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Brief Original Article

Detection and identification of coxsackievirus B3 from sera of an Indonesian patient with undifferentiated febrile illness

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Abstract

Introduction: Coxsackievirus B3 (CVB3) virus has been implicated as the causative agent of various outbreaks of clinical disease, including hand, foot, and mouth diseases, aseptic meningitis, acute myocarditis, and inflammatory cardiomyopathy.
Methodology: Two hundred and nine undiagnosed cryopreserved specimens obtained from factory workers in Bandung, Indonesia, who displayed symptoms of acute febrile illness were gathered. Total RNA was isolated from serum and tested by conventional polymerase chain reaction (PCR) using *Enterovirus* genus-level primers and confirmed by sequencing. Concurrently, the virus was isolated in LLC-MK2 cells.
Results: CVB3 virus was identified in an archived specimen from a patient who presented with symptoms of fever, headache, myalgia, and nausea. Sequencing results of the VP1 region from both the clinical sample and tissue culture supernatant showed 97% homology to a CVB3 virus isolate from Taiwan. Virus propagation in LLC-MK2 cell culture exhibited severe cytopathic effects two days post-inoculation.
Conclusions: We report the first case of CVB3 from an undifferentiated febrile illness specimen from Indonesia.

Key words: Coxsackievirus; Indonesia; febrile illness.

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Introduction

Coxsackievirus B3 (CVB3), a member of the enterovirus B group, is a non-enveloped virus of the family *Picornaviridae*, and order *Picornavirales*. It contains a ~7.4 kb genome of single-stranded positive-sense RNA genome that encodes 2,185 amino acids [1]. CVB3 is an important human pathogen, and has been reported to be associated with type 1 diabetes mellitus, myopericarditis, aseptic meningitis, herpangina, pancreatitis and hand, foot, and mouth diseases (HFMD) [2-7]. Infection can be fatal, especially among infants younger than three months of age and pregnant women, whose infection may also lead to fetal growth retardation and miscarriage [6,8]. During an outbreak of HFMD in China and France, CVB3 was reported to be co-circulating with other enteroviruses [9-12]. Until now, CVB3 infection has never been reported in Indonesia. Here, we report the detection and identification of a CVB3 infection in Bandung, Indonesia.

Methodology

Clinical specimens

Sera were collected from factory workers who experienced febrile illnesses during a dengue cohort study in Bandung from 2006–2009 undertaken by Padjadjaran University in collaboration with US-NAMRU#2 [13,14]. In total, 209 sera underwent various testing panels to evaluate the presence of dengue and chikungunya infections. The samples were cryopreserved in -80°C until they were used in the current study. Those archived specimens, available from non-dengue cases, were transported to the Eijkman Institute of Molecular Biology for screening using comprehensive molecular viral detection panels. Collections were made under institutional review board approvals from the National Institute of Health

Research and Development, Indonesian Ministry of Health, and the United States Naval Medical Research Unit No. 2, Jakarta, Indonesia, and written informed consent was obtained as described in the earlier study [13,14]. This follow-up study of archived specimens was reviewed and approved by the ethical committee at the Eijkman Institute for Molecular Biology, Jakarta, Indonesia.

Molecular detection and identification of coxsackievirus B3

Viral ribonucleic acid was extracted from 140 µL of serum from patients with acute febrile illness using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany). cDNA synthesis was done using Go Taq Green Master Mix (PROMEGA, Inc., Madison, USA), according to manufacturer's instructions. The cDNA was then used as a template for detection by *Enterovirus* genus-level polymerase chain reaction (PCR) targeting the 5' untranslated region (primers used were 5' UTR Long-F: GGT CAA GCA CTT CTG TTT CCC and 5'UTR L541-R: GAA ACA CGG WCA CCC AAA GTA STC G). PCR was undertaken as follows: denaturing at 95°C for 15 minutes, then 16 cycles of 94°C for 45 seconds, 65°C for 45 seconds (-1°C/cycle), and 72°C for 45 seconds. This was followed by 32 cycles of 94°C for 45 seconds, 48°C for 45 seconds and 72°C for 45 seconds, finished with 72°C for 5 minutes. A recombinant plasmid carrying a sequence fragment from *Enterovirus* families was constructed and used as the positive control. Positive amplicons were further verified via sequencing to determine viral species. Additional characterization was done by sequencing the VP1 region of this virus for species determination, as previously described [15]. The VP1 region sequence was analyzed and compared with a selection of reference strains from the GenBank database (GenBank accession numbers JQ390213, FJ000001, FJ357838, GQ329744, JQ364875, GQ329754, JN203587, AM711026, DQ093619, M165721, and AF231765) using Geneious Software R8 version 8.1. A phylogenetic tree was constructed on the basis of 820 bp nucleotide fragments of the VP1 region using the neighbor-joining method and applying the Kimura 2-parameter method with 1,000 bootstraps of the neighbor-joining model as previously detailed [16].

Virus isolation

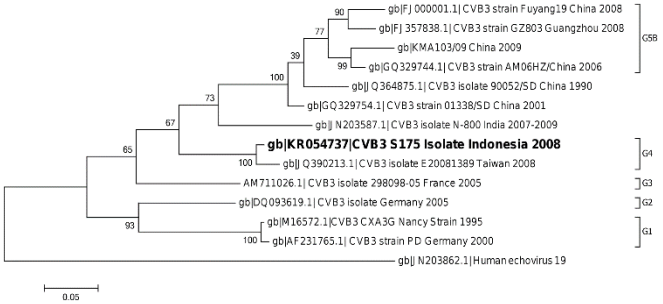
Virus isolation was performed on the PCR-positive sample by employing an LLC-MK2 cell line (rhesus monkey epithelial kidney cell, kindly provided by US-AFRIMS, Bangkok, Thailand) in Medium 199 (Life Technologies, Carlsbad, USA) supplemented with 7.5% fetal bovine serum (Life Technologies, Carlsbad, USA) [17]. In total, 100 µL of serum was inoculated onto each well of a 12-well plate containing 80% confluent cells, followed by incubation at 37°C. Presence or absence of cytopathic effect (CPE) was evaluated daily to assess viral infection.

Results

CVB3 was identified in one archived serum specimen from an adult female factory worker (sample S175) in Bandung, Indonesia, while molecular tests for other viruses were negative. This particular subject also tested positive for anti-dengue IgG in both acute and convalescent samples. The patient presented to the clinic following two days of fever (body temperature 38.3°C) with headache, nausea, and myalgia, but no arthralgia or rash. Physical examination showed hyperemic tonsil and epigastric tenderness. Hematology parameters such as hemoglobin levels, leukocyte, hematocrit and platelet counts, and other physical criteria were within normal limits.

PCR using enterovirus group-specific primers successfully amplified 400 bp of PCR product and revealed 96% similarity with enterovirus 84. The 820 bp of VP1 region sequence was analyzed by PCR sequencing. The VP1 region sequence demonstrated 92%–97% similarity with a CVB3 strain previously isolated in Taiwan (GenBank accession number JQ390213), which belongs to genogroup IV (Figure 1). The VP1 region sequence is considered the most reliable method for determining enterovirus genotypes and provides useful information for molecular epidemiology studies [18]

Figure 1. Phylogenetic tree analysis of CVB3 S175 isolated in Bandung based on VP1 gene sequence (820 bp) (GenBank accession number KR054737).
Human echovirus 19 was used as the outgroup. Genetic relationship was analyzed using Geneious Software R8 version 8.1. A phylogenetic tree was constructed on the basis of 820 length nucleotide fragments of the VP1 region by using the neighbor-joining method and applying the Kimura 2-parameter method with 1,000 bootstrap of the neighbor-joining model.



Virus inoculation of LLC-MK2 cells led to severe CPE after two days of incubation. Total destruction of the cell monolayer and severe changes in cell morphology were observed. Pyknotic shrinking of cells was observed under 200x magnification; furthermore, 95% of cells were detached from the flask compared to uninfected cells (Figure 2A-B). However, no vacuolization or syncytia formation was observed during CPE. Sequencing of the VP1 gene from supernatant RNA showed 100% homology to sequencing results from the direct clinical sample. Sequencing of the isolate was submitted to GenBank with accession number KR054737.

Discussion

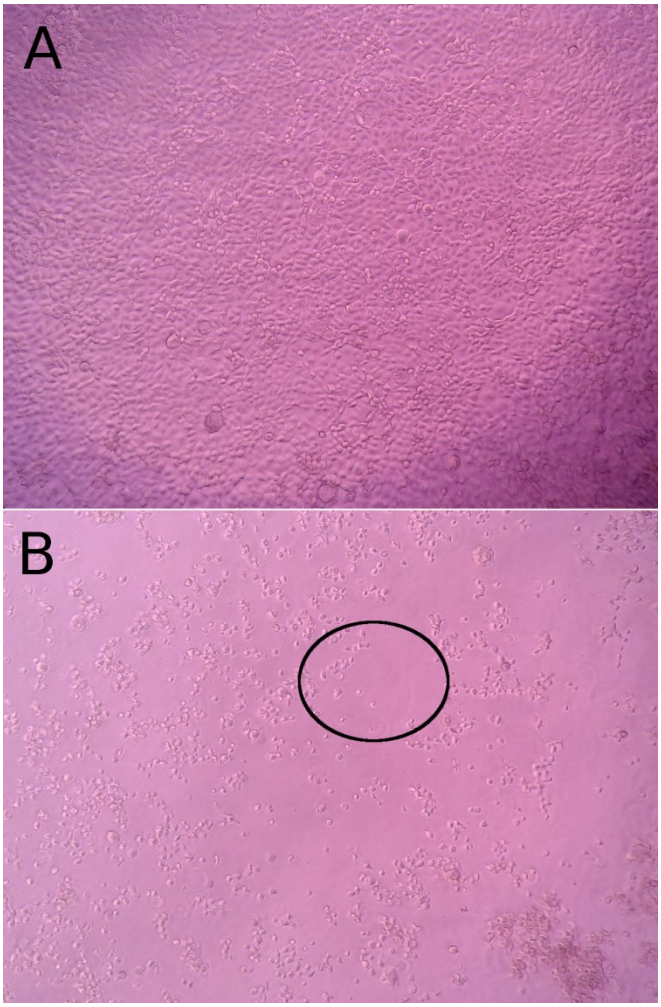
CVB3 is a known cause of severe complications in infected patients, especially in children and newborn infants [19]. CVB3 is usually not the predominant cause of enteroviral infections in humans, but is frequently identified together with other enteroviruses during aseptic meningitis, encephalitis, and HFMD outbreaks. Previously, It was reported that CVB3 was the fourth-most prevalent pathogen after EV-A71, CVA16, and CVA10 in patients presenting with HFMD in Shijiazhuang city, China, during 2010–2012 [9]. In the current study, we identified human coxsackievirus infection in a specimen from a 39-year-old female who showed mild clinical manifestations. Sequencing of the VP1 gene showed 92%–97% similarity with strains from Taiwan that were also isolated in 2008 [20]. Recently, Ni *et al.* reported that CVB3 isolated from patients with febrile rash illness in China was closely associated with the CVB3/MKP and CVB3/Macocy strains, which induce myocarditis and central nervous system disease [21].

This recent detection of CVB3 from a clinical specimen in Bandung confirms that CVB3 exists as an etiological agent in Indonesia. Further surveillance is needed to characterize circulation of the virus in Indonesia, particularly in undiagnosed viral infections, to determine if this is an isolated case or outbreak or if it is endemic.

Conclusions

We report the first case of CVB3 from sera of an undifferentiated febrile illness Indonesian patient. Although the number of cases is low and clinical symptoms are mild, CVB3 should be considered as one of the etiological agent for febrile illness cases.

Figure 2. Coxsackievirus B3 inoculation in LLC-MK2 cells
A: Uninfected LLC-MK2 cell monolayer (200x magnification);
B: Cytopathic effect observed on LLC-MK2 cells infected by CVB3 S175 after two days of incubation (200x magnification).



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Authors' contributions

AW, DS, UA, CM, and KSM conceived and designed the experiments. AW and CNM carried out the experiments. SFR, HD, BA, and HK collected the patient samples. AW, UA, BS, CNM, and DS performed the analysis. AW, UA, IMA, and HK wrote the paper. All authors read and approved the final manuscript.

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