spectra deposited in the database, thus enabling identification of the microorganism [26]. The main advantages of this technology include low cost, speed and simplicity of analysis (it requires minimal sample preparation) as well as the accuracy of the obtained results.

With MALDI-TOF MS, bacteria can be identified at the genus and species level, and sometimes down to the subspecies level. This technique enables the analysis of whole bacterial cells and their individual components [27]. Most often, identification of microbes occurs as a result of comparing their protein profiles with those recorded in a database, e.g. Bruker BioTyper, bioMérieux VITEK MS, and Andromas [13,27]. The simplest identification of bacteria using MALDI can be performed for intact microbial cells. However, protein extraction with formic acid is often carried out as well, which results in increased identification rates [28]. The development of easy-to-use, commercially available systems for bacterial identification by MALDI-TOF MS has become the standard of practice in many clinical microbiological laboratories around the world [27].

In addition, this technique also allows tracking changes in bacterial metabolism and detecting resistance to antibacterial agents such as antibiotics – e.g. a novel method of phenotypic antimicrobial susceptibility testing (AST) called direct-on-target microdroplet growth assay (DOT-MGA) [29]. The MALDI-TOF MS technique was also shown to be able to distinguish e.g. *Sindbis virus* [30] and influenza virus [31] by proteotyping. The proteotyping of all the mentioned viruses was performed with detection of peptide fingerprints of tryptic digests of the whole virus. In the case of influenza virus it was possible to differentiate the H1N1 strain unique for the vaccine from seasonal type A (H1N1) strains, showing high sensitivity of the method in virus typing and subtyping [31]. The study of *Sindbis virus* showed that 5-min tryptic digestion is enough to obtain specific peptide fingerprint spectra [30]. However, what is even more interesting is the possibility to apply the existing systems used in clinical practice for pathogen identification in viral discrimination. Musaji et al. demonstrated that the influenza virus can be detected by subjecting the intact virus to MALDI-TOF MS [32]. The authors used the spectra of corresponding neuraminidase and hemagglutinin in proteins as well as the entire influenza virus to make a comparison. The advantage of MALDI-TOF MS in virus identification may also be that laboratories can quickly adjust to the appearance of new viral strains, as the reagents are the same for all investigations. Moreover, changes in databases can be made on-line without any delay. In contrast, the PCR as well as immunoassay analyses require the presence of specific reagents (primers and antibodies), which prevents a quick reaction to the appearance of new viruses or new virus mutations. However, MALDI-TOF MS is successfully utilized in MassARRAY analysis, which is the substitution of fluorescent probes in genotyping, and makes it possible to overcome the problem of mismatch. MassARRAY can also be used for monitoring mutations. Still, utilization of MALDI-TOF MS for virus investigation is less reported in the literature, so this matter requires further investigation. Unfortunately, this method also has many limitations. One

of the greatest problems is the insufficient number of spectra deposited in commercial databases as the quality of reference databases is a basic condition for correct identification of microbial isolates. Currently, numerous works are underway to supplement the existing databases with new spectra obtained for pathogens accurately identified by other techniques, in particular rare bacterial species [33]. Many research groups also create their own databases that ensure quick and accurate identification of microbial species of interest [34]. Other disadvantages of MALDI-TOF MS include the fact that in order to obtain a good quality spectrum it is necessary to have clean or well isolated colonies and a relatively high concentration of cells in the sample (10^5 – 10^9 cells per spot), which lengthens the whole process due to the necessity of including the culturing step [35]. In addition, the composition of the culture medium and the type of the matrix used during the analysis may affect the identification efficiency [35]. Like molecular biology methods, MALDI may not be able to properly distinguish the spectra of some closely related bacterial species, which can lead to their misidentification. Such a problem concerns e.g. differentiation of *Shigella* species from *Escherichia coli* [36]. Regarding identification of viruses, the crucial limiting factors – besides poor spectral libraries – are the relatively low protein content and higher molecular weight of viral proteins [37]. Moreover, viruses are also known to be difficult to multiply even under ideal conditions.

Nevertheless, scientists predict that MALDI-TOF technique will quickly become a standard tool for routine identification in virology labs – as soon as researchers are able to overcome the major obstacles related to inconsistencies surrounding the extraction protocols or to low reproducibility of the results due to the matrix effect in the direct detection mode [38]. In the face of the current COVID-19 pandemic, pathogen identification via MALDI-TOF technique is proposed as an easy-to-use and robust approach for large-scale SARS-CoV-2 testing in developing countries which lack the necessary laboratory resources and access to PCR kits. Nachtigall et al. proved that MALDI-MS can be used to reliably detect SARS-CoV-2 in nasal swab samples if appropriate machine learning analysis (e.g. decision tree, DT; k-nearest neighbors, KNN; or support vector machine with a radial kernel, SVM-R) is applied [39]. The authors found a concordance level acceptable in clinical diagnostics between results obtained using routine RT-PCR and the proposed MALDI-ML approach (>80%). The study used nasal swab samples without prior purification; therefore, the proposed approach could be used in a follow-up confirmation test using the RT-PCR gold standard. In turn, Gould et al. suggested that MALDI-TOF MS technique also shows high potential in the identification of viral strains within breath samples since its coupling with electrospray ionization (ESI) allows analysis of such big molecules as DNA or RNA [37]. Since it is possible to detect viral RNA in tidal breath samples [40], ESI-MALDI-MS technique offers an ideal solution to rapid and non-invasive detection of viral infections, potentially also SARS-CoV-2.

In addition to identifying bacteria based on specific protein profiles, the MALDI technique also offers identification based on the analysis of profiles of other cellular components such as lipids or nucleic acids. Currently, a large increase in interest can be observed over the use of bacterial cell lipid profile analysis for taxonomic identification [41]. This approach allowed the differentiation of *Bacillus* and *Brevibacillus*, with the average correct identification rate of 62.23% [42]. Similarly, lipid fingerprinting enabled differentiation of the bacteria of *Bacillus* spp., difficult to distinguish by conventional techniques [43]. There have also been several reports on the use of the MALDI technique for bacterial DNA and RNA analysis [44]. MALDI mass spectrometry was used to detect PCR products obtained from *Legionella* after rapid purification to

remove salts and unreacted primers [45]. In addition, MALDI technique allowed the analysis of posttranscriptional modifications of bacterial RNA.

An innovative strategy able to improve the analysis of pathogens may be application of nanotechnology-assisted laser desorption/ionization time-of flight mass spectrometry (NALDI-TOF MS). This technique replaces traditional organic matrices with a nanostructured silicon-based target plate [46]. The main role of nanoparticles in this method is to enrich analyte particles and enable their efficient desorption and ionization [47]. For this purpose, nanomaterials with porous structure, large surface area, easy functionalization, high heat transfer efficiency and photo-absorption properties are highly preferred. Metal nanoparticles as well as metal oxide nanoparticles were found to efficiently transfer heat to the analyte molecules under laser irradiation, which favors more effective ionization. Other nanomaterials useful in NALDI are silicon based ones, particularly porous structure materials, which are very useful in enriching analyte molecules from the sample [47]. The virtue of NALDI systems is their strong hydrophobic surface, which may enhance the ionization of hydrophobic peptides and proteins from the sample [48]. Current reports indicate that this method can be successfully used to analyze a wide range of organic particles such as lipids or proteins, while the lack of need for organic matrices eliminates uneven distribution and different sizes of crystals as well as background peaks in the low mass range; it also has a positive impact on the reproducibility of analyses [46,47]. Structural proteins in viruses often contain hydrophobic regions, which help maintain protein-protein association [49]. Thus the NALDI approach may be helpful in their investigation.

2.4. Electromigration approach

Another group of analytical techniques which can find an application in microbial diagnostics are electromigration techniques. In recent years the use of capillary electrophoretic (CE) techniques such as capillary zone electrophoresis (CZE), capillary isoelectric focusing (CIEF), or isotachopheresis (ITP) for separation, characterization and identification of the wide range of microorganism types has been the focus of growing attention. In comparison with GC and LC, electromigration techniques demonstrate better separation efficiencies, the need for very low volumes of sample and reagents, as well as the ability to separate cations, anions and uncharged molecules in a single run. The main advantage of electromigration techniques is the possibility to exploit several microbial parameters – size, shape and charge – which are very advantageous to their separation, characterization and identification. This set of analytical techniques also offers many advantages, such as very short analysis time or the possibility of direct analysis of biological samples [50,51]. The literature provides several examples of CE used for determination of bacterial pathogens [52,53], yeast cells [54], various types of human and plant viruses [55] or even bacteriophages [56]. The separation of pathogens is possible on the basis of the difference in their electrophoretic mobilities or isoelectric points (pI).

The first report on the use of capillary electrophoresis for pathogen determination was published by Hjerten et al., in 1987; they described the migration of *Tobacco mosaic virus* and *Lactobacillus casei* bacteria in 20 mM Tris-HCl buffer [55]. In 1993, Ebersole and McCormick separated four bacterial species in TBE buffer and proved by collecting individual fractions after the electrophoresis process that most bacteria (80%) are alive [57]. A year later, Torimura published a paper on the electrophoretic behavior of nine bacterial species, determining their electrophoretic mobility [58]. In the 1990s, Pfetsch and Welsch [59] as well as Glynn [60] determined the electrophoretic mobility of bacteria in various buffer

solutions. The pioneering work of Buszewski and Kłodzińska showed that capillary zone electrophoresis is an effective tool for identification of *Staphylococcus aureus* and *Escherichia coli* as well as *Proteus vulgaris* [52]. Another paper of Buszewski and coworkers presented the clinical use of a fast screening test based on CZE analysis for identification of *E. coli* infection in biological samples such as infected wounds and ulcerations [53]. Kłodzińska et al. applied CZE for identification of *E. coli* and *Helicobacter pylori* in urine samples [61].

Although this analytical method provides many solutions in the field of pathogen identification, it is important to remember its limitations and drawbacks such as uncontrolled aggregation (clumping) of bacterial cells and their adhesion to the capillary surface [62,63]. This can also be a source of capillary clogging if aggregation occurs to a large extent. Moreover, factors such as pH, osmolarity or high electric fields can also affect the analysis of bacterial cells by CE [64]. According to the electrokinetic theory, microorganisms are often considered as biocolloids, mainly due to the complex structure of their cell wall and protonation equilibria formed at the interface; for such a system, understanding the electrophoretic process is more complicated. The cell wall composition is specific for individual types of bacterial species – they have different content of proteins, phospholipids, polysaccharides or another organic components [65]. The wall structure also determines the division of bacteria into two main types: Gram(+) and Gram(–). All compounds present in the bacterial cell wall structure strongly affect the surface charge of microorganisms. This can be explained by the presence of many functional groups undergoing the protonation process. Therefore, it is important to develop a method that minimalizes the uncontrolled aggregation and adhesion problem. Some research groups proposed the addition of poly(ethylene oxide) (PEO) to the buffer solution [63,66] which functioned as a focusing agent. Kłodzińska et al. identified the *E. coli* and *H. pylori* in human urine by using PEO in the CE analysis [61]. Another approach is capillary surface modification e.g. by divinylbenzene (DVB) or preparation of new types of materials for bio-separation [52,66]. Moreover, Yu and Li emphasized the importance of proper preparation of bacteria samples – the vortex and sonication processes seem to be crucial in decreasing the aggregation level of microorganisms [67]. However, over past years a new method based on microbial surface modification by divalent metal ions was suggested [54,62]. Application of this method may result in controlled aggregation of microorganism's cells. Rogowska et al. investigated the impact of the cell surface modification by Ca^{2+} ions on the control clumping of *Saccharomyces cerevisiae*, and the results of this study pointed out that during the CE analysis of cells modified by 5 mM Ca^{2+} , and with the increasing value of medium pH, the sharpening of the peaks and the reduction in the number of *S. cerevisiae* aggregates were observed [54]. This phenomenon was correlated with the role played by calcium ions in the controlled clumping of yeast cells by binding surface-carboxyl groups or lectin-like mechanisms of flocculation, in which Ca^{2+} enables the lectins to acquire a suitable active conformation and thus interact with carbohydrate residues of α -mannans present on the cell surface [68]. Moreover, results obtained by Rogowska et al. indicate that the proposed new sample preparation approach for wild microorganism strains may become in the future a foundation for the application of capillary electrophoresis in diagnostic laboratories [54]. Different pH and modification of the cells by Ca^{2+} influence the molecular profiles of yeast cells but do not affect the identification quality of MALDI-TOF MS equipped with the BioTyper database. Such data may provide a foundation for coupling capillary electrophoresis and the MALDI-TOF MS analysis. Another example can be evaluation of the Zn^{2+} aggregation activity on probiotic strains. Studies conducted by

Buszewski et al. described the electrophoretic determination of *Lactococcus lactis* modified by zinc ions at different concentration levels [69]. The referenced study shows the formation of microbial aggregates after the surface modification and, what is more, proposes the possible mechanism of this process.

2.5. The use of CE in modern hyphenated techniques approach

Due to very high demand for SARS-Cov-2 identification, alternatives to the standard PCR tests are potentially useful for increasing the number of samples screened. Gómez et al. describe a technical approach to SARS-Cov-2 testing by amplifying fragments of the viral genome with 5'-fluorescent primers followed by capillary electrophoresis [70]. The proposed method allowed the analysis of 96 samples in approximately 5 h. The two SARS-Cov-2 fragments were successfully amplified in the positive samples, while the negative samples did not render fluorescent peaks. The researchers validated 20 virus positive and 10 virus negative samples. The aim of that study was not to substitute the gold PCR technique, but to develop an alternative to facilitate the analysis when the demand for COVID-19 testing exceeds the capacity of the PCR of any lab that can implement the CE technique [70]. Already in 2008, Buszewski et al. proposed using capillary electrophoresis, PCR and physiological assays in differentiation of clinical strains of bacteria. The electrophoretic measurements involved the differential mobility of bacteria in a fused silica capillary under the direct current electric field. To perform coagulase gene typing, the repeated units encoding hypervariable regions of the *S. aureus* gene are amplified using the PCR technique, by restriction enzyme digestion, then followed by the analysis of restriction fragment length polymorphism patterns as well as sequencing. Finally, the results of electrophoretic measurements were compared with molecular analysis [71]. The same group utilized CE, zeta potential and coagulase gene polymorphism as a very specific combination for differentiation of the same species of clinical bacterial strains. The data presented in this contribution suggested that electrophoretic behavior and the values of zeta potential should be very useful in distinguishing between closely related strains which exhibited coagulase gene/protein polymorphism [72]. Coupling standard PCR or molecular biology methods with capillary electrophoresis seems to be a very powerful tool to overcome the limits imposed on many labs by the PCR requirements and thus increase the testing capacity.

Nowadays, also the lab-on-a-chip (LOC) technology has demonstrated its superior capability of rapid determination of pathogens. This type of technique combination is characterized by short analysis time, low sample and power consumption, high integration, high throughput and portability; thus it shows great potential in the areas of genetics, proteomic and cellular research. For example, it has been proven that a number of genetic analysis steps such as sample purification, nucleic acid amplification and amplicon separation can be done on microfluidic devices. Polymerase chain reaction–capillary electrophoresis microdevice for pathogen detection is another method which can be used for sample separation and purification. The combination of the polymerase chain reaction with microcapillary electrophoresis (mCE) has shown excellent performance in terms of sensitivity, speed and specificity. A fast static PCR reaction was performed in a nanoliter scale chamber and the resultant PCR amplicons were subsequently separated in the CE microchannel. Kim and coworkers utilized a capillary electrophoresis-based multiplex PCR (CEMP) panel to enable the detection of viral and bacterial pathogens associated with community-acquired pneumonia (CAP) [73]. They simultaneously determined 13 common unculturable CAP viral and bacterial pathogens within 4 h. They also evaluated the performance of

a commercially available panel with 314 samples collected from CAP patients. Finally, the authors compared the results to those obtained with the liquid chip-based Luminex xTAG Respiratory Viral Panel (RVP) Fast Kit (for viruses) and the agarose gel-based Seegene PneumoBacter ACE Detection Kit (for atypical bacteria). All positive samples were further verified by the Sanger sequencing method. The proposed procedure provides a rapid and accurate method for high-throughput detection of pathogens in patients with CAP [74].

Another significant and useful analytical solution is capillary electrophoresis coupled to MALDI mass spectrometry. While CE-MALDI interface has been explored by numerous groups in a variety of contexts, the coupling with MSI is a relatively recent and innovative technique. The increased number of identifications in biological samples is largely attributed to improved separation resolution. Other methods often couple CE with MALDI using fraction collection; however, by continuously collecting liquid (containing bacterial and/or viral cells) from the capillary onto the plate and performing MS imaging, the resolution can be dramatically expanded. The research presented by Delaney used larger injection volumes and enhanced separation resolution with a positively-charged capillary coating that reduces peptide adsorption and reverses electroosmotic flow (EOF) [75]. With this method, an increase in the number of detected neuropeptides in two tissue sample types was obtained with good reproducibility. Other studies presented by Horká et al. involved nano-etched fused-silica capillary used for on-line preconcentration and electrophoretic separation of bacteriophages from large blood sample volumes with off-line MALDI-TOF mass spectrometry [56]. This is very important because bacteriophages have a great potential for developing specific diagnostic and therapeutic tools against bacterial infections. The phages that exhibit a selective lytic effect towards bacterial cells can be applied directly into living tissues. The authors monitored staphylococcal phages using capillary electrophoretic methods on fused-silica capillaries with different morphology of surface roughness. After electrophoretic analysis, viability of the detected phages was verified by the modified “double-layer drop assay” method, and collected phage fractions were simultaneously analyzed off-line by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

Small viruses have diameters in the range of tens of nanometers. Bacteria are generally larger, some by a factor of 100, and the larger size leads to increased complexity. The microbial surface charge originates from the ionization of surface molecules and the adsorption of ions from solution. Bacterial cell walls and membranes contain numerous proteins, lipid molecules, teichoic acids, and lipopolysaccharides that contribute to this characteristic charge. The undesired effects occurring during electrophoretic analysis such as aggregation and/or adhesion of the cells have a strong effect on the separation process. For a better understanding of this phenomenon, Buszewski and coworkers decided to apply a fluorescence stereomicroscope as an in-line detection unit for observation of electrophoresis of microorganisms. It was the first such in-line combination of CE and fluorescence stereomicroscope which allowed direct observation of migrating zones of bacterial cells and determining the viability of these cells [76]. Taking into account the mechanism of bacterial migration in the direct current electric field the knowledge of the electrical properties of microorganisms is of benefit for the biofilms electrokinetic formation – aggregates with a defined structure. It also plays a very important function in detection of physical changes of bacterial cells and is emerging as a modern diagnostic tool to determine their phenotypical features. One of such changes is bacterial viability. It is widely known that when the cell dies, the membrane potential breaks down and the viable cells are more voluminous. These

changes are directly associated with alteration in zeta potential for particular bacterial species, strains or even viable or dead bacteria. In the case of medical diagnosis and pharmaceutical application, an analytical method capable of detecting viable or dead cells with a simple sample preparation procedure is also highly desirable. Zeta potential measurements are helpful in understanding the electrophoretic separation behavior and provide more information about the charge which is present on the cell surface of the examined bacteria. The electrophoretic data, the zeta potential measurements and microscope observations were compared and found to be consistent. Furthermore, the use of unique and very precise CE-NMR coupling helped to estimate which characteristic functional groups present on the surface of microbial cells play a key role in electrophoretic separation. The NMR spectrum represents all functional groups in a sample, and functional groups on the surface of the particle are not identified separately. On the other hand, the FTIR and XPS spectra obtained for microorganisms identified the impact of functional groups on their properties [77].

The separation of bacterial cells might be a basis of fast capillary electrophoretic cell sorting (fractionation). Here, any practical applicability of such a method would be directly connected with the viability of the cells which, after being electrophoretically sorted, could be further cultivated or directly examined by detection methods of choice. Capillary electrophoresis was used, for example, for the determination of bacteria viability in pharmaceutical products or as sterility test in food samples. The bacteria were stained with live/dead fluorescent dye test solutions before the electrophoretic process. Then with a fluorescence detector it was possible to assess the ratio of living and dead cells in the preparation, which corresponded to its quality. However, a much more sensitive method involving a flow cytometer (FC) to determine the viability of microbial cells can be used as well. Salzano et al. performed analysis by cytometry and capillary electrophoresis in ethanol-stressed *Oenococcus oeni* strains and assessed changes of membrane fatty acids composition. As malolactic bacteria play an essential role in wine production methods that use malolactic fermentation (MLF), efficient control of these microbiological processes requires increased knowledge of bacterial behavior under stress conditions that can affect bacterial viability and activities. Salzano et al. assessed the different physiological states of *O. oeni* strains by nucleic-acid double-staining flow cytometry assay, which allowed the researchers to differentiate between viable and damaged/membrane-compromised (dead) cells of *O. oeni* strains, and to study ethanol-induced variations of cell membrane fluidity and permeability with the application of capillary electrophoresis to evaluate the changes in the charging rates on cell surface [78].

3. Sample preparation techniques for pathogen determination

Another scientific challenge is pre-separation and pre-concentration of samples, particularly when pathogens are uncultured and their concentration in the matrix is low. According to the literature data [79], electrophoretic approach might be promising for both pre-concentration and subsequent separation of pathogens according to their properties. Furthermore, adhesion of bio-colloids such as microorganisms onto the inner capillary surface can also be used for their concentration and separation from large sample volumes of high conductivity matrices. Thus techniques such as capillary zone electrophoresis (CZE) [80], isoelectric focusing (CIEF) [81] and isotachopheresis (CITP) [82] deserve researchers' attention. For instance, CIEF is a great tool for separation of amphoteric analytes due to differences in their isoelectric points (pI); it also provides an opportunity to increase sample concentration in a single step [79]. Horká et al. used isoelectric focusing for

analysis of three microbial strains (*Micrococcus luteus*, *Dietzia* sp., and *Rhodotorula mucilaginosa*) [79]. The pre-separation and pre-concentration of microbial cells was possible due to cellulose-based separation medium. Then the collected fractions of cells were used for the MALDI-TOF MS analysis. Another work of Horká et al. presents the combination of capillary electrophoresis with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for the analysis of methicillin resistant *S. aureus* (MRSA) and *Pseudomonas aeruginosa* [83]. Bacterial strains were pre-concentrated from the rough part of the capillary surface with the use of isotachophoretic stacking, collected from the capillary, and then off-line analyzed by MALDI-TOF-MS. Thus the coupling of electrophoretic methods with matrix-assisted laser desorption ionization mass spectrometry seems to be pivotal for sample preparation and microbial analysis (Fig. 2).

Electromigration techniques can also be used when it comes to viruses. In 1987 Hjertén et al. published the first paper demonstrating the possibility of analysing virus samples with capillary zone electrophoresis [55]. They recorded a single, sharp peak of *Tobacco mosaic virus*. Schnabel et al. were the first to apply CIEF to determine the pI value of human rhinovirus serotype 2 – HRV2 [84]. Both of these techniques can be also used in pre-separation and pre-concentration of virus samples from culture media. Horká et al. examined the conditions for simultaneous separation and detection of phage K1/420 and *S. aureus* by CZE and CIEF [85]. First, they purified and pre-concentrated the bacteriophage from the matrix using preparative IEF – the harvested fraction of the phage was pre-concentrated approximately ten times. In the next step the authors detected separated phage particles in the presence of its host, *S. aureus*, with the use of CZE and MALDI-TOF MS.

Pre-separation and pre-concentration is particularly important in the case of biological samples, which are usually available in small volumes and have low concentration of the analyte in biological matrices. The team led by Horká conducted an experiment in a real environment represented by whole human blood and human plasma [56]. In the test they used five different staphylococcal phages, which were separated and pre-concentrated with

the use of CE techniques. CIEF allowed determination of pI values of the examined phages while micellar electrokinetic chromatography (MEKC) made it possible to choose the proper type of capillaries for separation of the phage mixture. The researchers then used off-line combination of MEKC in an FS capillary with a roughened part, and MALDI-TOF MS analysis of the collected fractions. A plasma sample was spiked with *S. aureus* and phage K1/420. The MALDI results showed that the bacteriophage was effectively separated from the remaining components of the sample. The authors also amplified phage K1/420 on the host cell directly in the MEKC capillary, which allowed them to pre-concentrate bacteriophages from very low initial concentrations. It is possible to increase concentration of the phages even more by performing two-step propagation [86]. In this process, both the phages adhered on the capillary and the phages generated by the propagation interacted again with the new host cells.

Field-flow fractionation (FFF) is another method which can be used for sample separation and purification. It is particularly suited to samples of biotechnological interest, where analytes range from macromolecules such as proteins or nucleic acids to micron-sized particles such as bacteria, viruses and whole cells. This technique is similar to LC but has no stationary phase, which makes it possible to avoid unwanted interactions of analytes with the phase. FFF relies on the interaction of analytes with an externally generated field, which is applied perpendicularly to the direction of the mobile phase flow. Due to differences in their density, size or surface properties, which result in different retention times, the field drives the analytes into different laminar flows [13,87]. The separation of viruses has been successfully achieved by the use of e.g. sedimentation FFF (which uses a sedimentation field generated by a centrifuge) and asymmetrical flow FFF.

After pre-concentration or separation, a sample can be identified by laser desorption/ionization techniques (LDI), such as the MALDI, NALDI, SELDI (surface enhanced laser desorption ionization) and DIOS (desorption/ionization on silicon) (Fig. 3). LDI methods allow analyzing many biomolecules, but they are rarely applied to the detection of viruses in complex biological samples.

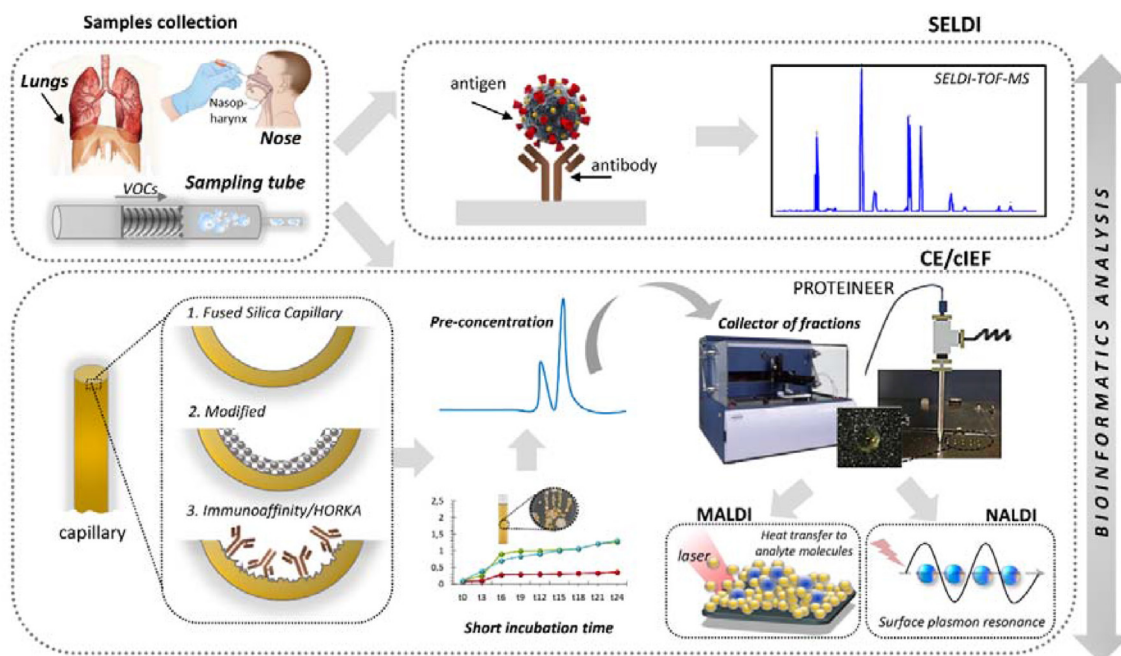


Fig. 2. Coupling electromigration techniques with laser desorption/ionization approach in virus analysis.

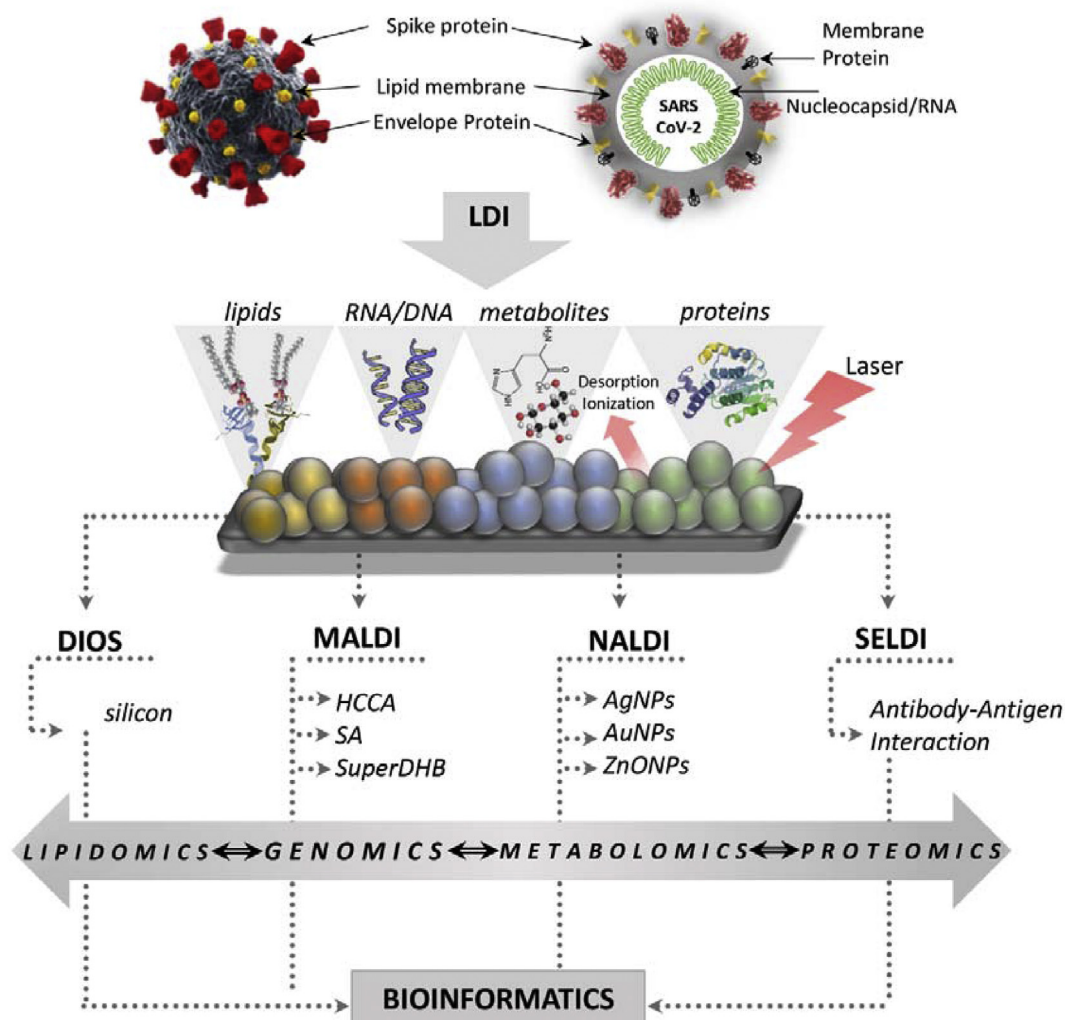


Fig. 3. Laser desorption/ionization analysis of virus molecular compounds.

MALDI is the most common of LDI methods and has been already used to investigate a wide variety of viruses, also from clinical samples. This method was applied to identify for instance polioviruses [88], viruses isolated from respiratory tract samples [89], human *Papillomavirus* [90], and SARS-CoV-2 [39]. A similar technique is SELDI, which was used by Golizeh et al. to compare serum proteome profiles at different stages of fibrosis in HCV and HIV/HCV infected patients [91]. Table 1 summarizes virus analyses by approach to pathogen identification.

4. Future directions in identification of microbial pathogens

The last decades saw the emergence of many modern techniques for determination and identification of microbial samples. Despite their limitations, culture and microscopy are still two of the staple techniques. PCR and other genetic approaches are particularly significant in case of non-culturable pathogens. MS has been shown to be a useful, quick and easy approach to identification of microbial samples and detection of microbial threats; however, it is reserved for pure isolates and cannot be used for complex samples since they may promote background interference. This may be circumvented through the use of separation-based methods, such as chromatography – HPLC, LC-MS or field flow fractionation (FFF). In the future, determining limits of detection for pathogens will

continue to be a key goal of clinical microbiology. Combination of the above (and possibly other) methodologies and instrumentation will surely improve the pathogen detection capability.

One of the most promising options is the lab-on-a-chip technology that can perform various laboratory operations on a miniaturized scale. Miniaturization of the devices such as A4F separation canal or LDI targets allows reduction of reagents, which results in reduction of analysis costs. Another important advantage is speed – by using chips it is possible to obtain simultaneously a multiplicity of parameters. What is more, this technology allows automation and high-throughput screening. However, it is mainly used for research purposes. Its usefulness for routine diagnostics is limited due to relatively high costs of equipment and insufficient standardization, which results in low reproducibility of the results. In the case of microarrays, the type of molecules immobilized in the array affects their application – DNA microarrays can be applied to testing for DNA from pathogenic organisms or to resequencing of a pathogen, antibody microarrays can detect pathogen proteins or antigens present in clinical or environmental samples, and small-molecule microarrays offer novel approaches for differentiating between pathogens. There are several texts in the literature describing the application of microarrays to detection of pathogens, such as viruses. Fig. 4 presents future perspectives of the lab-on-a-chip.

Table 1
Summary of virus analyses by approach to pathogen identification (n/a - not available).

Virus	Sample source	Analytical Method	No. of samples	Reference
HBV, HCV	serum	PCR	730	[92]
Enteric viruses	stool samples	monoplex or multiplex RT-PCR	227	[93]
HRSV	nasopharyngeal and oropharyngeal swab	real-time RT-PCR	334	[94]
DENV, ZIKV	serum	RT-qPCR	46	[95]
Respiratory viruses	throat swabs and sputum samples	RT-qPCR	408	[96]
Respiratory viruses	nasopharyngeal aspirates, nasopharyngeal and oral pharyngeal swab	rRT-PCR	8173	[97]
SARS-CoV-2	sputum, nose and throat swabs	real-time RT-PCR	297	[98]
LASV	serum, lung and spleen tissue, blood	RT-qPCR	82	[99]
Respiratory viruses	respiratory tract swabs and aspirates	MALDI-TOF MS	58	[89]
HR HPV	uterine cervix cytology	MALDI-TOF MS	356	[90]
Poliovirus	cerebrospinal fluid, throat swab, stool samples	MALDI-TOF MS	5	[88]
SARS-CoV-2	nasal swab	MALDI-MS + machine learning analysis	362	[39]
Enterovirus EV71	serum	LDI-MS with cellulose acetate membranę and Ag or Au NPs	n/a	[100]
HIV, HCV	serum	SELDI MS	151	[91]
Bacteriophages				
Triaviruses, Phietaoviruses, Biseptimaviruses, Kayviruses, Twortvirus, P68virus	reference and isolates	MALDI-TOF MS	n/a	[101]
vB-SdyS-ISF003	wastewater and sewage	PCR and sequencing DNA	n/a	[102]
Kayvirus K1/420	medicamento isolate	CZE, MALDI-TOF MS	n/a	[85]
Staphylococcal phages (K1/420, 11, P68)	physiological saline solution, human serum	MALDI-TOF MS	n/a	[86]
Staphylococcal phages (K1/420, 11, P68, 3A, 77)	blood, serum	MALDI-TOF MS	n/a	[56]

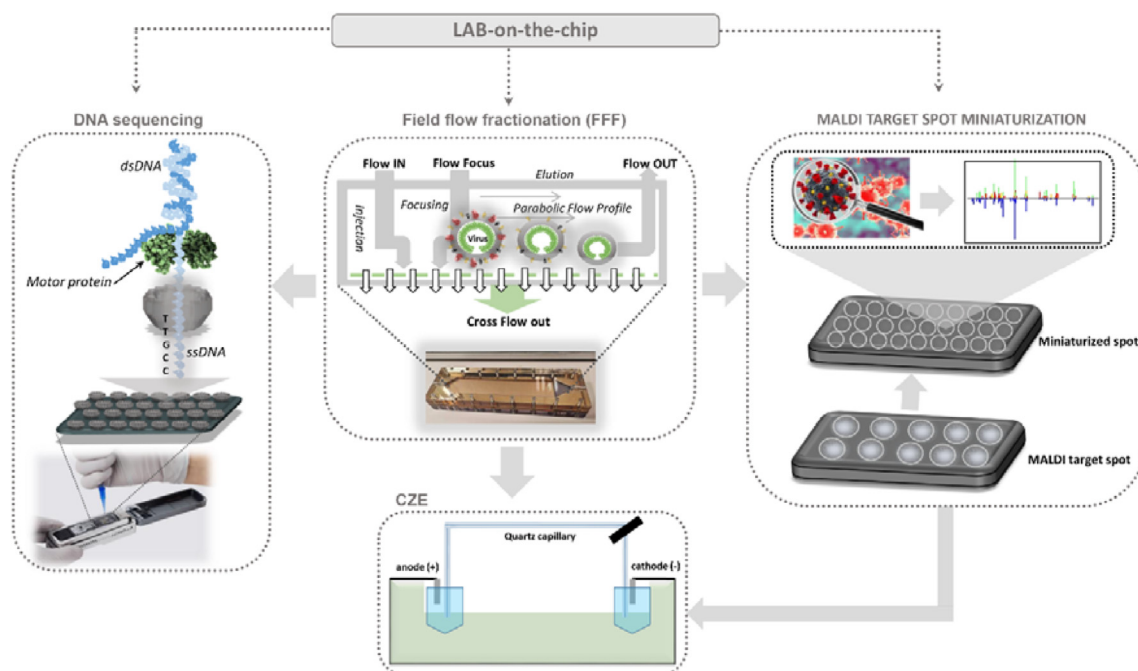


Fig. 4. Future perspectives of lab-on-a-chip.

The development of new, effective methods of microbial identification should be based on a modern interdisciplinary approach. In order to improve microbial identification, pathogens must be characterized comprehensively. A very important step in microorganism identification is the selection of culture conditions for clinically relevant pathogens as well as virus samples (e.g. *tobacco mosaic virus*, *hepatitis virus* or clinical isolates of CoVID-19). The optimization of virus growing should be performed on the models of viruses, e.g. *Tobacco mosaic virus* cultivated on the plant leaves or *hepatitis virus* cultivated on commercially available hepatocyte cells. After these procedures, the isolated pathogens can be pre-

concentrated by using electromigration techniques. For this step capillary zone electrophoresis (CZE) and capillary isoelectric focusing (CIEF) can be used in different capillary coatings modes. Another possibility is the use of optical and fluorescence microscopy and flow cytometry to verify the effectiveness of the method in the case of bacteria. Matrix-assisted laser desorption/ionization technique is the latest generation tool used for rapid identification and classification of microorganisms; it is characterized by high precision, low sample consumption, short analysis time and relatively low unit cost. Results obtained in this way will be compared with the results of sequencing of the conservative 16S rDNA gene,

which is now considered to be the gold standard in microorganism identification. Nowadays the nanostructure-assisted laser desorption/ionization (NALDI) approach complements the classic MALDI in the field of modern bioanalytics.

Coupling sample treatment devices to commercial CE systems is of special interest with regard to developing in vivo analysis, facilitated by the ability of electrophoretic systems to use small volumes of sample and provide rapid analyses. For example, a microdialysis needle on-line coupled to a commercial CE system enabled in vivo monitoring of the evolution of a drug during brain tissue analysis. Miniaturization of sample treatment devices will undoubtedly expand the potential of this combination further. As to interfacing non-integrated detection modes with commercially available CE equipment, the properties of a specific analyte dictate which method is best suited to its detection. For example, with analytes containing fluorophore/electroactive groups, fluorescence/electrochemical detections can be an appropriate choice, made with a view not only to enhancing sensitivity but also to improving the apparent selectivity through specificity. CE-PCR, CE-MALDI TOF MS, CE-fluorescence stereomicroscope, CE combination with flow cytometry, as well as very specific CE-NMR combinations seem to be very promising tools that can supplement the standard methods used in medical diagnostics, especially in the face of the present epidemiological threat. Additionally, these unique combinations make it possible to explain the phenomena occurring during electrophoresis of microorganisms and elucidate its full mechanism. It should be taken into account that the use of MALDI MS coupled with electromigration techniques is a very selective method of detection and constitutes a type of imaging. It is also very important to fully learn the mechanism of the separation process itself as well as the mutual interactions between the cells. Moreover, the 2020 pandemic has very clearly demonstrated the need for further developments in this field of analytics.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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