Journal of Clinical Virology 184 (2026) 105940

Contents lists available at ScienceDirect

Journal of Clinical Virology

journal homepage: www.elsevier.com/locate/jcv

Next generation sequencing approaches for the detection and characterization of enteroviruses in clinical, public health, and research settings: Expert view of the European non-polio enterovirus network (ENPEN)

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https://doi.org/10.1016/j.jcv.2026.105940
Received 10 September 2025; Received in revised form 5 February 2026; Accepted 22 March 2026
Available online 24 March 2026

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ARTICLE INFO

Editor: Benjamin A. Pinsky

Keywords:
Enteroviruses
Next Generation Sequencing
Viral metagenomics
Targeted PCR-based
Wet-lab
Dry-lab
Bioinformatics

ABSTRACT

Enteroviruses (EVs) are a common cause of a wide spectrum of infectious diseases, ranging from mild respiratory illnesses to severe neurological conditions, particularly affecting children. Current molecular methods, such as 5′UTR-based PCR for detection and (partial) VP1 gene sequencing for typing, are widely utilized. However, Next-Generation Sequencing (NGS), and bioinformatics offer a comprehensive alternative, enabling full-genome analyses for improved virus characterization, genomic epidemiological surveillance, and outbreak investigation. Despite its advantages, implementation of NGS poses challenges, particularly in standardizing and optimizing laboratory workflows (wet-lab) and bioinformatics analyses (dry-lab), methods that are not often readily accessible in many laboratories. Here, we discuss the potential of NGS as a tool for EV detection/characterization in clinical virology, public health, and research settings. We provide practical options for actions for implementing NGS to advance the understanding and management of enterovirus infections. These recommendations are based on expert discussions during the recent European non-polio enterovirus network (ENPEN) workshop

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held in Corfu, Greece, on 23–24 May 2024, aiming to guide harmonization of NGS practices across clinical, public health, and research settings.

1. Introduction

Enteroviruses (EVs) are a diverse group of non-enveloped RNA viruses that primarily spread through the faecal-oral and/or respiratory route. Although most infections are asymptomatic, EVs are the leading causes of aseptic meningitis and can cause a wide spectrum of other severe clinical conditions, including hepatitis, myocarditis, neonatal sepsis, respiratory infections, ocular infection, encephalitis, and acute flaccid paralysis, particularly in children.

The EV genome is on average 7.4 kb long and encodes four capsid proteins (VP1-VP4) and seven non-structural proteins (2A-2C and 3A−3D). There are currently over 300 EV types infecting humans classified into four species based on the VP1-gene (*Enterovirus alphacoxsackie* (EV-A), *E. betacoxsackie* (EV-B), *E. coxsackiepol* (EV-C) and *E. deconjunctivi* (EV-D) and the rhinovirus species *E. alpharhino (RV-A), E. betarhino (RV-B)* and *E. cerhino* (RV-C) [1,2]. The genetic diversity of EVs is shaped by their high mutation rates, immune pressure, and frequent recombination events in EV-A-C [3–6].

In clinical virology, the 5′UTR-based reverse transcription real-time polymerase chain reaction (RT-PCR) remains the gold standard for detecting EVs due to its high sensitivity, cost-effectiveness, and rapid turnaround time [7]. However, the high sequence conservation of the 5′UTR region limits typing, and partial sequencing of VP1, which exhibit greater genetic variability, is required [8]. Although partial VP1-sequencing suffices to identify the EV type [8], complete or whole-genome sequencing (WGS) can improve the accuracy of typing and provides deeper insights into virus evolution and recombination events, aiding in molecular epidemiological surveillance, and outbreak investigation [9–14]. Ascertainment of complete genome sequences play an important role in public health surveillance and early warning, in particular when recombination and antigenic changes contribute to the emergence of an EV outbreak [15–20]. Moreover, in the case of poliovirus (PV), the higher resolution of complete genome data contributes to PV eradication efforts by identifying circulating strains and transmission chains in higher detail [21–23].

Next-Generation Sequencing (NGS) and bioinformatics offer a comprehensive solution, enabling both partial and complete genome sequencing. NGS technologies further have the capacity to identify novel types/variants by avoiding primer-probe mismatches. Consequently, NGS data can be used to refine primers and probes, enhancing the performance of RT-PCRs. NGS also enables the simultaneous characterization of co-infections (e.g. with multiple EV types or other viruses) within a single assay further enhancing its utility in clinical and public health investigations. NGS also provides critical research opportunities to advance clinical care by deepening our understanding of the role specific EV types, specific mutations and co-infections in disease severity and outcomes.

The application of WGS for EVs is in alignment with the recommendations by The European Commission and ECDC support for the implementation of molecular testing and genomic sequencing for European public health surveillance of infectious diseases and improving the in-country WGS infrastructure [24].

However, there are currently no standardized NGS methods for EVs in the context of laboratory testing (wet-lab), and data analysis/interpretation (dry-lab). Furthermore, compliance with *In Vitro* Diagnostic Regulations (IVDR) and ISO15189 poses challenges for validation and adherence to high-quality standards, due to the increasing developments of NGS techniques. It should be noted that validation requirements for NGS differ substantially between clinical and public heath use and research. For research applications, protocols may be more flexible and validation focuses primarily on reproducibility and accuracy within the study context. In contrast, clinical diagnostics require strict adherence to these regulatory standards. In addition, implementing NGS can be challenging due to the limited access to NGS equipment, need for highly skilled laboratory personnel and specialized bioinformaticians, which is particularly difficult for smaller laboratories or low-resource settings.

This paper was initiated following an European Society of Clinical Virology (ESCV) supported European non-polio enterovirus network (ENPEN) [25,26] (https://escv.eu/european-non-polio-enterovirus-network-enpen) workshop held in Corfu, Greece, on 23–24 May 2024. This manuscript provides an updated expert view on the use of next-generation sequencing (NGS) for the detection and characterization of enteroviruses, building on the recommendations of Harvala et al. [25]. Here we provide a summary of wet-lab and dry-lab approaches for the characterization of EVs by NGS and make recommendations for future options for actions to improve the quality EV sequencing across clinical, public health, and research laboratories.

2. Application of NGS for enteroviruses

NGS for EVs can be applied using two approaches: viral unbiased/metagenomic sequencing (VIR-mNGS) or EV PCR-based targeted sequencing (EV-tNGS). VIR-mNGS is suitable for obtaining both partial and complete genomes without prior knowledge of the viral sequence. It is particularly advantageous in rare cases where targeted assays fail due to extensive genetic variability or novel EV variants that escape detection by existing primer sets, or when co-infections with other viruses are presumed. However, metagenomic techniques may suffer from lower sensitivity as compared to targeted techniques, especially when the virus is present in a rich background of non-viral nucleic acids (NA; e.g. host or bacterial), and several enrichment steps may be required (described below) to deplete the background or specifically enrich viral NA (Fig. 1). A targeted approach in which viral sequences are captured with generic virus-specific probes, can be used additionally to enrich exclusively for virus genomes prior to or after library construction.

EV-tNGS, on the other hand, specifically targets the detection and characterization of EVs and utilizes EV-specific primers to generate either partial or complete amplicons for subsequent sequencing. Complete EV genomes can be generated from a single ds-cDNA amplicon or multiple overlapping ones [27–30]. Longer amplicons are more informative, however at the cost of PCR sensitivity. It should be noted that for complete genomes generated from 2 or more amplicons or through the use of short read platforms, requires assembly in bioinformatics pipelines, which can create an artificial recombinant genome from a simple EV co-infection. Several research groups have made significant contributions to the development of longer PCR amplicons generating complete capsid or (near) complete genomes. Joffret *et al.* [28] published a complete genome assay encompassing two amplicons; one single PCR enables generation of 5'UTR, the complete P1 region and the 2 A, 2B and partial 2 C region of all EV types, while a second PCR that is species-specific generates an 2C-overlapping amplicon encompassing the 2C−3'UTR region. Isaacs *et al.* [29] described a method for amplification of a single near complete EV sequence. PV-specific assays have been developed by Arita *et al.* [30] generating a single capsid amplicon for poliovirus in a nested format. This assay was further developed for the Oxford Nanopore Technologies (ONT) platform by Shaw [22]. A near complete genome PCR assay for poliovirus is described by Stern et al. [31].

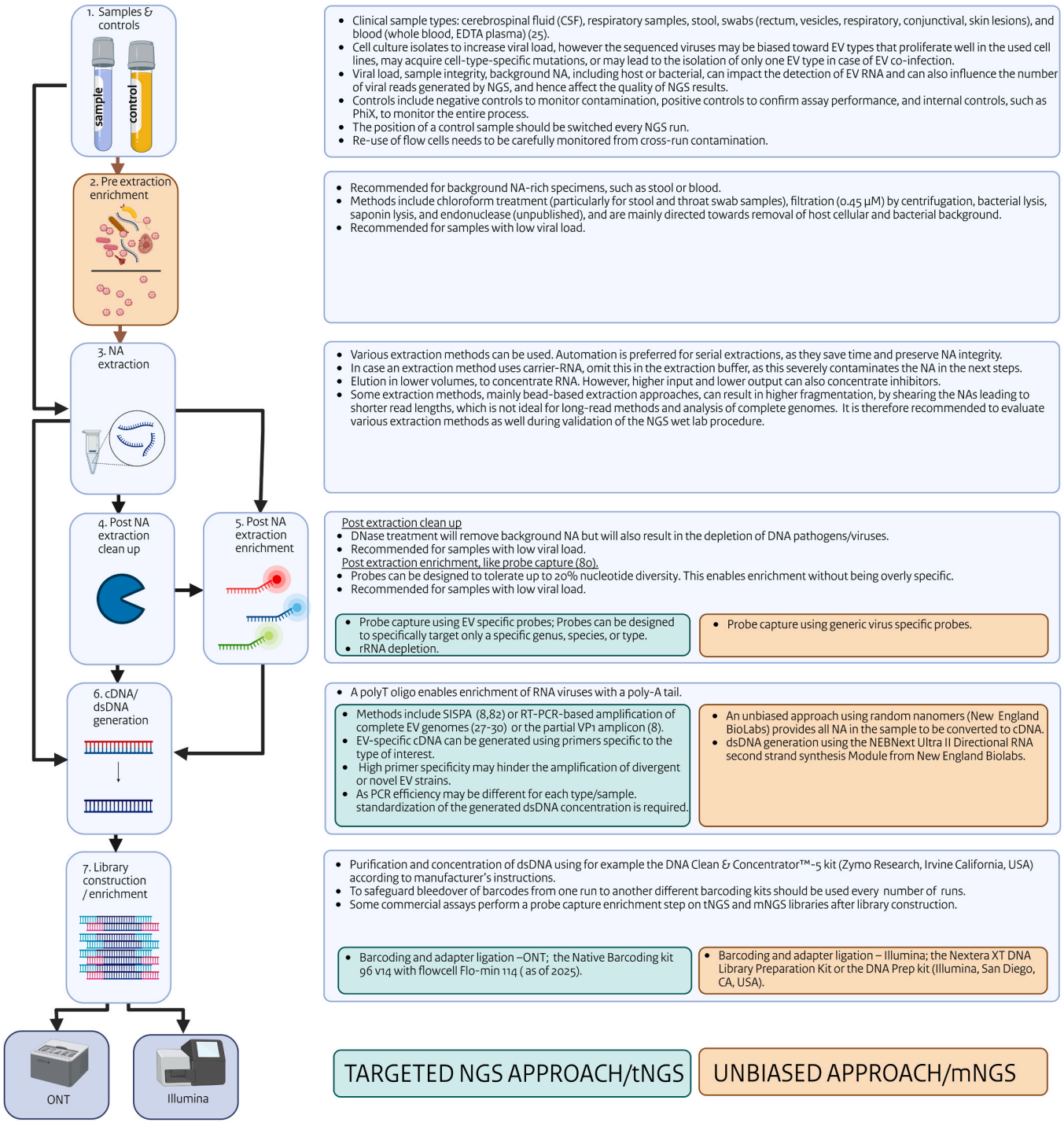

Fig. 1. From sample to raw sequencing data (wet-lab): Steps required to perform NGS on clinical samples with ONT and Illumina include (1) "Sample and control selection"; (2)"Pre-extraction enrichment" (only for unbiased approach - indicated by brown arrows); (3) "Nucleic acid (NA) extraction"; (4) "Post NA extraction clean up"; (5) "Post extraction enrichment"; (6) "cDNA/dsDNA generation"; and (7) "Library construction/ enrichment)". Black arrows can be used for both EV-targeted approach (EV-tNGS) and unbiased viral metagenomic approach (VIR-mNGS), where brown and green sections indicate specific considerations with the VIR-mNGS or EV-tNGS approach, respectively. The steps (4) "Post NA extraction clean up" and (5) "Post extraction enrichment" is optional and may be either or both included to enhance sequencing results. However, the time between NA extraction (step 3) and library construction (step 7) should be minimized as much as possible. RNA degrades rapidly, even when frozen, so it is advisable not to pause the process before obtaining cDNA or the library, especially if amplification and/or NGS of long fragments will be applied. Repeated freezing and thawing cycles should also be avoided. Steps (6) "cDNA/dsDNA generation" and (7) "Library construction/ enrichment" are also referred to as Library preparation and utilize barcoding to provide unique identifiers to each sample during library preparation and pooling the samples for multiplex analysis. Some commercial assays perform the probe capture on NGS libraries as a library enrichment step. Figure created with BioRender.com [8,27–30,80,82].

The use of targeted enrichment methods, such as probe capture with EV-specific probes, can further enhance EV-tNGS applications (Fig. 1). EV-tNGS is particularly effective for characterizing EVs in low-viral-load samples, such as cerebrospinal fluid (CSF) [32] and clinical specimens with rich background of non-viral NA (such as stool and nasopharyngeal specimens, or wastewater samples). A key advantage of EV-tNGS is its cost efficiency by allowing multiplexed sequencing of multiple samples with less sequencing depth.

3. NGS methodological framework for EV NGS

The methodological framework for EV NGS is described as a two-phase process: i) sample-to-raw sequence (wet-lab, Fig. 1) and ii) sequence-to-interpretation (dry-lab, Fig. 2). Figs. 1 and 2 provide guidance and the necessary key points to adopt and consider in each step. Both processes require quality control (QC) steps. The steps described are focused on the applications of NGS in clinical virology and public health.

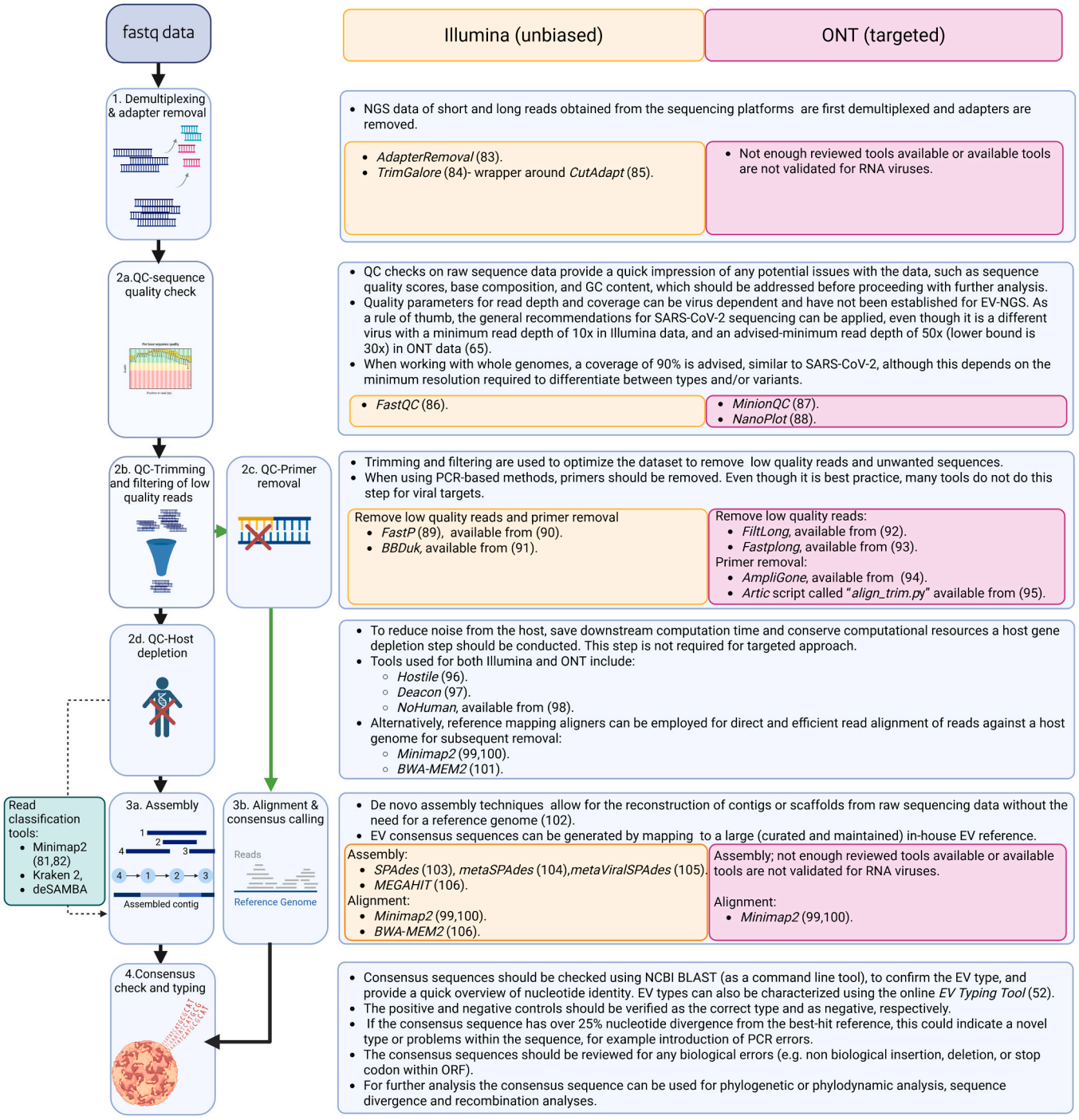

Fig. 2. From raw sequencing data to reportable results (dry-lab): Steps required to perform NGS analyses of sequences generated with Illumina and Oxford Nanopore Technologies (ONT) include (1) "De-multiplexing and adapter removal"; (2) "Quality control (QC)"; (3) "Assembly or alignment"; and (4) "Consensus check and typing". Green box: read classification tools can be used for preliminary virus identification in the viral NA pool, providing information on possible co-infections needed to be analyzed. Black arrows can be used for both targeted approach (EV-tNGS) and unbiased metagenomic approach (VIR-mNGS), with indicated tools using either Illumina generated data (orange sections) or ONT generated data (pink sections). Green arrows are specifically used for EV-tNGS. Read classification tools can additionally be used for preliminary virus identification in the viral NA pool (dotted arrows). Figure created with BioRender.com [52,65,81–106].

3.1. From sample to raw sequence (wet-lab)

The wet-lab process consists of several key steps, each presenting with specific choices, challenges and advantages: sample and control selection, pre-extraction enrichment (if required), NA extraction, post NA extraction clean-up/enrichment (if needed, by probe capture for example), cDNA/dsDNA generation and library construction and enrichment (Fig. 1). The wet-lab steps are focused on either depleting non-viral NA or enriching the targeted viral NA. Each step influences NGS success rates and data quality. Most importantly, high viral loads generally increase the likelihood of successful sequencing. If real-time PCR is available, the cycle threshold (Ct) value serves as a semi-quantitative indicator of viral load, and it can be used to select appropriate samples to improve the cost-effectiveness of the NGS-based method for subsequent typing. However, determining a Ct cut-off for all laboratories is challenging due to variations in assay design. In addition, several commercial "black box" diagnostic instruments and multiplex panels do not provide Ct-values or any semi-quantitative viral load estimates.

As Illumina and ONT are predominantly used among laboratories within ENPEN, this paper is focused on these technologies. Details of the characteristics, costs, suitability and limitation of these NGS technologies for EV applications are described in Table 1. While EV-tNGS and VIR-mNGS can be implemented with both Illumina and ONT, in practice VIR-mNGS is more frequently applied with Illumina sequencing. EV specific options for action per technology are indicated in Figs. 1 and 2.

3.2. From sequence data to reportable results (dry-lab)

A bioinformatics workflow follows several steps described in Fig. 2. Illumina-based software is fairly well established, whereas ONT-based software is still a rapidly developing field, and the proposed tools in Fig. 2 are current examples. Currently, there is no best practice for standardized ONT data analysis and there are no set quality parameters or criteria (e.g. for quality scoring or sequence mapping) for EVs and many other viruses.

The choice of bioinformatics tools depends on the application and approach (EV-tNGS or VIR-mNGS). Commercial tools offer user-friendly interfaces and robust support while open-source solutions provide flexibility and cost-effectiveness but may require more bioinformatics expertise. Laboratories should consider these strengths and limitations when selecting the most appropriate tool for their specific needs using an External Quality Assessment (EQA). A range of commercial software solutions are available to analyse genomic data, several that are frequently used in the field include *Genome Detective* [34], *Geneious Prime* [35], and *CLC Workbench* [36], however this list is non-exhaustive. A free software package includes Galaxy [37,38]. Many institutes have developed in-house tools, freely available, that can be used for EVs. A few examples are *Jovian* [39], DTU CC Metagen [40], *CZ ID metagenomics* [41,42], *pikavirus* [42,43], and *InsaFlu* [44]. More targeted examples include *ViroConstrictor* [45], *ViralRecon* [46], *CASTA-NET* [47] and the *ARTIC field bioinformatics workflow* [48]. Both commercial and in-house tools require regular updates to ensure accurate outcomes. In general, sequencers generate thousands to millions of reads per sample, each accompanied by a quality score to indicate base-calling accuracy.

For EV-tNGS, where primers define the target and reference sequences are known, reads are mapped to reference EV types. Depending on the threshold used to map sequences, reference-based tools may only account for known EV types, and additional parameter settings may be needed to identify new strains or types. In contrast, for VIR-mNGS filtered reads are *de-novo* assembled into contigs using their overlaps, enabling genotyping and detection of well-known or novel EVs. Many tools exist for this complex task both for short-read sequencing, and long-read sequencing, as reviewed by Holzer *et al.* [49] and Cosma *et al.* [50]. The assembly parameters can be usually customized with a preference for accuracy or for the length of the contigs, balancing between discovery of novel sequences *versus* certainty that the newly assembled sequence is correct. Alternatively, before assembly and mapping of fastq data, read classification tools can be used for

Table 1
Characteristics, costs, suitability, and limitations of Illumina Technology and Oxford Nanopore Technology (ONT) for enteroviruses applications.

	Illumina technology (2nd generation)	ONT technology (3rd generation)
Platforms	• Bench top: iSeq, MiniSeq, MiSeq, NextSeq 550, 1000, 2000. • Large-scale sequencers: NovaSeq 6000 and NovaSeq X.	• MinIOn (low throughput). • GridION (high throughput). • PromethION (ultrahigh throughput).
Sequencing methods	• Short read sequencing using reversible dye terminators.	• Long read sequencing that identifies the sequence of nucleotides by passing individual molecules through nanoscale pores and measuring changes in the electrical current.
Costs	• Costs per GB are low so ongoing costs can be lower especially for large scale projects.	• Instrumentation is relatively affordable.
Characteristics and suitability	• Highly accurate, high-throughput method. • Production scale sequencing capability in benchtop format (NextSeq 1000 and 2000). • Automated solutions to library preparation • Offers hybrid capture based sequencing panels. • Partial and complete genome generation. • Unbiased detection and typing.	• Low, high and ultrahigh throughput depending on the platform. • Automated solutions to library preparation. • Long read sequencing promotes complete genome assembly, analyses of recombination with mixed infections and also the detection of co-mutations. • Adaptive sampling possible (enrichment or depletion) during run. • Well adapted to field sequencing which is highly suitable for environmental and outbreak monitoring and for sequencing in low-resource countries and decentralized settings with limited laboratory infrastructure.
Limitations	• Sample preparation is complex. • Time consuming. • High G + C content areas, complex regions and long repeats can be problematic • Due to short read sequencing, recombination studies, in particular with multiple infection can be problematic. However, Illumina now also developed a "Complete Long Read technology", where long fragments were "land-marked" to capture single-molecule [33]. • High initial investment, costs of reagents and disposables and infrastructure, particularly problematic in low-resource settings.	• Constant changes in assay chemistry will complicate the necessary validation to comply with the IVDR standards. • High input of NA required, as such often PCR based methods or culture as input are applied. Hence, these do not allow for unbiased detection and typing of other viruses/pathogens (biased towards specific PCR primer-sets, best replicating strain in the culture, and can be problematic for recombination studies, in particular with multiple infections). • Less accurate and lower throughput when compared to Illumina, however accuracy has increased recently due to base calling advances and downstream analysis.

preliminary virus identification in the viral NA pool, providing information on possible co-infections [51].

Once a consensus sequence is generated, it can be characterized through NCBI *BLAST* [52] or using the online *EV Typing Tool* [53,54], which are often built in various bioinformatics tools. Phylogenetic analysis using user friendly tools as *MEGA* [55] or more sophisticated as *RAxML* [56] and *IQ-Tree* [57], and phylodynamic analysis using *Nextstrain* [58] can subsequently be performed. To detect any potential recombination events, phylogenetic analysis of the P1, P2 and P3 regions or phylogenetic comparison between the VP1 and 3Dpol gene could be utilized. Additionally, software, such as *Simplot + + /Bootscan* [59], *RDP4* [60]/*RDP5* [61], or *GARD* [62] can be used to plot similarities and detect recombination.

3.3. Quality control (QC)

The ISO15189 requires laboratories to implement appropriate quality controls (QCs). To ensure high-quality sequencing this would require QCs at each step in the process, including negative controls to monitor contamination, positive controls to confirm assay performance, and internal controls, such as PhiX, to monitor the entire process [63–65]. Failed NGS sequencing resulting from insufficient quality control can lead to contaminated data, misidentification of EV types, and missed detection of co-infections. Cross-contamination during library preparation can result in erroneous outbreak attribution, underscoring the need for robust QC at every step.

For wet-lab, internal controls can be spiked into different sample matrices to evaluate the impact of these matrices on the quality of RNA purification and NGS. Another control measure is to prevent sample contamination from one run to another, especially when ONT flowcells can be re-used which might create cross-run contamination issues. The position of controls and the barcodes set should be switched every run, if possible, so that no consecutive run uses the same barcodes. A challenge for validation and adherence to high-quality standards under ISO15189 are the ongoing changes in assay chemistry (particularly ONT), which require frequent re-validation.

QC should also be ensured in the dry-lab bioinformatics pipeline at the start to minimize errors throughout downstream analysis [66]. QC of the reads includes filtering (discarding those that do not meet the quality criteria), trimming (shortening the ends of the reads until the rest meets the criteria) or both. QC parameters provide a quick impression of any potential issues with the data, such as sequence quality scores including coverage of the genomic sequence and depth of coverage, base composition, and G + C content, which should be addressed before proceeding with further analysis. After QC checks, additional steps, such as non-viral/host read removal, are often applied to further optimize the dataset (Fig. 2). When using PCR-based methods for viral NA enrichment prior to sequencing, primer sequences should be removed (trimming) (Fig. 2).

4. Conclusions and options for action

The implementation of NGS for EVs holds great potential for advancing clinical diagnostics, epidemiological molecular surveillance, and research. Recent studies have highlighted the value of accurate typing for diagnosis [67–70], outbreak detection and surveillance and complete genome sequencing can enhance its effectiveness by providing higher resolution data for identifying variants, tracking transmission, and informing public health responses [15–20,71]. In case of poliovirus surveillance, it may be beneficial to have the higher resolution of complete genome data to more efficiently contribute to PV eradication efforts [71]. NGS adoption, however, necessitates carefully tailored approaches, depending on the benefits for routine diagnosis and public health application. While EV-tNGS is the most practical option for clinical diagnosis due to its ability to detect (known) viruses with high sensitivity and also for outbreak response due to its ability to define transmission chains more accurately, VIR-mNGS provides an unbiased approach for novel virus identification but involves additional steps for optimizing the output of viral reads, in particular, in cases where the etiological agent cannot be identified through targeted approaches.

The group emphasized the importance of harmonized wet-lab and dry-lab protocols, robust quality controls throughout the workflow, and the need for regularly updated reference databases. Standardization of protocols and guidelines across laboratories were identified as priorities for advancing NGS in clinical and public health settings.

To enable the effective implementation of NGS for EVs, the following actions are considered by this group:

- Standardization of NGS protocols and guidelines: Harmonized and validated wet-lab and dry-lab protocols and guidelines for standardization of quality parameters for laboratory procedures, bioinformatic analysis workflows, under IVDR and ISO15189 requirements [72–75], in particular for clinical use are emphasized as highly important. Clinical guidelines for early-stage and accurate sample collection by clinicians to ensure high-quality nucleic acid material [63] are crucial. Sample collection should be guided by clinical symptoms and the suspected EV type [25]. The adoption of harmonized protocols across multiple laboratories, can lead to improved data comparability and reliability, enabling more accurate tracking of circulating strains and timely identification of emerging variants over time as seen with other viruses [76,77].
- Maintenance and accessibility of reference databases: Regular updating and accurate annotation of EV/viral reference databases are considered crucial for detection and characterization of EVs. The use of cloud-based bioinformatics pipelines can improve data accessibility and collaboration between laboratories. [78].
- EQA and collaboration: Establishing an EQA program for EV NGS - both wet-lab and dry-lab assessment is a priority. This will allow direct comparison of protocols and tools suggested and address their strengths and weaknesses [66,79].

Collaboration among laboratories, clinicians, and bioinformaticians within ENPEN [25,26], the ESCV Network on NGS (ENNGS) [9,10] and ESCV is essential to support widespread implementation of EV NGS. Within the working group, comprehensive personnel training and EQAs can be provided, which is essential to ensure proper protocol implementation. In addition, procedures for the collection of clinical and epidemiological data and the integration thereof with NGS-generated data will enhance data sharing and collaborative analysis [15,74]. Improved accessibility to NGS-based EV data will facilitate early outbreak detection, clinical diagnostics, and research on EV evolution, epidemiology and disease burden – particularly concerning co-infections - on both regional and global scales.

Author contributions

The study was initiated by members of the ENPEN steering committee (KSMB, NB, TKF, HH, and CKJ) and co-written with all authors from the first draft to the final submitted version. The co-writing was coordinated by KSMB, together with FZ who structured the dry-lab section.

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Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Given their role as co-author, Prof Mario Poljak had no involvement in the peer-review of this article and has no access to information regarding its peer-review. Full responsibility for the editorial process for this article was delegated to another journal editor. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We would like to thank ESCV for supporting the ENPEN workshop held on 23–24 May 2024 in Corfu, Greece, that led to the initiation of this paper.

References

[1] M.S. Oberste, K. Maher, D.R. Kilpatrick, M.A. Pallansch, Molecular evolution of the human enteroviruses: correlation of serotype with VP1 sequence and application to picornavirus classification, J. Virol. 73 (3) (1999) 1941–1948, https://doi.org/10.1128/JVI.73.3.1941-1948.1999.

[2] M.S. Oberste, K. Maher, D.R. Kilpatrick, M.R. Flemister, B.A. Brown, M. A. Pallansch, Typing of human enteroviruses by partial sequencing of VP1, J. Clin. Microbiol 37 (5) (1999) 1288–1293.

[3] A.N. Lukashev, Role of recombination in evolution of enteroviruses, Rev. Med. Virol. 15 (3) (2005) 157–167, https://doi.org/10.1002/rmv.457.

[4] A.N. Lukashev, Recombination among picornaviruses, Rev. Med. Virol. 20 (5) (2010) 327–337, https://doi.org/10.1002/rmv.660.

[5] P. Simmonds, Recombination and selection in the evolution of picornaviruses and other Mammalian positive-stranded RNA viruses, J. Virol. 80 (22) (2006) 11124–11140, https://doi.org/10.1128/JVI.01076-06.

[6] P. Simmonds, J. Welch, Frequency and dynamics of recombination within different species of human enteroviruses, J. Virol. 80 (1) (2006) 483–493, https://doi.org/10.1128/JVI.80.1.483-493.2006.

[7] M. Nijhuis, N. van Maarseveen, R. Schuurman, S. Verkuijlen, M. de Vos, K. Hendriksen, et al., Rapid and sensitive routine detection of all members of the genus enterovirus in different clinical specimens by real-time PCR, J. Clin. Microbiol. 40 (10) (2002) 3666–3670, https://doi.org/10.1128/JCM.40.10.3666-3670.2002.

[8] W.A. Nix, M.S. Oberste, M.A. Pallansch, Sensitive, seminested PCR amplification of VP1 sequences for direct identification of all enterovirus serotypes from original clinical specimens, J. Clin. Microbiol. 44 (8) (2006) 2698–2704, https://doi.org/10.1128/JCM.00542-06.

[9] J.J.C. de Vries, J.R. Brown, N. Couto, M. Beer, P. Le Mercier, I. Sidorov, et al., Recommendations for the introduction of metagenomic next-generation sequencing in clinical virology, part II: bioinformatic analysis and reporting, J. Clin. Virol. 138 (2021) 104812, https://doi.org/10.1016/j.jcv.2021.104812.

[10] F.X. Lopez-Labrador, J.R. Brown, N. Fischer, H. Harvala, S. Van Boheemen, O. Cinek, et al., Recommendations for the introduction of metagenomic high-throughput sequencing in clinical virology, part I: Wet lab procedure, J. Clin. Virol. 134 (2021) 104691, https://doi.org/10.1016/j.jcv.2020.104691.

[11] X. Wang, S. Stelzer-Braid, M. Scotch, W.D. Rawlinson, Detection of respiratory viruses directly from clinical samples using next-generation sequencing: a literature review of recent advances and potential for routine clinical use, Rev. Med. Virol. 32 (5) (2022) e2375, https://doi.org/10.1002/rmv.2375.

[12] M. Duval, A. Mirand, O. Lesens, J.O. Bay, D. Caillaud, D. Gallot, et al., Retrospective study of the upsurge of Enterovirus D68 Clade D1 among adults (2014-2018), Viruses 13 (8) (2021), https://doi.org/10.3390/v13081607.

[13] K.E. Leon, R.D. Schubert, D. Casas-Alba, I.A. Hawes, P.S. Ramachandran, A. Ramesh, et al., Genomic and serologic characterization of enterovirus A71

brainstem encephalitis, Neurol. Neuroimmunol. Neuroinflamm. 7 (3) (2020), https://doi.org/10.1212/NXI.0000000000000703.
[14] A.L. Greninger, S.N. Naccache, K. Messacar, A. Clayton, G. Yu, S. Somasekar, et al., A novel outbreak enterovirus D68 strain associated with acute flaccid myelitis cases in the USA (2012-14): a retrospective cohort study, Lancet Infect. Dis. 15 (6) (2015) 671–682, https://doi.org/10.1016/S1473-3099(15)70093-9.
[15] K.S.M. Benschop, E.K. Broberg, E. Hodcroft, D. Schmitz, J. Albert, A. Baicus, et al., Molecular epidemiology and evolutionary trajectory of emerging Echovirus 30, Europe, Emerg. Infect. Dis. 27 (6) (2021) 1616–1626, https://doi.org/10.3201/eid2706.203096.
[16] E.C. McWilliam Leitch, J. Bendig, M. Cabrerizo, J. Cardosa, T. Hyypia, O. E. Ivanova, et al., Transmission networks and population turnover of echovirus 30, J. Virol. 83 (5) (2009) 2109–2118, https://doi.org/10.1128/JVI.02109-08.
[17] E.C. McWilliam Leitch, M. Cabrerizo, J. Cardosa, H. Harvala, O.E. Ivanova, S. Koike, et al., The association of recombination events in the founding and emergence of subgenogroup evolutionary lineages of human enterovirus 71, J. Virol. 86 (5) (2012) 2676–2685, https://doi.org/10.1128/JVI.06065-11.
[18] E.C. McWilliam Leitch, M. Cabrerizo, J. Cardosa, H. Harvala, O.E. Ivanova, A. C. Kroes, et al., Evolutionary dynamics and temporal/geographical correlates of recombination in the human enterovirus echovirus types 9, 11, and 30, J. Virol. 84 (18) (2010) 9292–9300, https://doi.org/10.1128/JVI.00783-10.
[19] M. Grapin, A. Mirand, D. Pinquier, A. Basset, M. Bendavid, M. Bisseux, et al., Severe and fatal neonatal infections linked to a new variant of echovirus 11, France, July 2022 to April 2023, Eur. Surveill. 28 (22) (2023), https://doi.org/10.2807/1560-7917.ES.2023.28.22.2300253.
[20] A. Piralla, A. Borghesi, A. Di Comite, F. Giardina, G. Ferrari, S. Zanette, et al., Fulminant echovirus 11 hepatitis in male non-identical twins in northern Italy, April 2023, Eur. Surveill. 28 (24) (2023), https://doi.org/10.2807/1560-7917.ES.2023.28.24.2300289.
[21] M. Majumdar, D. Klapsa, T. Wilton, J. Akello, C. Anscombe, D. Allen, et al., Isolation of Vaccine-Like Poliovirus strains in sewage samples from the United Kingdom, J. Infect. Dis. 217 (8) (2018) 1222–1230, https://doi.org/10.1093/infdis/jix667.
[22] A.G. Shaw, M. Majumdar, C. Troman, A. O'Toole, B. Benny, D. Abraham, et al., Rapid and sensitive direct detection and identification of Poliovirus from stool and environmental surveillance samples by use of Nanopore sequencing, J. Clin. Microbiol. 58 (9) (2020), https://doi.org/10.1128/JCM.00920-20.
[23] D. Klapsa, T. Wilton, A. Zealand, E. Bujaki, E. Saxentoff, C. Troman, et al., Sustained detection of type 2 poliovirus in London sewage between February and July, 2022, by enhanced environmental surveillance, Lancet 400 (10362) (2022) 1531–1538, https://doi.org/10.1016/S0140-6736(22)01804-9.
[24] European Centre for Disease Prevention and Control. Long-term surveillance framework 2021–2027. April 2023.Available from: ⟨https://www.ecdc.europa.eu/sites/default/files/documents/long-term-surveillance-framework-2021-2027.pdf⟩.
[25] H. Harvala, E. Broberg, K. Benschop, N. Berginc, S. Ladhani, P. Susi, et al., Recommendations for enterovirus diagnostics and characterisation within and beyond Europe, J. Clin. Virol. 101 (2018) 11–17, https://doi.org/10.1016/j.jcv.2018.01.008.
[26] H. Harvala, K.S.M. Benschop, N. Berginc, S. Midgley, K. Wolthers, P. Simmonds, et al., European non-polio enterovirus network: Introduction of hospital-based surveillance network to understand the true disease burden of Non-Polio Enterovirus and Parechovirus infections in Europe, Microorganisms 9 (9) (2021), https://doi.org/10.3390/microorganisms9091827.
[27] J.O. Akello, E. Bujaki, A.G. Shaw, A. Khurshid, Y. Arshad, C. Troman, et al., Comparison of eleven RNA extraction methods for Poliovirus direct molecular detection in stool samples, Microbiol. Spectr. 11 (2) (2023) e0425222, https://doi.org/10.1128/spectrum.04252-22.
[28] M.L. Joffret, P.M. Polston, R. Razafindratsimandresy, M. Bessaud, J.M. Heraud, F. Delpeyroux, Whole Genome Sequencing of Enteroviruses species A to D by high-throughput sequencing: application for viral mixtures, Front Microbiol 9 (2018) 2339, https://doi.org/10.3389/fmicb.2018.02339.
[29] S.R. Isaacs, K.W. Kim, J.X. Cheng, R.A. Bull, S. Stelzer-Braid, F. Luciani, et al., Amplification and next generation sequencing of near full-length human enteroviruses for identification and characterisation from clinical samples, Sci. Rep. 8 (1) (2018) 11889, https://doi.org/10.1038/s41598-018-30322-y.
[30] M. Arita, D.R. Kilpatrick, T. Nakamura, C.C. Burns, D. Bukbuk, S.B. Oderinde, et al., Development of an efficient entire-capsid-coding-region amplification method for direct detection of poliovirus from stool extracts, J. Clin. Microbiol. 53 (1) (2015) 73–78, https://doi.org/10.1128/JCM.02384-14.
[31] A. Stern, M.T. Yeh, T. Zinger, M. Smith, C. Wright, G. Ling, et al., The evolutionary pathway to virulence of an RNA virus, e19, Cell 169 (1) (2017) 35–46, https://doi.org/10.1016/j.cell.2017.03.013.
[32] R.P. Minnaar, G. Koen, K. de Haan, K.C. Wolthers, K.S. Benschop, Evaluation of two (semi-)nested VP1 based-PCRs for typing enteroviruses directly from cerebral spinal fluid samples, J. Virol. Methods 185 (2) (2012) 228–233, https://doi.org/10.1016/j.jviromet.2012.07.009.
[33] V. Marx, Method of the year: long-read sequencing, Nat. Methods 20 (1) (2023) 6–11, https://doi.org/10.1038/s41592-022-01730-w.
[34] M. Vilsker, Y. Moosa, S. Nooij, V. Fonseca, Y. Ghysens, K. Dumon, et al., Genome detective: an automated system for virus identification from high-throughput sequencing data, Bioinformatics 35 (5) (2019) 871–873, https://doi.org/10.1093/bioinformatics/bty695.
[35] M. Kearse, R. Moir, A. Wilson, S. Stones-Havas, M. Cheung, S. Sturrock, et al., Geneious basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data, Bioinformatics 28 (12) (2012) 1647–1649, https://doi.org/10.1093/bioinformatics/bts199.
[36] CLC workbench [Available from: ⟨https://digitalinsights.qiagen.com/⟩].
[37] C. Galaxy, The Galaxy platform for accessible, reproducible, and collaborative data analyses: 2024 update, Nucleic Acids Res. 52 (W1) (2024) W83–W94, https://doi.org/10.1093/nar/gkae410.
[38] Galaxy [Available from: ⟨https://github.com/OpenGene/fastplong⟩].
[39] D. Schmitz, F. Zwagemaker, S. Nooij, T.K.S. Janssens, J. Cremer, R. Verhagen, et al., Accessible viral metagenomics for public health and clinical domains with Jovian, Sci. Rep. 14 (1) (2024) 26018, https://doi.org/10.1038/s41598-024-73785-y.
[40] V.R. Marcelino, P. Clausen, J.P. Buchmann, M. Wille, J.R. Iredell, W. Meyer, et al., CCMetagen: comprehensive and accurate identification of eukaryotes and prokaryotes in metagenomic data, Genome Biol. 21 (1) (2020) 103, https://doi.org/10.1186/s13059-020-02014-2.
[41] CZ ID metagenomics [Available from: ⟨https://czid.org/⟩].
[42] C. Launes, J. Camacho, M. Pons-Espinal, F.X. Lopez-Labrador, C. Esteva, M. Cabrerizo, et al., Hybrid capture shotgun sequencing detected unexpected viruses in the cerebrospinal fluid of children with acute meningitis and encephalitis, Eur. J. Clin. Microbiol. Infect. Dis. 43 (5) (2024) 863–873, https://doi.org/10.1007/s10096-024-04795-x.
[43] Pikavirus [Available from: ⟨https://repositorio.uam.es/handle/10486/700289⟩].
[44] InsaFlu [Available from: ⟨https://insaflu.insa.pt⟩].
[45] ViroConstrictor [Available from: ⟨https://github.com/RIVM-bioinformatics/ViroConstrictor⟩].
[46] NFCore [Available from: ⟨https://github.com/nf-core/viralrecon⟩].
[47] R. Mayne, S. Secret, C. Geoghegan, A. Trebes, K. Kean, K. Reid, et al., Castanet: a pipeline for rapid analysis of targeted multi-pathogen genomic data, Bioinformatics 40 (10) (2024), https://doi.org/10.1093/bioinformatics/btae591.
[48] Artic network [Available from: ⟨https://github.com/artic-network/fieldbioinformatics⟩].
[49] M. Holzer, M. Marz, De novo transcriptome assembly: a comprehensive cross-species comparison of short-read RNA-Seq assemblers, Gigascience 8 (5) (2019), https://doi.org/10.1093/gigascience/giz039.
[50] B.M. Cosma, R. Shirali Hossein Zade, E.N. Jordan, P. van Lent, C. Peng, S. Pillay, et al., Evaluating long-read de novo assembly tools for eukaryotic genomes: insights and considerations, Gigascience 12 (2022), https://doi.org/10.1093/gigascience/giad100.
[51] J. Maric, K. Krizanovic, S. Riondet, N. Nagarajan, M. Sikic, Comparative analysis of metagenomic classifiers for long-read sequencing datasets, BMC Bioinforma. 25 (1) (2024) 15, https://doi.org/10.1186/s12859-024-05634-8.
[52] NCBI [Available from: ⟨https://blast.ncbi.nlm.nih.gov/⟩].
[53] A. Kroneman, H. Vennema, K. Deforche, H. v d Avoort, S. Penaranda, M. S. Oberste, et al., An automated genotyping tool for enteroviruses and noroviruses, J. Clin. Virol. 51 (2) (2011) 121–125, https://doi.org/10.1016/j.jcv.2011.03.006.
[54] Picornavirus study group [Available from: ⟨http://www.picornastudygroup.com/⟩ 1.
[55] MEGA12 [Available from: ⟨https://www.megasoftware.net/⟩].
[56] A. Stamatakis, RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies, Bioinformatics 30 (9) (2014) 1312–1313, https://doi.org/10.1093/bioinformatics/btu033.
[57] L.T. Nguyen, H.A. Schmidt, A. von Haeseler, B.Q. Minh, IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies, Mol. Biol. Evol. 32 (1) (2015) 268–274, https://doi.org/10.1093/molbev/msu300.
[58] J. Hadfield, C. Megill, S.M. Bell, J. Huddleston, B. Potter, C. Callender, et al., Nextstrain: real-time tracking of pathogen evolution, Bioinformatics 34 (23) (2018) 4121–4123, https://doi.org/10.1093/bioinformatics/bty407.
[59] S. Samson, E. Lord, V. Makarenkov, SimPlot++: a Python application for representing sequence similarity and detecting recombination, Bioinformatics 38 (11) (2022) 3118–3120, https://doi.org/10.1093/bioinformatics/btac287.
[60] D.P. Martin, B. Murrell, M. Golden, A. Khoosal, B. Muhire, RDP4: detection and analysis of recombination patterns in virus genomes, Virus Evol. 1 (1) (2015) vev003, https://doi.org/10.1093/ve/vev003.
[61] D.P. Martin, A. Varsani, P. Roumagnac, G. Botha, S. Maslamoney, T. Schwab, et al., RDP5: a computer program for analyzing recombination in, and removing signals of recombination from, nucleotide sequence datasets, Virus Evol. 7 (1) (2021) veaa087, https://doi.org/10.1093/ve/veaa087.
[62] S.L. Kosakovsky Pond, D. Posada, M.B. Gravenor, C.H. Woelk, S.D. Frost, GARD: a genetic algorithm for recombination detection, Bioinformatics 22 (24) (2006) 3096–3098, https://doi.org/10.1093/bioinformatics/btl474.
[63] J. Flipse, A.T. Tromp, D. Thijssen, N. van Xanten-Jans-Beken, R. Pauwelsen, H. J. van der Veer, et al., Optimization of the STARlet workflow for semi-automatic SARS-CoV-2 screening of swabs and deep respiratory materials using the RealAccurate Quadruplex SARS-CoV-2 PCR kit and Allplex SARS-CoV-2 PCR kit, Microbiol Spectr. 12 (2) (2024) e0329623, https://doi.org/10.1128/spectrum.03296-23.
[64] E. Bailly, S. Valot, A. Vincent, Y. Duffourd, N. Grangier, M. Chevarin, et al., Evaluation of next-generation sequencing applied to cryptosporidium parvum and cryptosporidium hominis epidemiological study, Pathogens 11 (8) (2022), https://doi.org/10.3390/pathogens11080938.
[65] C. Endrullat, J. Glokler, P. Franke, M. Frohme, Standardization and quality management in next-generation sequencing, Appl. Transl. Genom. 10 (2016) 2–9, https://doi.org/10.1016/j.atg.2016.06.001.

[66] van Walle I., Simon-Loriere E. European SARS-CoV-2 and influenza Bioinformatics External Quality Assessment (ESIB-EQA), 2023. 2022.Available from: ⟨https://www.ecdc.europa.eu/sites/default/files/documents/SARS-CoV-2-influenza-bioinformatics-external-quality-assessment-2023.pdf⟩.

[67] J. Wise, Myocarditis: Unusual cluster of cases in babies is identified in Wales, BMJ 381 (2023) 1151, https://doi.org/10.1136/bmj.p1151.

[68] K. Takats, B. Balazs, A. Boros, D. Sipos, Z. Peterfi, M. Harmat, et al., A meningoencephalitis outbreak associated with echovirus type 18 (E18) in south-western Hungary in mid-2023, Arch. Virol. 169 (11) (2024) 237, https://doi.org/10.1007/s00705-024-06166-5.

[69] S. de Schrijver, E. Vanhulle, A. Ingenbleek, L. Alexakis, C.K. Johannesen, E. K. Broberg, et al., Epidemiological and clinical insights into enterovirus circulation in Europe, 2018-2023: a multicenter retrospective surveillance study, J. Infect. Dis. 232 (1) (2025) e104–e115, https://doi.org/10.1093/infdis/jiaf179.

[70] H. Harvala, C.K. Johannesen, K.S.M. Benschop, E.V. Saxentoff, S. Huseynov, J. E. Hagan, et al., Enterovirus circulation in the WHO European region, 2015-2022: a comparison of data from WHO's three core poliovirus surveillance systems and the European non-polio enterovirus network (ENPEN), Lancet Reg. Health Eur. 53 (2025) 101292, https://doi.org/10.1016/j.lanepe.2025.101292.

[71] N.S. Zuckerman, E. Bucris, H. Morad-Eliyahu, L. Weiss, R. Vasserman, I.S. Fratty, et al., Environmental surveillance of a circulating vaccine-derived poliovirus type 2 outbreak in Israel between 2022 and 2023: a genomic epidemiology study, Lancet Microbe 5 (10) (2024) 100893, https://doi.org/10.1016/S2666-5247(24)00116-2.

[72] A.L. Greninger, The challenge of diagnostic metagenomics, Expert Rev. Mol. Diagn. 18 (7) (2018) 605–615, https://doi.org/10.1080/14737159.2018.1487292.

[73] L. Schuele, H. Cassidy, N. Peker, J.W.A. Rossen, N. Couto, Future potential of metagenomics in microbiology laboratories, Expert Rev. Mol. Diagn. 21 (12) (2021) 1273–1285, https://doi.org/10.1080/14737159.2021.2001329.

[74] E. Mutschler, T. Roloff, A. Neves, H. Vangstein Aamot, B. Rodriguez-Sanchez, M. Ramirez, et al., Towards unified reporting of genome sequencing results in clinical microbiology, PeerJ 12 (2024) e17673, https://doi.org/10.7717/peerj.17673.

[75] C.Y. Chiu, F.X. Lopez-Labrador, M.R. Wilson, J.J.C. de Vries, The regulatory landscape for clinical metagenomic testing, JAMA Neurol. (2025), https://doi.org/10.1001/jamaneurol.2025.0461.

[76] L.W. Meredith, W.L. Hamilton, B. Warne, C.J. Houldcroft, M. Hosmillo, A. S. Jahun, et al., Rapid implementation of SARS-CoV-2 sequencing to investigate cases of health-care associated COVID-19: a prospective genomic surveillance study, Lancet Infect. Dis. 20 (11) (2020) 1263–1272, https://doi.org/10.1016/S1473-3099(20)30562-4.

[77] Y. Shu, J. McCauley, GISAID: Global initiative on sharing all influenza data - from vision to reality, Eur. Surveill. 22 (13) (2017), https://doi.org/10.2807/1560-7917.ES.2017.22.13.30494.

[78] J.G. Chappell, T. Byaruhanga, T. Tsoleridis, J.K. Ball, C.P. McClure, Identification of infectious agents in high-throughput sequencing data sets is easily achievable using free, cloud-based bioinformatics platforms, J. Clin. Microbiol. 57 (12) (2019), https://doi.org/10.1128/JCM.01386-19.

[79] J.J.C. de Vries, J.R. Brown, N. Fischer, I.A. Sidorov, S. Morfopoulou, J. Huang, et al., Benchmark of thirteen bioinformatic pipelines for metagenomic virus diagnostics using datasets from clinical samples, J. Clin. Virol. 141 (2021) 104908, https://doi.org/10.1016/j.jcv.2021.104908.

[80] K. Mourik, I. Sidorov, E.C. Carbo, D. van der Meer, A. Boot, A.C.M. Kroes, et al., Comparison of the performance of two targeted metagenomic virus capture probe-based methods using reference control materials and clinical samples, J. Clin. Microbiol. 62 (6) (2024) e0034524, https://doi.org/10.1128/jcm.00345-24.

[81] K. Chrzastek, D.H. Lee, D. Smith, P. Sharma, D.L. Suarez, M. Pantin-Jackwood, et al., Use of sequence-independent, single-primer-amplification (SISPA) for rapid detection, identification, and characterization of avian RNA viruses, Virology 509 (2017) 159–166, https://doi.org/10.1016/j.virol.2017.06.019.

[82] M. Majumdar, D. Klapsa, T. Wilton, E. Bujaki, M.D. Fernandez-Garcia, T.O. C. Faleye, et al., High diversity of human Non-Polio Enterovirus serotypes identified in contaminated water in Nigeria, Viruses 13 (2) (2021), https://doi.org/10.3390/v13020249.

[83] M. Schubert, S. Lindgreen, L. Orlando, AdapterRemoval v2: rapid adapter trimming, identification, and read merging, BMC Res. Notes 9 (2016) 88, https://doi.org/10.1186/s13104-016-1900-2.

[84] Krueger F.J.F., Ewels P. FelixKrueger/TrimGalore: v0.6.10 2023 [Available from: ⟨https://github.com/FelixKrueger/TrimGalore⟩].

[85] Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet journal. https://doi.org/10.14806/ej.17.1.200.

[86] FASTQC [Available from: ⟨https://www.bioinformatics.babraham.ac.uk/projects/fastqc⟩/].

[87] R. Lanfear, M. Schalamun, D. Kainer, W. Wang, B. Schwessinger, MinIONQC: fast and simple quality control for MinION sequencing data, Bioinformatics 35 (3) (2019) 523–525, https://doi.org/10.1093/bioinformatics/bty654.

[88] W. De Coster, R. Rademakers, NanoPack2: population-scale evaluation of long-read sequencing data, Bioinformatics 39 (5) (2023), https://doi.org/10.1093/bioinformatics/btad311.

[89] S. Chen, Y. Zhou, Y. Chen, J. Gu, fastp: an ultra-fast all-in-one FASTQ preprocessor, Bioinformatics 34 (17) (2018) i884–i890, https://doi.org/10.1093/bioinformatics/bty560.

[90] FastP [Available from: ⟨https://github.com/boulund/adapter_benchmark⟩].

[91] BBDuk [Available from: ⟨https://sourceforge.net/projects/bbmap/⟩].

[92] FiltLong [Available from: ⟨https://github.com/rrwick/Filtlong⟩].

[93] FastP Long [Available from: ⟨https://github.com/OpenGene/fastplong⟩].

[94] Zwagemaker F., Hajji, K., & The RIVM-IDS Bioinformatics team. AmpliGone [Computer software]. https://doi.org/10.5281/zenodo.7684307.

[95] Artic trim [Available from: ⟨https://github.com/artic-network/fieldbioinformatics/blob/master/artic/align_trim.py⟩].

[96] B. Constantinides, M. Hunt, D.W. Crook, Hostile: accurate decontamination of microbial host sequences, Bioinformatics 39 (12) (2023), https://doi.org/10.1093/bioinformatics/btad728.

[97] B. Constantinides, J. Lees, D.W. Crook, Deacon: fast sequence filtering and contaminant depletion, bioRxiv (2025), https://doi.org/10.1101/2025.06.09.658732.

[98] NoHuman [Available from: ⟨https://github.com/mbhall88/nohuman⟩].

[99] H. Li, New strategies to improve minimap2 alignment accuracy, Bioinformatics 37 (23) (2021) 4572–4574, https://doi.org/10.1093/bioinformatics/btab705.

[100] H. Li, Minimap2: pairwise alignment for nucleotide sequences, Bioinformatics 34 (18) (2018) 3094–3100, https://doi.org/10.1093/bioinformatics/bty191.

[101] M. Vasimuddin, M.L.H. Misra, S. Aluru, Efficient architecture-aware acceleration of BWA-MEM for multicore systems. Proceedings of the IEEE International Parallel and Distributed Processing Symposium (IPDPS), 2019, 10.1109/IPDPS.2019.00041.

[102] A. Latorre-Perez, P. Villalba-Bermell, J. Pascual, C. Vilanova, Assembly methods for nanopore-based metagenomic sequencing: a comparative study, Sci. Rep. 10 (1) (2020) 13588, https://doi.org/10.1038/s41598-020-70491-3.

[103] A. Prjibelski, D. Antipov, D. Meleshko, A. Lapidus, A. Korobeynikov, Using SPAdes De Novo Assembler, Curr. Protoc. Bioinforma. 70 (1) (2020) e102, https://doi.org/10.1002/cpbi.102.

[104] S. Nurk, D. Meleshko, A. Korobeynikov, P.A. Pevzner, metaSPAdes: a new versatile metagenomic assembler, Genome Res. 27 (5) (2017) 824–834, https://doi.org/10.1101/gr.213959.116.

[105] D. Antipov, M. Raiko, A. Lapidus, P.A. Pevzner, Metaviral SPAdes: assembly of viruses from metagenomic data, Bioinformatics 36 (14) (2020) 4126–4129, https://doi.org/10.1093/bioinformatics/btaa490.

[106] D. Li, C.M. Liu, R. Luo, K. Sadakane, T.W. Lam, MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph, Bioinformatics 31 (10) (2015) 1674–1676, https://doi.org/10.1093/bioinformatics/btv033.