ISOLATION AND GENETIC IDENTIFICATION OF HUMAN NON-POLIO ENTEROVIRUSES AT THE NATIONAL REFERENCE LABORATORY OF ENTEROVIRUSES, BULGARIA

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ABSTRACT

Background: Human non-polio enteroviruses (NPEVs) are a diverse group of RNA viruses belonging to the genus *Enterovirus* within the subfamily *Ensavirinae* of the family *Picornaviridae*. They are responsible for a wide range of diseases, ranging from mild febrile illnesses to severe neurological conditions. Accurate isolation and genetic identification are critical for understanding their epidemiology, evolution, and pathogenic potential.

Aim: This study aimed to isolate NPEVs from clinical specimens, perform molecular identification through RT-PCR, and analyze viral sequences of parts the viral genome to elucidate their molecular genotyping.

Materials and methods:
Clinical specimens from patients presenting with neurological, febrile illnesses, or HFMD were collected between 2021 and 2024 and were sent to NRL "Enteroviruses" for testing. All samples were tested using viral isolation and microneutralization. Direct molecular detection of enterovirus RNA was performed by real-time RT-PCR. Partial VP1 gene sequences were used for genotyping after Sanger sequencing of the target region.

Results: Successful serotyping via microneutralization was obtained for 9 isolates, which resulted in the identification of 4 echovirus 11 (E11), one echovirus 2 (E2), two coxsackievirus B (CVB), one echovirus 25 (E25), and one echovirus 6 (E6) strains. Molecular genotyping revealed multiple genotypes predominantly belonging to *Enterovirus alphacoxsackie* species, including CVA6, CVA16, CVA4, CVA2, and EV-A71. From *Enterovirus betacoxsackie* species E11, E9, E3, and E2 were detected.

Conclusion: Despite the incomplete enterovirus surveillance in Bulgaria and nonsystematic approach for NPEV detection, we managed to identify different virus strains in various clinical settings. Our study underscores the importance of combining virus isolation with molecular techniques for accurate identification of NPEVs, as well as the necessity of continuous monitoring of NPEVs.

Keywords: Enteroviruses, isolation, real-time RT-PCR, genotyping

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INTRODUCTION

Enteroviruses (EVs) are one of the most abundant virus groups infecting humans worldwide [1, 2]. Although generally considered nonthreatening as largely asymptomatic or causing a mild disease, enteroviruses can be responsible for a wide range of clinical manifestations, including epidermal, myocardial, severe respiratory, and neurological diseases [3]. One of the most widely recognized enteroviruses are polioviruses, the etiological agent of poliomyelitis. With the establishment of the Global Polio Eradication Initiative (GPEI) in 1988 and global vaccination campaigns, polioviruses have been eliminated in many regions of the world, and currently remain endemic in only two countries [4, 5] As poliovirus cases tend to decrease globally, the focus is shifted to non-polio enteroviruses (NPEVs), which began to

emerge and to cause outbreaks and sporadic cases with significant impact on public health [6]. Enteroviruses are known to be one of the most rapidly evolving viral groups with high mutation rates [7]. The genetic diversity of NPEVs complicates diagnosis and epidemiological tracking, necessitating molecular methods for accurate identification. Although GPEI led to the establishment of a very good and well-coordinated worldwide polio surveillance network, this is not the case for non-polioviruses. Most countries lack NPEV surveillance and global surveillance activities are reduced to voluntary, case-based, and reportable. The European Non-Polio Enterovirus Network (ENPEN) was formed in 2022. This initiative involves more than 17 European countries, Bulgaria included, in an attempt to establish standardized surveillance of non-polio enteroviruses that are associated with hand-foot-and-mouth disease as well as respiratory and neurological infections [8]. These efforts include standardized and improved protocols for the detection and identification of NPEVs. Traditional detection assays rely on virus isolation and involve cell culture techniques; however, molecular methods such as RT-PCR targeting conserved regions of viral genome enable a rapid and sensitive detection [9]. Sequence analysis of the viral VP1 gene encoding a major capsid protein, provides insights into genotype classification and evolutionary dynamics [6, 10].

This study presents the isolation, molecular detection, and genotyping of NPEVs from various clinical specimens received at the National Reference Laboratory (NRL) for Enteroviruses, at the National Center for Infectious and Parasitic Diseases, Bulgaria, between 2021 and 2024.

MATERIALS AND METHODS

Sample Collection

Clinical specimens (respiratory samples, cerebrospinal fluid, stool samples) were collected from patients presenting with neurological, febrile illnesses, or hand-foot-and-mouth-disease (HFMD), at the Specialized Hospital for Active Treatment of Infectious and Parasitic Diseases "Prof. Ivan Kirov", Sofia, and the Infectious Diseases Clinic at Medical University Plovdiv, as well as from the Bulgarian AFP surveillance system. NRL for Enteroviruses is responsible for testing samples received via the sample flow mechanism under the case-based acute flaccid paralysis (AFP) surveillance system in the country. According to Bulgarian legislation, all AFP cases in children less than 15 years of age are notifiable, and samples are forwarded to the NRL for Enteroviruses, where they are tested both for polio and non-polio enteroviruses. The case definition of AFP, used by Bulgarian health authorities, includes facial nerve palsy. Samples from patients with facial nerve palsy were not included in the present study. The NRL also receives additional samples sent in by hospitals based on suspicion of enteroviral infection by the treating physicians.

All samples received at the NRL for Enteroviruses were tested using viral isolation and microneutralization. Additionally, samples of probable enteroviral infections with central nervous system (CNS) involvement, HFMD, as well as AFP cases with ambiguous isolation results, were tested using real-time RT-PCR for direct molecular detection of NPEVs.

Virus isolation and mocroneutralization

Samples were collected between 2021 and 2024. Upon receipt of clinical specimens, they were processed within 24 hours and inoculated onto RD (rhabdomyosarcoma) and L20B cell lines following standard protocols [11]. Briefly, culture tubes with confluent monolayers of RD and L20B (a cell line selective for polioviruses) cells were changed to maintenance medium and inoculated with 0.2 mL specimen extract and incubated for 5–7 days at 37°C and 5% CO_2 atmosphere. Cultures were examined daily using an inverted microscope for the appearance of a cytopathic effect (CPE).

Microneutralization assay with equine mixed hyper-immune antisera (RIVM enterovirus serotyping pools, National Institute of Public Health and the Environment, The Netherlands) was performed for serotype differentiation of samples with specific CPE in the RD cell line. This assay was performed with sets of RIVM enterovirus-typing antisera that contain anti-enterovirus pools allowing the identification of coxsackievirus B1–6 and coxsackievirus A9 and 20 echoviruses (including echovirus 30). Briefly, 50 µL previously diluted (1:20) anti-enterovirus pools were incubated with 50 µL appropriate dilution of the RD-positive samples for 2 h at 37 ◦C and 5% CO2. Subsequently, 100 µL of RD cell suspension of approximately 1.5 × 10−5 cells per mL was added. Cell monolayers were incubated at 37°C and 5% CO2 atmosphere, and cytopathic effects were scored daily by inverse light microscopy for 5–7 days. The virus was identified by the pattern of inhibition of CPE by antiserum pools [11].

RNA Extraction and real-time RT-PCR

Viral RNA was extracted directly from clinical samples using an automated extraction system (ExiPrep™ 16 Automated Nucleic Acid Extraction Systems, Bioneer, Republic of Korea) and commercial ExiPrep Dx Viral DNA/RNA kit (Bioneer, Republic of Korea) in accordance with

the manufacturer's instructions.

RT-PCR targeting the conserved 5′ untranslated region (5′ UTR) was performed using primers and probe as described by Dierssen et al. [12]. Both primers (EQ-1: *5′-ACATGGTGTGAAGAGTCTATTGAGCT-3′* and EQ-2: *5′-CCAAAGTAGTCGGTTCCGC-3′*, final concentration of 0.5 µM each) and the probe (EP: *6-FAM-5′-TCCGGCCCCTGAATGCGGCTAAT-3′-BHQ*; 0.125 µM) were added to the master mix (20µl) containing 12.5 µl of the reaction buffer and 1µl enzyme mix and 1.5µl detection enhancer from AgPath-ID One Step RT-PCR Kit (Applied Biosystems, ThermoFisher Scientific, USA). Five µL extracted RNA were added to the total of 25 µL reaction volume. Thermal cycling conditions were: reverse transcription at 45°C for 10 min, initial denaturation at 95°C for 10 min, followed by 40 cycles of [94°C for 15 s, 60°C for 45 s]. Positive and negative controls were included in each run. Amplification was performed using a Real Time PCR Detection System – Gentier 96R (Xi'an TianLong Science and Technology Co., Ltd, China). Samples with a cycle threshold (ct) value <38 were considered positive. Positive samples were selected for further analysis.

<u>Enterovirus genotyping</u>

Partial VP1 gene sequences were used for genotyping. The protocol was as previously described by Nix et al. [6]. The selected region resulted in ~330 bp amplicons, which were purified and Sanger sequenced bidirectionally, using primers AN232: *5′*- CCAGCACTGACAGCA-*3′* and AN233: *5′*- TACTGGACCACCTGG-*3′*. Sequencing was performed with the GenomeLab GeXP genetic analysis system (Beckman Coulter, USA). The web-based open-access Enterovirus Genotyping Tool, Version 1.0 was used for genotyping [13].

RESULTS

<u>Virus Isolation and Neutralization</u>

A total of 125 patients were enrolled in this study. Fecal samples and/or (cerebro-spinal fluid) CSF, and/or nasopharyngeal swabs or other suitable sample types from the participants in the study were received at the NRL for Enteroviruses in 2021 to 2024. Of those, a total of 171 samples were tested via the classical virus isolation technique, and 14 samples (11,2%) yielded cytopathic effects after two consecutive passages in RD cells, indicative of enterovirus infection. No CPE was observed in L20B cells, according to the WHO algorithm for polio identification, suggesting the absence of polioviruses,. Successful serotyping was obtained for 9 isolates, which resulted in the identification of 4 echovirus 11 (E11), one echovirus 2 (E2), two coxsackievirus B (CVB), one echovirus 25 (E25), and one echovirus 6 (E6) strains.

<u>Molecular Detection and Typing</u>

RT-PCR targeting the 5′ UTR confirmed enterovirus RNA presence in all CPE-positive samples. Additionally, 68 out of 171 samples were positive in RT-PCR molecular enterovirus detection. Those samples were CPE-negative in virus isolation assay.

PCR-positive samples with ct value <28 were selected for further analysis and genotyping. Only 27 of them were successfully amplified, yielding sequences of VP1 Nix fragments with quality good enough for genotyping. From cell-culture positive and serotyped isolates, only four yielded sequences with good quality, and some of the isolates could not be confirmed. Molecular genotyping revealed multiple genotypes predominantly belonging to *Enterovirus alphacoxsackie* species, including CVA6, CVA16, CVA4, CVA2, and EV-A71. From *Enterovirus betacoxsackie* species E11, E9, E3, and E2 were detected (Figure 1).

<u>Clinical manifestations</u>

Clinical diagnoses were obtained from the medical professionals sending the samples in a descriptive manner. For the purposes of the present study, diagnoses of successfully sequenced samples (n = 27) were coded as neurological (meningitis, encephalitis), hand-foot-and-mouth (HFMD), other rash (non-HFMD), fever, paralysis (facial nerve palsy not included), gastrointestinal, and herpangina. HFMD (n=12, 44%) and neurological infections (n=6, 22%) were the most frequently reported clinical presentations (Table 1). Coxsackievirus A6 (CVA6) showed a noticeable association with HFMD and was detected in 8 (66.7%) of the cases. E11 was detected in 2 out of 6 cases of neurological diseases, as well as in one case with paralysis.

DISCUSSION

In Bulgaria, there is no established system for surveillance of enterovirus infections. The only laboratory in the country responsible for enterovirus testing and typing is the NRL for Enteroviruses at the National Center of Infectious and Parasitic Diseases. The present study aimed to investigate the overall non-polio enterovirus circulation. Here, we combined classical virological assays for viral isolation and characterization via microneutralization and molecular detection and genotyping. The main limitation of our study was due by the fact that surveillance of non-polio enteroviruses is case-based and depends upon the decision of treating physicians. Nevertheless, non-polio enterovirus detection and typing can be informative about the general level of non-polio enterovirus infections and is an useful approach in routine ongoing surveillance to detect possi-

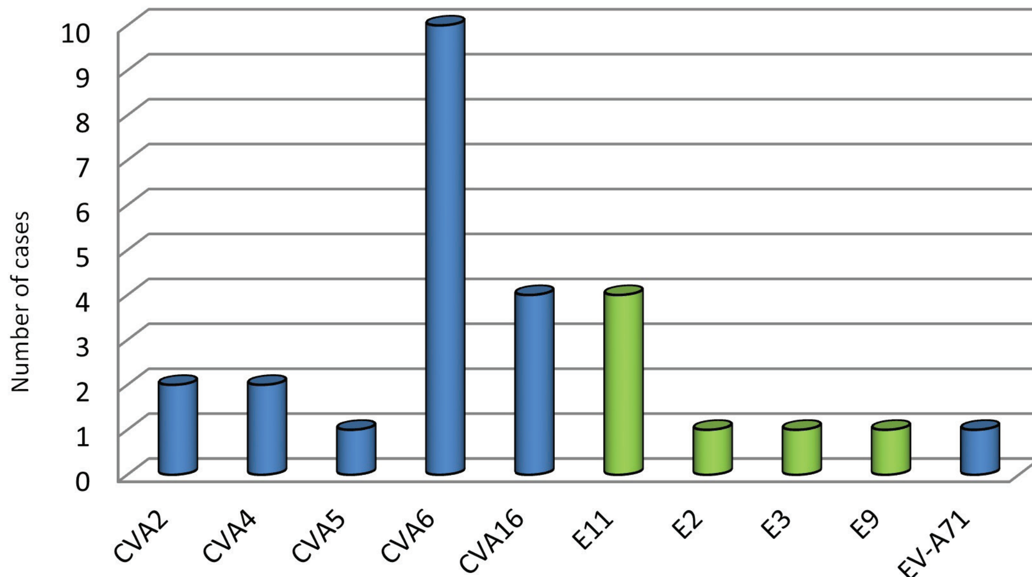

Figure 1. Number of enterovirus types divided into species. *Enterovirus alphacoxsackie* are marked in blue, *Enterovirus betacoxsackie* are marked in green.

ble outbreaks.

Out of 125 different cases of possible enterovirus infection, we detected non-polio enteroviruses in 68 of the samples (a total of 171 samples tested, positivity – 39.8%). Multiple sample types were received from some of the patients. Enterovirus detection was performed via both virus isolation and molecular PCR detection. The isolation assay yielded positive results only for 8 samples (4.7%). The higher detection rate of viral RNA through RT-PCR is not surprising, as this is a much more efficient and sensitive detection method than cell-culture isolation. The use of combined approach, including both diagnostic techniques, is important for a more accurate diagnosis of enterovirus infections.

Sequencing of the Nix fragments of the enterovirus VP1 gene allowed the genotyping of several of the enteroviruses. In total, 10 different EV types were identified, with CVA6, CVA16, and E11 most frequently detected.

With the eradication of polioviruses, there is an increasing focus on non-polio enteroviruses due to their potential as emerging pathogens [14]. Although there is no global non-polio enterovirus surveillance, the European Non-Polio Enterovirus Network (ENPEN) works actively to alert the community and conduct multiple investigations of outbreaks or overall circulation of these viruses. Thus, upsurges in circulation of E30 were detected in 2018, and an overall high basic level of CVA6 detection after a peak in 2018. Increased detections of E11 were observed in 2018, 2019 and 2022 [15]. Conclusions about possible outbreaks in Bulgaria and comparison with the epidemiological background of non-polio enterovirus circulation in the European region have to be interpreted with caution, due to the small number of samples received for testing. Previous studies suggest that E30 detections are rather sporadic, and no epidemic event was observed in the country during the epidemiological upsurge of the virus reported in Europe in 2018 [16].

Most samples from patients presenting with neurological infections contained *Enterovirus betacoxsackie* like E2, E9, and E11.These results are in agreement with previous studies [15, 17]. A high prevalence of *Enterovirus alphacoxsackie* was largely reported in Asia, in particular CVA6, EV-A71, and CVA16 in cases of HFMD, where the disease is reportable [18, 19]. These viruses were also detected in Europe, North America, South America and Oceania, indicating their worldwide spread [15, 19, 20]. In our study, HFMD was the most often reported clinical manifestation of enterovirus infection, with CVA6 most frequently detected in the samples. This reveals the circulation of the virus in the Bulgarian population as well. HFMD is not notifiable in our country, so the real burden of the disease could not be determined, and most of the cases probably remained undiagnosed. NRL for Enteroviruses is responsible for testing samples received via the sample flow mechanism under the case-based acute flaccid paralysis (AFP) surveillance system in the country. According to Bulgarian legislation, all AFP cases in children less than 15 years of age are notifiable, and samples are forwarded to NRL for Enteroviruses, where they are tested both for polio and non-polio enteroviruses. In order to expand and

Table 1. Enterovirus detections in various clinical conditions. Number of smples (N) (% of total).

Clinical presentation	Neuro	HFMD	Other rash	Fever	Paralysis	GI	herpangina	Total
EV Types	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N
CVA2	NA	NA	NA	NA	NA	2 (100%)	NA	2
CVA4	1 (16.7%)	NA	NA	1 (100%)	NA	NA	NA	2
CVA5	NA	NA	1 (33.3%)	NA	NA	NA	NA	1
CVA6	NA	8 (66.7%)	1 (33.3%)	NA	NA	NA	1 (100%)	10
CVA16	1 (16.7%)	2 (16.7%)	1 (33.3%)	NA	NA	NA	NA	4
E2	1 (16.7%)	NA	NA	NA	NA	NA	NA	1
E3	NA	NA	NA	NA	1 (50%)	NA	NA	1
E9	1 (16.7%)	NA	NA	NA	NA	NA	NA	1
E11	2 (33.3%)	1 (8.3%)	NA	NA	1 (50%)	NA	NA	4
EV-A71	NA	1 (8.3%)	NA	NA	NA	NA	NA	1
Total N (%)	6 (22%)	12 (44%)	3 (11%)	1 (4%)	2 (7%)	2 (7%)	1 (4%)	27

NA - not available, Neuro - neurological disease, HFMD – hand-foot-and-mouth disease, GI - gastrointestinal.

improve our enterovirus detection capability, in recent years, we started to apply additional molecular diagnostic for specimens from severe paralysis. NPEVs identified as E3 and E11were associated with two cases of paralysis in this study. Those findings were not surprising as the capacity of E3 and E11 to cause acute flaccid paralysis was previously reported [21, 22]. Therefore, our data highlight the clinical and public health threat posed by NPEVs and emphasize the necessity for the establishment of an enterovirus molecular surveillance system in Bulgaria.

In conclusion, the present study reports data from enterovirus surveillance in Bulgaria, though incomplete it may be. The successful isolation and molecular characterization of NPEVs highlight their ongoing circulation in the country. The genetic diversity observed aligns with previous studies demonstrating the extensive variability of NPEV genotypes and the simultaneous circulation of multiple genotypes. The predominance of *Enterovirus alphacoxsackie* species is consistent with their worldwide spread, and relatively high basic level of CVA6 detection in the European region, in particular.

The detection of *Enterovirus betacoxsackie* species in some medical conditions is consistent with their known prevalence in neurological diseases [23], and they were identified in samples from meningitis or paralysis cases. A limitation of the study is the relatively small sample size and focus on symptomatic cases, possibly underestimating asymptomatic circulation. Sequence analysis of partial VP1 provides a valuable instrument for molecular genotyping, which is crucial for epidemiological surveillance of circulating genotypes.

This study underscores the importance of combining virus isolation with molecular techniques for accurate identification of NPEVs. The detection of different strains in various clinical conditions highlight the importance of continuous monitoring of NPEVs.

Institutional Review Board Statement: We present surveillance data collected routinely by the national reference laboratory and surveillance units in the country. Data is presented in an aggregated and anonymous format. Publication of this analysis does not harm or influence either cases or institutions. Ethical committee approval was therefore not required.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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