Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry applied to virus identification

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Virus detection and/or identification traditionally rely on methods based on cell culture, electron microscopy and antigen or nucleic acid detection. These techniques are good, but often expensive and/or time-consuming; furthermore, they not always lead to virus identification at the species and/or type level. In this study, Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) was tested as an innovative tool to identify human polioviruses and to identify specific viral protein biomarkers in infected cells. The results revealed MALDI-TOF MS to be an effective and inexpensive tool for the identification of the three poliovirus serotypes. The method was firstly applied to Sabin reference strains, and then to isolates from different clinical samples, highlighting its value as a time-saving, sensitive and specific technique when compared to the gold standard neutralization assay and casting new light on its possible application to virus detection and/or identification.

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) is a high throughput technology based on the comparison of the protein fingerprint obtained by microbial cells with a database of reference spectra by means of the use of various algorithms integrated in systems recently made commercially available. In the last few years this tool has been increasingly studied and applied for the identification and typing of microorganisms1–4. MALDI-TOF MS is referred to as a “soft” ionization technique, because it causes minimal or no fragmentation and allows the molecular ions of analytes to be identified, even in complex mixtures of biopolymers3–8.

Many studies investigating the application of MALDI-TOF MS in clinical microbiology laboratories or addressing its use in experimental approaches related to bacteria and fungi have been published; however, very few studies have been conducted about its application to virus research and diagnostics8–12. This could be a consequence of the high molecular weight of the viral proteins (> 20 KDa); in microbiology diagnosis the identification is based on a low molecular weight range (2–20 KDa), where the majority of the peaks detected corresponds to microorganism ribosomal proteins8. Moreover, while for bacteria there is a simple and standardized protocol of protein extraction8, for viruses, requiring a cell substrate to be in vitro cultivated, the identification of specific viral proteins can be affected by cellular debris14.

Traditionally, the gold standard method for the direct detection of viruses is cell culture, although the use of this method often requires several days or weeks before the results can be obtained. Electron microscopy and direct antigen detection methods based on enzyme immunoassay or immunofluorescence are widely used for virus detection and/or identification, although some of these techniques are less sensitive than cell culture14. Finally, generic amplification techniques based on the polymerase chain reaction (PCR) provide a rapid and sensitive alternative for virus detection and/or identification15.

Considering the well-founded ability of MALDI-TOF MS to determine the molecular weight of individual specific polypeptides, the aim of the present study was to develop a simple method exploiting the MALDI-TOF MS platform application for the identification of viruses and the detection of specific viral biomarkers in order to obtain a profile of identification useful to differentiate between virus-infected and uninfected cells.

We focused on three members of the Picornaviridae family, Enterovirus genus, Enterovirus C species (www.ictvdb.org), that includes poliovirus serotypes 1, 2 and 316.
Poliovirus serotype presents slightly different capsid proteins, conferring cellular receptor specificity and virus antigenicity. Poliovirus type 1 is the most common serotype encountered in nature; however, all the three serotypes are extremely infectious and dangerous, being the most frequent causative agents of poliomyelitis.

The choice of poliovirus as an experimental model was firstly based on the relevance of this viral agent in causing one of the most severe human pathologies of the central nervous system and on the importance in the globalization context: thus, their efficient identification and, most importantly, their eradication represent one of the main goals for the World Health Organization.

Furthermore, poliovirus provides an ideal experimental viral model, considering its very simple structure: the virion is composed by a non-enveloped icosahedral protein coat built up of 60 copies of 4 structural proteins (VP4, VP2, VP3, VP1, listed as mapped in the viral genome) and a viral genome-linked protein, VPg, covalently attached to the 5′ end of viral RNA, which acts as a primer during RNA synthesis.

**Results**

**MALDI-TOF MS analysis of type 1, 2 and 3 Sabin poliovirus strains.** In order to develop a simple method exploiting the MALDI-TOF MS platform application in the virologic field we used poliovirus type 1, 2 and 3 Sabin reference strains and LLC-MK2 confluent cell monolayers (Rhesus monkey kidney epithelial cell line LLC-MK2; ATCC CCL-7) for their cultivation and titration, using the limit dilution method ("tissue culture infectious dose50", "TCID50"); the experimental infections were performed using a multiplicity of infection of 0.01 TCID50/cell.

LLC-MK2 infected cells and the respective culture media were harvested when a robust cytopathic effect was clearly observable and the recovered viral particles were subjected to a linear sucrose gradient.

The gradient profile showed a peak that spans through fractions 2 to 6, at the expected gradient region (Supplementary Fig. 1a). These fractions were pooled and subjected to ultracentrifugation. Poliovirus concentration, morphology and purity were confirmed by electron microscopy analysis after negative staining, that also enabled us to exclude any evident cellular contamination (Supplementary Fig. 1b). The highly purified poliovirus particles were subjected to protein extraction for subsequent MALDI-TOF MS analysis of low (2–20 KDa range) and high molecular weight (17–90 KDa) proteins.

The purified virion spectra analysis, performed in the low molecular weight range (2–20 KDa) (Fig. 1a), showed only two significant peaks of about 3,800 and 7,600 m/z (common to the three purified poliovirus strains, corresponding to the mass of VPg (genome-associated viral protein) and VP4 poliovirus proteins (Table 1). The analysis of the spectra obtained from purified virions performed in the high molecular weight range (17–90 KDa) (Fig. 2b) showed three peaks around 26,000, 30,000 and 33,000 m/z corresponding to VP3, VP2 and VP1 poliovirus capsid proteins (Table 1).

In order to exclude any non-specific peaks the non-inoculated LLC-MK2 infected cell debris and uninfected LLC-MK2 spectra (Fig. 2). In order to verify whether the peaks observed in the spectra derived from MALDI-TOF MS analysis of purified virions corresponded to viral proteins, the poliovirus protein pattern obtained after sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was analyzed. Three major bands, corresponding to the viral capsid proteins VP1, VP2, VP3, were clearly observable: two of them (VP1 and VP2) migrated quite closely and were cut together, while the VP4 protein band was very faint and the protein VPg band was not detectable (data not shown).

The spectra obtained by MALDI-TOF MS analysis of VP1, VP2 and VP3 bands directly excised from Coomassie stained gel revealed a mass/charge ratio matching the corresponding molecular weights (Supplementary Fig. 3a and Table 1). For the VP4 band a very weak signal on the gel after SDS-PAGE was observed, so that after the excision of the band the concentration of the protein was lower than the detection limit of MALDI-TOF MS and than not detectable. The expected correspondence between the molecular weights obtained by SDS-PAGE and MALDI-TOF MS analysis observed for the viral proteins was corroborated by the correspondence of the molecular weight marker bands in the range of interest (6,5 to 36,0 KDa) observed when excised and subjected to the same protein extraction protocol followed by mass spectrometry analysis (Supplementary Fig. 3b and Table 1).

**MALDI-TOF MS analysis of Sabin poliovirus protein bands extracted from polyacrylamide gel.** In order to assess whether the peaks observed in the spectra derived from MALDI-TOF MS analysis of purified virions corresponded to viral proteins, the poliovirus protein pattern obtained after sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was analyzed. Three major bands, corresponding to the viral capsid proteins VP1, VP2, VP3, were clearly observable: two of them (VP1 and VP2) migrated quite closely and were cut together, while the VP4 protein band was very faint and the protein VPg band was not detectable (data not shown).

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**MALDI-TOF MS analysis of Sabin poliovirus-infected cells: detection of differentiating viral peaks.** In order to verify whether substantial differences exist between viral and cellular protein peak patterns, concentrated stocks of the three poliovirus Sabin reference strains were also prepared, not subjected to sucrose gradient purification, in order to obtain a pellet of poliovirus particles together with residual infected cell debris. Similarly, we collected uninfected LLC-MK2 cells by applying the same procedure.

To identify the protein mass profiles of the three poliovirus types, the corresponding spectra were automatically acquired. The macroscopic comparison of the different spectra in the "stack view mode" indicates that significant differences do exist between viral particles (poliovirus types 1, 2 and 3) combined with the residual infected cell debris and uninfected LLC-MK2 spectra (Fig. 2).

The mass spectra of poliovirus strains with residual infected cell debris compared at both low (Fig. 2a,b,c) and high (Fig. 2e,f,g)
Figure 1 | Representative MALDI-TOF mass spectra of purified poliovirus Sabin reference strains and ClinPro Tools statistical analysis. (a,b) Spectra of human poliovirus type 1, 2, 3 purified particles, shown in the m/z range of (a) 2 to 20 KDa (low molecular weight) and (b) 17 to 90 KDa (high molecular weight). Molecular weight values of poliovirus significant peaks are indicated on peak tops. (c,d) Analysis of discriminating peaks within the three poliovirus serotypes by using ClinPro Tools software (“Poliovirus type 1”, blue; “Poliovirus type 2”, red; “Poliovirus type 3”, green): “2D Peak distribution view” with the details at low (c) and high (d) molecular weight of the two best separating peaks; “PCA-3D dot plots window” of the spectra showing in 3 separate clusters the spectra of the 3 different poliovirus serotypes in the low (c) and high (d) molecular weight range; “Spectra view” in the low (c) and high (d) molecular weight range of the average spectra of the replicates of each poliovirus serotype. The red rectangles highlight the three discriminating peaks revealed in the mass range 2–20 KDa (7,522; 7,579; 7,635 m/z), and the four discriminating peaks in the mass range 17–90 KDa (30,197; 33,257; 33,554; 38,601 m/z). The blue rectangles are not discriminating peaks according to the applied Supervised Neural Network (SNN) statistical method.
molecular weight with those of uninfected cells (Fig. 2d,h) revealed the presence of specific viral proteins.

In order to verify the discriminant viral peaks also in cells infected with poliovirus Sabin reference strains, the “Spectra view” in the range 7,400 to 7,900 Da (Fig. 2i) and in the range 26,000 to 35,000 Da (Fig. 2j) was created with ClinPro Tools software. From this analysis, performed directly on the infected and uninfected cells, only the VP4 protein (with a mass included in the low molecular weight range) corresponding peak, resulted a discriminant peak among the three different poliovirus types.

MALDI-TOF analysis of poliovirus isolates from different clinical samples. In order to further explore the applicability of this technology an in-house viral database was created. A Main Spectrum Profile (MSP) was generated, at low molecular weight, for each of the three poliovirus serotypes and for the uninfected LLC-MK2 cells. The four MSP spectra obtained by this procedure for each of the three poliovirus serotypes and for the uninfected samples were imported into ClinPro Tools software. The average spectra of each clinical isolate (Fig. 3e,f,g), previously identified by neutralization assay. As observed for purified Sabin reference strains and for Sabin reference strain-infected cells, also for the polioviruses identified from clinical samples the VP4 protein showed mass differences among the three serotypes.

Table 1 | Proteic peaks detected for poliovirus 1, 2 and 3 and their statistical significance

| Protein | Sabin Strains | Theoretical Da* | Observed** m/z | SD | p-value |
|--------|---------------|----------------|---------------|----|---------|
| VPg    | Poliovirus1    | 3,890          | 3,807 Da      | +/− 2 Da | 0.0000008 |
|        | Poliovirus2    | 3,720          | 3,788 Da      | +/− 1 Da | 0.000004  |
|        | Poliovirus3    | 3,530          | 3,760 Da      | +/− 2 Da | 0.000002  |
| VP4    | Poliovirus1    | 7,530          | 7,613 Da      | +/− 3 Da | <0.000001 |
|        | Poliovirus2    | 7,370          | 7,578 Da      | +/− 4 Da | <0.000001 |
|        | Poliovirus3    | 7,450          | 7,522 Da      | +/− 2 Da | <0.000001 |
| VP3    | Poliovirus1    | 26,570         | 26,622 Da     | +/− 25 Da | 0.98      |
|        | Poliovirus2    | 26,430         | 26,759 Da     | +/− 30 Da | 0.85      |
|        | Poliovirus3    | 26,270         | 26,320 Da     | +/− 25 Da | 0.87      |
| VP2    | Poliovirus1    | 30,090         | 30,240 Da     | +/− 35 Da | 0.94      |
|        | Poliovirus2    | 30,050         | 30,130 Da     | +/− 40 Da | 0.94      |
|        | Poliovirus3    | 30,150         | 30,197 Da     | +/− 30 Da | 0.9800012 |
| VP1    | Poliovirus1    | 33,460         | 33,530 Da     | +/− 45 Da | 0.000008  |
|        | Poliovirus2    | 33,150         | 33,257 Da     | +/− 45 Da | <0.000001 |
|        | Poliovirus3    | 33,480         | 33,555 Da     | +/− 50 Da | 0.000008  |

Da: Dalton.
*: theoretical mass available by GenBank accession numbers AAN85442.1, AAN85443.1, AAN85444.1 for poliovirus type 1, 2 and 3, respectively.
**: average value of the multiple measurements.
SD: Standard Deviation of the multiple measurements (see Methods section).
p-value: significance of the mass differences of each protein evaluated with ANOVA test (see Methods section).

Poliovirus clinical isolates serotypes differentiation. In order to detect and visualize the specific viral biomarker VP4 of the three different poliovirus serotypes isolated from the five clinical samples, all the spectra used to create the MSP spectra (the same that were previously used for the creation of the in-house database) of Sabin reference strains-infected cells, and those of uninfected LLC-MK2 cells, were imported into ClinPro Tools software. The average spectra of the cells infected with the three Sabin reference strains, as well as those of uninfected LLC-MK2 cells, were compared with the average spectra of each clinical isolate (Fig. 3e,f,g), previously identified by neutralization assay. As observed for purified Sabin reference strains and for Sabin reference strain-infected cells, also for the polioviruses identified from clinical samples the VP4 protein showed mass differences among the three serotypes. The molecular weight of VP4 protein of the five poliovirus clinical isolates overlapped the mass value of the corresponding Sabin reference serotype. In order to evaluate the reliability of VP4 protein as a discriminating peak at the serotype level, the Receiver Operating Characteristic (ROC) curve was created and the Area Under Curve (AUC) value was calculated. The VP4 protein of each poliovirus clinical isolates was compared to the VP4 of each Sabin reference strain-infected cells so that 3 ROC curves for each poliovirus clinical isolates were created; when correspondence between the serotype of poliovirus clinical isolate and the serotype of Sabin reference strain-infected cells was found the ROC curve gave an AUC values of 0. Otherwise the AUC values were >0.9.

VP4 protein as a biomarker for Enterovirus. In order to verify the absence of misidentification with members of the *Picornaviridae* family other than polioviruses, human coxsackievirus B1 and human echovirus 9 (both members of *Enterovirus B* species) were analyzed by MALDI-TOF MS. The protein spectra obtained in low molecular weight range (2–20 KDa) of human coxsackievirus B1 and echovirus 9 infected- and uninfected-LLC-MK2 cells are shown in Figure 4a. As expected for *Enterovirus* genus, the spectra showed also the specific peak of about 7,500 Da related to VP4 protein.

The VP4 proteins of human coxsackievirus B1 and human echovirus 9 were compared with those of the three Sabin poliovirus serotypes in the molecular range 7,400 Da to 7,900 Da (Fig. 4b,c) giving AUC values > 0.9 in all the cases.

Discussion

This study aimed to evaluate the application of MALDI-TOF MS to virus identification, and to the detection of specific viral biomarkers, differentiating between virus-infected and uninfected cells, thus extending the use of this technology to a novel application.

The mass spectrometry analysis applied to the study of viral structure and identification, although far from conclusive, has received increasing amounts of consensus, due to its high level of accuracy, rapidity and low operational costs. Nevertheless, applications of MALDI-TOF MS for virus identification, in particular those with diagnostic purposes, couple this technique with PCR, often resulting in very expensive procedures. On the other hand, other studies that have used the spectra obtained from infected cells with the aim of detecting specific viral peaks have neither compared them with those obtained with purified virions, nor made an accurate analysis of the
uninfected cells protein peaks, thus rendering the results often unclear and inconclusive\(^1\).

In this study, the very efficient technique adopted to obtain highly purified poliovirus preparations combined with SDS-PAGE analysis allowed us to confirm the specificity of the peaks detected by MALDI-TOF MS analysis, confirming its ability to discriminate viral protein peaks from uninfected cells peaks and to detect specific poliovirus protein biomarkers.

Moreover, MALDI-TOF MS analysis applied to the three Sabin poliovirus serotypes revealed characteristic peak profiles for each of them, as demonstrated by statistical analysis, showing three independent clusters for the three serotypes, as expected considering that the genes coding for poliovirus capsid proteins widely diverged\(^2\).

The results showed a significant match between the molecular weight of the VP4 protein of each of the reference poliovirus strains and the corresponding clinical isolates at the serotype level. Moreover, no significant match between the molecular weight of the VP4 protein of each of the reference poliovirus strains and human coxsackievirus B1 and human echovirus 9 was found. For this reason the VP4 protein may be considered a good viral biomarker to identify poliovirus strains at the serotype level.

Figure 2 | Representative MALDI-TOF mass spectra of Sabin poliovirus-infected and uninfected LLC-MK2 cells and comparative analysis by ClinPro Tools software. The spectra of LLC-MK2 cells infected with human poliovirus types 1, 2, 3 ("Poliovirus type 1-infected cells", "Poliovirus type 2-infected cells", "Poliovirus type 3-infected cells"), with pointed average masses, are compared in the m/z range 2 to 20 KDa (a–c) and 17 to 90 KDa (e–g) with the spectra of LLC-MK2 uninfected cells (d,h). (i,j) Spectra view of the Average spectra profiles in the mass range 7,400–7,900 Da (i) and in the mass range 26,000 to 35,000 Da (j) of LLC-MK2 cells infected with the 3 different poliovirus serotypes with LLC-MK2 uninfected cells.
One of the main advantages of this approach is that the identification of poliovirus strains by MALDI-TOF MS analysis can be obtained after a 5-days-procedure (starting from the cytopathic effect observation upon sample cultivation), significantly shortening the time needed to perform the gold standard neutralization test (about 20 days). This confirms the ability of MALDI-TOF MS platform of shortening significantly the times for conventional identification methods in some specific cases (in the case of polioviruses, the neutralization assay) and, moreover, the significant reduction of reagent costs and the need of experienced personnel.

Enzyme immunoassays and immunofluorescence assays for poliovirus identification have been described, but not yet recommended for laboratory diagnosis, because the WHO gold standard is the neutralization assay.

Furthermore, as compared to the molecular methods used for poliovirus identification at the species and at the serotype level (such as reverse transcription and PCR [RT-PCR], Real Time RT-PCR and oligonucleotide microarray hybridization), MALDI-TOF MS is less cumbersome and cheaper, not requiring separate areas of manipulation of the samples in the different phases, preventing from risk of genome contamination not requiring expertise by technicians, and could be considered the best method for protein identification.

These results, though they need to be further refined, support the potential of MALDI-TOF MS technology for virus identification and provide a promising basis to extend its application to many other RNA and DNA viral agents of medical interest.

**Methods**

**Cell cultivation.** Confluent cultures of Rhesus monkey kidney epithelial cell line (LLC-MK2; American Type Cell Culture, ATCC CCL-7) were propagated in different lots of Earle's modified Minimum Essential Medium (E-MEM), supplemented with 2 mM L-glutamine, antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin), and 10% (growth medium) or 1% (maintenance medium) fetal bovine serum (FBS). Culture media and supplements were from PAA (The cell culture company, Velzy-Villacoublay, France).

**Reference viral strains.** Sabin poliovirus type 1, 2 and 3 reference strains (PHLS, Central Public Health Laboratory Service, London, UK), human coxsackievirus B1 (ATCC VR28) and human echovirus 9 (ATCC VR1050) were cultivated and titrated in LLC-MK2 cells.

**Poliovirus clinical isolates.** Human poliovirus strains representative of the three serotypes isolated from five different biological samples, identified by neutralization assay according to standard procedures and stored at 280°C in our laboratory collection were included in the study: one poliovirus type 1 isolate (from cerebrospinal fluid), two poliovirus type 2 isolates (from stools and throat swab) and two poliovirus type 3 isolates (from stools and throat swab) strains, derived from patients with different clinical signs and symptoms referred to viral meningitis, gastroenteritis and pharyngitis.

**Virus titration.** For poliovirus titration, the 50% tissue culture infectious dose (TCID<sub>50</sub>) was calculated by using the Reed and Muench formula on the basis of microscopic observations (DPC 320, Leica, Milan, Italy) of the cytopathic effect in LLC-MK2 cell monolayers infected with the poliovirus strains (10-fold viral dilutions, from 10<sup>-2</sup> to 10<sup>-5</sup>; 6 replicas/dilution using a 96 - well microtiter plate for each viral serotype).

**Virus cultivation.** Sabin reference polioviruses, human coxsackievirus B1 and human echovirus 9 were cultivated in LLC-MK2 cells: viruses were inoculated at a
multiplicity of infection (MOI) of 0.01 TCID50/cell in flasks with confluent cell monolayers. After 1 hour-absorption, the viral inoculum was removed and the cells re-fed with maintenance medium (E-MEM) without serum. Infected cells were incubated at 37°C humidified incubator for 48 h post infection (p.i.). Then the maintenance medium was collected in 50 ml conical sterile centrifuge tubes and stored at 2°C. The cells monolayers were re-fed with fresh E-MEM without serum and this step was repeated till the complete cytopathic effect had developed (usually at 96 h p.i.). Finally, the cells were scraped off the flasks and collected in 50 ml conical sterile centrifuge tubes and added to the previously frozen culture medium.

Sabin poliovirus strains isolation, purification by sucrose gradient centrifugation. Poliovirus particles released from infected cells were obtained by a freezing-thawing cycle and cell removing by centrifugation at 2,000 × g for 15 minutes (3,153 rpm, Centrifuge 5804 R, Eppendorf, Milan, Italy). Ammonium sulfate (Sigma-Aldrich, Milan, Italy) was then added to the supernatant at a concentration of 40 g/100 ml, and the viral particles and the residual cell debris were pelleted by centrifugation at 3,000 × g (3,861 rpm, Centrifuge 5804 R, Eppendorf, Italy) for 2 hours at 4°C. The pellet was

Figure 4 | Representative MALDI-TOF mass spectra of LLC-MK2 cells infected with the Picornaviridae family members human coxsackievirus B1 and human echovirus 9 and uninfected cells, and ClinPro Tools comparative analysis with the Sabin poliovirus type 1, 2, and 3 strains. (a) Spectra of LLC-MK2 cells infected with human coxsackievirus B1 and human echovirus 9 ("Coxsackie B1-infected cells" and "Echovirus 9-infected cells") compared with the protein profile of LLC-MK2 uninfected cells in the m/z range of 2 to 20 KDa. Molecular weight values are indicated on peak tops. (b,c) Spectra view of the average spectra of human coxsackievirus B1 (b) and human echovirus 9 (c) compared by ClinPro Tools in the mass range 7,400–7,900 Da with the average spectra of the 3 Sabin poliovirus strains (poliovirus type 1; poliovirus type 2; poliovirus type 3) and uninfected LLC-MK2 cells.
dissolved in 2 ml phosphate buffer saline (PBS solution) with 1% Nonidet P-40 detergent (Sigma-Aldrich, Milan, Italy) to “clean” the viral particles.  
Sucrose gradient solutions were prepared in 13.2 ml-Ultraclear tubes (Beckman Coulter, Milan, Italy) by using ultrapure analytical grade sucrose (Sigma-Aldrich, Milan, Italy) and sterile Tris-HCl 10 mM (pH 7.4), NaCl 0.05 M 42.  
Solute preparations were studied at a final concentration of a) 15% (w/v): sucrose 15 g per 100 ml Tris-HCl 10 mM (pH 7.4), NaCl 0.05 M, and b) 45% (w/v): sucrose 45 g per 100 ml Tris-HCl 10 mM (pH 7.4), NaCl 0.05 M, respectively. Gradients were prepared by using a peristaltic pump (Minipuls™3, Gilson, Middleton, USA) and a gradient maker (PBI, Milan, Italy).  
The viral pellet, suspended in PBS as described above, was carefully layered onto the gradient tubes (1 ml of virus suspension/tube) and centrifuged at 303,700 × g for 4 hours at 4°C.  
Sucrose gradient fractions were collected and evaluated for their protein content by spectrophotometric analysis (Beckman, DU-85, Milan, Italy) at 750 nm, following Lowry assay. For MALDI-TOF MS analyses, using a commercial kit (TOC Protein Assay, Bio-Rad, Milan, Italy) according to the manufacturer’s instructions. Finally, the fractions corresponding to the purified viruses were pooled and centrifuged at 154,300 × g for 70 minutes at 4°C. The pellet was resuspended in 50 μl of E-MEM without serum and further analyzed by electron microscopy, as previously described 46, before protein extraction.  
Protein extraction and target preparation for MALDI-TOF MS analysis.  
A short protein extraction protocol was used. Each of the poliovirus preparations (puriﬁed Sabin reference strains; Sabin strains combined with residual infected cell debris; polioviruses from clinical samples), human coxsackievirus B1, and echovirus 9 strains were extracted using the cell debris and were suspended on PBS solution with added with 30 μl 70% formic acid (HCOOH) (Sigma-Aldrich, Milan, Italy) by vigorous mixing, than with 30 μl 100% acetonitrile (CAN) (Sigma-Aldrich, Milan, Italy) and further vigorous mixing. This mixture was subjected to centrifugation at 16,100 × g for 2 minutes (13,200 rpm; Centrifuge 5415R, Eppendorf, Italy). MALDI-TOF MS MS was performed as in a low (1-20 KDa) and high (17-90 KDa) molecular weight range using by using a MicroFlex LT mass spectrometer (Bruker Daltons, Germany) supplied by Becton Dickinson (Italy) instrument. For the analysis in the low molecular weight range, 1 μl of the supernatant was spotted (20 spots for each strain) onto a MSP 96 polished steel target plate (Bruker Daltons, Germany), air-dried at room temperature, and over with 1 μl of matrix solution (alpha-cyano-4-hydroxycinnamic acid (4HCCA), diluted in 50% CAN and 2.5% trifluoroacetic acid (TFA), Sigma-Aldrich, Milan, Italy) followed by air-drying. For the analysis in the high molecular weight range, a saturated solution of synapinic acid (SA) was used as the matrix (50 mg/ml SA in 30-70% TFA acid 0.1%). Equal volumes (2 μl) of supernatant and matrix solution were mixed. One microliter of this solution was spotted onto the target plate (20 spots for each strain) and air-dried at room temperature.  
SDS-PAGE and protein extraction from polyacrylamide gel. The methods used for SDS-PAGE and protein extraction were modiﬁed from those originally described by Susnea et al. 34 and by Mirza et al. 35, respectively.  
Briefly, viral peptides from puriﬁed virions, treated with Laemmli buffer (50 mM Tris-HCl pH 6.8; 100 mM dithiothreitol; 25% (w/v) glyceral; 2% SDS; 0.1% bromo-phenol blue) and heated at 95°C for 5 minutes, were resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% acrylamide gel with the following concentration conditions: 4% acrylamide/N,N-Bisacrylamide for the stacking gel and 20% for the running gel. A low range protein standard (Sigma-Aldrich, Milan, Italy) (MW 6,500-66,000) was used as the molecular weight marker.  
Coomassie brilliant blue (Bio-Rad, Milan, Italy) gel staining was used according to Mirza et al. 35.  
For protein extraction, the individual bands were excised and put into 1.5 ml colorless tubes. After washing with 300 μl of high purity liquid chromatography (HPLC) grade water, each excised gel band was vortexed for 10 minutes in 100 μl 10% acetic acid solution. The solution was removed and the gel piece washed thoroughly with 300 μl of 100% acetonitrile (CAN) grade water, then with 300 μl of 100% CAN, then with HPLC grade water, and ﬁnally with methanol for 20 minutes. To destain the gel completely, each gel piece was dipped into 100 μl of solution of formic acid:HPLC grade water: isopropanol (1 : 3 : 2, v/v/v) and vortexed for 30 minutes (until the gel piece turned colourless). Finally, each gel piece was partially dried, crushed into small pieces, and the proteins were extracted by adding 10 μl 4HCCA, when used for MALDI-TOF MS MS analysis in the low molecular weight range and 10 μl of 50 mg/ml saturated SA solution when used for MALDI-TOF MS MS analysis in the high molecular weight range. Molecular weight marker bands in the range of interest (6,500 to 36,000 Da) were visualized by using a 24W1 Ti Rotor (24,600 rpm, Optima™XPN, Beckman Coulter, Milan, Italy) for 4 hours at 4°C.  
Data analysis began with raw data pre-treatment, including baseline substraction, normalization of a set of spectra, internal peak alignment using prominent peaks, and a peak picking procedure. Subsequently, the data derived from purified polioviruses (Sabin reference strains) were imported into ClinProTools Software version 2.2 (Bruker Daltons, Germany) for statistical analysis. This software was used for the statistical comparison of the loaded spectra, as well as for identifying speciﬁc peaks to discriminate among the three analyzed polioviruses serotypes.  
To compare individual serotypes, the same number of spectra for each strain was needed to be analyzed by using ClinProTools. Two different analysis were performed for the low (2 to 20 KDa) and the high (17 to 90 KDa) molecular weight range. In order to evaluate each statistical analysis, a total of 30 spectra (speciﬁcally ten replicates for each of the investigated viral serotypes), obtained by the MFT-FC method (low molecular weight), and a total of 30 spectra (speciﬁcally ten replicates for each of the investigated viral serotypes) obtained by the LP-44 KDa method (high molecular weight), were uploaded into the software and automatically recalibrated.  
As reported in the manufacturer’s procedure, the task of recalibration is to reduce mass shifts occurred during the multiple measurement of the same sample obtained in a single or in different experiments. The recalibration was carried out with a speciﬁc algorithm already present in ClinProTools software. In this study the most important parameter (Maximum Peak Shift) is set to 1,000 ppm.  
Moreover, as part of recalibration step, a list of masses which occur very frequently within the entire data set is generated. The number of these masses is called the Maximum Quality Value. After recalibration it is checked how many of these masses can be found in each spectrum using the Maximum Peak Shift parameter as maximum shift. The number of found masses is called the Spectrum Quality Value. The Spectrum Quality Threshold is computed as the product:  

$$\text{Spectrum Quality Threshold} = \text{Maximum Quality Value} \times \% \text{ Match to Calibrant Peaks}$$  

All spectra with a Spectrum Quality Value < Spectrum Quality Threshold are marked as “Not Recalibratable” and excluded. The value of the % Match to Calibrant Peaks parameter is set to 30%.  
After the recalibration process, the software automatically created an average spectrum from all the replicates of each poliovirus serotype. The obtained average spectra were compared with each other and a report of peaks was available.  
The data included in this peak report were used to assess statistical information. To give a signiﬁcance value for differences in each m/z peaks ANOVA test was performed and a p-value was calculated. A p-value < 0.05 was considered significant (discriminating peak).  
Statistical testing of the datasets was performed on the basis of multivariate unsupervised principal component analysis (PCA) and the results were displayed in a three-dimensional score plot, which was generated automatically by the software. PCA reduces the variables of the complex dataset, generating a set of new variables (principal components) that explain most of the variance of the same spectra. A supervised algorithm to find the peaks with the probably highest serotypes separation capability under a uni/multivariate view of the data. In this study 3 statistical algorithms included in the software are used: QuickClassifier (unsupervised supervised algorithm), Supervised Neural Network and Genetic Algorithm (multivariate supervised algorithms).  
These algorithms automatically determined the best number of peaks to be integrated in the statistical model, in order to differentiate the different serotypes, on the basis of the Recognition Capability (RC) parameter. Moreover the algorithms evaluated the reliability of the created statistical model by the Cross Validation (CV) parameter; this parameter can be used also to predict how this model will behave in the future.  
The algorithm with the highest score of RC and the highest value of CV, also taking into account the peaks number used to obtain the model, was chosen as a reference model in order to analyze the spectra of the clinical isolates. The presence/absence of each discriminating peak was evaluated by comparing the average spectra automatically recalibrated with the same spectra analyzed by the MBT-FC method. The discriminative power for each putative biomarker in the model was further described via analysis of area under the receiver operating characteristic (ROC) curve (AUC) using ClinProTools software.
The ROC curve gives a graphical overview about the specificity and the sensitivity of a test, and in this case an evaluation of the discrimination quality of a peak. The ROC curve view takes into account only one peak as a test criterion and indicates this score of a test, and in this case an evaluation of the discrimination quality of a peak. The software calculates an arbitrary unit score value between 0 and 3, indicating the similarity between sample and reference spectra and finally displays the top ten matching database records. The results were displayed into the “Detected species” window as specified by the manufacturer with an identification score value ≥ 1.7 for a reliable identification. Scores < 1.7 were considered as unreliable.

1. Welker, M. & Moore, E. R. B. Application of whole-cell matrix-assisted laser desorption/ionization time-of-flight mass spectrometry in systematic microbiology. Syst. Appl. Microbiol. 34, 2–11 (2011).
2. Fenselau, C. & Demirev, P. A. Characterization of intact microorganisms by MALDI mass spectrometry. Mass Spectrom. Rev. 20, 157–171 (2001).
3. Lavigne, J. P. et al. Mass spectrometry: a revolution in clinical microbiology? Clin. Chem. Lab. Med. 51, 2270–2273 (2013).
4. Wieser, A., Schneider, L., Jung, I. & Schubert, S. MALDI-TOF MS in microbiological diagnostics-identification of microorganisms and beyond (mini review). Appl. Microbiol. Biotechnol. 93, 965–974 (2012).
5. Susnea, I. et al. Application of MALDI-TOF-Mass Spectrometry to Proteome Analysis Using Stain-Free Gel Electrophoresis. Top. Curr. Chem. 331, 37–54 (2013).
6. Calderaro, A. et al. Identification of Barreliella species after creation of an in-house MALDI TOF database. PLoS One 9, e88895 (2014).
7. Bizzini, A. & Greub, G. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry, a revolution in clinical microbial identification. Clin. Microbiol. Infec. 16, 1614–1619 (2010).
8. Croxatto, A., Prod’hom, G. & Greub, G. Applications of MALDI-TOF mass spectrometry in clinical diagnostic microbiology. FEMS Microbiol. Rev. 36, 380–407 (2012).
9. Cobo, F. Application of MALDI-TOF Mass Spectrometry in Clinical Virology: A Review. Open Virol. 7, 84–90 (2013).
10. Liu, T. et al. Proteomic profiling of hepatitis B virus-related hepatocellular carcinoma with magnetic bead-based matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Acta Biochim. Biophys. Sin. 43, 542–550 (2011).
11. Erkutkivitchov, V., Karpasaa, M. & Hulehile, M. Spectroscopic detection and identification of infected cells with herpes viruses. Biopolymers 91, 61–67 (2009).
12. Chou, T. C., Hsu, W., Wang, C. H., Chen, Y. J. & Fang, J. M. Rapid and specific influenza detection by functionalized magnetic nanoparticles and mass spectrometry. J. Nanotechnol. 9, 52 (2011).
13. Calderaro, A. et al. MALDI-TOF MS analysis of human and animal Brachyspira species and benefits of database extension. J. Proteom. 78, 273–283 (2010).
14. Storch, G. A. Diagnostic Virology. Clin. Inf. Dis. 31, 739–751 (2000).
15. Piqueur, M. A., Verstrepen, W. A., Bruynseels, P. & Mertens, A. H. Improvement of a real time RT-PCR assay for the detection of enterovirus RNA. J. Virol. 6, 1–3 (2009).
16. Knowles, N. J. et al. Picornaviridae. In: Virus Taxonomy: Classification and Nomenclature of Viruses: Ninth Report of the International Committee on Taxonomy of Viruses. Eds King, M. Q. O., Adams, M. J., Carstens, E. B. & Lefkowitz, E. J., 855–880 (Elsevier, San Diego, USA, 2012).
