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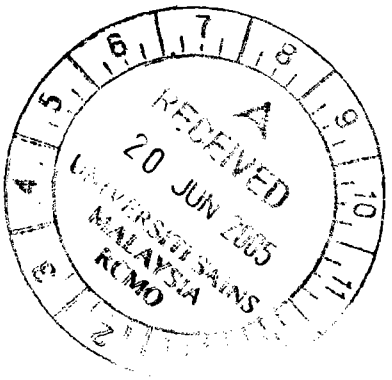

PROJECT TITLE

'ESTABLISHMENT OF MULTIPLEX PCR FOR THE DETECTION OF COMMON NEUROTROPIC VIRUSES'

NAME OF INVESTIGATOR

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BAHAGIAN PENYELIDIKAN
PUSAT PENGAJIAN SAINS PERUBATAN

SALINAN :

- [ ] Bhg. Penyelidikan, PPSP
- [ ] Perpustakaan Perubatan, USMKK
- [x] RCMO

Tarikh : 2/7/05

4) (a) Penemuan Projek/Abstrak

(Perlu disediakan makluman di antara 100 – 200 perkataan di dalam Bahasa Malaysia dan Bahasa Inggeris. Ini kemudiannya akan dimuatkan ke dalam Laporan Tahunan Bahagian Penyelidikan & Pembangunan sebagai satu cara untuk menyampaikan dapatan projek tuan/puan kepada pihak Universiti).

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. These methods are also 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 2% agarose gel stained with ethidium bromide. Sensitivity of the PCR has been ascertained. Further, 3 sets of primers were used to perform a second PCR (nested), which confirms the product specificity, and also helps in increasing the sensitivity of the assay. The RT multiplex PCR standardized can be employed to detect herpes, varicella and enteroviral infections.

ABSTRAK

Infeksi system saraf oleh virus boleh menyebabkan sindrom klinikal seperti meningitis aseptik, enkephalitis dan myelitis. Infeksi ini biasanya tidak dapat dikesan dengan mudah melalui teknik ujian makmal konvensional seperti pengkulturan virus dan serolgi. Tambahan pula, teknik diatas memerlukan banyak masa dan keputusannya tidak memuaskan. Oleh itu, satu kajian telah dilakukan untuk membangunkan ujian *rapid* / cepat bagi mengesan kehadiran virus. Di dalam kajian ini, multiplek PCR *reverse transcriptase* (RT) telah dipiawiakan bagi mengesan virus yang menyebabkan infeksi sistem saraf. Multiplek PCR RT telah direka bentuk bagi mengesan viru entero, herpes simplex dan varicella zoster dengan menggunakan tiga set primer. Amplifikasi rangkaian target telah dianalisa secara qualitatif dengan melihat kehadiran amplikon pada gel agaros 2% yang telah diwarnai dengan ethidium bromid. Sensitiviti bagi ujian PCR ini telah ditentukan. Selanjutnya, tiga set primer digunakan didalam PCR kedua (*nested*)bagi mengesahkan spesifisiti produk, dan ianya telah meningkatkan sensitiviti asay telah direka bentuk ini. Multiplek PCR RT yang telah dipiawaikan boleh digunakan untuk mengesan infeksi yang disebabkan oleh virus entero, herpes simplex dan varicella zoster.

Laporan Komprehensif

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Title:

ESTABLISHMENT OF MULTIPLEX PCR FOR THE DETECTION OF COMMON NEUROTROPIC VIRUSES

Name of the Investigator: Dr.V.Gopalakrishnan

Co-Iinvestigator: En. Chan Guan Thong

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. These methods are also 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 2% agarose gel stained with ethidium bromide. Sensitivity of the PCR has been ascertained. Further, 3 sets of primers were used to perform a second PCR (nested), which confirms the product specificity, and also helps in increasing the sensitivity of the assay. 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. 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 (HSV), varicella zoster (VZV), cytomegalo, Epstein Barr, human herpes virus 6, mumps, measles and adenovirus. To detect the aetiologic agent 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 (1).

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 (2). Hence, a multiplex PCR for

detection of viral agents in meningitis and encephalitis (3,4,5) will be most appropriate and useful in clinical management of the patients.

Against this background, it is proposed that work to establish and standardize multiplex PCR for the detection of the 4 principal viral antigens in this institution. Previous work has already established protocol for detection of enteroviruses (6,7). The present work is an extension of that study.

OBJECTIVE OF THE STUDY

1) To design a multiplex PCR for the detection of herpes simplex, varicella zoster and enteroviruses.
2) To standardize the multiplex PCR to local conditions.

MATERIALS AND METHODS

For establishing a multiplex PCR for HSV –1, HSV-2, enterovirus and varicella zoster virus, which are the most common causative agents of viral infection in the central nervous system, the following were procured. Viral stocks were procured as follows. Dr.Nor Shahidah Khairullah, Institute of Medical Research (IMR), kindly provided HSV1 virus stock. Enteroviral stock available at the department was utilized. Varicella Zoster and Polio viral stocks were prepared by inoculating live vaccines into Vero cell line. The viruses were cultured using Vero cell line available at the department of Microbiology and Parasitology, School of Medical Sciences, USM. Molecular biology Chemicals for PCR were procured from MBI Fermentas. QIAamp MinElute^{TM} Virus Spin kit was used for extraction of viral nucleic acid.

Viral culture and nucleic acid extraction:

Vero cells (African Vervet Green Monkey Kidney cells) were used to grow the viruses. Confluent mono-layers of the cell cultures were grown using MEM (minimal essential media) with 10% fetal calf serum. On the third, when the mono-layer was confluent, they were seeded with viruses/live vaccines. For this, medium in the flask is decanted and viral suspension/live vaccine was added and left for 1 hour for the viral adsorbtion. Then, maintenance media was added and left in the incubator. On observing cytopathic effect, the flasks were subjected twice to freeze-thaw cycles. Supernatants were clarified by centrifugation at 5000 rpm /5 min were diluted 10 folds and nucleic acid will be extracted from100µl of the sample QIAamp MinElute^{TM} Virus Spin kit. Extraction and purification

of viral nucleic acid with virus spin kit was performed according to the manufacturer's instructions.

Multiplex PCR:

Multiplex PCR was standardized to detect the nucleic acid of various viruses using the 3 sets primer sequences. The primers selected are specific for the respective agent and selected in such a way that, the products formed are well spaced for easy identification (References 1,2,3 and 4). 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 Res QIAamp MinElute™ Virus Spin kit earch PTC thermal cycler.

Amplified products was identified by their molecular weights following electrophoresis of 10µl of the reaction mixture using an ethidium bromide-stained 2% agarose gel and UV transillumination.

Molecular sensitivity was carried out as follows. Doubling dilution of pre-quantitated total nucleic acid extracted from the infected cell lines were used in the study. Following this the end point was ascertained, by looking for the presence of bands for the respective viruses in the highest dilution, and the quantity of nucleic acid used in this dilution was equated.

Nested PCR:

Secondary amplifications with the nested primers were performed with 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 standardization of RT multiplex PCR was successful in that, the required portions of the sequences were well amplified. Three bands of amplifications (Figure 1& 4) were perceived with sizes of 415, 271 and 193 base pairs for detection of amplification for HSV, Varicella zoster and enterovirus gene sequences respectively.

Subsequently the second PCR (nested PCR – Figure 3, & 4) carried out, which yielded amplicons with size of 280, 200 and 144 base pairs respective to the amplification of HSV, VZV and entroviral genomes.

The RT multiplex PCR standardized is aimed to have a high 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 multiplex PCR developed was 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 is more sensitive.

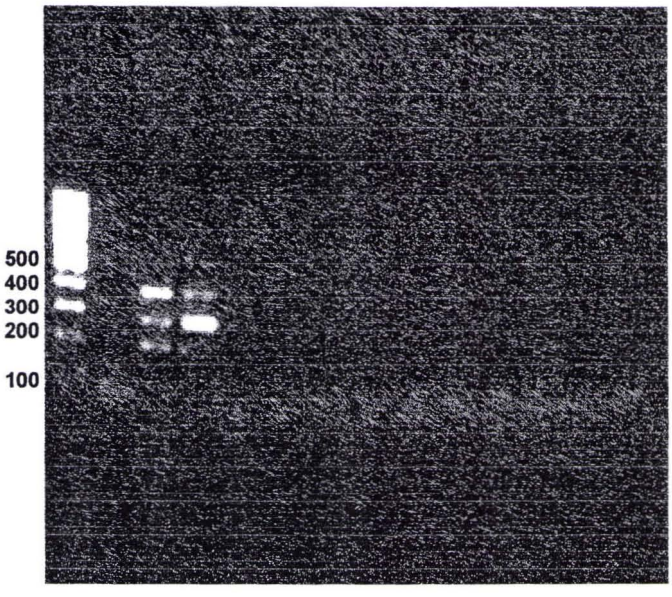

Figure 1 showing the RT multiplex PCR gel picture. Lane 1 is loaded with 100 bp ladder; Lane 2 & 5 is the amplification control and negative nucleic acid control, Lane 3 & 4 are control mixture containing nucleic acids for HSV, VZV & enterovirus (HSV -415 bp, Varicella-271 bp and enterovirus-193 bp).

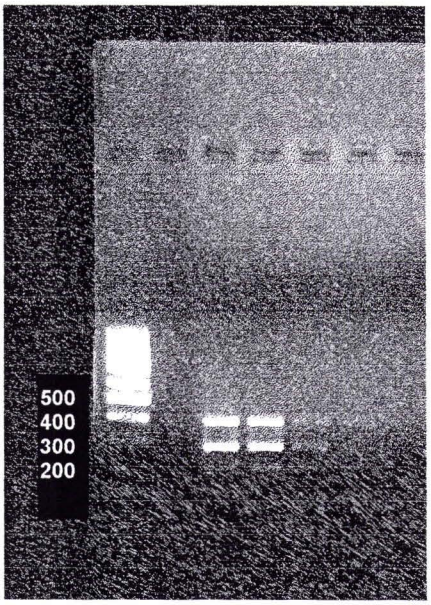

Figure 2 showing Multiplex PCR amplicons
Lane 1 100bp ladder, Lane 2 negative amplification control, Lane 3 - Lane 7 of double dilution of template nucleic acid

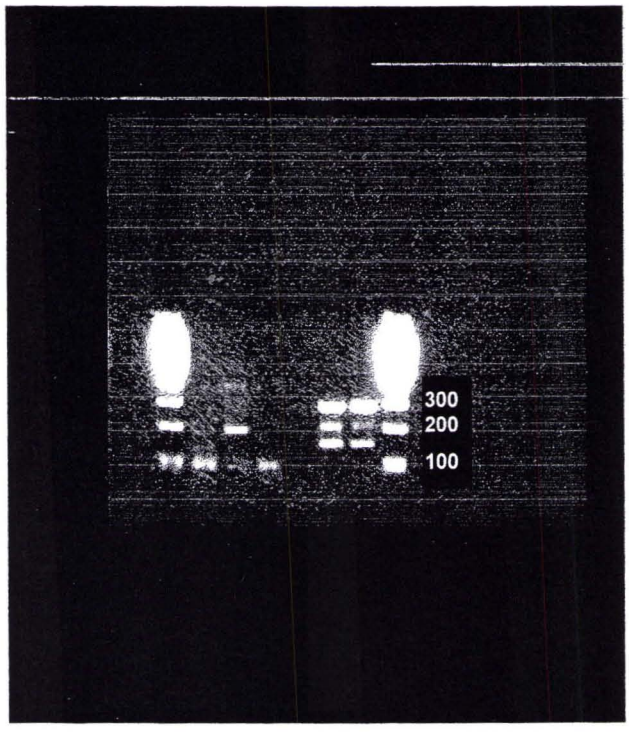

Figure 3 showing second PCR (nested) amplicons –
Lanes 1 & 8 100 bp ladder, Lanes 2 & 4 negative nucleic acid extraction and amplification control, Lane 3 first PCR products. Lane 5 negative amplification control and Lanes 6 & 7 positive control with amplicons for second PCR -HSV (280 bp), Varicella (200 bp) and enterovirus (144 bp).

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