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Application of immunomagnetic separation for detection of human coxsackievirus group b using polyclonal antibodies.

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Abstract

Infection of Human Enterovirus (HEV) containing coxsackievirus has been associated with wide variety clinical illnesses, including aseptic meningitis and central nervous system pathologies. Although diagnosis of HEV infection relies on detection of the virus in the Cerebrospinal fluid (CSF), the virus loads in such specimens tend to be quite low. To improve the sensitivity of HEV diagnosis, the aim of this study was to apply an Immunomagnetic Separation (IMS) technique using affinity polyclonal antibodies against HEV-B to determine the concentration of HEVs in CSF specimens. The difference in the average cycle threshold values between CSF-only samples and CSF samples containing an antibody complex was 1.55. IMS improved the HEV concentration by 3.1-fold, and thus appears to be an effective method for concentrating HEV in the CSF. Therefore, IMS is a useful technique for detecting HEV in the CSF from patients with enteroviral diseases.

Keywords: Human enterovirus, Immunomagnetic separation, Cerebrospinal fluid.

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Introduction

Human Enterovirus (HEV) including human Coxsackievirus group B (CV-B) is a primary cause of aseptic meningitis and encephalitis, leading to high levels of hospitalization from the summer to fall [1]. In addition, HEV infections of the Central Nervous System (CNS) have been associated with an increased risk of adult-onset schizophrenia or psychosis [2]. In some cases, these pathologies of the CNS have a detrimental impact on other organs, such as fatal cases of cardiomyopathies caused by HEV-71 affecting the CNS [3]. HEV infections leading to severe injuries of the CNS or heart tissue are associated with an adverse prognosis; therefore, accurate diagnosis is important for initiating effective treatment [4]. In routine medical practice, CNS and myocardial viral pathologies are typically managed with only standard symptomatic therapy as a widely accepted strategy, although antiviral and specific therapies can also be used for improving the cause and outcome of the disease. For diseases with an HEV-related etiology, virus detection and identification are particularly important, because distinct serotypes and their genogroups can differ in pathogenicity and virulence [5,6]. Moreover, since the efficacy of antiviral drugs can differ depending on certain serotypes and genogroups [7], this detailed information of the virus should be considered in the choice of antiviral and specific therapies for effective patient management [4]. HEV culture is currently the "gold standard" used to diagnose HEV infections in Cerebrospinal fluid (CSF) specimens, but the test requires several days to be conclusive. Development of a rapid diagnostic test may therefore have a strong impact on the diagnosis and clinical management of viral aseptic meningitis [8]. During the last decade, various Reverse Transcription-Polymerase Chain Reaction (RT-PCR) assays were developed as a more convenient alternative to viral culture [9-13]. The precise intravital diagnosis in the CNS can be established only by detection of the virus in the CSF and subsequent virus serotyping. Although RT-PCR assays for HEV diagnosis have been developed, the virus loads in such clinical specimens are quite low in many cases [14]; therefore, a new approach to overcome this problem is required. Toward this end, the aim of this study was to evaluate a method to improve the sensitivity of HEV diagnosis. We applied Immunomagnetic Separation (IMS) using affinity polyclonal antibodies against CV-B to concentrate viruses in the CSF specimens of aseptic meningitis patients.

Materials and Methods

CSF samples

Eleven CSF specimens were obtained from patients with aseptic meningitis admitted to the Soonchunhyang University Hospital in Cheonan Korea during 2010 [15]. The general demographic and clinical characteristics of the included patients are shown in Table 1. This study was conducted in accordance with ethical principles as formulated in the World Medical Association Declaration of Helsinki and approved by the institutional review board (IRB No. 2012-48) of the Ethical Committee of Soonchunhyang University Hospital in Cheonan Korea. Informed consent was obtained from the parents of the patients who participated in the study, and the parents of participants also gave their consent to publish the data.

Virus identification

The samples were subjected to semi-nested RT-PCR in the VP1-coding region for molecular typing as described previously [16]. The VP1 amplicons generated by semi-nested RT-PCR were then sequenced using internal primer sets. The molecular type of each isolate was determined by the serotype of the highest scoring strain in GenBank using the Basic Local Alignment Search Tool (BLAST); i.e., the sequence of the HEV strain that gave the highest nucleotide similarity value with the query sequence [17].

Combination of magnetic beads with polyclonal antibodies for HEV-B

Thirty microliters of magnetic beads (Dynabeads M-280, tosyl-activated; Dynal, Oslo, Norway) were washed 3 times with 0.1 M sodium phosphate buffer (pH 7.4) at 20˚C. The washed beads were suspended gently in 10 µl antisera of Coxsackie virus type B-1, B-2, B-3, B-4, and B-5 (1:320; Denka Seiken, Tokyo, Japan) and mixed with CSF. The samples were incubated for 2 h at 20˚C with slow-tilt rotation on an MX4 sample mixer (Dynal). To remove unbound polyclonal antibody, the virus-bound immune-magnetic beads were washed four times with phosphate-buffered saline (pH 7.4) containing 0.1% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) at 20˚C. Antibody-bound magnetic beads were carefully collected with 500 µl of NuPAGE buffer (Thermo Fisher, Waltham, MA, USA) with rotation on an MCB1200 processing system (Sigris Research, Brea, CA, USA) for 10 min at room temperature. The total antibody-bound beads complex was submitted to RNA extraction and real-time RT-PCR.

Comparison of concentration methods for the detection of HEV

For each CSF-alone and CSF-with-antibody-complex sample, the viral RNA was extracted under the same conditions at the same time. Viral RNA was extracted using the Maxwell 16 viral total nucleic acid purification kit (Promega, Madison, WI, USA) per the manufacturer's instructions. Viral RNA was eluted in 50 µl of elution buffer and stored at -70˚C. Real-time RT-PCR amplification was performed using an ABI 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA, USA). To detect HEV RNA, the AccuPower® EV Real-Time RT-PCR kit (Bioneer, Daejeon, Korea) was used based on the 5′ non-coding region of a highly conserved region in the HEV genome per the manufacturer's instructions. Five microliters of nucleic acid was added per well, and the final total volume in each well was 25 µl. The temperature and time parameters were as follows: RT for 15 min at 45˚C, denaturation for 5 min at 95˚C, and amplification (45 cycles of 10 s at 95˚C and 1 min at 55˚C). The results of quantitative analyses with real-time RT-PCR for control CSF and virus-bound immunomagnetic beads were compared.

Results

Eleven CSF samples obtained from patients with aseptic meningitis were subjected to diagnostic real-time RT-PCR. The VP1 amplicons generated in the semi-nested PCR were sequenced and were determined to correspond to a 372-bp VP1 region for molecular typing. Gapped BLAST analyses were carried out, and each virus was assigned to the type with the highest VP1 identity score. The obtained enterovirus sequences were deposited in the GenBank sequence database, and the accession numbers are shown in Table 1. CSF samples with and without the antibody complex were used to evaluate the feasibility of the IMS method for improving the sensitivity of HEV diagnosis, and the threshold cycle (Ct) values for the amplification of HEV-specific sequences are shown in Table 2. The average Ct values of HEV real-time RT-PCR for the CSF samples without and with the antibody complex were 29.54 and 27.88, respectively. Therefore, the difference in the average Ct values (ΔCt) was 1.55, indicating that the IMS method resulted in a 3.1-fold improvement in the enteroviral RNA concentration. In addition, Ct value showed same range to the result of CSF only sample in this CSF test using previously made norovirus antibody complex [18], and the results were negative in five CV-B negative CSF samples.

Discussion

Precise intravital diagnosis of a viral infection in the CNS can be established only by virus detection in the CSF and subsequent virus serotyping. In many cases, the HEV loads in such clinical specimens are quite low [14], and therefore addition of a concentrating step of HEV in the CSF is required to improve diagnostic sensitivity. Previously, we explored the feasibility of the IMS method for improving norovirus detection in food [18]. In the present study, we applied this method to increase the efficiency of HEV detection in the CSF. The principles of magnetic separation aided by antibodies or other specific binding molecules have been exploited to isolate specific viable whole organisms, antigens, or nucleic acids. This technique has also been shown to be suitable for detecting prokaryotic organisms such as bacteria and viruses in food samples [19]. This method employs antibodies specific to a particular microorganism, resulting in high specificity for

concentrating and purifying microorganisms in food and other environmental samples [20]. The amount of product should double during each cycle of PCR [21], and therefore the ΔCt method assumes that the amount of viral RNA copies for each sample will be 2. In the present study, the average ΔCt value between the two samples (only CSF and CSF with antibody complex) was 1.55, indicating a 3.1-fold difference of the enteroviral RNA concentration. That is, IMS can result in an improvement of the HEV concentration of 3.1 times. This is the first study to successfully apply the IMS concentration technique for detecting CV-B in CSF samples from patients with enteroviral diseases. Future work should explore the combination of antibodies with magnetic beads of various HEV serotypes for the optimization of IMS in detecting HEV in specimens that have a low virus load, such as the CSF.

Table 1. *Candidate enteroviruses isolated from patients with aseptic meningitis in 2010.*

	Isolate	Gender	Age (years)	Month of isolation	Specimen	Accession no.	Serotype
Sample 1	Kor10-CVB1-662cn	F	0	July	CSF/Stool	KX256186	Coxsackievirus B1
Sample 2	Kor10-CVB2-589cn	F	0	July	CSF	KX256187	Coxsackievirus B2
Sample 3	Kor10-CVB2-757cn	F	1	September	CSF/Stool	KX256188	Coxsackievirus B2
Sample 4	Kor10-CVB2-895cn	M	0	November	CSF/Stool	KX256189	Coxsackievirus B2
Sample 5	Kor10-CVB3-522cn	M	0	July	CSF	KX256190	Coxsackievirus B3
Sample 6	Kor10-CVB3-583cn	F	2	July	CSF/Stool	KX256191	Coxsackievirus B3
Sample 7	Kor10-CVB4-692cn	F	3	August	CSF/Stool	KX256192	Coxsackievirus B4
Sample 8	Kor10-CVB4-725cn	F	0	August	CSF/Stool	KX256193	Coxsackievirus B4
Sample 9	Kor10-CVB4-811cn	F	0	September	CSF/Stool	KX256194	Coxsackievirus B4
Sample 10	Kor10-CVB5-697cn	F	0	August	CSF/Stool	KX256195	Coxsackievirus B5
Sample 11	Kor10-CVB5-752cn	M	1	September	CSF/Stool	KX256196	Coxsackievirus B5

Table 2. *Average (± standard deviation) threshold cycle (Ct) values of real-time RT-PCR for CSF samples with and without the antibody complex.*

	Ct values		ΔCt
	CSF	CSF with antibody complex	
Sample 1	29.34 ± 0.10	28.12 ± 0.19	1.22
Sample 2	25.72 ± 0.18	23.89 ± 0.44	1.83
Sample 3	30.23 ± 0.26	28.84 ± 0.15	1.39
Sample 4	26.68 ± 0.23	25.48 ± 0.21	1.2
Sample 5	27.08 ± 0.13	26.01 ± 0.38	1.07
Sample 6	32.48 ± 0.03	30.26 ± 0.25	2.22
Sample 7	30.36 ± 0.06	28.42 ± 0.33	1.94
Sample 8	28.82 ± 0.14	27.55 ± 0.26	1.27
Sample 9	30.07 ± 0.40	28.51 ± 0.03	1.56
Sample 10	33.73 ± 0.42	31.95 ± 0.31	1.78
Sample 11	30.39 ± 0.27	28.83 ± 0.08	1.56
Average	29.54 ± 0.20	27.99 ± 0.24	1.55

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