REVERSE TRANSCRIPTASE MULTIPLEX NESTEDPCR FOR CENTRAL NERVOUS SYSTEM INFECTIONS

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ABSTRACT

Viral infections of the central nervous system (CNS) may result in clinical syndromes like aseptic meningitis, encephalitis and myelitis. These infections are often difficult to diagnose using conventional laboratory techniques like viral culture and serology, which are time consuming and unsatisfactory. Hence a study was designed to develop a rapid technique to detect the viral etiology. In this study a reverse transcriptase (RT) multiplex PCR to detect viral etiologies in CNS infections was standardized. The RT multiplex PCR was designed to detect enterovirus, herpes simplex and varicella zoster viruses. Three sets of primers were employed for their detection. Amplification of target sequences was qualitatively analyzed by looking for the presence or absence of amplicons on a 1.5% agarose gel, stained with ethidium bromide. Sensitivity of the PCR has been ascertained. Further, a second PCR (nested), which confirms the product specificity and also helps in increasing the sensitivity of the assay, was performed using internal primers. The RT multiplex PCR standardized can be employed to detect herpes, varicella and enteroviral infections.

INTRODUCTION

Viral infections of the central nervous system (CNS) result in the clinical syndromes of aseptic meningitis, encephalitis, and myelitis. A wide variety of different viruses can infect the CNS. Viral infections of the CNS are often difficult to diagnose because conventional laboratory methods, such as viral culture and serology, are time consuming and unsatisfactory. Identifying the causative organisms in CNS infections help in providing a rational basis for chemotherapy and prognosis, in limiting unnecessary investigations and also to provide epidemiological information (Read et al.,). Enteroviruses are most frequently cultured from CSF, and viral culture and serology usually provide only retrospective evidence of viral infection, which has no influence and on the acute management of patients. Other etiologic agents include herpes virus, varicella zoster, cytomegalo, Epstein Barr, human herpes virus 6, mumps, measles and adenovirus. To detect the aetiology for acute management of the patient, a technique rapid enough should be employed. Improved diagnostic techniques, particularly the use of the polymerase chain reaction (PCR) and other DNA signal and target amplification techniques have resulted in these molecular diagnostics becoming key procedures in viral detection and have helped in the identification of a pathogen in 55 to 70 percent of cases of aseptic meningitis and an increasing number of patients with viral encephalitis (Rotbart, 1991).

Among the various molecular techniques PCR is the most preferred for its feasibility and rapidity and ability to detect the pathogen. Use of multiplex PCR is more advantageous and it overcomes the shortcomings of PCR and increases the diagnostic capacity of PCR. Multiplex PCR has the potential to produce considerable savings of time and effort within the laboratory without

compromising test utility (Elfath M. et al., 2001). Hence, a multiplex PCR for detection of viral agents in meningitis and encephalitis (Glimaker M, et al., 1993, Echevarria J M et al., 1994, Read and Kurtz, 1999) will be most appropriate and useful in clinical management of the patients.

Against this background and previous work that had established protocol for detection of enteroviruses (Nicholson, F et al., 1994, Aiyar and Leong 1996) a study was proposed to establish and standardize RT multiplex nested PCR for the detection of the viral agents in CNS infections.

MATERIALS AND METHODS

For this study that was aimed at establishing a multiplex PCR for HSV –1 HSV-2, polio and varicella zoster viruses, the materials were procured as follows. Viral stocks were procured as follows. Dr.Nor Shahidah Khairullah, IMR, kindly provided HSV1 virus stock. Enteroviral stock was already available in the department. Varicella Zoster and Polio viral stocks were prepared by inoculating live vaccines into Vero cell line. The viruses were cultured using Vero cell lines available in the department of Microbiology and Parasitology. Molecular biology Chemicals for PCR, enzymes and DNA ladder were procured from MBI Fermentas. Qiagen's QIAamp MinElute^{TM} Virus Spin kit was used for extraction of viral nucleic acid.

Viral culture and nucleic acid extraction: Vero cells were used to grow the viruses. Confluent cell cultures were seeded with viral stocks/live vaccines, and were observed 50% cytopathic effect. They were then subjected to two freeze-thaw cycles. Freeze-thawed solutions were clarified by centrifugation at 5000 rpm /5 min and nucleic acid was extracted from100µl of the sample using QIAamp MinElute Virus Spin kit. The protocol of extraction was strictly followed as mentioned in the kit. On extraction, the nucleic acid quantities were estimated using Eppendorf Biophotometer.

Multiplex PCR was standardized to detect the nucleic acid of the above viruses using 3 sets of primers. PCR amplification was performed in a solution with total volume of 50µl containing 5µl of each template (viral nucleic acid), 16 mM $(NH_4)_2$ SO_4, 67 mM Tris –HCl, 0.01% Tween 20, 1.5mM $MgCl_2$, 0.25mM of each deoxynucelotide triphosphate, 0.625U of Taq polymerase, 0.1U MMLV RT and 0.1 µM each nucleotide primer.

PCR thermal cycling incubations were carried out as follows: Reverse transcription and amplification were performed in a single reaction by incubation at 37°C for 15 minutes and 94°C for 40 seconds, preceding 33 cycles of incubation at 94, 60 and 72°C for 40 seconds each, followed by a final extension step at 72°C for 10 minutes. The thermal cycling was performed with MJ Research PTC thermal cycler.

Amplicons were identified by their molecular weights following electrophoresis of 10µl of the reaction mixture using an ethidium bromide-stained 1.5% agarose gel and photographed over UV transillumination using Alpha Innotech Image analyzer.

Nested PCR:

Secondary amplifications with the internal primers, to amplified regions of the respective viral gene sequences, were performed using 1 µl of the primary reaction solution. Reagent concentrations were identical to those used in the multiplex PCR, except that reverse transcriptase enzyme was omitted.

PCR thermal cycling incubations for the primary amplification was at 37°C for 15 minutes and 94°C for 20 seconds, preceding 20 cycles of incubation at 94, 60 and 72°C for 40 seconds each, followed by a final extension step at 72°C for 10 minutes. The secondary amplification was carried out as follows; 20 cycles of incubation at 94, 60 and 72°C for 20 seconds each, followed by a final extension step at 72°C for 10 minutes. The products were viewed as in the multiplex PCR.

RESULTS AND DISCUSSION

The use of PCR for the diagnosis of CNS infections has been evaluated for HSV encephalitis (Guffond et al.,) and enterovirus meningitis (Tanel et al.,). These techniques have increased the understanding the etiological roles of viruses in CNS infection.

The standardization of RT multiplex PCR was successful in that, the required portions of the sequences were well amplified. Three bands of amplifications were perceived with sizes of 415, 271 and 193 base pairs for detection of amplification for HSV, Varicella zoster and enterovirus gene sequences respectively.

The RT multiplex PCR standardized is aimed to have a higher molecular sensitivity in detection of HSV, varicella zoster and enteroviruses. The molecular sensitivity (Figure 2) ascertained showed that, the quantity of total nucleic acids (nucleic acid from the cells of the cell line and that of the virus) from which the viral agents could be detected was as follows – HSV-66 pg, VZV-104 pg, EV-15 pg and Polio-18 pg.

The PCR developed will be more sensitive than testing for viral sensitivity. As detection of these viruses is being performed in single nucleic acid extraction and amplification screen, it is time saving, convenient and cost effective for use of routine detections in the lab. The whole procedure takes approximately 4 hours to complete. Further in cases of ambiguity the second (nested) PCR can be performed to confirm the presence or absence of the viral agent, which increases the sensitivity and specificity of the assay.

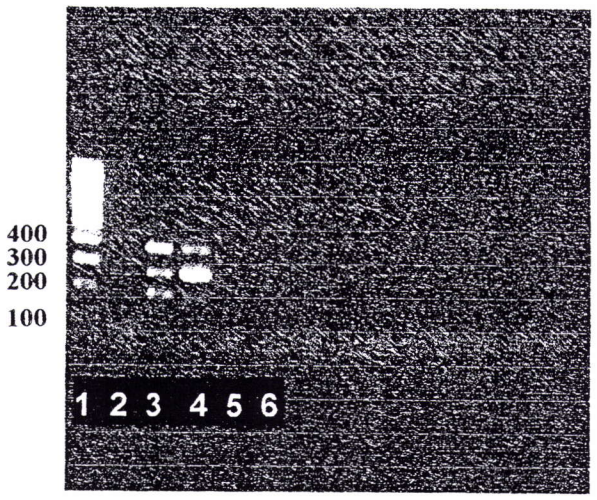

Figure 1 showing the RT multiplex PCR gel picture. Lane 1 is loaded with 100 bp ladder; Lane 2 & 5 is the amplification control and negative nucleic acid control, Lane 3 & 4 are control mixture containing nucleic acids for HSV, VZV & enterovirus (HSV - 415 bp, Varicella-271 bp and enterovirus-193 bp).

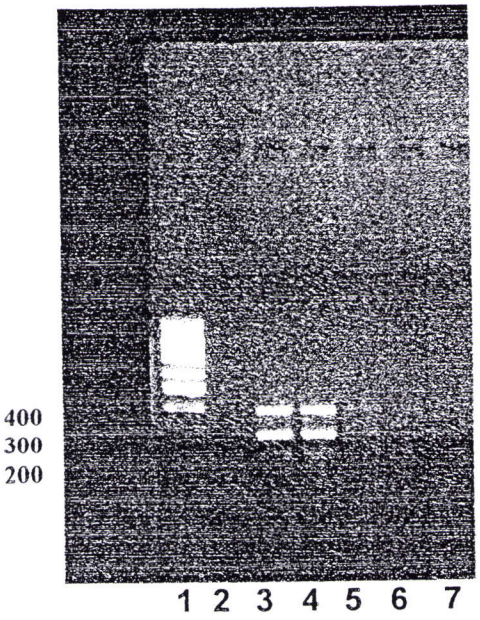

Figure 2 showing Multiplex PCR amplicons

Lane 1 100bp ladder, Lane 2 negative amplification control, Lane 3 - Lane 7 of double dilution of template nucleic acid

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