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ORIGINAL ARTICLE

A 5-year study of human parechoviruses in children living in bad sanitation conditions and non-polio acute flaccid paralysis children from Greece

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Abstract

In Greece, data for human parechoviruses (HPeVs) are scarce and our aim was to conduct a large scale study to determine for the first time their occurrence. Under the spectrum of surveillance, we retrospectively screened stool specimens obtained from 71 children with acute flaccid paralysis (AFP) symptoms and from 311 individuals in high-risk population groups such as children living in bad sanitation conditions for HPeVs presence by rRT-PCR targeting the 5′ UTR. All positive samples were then genotyped by targeting the HPeVs VP1 region. Totally, 15/311 (5%) stool samples from children living in bad sanitation conditions and 4/71 (6%) from the non polio AFP children were positive for HPeVs. Sequencing analysis revealed that genotypes HPeV1 ($n = 4/15$), HPeV5 ($n = 2/15$), and HPeV6 ($n = 2/15$) were circulating among Roma children population whereas HPeV1 ($n = 1/4$) and HPeV5 ($n = 1/4$) were circulating in children with AFP-like symptoms. We did not obtain a seasonality motive among HPeV1 or HPeV5 genotypes whereas HPeV6 was detected only in July. Phylogenetic analysis showed that Greek HPeVs strains are clustered together with HPeV strains circulating in other European countries during the same period. We describe the presence of HPeVs in Greece, and we enforce that their diagnosis should be considered in children with neurological outcome such as non-polio AFP.

Keywords Human parechoviruses · Non-polio acute flaccid paralysis · Nomadic Roma · Greece

Introduction

Human parechoviruses (HPeVs) are members of Picornaviridae family; they were initially named as echovirus -22 and -23 within the Enterovirus genus but in the late 90s renamed and reclassified as HPeV1 and HPeV2 respectively, based on differences in their genome organization, structure, and biological properties [1]. The biomedical interest in HPeVs was increased after the discovery that these viruses have been linked with central nervous system (CNS) infections, transient paralysis, neonatal sepsis, and sudden death in infants [2, 3]. Recent data indicate that HPeVs are at least as prevalent as enteroviruses, with HPeV1 to be the most prevalent type followed by the most important one that is HPeV3 [4, 5]. Infections by HPeVs are commonly associated with mild gastrointestinal and respiratory symptoms in young children, but are also linked with more severe conditions, such as acute flaccid paralysis (AFP) and encephalitis [2, 3]. Indeed, HPeVs have been involved in outbreaks of AFP since 1989 [6] and it is estimated to the degree that the non-polio AFP cases rate for 2017 globally is 5.47, in Europe 1.04, and in Greece 0.65 [7].

In Greece, there are several studies regarding enterovirus surveillance [8] although data for HPeVs are scarce [9, 10]. Even though Greece is considered as a "polio-free" country since 2002, the last few years due to increased immigration, enterovirus laboratory surveillance is reinforced. From 2008, the Hellenic Polioviruses/Enteroviruses Reference Laboratory performed detailed supplementary surveillance of circulating enterovirus among healthy individuals in high-risk population

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groups such as people that live in bad sanitation conditions. The main strategy that is recommended by the World Health Organization (WHO) includes the investigation of AFP cases in hospitalized children < 15 years of age throughout Greece which is a sensitive marker for poliomyelitis. In this study, for the first time in Greece, we determined the occurrence of HPeVs in stool samples of children living in bad sanitation conditions along with the stool samples from children with AFP-like symptoms. All samples were received to our Reference Laboratory under the spectrum of surveillance of enteroviruses presence in Greece.

Materials and methods

Population studied

From November 2010 to May 2014, stool samples were collected from children < 15 years old belonging to high-risk population groups with low vaccination coverage and poor sanitation and living conditions in representative geographical areas of Greece. The study was performed in collaboration with the HCDCP that provides medical and social services to local (stable and mobile) Roma populations. All participants provided samples voluntarily. Samples were collected from regions throughout Greece and sent to our reference laboratory for further processing.

Supplementary enteroviruses surveillance was initially implemented in Greece in 2008 as a pilot study until its establishment at the end of 2010 to complement AFP surveillance. As a result, from January 2010 to April 2015, we studied stool samples from children referred from national hospitals with AFP-like symptoms. Indeed, we tested children less than 15 years of age with a sudden onset of paralysis/weakness in any part of their body that were negative for enteroviruses presence (Fig. 1).

Sample collection and treatment

We collected one fecal specimen for the children belonging to high-risk population groups, and two adequate fecal specimens were collected 24–48 h apart and ≤ 14 days after the disease onset for the children with AFP-like symptoms. All stool samples were kept at − 20 °C until they were processed. Two grams of each fecal sample were homogenized in a plastic tube containing 90% Dulbecco's modified Eagle medium (DMEM), 10% chloroform, and sterile glass beads. After homogenization and centrifugation at 2000 rpm for 20 min, viral RNA was extracted from 140 μl of the supernatant using the QIAamp viral RNA minikit (Qiagen, Hilden, Germany) according to manufacturer instructions.

Molecular assays

Screening

Stoll samples were analyzed for HPeVs by real-time reverse-transcription PCR (rRT-PCR) targeting the 5′ UTR. The HPeVs group-specific primers and probe were used as described [11]. To detect inhibitors presence in the RNA extracts as well as to indicate template loss during handling, the potato *Solanum tuberosum* phyB gene was copurified and coamplified with each sample as previously described [8]. To avoid probable contamination, all necessary controls and precautions were taken, including use of a three-room separation system.

Typing and phylogenetic analysis

Positive HPeV samples were genotyped for detecting the parechovirus strains by seminested RT-PCR amplification in their VP1 region [12]. PCR products were purified by using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and the MinEluteTM Gel extraction Kit (Qiagen, Hilden, Germany) and then sequenced in both directions by using the BigDye™ Direct Cycle Sequencing Kit (Thermo Fisher Scientific, Massachusetts, USA). The electrophoresis was carried out in ABI3730xl DNA Analyzer (Thermo Fisher Scientific, Massachusetts, USA). The sequences obtained from the samples were identified by closest homology sequence using BLAST. Multiple sequence alignments with the respective reference strains from GenBank® database sequences were made by BioEdit Sequence Alignment Editor. Molecular evolutionary genetics analysis (MEGA) software, version 6 was used for the phylogenetic analysis. The phylogenetic trees were created by the neighbor-joining method (bootstrap resampling with 1000 replicates).

Statistical analysis

Epi Info version 6.0 software (Centers for Disease Control and Prevention, Atlanta, GA, USA) was used for calculating the variations of significance in the number of positive specimens between two consecutive months, nonconsecutive months, and seasons ($p < 0.05$). Seasons were defined as winter (January–March), spring (April–June), summer (July–September), and autumn (October–December). The Mantel-Haenszel test or the Fisher exact test was used to test their significance.

Accession numbers

Distinct partial VP1 sequences from positive HPeV samples were submitted to GenBank under the accession numbers MH599072–081.

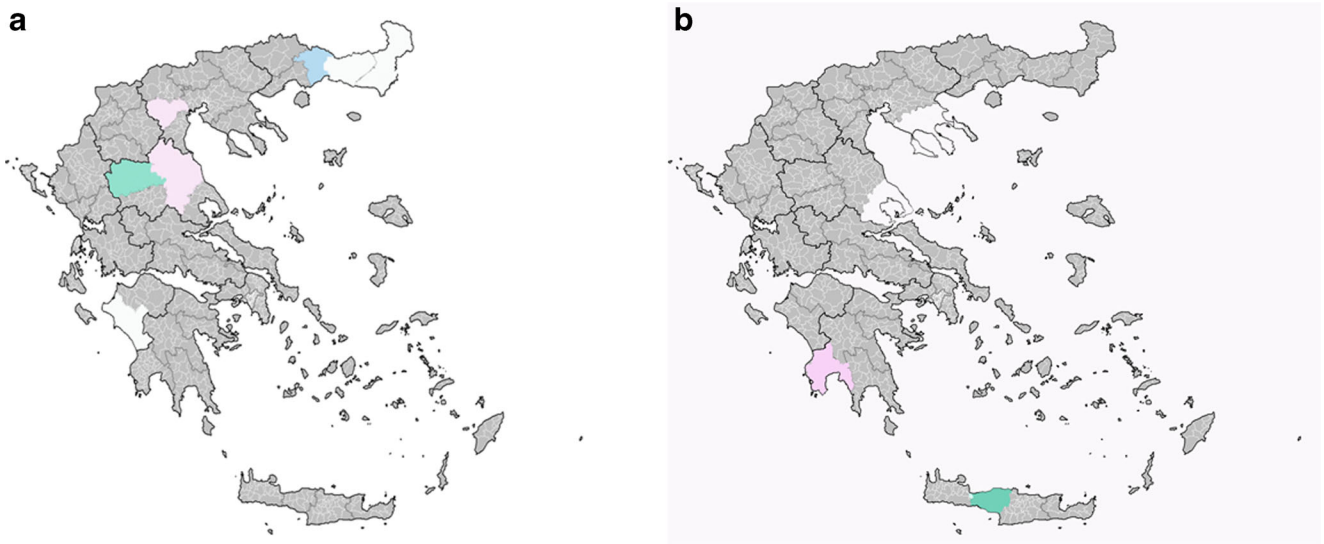

Fig. 1 Map of Greece with regions positive for HPeVs. **a** Roma children, **b** AFP-symptoms children

Results

We tested 311 stool samples obtained from 311 Roma children [median age ± standard deviation (SD) 6.2 ± 3.9] and 71 samples from children with AFP-like symptoms (Table 1). Totally, 15 (5%) stool samples stools from the Roma children (median age ± SD 5.2 ± 2.1) and four (6%) from children with AFP-like symptoms (median age ± SD 7.3 ± 3.3) were positive for HPeVs.

Genotyping analysis

Genotyping analysis of the VP1 region was achieved for eight samples obtained from the Roma population and for two from the AFP children due to a sufficient RNA load. We detected three different genotypes circulating among the Roma children including HPeV1 ($n = 4$), HPeV5 ($n = 2$), and HPeV6 ($n = 2$). Indeed, in North Greece, the genotypes circulated were HPeV1 ($n = 3$) and HPeV6 ($n = 2$), whereas in Central Greece, the genotypes were HPeV5 ($n = 2$) and HPeV1 ($n = 1$). The two cases of HPeV5 in Central Greece derived from two different members of the same family. Similarly, the two cases of HPeV6 in North Greece derived from different members of the same Roma family. RNA load was not enough to achieve genotyping in samples from South Greece.

In children with AFP-like symptoms, we identified two diverse genotypes including HPeV1 and HPeV5. Both genotypes detected in children from South Greece.

Seasonality

Positive HPeVs cases were plotted for each month (Fig. 2a) to identify seasonal distributions of HPeVs from 2010 through 2015. The monthly mean numbers of all HPeVs presented a significant increase during December–January ($p = 0.01$). However, when we looked for seasonality concerning genotypes to the samples with a sufficient RNA load, we did not find any seasonality motive for HPeV1 as HPeV1 cases were detected throughout the year. In addition, HPeV5 cases occurred during March and December whereas HPeV6 cases only during July (Fig. 2b).

Phylogenetic analysis

Phylogenetic analysis of HPeVs was achieved for ten samples, and we did not find differences among HPeVs circulating in the Roma children and the children with AFP-like symptoms (Fig. 3a). Moreover, we created a phylogenetic tree to determine if the Greek HPeVs were closely related to strains circulated during the same period in other countries. As the presence of HPeVs has not previously been described in countries close to Greece, we compared our results with other European countries where HPeVs sequences are available and as well from countries from other continents (Fig. 3b). We found that the HPeV1 circulated in North Greece was clustered together with the viruses of the same type from Central and South Greece and with the HPeV1 strains circulating in other European countries. Likewise, a cluster was created for HPeV5s that derived from samples of Central and South Greece and the HPeV5s strains from Germany and Denmark. Finally, HPeV6 that was only derived from North Greece clustered together with HPeV6 that was identified in Germany, Netherlands, and Italy.

Table 1 Number of parechovirus positive samples and genotypes in each children group

	Region of Greece	Asymptomatic Roma children	Acute flaccid paralysis children
HPeVs 1 positive (%)	North	3/15 (20%)	0
	Central	1/15 (6%)	0
	South	0	1/4 (25%)
HPeVs 5 positive (%)	North	0	0
	Central	2/15 (13%)	0
	South	0	1/4 (25%)
HPeVs 6 positive (%)	North	2/15 (13%)	0
	Central	0	0
	South	0	0
Total HPeVs positive (%)		15/311 (5%)	4/71 (6%)

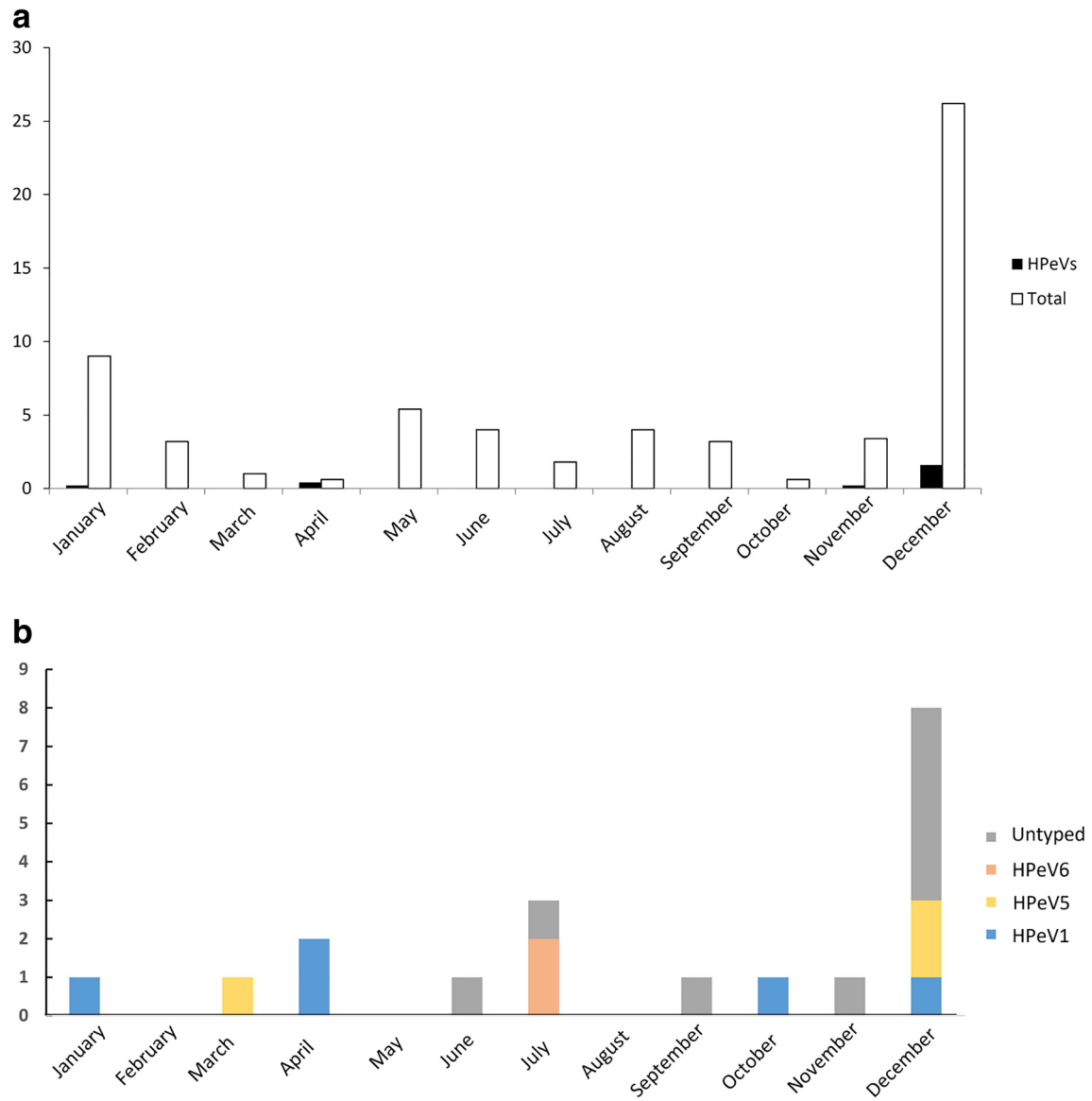

Fig. 2 **a** Mean numbers of children tested and for whom HPeVs was detected. **b** number of HPeVs genotypes detected

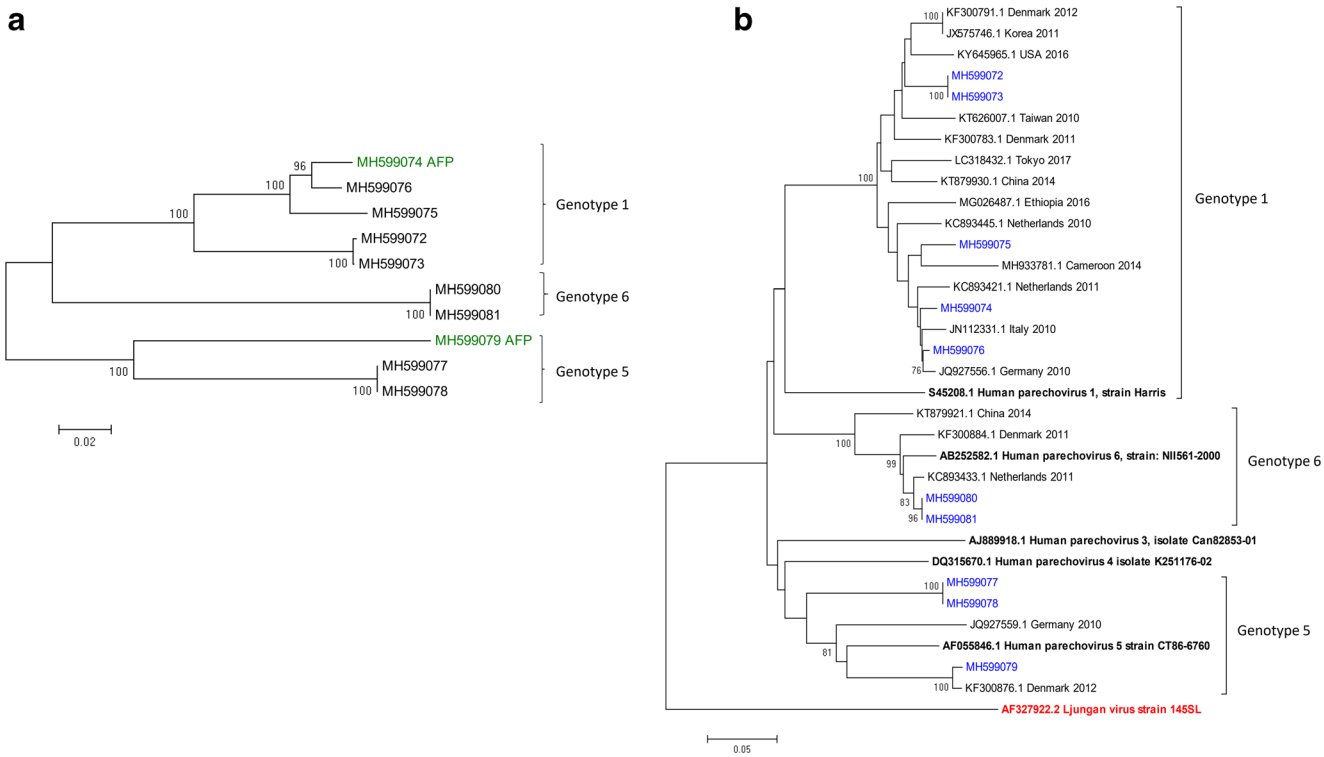

Fig. 3 Phylogenetic analysis of HPeVs-positive samples. **a** Among Roma and AFP children

Discussion

We detected the presence of HPeVs in Roma children living in bad sanitation conditions as well as in children with AFP-like symptoms from Greece. Although this was a study in stool specimens, HPeVs can also be detected in respiratory and CSF samples [13]. Indeed, the absence of HPeVs in stool does not imply that HPeVs was not a causative agent for the AFP cases. The HPeVs found in our study were genetically similar with the HPeVs isolates circulating in other European countries. To the best of our knowledge, our study is the first describing the presence of HPeVs in Greece. To accomplish this, we targeted the 5′ UTR region of all known HPeVs and their conserved VP1 region for the typing of their genotypes as previously described [11, 12]. Although our study took place during a 5-year period, all samples were analyzed with the same extraction and molecular assays protocols. Also, we routinely included many negative and positive controls in each assay, processed in the same way as the test samples. However, we cannot infer attack rates from this study due to the relatively small numbers of cases.

HPeV presented a seasonality in Greece with the majority of cases detected during autumn. The seasonality of HPeV infections shows considerable variability and appears to depend on the predominant genotype [4]. We did not find a seasonality motive for the HPeV1 circulation and similarly to our study is believed that HPeV1 circulates throughout the year [4]. National surveillance studies from Denmark revealed that both HPeV1 and HPeV3 infections occurred in all months of the year, with a small increase in the rate in the summer and autumn months [14]. Similarly, a national surveillance study from the USA during a 35-year period described a small increase in the rate of HPeV1 infections in the summer and autumn months [15]. In contrast, in Japan, all positive samples of HPeV1 were collected between July and December during a 2-year study [16]. Finally, during the years of high HPeV3 frequency, the annual distribution of HPeV presented a peak during the summer months in the Netherlands which resembled to that of enterovirus infections [17]. A limitation of our study was that due to a not sufficient RNA load, we did not achieve genotyping for nine HPeV positive samples by RT-PCR, and as a result, it is difficult to make conclusions regarding the seasonality of genotypes due to a very limited number of available sequences.

We detected children with AFP-like symptoms positive for HpeV1 and HpeV5. Previously, HpeV1 was associated with an AFP outbreak in Jamaica in 1986 where most of AFP patients with HPeV1 positive stool samples also presented a significant antibody titer increase [6]. In addition, HPeV3 was also detected in a 1-year-old female with fever, diarrhea, and transient paralysis [18]. Other HPeV types associated with acute flaccid paralysis include 1 and 6 [19, 20] whereas there are scare studies that have

associated the HPeV5 with non-polio AFP patients under the age of 3 years [2, 21].

In this study, Roma children throughout Greece reveal HPeVs' presence in their stool samples. In a previous study from our reference laboratory, we detected that in 25% of stool samples obtained from the same group of asymptomatic children that live in bad sanitation conditions with low vaccination coverage presented non-polio enteroviruses [8]. Similarly, HPeVs' presence was found on healthy children from Finland (6%) [22], Norway (11%) [23], Japan (4%) [24], the Netherlands (2.5%), and Scotland (14%) [25]. We also detected that the genotypes HpeV1, HpeV5, and HPeV6 were circulating among these Roma children. Previously, the genotypes HpeV1, HpeV3, and HpeV6 were found in healthy children from Finland [22], whereas the genotype HPeV3 was detected in the stools of healthy asymptomatic children in Japan [26]. There are no reports about HPeVs in countries close to Greece to compare our result; however it is considered that the genotypes HPeV1 and HPeV6 are the most commonly observed in Europe [4]. In contrast, we detected three children both from the Roma and AFP groups that the detected genotype was HPeV5 that is considered as an uncommon HPeV [27].

In conclusion, we describe for the first time the presence of HPeVs in the stool samples of Roma children living in bad sanitation conditions and in children with AFP-like symptoms from Greece. The last years, HPeV infections are increasingly considered as an important cause of sepsis-like illness and CNS infections in infants but until now no effective treatment for HPeV infections has been identified. Clinicians in Greece should consider HPeVs in the diagnosis of children with neurological outcome such as non-polio AFP, and we believe that an extensive monitoring and surveillance of picornaviruses is crucial in Greece to determine if the virus presence is a public health problem.

Green color, HPeV5 positive; Pink color HPeV1 positive; Blue color, HPeV6 positive; White color, untyped HPeV samples

Green color, strains detected in AFP children; black color, strains detected in Roma children.

b In comparison to HPeVs reference strains and strains circulating the same period in Europe.

Bold black color, reference strains; Black color, strains detected in other European countries; Blue color, the strains observed in our study.

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Compliance with ethical standards

Ethics policy This study was approved by the institutional ethics committee of the Hellenic Centre for Disease Control & Prevention (HCDCP). Patient records were coded prior to analysis and no identifying details are included.

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval Not necessary.

Informed consent Not necessary.

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