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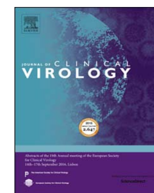

Review

Recommendations for enterovirus diagnostics and characterisation within and beyond Europe

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ABSTRACT

Enteroviruses (EV) can cause severe neurological and respiratory infections, and occasionally lead to devastating outbreaks as previously demonstrated with EV-A71 and EV-D68 in Europe. However, these infections are still often underdiagnosed and EV typing data is not currently collected at European level. In order to improve EV diagnostics, collate data on severe EV infections and monitor the circulation of EV types, we have established European non-polio enterovirus network (ENPEN). First task of this cross-border network has been to ensure prompt and adequate diagnosis of these infections in Europe, and hence we present recommendations for non-polio EV detection and typing based on the consensus view of this multidisciplinary team including experts from over 20 European countries. We recommend that respiratory and stool samples in addition to cerebrospinal fluid (CSF) and blood samples are submitted for EV testing from patients with suspected neurological infections. This is vital since viruses like EV-D68 are rarely detectable in CSF or stool samples. Furthermore, reverse transcriptase PCR (RT-PCR) targeting the 5′noncoding regions (5′NCR) should be used for diagnosis of EVs due to their sensitivity, specificity and short turnaround time. Sequencing of the VP1 capsid protein gene is recommended for EV typing; EV typing cannot be based on the 5′NCR sequences due to frequent recombination events and should not rely on virus isolation. Effective and standardized laboratory diagnostics and characterisation of circulating virus strains are the first step towards effective and continuous surveillance activities, which in turn will be used to provide better estimation on EV disease burden.

1. Introduction – why should we diagnose enterovirus infections?

Enteroviruses (EVs) and human rhinoviruses (HRVs) are members of the *Enterovirus* genus of the virus family *Picornaviridae.* EVs infecting humans are classified into four species (A–D) and HRVs into three species (A–C) on the basis of genetic divergence [1]. The most well-known are polioviruses (PV), classified within species C enteroviruses and the target of a global polio eradication program. This guideline focuses on the detection and characterisation of non-polio enteroviruses.

EVs are a common cause of self-limiting febrile illnesses in infants and young children but can occasionally cause severe disease including meningoencephalitis, myelitis, paralysis, myocarditis, sepsis-like syndrome, respiratory disease and acute hepatitis [2–4]. EVs are now recognised as the most common cause of meningitis, and infections with some EV types also been linked to acute flaccid myelitis (AFM) and paralysis (AFP) [5]. Whereas both AFM and AFP involve clinical signs of rapid limb weakness with low muscle tone, changes in the gray matter of the spinal cord demonstrated by magnetic resonance imaging [MRI] are typical for AFM cases only [6–13]. Certain EV types, notably EV-A71 and EV-D68, have also been associated with outbreaks, occasionally resulting in significant morbidity and mortality [6,9–18]. EV-A71 has been responsible for large hand, foot and mouth disease (HFMD) outbreaks associated with rare but severe cases of rhombencephalitis in Asia and, more recently, in Europe [9,10], whereas an EV-D68 epidemic has been associated with severe respiratory disease, occasionally leading to AFM in the Northern America and Europe [6,11–18]. The potential neurotropism of EVs and their ability to cause severe infections reinforces the need for the monitoring and characterization of EV types associated with these different clinical presentations.

Enteroviruses have been recognised since the first image of poliovirus (PV) was taken in 1952 by electron microscopy (EM) [19]. Since then, we have witnessed the transition from the use of EM or suckling mice to cell culture for propagation and detection of EV, followed by the replacement of cell culture by molecular methods. With every step, sensitivity has been improved, and faster turnaround times for establishing a clinical diagnosis have been gained. However, the virus culture is still used for detection of PV [20].

Proper and early detection, combined with genetic characterization of EVs in appropriately collected specimens, is essential both at the individual and the community levels for several reasons [21,22]. Prompt laboratory diagnosis of an EV infection may reduce antibiotic usage, limit unnecessary and costly investigations, shorten the length of hospitalisation and minimise the risk of complications [23,24]. In addition, it enables health care providers to respond in a timely fashion with infection control measures as well as to evaluate the usefulness of potential therapies such as novel antiviral or immunotherapies and provide clues for the further vaccine development. Objectives for identification of the EV types involved, especially in patients with severe clinical presentations and for investigating outbreaks, include:

1) To identify EV types associated with more severe conditions and monitor their associated disease burden;
2) To identify the specific EV type associated with outbreak;
3) To confirm the absence of circulation and importation of poliovirus;
4) To monitor the molecular epidemiology and geographical distribution of EV types or strains known to be associated with severe or specific disease, such as EV-A71 and EV-D68;
5) To detect and monitor the appearance of new EV types or new recombinant forms of known EV types.

Continuous surveillance will provide knowledge of EV infection outcomes and recognition of their disease presentations, allow monitoring the emergence of new EV strains, better estimation of EV disease burden and is also required for potential implementation of vaccination programmes (e.g. EV-A71 in China [25]). Although over 7000 EV-positive samples were successfully typed by 24 EU/EEA countries in 2015, the extent of EV surveillance is variable across the region and the typing data is not currently collected at European level [26].

To achieve the objectives listed above, we have established the European Non-Polio Enterovirus Network (ENPEN) under the auspices of the European Society for Clinical Virology (ESCV) with founder participants from 22 European countries representing public health institutions, clinical specialities (paediatrics, neurology, internal medicine, microbiology and virology), national reference laboratories as well as academia. The ENPEN network aims to raise awareness across professional backgrounds as a prerequisite for more effective detection, and guide surveillance activities. In addition, a cross-border non-polio EV surveillance network is useful for harmonising diagnostic methods, obtaining and sharing epidemiological data as well as for the detection of outbreaks and emerging EVs. Such a network enables not only the exchange of knowledge and experience but also real-time alerts at the European level, as has been shown recently in the context of the global emergence of EV-D68 [12]. The development of these guidelines is the first task undertaken by the ENPEN.

2. Aim and scope

We summarized standardized recommendations for the detection and characterization of EVs based on a consensus view of the experts in the field.

3. Recommendations

3.1. What clinical specimens should be obtained for testing if EV infection is suspected and why?

1 Sampling should be performed according to the clinical manifestations, and as soon as possible after the symptom onset (Table 1).
2 In cases of neurological infections, respiratory specimen and stool in addition to the CSF sample and blood should be submitted for EV testing.

Diagnostic stewardship consists of agreement on a diagnostic algorithm according to different clinical presentations and standardized collection of clinical, epidemiological and virological data. Close collaboration with the clinician raising a suspicion of EV infection and obtaining appropriate samples for diagnostic testing is vital so that these algorithms will be followed. Clinical virologists have an important role in promoting collection of appropriate samples in cases of EV infection, including a close liaison with several clinical specialities such as neonatology, paediatrics, neurology, dermatology and cardiology.

EVs are transmitted via the faecal-oral or respiratory route and symptoms may develop after an incubation period of 3–21 days. Sample collection should be performed according to clinical manifestations (Table 1) and as soon as possible after the onset of symptoms. Specimen materials may include stool, CSF, nasopharyngeal aspirates (NPA), nasopharyngeal swabs, vesicular fluid, bronchoalveolar lavage (BAL), blood, conjunctival swabs, biopsy specimens (including nail tissue) and urine.

Reasons for sampling include diagnosis of acute illnesses, outbreak investigations and surveillance, where specimens suitable for further typing are required. In case of suspected EV infection, multiple samples should be collected from different sites. The type of specimen that would be most suitable to detect EVs will vary according to the time from the symptom onset and clinical manifestations

Since young age is associated with an increased risk of severe outcome of EV infection [27], patient age should be taken into account when considering the value of diagnostic sampling. In the neonate, EV infections can present as a sepsis-like illness with or without specific manifestations such as myocarditis, hepatitis and encephalitis [27]; EV can be detected in plasma, stool, respiratory secretions, as well as in CSF or tissue biopsies.

Table 1
Recommended clinical sample types for EV diagnosis in different clinical presentations.

Presentation	Sample types*	Comments
Meningitis/meningoencephalitis	**CSF**, stool AND respiratory sample, possible blood	EV RNA detectable in CSF in the majority of meningitis cases by PCR but inconsistently in encephalitis cases; EV excretion in throat and stool samples is prolonged but detection does not automatically demonstrate aetiological link.
Neonatal sepsis	**CSF**, stool, blood AND respiratory sample	Often difficult to distinguish from meningitis. Viral load can be higher in blood than in CSF, but requires further studies. Human parechovirus testing should also be performed, either as a first line investigation or if EV testing is not diagnostic.
Acute flaccid paralysis/myelitis	**Respiratory**, CSF, **stool** AND blood sample*	CSF sample should be tested for enteroviruses, but in many cases of EV-D68 and EV-A71 virus has been only detectable in respiratory tract specimen and/or stool specimen. Therefore testing of respiratory specimen are necessary for any clinical case with CNS/paralysis/myelitis involvement. In cases of classical AFP (without myelitis), consider also possibility of polio and obtain stool sample for the virus detection.
HFMD/other rash	**Vesicle fluid**, respiratory sample and/or stool	Usually high viral loads in vesicle fluid. CV-A6 has been associated with onychomadesis (nail shedding) and virus has also been recovered from finger nail in HFMD cases.
Respiratory disease	**Respiratory sample**, possibly stool	Usually nasopharyngeal aspirates or swabs recommended. Consider testing for both HRV and EV, and consider also cross-reactivity of PCR used.
Myocarditis	Stool and respiratory sample, blood and/or **heart biopsy**	Typing might be helpful as CV-Bs are typically associated with myocarditis. Tissue biopsy can be used to confirm diagnosis.
Conjunctivitis	**Eye swab**	Viral haemorrhagic conjunctivitis caused by enteroviruses is highly infectious.

CNS, central nervous system; CSF, cerebrospinal fluid; CV, coxsackie virus; EV, enteroviruses, HFMD, hand, foot and mouth disease; PCR, polymerase chain reaction. * Primary sample type has been marked as bold. ** Base line blood sample should to be collected before any blood products (i.e. intravenous immunoglobulin) are administered.

Pleocytosis with increased lymphocyte count is often, but not always, present in patients with EV meningitis but acellular CSF is also common, especially in children under 3 months old [28]. Patients suffering from aseptic meningitis, encephalitis or myelitis have detectable virus in stool and respiratory secretions for a longer period than spinal fluid, where virus remains sometimes undetectable. Furthermore, as viral loads are usually higher in stool, blood and respiratory samples than in CSF, these samples should be collected for EV identification. Respiratory specimens should always be collected from all AFM cases to exclude EV-D68 infection, as this virus has only rarely been detected in CSF or stool samples [29,30]. Furthermore, stool samples should be obtained from patients with suspected PV infection. For respiratory illnesses, virus can be found in throat swabs and NPA or BAL specimens. As EVs colonize the throat and gut for weeks to months, detection in these sites must be interpreted cautiously. In acute hemorrhagic conjunctivitis, a swab from conjunctiva can allow for detection of EV-D70 or coxsackievirus A24 (CV-A24) and, in HFMD cases, CV-A6, EV-A71 and CV-A16 can be identified in vesicular lesions, throat and stool. In epidemic pleurodynia (Bornholm disease), pleural fluid and stool samples should be investigated. In immunocompromised individuals, clinical presentation can be particularly non-specific and severe; therefore, the threshold for investigation should be low (especially in patients on IgG-depleting therapies such as rituximab). It should be noted that immunocompromised patients can shed EVs in stools for years [30].

3.2. Methods used for primary diagnosis

1. Reverse transcriptase PCR (RT-PCR) assays targeting the 5′non-coding region (NCR) should be used for diagnosis of enteroviruses due to their sensitivity, specificity and a short turnaround time (Table 2). However, it is vital to ensure that the method used will detect all EV types and is frequently updated. All laboratories performing EV testing should be accredited.
2. Virus isolation should not be used for routine diagnosis but can be used for further characterization of EVs. Virus culture skills should be maintained at the national level.
3. Serological methods such as ELISA and neutralisation tests should not be used for diagnosis of acute EV infection routinely.

The number of virology laboratories performing EV testing in each country varies from just one laboratory to over 100 laboratories per country [26], and hence the standardisation of testing as well as methods is vital. It is important that all laboratories have sensitive broad-spectrum molecular diagnostic assays for detection of all EVs to allow rapid detection of potential outbreaks (Table 2).

3.2.1. Molecular methods

RT-PCR has been shown to be far more sensitive than cell culture [20,31,32] for detection of EVs in clinical samples. RT-PCR-based assays for the detection of EV RNA mainly target the highly conserved 5′NCR and are, therefore, suitable for all EV types [32–36]. Correct sample preparation prior to nucleic acid extraction and detection is important, especially for stool samples, which should be disaggregated in buffer (e.g. saline) and clarified by centrifugation to avoid PCR inhibition. To identify the presence of inhibitory compounds in the extracted RNA, it is important to include an internal control. Any EV detection in stool sample should be carefully evaluated as it does not automatically imply causation, and might simply reflect prolonged extretion.

Despite their increased sensitivity and speed, some molecular detection methods may fail to detect certain EV types such as EV-D68, EV-C105 and EV-C109 [37–39]. It is important that primer and probe sequences are regularly reviewed to reflect the evolutionary and genomic changes of the viruses, and any assay used should be validated for the detection of a variety of different EV types (including at least one virus from each EV species). Several commercial real-time RT-PCR kits are now also available for the detection of EVs. More data is needed to confirm the sensitivity of commercial assays for the detection of EV types with divergent 5′NCR sequences [37–39]. Another complication of some 5′NCR-based RT-PCR assays is that their detection range may extend to human rhinoviruses, which potentially limits their usefulness for respiratory sample screening for EVs. For practical reasons, laboratories should use HRV and/or EV-specific assays already at the screening stage as typing all EV/HRV positive samples in order to identify EV-D68 is labour intensive.

During large EV outbreaks, such as those caused by EV-A71 in Asia, and EV-D68 in Europe and USA, type-specific RT-PCR assays have been developed to identify and type these EVs [12,40]. Such type-specific assays allow rapid and effective identification of the emerging virus

Table 2
Diagnostic and typing assays for non-polio enteroviruses.

Test	Target	Characteristics
Screening PCR	5′UTR	Recommended as primary assay for EV detection. Typically detects all EV types/species with equal sensitivity (but also potentially HRVs). Can provide semi-quantitation of viral loads.
	VP4, VP1	Rapid screening for defined EV types only (*e.g.* EV-A71, EV-D68), most likely to be used in outbreak situations or for surveillance purposes.
Virus typing	VP1	A minimum of 350nt long VP1 sequence is required for surveillance by reference laboratories. The complete VP1 sequence (~900nt) is necessary when assigning new EV types. Anyone describing new EV types and species should consult with the Picornavirus Study Group (http://www.picornaviridae.com/) prior to publication to ensure use of correct nomenclature.
	VP4, VP2	In case of VP1 region fails to amplify, other regions including VP2 and VP4 can be also utilized as these are generally simpler to amplify. However, recombination within the VP4 region limits their efficacy for identification of species B EV types.
	Whole genome	Next Generation Sequencing (NGS) methods are being developed in order to obtain the whole genome sequence of EVs, with or without prior amplification of genome. It is likely going to be used for EV typing in the near future. Sequencing the whole genome (or as a minimum the 3D polymerase gene region) is required for identification of new recombination events and the emergence of recombinant EVs. NGS method will also enhance the detection of mixed infections, especiallly caused by two EV types of same species.
Virus isolation	Whole virus	**Not recommended** as a front line screen. Competency in virus culture is required, useful for reference laboratories.
Virus neutralisation	Isolated virus	**Not recommended** as typing antisera are increasingly unavailable and limited in range. Requires prior virus isolation.
Serology	IgM, IgG	**Not recommended for non-polio EVs** as an IgM antibody response is not always detectable during acute disease, a second sample is often required to demonstrate the increase in IgG levels. Furthermore, there is a high prevalence of EV antibodies in the general population from previous exposure and, thus, serologic testing lacks clinical specificity. However, the diagnostic utility of intrathecal antibody detection using these available assays should also be explored.

EV, enterovirus; HRV, human rhinovirus; Nt, nucleotides; VP, viral protein.

strain during outbreaks, which is crucial for clinical and public health responses. Type-specific assays need also to be updated and re-validated regularly.

3.2.2. Cell culture

Although cell culture should no longer be used for routine EV diagnosis, we encourage reference laboratories to maintain virus culture facilities, to collect EV strains and make them available for diagnostics developmental work. Cell culture can be used for confirmation of PCR-negative specimens in case of a suspected new variant, for detection of more than one viral strain in a specimen, for typing by neutralisation, for microneutralisation assays to measure serum neutralizing antibodies, for assessment of effects of antivirals as well as for quality control purposes. For specimen collection, preparation for cell culture and methodology in virus isolation see guidelines by the WHO [41,42]. Antibody panels previously used to confirm EV serotype will no longer be provided by WHO collaboration with the National Institute for Public Health and the Environment, the Netherlands [26], but are still available via commercial routes.

Many EVs can be isolated in cell cultures of mammalian cell lines [43]. Often, at least three different cell lines, usually including monkey kidney cells and human fibroblasts are used [42]. It should be noted that species A EVs including EV-A71 grow poorly in cell cultures [36], and EV-D68 requires lower incubation temperatures than normally applied for EVs [44].

3.2.3. Serology

For the laboratory-confirmation of acute EV infection, serological methods such as ELISA and neutralisation tests are obsolete. Although specific IgM and IgG assays for EVs have been described and are available, their clinical utility is limited due to the cross-reactivity of the antigens used between the different serotypes. The two week interval between acute and convalescent samples makes diagnosis slow and often clinically irrelevant. However, when myocarditis, pericarditis or cardiomyopathy is suspected and direct sampling impossible, antibody detection can be useful for diagnosis.

Neutralization assays can be used to quantify neutralizing antibodies against selected EV type [34]. They are useful for seroprevalence studies to assess population immunity or for individual serological examination of paired serum samples. A 4-fold increase in EV-specific antibody concentrations between the acute and convalescent samples is diagnostic of recent infection, and 1:8 titre in neutralisation test would indicate protective antibody levels. However, as neutralisation assays are necessarily serotype-specific, testing for several EV antibodies is time-consuming and laboratory-intensive due to inherent requirements for different EV types.

3.3. State-of-the-art – enterovirus typing

1. Sequencing part of the VP1 capsid protein gene is the gold standard for EV typing, and should be performed according to the WHO recommendations [45].
2. 5′NCR sequencing should not be used for EV type identification
3. Use VP2 and VP4 capsid protein gene sequencing for EV typing if VP1 sequencing fails
4. Consider sequencing additional parts of the genome or obtaining full genome sequence if a new recombinant form of EV is suspected.

Within the EU/EEA region, EV typing is performed in most national reference laboratories (26/29 countries) but also at local virology laboratories (11/29 countries) [26]. The referral of samples to reference laboratories for virus typing or sharing typing data is essential for effective monitoring of EV circulation and the early detection of emerging virus types at a national level. Effective monitoring additionally requires that both screening assays and typing methods used are of comparable sensitivity and effectiveness for detection of different EV types and species.

Sequence relationships in any genome region are predictive of EV species except the 5′NCR. Originally, the assignment of EV types within species was based on cross-neutralisation properties. Later, it was shown that these correlate well with sequence divergence in VP1 region [46]. The term ”type” can be used to describe both genotypic assignments and those based on neutralisation properties.

Recombination is a frequent event between EV types within the same species and usually occurs between structural and non-structural regions in EV species A-C (but rarely if at all in EV-D or human rhinovirus species A–C) [47]. There are also further recombinations in all species between the structural gene region and the 5′NCR. For these reasons, type identification should be based on VP1 (or potentially VP2 or VP3) sequences EV species A–C (and recommended for EV-D and HRVs). The use of VP4 for type identification is also acceptable for all species except EV-B.

Sequencing all or part of the VP1 capsid protein gene is the gold standard for EV typing [45,48]. A generic assay based on WHO recommendation is widely used in national laboratories [45,49]. Molecular typing assays based on species-specific and type-specific primers, or on other genomic regions (capsid proteins VP4-VP2) are also used [50–52], some of which have increased sensitivity. However, this will limit the comparability of the data between other centres using the VP1 typing. The complete VP1 sequence (~900 nucleotides) is used for formal type identification, and is required for assignment of new types. Sequencing multiple parts or the full-length genome is used to address specific epidemiological and research questions, including the emergence of recombinant EVs and the identification of recombination events [47,53–55]. Several bioinformatics typing tools are available for EV type identification (Table 1, Supplementary Data).

4. Future directions for enterovirus detection and characterisation

It is crucial that, as technology advances, laboratories progress towards implementation of high throughput state-of-art genome sequencing assays to rapidly characterise and respond to EVs. By doing so, we may avoid the burden of design and implementation of type-specific RT-PCR assays. Many university hospitals are well equipped to enable them to detect and characterise EV strains collected from diagnostic specimens. These data should be collectively shared among the community. The roles of national laboratories, in this context, are: (1) coordinated response and support in management of outbreaks by rapid identification and characterisation of emerging EV strains; (2) continuous support to general clinical laboratories through development, standardisation, dissemination and support in quality control of first line broad-spectrum EV molecular diagnostics; and (3) maintenance of a repository of relevant up-to-date methods for detection and characterization of EVs. However, specific diagnostic skills including virus isolation and histopathological investigations should not be forgotten as they can provide vital tools to investigate newly emerging, less well-characterised or rarer enteroviruses. Histopathological methods including in situ hybridisation or immunohistochemistry can also be used to investigate the pathogenesis and/or to confirm diagnosis (e.g. meningoencephalitis, myocarditis) in tissue biopsies [56–58].

With the establishment of the ENPEN and the successful collaborative efforts documented here, the goal of a strong future network is to provide Europe with a platform for EV surveillance and data sharing. A network with up-to-date-guidelines and real-time sharing of information (including sequences) on emerging EVs causing outbreaks and/or severe disease will strengthen cross-border threat detection and, hopefully, ensure rapid control of disease spread. Control of EVs is important in the era of future polio-eradication where other EVs than poliovirus are likely to become an increasing important cause of severe infections associated with potential devastating consequences.

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Competing interest

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Ethical

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Conflicts of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jcv.2018.01.008.

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