

Widespread circulation of echovirus 6 causing aseptic meningitis in paediatric patients in the area of Modena, Italy, in 2011

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Summary

Introduction: Between May and November 2011, enterovirus RNA was detected in the cerebrospinal fluids (CSFs) of 72 children with signs of aseptic meningitis admitted to paediatric departments of different Hospitals of the prefecture of Modena, Emilia Romagna region, Italy. Enterovirus RNA was detected in 34 CSFs by commercial reverse transcriptase-polymerase chain reaction (RT-PCR). Twenty-one samples, resulted human enterovirus B by species-specific RT-nested PCR, were submitted to sequencing of the 3' terminus of the *VPI* gene.

Materials and Methods: Upon sequencing and interrogation of the National Center for Biotechnology Information database, all 21 viruses were characterized as echovirus 6 (E6), and possess a 100% nucleotide identity each other.

Results: This study reports the molecular detection and typing of E6 isolated from clinical specimens from paediatric patients with aseptic meningitis in the wide area of Modena, Italy, in 2011.

Introduction

Human enteroviruses (HEVs) are small non-enveloped viruses with a single-stranded positive sense RNA genome belonging to *Picornaviridae* family. Serotypes of HEVs have traditionally been classified into echoviruses (E), coxsackieviruses group A and B and polioviruses by neutralization test. In 1999, molecular typing method was introduced, which suggests strains with 70% *VPI* nt similarity are classified as different types and the strains with 75% *VPI* nt similarity are classified as members of the same type, and led to the discovery of a large number of new enterovirus (EV) types. Currently, HEVs comprise more than 100 types, which are classified into 4 species, HEV-A to HEV-D (11,15,18).

The HEVs infect millions of people worldwide each year with a seasonal peak in summer and autumn in temperate climates causing epidemic or endemic infections in individuals of any age, especially children. HEVs are transmitted mainly through fecal-oral route. They are ubiquitous and can be found in natural and in aquatic environmental samples. Most of these infections are asymptomatic or result in mild illness. They have been also associated with the common cold, acute haemorrhagic conjunctivitis, myocarditis, aseptic meningitis, encephalitis and acute flaccid paralysis. Aseptic meningitis is the most common illness associated with HEVs infections in young children and often occurs as outbreaks. E is the major causative agent of aseptic meningitis and E6 and E30 have been frequently associated with worldwide countries' outbreaks (5-9,14,16,17). In Italy, no outbreak due to E6 has been reported until now. We report an outbreak of E6 causing aseptic meningitis in paediatric patients admitted to paediatric departments of different Hospitals of the prefecture of Modena, Emilia Romagna region. It was identified by determining the partial sequence of the *VPI* gene of HEVs detected and analysing the viral sequences with cognate sequences available in the National Center for Biotechnology Information (NCBI) database (10).

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Materials and Methods

Between May 30 and November 14, 2011 the Laboratory of Microbiology and Virology of the Polyclinic Hospital of Modena has received 72 samples of cerebrospinal fluid (CSF) from as many paediatric patients with signs of aseptic meningitis admitted to four different paediatric Hospitals distributed in the prefecture of Modena (Figure 1). The age of the patients ranged from 11 days to 14 years.

Nucleic acids were extracted from 1 mL of CSF by using NucliSENS easyMag instrument (bioMérieux, Marcy L'Etoile, France) and submitted to amplification by Real Time polymerase chain reaction (PCR) for cytomegalovirus (CMV), Epstein-Barr virus (EBV), herpes simplex virus 1/2 (HSV1/2), varicella zoster virus, human herpes virus 6

(HHV6), adenovirus (AdV) (ELITechGroup, Turin, Italy). To detect EVs, the extracted RNA was converted into cDNA by using RT-kit plus and amplified by Real Time PCR (ELITechGroup). HEVs typing was carried out by using a species-specific nested PCR producing a fragment in the 3'-half of the *VP1* coding region of HEVs. Briefly 10 μ L of cDNA for each sample were amplified in a total volume of 50 μ L containing 2 \times of Dream Taq Green PCR Master Mix (ThermoFisher Scientific, Waltham, MA, USA) and 40 pmoles of each external primer (VP1-1S GGTTYGAYITGGARITACITTYGT; VP1-1A: TGIGAYTGRITAYCTIKYKGGRTARTA) as previously described (4). Nested PCR was performed in 50 μ L containing 1 μ L of first PCR product, 2 \times of Dream Taq Green PCR Master Mix, and 60 pmoles of each species-specific primer pair. In particular three sets of specific primers for HEV-A (487: ATGTWYGYICCCIGGICGNCC; 489: AYIGCICISWITGYTGNC), HEV-B (491: TGTAYRTICCCIGGNGG; 493: TCNACIANICCCIGGICCYTC), and HEV-C (495: ATGTAYRTICCCIGGICGNCC; 497: GCITTTTITGRTGICCRANCC) species were used. The sizes of the fragments visualized by standard agarose gel electrophoresis were about 454, 758, and 458-bp, for HEV-A, -B, and -C species, respectively (3,12). The amplicons were purified using High pure PCR product purification kit (Roche Diagnostics AG, Rotkreuz, Schweiz), and sequenced with the nested PCR primers, using BigDye Terminator v 3.1 Cycle Sequencing Kit and an automated sequencer ABI 3100 (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions.

The obtained nucleotide sequences were compared with cognate HEV sequences available in GenBank database (NCBI) to assign the serotype on the basis of a 75% nucleotide identity cut-off (10).

Results

All the 72 CSFs tested negative for CMV, EBV, HSV1/2, HHV6, AdV. HEV typing of the 34 HEVs detected gave positive results by species-

specific reverse transcriptase-nested PCR for HEV-B (758-bp) in 21 (61.8%) cases. Six (17.6%) samples did not generate *VP1* amplicons and were therefore considered untypable. The remaining 7 (20.6%) samples were not processed for HEV typing due to the lack of biological material. All 21 partial *VP1* sequences showed a 100% nt identity each other and by comparison with sequences available in the NCBI database were classified as E6 virus (78% nt identity respect to the E6 prototype strain, GenBank accession number AF081321). In addition, the 21 sequences showed an identity from 83 to 99% nt with sequences of human isolates in different geographical area, including a Chinese isolate with 83% nt identity (accession number KF042342), a Romanian isolate with the 91% nt identity (accession number HE616672), a Tunisian isolate with 98% nt identity (accession number LN713456) and with different sequences of French isolates with 99% nt identity (accession numbers HG793683-88 and HG793690-98). By contrast, it was not possible to compare our sequences with other sequences (accession numbers KP262024, KM598863, KC880340, KC852176, KF931635, KF006264) of E6 Italian human isolates because obtained by sequencing of other genic region.

Overall, 26/34 patients (76.5%) E6 positives were aged between 2 and 14 years, the remaining 8/34 patients (23.5%) had less than one year of age (from 11 days to 4 months). The most of patients (21/34; 61.76%) were admitted to a single Hospital. Temporal distribution of the E6 showed an obvious seasonal pattern with the highest number of positive in June and July (25/34; 73.5%) (Figure 2).

Discussion

In our study, it has been possible to identify E6 as the responsible for a vast outbreak between summer and autumn 2011 in paediatric patients with fever and meningitis symptoms admitted to paediatric

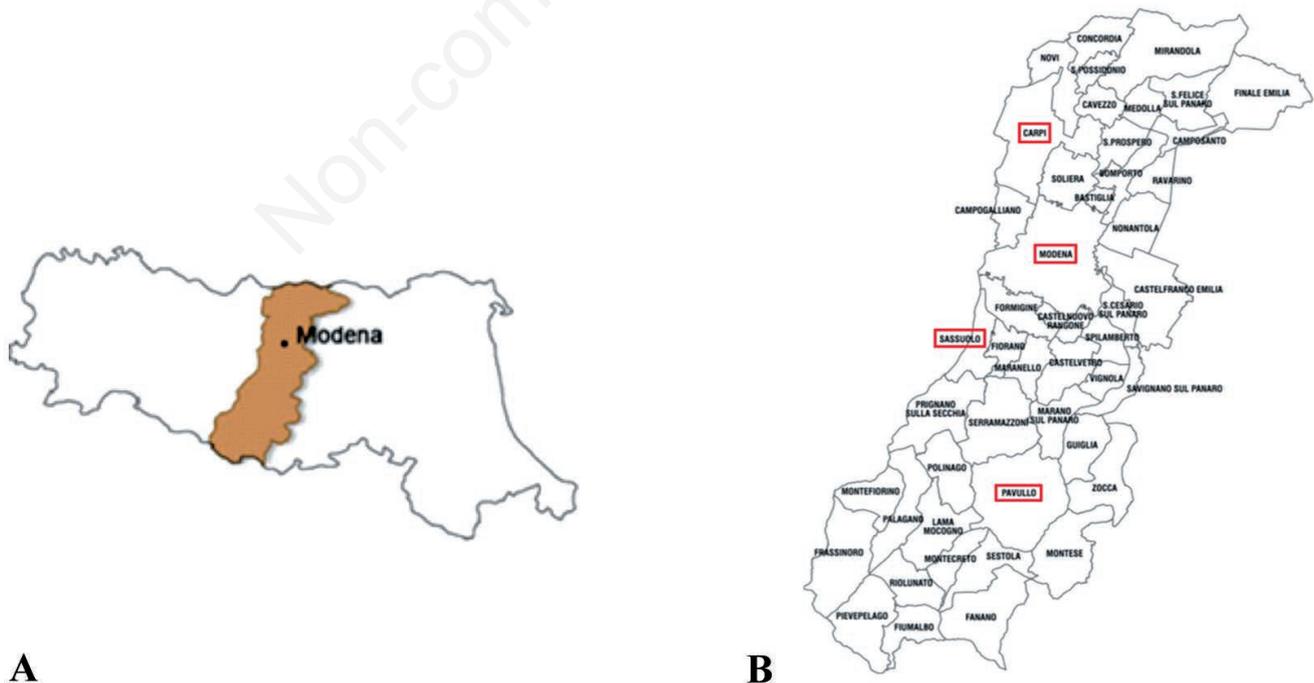


Figure 1. A) Emilia Romagna region: Modena province; B) Municipalities involved in the outbreak of echovirus 6 infection: Modena (n. 11), Carpi (n. 1), Sassuolo (n. 21), Pavullo (n. 1).

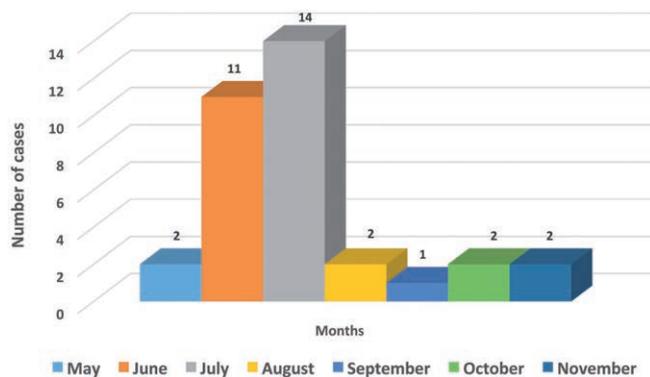


Figure 2. Distribution of echovirus 6 cases during 30 May-14 November, 2011.

departments of four Hospitals of the prefecture of Modena, Emilia Romagna region.

The use of a molecular diagnostic kit has allowed identifying HEV and the partial sequencing of *VP1* gene has allowed identifying E6. The HEV molecular detection was directly applied to all CSFs of patients and the positive samples were submitted to genotyping. The homology of 78% with a reference isolate permitted us to characterize the positive samples as E6. As expected, the comparison with other E6 sequences deposited in GenBank has revealed that the E6 isolated in patients admitted in the Hospitals of prefecture of Modena were more similar to European E6 isolates compared to those of other parts of the world, such as China. The obtained sequences, compared to each other showed a homology degree of 100% permitting to consider all the E6 as part of a single outbreak. Since the samples belonged to patients admitted in different Hospitals of the prefecture of Modena it was not clear the link among them.

E6 is one of the most commonly HEV associated with aseptic meningitis outbreaks in Europe, in fact has been identified in Spain (19), Finland (2), Greece (20) and France (1). To our knowledge, this is the only outbreak reported in Italy. So far, an Italian study showed that E6 was the most frequently isolated in sewage samples collected from treatment plants located in central-eastern area of Milan and its hinterland (13).

Conclusions

In conclusion, the present work reported the circulation of a same E6 type during the single outbreak occurred in a large area of the prefecture of Modena. The wide distribution of the same E6 type in several districts of Modena prefecture may be considered a prerequisites for others potential E6 outbreaks. For this reason, a continuous surveillance of HEVs circulation is necessary to control their transmission both community and individual levels.

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