RESEARCH NOTE

DETECTION AND GENETIC CHARACTERIZATION OF HUMAN ENTEROVIRUS A71 FROM HAND, FOOT AND MOUTH DISEASE CASES IN BANJARMASIN, INDONESIA

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Abstract. Enterovirus A71 (EV-A71) infection associated with hand, foot and mouth disease (HFMD) has been recognized as a major health threat especially in children. The aim of this study was to identify and genetically characterize the viral etiological agent associated with HFMD. Using molecular methods, EV-A71 sub-genogroup B5 was detected as the causative agent in 7 out of 13 archived throat swab specimens collected during the 2016 HFMD outbreak in Banjarmasin, South Kalimantan, Indonesia.

Keywords: enterovirus A71, hand, foot and mouth disease, Indonesia

INTRODUCTION

Enterovirus 71 (EV-A71), an RNA virus of family Picornaviridae, is a common cause of hand, foot and mouth disease (HFMD) (Solomon *et al*, 2010). The infection usually manifests with fever, rash, blisters and oral lesions, and in severe cases may proceed to encephalitis, aseptic meningitis, pancreatitis, cardiopulmonary complications and poliomyelitis-like paralysis (Wu *et al*, 2002; Ooi *et al*, 2010; Zhang *et al*, 2016). The virus is highly transmissible via a fecal-oral route and respiratory droplets and is recognized as a global public health threat with a potential to cause pandemics similar to poliovirus (Yip *et al*, 2013). EV-A71, together with coxsackievirus CV-A6 and CV-A16, attract the most public health because these three viruses are the major common causes of HFMD. However, compared to CV-A6 and CV-A16, EV-A71 has a stronger association with CNS involvement, such as acute flaccid paralysis (Long *et al*, 2016; Suresh *et al*, 2017).

EV-A71 epidemics have occurred with increasing numbers in the Asia-Pa-

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cific region, including Hong Kong, Japan, mainland China, Malaysia, Singapore, Taiwan, and Thailand for the past two decades (Shimizu *et al*, 1999; Fujimoto *et al*, 2002; Lin *et al*, 2003; Chua *et al*, 2007; Wu *et al*, 2010; Mauleekoonphairoj *et al*, 2015). In the Southeast Asian region, HFMD usually occur during the transition from rainy to dry season and the numbers have increased with large temperature fluctuations (Hii *et al*, 2011). A study in Indonesia between 2008-2012 detected EV-A71 as a cause of mild HFMD across the country with a prevalence of 6.5% (Susanti *et al*, 2014).

Virulence of EV-A71 depends on multiple factors, with host and environmental factors appearing to be the most significant (Shih *et al*, 2011; Chang *et al*, 2012). Mutations in VP1 gene, which encodes the capsid protein has reported to be associated with EV-A71 virulence, resistance and adaptation in *in-vitro* studies (Shih *et al*, 2004; Chen *et al*, 2008; Kelly *et al*, 2015). Despite being endemic, studies on the etiological agent of HFMD and genetic characterization of the virus are limited in Indonesia. This study aimed to describe the investigation on HFMD cases caused by EV-A71 in Banjarmasin, Indonesia in 2016.

MATERIALS AND METHODS

Samples

This study was conducted retrospectively on specimens from HFMD cases collected between November 2015 to February 2016 at pediatric wards in Ulin General Hospital and Islamic Hospital, Banjarmasin, South Kalimantan, Indonesia. Throat swab specimens were from 13 hospitalized patients presenting with fever (≥38.5°C) and typical HFMD symptoms, viz. rash and blisters in the mouth, hands and feet. Samples were eluted from swabs using Viral Transport medium prepared from brain heart infusion (Oxoid, Hampshire, UK) and penicillin-streptomycin (Gibco, Carlsbad, CA) and stored at -80°C until shipped to the Eijkman Institute, Jakarta for viral molecular testing.

The study was approved by the Eijkman Institute for Molecular Biology Research Ethics Commission (no. 66, 18 November 2013). Residential locations of the cases were based on street addresses provided by the patients.

PCR-based virus genotyping assay

Viral RNA was extracted using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) to obtain 60 μl of RNA, from which 5 μl aliquot was taken for cDNA synthesis employing Go Script Reverse Transcription System (PROMEGA, Madison, WI) followed by PCR with Go Taq Green Master Mix (PROMEGA) pan-enterovirus primers targeting the 5′-untranslated region (Wiyatno *et al*, 2016) and primers targeting β-actin gene as internal control. In brief, the reaction mixture (25 μl) contained 2 μl cDNA template, 400 μM forward and reverse primers, 1.5 mM $MgCl_2$, 200 μM dNTPs, and buffer containing DNA polymerase (PROMEGA, Madison, WI). Thermocycling was conducted in Proflex PCR instrument (Applied Biosystems, Carlsbad, CA) as follows: 95°C for 15 minutes;16 cycles of 94°C for 45 seconds, 65°C for 45 seconds (-1°C/cycle) and 72°C for 45 seconds; 32 cycles of 94°C for 45 seconds, 48°C for 45 seconds, and 72°C for 45 seconds; and a final step at 72°C for 5 minutes. Amplicons (~400 bp) were detected by 1.5% agarose gel-electrophoresis and staining with SYBR Safe (Invitrogen, Carlsbad, CA), directly sequenced at Eijkman Institute,

Jakarta and analyzed using Geneious Software V.8. (Biomatters, Auckland, NZ). The presence of EV-A71 was confirmed by amplifying a 891-bp region of the VP1 gene followed by sequencing for phylogenetic analysis (Ap *et al*, 2011; Ling *et al*, 2014). In short, the PCR mixture (25 µl) contained 2 μl cDNA template, 400 μM upstream and downstream primers, 1.5 mM $MgCl_2$, 200 μM dNTPs and buffer containing DNA polymerase (PROMEGA, Madison, WI), and amplified using the following thermocycling conditions: 95°C for 10 minutes; 44 cycles of 94°C for 45 seconds, 50°C for 45 seconds and 72°C for 1 minute; with a final step at 72°C for 5 minutes. Amplicons were processed for sequencing as described above.

All sequences were submitted to GenBank database with accession numbers KY041858, KY041859, KY041860, KY041861, KY041862, KY041863, and KY041864. All enterovirus negative samples were also tested using consensus PCR for other viral causes, such as herpesvirus (terminase gene; 419 bp) and paramyxoviruses (polymerase gene; 561 bp) (Chmielewicz *et al*, 2001; Tong *et al*, 2008).

RESULTS

Throat swab specimens came from six male and seven female children with median age of 16 months (range 8-37 months). In addition to the characteristic HFMD lesions, other symptoms, such as cough, conjunctivitis, diarrhea, seizure and vomiting were also observed in some patients. There were no CNS complications or mortality. Nine HFMD cases resided in southeastern Banjarmasin, with the remaining in the northwestern region of the city.

Seven HFMD patients were positive by pan-enterovirus PCR and one by paramyxovirus PCR (data not shown). Sequence analysis of the enterovirus-specific amplicons revealed all seven positives were EV-A71. Further amplification and sequencing of VP1 region demonstrated that all EV-A71 sequences had 96-98% similarity with strains from Malaysia, Taiwan, Thailand, and Vietnam, with all isolates belonging to the subgenogroup B5. Intratypic similarity among the sequences ranged from 93.7% to 99.9%. A dendogram constructed from 891 nt fragments of the VP1 region showed five isolates clustered with strains from mainland China and Taiwan, and the remaining with strains from Malaysia, Thailand and Vietnam (Fig 1). No co-detection with other enteroviruses were found.

DISCUSSION

In this study, we identified EV-A71 associated with a 2016 outbreak of HFMD in Banjarmasin, South Kalimantan. Although both hospitals where the patients were admitted were located in northeast Banjarmasin, cases came from other parts of the city. Human EV-A71 is considered endemic in Southeast Asia, with outbreaks occurring in cycles every 2-3 years in Japan, Malaysia and Taiwan and annually in China (Sabanathan *et al*, 2014). CV-A6 and CV-A16 are known to co-exist with EV-A71 especially in cases with CNS manifestations (Solomon *et al*, 2010; Ling *et al*, 2014; Gaunt *et al*, 2015) and recombination between EV-A71 and CV-A16 is linked with pathogenicity of the virus (Zhang *et al*, 2010). Only a single enterovirus strain was detected from swab of each HFMD patient.

The EV-A71 strains isolated in this study belonged to subgenogroup B5, the most prevalent strain in the region (Ooi *et al*, 2007; Ap *et al*, 2011; Hassel *et al*, 2015).

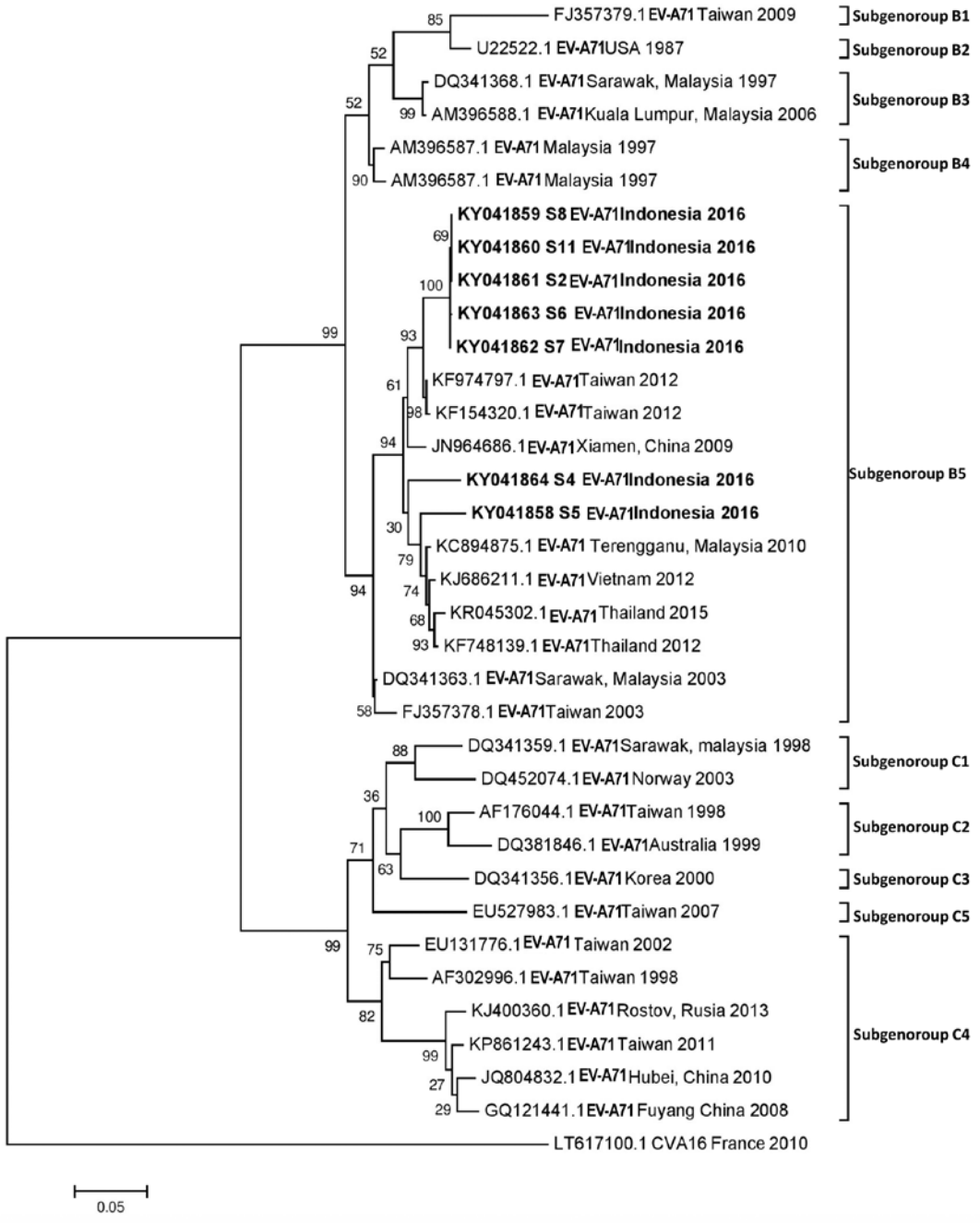

Fig 1-Phylogenetic tree of 891-bp enterovirus (EV)-A71 VP1 gene fragments isolated from seven hand, foot and mouth disease (HFMD) samples from Banjarmasin, South Kalimantan, Indonesia. Human coxsackievirus (CVA)A16 is used as the outgroup. GenBank accession number is indicated for each EV strain. Genetic relationship was analyzed using Molecular Evolutionary Genetics Analysis (MEGA) version 6.06 (http://www.megasoftware.net/mega.php) using neighbor-joining method and applying Kimura 2-parameter model with 1,000 bootstraps. Number at branch point denotes percent similarity. Scale barindicates percent nucleotide substitution chanbge.

In the Asia-Pacific region, subgenogroup B5 together with C4 have recently replaced subgenogroups B3 and B4 (Chang *et al*, 2016). Subgenogroup C4 is now recognized as the major subgenogroup causing severe illness particularly associated with CNS manifestations and high mortality.

To the best of our knowledge, this study is the first genetic characterization of EV-A71 from a HFMD outbreak in Banjarmasin, South Kalimantan, Indonesia. Our study should increase the awareness of EV-A71 as an etiological agent of HFMD in Indonesia. Further studies of genetic characterization are important to identify additional causative agents of HFMD and subgenotypes to understand the epidemiology of outbreaks.

ACKNOWLEDGEMENTS

The authors thank Ms Aghnianditya Dewantari for assistance in molecular testing of viral panels.

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