AJPS, 2008, Vol. 5, No. 1

Title: Diagnosis of Enteroviral-associated-Meningitis by RT-PCR for the First Time in Iraq

Faiza, L. Tuama, Yassen, I. Omeran, Manal Khalid Abdul Ridha, Walid, K. Ridha

Central Public Health Laboratory/Molecular Biology Department, Al-Nahrain university/Collage of Science/ Biotechnology Department, University of Almustansiriyah,College of Pharmacy, Pharmacotheraputics dept., Central Public Health Laboratory/Virology Department.

الخلاصة

مقدمة : لفحص نماذج سائل النخاع الشوكي, استخدم تقنيةٌ RT-PCR لتشخيص إصابات الجهاز العصبي المركزي وقد تم فحص عشرين نموذج من سائل النخاع الشوكي.

الهدف :

أ- تقييم كفاءة الخليط الثلاثي للمواد(TRI-Reagent) في استخلاص الحامض النووي المنقوص الأوكسجين RNA

ب - إمكانية مضاعفة Enteroviral-RNA-genome باستخدام تقنيةٌ Single step-RT-PCR.

طريقة العمل: تضمن البحث (20) نموذج سائل النخاع الشوكي (حجم 250 مايكروليتر من سائل النخاع الشوكي) واستخدم في استخلاص الحامض النووي RNA تبعا لطريقة Chomcynki,1993 وحضرت تقنية EV-RT-PCR لفحص جميع نماذج الدراسة.

النتائج: لقد نجحنا باستخلاص الحامض النووي RNA من عشرين نموذج سائل نخاع شوكي من حالات التهاب السحايا aseptic meningitis, عشرة منها كانت موجبة في فحص EV-RT-PCR .

الاستنتاج: أظهرت طريقة الاستخلاص ذات الخطوة المنفردة قابليتها على استخلاص RNA خلال أربع ساعات فقط باستخدام الخليط الثلاثي للمواد (TRI-Reagent) وأعطتنا منتوج جيد والحامض النووي المستخلص ذا نقاوة عالية. يعتبر EV-RT-PCR فحص ملائم للتشخيص الطبي لالتهاب السحايا المتسبب من الإصابة بالفيروسات المعوية وباستخدامه يطور ويحسن معالجة المتلازمات العصبية المتسببة عن الفيروسات المعوية.

Abstract

Introduction: A protocol for testing cerebrospinal fluid specimens using RT-PCR for the diagnosis of central nervous system infections was developed [2] and used to test 20 CSF specimens.

Objectives: A-Evaluation of efficiency of In-house prepared Extraction reagent. B-Amplified of Enteroviral-RNA genome by single-step RT-PCR. Methods: 20 cerebrospinal fluid specimens were included. RNA was extracted from 250µl by method Chomcynki, 1993. EV-RT-PCR was performed for all specimens studied.

Results: We successfully isolated total RNA from 20 CSF specimens of AM cases. Ten of 20 specimens were positive by RT-PCR.

Conclusions: Single-step extraction method was allowed isolation of RNA in 4hr. and provided both high yield and purity of un-degraded RNA preparation. EV-RT-PCR detection test is suitable for clinical diagnosis of EV-related meningitis and may improve the management of EV-related neurologic syndromes.
Keywords: Enteroviral-associated- meningitis, EV-RT-PCR

Introduction

EVs are a divers group of small, non-enveloped RNA viruses that are transmitted largely by a fecal-oral route, that replicate in high titer in the enteric tract and carried by the blood to target organs. Non-polio enteroviruses, included Echoviruses and Coxsackieviruses A and B are the etiologic agents of aseptic meningitis[9].

Laboratory diagnosis of enteroviral infection relies on virus isolation in cell culture. However, this technique has several limitations, include a period of days to weeks to obtain results and the limited sensitivities of cell cultures to certain enteroviruses[4]. The detection of enteroviral RNA in clinical samples by PCR has recently been developed[10, 11, 14] by taking advantage of the conserved sequences in the 5'end of the majority of enteroviruses which infect humans have been developed[14, 16]. This report described a method for isolation of RNA. The method based on the use of a reagent containing phenol and guanidine thiocyanate. A biological sample is homogenized in the reagent and isolation by a liquid phase separation. The isolation of RNA can be completed in 1hr[6]. Enzymatic amplification of viral cDNA synthesized by reverse transcription (RT-PCR) is a sensitive method for the detection of EV directly from CSF specimens[11, 14, 16].

Materials and Methods

Twenty children with meningitis syndrome were included in our study. Lumber punctures reveled a pleocytosis (>= 6 cell per mm3) and negative gram stain. Cultures of CSF were negative for bacteria and fungi. All of these children were considered to have aseptic meningitis.

Reagent:

In-house extraction reagent (TRI-Reagent) was prepared (according to procedure described by Chomcynki, 1993) by mixing the components at the concentration given versus each one as shown in the table (1).

Experimental procedure:

TRI-Reagent was used to isolate RNA from both control (Enteroviral infected tissue was prepared in National Polio Lab./Central Public Health Lab.) and CSF specimens. Table (2) described isolation of RNA.

Enteroviruses RNA were detected by a single-step RNA, with the primers from the highly conserved 5' NTR of the enterovirus genome:
Upstream primer (TCCGGCCCCTGAATGCGGTAAT).
Downstream primer (ACACGGACACCCAAAGTAGTCGGTT).

RT-PCR (Promega, Medison, Cat. No.A1260) was performed with one tube containing 2µl of RNA, 1µM concentration each of primers (Downstream and Upstream primers), 0.2 mM deoxynucleoside triphosphate, 1X of AMV/Tfl Reaction Buffer, 1.5 mM MgSO4, 2U/µl Ribonuclease inhibitor, 0.1U/µl of AMV reverse transcriptase, 0.1U/µl of Tfl polymerase under standard conditions to avoid the risk of contamination and inhibition [12].

The reactions were performed with thermal Cycler (GeneAmp model 9700, Perkin Elmer). First strand cDNA synthesis was performed for 45min. at $48C^0$, followed by denaturation at $94C^O$ for 2min. and then 40 cycles of denaturation at $94C^0$ for 30 S, annealing at 62 C^0 for 1min. and extension at $68C^0$ for 2min.

The PCR products were separated on a 2% agarose gel stained with ethidium bromide and visualized under UV light. Negative (water) and positive (Enterovirus culture in RD cells) controls were included in all reactions from the extraction step, were run in parallel with test samples.

Results and discussion

The single-step (TRI-Reagent) method has become a predominant choice for isolation of total RNA from a wide variety of biological material.

Total RNA yield: We successfully isolated total RNA from 20 specimens of AM cases. Since it is generally held that a solution containing 40 µg/ml of RNA gives a value at $A_{260}=1$[13], accordingly, when the RNA concentration was determined by measuring the A_{260} of an aliquot of the total RNA extract preparation, the total RNA yield obtained ranged from 0.36 to 0.40 µg/µl, as shown in the table (3).

The purity of RNA extracts measured as the ratio A_{260}/A_{280} was found to range between 1.5-1.65 with a mean value of (1.57), this value fell just short of the recommended value for this ratio of 1.7 this indicates an acceptable purity of the RNA preparation obtained with only scarce protein contamination.

On the other hand, when the A_{260}/A_{230} ratio was measured, the extracted RNA purity gave a value in the range of 1.3-1.5 and a mean of (1.38), which was lower than the recommended 2.0 ratio, indicating carryover contamination by Guanidium Thiocyanate during the precipitation steps of the RNA extraction process.

The problem of RNases contamination was overcome when the TRI-reagent method was used where the single-step procedure kept sample manipulations to a

minimum, and the fact that the TRI-Reagent itself rapidly inactivated the Ribonuclease and therefore improved the stability of the extracted RNA genomes and is useful for isolation RNA from minute quantity of cells[6].

Previous studies used this method with successful result for the extraction of enteroviral RNA from CSF and different clinical samples[3,4,8].

RT-PCR Reaction:

The appearance of visually clear, distinct and interpretable bands when target Amplicons were run in gel Electrophoresis; considerable reduction of non-specific priming and the appearance of non-target bands as determined by differences in molecular size; and finally the consistent appearance of internal control Amplicons (figure 12), all of which testified for accurate test performance, Successful amplification of EV positive control samples included in the runs affirmed the specificity of EV target sequence priming. The sensitivity of EVs-primer with Access RT-PCR system (Promega comp.) was pre-evaluated by Pozo and coworkers (1998) using different (serial) dilutions of EVs-RNA containing 5′-NTR sequence of EV, they found that the lowest detection limit of the test ranged between 0.2 and 0.02 TCID50/0.1ml

The molecular size (or band length) was calculated by the application of (PhotoCapt Analysis program) software system with a resolving power capable of discriminating two bands (100 and 200) base pair different in length[1]. Using this software, the calculated molecular size of the target band was 114 bp. This was further confirmed by computation of the band size using the modified software (Modified Scion Image System), which gave similar values for the target product (by Dr. Mahmod/ Collage of Medicine / Al-Nahrain University/ personal contact).

Similar results in terms of specificity were observed when another commercially available RT- PCR test (Penter RT-PCR) recognized all 64 prototypes of EVs, this latter assay system has also proved to be suitable for use in clinical diagnosis [2]. The value of the Enteroviral-RT-PCR test lies mainly in its higher sensitivity, the shorter test duration and the sensitivity for the different EVs when multiplex primer set is used to prime the target template, therefore, this test (Enteroviral-RT-PCR) was evaluated for diagnosis EV-associated-meningitis in this country for the first time.

Materials	Quantity
4.0M Guanidium Thiocyanate	10ml*
0.75M Sodium citrate (pH 7.0)	352µl
10% Sodium Dodecyl Sulfate (SDS)	528µl
14.3 M β-Mercaptoethanol	76µl*

10ml of this mixture was placed in a new tube and added	
2.0M Sodium acetate (pH 4.0)	1.0 ml
Water-saturated phenol	10ml
This solution is good for at least 2 months at $4C^0$	

Table 1: Preparation of Extraction-Reagent (TRI-Reagent)[6]

Test/Step	Standard value and procedure
Total RNA Isolation using In-house Prepared Extraction Reagent	It was used to isolate RNA from all specimens included this study
Input samples	100µl Control or 250 µl specimen (CSF)
Lysis solution	(0.25ml) TRI-Reagent mixed with input samples
Separation of two phases; RNA in the aqueous phase where as DNA and protein in inter-phase and organic phase	Centrifugation; 12000g/15mint./$4C^0$
	To aqueous phase was added; 100µl phenol-chloroform
	Centrifugation; 12000g/15mint./$4C^0$
RNA precipitation	To aqueous phase was added; 2.5volume 100%cold ethanol
	Incubation; overnight/$-20C^0$
	Centrifugation; 12000g/30mint. /$4C^0$
RNA wash/twice times; to removal residual ethanol	Each time; mixed with 100µl 75%ethanol/vortexed/centrifugation (12000g/10mint. /$4C^0$).
Pellet/air dried	For 1-3 minutes
RNA solubilization	-pellet re-suspended in 20µl of nuclease-free-water and added 1µl of Recombinant RNasin inhibitor
Storage	At $-70C^0$ until used
Determination of RNA yield and purity	Spectrophotometrically
Dilution RNA sample	5µl RNA sample +495µl nuclease-free-water
Measurement absorbency	At wave length 260,280and 230 nm
Determination purity by formulas:	-RNA purity = A_{260} / A_{280} -RNA purity = A_{260} / A_{230}

Calculation of RNA concentration (µg/µl) by formula:	[A_{260} x40x(Dilution factor)] /1000

Table 2: Summary of the Standard RNA Extraction procedure and steps in the RT-PCR Technique followed in this study.

Case Code	Purity of RNA extracts		RNA concentration µg/µl
	A_{260}/ A_{280}	A_{260}/ A_{230}	
4F	1.56	1.34	0.38
8F	1.53	1.38	0.37
9F	1.5	1.35	0.38
10F	1.6	1.40	0.39
11F	1.57	1.34	0.40
12F	1.58	1.35	0.38
19F	1.53	1.31	0.39
20F	1.61	1.50	0.40
22F	1.61	1.30	0.39
35F	1.50	1.32	0.39
54F	1.50	1.40	0.36
56F	1.56	1.33	0.40
58F	1.65	1.51	0.38
61F	1.57	1.36	0.39
64F	1.61	1.40	0.40
65F	1.50	1.30	0.36
120F	1.61	1.40	0.38
121F	1.60	1.40	0.39
129F	1.57	1.37	0.38
130F	1.65	1.50	0.40
Mean	1.57	1.38	0.38

Table 3: The yield of total RNA isolated from 20 CSF-specimens and their purity ratios.

References

1. Al-Gafari R.N., (2004) Characterization, Classification, Genomic Analysis and Establishment of phylogenetic Tree among Local Isolates of Antibiotic Producing Streptomycetes, Thesis, Al-Nahrain University, Baghdad, Iraq.
2. Bourlet T., Caro V., Minjolle S., Jusselin I., Pozzetto B., Crianic R. and Colimen R. (2003) New PCR Test that Recognize all Human Prototype of Enterovirus :Application for Clinical Diagnosis, J. Clin. Microbiol. 41(4): 1750-1752.
3. Bourlet T., Gharbi J., Omar S., Aouni M. and Pozzetto B. (1998) Comparison of a Rapid Culture Method Combining an Immunoperoxidase Test and a Group Specific Anti- VP1 Monoclonal Antibody with Conventional Virus Isolation Technique for Routine Detection of Enteroviruses in Stool, J. Med. Virol. 54: 204-209.
4. Casas I., Powell L., Klapper P.E., and Cleator G.M. (1995) New Method for the Extraction of Viral RNA and DNA from Cerebrospinal Fluid for use in the Polymerase Chain Reaction assay, J. Virol. Methods 53(1): 25-36
5. Chesky M., Scalco R., Failace L., Read S. and Jobim L.F. (2000), Polymerase Chain Reaction of the Laboratory Diagnosis of Aseptic Meningitis and Encephalitis, Arq. Neuro. Psiquiatr. 34 (3B).
6. Chomczynski P., (1993) Manufacturer protocol: TRI Reagent-RNA/DNA/Protein Isolation Reagent, Molecular Research Center, Cincinnati, OH. (http://www.mrcgene.com/)
7. Chonmaitree T., Ford C., Sanders C. and Lucia H. (1988), Comparison of Cell Cultures for Rapid Isolation of Enteroviruses, J. Clin. Microbiol. 26(12): 2576-2580.
8. Craig M.E., Roberston P., Howard N., Silink M. and Rawlison W.D. (2003) Diagnosis of Enteroviruses Infection by Genus-Specific PCR and Enzyme-Linked Immunosorbent Assay, J. Clin. Microbiol. 41(2): 841-844.
9. Melnick J.L. (1996), Enteroviruses: Polioviruses, Coxsackieviruses, Echoviruses and newer Enteroviruses In: Field B.N., Knipe D.M. and Howley P.M. (eds.) Field Virology, 3rd ed. Lippincott-Raven Publishers, New York. PP: 655-712.
10. Muir P., Nicholson F., Jhetam M., Neogi S., and Banatvala J.E. (1993), Rapid diagnosis of Enterovirus infection by magnetic bead extraction and polymerase chain reaction detection of Enterovirus RNA in clinical specimens, J. Clin. Microbiol. 31:31-38
11. Petitjean J., Freymuth F., Kopecka H., Brouard J., Boutard P. and Lebon P. (1994), Detection of Enteroviruses in cerebrospinal Fluid: Enzymatic

amplification and Hybridization with a Biotinylated riboprobe, Molecular cellular Probes 8(1): 15-22.

12. Promega, Access RT-PCR and Access RT-PCR Introductory System, Technical Bulletin #TB220, Promega Corporation, U.S.A. (http://www.promega.com/tbs/tb220/tb220.html)
13. Pozo F., Casas I., Tenorio A., Trallero G. and Echevarria J.M. (1998), Evaluation of a Commercially available Reverse Transcription-PCR Assay for Diagnosis of Enteroviral Infection in Archival and Prospectively Collected Cerebrospinal Fluid Specimens, J. Clin. Microbiol. 36:1741–1745.
14. Rotbart H.A., Sawyer M.H., Fast S., Lewinski C., Murphy N., Keyser E.F., Spadoro, Kao S.Y. and Loeffelhloz M. (1994), Diagnosis of enteroviral meningitis by using PCR with a colorimetric microwell detection assay, J. Clin. Microbiol. 32: 2590-2592
15. Rotbart H.A. (1990) Diagnosis of enteroviral meningitis with polymerase chain reaction, J. Pediatr. 117:85-89
16. Sambrook J., Fritsch E.F. and Maniatis T. (1989), Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory, New York.
17. Sawyer M.H., Holland D., Aintablian N., Conner J.D., Keyser F. and waecker N. J. (1994) Diagnosis of enteroviral central nervous system infection by polymerase chain reaction during a large community outbreak, J. Pediatr. Infect. Dis., 13:177-182.

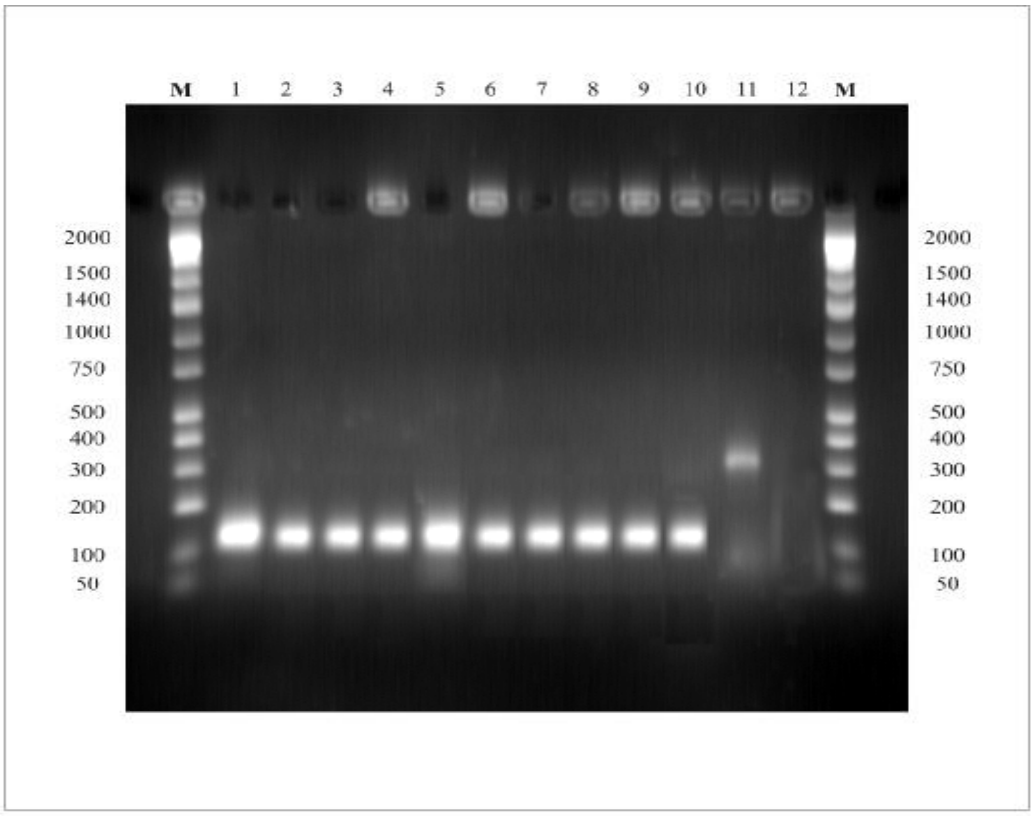

Figure 12

Visualization of Enteroviral RNA by gel electrophoresis after the RT-PCR protocol.
Lane 1: Specific-positive control.
Lanes 2 to 10 : Amplicor RT-PCR- positive in test samples. Lane 11: Internal Control.
Lane12: Negative control.
M: molecular weight standard (Direct Load ™ 50-10000 bp DNA Marker, Sigma).