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Multiplex PCR detection of bacterial and viral meningitis in Cerebrospinal Fluid

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M.Sc. Thesis

Jerusalem – Palestine

1434 Hijri / 2013 AD

Multiplex PCR detection of bacterial and viral meningitis in Cerebrospinal Fluid

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A thesis submitted in partial fulfillment of requirements for the degree of Master of Medical Laboratory Sciences –Diagnostic Microbiology and Immunology Track/ Faculty of Health Professions/ Al-Quds University.

1434 Hijri/ 2013 AD

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Thesis Approval

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Master thesis submitted and accepted, Date: 8 Jan 2013

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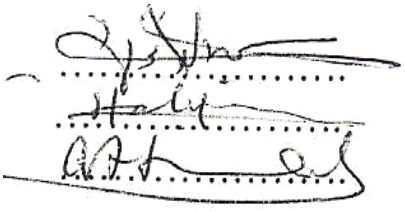

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الإهداء

أهدي هذا العمل المتواضع إلى سيد الخلق والمرسلين, سيدنا محمد صلى الله عليه وسلم.

كما وأهديه إلى زوجي الغالي السيد موسى حراحشة, والى والدي الحبيبين علي ونوفه مناصرة, وإلى أختي الغالية شيرين, وإلى والدة زوجي أم موسى, وإلى ابنتي الغاليتين ديار و ميار وإلى إخوتي وأخواتي في الله وفي طلبه العلم, و أهديه إلى دولة فلسطين شهداء وأرضا وشعبا,

كما وأهديه إلى أستاذي وموجهي ومعلمي الدكتور سمير البرغوثي

Declaration

I certify that this thesis submitted for the degree of Master in medical laboratory sciences / microbiology and immunology track, is the result of my own research, except where otherwise acknowledged, and that this thesis (or any part of the same) has not been submitted for a higher degree or to any other university or institution.

Signed…….

Iman Ali Mohamed Manasrah

Date: 8\01\2013……

Acknowledgements

I would like to express my profound thanks and indebtedness to Prof. Dr Sameer Bargouthi, my supervisor, for his supervision, encouragement, guidance and inspiration throughout this study.

I would like also to express my sincere gratitude to Mohamed Ayesh for their valuable comments and time in reviewing this manuscript.

Special thanks are due to all my colleagues, especially. Dr. mahmoud srour, Dr. Rsmi abu alhlu, Dr. Hatem Eidih.

I greatly appreciate the help from the Princess Alia Hospital staff, Yata Government Hospital.

Special thanks to Mr Osama Doudeen For his support in the provision of CSF samples.

I wish to express my gratitude to my husband Mousa harahsha and to my daughters for their encouragement and endless support.

Abstract

Bacterial meningitis is a serious infection of the fluid in the spinal cord and the fluid that surrounds the brain**.** A multiplex assay was devised to provide simultaneous detection and discrimination of bacterial and Herpes viruses as pathogenic agents of meningitis.

A DNA extraction method that was suitable for both bacterial and viral meningitis was devised; the method was designed to allow concentration of viral particles by ammonium sulfate precipitation, for bacterial pathogens, simultaneous treatment of the sample with 2-mercaptoethanol. The prepared samples were tested for amplifiable bacterial or Herpes DNA. Detection was successful when samples were fresh, or when DNA was correctly preserved. Old samples with crude DNA extracts usually produced negative PCR results indicating the unstable nature of crude DNA extract and old samples.

The PCR amplification of bacterial DNA was based on previous studies by Barghouthi (2009) where the universal method bacterial multiplex (G7) was utilized directly. A new multiplex (IM) targeting Human Herpes Viruses 1-6 and 8 was designed in this study. IM multiplex was capable of detecting HHV DNA when extracted from water or several dilutions of adulterated CSF (HHV 3/VZV). Epstein-Barr virus was refractory to detection even when two additional primer pairs were introduced, EBV was undetectable from DNA and sera of patients with mononucleosis.

Simultaneous detection of bacterial and VZV virus or CMV was possible when IM was combined with G7 in a single multiplex (IMG7). The results were promising since the combined IMG7 multiplex effectively had detected VZV or CMV DNA extracted from CSF. IMG7 was also efficient in detecting *Escherichia coli* from adulterated CSF. The results were encouraging with the exception of EBV.

Clinically, 230 potential meningitis samples from hospitals in different cities in the West Bank, Palestine were collected and tested. Three samples were reported by the hospitals to be viral meningitis. Only one sample was found to be caused by CMV in this study, the other two samples were most likely caused by viruses other than HHV 1-3, 5,6, or 8. The positive sample was confirmed to contain CMV as shown by DNA sequencing and BLAST results determined for the PCR amplicon.

Although this study did not solve the issue of viral detection, it has brought us closer to identifying etiological agents of meningitis; it had shown a new method for DNA extraction and the possible mixing of bacterial and viral multiplexes. In addition, the study showed that EBV was refractory to detection by conventional PCR and that samples better be processed immediately (bedside testing) to overcome the potential loss of viral pathogens in the sample; a result that dictate to hospital directors to move in the direction of adopting new technologies and enter the realm of applied research. The study has raised questions regarding the detection of over 150 viruses implicated in central nervous system pathology including the RNA and DNA known and unknown viruses. This study should encourage others to search for solutions for this health problem; detection of viruses in general is a health requirement in different settings including organ transplants, transfusion, cancer, surgery, immune deficiency cases, and others.

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Chapter I

Introduction

Introduction:

Meningitis is a an inflammation of the meninges (1), which form the thin protective membrane covering the brain and spinal cord (1, 2). Meninges have three layers; the outermost layer is dura mater, is composed of tough, nonelastic, dense connective tissue and adheres to the skull and vertebral column, it is covered on its inner most surface by squamous epithelial cells. The arachnoid is the middle layer composed of collagenous and elastic connective tissue, adheres to the dura mater, and has trabeculae to connect it to the third inner layer (pia mater). The pia mater covers the brain and the spinal cord.

Meningitis may develop in response to a number of causes, usually bacterial or viral infection, or can also be caused by other agents. Bacterial meningitis is the most common and notable of central nervous system infections, with high rate of mortality and many cases of severe sequelae (1, 3, 4). Viral meningitis of the central nervous system (CNS) (5) include DNA and RNA viruses. procedures currently used in clinical laboratories are microscopic examination of gram stained CSF, this simple method, is rapid, accurate, and inexpensive for detecting bacterial meningitis but it is not useful in other meningitis (1), Culture is used in detecting bacteria in CSF. Culturing requires at least 8 hours of incubation in culture medium under aerobic and anaerobic conditions, and with several types of media (1, 6, 7). Moreover, culture frequently remains negative, especially if the CSF is taken after initiation of antimicrobial therapy (6, 7). Bacteria is an important cause of meningitis and have a high morbidity and mortality rates. The early administration of antibiotics correlates with reduced rate of mortality and morbidity. Antibiotic resistance is a rising problem and increasing with increase

use of Empirical therapy without accurate and rapid detection (3, 4, 7). Antibiotics have no role to play in the treatment of viral meningitis, as they neither kill nor stop the multiplication of viruses (8). The severity of meningitis and its treatment depends on the causative virus, this shows the importance of accurate identification and diagnosis of the causative agent of the disease (2).

Effective treatment of bacterial or viral meningitis often requires the rapid and accurate detection and identification of causative in cerebrospinal fluid (CSF) (7). Recently; developed polymerase chain reaction (PCR) method using different target genes, has made it possible to detect low number of infectious agents or even fragments of their DNA (4, 9, 10, 11, 12). PCR use of broad-range 16SrRNA gene, with subsequent sequencing has been reported, then we use develop system that uses a specific-specific multiplex PCR focused on the bacterial and viral micro-organism most frequently found in CSF (3, 13).

1.2 Types of meningitis

1.2.1. Bacterial meningitis

Bacterial meningitis is an infection with a high rate of morbidity and mortality, this meningitis type is mostly severe, and can cause brain damage, hearing loss, and learning disabilities. Approximately 95% of bacterial meningitis cases are caused by *Neisseria meningitides, Streptococcus pneumoniae, Streptococcus agalactiae, Staphylococcus spp, Escherichia coli, Haemophilus influenzae, or Listeria monocytogenes* (3, 6, 14). Bacterial meningitis cause sudden fever, headaches, neck stiffness, nausea, vomiting, increased sensitivity to light and confused mental status (9, 15).

Lyme meningitis is the direct result of invasion of the nervous system by *Borrelia burgdorferi***,** occurring within the first few months of infection. It initially presents as a chronic basilar meningitis, erythema migrans within the first few weeks after injection and still present from 1-6 months later (16, 17).

1.2.2. Viral meningitis

Aseptic meningitis is a central nervous system infection. It is the most common type of meningitis that occurs in sporadic outbreaks. It is generally less severe than bacterial meningitis and resolves without specific treatment (16). Over 100 viruses are known to cause viral meningitis in human (17). These viruses that records under many viruses family (Enteroviruses, Arboviral, Mumps, Lymphocytic choriomeningitis(LCM) virus and Herpesviruses) (16). As shown:

1.2.2.1 Enteroviruses

Enteroviruses are the most frequent cause of life threatening meningitis (15, 17). Enteroviruses are small, nonenveloped viruses with +ssRNA genomes polyadenylated molecule and one long open reading frame. Human enteroviruses (HEV) types are classified into four species including polioviruses which belongs to picornavirus (18), 80% of Enteroviruses cause meningitis. They are transmitted from person to person by fecal-oral or oral-oral routs. They depend on temperate climate, the typical season occurring during June-October (16).

1.2.2.2. Arboviral meningitis

They are RNA virus and have the same seasonal pattern as enteroviruses. Arbovirviruses are maintained in nature through biological transmission between vertebrate host and mosquito vectors. These viruses cause neurologic signs and symptoms of aseptic meningitis. These types of viruses mostly infect elder people (16).

1.2.2.3. Mumps

It is a ssRNA virus and is a serious causative agent of aseptic meningitis in an unimmunized population (16). Its threat has been greatly reduced due to vaccination programs.

1.2.2.4. Lymphocytic choriomeningitis (LCM).

It is a single strand RNA virus and is rarely reported. This type is transmitted to human by contact with rodents or their excretions (2, 16).

1.2.2.5. Herpes viruses.

The Herpes group of viruses encompasses double stranded DNA virus with linear genomes, and are the most common cause of human meningitis; Human herpes simplex virus type-1 (HHV-1) and type-2 (HHV-2), Vericella-Zoster Virus VZV (HHV-3), Epstein-Barr virus EBV (HHV-4), Cytomegalovirus CMV (HHV-5) and human herpes 6,7, &8 (HHV-6,7, & 8) (13, 16, 17, 19).

The Human herpes virus-(1&2) is a large virus. Herpes simplex 1&2 are frequently benign but can cause severe disease including skin lesions and cold sores, mucous membranes and nervous system infection (20, 21). The complications of

neurological system appear with or without mucocutaneous lesion. The initial lesions appear as clear vesicles containing infectious viruses with a base of red (erythematous) lesions at the base of the vesicle (21). After the primary infection, the virus remains latent in the nervous ganglia, where it can reactivate. This type of disease called Mollaret´s disease or meningitis (19, 21). It is the most recurrent aseptic meningitis (19). HHV 1&2 cause more disease as oral herpes-Cold sores, herpes keratitis, herpes whitlow, herpes gladiatorum, Eczema herpeticum, genital herpes, HSV proctitis and HSV encephalitis, HSV meningitis (13, 16, 19, 20, 21, 23) its Transmissible by skin-to-skin contact.

Varicella Zoster virus (VZV, HHV-3) is known as chicken pox virus. It is characterized with rash (22), skin lesion infection. If this virus reaches blood circulation then it can cause aseptic meningitis with or without typical skin lesions (13, 16, 17, 19, 20, 21).

Epstein-Barr virus EBV (HHV-4) causes central nervous system (CNS) infection. This infection occurs in up to 18% of patients with infectious mononucleosis and may include encephalitis, meningitis, and other neurological disease especially in immunocompetent patients (13, 16, 17, 19, 20, 21, 22, 23). EBV infection is associated with AIDS related CNS lymphoma in HIV-infected patient (16, 17).

Cytomegalovirus Or CMV(HHV-5) (13, 16, 17, 20, 21), has the largest genome of all herpes viruses and appears only to replicate in human cells (21). CMV meningoencephalitis is a disease of congenitally infected newborns and immunocompromised individuals. It plays an important role in organ transplants and in patients with advanced HIV infection (17).

Human herpes 6 and 7 (exanthum subitum or roseola) infantum are found worldwide and found in saliva of the majority of adults and children are infected and remain infected for life. It is latent infection in B and T lymphocytes, which can be activated when the cells stimulated to divide (17, 21).

Human herpes 8 **(Kaposi's** sarcoma-associated herpes virus**),** is found in the saliva of many AIDS patient. It infects peripheral blood lymphocytes**.**

1.2.3. Fungus.

Fungal meningitis is much less common than the bacterial and viral meningitis. Fungus is rare but can be life threatening, Fungal meningitis rarely occurs in healthy people; however, at higher risk are those with AIDS, leukemia, or other forms of immunodeficiency and immunosuppressed patients. The most common cause of fungal meningitis is *Cryptococcus neoformans, Histoplasma capsulatum, Coccidioides immitis*, *Candida albicans*, *Blastomyces dermatitidis*, *Aspergillus species* and others (2).

1.2.4. Parasitic meningitis

Parasitic meningitis is rare but may be fatal. *Naegleria fowleri* (2), *Toxoplasma gondii* and *Trypanosoma cruzi* are common**.**

1.2.5. Non infectious meningitis

Non–infectious meningitis may result from cancers, systemic lupus erythematosus, some drugs, head injury, and brain surgery. This type of meningitis is non transmissible from patient to another as the name suggests (2).

1.3. Sign and symptoms

Clinical manifestation of meningitis include sudden onset of fever, chills, severe headaches, nausea, vomiting, neck stiffness, back pain, diffuse paresthesia, mental status, increase sensitivity to light and focal neurological deficits (5, 9, 13, 15, 17, 25, 26).

1.4. Lab findings

Normal CSF specimens are color less, whereas bacterial meningitis CSF specimens will have a turbid appearance, elevated white blood cell count > 3x107 cells/l, elevated protein > 1.0g/l, and decreased glucose concentration <2.22 mmol/l. Neutrophils more than 70% of the total WBCs in CSF. In viral meningitis it shows mild increase in protein, normal level of glucose and pleocytosis (13, 26, 27).

1.5. Diagnostic Laboratory tests

CSF samples are collected by lumber puncture, need tests are biochemical tests such as (glucose and protein concentration), WBC and neutrophils counts, bacterial Gram stain, microscopy, and bacterial cultures should be carries out. Cultures frequently remain negative, especially if the CSF samples were taken after antimicrobial therapy (1, 4, 6, 7, 9). Antimicrobial susceptibility testing is done for bacteria from CSF to determine their antibiotic spectrum (1, 27). CSF samples can be

seen directly after lumber puncture, but do not give an accurate result, because the diagnostic usefulness of the procedures depends on the concentration of bacterium in the CSF of patients with bacterial meningitis ranging from 10^1 to 10^9 colony forming unit (CFU/ml) (1,27), a false negative result may prevail when bacterial concentration is low, or if antibiotic had been administered to the patient before CSF collection.

Alternative methods of diagnosis of bacterial meningitis include antigen detection by Counter immune electrophoresis (CIE), coagglutination (COAG), latex agglutination (LA) which have been established as rapid and direct detection of soluble bacterial antigen in CSF. These methods are rapid antigen detection test that may provide true positive result. Most CNS bacterial pathogens have been detected by antigen-antibody reactions except *Streptococcus* group B because it has a soluble, type specific, capsular, polysaccharide antigen that is released into surrounding body tissues and fluid as the bacterium proliferates. This method can be complicated by non-specific reactions, cross-reactions and /or low concentration of antigen in clinical specimens (1).

Previous methods cannot improve the diagnoses of meningitis, if the causative agent is not a bacterium. Other ways to diagnose bacterial meningitis and to determine the causative agent were sought. Enzyme immunoassay (EIA) and enzyme-linked immunosorbent assay (ELISA), for the detection of bacterial antigens in CSF use specific antibodies (1, 28). The tests can detect bacterial antigens in concentrations as low as 0.1 to 5 ng/ml (1). This method needs several hours to detect the antigen-antibody complex and is expensive (1, 28). Other methods depend on molecular component is polymerase chain reaction (PCR). Recently, developed polymerase chain

reaction (PCR) method using different target genes, has made it possible to detect low numbers of infectious agent or even fragments of DNA (4, 5, 9, 11, 12).

Multiplex PCR use for the detection of nine targeted pathogens has been reported to have excellent sensitivity and specificity; specific-Specific multiplex PCR focused on a limited number of bacterial and viral microorganism most frequently found in CSF was illustrated (3, 13). In a most recent study, The Method was applied to the detection of bacteria in CSF. The study showed that the universal method could detect bacteria that was missed by culture and bacteria that was obtained after antibiotic administration to the patient (29, 30).

1.6. The universal method.

Tow study that was developed to take advantage of the multiplex approach, probability, and conservative sequences of the 16S rDNA (29, 30), the universal method that includes a number of multiplex mixture of primer that contain G5, G7 and G11 that can detect and give PCR positive result to any bacterium, G7 has been tested and is believed to detect any bacterium, The Universal Method (Barghouthi 2009) had integrated several general primers, PCR amplification, DNA sequencing, and sequence alignment (BLAST) in one system designed for the detection and identification of that bacterium, some works have used the 16S rDNA gene as a target for bacterial detection. PCR can be used as a method for the rapid detection of bacteria in clinical samples and would be useful in differentiating bacterial from viral infections (29).

Strategic application of broad range primers has been found to be superior to conventional techniques for detection of bacteria in sterile body fluids (30). When antibiotics were introduced to the patient, culture technique cannot be used since it will

have poor sensitivity, there for it would be better to use PCR technique for rapid diagnosis of bacterial meningitis (1). The specificity of broad range universal PCR has been satisfactorily improved by further analysis of the PCR products by different methods (30). The assay is valuable for initial screening to confirm or rule out bacterial meningitis (29).

1.7. Treatment.

Accurate and immediate therapeutic intervention in cases of meningitis may prevent undesirable outcomes of the infection. In addition, accurate determination of the absence of bacterial meningitis will prevent administration of ineffective antibacterial drugs and allow the continuous search for the true pathogen. Meningitis is a medical emergency requiring immediate diagnosis and immediate treatment. Cerebral imaging and repeated lumbar puncture should be considered in patients who fail to improve clinically after 48 h of treatment. Acyclovir and Ganylovire are the drugs useful in the treatment of viral infections. Corticosteroids reduce brain edema, intracranial hypertension and meningeal inflammation in experimental models of bacterial meningitis (27). Analgesic drugs may be used to reducing the meningitis symptoms (4).

1.8. literature Review.

The most recent study: The Universal Method Developed by Barghouthi 2009, universal method was designed to identify all bacteria. This method includes a number of multiplex mixture of primers (29).

Universal Method Was successfully applied in the detection of any bacterial DNA in CSF samples (30)

A series of studies have developed species-specific multiplex PCR focused on the bacterial and viral microorganisms most frequently found in CSF. *Haemophilus influenzae* strains that are detected and identified by Real Time PCR (3, 32).

Streptococcus pneumoniae is an important causative pathogen of acute bacterial meningitis, the traditional method need a minimum of 10^4-10^5 bacilli/ml to detect it; PCR provides a quick detection of *Streptococcus pneumoniae* (9). 45% of meningitis cases involve bacterial loads of less than 10^5 CFU per/ml (7). Multiplex PCR applied in sero-typing of *Streptococcus pneumoniae* directly on clinical sample has sensitivity higher than cultural methods (33). Multiplex PCR succeeded in detecting, *Chlamydia pneumoniae* DNA in cerebrospinal fluid and in detecting fungi in blood stream infection (31, 36).

The use of PCR for rapid diagnosis of microorganisms that cause meningitis has the potential to overcome the poor sensitivity and slow growth of pathogens when using cultures (35). In addition to the fact that some pathogens are not culturable, diagnosis of viral CNS infection has been revolutionized by the advent of new molecular diagnostic technologists such as PCR to amplify viral nucleic acid (17). The

use of multiplex PCR for the diagnosis of CNS diseases has been well evaluated for human herpes virus encephalitis (34). Multiplex PCR used in serotyping and CSF based PCR had at least 80% sensitivity and 100% specificity. For many years many studies developed to detect one or few of the six major human herpes virus (HHSV) from cerebrospinal fluid by single tube (3, 16).

1.9. Specific Aims and Justification:

Specific Aims and Justification: Simultaneous detection of bacteria & HHV1-6 & 8 in CSF, to reduce the time required for detection, enhance disease treatment, management, and minimize administration of empirical antibiotic therapy. Evaluation of clinical CSF bacterial and viral infection is amply justified for treatment and epidemiological studies which can be applied upon identification of etiological agents.

The specific aim of the present study was to develop a single-tube multiplex PCR assay for the simultaneous detection of bacteria and human herpes viruses for diagnostic application as a multiplex PCR in hospitals with the potential for identifying any bacterial agent and most HHVs from CSF- meningitis infections. The Clinical Significance of the single-tube assay is to reduce the time required for detection of bacterial or HHV meningitis, in order to enhance treatment and disease management as well as minimizing the administration of empirical antibiotic therapy. The study will contribute to epidemiological studies and prevention of meningitis that will be a normal extension of successful diagnosis of bacterial and Herpes pathogenesis.

1.10. Statement of the Problem

Molecular method is a new method with some specifications fast, sensitive and specific. This research is designed to test the ability to detect the seven human herpes viruses in a single test in addition to any bacterial pathogen in CSF sample according to The Universal Method Developed by Barghouthi 2009, and tested by Barghouthi and Zughayyer 2012. in the same reaction.

1.11. Hypothesis and Principle:

A multiplex can be formulated that will allow the simultaneous detection of all bacterial pathogens and all or most of the Human Herpes viruses from CSF..

Chapter 2

Materials and methods

Materials and methods:

The method being developed should be sensitive, specific for bacterial and Herpes viral infections, easy to apply and interpret, inexpensive, and most importantly time saving. A multiplex that can be formulated as a single-tube test may represent a good choice and advancement of CSF diagnosis.

2.1 Clinical samples and Patient:

230 CSF samples obtained from pediatric and adult patients by LB (lumber puncture), suspected of suffering from meningitis, were collected over a period of one year from hospitals in the west bank; Hebron Governmental Hospital , Yata Hospital, Jenin Governmental Hospital, and Nablus Governmental Hospital. The clinical CSF samples were collected in DNA free sterile screw cap microfuge tubes and stored at -40˚C. These samples were tested for bacterial and viral meningitis in the hospitals, with conventional method.

2.1.1 Target pathogens to be detected in this research:

Human herpes virus (1&2), Human herpes virus 3 (VZV), Epstein Barr virus (HHV 4), Human herpes virus 5 (CMV), human herpes six and eight (HH6&8), and all pathogenic bacteria. The procedure depend on the DNA extraction from the CSF samples, do the PCR reaction with thermocycler cycles, and then identify gene according to molecular weight on gel electrophoresis.

2.2 Primer design:

Bacterial Universal detection multiplex was as described for G7 multiplex (Barghouthi 2009). Viral primer pairs (Table 2.1) were selected from published DNA sequences. The selected DNA sequence that was targeted for amplification was BLAST analyzed against all published sequence to insure its uniqueness and lack of cross reactivity. Similarly, selected primer sequences were analyzed using BLAST. The primers were designed to allow their use at a fixed annealing temperature (58-60˚C). The primers were also designed to produce a specific size amplicon that did not match any other amplicon; each of the potential amplicons should produce a unique size

Table 2.1: Primer pairs of Herpes viruses designed in this study

Human Herpes Virus	**Viral Primer**	**Length**	***Tm***	**PCR product size**
HHV1&2	F 5'- CCTCATGATCCTCATCGAGGGCG -3'	23 mer	61ºC	213
	tR 5'- CCGAAACAGCCGGTACACGCG-3'	21 mer	60 ºC	
HHV 3 VZ	F 5'-GCTCACCCCAAAGGATGACCC-3'	21	58 ºC	679
	tR 5'- CGTCGCATACACCGTGTGTACC-3'	22	59 ºC	
HHV4	F 5'-CCACCTCCAGGTGCGCCTAGG-3'	21	62 ºC	346
	tR-5'- TTGGTGCCACCATCTGGCAGC-3'	21	58 ºC	
HHV6 A&B	F 5'-AGACCGCTGCTGGACGAACTG-3'	21	58 ºC	850
	tR-5'- TTCCTCCGCGGGACCTGGC-3'	19	60 ºC	
HHV 5 CMV	F 5'- GCGTGGAGTCGACGGCCTCC - 3'	19	62 ºC	395
	tR-5'- TGGTGGACATCACGGATACCGAG-3'	23	59 ºC	
HepB	F- 5'- GGACTGGGGACCCTGCACCG-3'	19	62 ºC	589
	tR-5'- GTGGGGGAAAGCCCTACGAACC-3'	22	60 ºC	

Additional primer for Epstein-Barr virus

Table 2.2: Epstein Barr Virus primers.

Name	Sequence 5'→3'	TM
EB-F	**CCACCTCCAGGTGCGCCTAGG**	**62˚C, 21-mer , 346bp**
EB-R	**TTGGTGCCACCATCTGGCAGC**	**63˚C, 21-mer**
BARF QF*	**GACCGGGGTCCTCACAAACACAG**	**62˚C, 23-mer , 497bp**
BARF QR	**CTGCTTGGTGACCTCGGTCTC**	**60˚C, 21-MER**
EB15F	**GAACAGCTCCTGGACGTTGCG**	**61˚C, 21-mer, 507bp**
EB15R	**GTGGCGGCGGCCTTCCTC**	**64˚C, 18-mer**

(35, 36).

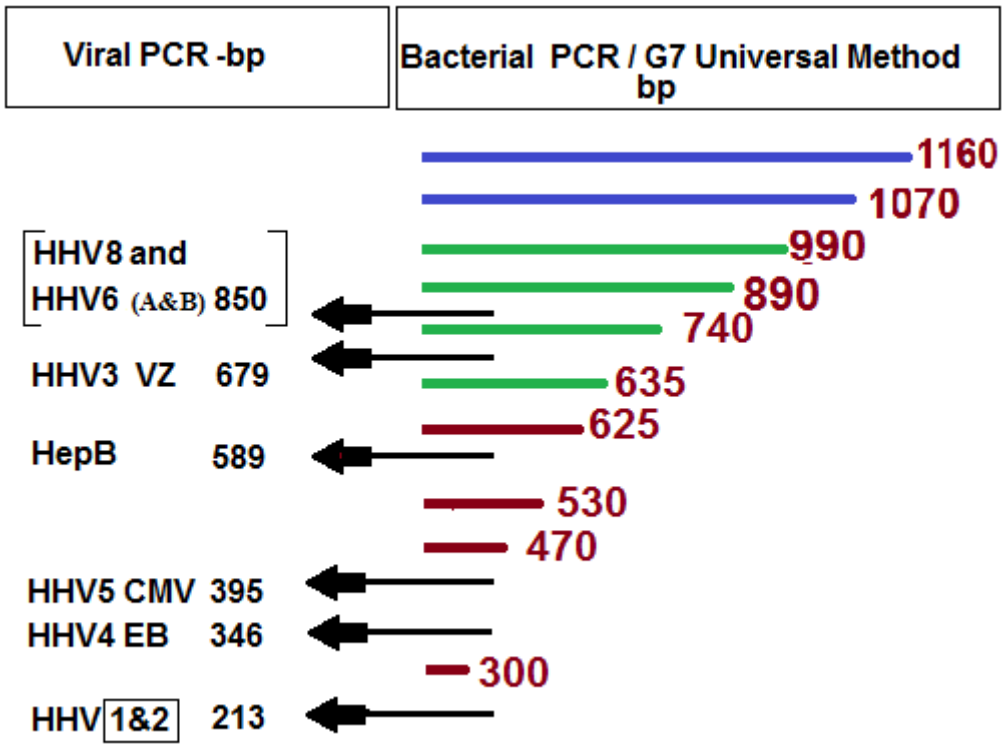

Figure 2.1: A representation of predicted PCR amplicons that may be produced by the single multiplex. The predicted sizes are shown.

2.3 Sample preparation:

Dry skin from viral lesions was weighed (~ 30mg) and soaked for 10min at 36˚C in 190µl sterile pure water in a sterile microfuge tube.

The skin lesion was disrupted and squashed using the plastic pipette tip and vigorous vortexing. A turbid milky suspension was obtained, centrifuged for one minute at 5,000 rpm and the supernatant was used in DNA extraction as detailed below.

2.4 DNA Extraction from pure stock sample

Stock sample was collected that contained human herpes viruses; HHV-1 was obtained from cold sore volunteer patients showing Herpes oral-lesions, Human herpes virus-3 (VZV) was obtained from several children recovering from chicken pox; mature dried lesions were collected. Human herpes virus-4, 6 and 8 (EBV) DNA were a gift from Dr. Rania Abu Sir, human herpes virus-5 (CMV) was a gift from Dr. Maysa Al-Azza and was obtained as a tissue-culture supernatant.

DNA extraction from bacterial or viral suspension was optimized; a new method was invented for the purpose of satisfying the conditional and simultaneous extraction of bacterial and viral DNA. The new extraction method took into consideration possible concentration of both bacteria and viruses. It was based on ammonium sulfate precipitation of viruses followed by centrifugation to ensure the concentration of bacteria and the precipitated viruses. The method is detailed as follow:

To 100µl of viral sample as CSF, or water suspension placed in a labeled sterile screw- cap microfuge tube, 190µl of saturated ammonium sulfate (Sigma Chemical Company) containing (0.1% 2-mercapto ethanol) were added. After vigorous mixing, and standing for 5 min at ambient room temperature, it was centrifuged (MIKRO 12-24) for two minutes at 13,000 rpm. The pellet (visible or invisible) was dissolved in 25µl of 1% sodium dodecyl sulfate (SDS) then diluted with175µl pure sterile water, and steamed over a boiling water bath for 15 minutes, centrifuged to remove insoluble components, the clear supernatant was collected, used in PCR and stored at -20ºC.

2.5 Multiplex Primer preparation.

A mixture of (10 pmol) of both forward and reverse primer pairs of each target virus (Table 2.1) was prepared. . The Multiplex was prepared by mixing all primer pairs (10 pm each) in one mixture; which contained primers for human herpes virus1 (HHV-1 / 2), human herpes virus-3 (VZV), human herpes virus-4 (EBV), human herpes virus-5 (CMV), and human herpes virus-6 / 8 (HHV-6&8) The viral multiplex was labeled as IM primers.

Bacterial DNA was detected using G7 multiplex of the Universal method which was provided by Dr Barghouthi.

The single-tube multiplex was prepared by mixing IM and G7 (1:1) ratio which produced a solution containg 5 pmol of each viral and bacterial primers (IMG7).

2.6 Specificity testing

All viruses obtained as DNA or as live viruses from confirmed cases of infection (VZV and HHV1/2) were tested with their specific primer pairs, the viral multiplex primer (IM), G7, and the G7-IM mix.

Bacterial DNA and *Escherichia coli* DNA was tested with G7 and G7-IM mixture.

2.7 PCR reaction preparation

A final PCR reaction of 25 µl contained:

12.5 µl green master mix (Promega, Madison, W1 USA), 11.5 µl water,0.8 µl primer mix, 0.2 µl sample DNA. PCR parameters were :

Hot start at 95°C for 3 minutes, followed by 33 cycles of (94°C for 90 sec, 60°C for 90sec, 72°C for 70 sec), followed by a final extension step at 72°C for 3 minutes.

2.8 Agarose gel electrophoresis.

Agarose gel buffer, was prepared(250 ml of Tris-acetate EDTA buffer (TAE;48.4 g Tris base, l1.4 ml glacial acetic acid, and 19 ml of 0.5M EDTA; PH 8.0). The buffer was used to prepare 1.5% (w/v) agarose gel and as electrophoretic buffer. Ethidium bromide was added (0.5 µg /ml), and the gel was solidification. The gel was electrophoresed for 30 min approximately at 60 volts; 0.6mAmp and visualized under UV. A digital camera with sepia filter, was used to document the gel. The size of DNA bands was determined relative to 100bp-ladder markers (New England BioLabs).

2.9 DNA Sequencing

Amplified DNA was purified using DNA purification kit (Nucleo Trap, Macherey –Nagel) according to supplier instructions. The purified product was checked on agarose gel and sent for sequencing (DNA sequencing Services, Bethlehem University). The received DNA sequence was analyzed using online BLAST.

Chapter 3

Results

Result

3.1 Assay development:

This method was developedt to increase the range of PCR identification of all bacteria and most human herpes viruses in one PCR test; G7 which contains primers that amplify different regions of the bacterial 16SrDNA gene was prepared. Five different primer pairs for human herpes virus (HHV1/2, 3, 4, 5,and 6/8) that can cause meningitis (Table 2.1). Bacterial (G7) and viral (IM mix) primers were all mixed to generate a single-tube mix (IMG7), G7 and IM mix were used separately as well. The results showed that IMG7 was capable of producing positive results when bacterial DNA or relevant viral DNA was added to the PCR reaction.

3.2 Detection of viral DNA using specific primer pairs or multiplexes

The newly established DNA extraction was useful in the detection of viral DNA with specific primer pairs or multiplexes; IM or IMG7. Figure 3.1shows the PCR product of HHV1&2 with 213bp (lane1), human herpes virus 3 (varicella zoster virus) appeared as 679 bp, human herpes virus 5 (cytomegalo virus; CMV) appeared as 395 bp. Human herpes virus 4 (Epstein Barr virus A&B) with PCR product 346 could not be detected (see discussion). IM multiplex and IMG7 were successful in detecting HHV1/2, 3, and 5 but not EB (HHV4) which was refractory to detection throughout the study even when pure DNA or clinical serum samples from mononucleosis patients (diagnosed positive via serological testing) was utilized several times while changing

the DNA extraction method or designing new primers (see discussion). HHV6/8 which was obtained later in the study, it was detectable with IM and IMG7 as well. The DNA was obtained as pure samples or as water suspension viral particles in water that was extracted as described in methods.

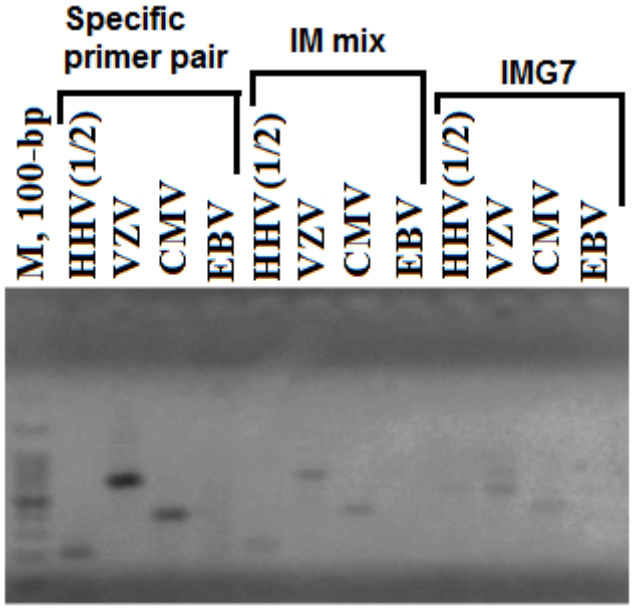

Figure 3.1: M, 100-bp marker ladder; Specific Primer pairs (lanes 1, 2 , 3 and 4); 231bp amplicon of HHV(1/2) lane1; 679 bp amplicon of VZV/HHV3 lane 2; 395 bp amplicon of CMV/ HHV5 lane 3; EBV/HHV4 lane 4 (no amplification); IM mix (lanes 5, 6, 7 and 8); IMG7 mix (lanes 9, 10,11, and 12).

3.3 Efficacy of DNA extraction from CSF and detection with Multiplexes IM and IMG7

CSF samples that produced negative results with IM and IMG7 were pooled. Viral particles and or Bacteria were then added to the CSF. The adulterated CSF was

used to extract DNA and test for the presence of bacterial and or viral DNA. The results are shown in Figure 3.2.

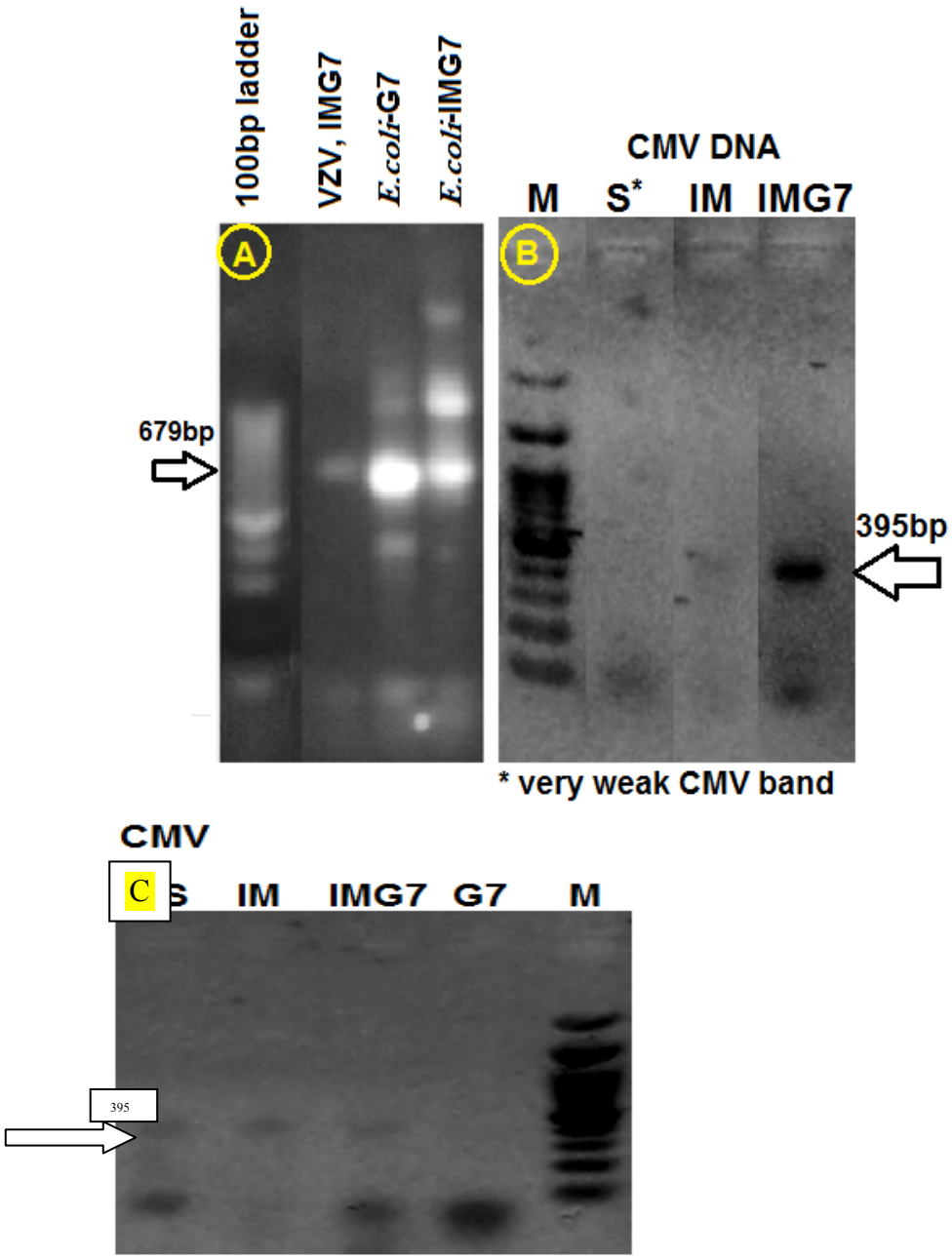

Figure 3.2: Adulterated CSF; VZV water -suspension particles (obtained from healing scales of a 3-year old girl) and/or *Escherichia coli* colonies were mixed with CSF then extracted. VZV 679 bp amplicon was detectable with IMG7. Bacterial amplicons

(usually more than one amplicon) was detected with G7 and IMG7 (**panel A**). CMV particles were used to adulterate CSF, the DNA was extracted and was subjected to detection with CMV specific primer pair (S*), IM multiplex, or IMG7 (**panel B,C**).

3.4 Sensitivity of PCR detection of Varicella zoster virus in CSF :

Varicella zoster virus (section 2.3) was diluted by 10-folds serial dilutions in CSF (pooled from negative samples). After mixing, the DNA was extracted from each dilution (**10-10^5**). The extracted DNA was then tested with IM mix. The results are depicted in Figure 3.3. The virus was detectable after 10^3 folds dilutions but was not detected at higher dilutions.

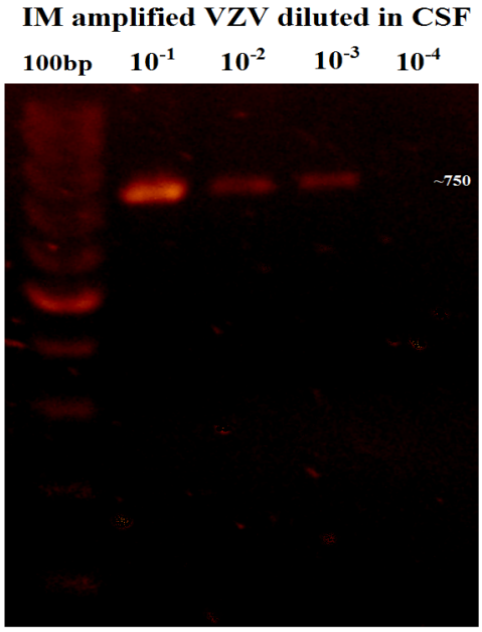

Figure 3.3: Ten-fold serial dilutions of VZV in CSF, a band at 750 bp appeared that is longer than the theoretically predicted 679 bp was clearly detected (lanes 10^{-1}, 10^{-2}, 10^{-3} , but not at 10^{-4}, and 10^{-5} .

3.5 CSF Clinical Samples

Clinical CSF samples (230 samples) were collected, kept frozen or were utilized as fresh samples for DNA extraction and PCR analysis. To ensure the reliability of results, each sample was tested (screened) separately with both IM and G7 mix; any positive sample was retested using the IMG7 mix. Only one sample showed a positive reaction with IM mix, it was suspected for being a CMV meningitis case. The sample was retested with CMV specific primer pair (Figure 3.4). Which gave a positive result, that result was viral meningitis, the PCR product gave positive on 395 kd by used multiplex primers (IM primers) and specific CMV primer.

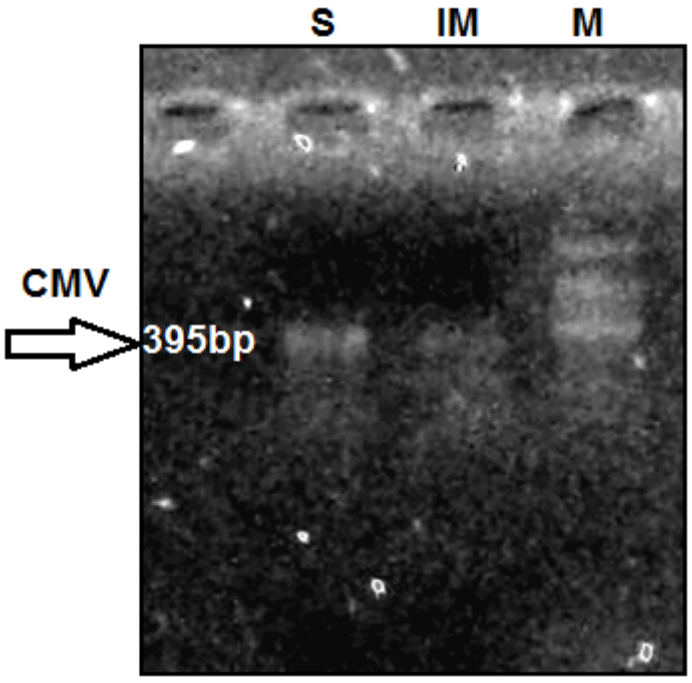

Figure 3.4 . Clinical sample showing positive results with CMV specific primer pair (S) and with the viral multiplex IM mix, the remaining samples (229 were negative with IM) and all 230 samples were negative for bacteria as judged by G7.

The DNA sample was re-subjected to amplification with CMV primer pair, the amplicon was purified using Nucleo Trap binding matrix. The purified DNA was checked on agarose before it was sent for sequencing (Bethlehem University DNA sequencing services). The obtained sequences (Figure 3.5F & R) were aligned against available Nucleotide Data Base using online alignment BLAST.

5'.AGCCCTCATGACCGTGGTGACGCTGTATTTGAGAGCCGTGGAAGTCGTAGGCATCCTG AAGGGATTGTAAGCCAGGTGAGGATTCTTGAGGGCCCACGCGCGTTCGCGCGGCCAGTT GGCGGGGTTCATATCCCCGGGCAACGGCGCCGTCGGAGCCCAGGGCGAGTTACCGTTGA CCGGGGTTTGGGTACCCGCGAAGGTAGGTGTCGGGGCCGGAGCGGGGGCCGTGGAAGG ATTGACAGGCGTCGGCGTGAGGATGGCAGCGCCGGCGCCAGCAGGAACGTTAACTCCGG CGCCGAACGTCAACGTCGGTTGCTCGAACTTGTACGCGGTGGTGACGGGCGGTTTGGCG CTCGTCTCGGTATCCGTGATGTCCACCACCCCTCGGGG3'

gb|GU179289.1| **Human herpesvirus 5 strain VR1814**,

complete genome Length=235233, Score = 651 bits (352),

Expect = 0.0; Identities = 359/362 (99%), Gaps = 2/362(1%),Strand=Plus/Plus

```
Query  19     ACGCTGTATTTGAG-AGCCGTGGAAGTCGTAGGCATCCTGAAGGGATTGTAAGCCAGGTG  78
              ||| ||| |||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  41189  ACGGTGT-TTTGAGAAGCCGTGGAAGTCGTAGGCATCCTGAAGGGATTGTAAGCCAGGTG  41247

Query  79     AGGATTCTTGAGGGCCCACGCGCGTTCGCGCGGCCAGTTGGCGGGGTTCATATCCCCGGG  138
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  41248  AGGATTCTTGAGGGCCCACGCGCGTTCGCGCGGCCAGTTGGCGGGGTTCATATCCCCGGG  41307

Query  139    CAACGGCGCCGTCGGAGCCCAGGGCGAGTTACCGTTGACCGGGGTTTGGGTACCCGCGAA  198
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  41308  CAACGGCGCCGTCGGAGCCCAGGGCGAGTTACCGTTGACCGGGGTTTGGGTACCCGCGAA  41367

Query  199    GGTAGGTGTCGGGGCCGGAGCGGGGGCCGTGGAAGGATTGACAGGCGTCGGCGTGAGGAT  258
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  41368  GGTAGGTGTCGGGGCCGGAGCGGGGGCCGTGGAAGGATTGACAGGCGTCGGCGTGAGGAT  41427

Query  259    GGCAGCGCCGGCGCCAGCAGGAACGTTAACTCCGGCGCCGAACGTCAACGTCGGTTGCTC  318
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  41428  GGCAGCGCCGGCGCCAGCAGGAACGTTAACTCCGGCGCCGAACGTCAACGTCGGTTGCTC  41487

Query  319    GAACTTGTACGCGGTGGTGACGGGCGGTTTGGCGCTCGTCTCGGTATCCGTGATGTCCAC  378
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  41488  GAACTTGTACGCGGTGGTGACGGGCGGTTTGGCGCTCGTCTCGGTATCCGTGATGTCCAC  41547

Query  379    CA  380
              ||
Sbjct  41548  CA  41549
```

Figure 3.5F: Sequencing results with the forward primer of CMV virus applied to the Clinical sample amplicon. Showing the sequence and the BLAST results identifying Human herpesvirus 5 strain VR1814 (99.17% similarity).

5’AGGGCGCCAACCGCCCGTACACCACCGCGTACAAGTTCGAGCAACCGACGTTGACGTTCGGCGCCGGAGTTA
ACGTTCCTGCTGGCGCCGGCGCTGCCATCCTCACGCCGACGCCTGTCAATCCTTCCACGGCCCCCGCTCCGGCCC
CGACACCTACCTTCGCGGGTACCCAAACCCCGGTCAACGGTAACTCGCCCTGGGCTCCGACGGCGCCGTTGCCC
GGGGATATGAACCCCGCCAACTGGCCGCGCGAACGCGCGTGGGCCCTCAAGAATCCTCACCTGGCTTACAATCC
CTTCAGGATGCCTACGACTTCCACGGCTTCTCAAAACACCGTGTCCACCACCCCTCGGAGGCCGTCGACTCCACG
CA-3’

gb|GU179289.1| **Human herpesvirus 5 strain VR1814**, complete genome

Length=235233, Score = 669 bits (362), Expect = 0.0, Identities = 367/369 (99%), Gaps = 2/369 (1%), Strand=Plus/Minus

```
Query  4      GCGCC-AACCGCCCGTACACCACCGCGTACAAGTTCGAGCAACCGACGTTGACGTTCGGC  62
              ||||| |||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct  41522  GCGCCAAACCGCCCGT-CACCACCGCGTACAAGTTCGAGCAACCGACGTTGACGTTCGGC  41464

Query  63     GCCGGAGTTAACGTTCCTGCTGGCGCCGGCGCTGCCATCCTCACGCCGACGCCTGTCAAT  122
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  41463  GCCGGAGTTAACGTTCCTGCTGGCGCCGGCGCTGCCATCCTCACGCCGACGCCTGTCAAT  41404

Query  123    CCTTCCACGGCCCCCGCTCCGGCCCCGACACCTACCTTCGCGGGTACCCAAACCCCGGTC  182
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  41403  CCTTCCACGGCCCCCGCTCCGGCCCCGACACCTACCTTCGCGGGTACCCAAACCCCGGTC  41344

Query  183    AACGGTAACTCGCCCTGGGCTCCGACGGCGCCGTTGCCCGGGGATATGAACCCCGCCAAC  242
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  41343  AACGGTAACTCGCCCTGGGCTCCGACGGCGCCGTTGCCCGGGGATATGAACCCCGCCAAC  41284

Query  243    TGGCCGCGCGAACGCGCGTGGGCCCTCAAGAATCCTCACCTGGCTTACAATCCCTTCAGG  302
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  41283  TGGCCGCGCGAACGCGCGTGGGCCCTCAAGAATCCTCACCTGGCTTACAATCCCTTCAGG  41224

Query  303    ATGCCTACGACTTCCACGGCTTCTCAAAACACCGTGTCCACCACCCCTCGGAGGCCGTCG  362
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  41223  ATGCCTACGACTTCCACGGCTTCTCAAAACACCGTGTCCACCACCCCTCGGAGGCCGTCG  41164

Query  363    ACTCCACGC  371
              |||||||||
Sbjct  41163  ACTCCACGC  41155
```

Figure 3.5R: Sequencing results with the reverse primer of CMV virus applied to the Clinical sample amplicon. Showing the sequence and the BLAST results identifying Human herpesvirus 5 strain VR1814 (99.46% similarity).

Chapter 4

Discussion

4.1 General aspects of diagnosis:

It was possible to formulate a few multiplexes (G7 and others; Barghouthi, 2009) that allows the detection of any eubacterium based on a common gene shared by all eubacteria. However, viruses are diverse and do not have common genetic features that lends itself well to the development of a multiplex for even a group of viruses. This is mainly because viruses have small genomes, yet they are distributed among the RNA and DNA domains of nucleic acids.

In order to have a comprehensive robust assay for detecting any virus, different approach must be considered. Such approach most likely will depend on microarray design rather than multiplex, a combination of molecular methods, or development of new methods. Over 150 different viruses have been implicated in the pathogenesis of encephalitis; however, due to limitations with diagnostic testing, etiologies of over half of the cases remain unknown. Deficient in traditional laboratory techniques with regard to the diagnosis of viral CNS infection have meant that in many patients, a clinical diagnosis of viral meningitis is made without supportive laboratory evidence of viral etiology.

Laboratory diagnosis of viral infections of the CNS relied on virus isolation in cell culture, and detection of specific antibody production in CSF. The impact of laboratory diagnosis on acute patient management was relatively small because of the time taken for virus replication to produce a characteristic cytopathic effect in cell culture or for development of a specific antibody response.

PCR can be applied to the diagnosis of any disease in which nucleic acid DNA and RNA or their expression as mRNA plays a role. PCR techniques allow for the in vitro synthesis of millions of copies of specific gene segment, and allow the rapid detection of as few as 1 to 10 copies of target DNA from the original sample. One of the most successful application of CSF PCR is the diagnosis of viral nervous system infections. PCR is ideally suited for identifying fastidious organisms that may be difficult or impossible to culture, CSF PCR is previously the gold standard for diagnosis of many CNS herpes virus infections (17, 37). HHV-6 has been associated with a wide spectrum of neurological disorders including febrile seizure epilepsy, multiple sclerosis, and encephalitis (39).

Many HHV including HHV1/ 2 , CMV, and HHV 6 possess the characteristic property of becoming latent in the host following primary infection (39).

Enteroviruses may cause up to 90% of aseptic meningitis cases for which a specific etiology is identified, Enteroviral meningitis leads to a large number of hospitalizations of both children and adults (40).

4.2 Human Herpes Viruses

In this study, only the Human Herpes Viruses associated with Meningitis were targeted for detection (HHV1-6 &8). The developed multiplex IM was able to experimentally detect all targeted viruses except HHV4 (Epstein Barr Virus) for unknown reasons. Additional primers listed in table (2.2) were tested with old and new DNA samples as well as with sera from mononucleosis patients, yet the results were indifferently negative. Similar negative PCR results were seen with hepatitis B sera

when repeatedly tested with Hepatitis B primer pairs directed to the large S gene and two additional nested pairs in a nested DNA reaction (Barghouthi, 2012, unpublished data).

4.3 The development of a simple new method for simultaneous DNA extraction from bacteria and viruses

Simple method such as exposure to high temperature or repetitive freeze-thawing have been utilized to release nucleic acids from viruses or bacteria. Nucleic acid extraction and purification techniques, such as deposition methods to precipitate DNA, this method provides a pure nucleic acid for entry into the amplification reaction, along with removal of potential inhibitors of the PCR amplification reaction as well as substances that may degrade nucleic acid and reduce yields. In deposition method used in this study; 2-mercaptoethanol along with ammonium sulfate to deposit whole viruses and concentrate them since they are largely made of proteins, to isolate the pure DNA, then add sodium dodecyl sulfate (SDS 1%) and then boil the sample to denature the proteins and release single strand DNA. The method was shown to be efficient, simple, reliable, time saving, and inexpensive. It can be used in screening tests similar to the IMG7 screening.

4.4 CSF sample screening with IMG7

In cases of positive samples; bacteria can be suspected when the PCR contains one, two, or multiple bands similar to those shown in Figure 2.1. Herpes viruses are suspected when a band of the sizes depicted in Figure 2.1 appears. In all cases, confirmation of the identity of amplicon source will be made by retesting the sample with specific primer pairs, IM multiplex, and G7. Sequencing of amplicon follows; this

was done with the CSF sample that revealed CMV amplicon when amplified with multiplexes or specific primer pair (Figures 3.4, 3.5F and 3.5R)

Although this study did not solve the issue of viral detection, it has brought us closer to identifying etiological agents of meningitis; it had shown a new method for DNA extraction and the possible mixing of bacterial and viral multiplexes. In addition, it showed that EBV was refractory to detection by conventional PCR and that samples better be processed immediately (bedside testing) to overcome the potential loss of viral pathogens in the sample; a result that dictate to hospital directors to move in the direction of adopting new technologies and enter the realm of applied research. The study has raised questions regarding the detection of over 150 viruses implicated in central nervous system pathology including the RNA and DNA known and unknown viruses. This study should encourage others to search for solutions for this health problem; detection of viruses in general is a health requirement in different settings including organ transplants, transfusion, cancer, surgery, immune deficiency cases, and others. It is strongly recommended that molecular diagnosis of viral pathogens and other pathogens be adopted, supported, encouraged, and funded by the State, public, and private businesses and firms.

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Appendixes:

A:TABLES

Primer Pair (F&R) for G7 .

Bacterial 16S	
F3R1	719-740
F3R2	625
F3R3	00
F4Rn1	**635**
F4Rn2	**530**
F4Rn3	**00**
F5R1	1160
F5R2	1070
F5R3	470
F6Rn1	**990**
F6Rn2	**890**

F6Rn3	**300**

PCR prodct size for each pair of primer.

Forward primer Reverse primer	QUGP-Fn3 HP/PF	QUGP-F4 HP/PF	QUGP-Fn5 HP/PF	QUGP-Fn6 HP/PF
QUGP-Rn1	721/743	633/639	1142/1183	995/991 1277 Φ
QUGP-Rn2	623/636	532/536	00/1073	00/893 1097 Φ
QUGP-Rn3	NP¶	NP	465/473	287/298

* The exact PCR product size for each primer pair may vary from one bacterium to another. Some primer sites are missing and their products are indicated as zero (00bp).

Appendex B: Figures.

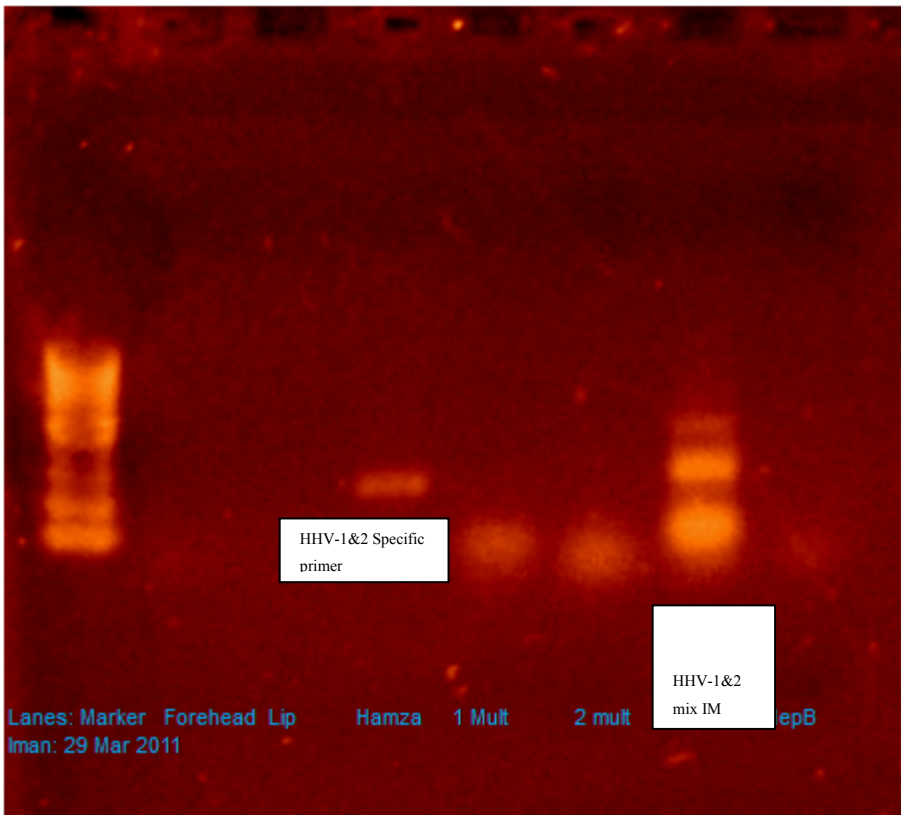


Fig (1)

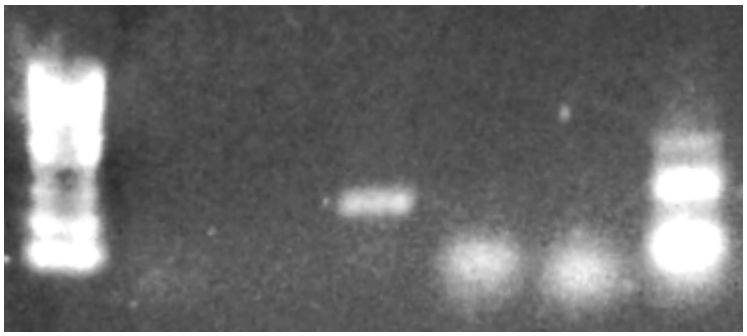


Fig (2)

1&2 the same figure, HHV(1&2), first band with specific primer , the second with mix IM primer

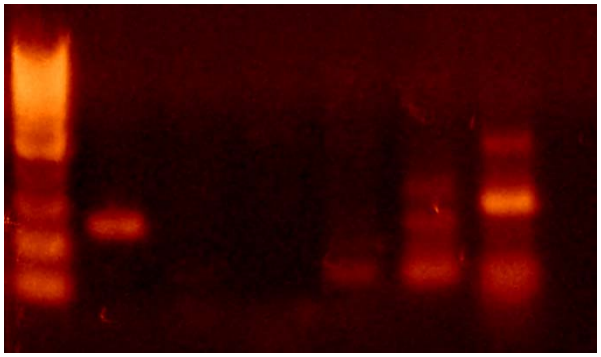


Fig (3) Clinical cold sore (lane 1, 213bp HHV1/2), lane 5(single primer) and 6 (IM), 395bp CMV.

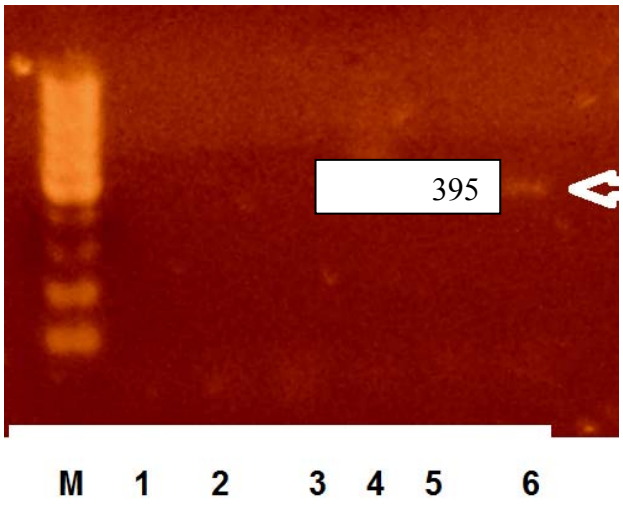
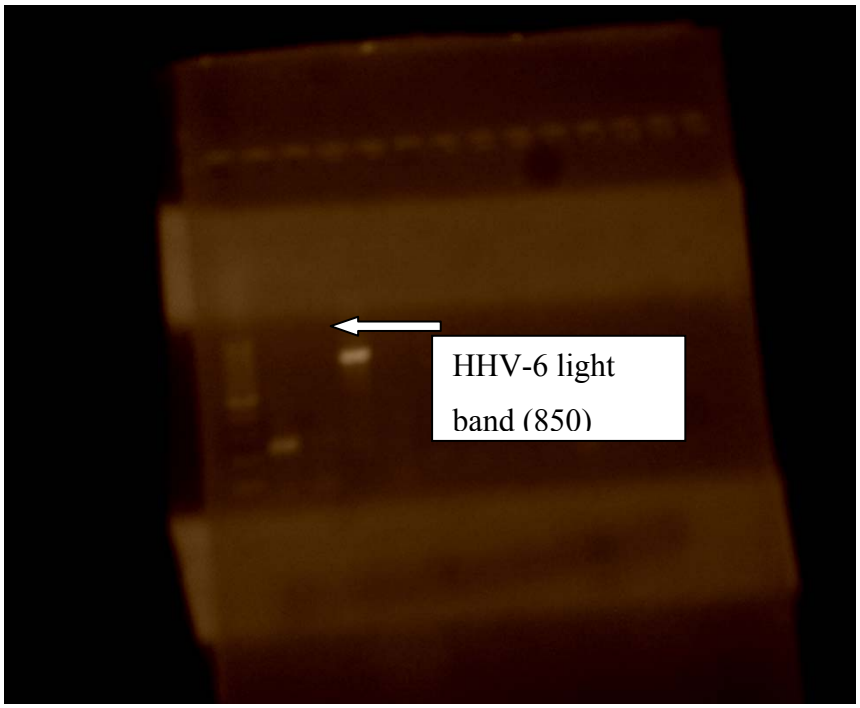


Fig (4) 395 CMV virus.


HHV-6 light
band (850)

إستخدام متعددة PCR الكشف عن التهاب السحايا البكتيرية والفيروسية في السائل المخي الشوكي.

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إشراف : الدكتور سمير البرغوثي

الملخص

التهاب السحايا أو **الحمة الشوكية**) باللاتينية(MENINGITIS :، هو التهاب الأغشية الدماغية (السحايا) المغلفة للدماغ والحبل الشوكي .على الرغم من أن غالبية أسباب الإصابة تكون عدوية (جرثومية، فيروسية، فطرية وطفيلية)، إلا أن هناك عوامل كيميائية وخلايا ورمية قد تسبب ذلك أيضاً.

ولقد ابتكر طريقة استخراج الحمض النووي التي كانت مناسبة لالتهاب السحايا البكتيرية والفيروسية على حد سواء, وقد صمم للسماح الأسلوب تركيز الجسيمات الفيروسية عن طريق الترسيب كبريتات الأمونيوم، لمسببات الأمراض البكتيرية، والعلاج في وقت واحد من العينة مع المركابتويثانول-2 لمنع النشاط الأنزيمي وتعطيل مثبطات PCR. ومن ثم إذابه للراسب وبعدها غلي. تم اختبار العينات البكتيرية المعدة للكشف أو الهربس DNA. وكان الكشف عن عينات ناجحة عندما كانت جديدة، أو عندما تم الحفاظ عليها بشكل صحيح DNA. العينات القديمة كانت تعطي نتائج سلبيه وهذا يشير إلى طبيعة الحمض النووي الغير مستقره اذا كانت العينة قديمة.

واستند التضخيم PCR من DNA البكتيرية على الدراسات السابقة التي كتبها البرغوثي (2009) التي استخدم فيها أسلوب متعدد من باديات عالمية البكتيرية (G7) مباشرة. تم تصميم متعدد الباديات الجديدة (IM) تستهدف الإنسان فيروسات الهربس 1-6 و 8 في هذه الدراسة. كان IM قادرة على اكتشاف الحمض النووي عندما HHV المستخرجة من المياه أو التخفيفات العديد من CSF المغشوشة (HHV 3/VZV). كان EBV فيروس ابشتاين بار لا يستجيب للكشف حتى عندما تم إدخال اثنين من أزواج التمهيدي إضافية، EBVلا يمكن الكشف عنه من DNA والأمصال من المرضى الذين يعانون من عدد كريات الدم البيضاء.

وكان الكشف في وقت واحد من ممكن الفيروسات البكتيرية وVZV أو CMV جنبا إلى جنب مع متعدد الباديات G7IM متعدد في فحص واحد (IMG7). وكانت النتائج واعدة منذ مجتمعة IMG7 متعددة بشكل فعال والكشف عن VZV أو DNA المستخرج من CMV CSF. كان IMG7 أيضا كفاءة في الكشف عن الإشريكية القولونية (Escherichia coli) من CSF المغشوشة. وكانت النتائج مشجعة باستثناء EBV.

230 عينات التهاب السحايا المحتملة من المستشفيات في مدن مختلفة في الضفة الغربية، تم جمع واختبارها. تم الإبلاغ عن ثلاث عينات من المستشفيات محتملة التهاب السحايا الفيروسي. عينة واحدة تم التأكد منها في هذه الدراسه وكانت تحوي CMV، والعينتين الأخريات من الممكن أن تحتوي على الفيروسات الأخرى من -1 HHV 3، 5،6 أو 8. وأكد العينة إيجابية لاحتواء CMV كما يتضح من تسلسل الحمض النووي وتحديد النتائج BLAST لAMPLICON PCR.

على الرغم من أن هذه الدراسة لم تحل مسألة الكشف الفيروسية، و ذلك يجعلنا أقرب إلى تحديد العوامل المسببة لالتهاب السحايا، بل قد أظهرت طريقة جديدة لاستخراج الحمض النووي واختلاط ممكن من متعدد الباديات البكتيرية والفيروسية. والتي تتم معالجتها على نحو أفضل العينات فورا (اختبار السرير) للتغلب على الخسائر المحتملة من مسببات الأمراض الفيروسية في العينة, وهي النتيجة التي تملي على مديري المستشفيات للتحرك في الاتجاه اعتماد تكنولوجيات جديدة وأدخل عالم الأبحاث التطبيقية. وأثارت الدراسة تساؤلات بشأن الكشف عن أكثر من 150 من تسبب التهاب السحايا بما في ذلك علم الأمراض RNA والحمض النووي المعروف وفيروسات غير معروفة.

هذه الدراسة يجب أن تشجع الآخرين على البحث عن حلول لهذه المشكلة الصحية؛ الكشف عن الفيروسات بشكل عام هو شرط الصحة في البيئات المختلفة بما في ذلك عمليات زرع الأعضاء، ونقلها، والسرطان، والجراحة، وحالات نقص المناعة، وغيرها.