Complete Sequence Analysis and Antiviral Screening of Medicinal Plants for Human Coxsackievirus A16 Isolated in Korea

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Abstract
Objectives: Coxsackievirus A group 16 strain (CVA16) is one of the predominant causative agents of hand, foot, and mouth disease (HFMD).
Methods: Using a specimen from a male patient with HFMD, we isolated and performed sequencing of the Korean CVA16 strain and compared it with a G10 reference strain. Also, we were investigated the effects of medicinal plant extract on the cytopathic effects (CPE) by CPE reduction assay against Korean CVA16.
Results: Phylogenetic analysis showed that the Korean CVA16 isolate belonged to cluster B-1 and was closely related to the strain PM-15765-00 isolated in Malaysia in 2000. The Korean CVA16 isolate showed 73.2% nucleotide identity to the G10 prototype strain and 98.7% nucleotide identity to PM-15765-00. Next, we assessed whether the Korean CVA16 isolate could be used for in vitro screening of antiviral agents to treat HFMD infection. Vero cells infected with the Korean CVA16 isolate showed a cytopathic effect 2 days after the infection, and the treatment of cells with Cornus officinalis, Acer triflorum, Pulsatilla koreana, and Clematis heracleifolia var. davidiana Hemsl extracts exhibited strong antiviral activity against CVA16.
Conclusion: Collectively, our work provides potential candidates for the development of vaccine and novel drugs to treat the CVA16 strain isolated from a Korean patient.

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1. Introduction

Coxsackievirus A16 (CVA16) is a serotype of the genus Enterovirus belonging to the family Picornaviridae, and one of the causative agents of hand, foot, and mouth disease (HFMD) in humans. In rare instances, it may cause neurological diseases, including aseptic meningitis, enanthems, and herpangina. HFMD is a self-limiting exanthematosus eruption characterized by vesicles in the oral cavity and peripherally distributed cutaneous lesions on the hands and feet [1,2]. Recently, large outbreaks of HFMD by CVA16 and enterovirus 71 (EV71) were reported, especially in the Asia-Pacific area [3,4]. Outbreaks of HFMD by CVA16 occurred in Taiwan (1996–2006) and in Singapore (2001–2007), as well as in England (1994) [1,5]. In Korea, outbreaks of HFMD caused by human enterovirus (HEV) infections have been consistently reported since 2009 [6–8]. HFMD has become a serious public health problem in South-East Asia, with periodic large epidemics occurring in recent decades. Most of therapeutic and vaccine strategies against HFMD have focused on EV71 [9,10]. In China, Phase III trials of antiviral vaccines against EV71 were recently successfully completed [11]. In addition, no HEV-specific antiviral drugs are yet available for clinical use. Many synthetic anti-HEV compounds have been described in vitro, but only a few of them are effective in vivo [12,13]. However, no vaccines are currently available to prevent CVA16 infections. Thus, research and development of vaccines and antiviral drugs to prevent CVA16 infections and treat HFMD must be spurred on.

A suitable alternative to antiviral drugs may be traditional medicinal plants. They have multiple targets, minimal side effects, low potential to cause resistance, and are less expensive. Screening of medicinal plants has led to the discovery of potent in vitro inhibitors of viral replication [14,15].

In this study, the molecular biological characteristics and genetic diversity of a Korean isolate of CVA16 were analyzed through complete nucleotide sequencing and phylogenetic analysis, respectively. Finally, Korean medicinal plants were screened for their potential anti-CVA16 effects.

2. Materials and methods

2.1. Virus isolation and identification

The Korean CVA16 strain was isolated from the stool sample of a male patient with HFMD admitted to the Department of Pediatrics at the Soonchunhyang University Cheonan Hospital. The pretreated sample was inoculated into Vero cells and incubated at 37°C in an atmosphere of 5% CO₂ until the appearance of cytopathic effects (CPE). Basic Local Alignment Search Tool (BLAST) search of VP1 sequences verified the identity of the Korean isolate. The VP1 sequences of the Korean isolate had the maximum nucleotide similarity with CVA16 serotype strains [16].

2.2. Nucleotide sequencing and sequence analysis

The complete nucleotide sequence of the Korean CVA16 strain was determined using a primer walking strategy; the sequences of the genome termini were determined by random amplification of cDNA ends system (Invitrogen, Carlsbad, CA, USA). Polymerase chain reaction (PCR) products were purified using a QIAquick PCR Purification Kit (Qiagen, Hamburg, Germany). The purified DNA was added to a reaction mixture containing 2 μL of BigDye terminator reaction mix (ABI Prism BigDye Terminator Cycle Sequencing Kit; Applied Biosystems, Foster, CA, USA) and 2 pmol of each primer. Sequencing reactions were subjected to an initial denaturation at 96°C for 1 minute and 25 cycles consisting of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes in a Gene Amp PCR system 2700 (Applied Biosystems). The products were purified by precipitation with 100% cold ethanol and 3M sodium acetate (pH 5.8), and then loaded on an automated 3100 Genetic Analyzer (Applied Biosystems). Nucleotide sequences of CVA 16 strains were constructed to contig and compared with the reference G10 strain (accession no. U05876). G10 strain, obtained from Genbank databases, was isolated in South Africa in 1954 and subsequently sequenced in 1994 [17,18]. The complete sequences of the Korean CVA16 isolate are deposited in the GenBank sequence database under the accession number JX839965. Complete nucleotide sequences of CVA16 isolate were compared with the reference strains by using CLUSTAL W (version 1.81) [19]. The phylogenetic relationships of each virus isolate were inferred using free MEGA software version 5.05 (Available from www.megasoft.net). The maximum composite likelihood method was used as the substitution method, while the neighbor-joining method was used to reconstruct the phylogenetic tree [20]. The reliability of the phylogenetic tree was determined by bootstrap resampling of 1000 replicates.

2.3. High-throughput screening of medicinal plant extracts for antiviral activity against CVA16

A medicinal plant extract library was supplied by the Ginseng Research Division, National Institute of Horticultural and Herbal Science, Eumseong-gun, Chungcheongbuk-do, Korea. To screen 492 medicinal
plant extracts, Vero cells were seeded in 96-well plates. After CVA16 infection, the plates were incubated with individual plant extract at 10 μg/mL for 48 hours. The antiviral activity was measured using a sulforhodamine B (SRB) assay.

2.4. Antiviral activity and cytotoxicity assays

Antiviral activity and cytotoxicity were evaluated by the SRB method using CPE reduction recently reported [21]. The effect of Cornus officinalis, Acer triflorum, Pulsatilla koreana, and Clematis heracleifolia var. davidiana extracts on CVA16-induced CPE was observed. Briefly, Vero cells were seeded onto a 96-well culture plate at a concentration of 2 × 10^4 cells/well. After CVA16 infection, the plates were incubated with plant extracts, Vero cells were seeded in 96-well plates. After CVA16 infection, the plates were incubated with individual plant extract at 10 μg/mL for 48 hours. The antiviral activity was measured using a sulforhodamine B (SRB) assay.

2.5. Real-time reverse transcription-PCR

Total RNA was isolated from Vero cells. Reverse transcription was performed using SuperScript II Reverse Transcriptase (Invitrogen, Grand Island, NY, USA) according to the manufacturer’s instructions. For real-time PCR analysis, the cDNA was serially diluted 10-fold and amplified using the 7500 Real Time PCR System (Applied Biosystems, Grand Island, NY, USA) with Power SYBR Green PCR Master Mix (Applied Biosystems, Grand Island, NY, USA) with 95°C for 10 minutes, amplification was performed for 40 cycles at 95°C for 15 seconds, 55°C for 15 seconds, and 72°C for 40 seconds. The final cycle was followed by the dissociation stage. The following primers were used: 5′-NCR gene, 5′-CCG GCC CCT GAA TGC GG-3′ and 5′-ATT CTT TAA TTG TCA CCA TAA GCA GCC A-3′ and β-actin gene, 5′-CAA TCA TGA AGT GTG ACG TGG-3′ and 5′-GTC CGC CTA GAA GCT GGC TTT GCG-3′.

2.6. Statistical analyses

Differences across more than three groups are analyzed using one-way Analysis of Variance (ANOVA) (Graphpad P, version 5.01). All results were expressed as means ± standard deviation. Significant differences in direct comparisons were determined using Tukey’s post hoc test. Differences with p < 0.05 were considered statistically significant.

3. Results

3.1. Analysis of nucleotide sequence of Korean CVA 16

The complete sequences of the Korean CVA16 isolate are deposited in the GenBank sequence database under the accession number JX839965. The genome is 7,411 nt in length, excluding the poly(A) tail. The 5′NCR contains 745 nt, followed by an ORF that encodes a viral polyprotein consisting of 2,194 codons, between a start codon (AUG) at position 746 and a stop codon (UGA) at position 7,327. The 3′NCR is 84 nt in length. These nucleotide sequences were used to construct a phylogenetic tree with 39 reference strains of the same serotype extracted from GenBank database. The CVA16 strains including Korean CVA16 isolate were segregated into four distinct genetic groups, which were supported by high bootstrap values (Figure 1). In phylogenetic relationships, the Korean CVA16 isolate belonged to cluster B-1 and was closely related to strain PM-15765-00 isolated in Malaysia in 2000. The Korean CVA16 isolate showed 73.2% nucleotide identity to the G10 prototype strain and 98.7% nucleotide identity to the PM-15765-00 strain.

3.2. Identification of antiviral activity of four medicinal plant extract against CVA16

We sought to identify the antiviral activity of medicinal plant extracts against CVA16. Four extracts were identified from 492 medicinal plant extracts screened that showed significant cell viability of > 50%, indicating antiviral activity against CVA16 (data not shown). The four extracts are Cornus officinalis, Acer triflorum, P. koreana, and C. heracleifolia var. davidiana Hemsl.

3.3. Antiviral activity of four medicinal plant extracts against CVA16

To determine drug potency, CVA16-infected Vero cells were treated with various doses of the four medicinal plant extracts. The antiviral assays demonstrated that P. koreana and C. heracleifolia var. davidiana Hemsl possessed strong antiviral activity of > 60% against CVA16 at concentrations of 2 μg/mL, 10 μg/mL, and 50 μg/mL. C. officinalis and A. triflorum showed 80% and 70% antiviral activity, respectively, against CVA16 at the concentration of 50 μg/mL (Figure 2A). The values of concentration required to inhibit virus-induced cytopathic effects by 50% were 32.9 μg/mL, 32.3 μg/mL, 1.51 μg/mL, and 2.55 μg/mL for C. officinalis, A. triflorum, P. koreana, and C. heracleifolia var. davidiana Hemsl extract, respectively. The CC_{50} values of C. officinalis, A. triflorum, P. koreana, and C. heracleifolia var. davidiana Hemsl extracts were superior to 50 μg/mL (Table 1). In addition, we confirmed the antiviral activity of the four medicinal plant extracts against CVA16 by real-time PCR analysis. The highest
inhibition of CVA16 replication was observed at 50 μg/mL for the *C. officinalis* and *A. triflorum* extracts, while the *P. koreana* and *C. heracleifolia* var. *davidiana* Hemsl extracts inhibited CVA16 replication only marginally at all concentrations (Figure 2B).

### 3.4. The effect of four medicinal plant extracts on CVA16 virus-induced CPE

After Day 2 post infection with CVA16, there was no difference between mock or Vero cells treated with 50 μg/mL of *C. officinalis*, *A. triflorum*, *P. koreana*, or *C. heracleifolia* var. *davidiana* Hemsl, in terms of typical spread-out shapes and normal morphology. Infection of Vero cells with CVA16 resulted in a severe CPE, whereas CVA16-infected Vero cells treated with the medicinal plant extracts exhibited noticeably reduced CPE compared with untreated CVA16-infected cells (Figure 3). These results indicate that the CPE of CVA16 infection is prevented by *C. officinalis*, *A. triflorum*, *P. koreana*, or *C. heracleifolia* var. *davidiana* Hemsl.

### 4. Discussion

CVA16 and EV71 infections are both responsible for widespread HFMD and present serious public health problems in the Asia-Pacific region [22,23]. However,
CVA16 is now considered as a serious public health threat in young children, especially in the Asia-Pacific region. In the current study, the first complete nucleotide sequence of a CVA16 isolate from a HFMD patient in Korea was described and its genetic diversity explored by phylogenetic analysis against 39 reference strains. In addition, a Korean medicinal plant library of 492 extracts was screened to find candidates with antiviral properties against the Korean CVA16 isolate.

CVA16 usually has a high mutation rate during viral replication due to the deficiency of proofreading activity [28], and there was >20% genetic difference between G10 prototype strain and the current widespread strain. Therefore, it would be difficult to develop a therapeutic antiviral drug against the current epidemic strain [29]. In the current study, we isolated a CVA16 from Korean HFMD patients, and confirmed that the CVA16 isolate could be used for development and screening of antiviral drugs in vitro.

Many plant-derived natural products have been used in traditional medicine for the treatment of various diseases, including viral infection. In the current study, we found that CVA16 infection in Vero cells could be prevented by extract of *C. officinalis*, *A. triflorum*, *P. koreana*, and *C. heracleifolia* var. *davidiana* Hemsl. Among them, *C. officinalis* is a species of dogwood and a known edible plant. Particularly, the fruit of *C. officinalis* is well known for its chemotherapeutic benefits. Recently, cornuside, isolated from the fruit of *C. officinalis*, was reported to have anti-inflammatory activity by inhibiting tumor necrosis factor-α production [30]. Cornuside has also been reported to inhibit lipopolysaccharide-induced inflammation by inhibiting nuclear factor-κB pathway [31]. Next, *P. koreana* is a perennial plant found in South Korea, and its roots have been used as traditional medicine for the treatment of dysentery, malaria, chills, and fever [32]. In addition, previous studies suggested that it has antifungal and antibiotic properties and antitumor effects and that it can lower blood pressure [33,34]. Unlike *C. officinalis* and *P. koreana*, there are few reports regarding the biological activities or medical uses of *A. triflorum* and *C. heracleifolia* var. *davidiana* Hemsl. More interestingly, there are no reports suggesting an antiviral activity of the *C. officinalis*, *A. triflorum*, *P. koreana*, or *C. heracleifolia* var. *davidiana* Hemsl extracts, and thus, further studies to elucidate the antiviral constituents of those plants are needed.

In this study, the CC50 values of all extracts were >50 μg/mL, suggesting that the extracts of those plants are nontoxic to Vero cells even in high concentrations. In addition, when their antiviral activities were assessed after CVA16 infection, all of the extracts showed significant antiviral activity against the Korean CVA16 isolate. In particular, *C. officinalis* and *A. triflorum* revealed high suppression potency against viral gene replication as assessed by real-time PCR, which

| Medicinal plant extract | CC50  (μg/mL) | IC50  (μg/mL) | TI |
|-------------------------|---------------|---------------|----|
| *C. officinalis*         | >50           | 32.9 ± 1.30   | 1.52 |
| *A. triflorum*           | >50           | 32.3 ± 1.67   | 1.55 |
| *P. koreana*             | >50           | 1.51 ± 0.05   | 33.11 |
| *C. heracleifolia* var. *d. Hemsl* | >50 | 2.55 ± 1.06   | 19.61 |

Results are presented as the mean IC50 values ± standard deviation obtained from three independent experiments carried out in triplicate. CC50 = concentration required to reduce cell growth by 50% (μg/mL); IC50 = concentration required to inhibit virus-induced cytopathic effects by 50% (μg/mL); TI = therapeutic index = CC50/IC50.
correlates with their antiviral activity tested by SRB assay. Although the extracts of *P. koreana* and *C. heracleifolia* var. *davidiana* Hemsl also showed high antiviral activity, their suppressive ability on viral replication was moderate.

In conclusion, this study is the first report of the complete nucleotide sequence of the Korean CVA16, and shows the possibility of using the virus for the screening of antiviral drug candidates against HFDM infection in Korea. Antiviral activity screening of 492 medicinal plant extracts showed that *C. officinalis*, *A. triflorum*, *P. koreana*, and *C. heracleifolia* var. *davidiana* Hemsl possessed strong antiviral activity against this Korean CVA16 isolate. The data would be useful in preventing future outbreaks of CVA16 and in treating patients infected with the strain. Prospectively, identification of antiviral constituents included in the medicinal plant extracts and study of the mechanisms underlying their antiviral activity is necessary for the development of antiviral therapeutics to treat Korean HFDM patients infected with CVA16.

Conflicts of interest

All contributing authors declare no conflicts of interest.

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