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SHORT COMMUNICATION

Genetic Characterization of Human Enteroviruses Associated with Hand, Foot and Mouth Diseases in Poland, 2013–2016

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Abstract

The objective of the present study was to describe the molecular characteristics of enteroviruses associated with hand, food, and mouth disease (HFMD) in Poland. Clinical material from HFMD cases, that occurred during 2013–2016 were examined. It has been showed that coxsackievirus A6 (CVA6), CVA10 and CVA16 were circulating in the country. Phylogenetic analysis showed that Polish CVA6 strains were divided into two distinct clusters suggesting two independent introductions. This is the first report of CVA6 infections associated with HFMD in Poland. These results emphasize the need for continuous monitoring of HFMD and facilitation of the diagnosis using molecular approaches.

Key words: coxsackieviruses, genotyping of HFMD enteroviruses, hand, foot and mouth disease (HFMD), human enteroviruses (HEVs)

Hand, food and mouth disease (HFMD) is a common infection characterized by fever, stomatitis and a vesicular rash affecting the hands, feet, and occasionally the buttocks. The disease is usually mild and self-limiting, but serve neurological and systemic syndromes that can be fatal occur in some patients. HFMD is common in children younger than 5 years old, but can also occur in older children and adults. It is caused by human enteroviruses (HEVs) belonging to the *Picornaviridae* family. The most common etiologic agents are coxsackievirus A16 (CVA16) and enterovirus 71 (EV71), but other enteroviruses, mainly belonging to the species HEV-A (CVA2, CVA4-8, CVA10, CVA12), but also HEV-B (echoviruses: E4, E7, E9, E11, E25, E30 and CVB1-5, CVA9, EV84) have been associated with illness (Lei *et al.*, 2015).

Recently, the switch of HFMD etiology has been suggested by the increased epidemics of serotypes other than EV71 and CVA16, including CVA6, CVA10, and CVA12. Serve HFMD in children and atypical HFMD in adults has been reported in association with CVA6 (Lott *et al.*, 2013). The switch of HFMD etiology requires a precise virus genotyping in the surveillance for a better HFMD control.

There are no published data available on the incidence of HFMD in Poland, there is no active public health surveillance for HFMD and it is not a notifiable infection. Whereas EV71 has been occasionally detected in clinical samples (Wieczorek and Krzysztoszek, 2016). To better understand the molecular characteristics of enteroviruses circulating in Poland, particularly the diversity of those viruses associated with HFMD, we examined clinical material from HFMD cases, that occurred during the period 2013–2016, in three regions of Poland.

Since November 2013 to March 2016, a total of 28 specimens (10 stools, 18 throat swabs) were collected from 27 clinically diagnosed HFMD cases (Fig. 1) from 3 voivodoships. Patient's age ranged from 5 months to 17 years (Table I). The clinical samples were tested with diagnostic pan-enterovirus RT-PCR. Viral RNA was extracted from 140 µl of the sample using QIAamp Viral RNA Mini Kit (Qiagen) following the manufacturer's instructions. RT-PCR was carried out using pan-enterovirus primers based on the WHO manual (WHO, 2004). This set of primers produces a product of 114 bp and has been designed to detect and amplify a genome segment present at the 5'NCR of the enterovirus genomes. Out of

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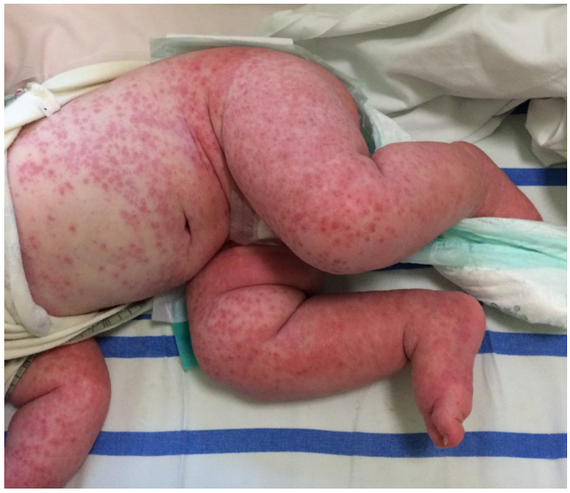

Fig. 1. Child with atypical hand, food and, month disease associated with coxsackievirus A6 infection, Wroclaw, Poland, February 2016.

28 samples, 20 (71.4%) were positive for enteroviruses using RT-PCR, 10 stools (100%) and 10 swabs (55.5%).

Viruses have been isolated from throat swabs and stool specimens by conventional cell culture method using WHO recommendations. Isolation was performed on RD cells (human rhabdomyosarcoma). A volume of 200 µl of the sample was inoculated into tubes with RD cells. The tubes were incubated at 36°C and were examined daily. After 7 days, the tubes were frozen and thawed and re-passaged, and anther 7-day examination was performed. Each specimen underwent three passages. The study found that among the samples with positive RT-PCR results 6 (30%) were positive. Enterovirus was isolated from the stools of 6 patients and not even one throat swab. Complete cytopathic effect (CPE) has occurred after 12 to 23 days post-inoculation (Table I).

To identify the enterovirus type in positive samples, RT-PCR specific for a partial sequence of the viral pro-

Table I
Laboratory findings in clinical specimens and epidemiologic data for patients with HFMD, Poland 2013–2016.

No.	Sample identification number	Sex/age	Region	Sampling date	Specimen type	Isolation result	5'NCR RT-PCR	HEV-A RT-PCR
1	672/PL30/2013	f/1 y	wielkopolskie	XI.2013	stool	+ (23d)	+	+ CVA10
2	1HFMD/PL14/2014	f/9 m	mazowieckie	VII.2014	throat swab	–	+	–
3	2HFMD/PL14/2014	m/9 y	mazowieckie	VII.2014	throat swab	–	–	–
4	3HFMD/PL14/2014	f/2 y	mazowieckie	VII.2014	throat swab	–	+	–
5	4HFMD/PL14/2014	f/17 y	mazowieckie	VII.2014	throat swab	–	–	–
6	0610/PL14/2014	m/2 y	mazowieckie	X.2014	stool	+ (17d)	+	+ CVA6
7	5HFMD/PL14/2014	f/1 y	mazowieckie	X.2014	stool	+ (14d)	+	+ CVA6
8	6HFMD/PL14/2014	m/3 y	mazowieckie	X.2014	stool	+ (15d)	+	+ CVA16
9	7HFMD/PL14/2014	m/9m	mazowieckie	X.2014	stool	–	+	+ CVA6
10	8HEMD/PL14/2015	f/5 y	mazowieckie	III.2015	stool	+ (13d)	+	+ CVA16
11	9HFMD/PL14/2015	f/2 y	mazowieckie	III.2015	stool	+ (12d)	+	+ CVA16
12	20HFMD/PL02/15	m/11 y	dolnośląskie	IV.2015	throat swab	–	–	–
13	10HFMD/PL14/2015	m/4 y	mazowieckie	VI.2015	stool	–	+	+ CVA6
14	11HFMD/PL14/2015	m/2 y	mazowieckie	VI.215	stool	–	+	+ CVA6
15	19HFMD/PL02/2015	f/15 y	dolnośląskie	VI.2015	throat swab	–	+	–
16	18HFMD/PL02/2015	m/7 y	dolnośląskie	VII.2015	throat swab	–	+	–
17	16HFMD/PL02/2015	f/5 m	dolnośląskie	VIII.2015	throat swab	–	–	–
18	17HFMD/PL02/2015	m/11 y	dolnośląskie	VIII.2015	throat swab	–	+	–
19	24HFMD/PL02/2015	m/1 y	dolnośląskie	XI.2015	stool	–	+	–
20	24HFMD/PL02/2015	m/1 y	dolnośląskie	XI.2015	throat swab	–	–	–
21	28HFMD/PL02/2015	m/1 y	dolnośląskie	XI.2015	throat swab	–	+	+ CVA6
22	13HFMD/PL02/2015	m/1 y	dolnośląskie	XI.2015	throat swab	–	+	+ CVA6
23	14HFMD/PL02/2015	m/2 y	dolnośląskie	XI.2015	throat swab	–	+	+ CVA6
24	15HFMD/PL02/2015	m/1 y	dolnośląskie	XI.2015	throat swab	–	+	–
25	12HFMD/PL02/2015	m/1 y	dolnośląskie	XII.2015	throat swab	–	–	–
26	21HFMD/PL02/2016	m/7 m	dolnośląskie	II.2016	throat swab	–	+	+ CVA6
27	22HFMD/PL02/2016	m/13 y	dolnośląskie	II.2016	throat swab	–	–	–
28	23HFMD/PL02/2016	f/16 y	dolnośląskie	III.2016	throat swab	–	–	–

tein 1 (VP1) region, was performed by using Superscript III (Invitrogen) followed by a second amplification reaction with nested primers for species A and B VP1 sequences and PCR cycling times and temperature as previously described (Leitch *et al.*, 2009). Amplified products were analysed in 1.5% agarose gels, GelRed-stained, and examined under a UV DNA transilluminator. The resulting DNA templates were processed in cycle sequencing reaction with a BigDye 3.1 according to manufacturer's protocol. The product of sequencing reaction was run in an automated genetic analyser (Applied Biosystems, model 3730). The resulting sequences were manually edited using BioEdit program and examined in terms of closest homologue sequence using BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/). The sequences of isolated strains (region VP1) were aligned with the reference strains. A phylogenetic tree was computed using the neighbor-joining method with bootstrap 1000 replicates. Molecular and phylogenetic analyses were conducted using MEGA version 6.06 (Tamura *et al.*, 2013) (http://www.megasoftware.net/). Sequences have been assigned GenBank accession numbers KX865266 to KX865274.

Out of 20 positive samples, 13 were positive in reaction characteristic for species A and none in reaction for species B. A total of 3 different serotypes of species A were identified by partial VP1 sequencing (CVA10, CVA6, CVA16). The typed strains included 6 cell culture

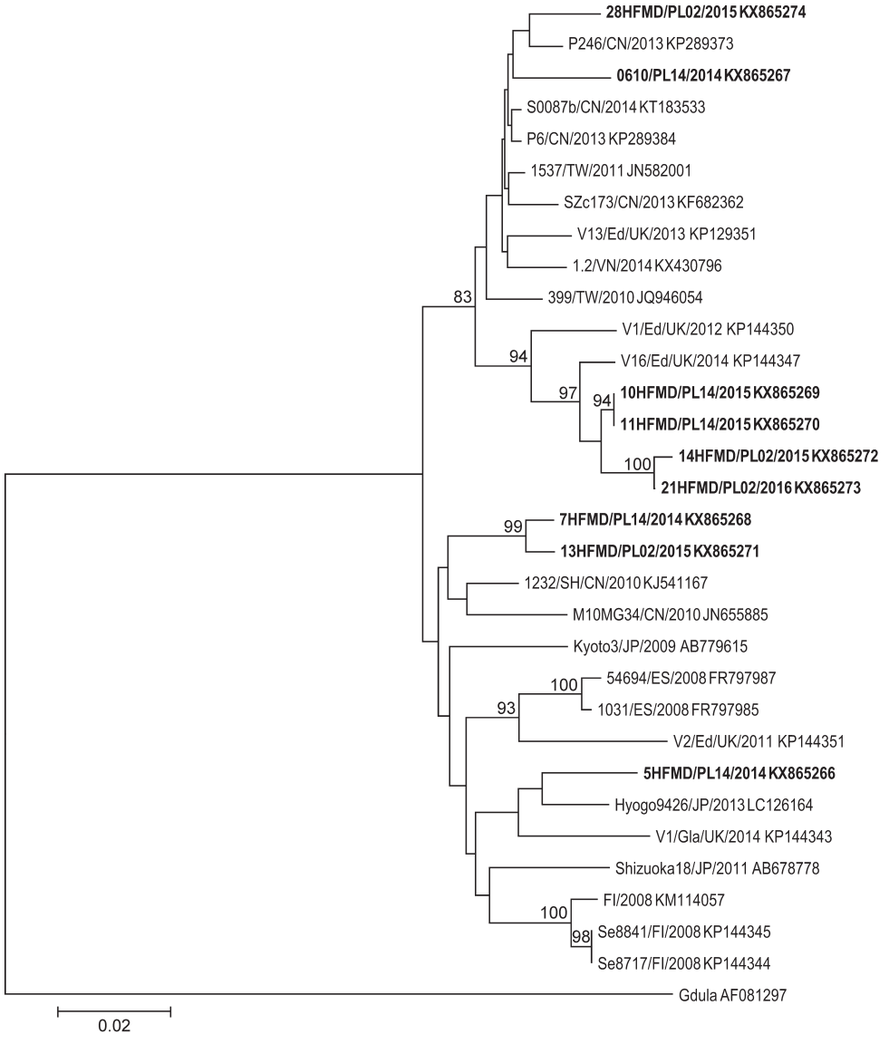

Fig. 2. Phylogenetic tree depicting the relationships between partial capsid gene sequences of 9 Polish CVA6 strains isolated from 2013 to 2016 and 23 sequences from GenBank (609 nt). Each strain is referenced by its geographical origin and its accession number.
The tree was constructed by neighbour-joining method and evaluated with 1000 bootstrap pseudoreplicates. Only bootstrap values ≥ 80% are indicated. In the analyses, genetic distances were calculated with Kimura 2-parameter algorithm. Analyses were conducted in MEGA 6.06 (Tamura *et al.*, 2013).

isolates and 7 strains primarily detected by RT-PCR. CVA6 was identified in 9 and CVA16 in 3 typed cases, respectively. One patient was positive for CVA10.

Genetic analysis of viral sequences showed that CVA6 strains were divided into two clusters (Fig. 1). In general, nucleotide sequence divergence in pairwise comparisons among isolates range from 0.0% to 7.5% (0.0–2.5% amino acid divergence). Compared with the prototype strain Gdula, the genetic divergence increased to 18.4–20.0%. Cluster I includes three sequences of CVA6 isolated in 2014–2015, presenting 1.0–5.6% nucleotide divergence (0.5–2.0% amino acid divergence). These strains had closest genetic relationship with isolates previously identified in various geographical origins (China 2010–2013, United Kingdom 2013, Japan 2013). Cluster II, comprised six strains from 2014–2016, presenting 0.0–5.6% VP1 nucleotide sequence divergence (0.0–2.0% amino acid divergence). Virus strains belonged to cluster II grouped together with strains isolated in the United Kingdom (2014) and China (2009–2014) (Fig. 2). Three Polish CVA16 strains from 2014–2015, were genetically homogeneous, presenting 0.0–2.0% nucleotide divergence and were closely related to Chinese strains isolated in 2011. One strain CVA10 grouped together with those from Russia isolated in 2009–2013 and Spain (2008).

In Poland, there is no the surveillance of HFMD. Therefore, little is known about the pathogenic roles of enteroviruses, their geographic distributions, and epidemiological data. The results demonstrated that CVA6, CVA10, and CVA16 emerged and co-circulated in Poland. To the best of our knowledge, this is the first report of CVA6 infections associated with HFMD in Poland. As an emerging pathogen, CVA6 increasingly became as common a causative agent of HFMD in Poland as was CVA16. CVA6 has been associated with more severe and extensive rash than HFMD caused by other enteroviruses (Wei *et al*., 2011). Since 2008, international outbreaks of CVA6 HFMD in children and adults have been described (Osterback *et al*., 2009; Blomqvist *et al*., 2010; Wu *et al*., 2010; Wei *et al*., 2011; Fujimoto *et al.*, 2012; Mirand *et al.*, 2012; Puenpa *et al*., 2013; Cabrerizo *et al.*, 2013; Sinclair *et al*., 2014), but no outbreaks had been reported in Poland previously. Although all 9 of the CVA6 strains identified in the Polish cases were genetically closely related (based on partial VP1 gene sequences) to CVA6 strains identified in recent international outbreaks. Two genetically distinct CVA6 clusters were co-circulating in Poland suggesting two independent introductions of the virus to Poland. It may be noted that the analysis of data obtained in this study was limited due to the small size of samples available.

In mild cases of HFMD laboratory testing is not necessary. Testing is usually reserved for severe cases and public health investigation of outbreaks. Of the 20 samples identified as HEV on the basis of the 5'NCR, only 13 could be typed using primers specific to the VP1 region. The failure of amplification of typing regions in seven specimens may have been due to a low viral load. Genotyping was performed directly in clinical specimens for 7 samples and for 6 isolates. HEV identification was effective for 50% of the EV-positive clinical samples and for 100% of the isolates. During the 2008 Finnish outbreak, EV identification was effective for 55% of the EV-positive clinical samples (Blomqvist *et al.*, 2010). In most epidemiological studies of HFMD, enterovirus genotyping relies on virus isolates despite the difficulty of growing HEV-A serotypes in cell culture.

CVA are generally difficult to grow in culture (Nsaibia *et al.*, 2007). Because of this feature, isolation from clinical material is often unsuccessful. Most CVA could propagate in RD cells, but they generally require more than one passage before inducing a detectable cytopathic effect. In this study, all the isolates were obtained on RD cell in the second or third passage and only from stool specimen. All identified CVA10 and CVA16 strains were able to multiply in RD cells, whereas only 40% of identified CVA6 strains showed successful propagation in RD cells, suggesting that CVA6 is more difficult to isolate than CVA16 or CVA10.

As HFMD is not a notifiable disease in Poland, the actual number of HFMD cases is not known. Enteroviral infections are underdiagnosed as a result of the lack of routine surveillance and also frequently atypical presenting symptoms and signs. In addition, increasing awareness will help to improve laboratory diagnosis and management of infected children. Improved HFMD surveillance is required, with virus genotyping as a key element.

In summary, the present investigation highlights the co-circulation of CVA6, CVA10 and CVA16 types causing HFMD in Poland. These results emphasize the need for continuous monitoring of HFMD in Poland and facilitation of the diagnosis of the associated HEV infections using molecular approaches.

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