

Detection of Enteroviruses in Clinical Samples of Patients with Aseptic Meningitis by Rapid Antigen Detection Assay

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SUMMARY

Introduction Human enteroviruses represent the most common etiological agents of aseptic meningitis. Rapid diagnosis of aseptic meningitis affects the management of patients.

Objective The aim of this work was to assess the efficacy of rapid antigen detection (RAD) assay for the confirmation of human enteroviruses in comparison to that of the conventional cell culture (CCC), and to identify the serotypes associated with aseptic meningitis cases in the Autonomous Province of Vojvodina.

Methods RAD assay was carried out using centrifugation of inoculated Vero, RD and HEp-2 cell cultures and indirect immunofluorescence with Pan-Enterovirus 2E11 reagent (Millipore-Chemicon). CCC was performed using the same type of cells and the same immunofluorescence reagent for enterovirus confirmation.

Results Out of 70 analyzed samples (29 cerebrospinal fluid specimens, 27 stool specimens, 9 rectal and 5 throat swabs), 36 (51.4%) were positive by immunofluorescence after CCC and 34 (48.6%) by the RAD assay. The sensitivity of RAD assay was 94.4% and specificity 100%. Detection time of enteroviruses by the RAD assay was 3 days, and by CCC varied from 3 to 13 days (mean time 6.1 days). Within 72 h from inoculation, a cytopathic effect (CPE) occurred in only 19 (47.5%) of 40 CPE positive samples. Serotyping revealed 11 types of enteroviruses: coxsackievirus A16, B3, B4, B5, and echovirus 2, 4, 6, 11, 13, 16 and 30.

Conclusion The RAD assay was slightly less sensitive than CCC and significantly shortened the detection time of enteroviruses, so it may be useful in rapid diagnosis of enteroviral meningitis.

Keywords: enterovirus; aseptic meningitis; cell culture

INTRODUCTION

Genus Enterovirus belongs to the family Picornaviridae Picornavirales. Human enteroviruses (HEVs) are classified into four species: HEV-A (coxsackie A2-8, 10, 12, 14, 16, enteroviruses 71, 76, 89-92), HEV-B (coxsackie A9, coxsackie B1-6, echoviruses 1-7, 9, 11-21, 24-27, 29-33, enteroviruses 69, 73-75, 77-88, 93, 97, 98, 100, 101, 106, 107), HEV-C (polioviruses 1-3, coxsackie A1, 11, 13, 17, 19-22, 24, enteroviruses 95, 96, 99, 102, 104, 105, 109) and HEV-D (enteroviruses 68, 70, 94) [1]. HEVs have been associated with a wide variety of clinical syndromes, from mild respiratory illness to severe diseases such as myocarditis, neonatal sepsis, type I diabetes and aseptic meningitis. They account for approximately 80-92% of aseptic meningitis cases for which the etiologic agent is identified [2]. Isolation of HEVs in conventional cell culture (CCC), followed by identification with indirect immunofluorescence (IFA) or neutralization test, is still regarded as the traditional diagnostic gold standard [2, 3]. The main drawback of this approach comes from the fact that it takes an average of 3-14 days before cytopathic effect (CPE) of HEVs is detectable [4]. Therefore, results obtained in this way have no influence on the treatment of patients, but only enable retrospective diagnosis. This disadvantage can be overcome by using the method of

rapid antigen detection (RAD) [4, 5, 6, 7]. This method is based on centrifugation of inoculated cell cultures, which increases their sensitivity to the virus. In conjunction with the IFA it allows detection of viral antigens before the appearance of CPE, which significantly reduces the time required to obtain final results. The method is not standardized, thus different numbers and types of cells, monoclonal antibodies, protocols of centrifugation and incubation are in use.

OBJECTIVE

The aim of this work was to assess the efficacy of RAD assay for the detection of human enteroviruses in 70 clinical samples from patients with suspected aseptic meningitis, in comparison to that of CCC. Also, we wanted to identify the predominant HEV serotypes included in aetiology of aseptic meningitis in the Autonomous Province of Vojvodina.

METHODS

Samples and their processing

A total of 70 samples (29 cerebrospinal fluid specimens, 27 stool specimens, 9 rectal swabs and 5 throat swabs) were obtained from paedi-

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atric patients suspected of having aseptic meningitis. Rectal and throat swabs were collected in viral transport medium (Earl's balanced salt solution with 1% bovine serum-albumin, penicillin 1000 U/ml, streptomycin 1000 µg/ml and amphotericin B 5 µg/ml). Transport mediums and 10-20% suspensions of faecal samples in viral transport mediums were centrifuged (20 min, 600 g), filtered (pore diameter – 0.45 µm), and inoculated into the cell cultures. Cerebrospinal fluid (CSF) samples were inoculated without prior treatment. If it was not possible to perform processing within 24 h of sampling, the samples were stored at -76°C.

Cell cultures

Three different continuous cell lines were used for isolation of HEVs: RD (human embryonic rhabdomyosarcoma cells), HEp-2 (human larynx carcinoma cells) and Vero (African green monkey kidney cells). Cell cultures were in-house prepared according to the standard protocols [8]. Cells were 2 to 3 days old at the time of inoculation.

Conventional cell culture

One tube of each of the cell culture was inoculated with a sample in accordance with WHO recommended protocols [8]. Tubes were examined daily for the presence of CPE by inverted microscope for 7 days postinoculation. Cultures showing characteristic enterovirus CPE were subpassaged for confirmation, and if positive, they were marked as CPE positive. In absence of a CPE, two blind subpassages were performed before reporting the culture as CPE negative. The presence of HEVs in CPE positive tubes was confirmed using IFA. All cultures without CPE were also submitted to IFA testing before reporting them as definitive negative to HEVs.

Rapid antigen detection assay

Cell cultures were seeded separately in 6-well microtitre plates. After discarding the growth medium – Eagle's minimum essential medium with 10% FBS, they were inoculated with 100-200 µl of specimens and centrifuged at 600 g for 60 min at 36°C as described by Lipson et al. [7]. After the addition of maintenance medium with 2% of FBS, incubation was performed for 72 h at 36°C. Then, regardless of the presence or absence of CPE, the cultures were subjected to IFA.

Indirect immunofluorescence

When about 75% of cells exhibited CPE, or at the end of the incubation period (for CPE negative cultures and in RAD assay) the cells were harvested and fixed in cold acetone (4°C) for 10 min. Uninfected cells were used as a negative control and cells infected with laboratory strain of

coxsackievirus B3 served as a positive control. Staining was performed in accordance with the manufacturer's instructions, using Pan-Enterovirus 2E11 reagent (Millipore-Chemicon) which contained mouse monoclonal antibodies against enteroviruses. After incubation (30 min, 37°C) in a humidity chamber and rinsing with phosphate-buffered saline (PBS), anti-mouse immunoglobulin G FITC-labelled conjugate (Millipore-Chemicon) was added. Slides were incubated (30 min, 37°C) in a humidity chamber, in dark, then washed with PBS and dried in air. The slides were examined under a fluorescence microscope (Olympus BH-2) at 200x magnification. A bright apple-green fluorescence in the nucleus and/or cytoplasm indicated a positive result. The sample that gave a positive fluorescence signal on at least one cell type was considered a true positive for the presence of HEV.

Neutralization assay

Serotyping of HEV isolates was performed by neutralization of CPE using Lim, Benyesh-Melnick (LBM) pools of specific antisera A to H (Statens Seruminstitut, Copenhagen, Denmark). These pools allowed the identification of 42 antigenic different HEVs. Each isolate was tested against all antiserum pools. Equal volumes (100 µl) of isolate and each pool were mixed and incubated for one hour at 36°C. Mixtures were inoculated on cell culture tubes and incubated at 37°C. Virus controls and cell controls were run along for comparison. The tubes were examined daily until the virus control showed complete CPE. Interpretation of the results was carried out by analyzing the pattern of inhibition of CPE by the antiserum pools, according to scheme enclosed with the pools.

Statistical analyses

Differences in sensitivity of cell cultures in the detection of HEVs were tested by McNemar test. They were considered statistically significant when *p* was less than 0.05.

RESULTS

Results of CCC

Data from isolation of HEVs by CCC are summarized in Table 1. Out of 70 samples analyzed, 40 (57.1%) induced CPE. Nineteen (47.5%) positive samples produced CPE in only one type of cells. Eight samples were positive only in RD, 6 samples only in Vero, and 5 samples only in HEp-2 cell cultures. The differences in sensitivity to HEVs between cell cultures were not statistically significant (Vero versus RD, *p*=1.0; Vero versus HEp-2, *p*=0.28; RD versus HEp-2, *p*=0.42). Detection times of HEVs by CCC varied within the range of 3-13 days (mean time 6.1 days). Within 3 days after inoculation, CPE was observed in 19 (47.5%), and by the 7th day in 31 (77.5%) of 40 finally

Table 1. Results of isolation of HEVs by CCC

Specimen	Number of specimens CPE positive								
	Positive only on			Total positive on			Positive by day		
	Vero	RD	HEp-2	Vero	RD	HEp-2	3rd	7th	21st
Liquor (n=29)	2	3	1	10	9	8	6	9	15
Faeces (n=27)	2	4	3	13	13	9	11	17	20
Throat swab (n=5)	2	0	1	2	1	2	2	4	4
Rectal swab (n=9)	0	1	0	0	1	0	0	1	1
Total (n=70)	6/40 (15%)	8/40 (20%)	5/40 (12.5%)	25/40 (62.5%)	24/40 (60.0%)	19/40 (47.5%)	19/40 (47.5%)	31/40 (77.5%)	40/70 (57.1%)

n – number of specimens analyzed; CPE – cytopathic effect

Table 2. Detection of HEVs by IFA after CCC and by RAD assay

Specimen	Number of specimens IFA positive							
	CCC				RAD assay			
	Vero	RD	HEp-2	Total	Vero	RD	HEp-2	Total
Liquor (n=29)	9	9	8	15	8	6	7	13
Faeces (n=27)	12	12	7	17	11	12	6	17
Throat swab (n=5)	2	1	2	4	2	1	2	4
Rectal swab (n=9)	0	0	0	0	0	0	0	0
Total (n=70)	23 (63.9%)	22 (61.1%)	17 (47.2%)	36 (51.4%)	21 (61.8%)	19 (55.9%)	16 (47.0%)	34 (48.6%)

n – number of specimens analyzed; CCC – conventional cell culture; RAD assay – rapid antigen detection assay; IFA – indirect immunofluorescence

positive samples. The remaining 9 (22.5%) samples were CPE positive before the end of the second passage.

Comparison of CCC and RAD assay

Results from IFA after CCC and the results of RAD assay are reported in Table 2. Out of 70 samples analyzed, 36 (51.4%) were positive by IFA after CCC and 34 (48.6%) were positive by RAD assay. No CPE negative sample was IFA positive. Three CPE positive stool samples and one positive rectal swab failed to give a positive signal in IFA both after CCC and in RAD assay. Within the period of 3 days from inoculation (time needed for RAD) CPE provided only 52.7% (19/36) positive samples. Compared to the results of IFA after classical isolation, RAD assay had a 94.4% (34/36) sensitivity and 100% (34/34) specificity. According to the individual type of samples, the sensitivity of RAD assay for CFS was lower – 86.7% (13/15), compared to stool samples and throat swabs which amounted to 100% (17/17 or 4/4). No statistical differences were observed among cell cultures regarding the sensitivity to HEVs, comparing CCC and RAD assay (Vero, $p=0.48$; RD, $p=0.25$; HEp-2, $p=0.48$).

Results of neutralization test

Results of serotyping are presented in Table 3. Neutralization test allowed typing 32 of 36 (88.9%) HEV isolates. The remaining 4 (11.1%) isolates could not be serotyped. Neutralization revealed the presence of 11 different HEV serotypes: coxsackievirus A16, coxsackievirus B3, 4 and 5, and echovirus 2, 4, 6, 11, 13, 16 and 30. Nineteen (52.8%) isolates were identified as echoviruses, 10 (27.8%) were from group B of coxsackievirus, and 3 (8.3%) isolates were

Table 3. Enterovirus types detected by CCC according to cell culture and by RAD assay

Enterovirus type	Number of specimens positive				
	CCC				RAD assay
	Vero	RD	HEp-2	Total	
CV-A16	0	3	0	3	3
CV-B3	2	0	1	2	2
CV-B4	2	0	2	2	2
CV-B5	6	0	2	6	5
ECV 2	2	1	0	2	2
ECV 4	1	1	1	1	0
ECV 6	0	0	4	4	4
ECV 11	4	5	4	5	5
ECV 13	0	0	1	1	1
ECV 16	4	4	0	4	4
ECV 30	0	2	2	2	2
NT	0	4	0	4	4
Total	21	20	17	36	34

CV-A – coxsackievirus A; CV-B – coxsackievirus B; ECV – echovirus; NT – not typeable

typed as coxsackieviruses A16. In the present study, all non-typable and coxsackievirus A16 isolates were recovered only on the RD cell culture, but none of the coxsackie B viruses was detected on this type of cells. The Vero cell culture proved to be more sensitive than HEp-2 cell line for coxsackie B virus isolation. The replication of echoviruses was supported by all three cell lines without significant differences, but there were differences in sensitivities according to the particular serotype. Four echovirus 11 isolates and the only echovirus 4 isolates were recovered from all three types of cells, while there were 6 isolates that gave CPE only on one type of culture. Among them, one echovirus 2, and one echovirus 11 were isolated only on Vero and RD cells, respectively, while the HEp-2 cell culture was the only culture that supported the growth of echovirus 6 and echovirus 13 isolates.

DISCUSSION

There is still no consensus concerning the optimal combination of cell cultures for the isolation of HEVs. The results of this study confirmed that Vero cell culture is not sufficient for the isolation of all HEVs and that the use of multiple cell lines increases their recovery. A significant number (47.5%) of positive samples CPE was found in only one type of cells. The use of two cell cultures alone would have resulted in the failure to detect from 12.5% (without HEp-2) up to 20% (without RD) of positive samples. Similar to this finding, She et al. [9] reported that 52.6% of 1047 HEV isolates were recovered from only one out of five cell lines used. In the study conducted by Lin et al. [10] as much as 91% of 238 HEV isolates grew in only one of the four different cell lines. In this work, cell cultures did not show significant differences in sensitivity to HEVs. Concerning the sensitivity of cell lines to HEVs, data from previous studies are not consistent because individual serotypes have different seasonal and geographic patterns of circulation and differ in ability to replicate in various cell types [11].

Out of 40 CPE positive samples, 4 failed to give positive signal in IFA. This may be due to the fact that the monoclonal antibodies from 2E11 reagent had been prepared using the prototype strains, thus maybe they could not detect all antigenic variants or possibly new types of HEVs. The sensitivity and specificity for the RAD assay versus IFA after classical isolation were 94.4% (34/36) and 100% (34/34), respectively. Two samples of CSF gave positive IFA signal after CCC, but were negative by the RAD assay. This is probably due to low amounts of virus present in these samples. A longer incubation period during the classical isolation gave viruses the ability to multiply and produce CPE. However, three days of incubation in the RAD assay were too short for the production of a sufficient number of viral particles with antigens that could be proven by IFA. Good correlations of 94% and 100% of rapid and conventional culture were reported previously by Perez-Ruiz et al. [2] and Terletskaia-Ladwig et al. [4], respectively. Out of 36 specimens positive within 3 days by the RAD assay, CPE provided only 19 (52.7%) in the same period of time. Previous studies also demonstrated a significant advantage of rapid methods for HEVs detection within 3 days after inoculation over classical isolation. When comparing the results from different studies, the influence of type of the sample, serotype of HEV, assay performances (duration of incubation, efficacies of monoclonal antibodies, etc.) should be considered. Lipson et al. [7] reported that centrifugation-enhanced antigen detection assay with 2E11 monoclonal antibodies, attained a sensitivity of 73% (37/51) and 92% (47/51) after 3 and 5 days postinoculation, respectively. On the 3rd and 5th day of incubation, classical isolation attained a sensitivity of only 23% (12/51) and 52% (27/51), respectively. Klespies et al. [5] detected 93% (40/43) of the positive samples by spin-amplified shell vial IFA assay with blend of 2E11 and 9D5 monoclonal antibodies (Milipore-Chemicon) within 3 days of incubation, and 51% (22/43) of them by CCC in the same period of time. In a study conducted by Van Doornum and De Jong [6] the proportion of specimens positive for HEVs

by rapid shell vial culture assay with 5-D8/1 monoclonal antibodies (DAKO) after 2 to 3 days of incubation was 57% (55/74), while 34% (25/74) of positive specimens showed a CPE until the 3rd day from inoculation.

In this study the neutralization test failed to identify 4 (11.1%) isolates which is a common situation in a number of studies [3, 12]. This may be due to the lack of homologous antisera or the presence of multiple serotypes, aggregations of virions, prime-strains or new serotype in these samples. Our results confirmed the importance of HEVs as the etiological agents of aseptic meningitis and indicated the serotypes associated with this disease in our Province. The most prevalent HEV serotypes were echoviruses (52.8%), which have been described as the most common etiological agents of aseptic meningitis, causing outbreaks and sporadic cases worldwide [13, 14]. Less numerous were the isolates from the group B of coxsackieviruses (27.8%), which are also well known as causative agents of meningitis [10, 15]. From the group A of coxsackieviruses only serotypes A16 were identified in three specimens (8.3%). It should be mentioned that LBM antiserum pools allowed the detection of only three coxsackie A serotypes: 7, 9 and 16. Although coxsackie A16 is usually associated with hand, foot and mouth diseases, sporadic cases of meningitis caused by this type have also been reported [10, 16]. Data from previous studies regarding the sensitivity of cell cultures to different HEV serotypes are often contradictory. The type of circulating HEV influence isolation from cell culture, and type distribution varies from season to season [11]. Van Loon et al. [17] have conducted the quality assessment of methods for the isolation and identification of HEVs in 11 laboratories. With few exceptions, it was not possible to find a clear pattern with regard to the sensitivity of certain cells to a particular serotype. Some serotypes gave positive CPE on one cell culture in one laboratory and a negative result on the same type of cells in another. In the present study, coxsackievirus A16 isolates were isolated only on the RD cell culture, but none of coxsackie B viruses was detected on this type of cells. This finding agrees with the results from previous studies according to which the RD cell line is generally considered as the culture of choice for the isolation of coxsackie A viruses [4, 9, 11, 18]. It has been reported that coxsackie B viruses replicate poorly on RD cells [9, 19]. They grow better on Vero and HEp-2 cell cultures [12, 19]. In our study, there was no significant difference in the number of echoviruses isolated from three cell cultures. The results obtained in previous studies indicate that the RD cell line is susceptible to most types of echoviruses [3, 12], but some serotypes replicate in HEp-2 [10, 20] and Vero cells also [21].

More than 90% of cases of viral meningitis are associated with HEVs [14]. Unlike bacterial and herpes virus meningitis, HEV meningitis is generally a benign disease which resolves within a few days. Rapid diagnosis of HEV meningitis improves the management of patients by reducing the unnecessary use of antibiotics, anti-herpes virus drugs and the cost in clinical practice. Our findings suggest that the RAD assay with Vero, HEp-2 and RD cell cultures in combination with immunostaining with 2E11 monoclonal antibodies 72 h after inoculation period was effective

in the detection of HEVs in clinical samples in our laboratory. It attained a slightly lower sensitivity (94.4%) than CCC, and turnaround time was significantly reduced from an average of 6.1 to 3 days. Recently, laboratory diagnosis of viral infections by molecular methods has become more and more important [22, 23]. Although the real-time RT-PCR has been demonstrated to be the most sensitive and fastest method for the diagnosis of enterovirus [4, 20], molecular techniques are not yet available to all laboratories. Their introduction into the routine laboratory setting requires expensive equipment, special technology and trained personnel [18]. In many virologic laboratories cell culture is still used as the primary method for the diagnosis of HEV infections [9, 10, 18, 19]. It offers the ability to type HEV isolates using IFA or neutralization test. Although serotyping has no influence on the clinical management of patients, it enables identification of circulating virus types, types responsible for an outbreak and monitoring of changes in virulence and the epidemic potential of HEV types. Molecular typing of HEVs is possible, but not widely or commercially available [3, 9]. Diagnostic RT-PCR assays commonly used are based on the amplification of the highly conserved 5' non-coding region of the HEV genome. These assays are sensitive and able to detect enterovirus strains that do not grow in conventional cell cultures, but they do not allow identification of the

detected viruses beyond the genus level [3, 11, 15]. Serotype specific sequences are situated in the VP1 coding part of the genome, so the RT-PCR and sequencing of the VP1 - encoding gene is applicable for virus typing [13, 15, 23]. However, these assays usually require a cell culture prior to extraction due to a lack of sensitivity [11, 15]. Until a reliable and sensitive nucleic acid-based typing method is commercially available, cell culture will remain necessary for epidemiological purposes. A good alternative for the molecular methods in the diagnosis of HEV meningitis is the method of rapid detection of viral antigens, which represents a significant advance over traditional isolation [2, 4, 7].

CONCLUSION

The results of this study have confirmed that HEVs are important etiological agents of aseptic meningitis and can indicate the serotypes associated with this disease in our Province. The RAD assay with Vero, HEp-2 and RD cell cultures in combination with immunostaining with 2E11 monoclonal antibodies 72 h after the inoculation period was slightly less sensitive than CCC and significantly shortened the detection time of enteroviruses; thus, it may be useful in a rapid diagnosis of enteroviral meningitis.

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Доказивање ентеровируса у клиничким узорцима болесника са асептичним менингитисом тестом за брзо откривање антигена

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КРАТАК САДРЖАЈ

Увод Хумани ентеровируси су најчешћи етиолошки агенси асептичног менингитиса. Брзо дијагностиковање асептичних менингитиса има велики утицај на лечење болесника.

Циљ рада Циљ рада био је да се испита ефикасност теста за брзо откривање антигена у доказивању ентеровируса у поређењу с конвенционалном изолацијом на ћелијским културама и одреде серотипови који су у вези са случајевима асептичног менингитиса на подручју Војводине.

Методе рада Тест за брзо откривање антигена изведен је центрифугирањем инокулисаних *Vero*, *RD* и *HEp-2* ћелијских култура и индиректном имунофлуоресценцијом са реагентом *Pan-Enterovirus 2E11* (*Millipore-Chemicon*). Конвенционална изолација је изведена употребом истих врста ћелија и истог имунофлуоресцентног реагенса за доказивање ентеровируса.

Резултати Од 70 анализираних узорака (29 узорака церебралне течности, 27 узорака фецеса, девет ректалних

брисева и пет брисева грла), 36 (51,4%) је било позитивно у имунофлуоресценцији након конвенционалне изолације, док су 34 узорка (48,6%) била позитивна након теста за брзо откривање антигена. Осетљивост овога теста била је 94,4%, а специфичност 100%. Време потребно за откривање ентеровируса овим тестом било је три дана, а у конвенционалној изолацији између три и тринаест дана (просечно 6,1 дан). Током 72 часа од инокулације цитопатогени ефекат (ЦПЕ) се јавио само код 19 (47,5%) од 40 ЦПЕ-позитивних узорака. Серотипизацијом је откривено 11 типова ентеровируса: коксаки вирус А16, Б3, Б4 и Б5 и еховирус 2, 4, 6, 11, 13, 16 и 30.

Закључак Тест за брзо откривање антигена био је незнатно мање осетљив од конвенционалне изолације на ћелијским културама и значајно је скратио време потребно за доказивање ентеровируса, те може бити користан у брзој дијагностици ентеровирусних менингитиса.

Кључне речи: ентеровирус; асептични менингитис; култура ћелија

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