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SHORT COMMUNICATION

A cluster of Enterovirus 71 subgenogroup C2 in a nursery school, Italy, 2014

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SUMMARY

During October 2014, enterovirus (EV) RNA was detected in the stools of four children attending the same class in a nursery school, and hospitalized with mild febrile and vomiting disease in Parma, Italy. Upon sequencing, the viruses were characterized as EV71 subgenogroup C2. Phylogenetic analysis of the four EV71 C2 viruses allowed the distinction of a diverging lineage within subgenogroup C2, containing the Italian EV71 C2 strains and viruses detected in France in 2013. The identification of an outbreak of EV71 C2 in Italy extended information on the geographic diffusion and clinical relevance of these viruses in Europe.

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Enterovirus 71 (EV71) is a small, single-stranded, positive-sense non-enveloped RNA virus of the genus *Enterovirus,* family *Picornaviridae* (Solomon *et al.,* 2010; McMinn, 2012). EV71 possesses a 7.5 kb RNA genome with a single open reading frame (ORF) encoding a polyprotein, flanked by 5' and 3' untranslated regions (UTRs). The polyprotein is cleaved into 11 proteins, i.e. four capsid proteins (VP1, VP2, VP3, VP4), and seven non-structural proteins (2A, 2B, 2C; 3A, 3B, 3C, 3D).

In children, EV71 mainly causes asymptomatic or benign infections, such as neonatal fever and hand-foot and mouth disease (HFMD); less frequently, EV71 causes neurologic complications, such as encephalitis and poliomyelitis-like paralysis (Solomon *et al.,* 2010).

Sequencing of VP1 has been used for genotyping and phylogenetic analyses, and six genogroups (A-F) have been classified in EV71. Genogroups B and C are further divided into five subgenogroups (B1-B5 and C1-C5) (Deshpande et al., 2003; Solomon *et al.,* 2010; Bessaud *et al.,* 2014). Subgenogroups B4, B5, and C4, and, to a lesser extent, C2 were the main EV71s co-circulating in Asian countries while, subgenogroups C1 and C2 were the most frequent EV71s found in Europe between 1990-2009 (McMinn, 2012; Mizuta *et al.,* 2014).

In the Asian–Pacific region, EV71 has emerged as a major public health concern over the past 15 years (Shih *et al.,* 2000; McMinn, 2012; Sanders *et al.,* 2006; Kung *et al.,* 2007; Solomon *et al.,* 2010). Large outbreaks have been reported, associated with the emergence of new genogroups and subgenogroups, high rates of illness, and fatal cases of encephalitis (Solomon *et al.,* 2010; Yip *et al.,* 2013). No particular genotype has been conclusively associated with an increased risk of acute neurological disease (Solomon *et al.,* 2010), although large epidemics were in general associated with genotype replacement (Wang *et al.,* 2002; 2010; van der Sanden *et al.,* 2010). Conversely, epidemic activity of EV71 was low in Europe and America, where only few outbreaks have been reported over the last 30 years (Witsø *et al.,* 2007; Bible *et al.,* 2008; Schuffenecker *at al.,* 2011). Recent information on the epidemiology of EV71 C2 strains in European countries are not available and only limited sequence data have been recorded during the last five years. Overall, many aspects of the epidemiological and evolutionary dynamics of circulating EV71 strains remain unknown (Tee *et al.,* 2010).

Here we report the findings of a cluster of EV71 subgenogroup C2 strains detected in Italy during October 2014 in children attending the same class in a nursery school. We determined the partial sequence of the VP1 gene and analyzed the virus sequences with cognate sequences available in the NCBI database.

From October 21 to 27, 2014, four children were hospitalized at the Maternal Infantile Department of the University Hospital of Parma, Northern Italy, with mild febrile, vomiting disease and in three cases neck stiffness. The symptoms had appeared 24 hours before hospitalization in all children, except one where symptoms appeared some hours before. No severe neurological complications were observed. Stools, pharyngeal swabs, and cerebrospinal fluid (CSF), collected at admission, were submitted to routine bacteriologic and virologic examinations. The clinical features and the results of laboratory investigations for the four children infected with EV are summarized in Table I. EV was detected by real-time PCR (ELITechGroup Molecular Diagnostics, Italy) in the stools of the four children and in the pharyngeal swabs of three of the four children. EV was not detected in the CSF. The leukocytes count in the four patients ranged from 57 to 588 cells/mm^3.

Key words:
Enterovirus 71, Subgenogroup C2, Italy, Outbreak, Phylogenetic analysis.

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Table 1 - *Clinical and laboratory findings in four children with enterovirus 71 C2 infection.*

						Enterovirus detection					
Case ID	*Collection Date*	*Gender*	*Age (years)*	*Clinical manifestation*	*Leukocytes/ mm³*	*Stool*		*Pharyngeal swabs*		*CSF*	
						real-time PCR	*Culture*	*real-time PCR*	*Culture*	*real-time PCR*	*Culture*
PR4536/2014 (1st case)	21/10/2014	M	3	Fever, vomiting, drowsiness, neck stiffness, abdominal pain	588	Positive	Negative	Positive	Negative	Negative	Negative
PR4596/2014° (2nd case)	25/10/2014	M	4	Fever, vomiting, neck stiffness	57	Positive	Negative	Positive	Negative	Negative	Negative
PR4597/2014 (3rd case)	25/10/2014	M	5	Fever, headache, vomiting, arthralgia, neck stiffness, ataxia, spinal pain	116	Positive	Negative	Positive	Negative	ND	ND
PR4620/2014° (4rd case)	27/10/2014	M	5	Fever, vomiting, diarrhea, headache	ND	Positive	Negative	Negative	Negative	ND	ND

°Stool positive for Campilobacter jejuni by Matrix-Assisted laser desorption/ionization.
*Pharyngeal swab positive for adenovirus by real-time PCR. ND: Not done; CSF: cerebrospinal fluid; M: male.
Stools were examined for Salmonella spp, Shigella spp, Staphylococcus aureus, and Campylobacter spp, by culturing with selective and differential media and for enteric viruses by electron microscopy, latex agglutination (for adenovirus and rotavirus), real-time RT- PCR (for norovirus) and cell cultures. Pharyngeal swabs were examined for antigen detection of influenza A and B viruses, parainfluenza 1, 2, 3 viruses; respiratory syncytial virus and adenovirus in cell culture by immunofluorescence, 18 hours after inoculation, for nucleic acid detection of influenza A and B viruses, parainfluenza 1, 2, 3 viruses, respiratory syncytial virus, adenovirus, metapneumovirus, bocavirus, coronavirus by real-time PCR and for virus isolation in cell culture. CSFs were submitted to bacteriscopic examination and culture for bacteria as well as real-time PCR for adenovirus, parvovirus, cytomegalovirus, herpes simplex 1-2, varicella zoster, human herpes 6, and Toscana viruses.

Three children were treated with antibiotics (ceftriaxone, 100 mg/kg/day for 5 days, until negative response for bacteria by culture in CSF) and antivirals (acyclovir, 30 mg/kg/day for 2 days, until negative response for herpesvirus DNAs in CSF) and the symptoms disappeared in two weeks. The duration of hospital admission was ten days for the first, three days for the second and four days for the third child. The fourth child was also infected by norovirus and presented with diarrhea. This patient was treated with rehydration and maintenance therapy (balanced glucose-electrolyte solutions), and completely recovered 24 hours after three days of hospitalization.

EV typing was performed with a semi-nested RT-PCR amplification of the VP1 gene and direct sequencing of a 375 bp amplicon between nucleotide positions 2602 and 2977 of EV71 genome (Nix *et al.,* 2006), using a BigDye Terminator v3.1 Cycle Sequencing Kit and an automated sequencer ABI 3730 (Applied Biosystems, USA).

Partial EV VP1 nucleotide sequences were generated only from the stool samples (GenBank accession numbers KT834994-KT834997). The four EV strains were characterized as genogroup 71, subgenogroup C2 and were identical to each other (100% nucleotide identity). For sequence and phylogenetic analyses, sequences were retrieved from the NCBI database for a selection of EV71 C2 circulating in Europe and Eastern and Southeastern Asia from 1995 to 2009 and for all C2 strains circulating from 2010 to 2015. Phylogenetic analysis was performed using MEGA v.6.0 (Tamura *et al.,* 2013).

The Italian C2 strains showed the closest nucleotide identity (96.2%) to a C2 strain detected in France in 2013 (GenBank number HG934279), which, in turn, was closely related to other French C2 strains detected in 2013 (*Fig.* 1). Conversely, the four Italian C2 EVs were more distantly related (4.8% nucleotide difference) to EV71 C2 strains detected in 2014 in Central Italy (GenBank number KM079156) and in Russia (GenBank number KR827498). Overall, the vast majority of the recent EV71 C2 sequences available in the database (93.54%, 29 out of 31, including the four Italian EV71 C2 strains) were detected in European (Italy, France, Germany, and United Kingdom) and Asian (Japan, Taiwan, and Russia) countries in 2010-2014, and formed a major lineage. Nucleotide variation within this lineage ranged from 3.8 to 4.8%.

Interestingly, the recent EV71 C2 strains were genetically unrelated to older strains dating back to the years 1995-2009, which showed an intertwining pattern of segregation, with the 1990s strains located at a basal level in the phylogenetic tree. The extent of nucleotide variation among the EV71 subgenogroup C2 strains reached 8.1%.

The identification of an Italian outbreak of EV71s C2 in children attending a nursery school, and hospitalized with mild to moderate symptoms has extended information on the geographic diffusion and clinical relevance of these viruses in Europe, where the epidemiologic and molecular data on EV71 strains are limited and scattered. In Italy, there is no specific surveillance for EV infections, and the extent of EV71 diffusion is not known as no report exists on the circulation of this virus, which is documented only by a sequence submitted to the NCBI database.

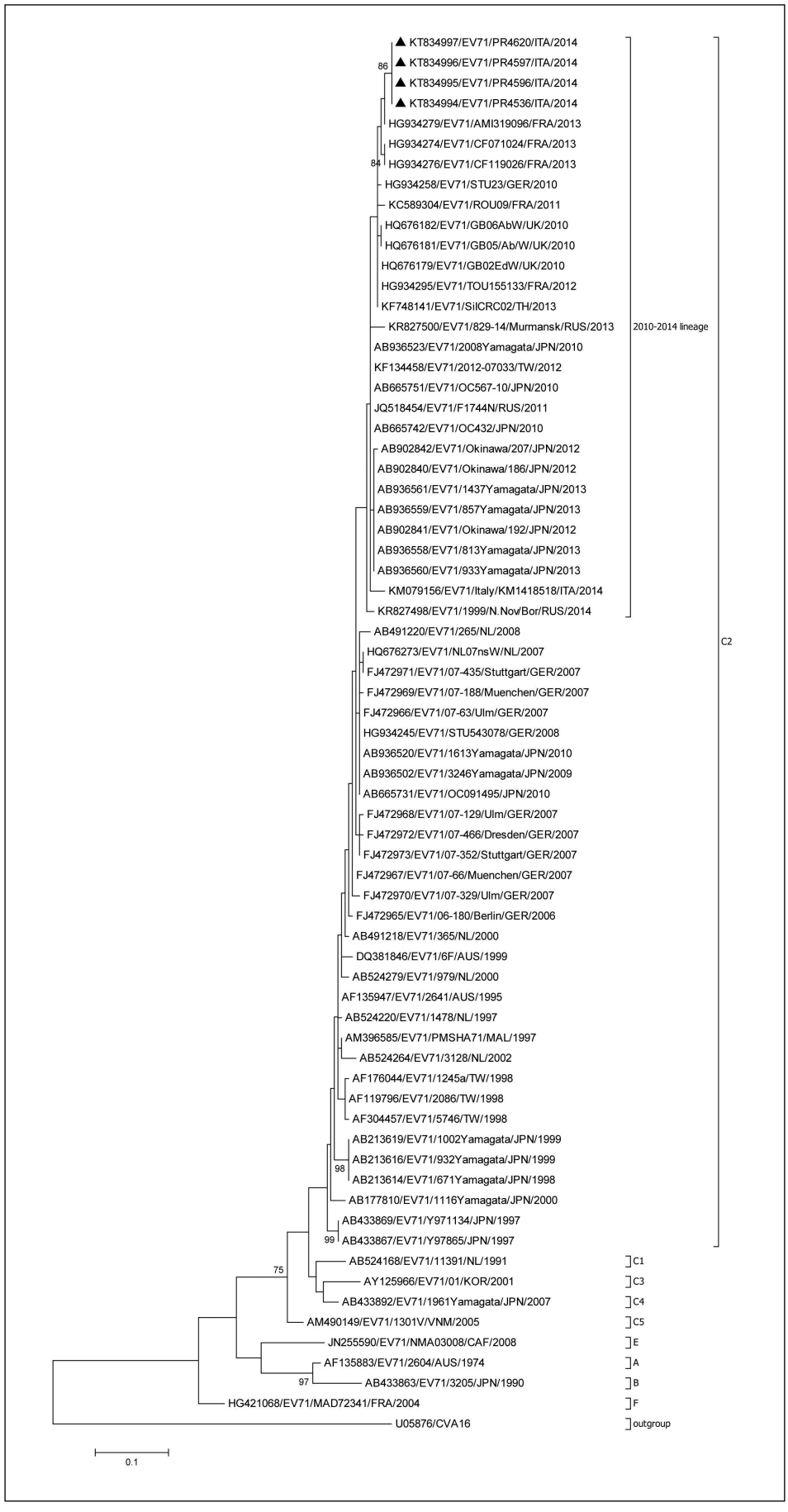

Figure 1 - *Phylogenetic analysis based on partial VP1 (375 nt) of the four Italian enterovirus 71 subgenogroup C2 strains (▲). The reference sequences were retrieved from the GenBank database. The tree was built with the maximum-likelihood method, and bootstrapped with 1000 repetitions. Bootstrap values >70% are indicated. The scale bar indicates the number of nucleotide substitutions per site.*

Comprehensive analysis of a large dataset of EV71 C2 allowed us to obtain hints on the evolution of these viruses and characterize the Italian C2 strains. Phylogenic analysis revealed a novel genetic signature for EV71 C2 viruses detected in different geographic settings after 2010 and suggests that C2 isolates are evolving and spreading worldwide. Although other EV71 C2 sequences detected in some European countries in recent years (2010-2014) are available in the database, the molecular trends of EV71 C2 in Europe have not been investigated thus far. The circulation of different EV71 C2 lineages highlights that the subgenogroup C2 is undergoing a fast diversification. Switching among lineages seems a common mechanism in the evolution of RNA viruses, and, in the case of EV71, the emergence of novel viral strains seems to correlate with the onset of epidemics.

It is possible that the simple scenario depicted in our analysis will be challenged and confuted as more sequence data are made available for recent EV71 C2 viruses. Nonetheless, the observed genetic heterogeneity likely reflects the results of processes of selection and diversification acting on a global scale, rather than the results of local selection. Monitoring the circulation of EV71 will be useful to generate a national database and integrate the data generated by European and extra-European laboratories.

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