Deanship of Graduate Studies
Al-Quds University

Detection of Bacteria in Cerebrospinal Fluid Using The Universal Method Based on 16S rDNA

Dima Karam Abdelmunem Al Zughayyar

M.Sc. Thesis in Medical Laboratory Sciences
Diagnostic Microbiology and Immunology Track

Jerusalem – Palestine

1431 Islamic / 2010 AD

Detection of Bacteria in Cerebrospinal Fluid Using The Universal Method Based on 16S rDNA

Prepared by:

Dima Karam Abdelmunem Al Zughayyar

B.Sc: Medical Laboratory Sciences

Al-Najah National University-Nablus

Supervisor: Dr. Sameer Barghouthi

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

1431 Islamic /2010 AD

Al-Quds University
Deanship of Graduate Studies
Diagnostic Microbiology and Immunology track/Faculty of Health Professions

Thesis Approval

Detection of Bacteria in Cerebrospinal Fluid Using The Universal Method Based on 16S rDNA

Prepared by: Dima Karam Al Zughayyar
Registration No.: 20724034

Supervisor: Dr. Sameer Barghouthi

Master thesis submitted and accepted, Date:18/12/2010

The names and signatures of the examining committee members are as follows:

1-Head of committee:	Signature
Dr. Sameer Barghouthi	
2-Internal Examiner:	Signature
Dr. Ibrahim Abbasi	
3-External Examiner:	Signature
Dr. Kamel Adwan	

Jerusalem-Palestine

1431 Islamic
2010 AD

Dedication

Dedicated to…

Our prophet Mohammad peace be upon him

My husband Eng. Alaa Al zughayyar

My parents Dr. Karam and Sawsan Naserideen

My children, Omar, Amal, Mustafa

And to all the people who helped me to overcome all difficulties.

Declaration

I certify that this thesis submitted for the degree of Master of 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……..

Dima Karam Abdelmunem Al zughayyar

Date : 18-12-2010

Acknowledgment

"والعصر. ان الانسان لفي خسر . الا الذين امنوا وعملوا الصالحات و تواصوا بالحق وتواصوا بالصبر"

Grateful thanks for God for giving me the patience and power to complete this work. I'm deeply grateful to my supervisor, Dr. Sameer Barghouthi for his valuable guidance, continuous support, and highly experienced advices. Thanks for Al- Quds University for their extended institutional support. My sincere thanks to all the doctors who tough me in the M.Sc. program, particularly Dr. Akram kharroubi, Dr. Hatem Eideh and Dr. Mahmoud Srour for their extended support. Appreciation is extended to Al-Ahli hospital staff and Alia Hospital staff in pediatric word for helping in CSF sample collection and data gathering. Special thanks to Dr. Musa hindyeh, Dr. Ahmad Saafen and Dr. Iyad Arafeh for their valuable advices and information. Finally, my endless thanks to my husband for his never ending enthusiasm, to my family and to my husband's parents and family for their encouragement and support.

Abstract

Meningitis is an inflammatory disease of the tissue surrounding the brain and spinal cord; the lepto-meninges. Although meningitis may be caused by viral or bacterial infection, bacterial meningitis is the most serious and notable infection of the central nervous system. It can progress rapidly with high mortality. The detection and identification of a bacterial pathogen is often essential to the physician in choosing appropriate antimicrobial therapy and managing the infection. The current standard for diagnosis is a microscopic examination and culturing of CSF. The definitive identification of a bacterium responsible for meningitis depending on CSF culture should be obtained from patients presenting with clinical symptoms of meningitis is essential for a successful management of meningitis. *Neisseria meningitidis, Streptococcus pneumoniae, and Haemophilus influenzae* are fastidious organisms, and are the main causative agents of bacterial meningitis, they may not survive long transit times or variations in temperature. As laboratories are the front line for detecting bacterial meningitis, molecular based diagnosis which is rapid and accurate, should be adapted for the detection of infectious diseases.

This study represents a two-stage molecular approach for detection and identification of bacterial meningitis. The first stage depends on the application of the Universal Method for bacterial detection, the amplification of rDNA from any bacterium leading to accurate determination of bacterial meningitis within 3 hours of sampling. In case of negative results bacterial meningitis can be ruled out. The samples are simultaneously tested with multiplex containing specific forward primers together with an anchored general reverse primer. The multiplex contained four species-specific forward primers; it should detect *Haemophilus influenzae*, *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Listeria monocytogenes.* These can be identified based on PCR product size. This anchored multiplex was formulated to cover the most common bacterial agents of meningitis, in case if the multiplex failed to detect the positive cases, the Universal Method will, allowing possible identification of the pathogen as the amplified polymerase chain reaction (PCR) product is sequenced and analyzed using BLAST alignment analysis.

This work focused on the importance of early detection of bacterial meningitis using the Universal Method developed by Barghouthi 2009. The presented approach should contribute to better diagnosis of meningitis while saving time, reducing hospitalization days, and increasing both specificity and sensitivity of bacterial detection, effectively enhancing treatment of bacterial meningitis. The work presented here will increase awareness of the progression and emergence of bacterial resistance for antibiotics in hospitals. The work may also guide researchers and health professionals to adopt the most robust Universal Method over the limited multiplex procedures. An important issue that was not addressed in this work is the capacity of molecular approach to identifying markers of bacterial resistance to antibiotics. This should be the subject of future investigations.

Table of Contents

List of tables

List of Figures

List of Appendices

Chapter 1: Introduction

1.1 Meningitis

Meningitis, an inflammation of the meninges, the thin anatomical structure; three layers or "membranes" that intimately and delicately cover the brain and spinal cord (Gray, 1992). Meningitis affecting the pia, arachnoid, and subarachnoid space may be caused by bacteria or viruses (Gray, 1992; Thomas *et al*., 2002). Specifically, meningitis is an infection within the subarachnoid space, a space between the middle and innermost Layer (Gray, 1992; Kim, 2010; Taha, 2004). Bacterial or septic meningitis and viral or aseptic must be distinguished clinically and diagnostically (Tamimi *et al.,* 2008). The manifestations of viral meningitis are generally similar to those of bacterial meningitis, but less sever (Taha, 2004). Although most infections occur in infants, the societal impact is also important because of the continued high incidence in healthy older children and adolescents (Saravolatz *et al.*, 2003). In spite of many improvements in health care system, acute bacterial meningitis remains a life threatening infectious emergency (Chakrabarti *et al.,* 2009). Successful and adequate treatment requires rapid detection then identification of the bacterium (Saravolatz *et al.*, 2003). Bacterial meningitis may lead to permanent neurological sequelae such as hearing loss, mental retardation, seizures and behavioral changes may occur in up to 50% of survivors, especially when the diagnosis and antibiotic administration are delayed (Dubos *et al.*, 2008; Saravolatz *et al.*, 2003; Welinder-Olsson *et al.*, 2007). Potential long-term neurological sequelae include cranial nerve paralysis, hemi paresis, hydrocephalus, and seizures as well as visual and hearing impairment which can have a profound impact on the quality of life of the survivors (Kim, 2003; Tebruegge and Curtis, 2008).

1.2 Bacterial meningitis:

Bacterial meningitis is the most notable infection of the central nervous system, it can progress rapidly and may result in death or permanent debilitation (Gray, 1992; Failace *et al.* 2005). It is a serious disease with high morbidity and mortality all over the world (Rafi *et al*., 2010; Schuurman, *et al*. 2004; Ceyhan *et al.*, 2010; Gray, 1992; Kim, 2009; Pandit *et al*., 2005; Rafi

et al., 2010; Tzanakaki *et al*., 2005). Bacterial meningitis can be difficult to diagnose, as the symptoms and signs are often non-specific (Rafi *et al.*, 2010). In recent years, despite improvements in antimicrobial therapy and intensive care support, overall mortality rates related to bacterial meningitis were around 20% to 25% have been reported by major centers (Tebruegge, 2008). Worldwide, an estimated 171,000 deaths are reported annually (Ceyhan *et al.* 2008). Delays in initiation of antibiotic therapy can adversely affect clinical outcome (Mishal *et al.*, 2008). The majority of patients with bacterial meningitis survive, but neurological sequelae occur in as many as one-third of all survivors especially newborns and children (Gray, 1992; Ceyhan *et al,.* 2008). Because distinguishing between bacterial and aseptic meningitis in the emergency department is difficult, it is recommended that antibiotics should be started immediately in children with clinical evidence of acute meningitis cerebrospinal fluid (CSF) and continued at least until bacterial culture results become available 48 to 72 hours later (Dubos *et al.*, 2008). Moreover, the culture may lead to false-negative results when fastidious or slowly growing bacteria are involved (Chen *et al.*, 2009). Many studies have revealed the ability of both Gram-positive and Gram-negative bacteria to go into a viable but non-culturable (VBNC) state (Rivers, 2001; Y. N. Sardessai, 2005). The viable but non-culturable state is defined as one in which cells are viable yet do not undergo sufficient division to give rise to visible growth on nonselective growth medium (Rivers, 2001) .

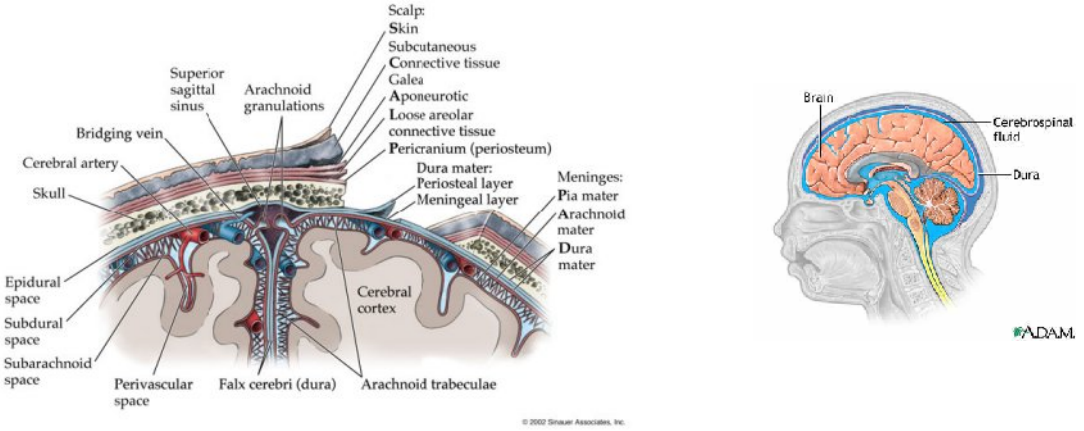

Figure1.1: ***Major anatomical features of the meninges. The meninges surrounds the brain and spinal cord and spinal cord and is composed of three distinct layers adapted from reference No. 28 and downloaded from the net.***

1.3 Etiological agents of Bacterial meningitis:

Almost all microbes that are pathogenic to human beings have the potential to cause meningitis, but a relatively small number of pathogens account for most cases of acute bacterial meningitis in neonates and children, although the reasons for this association remain incompletely understood (Kim, 2010). Determination of the etiology of bacterial meningitis and estimating cost of disease are important in guiding vaccination policies (Ceyhan, e*t al.,* 2008; Kaijalainen, *et al*., 2008). Distinguishing between bacterial and aseptic meningitis in children in the emergency department could contribute to limiting unnecessary antibiotic use and/or hospital admissions (Dubos, *et al.,* 2008). The results of national surveillance studies have shown that both the etiological agents and mortality rates (0 to 54%) of bacterial meningitis depend on the season of the year and the age, sex, ethnic background, medical conditions and geographic location of the patient (Chiba *et al.,* 2009; Gray, 1992; Rafi *et al.,* 2010). These are heavily affected by (i) the availability of vaccination against *Haemophilus influenzae type b* (Hib), *Streptococcus pneumoniae*, (ii) the availability of a medical insurance system, and (iii) the hygienic and sanitary conditions (Chiba *et al*., 2009). Worldwide, the three main pathogens *Neisseria meningitides, Haemophilus influenzae type b (Hib) and Streptococcus pneumoniae* are the main bacterial pathogens that account for 75–80% of cases after the neonatal period and cause invasive infections such as meningitis, sepsis and pneumoniae in children under 5 years of age (Kaijalainen *et al.*, 2008; Rafi *et al.*, 2010; Tebruegge, 2008; Tunkel, 1993). According to some studies, *S. pneumoniae* was the most common causative agent accounting for 89% of all culture positive episodes, followed by *H. influenzae* (6%) and *N. meningitidi*s (4%; Gray, 1992; Tebruegge, 2008; Ceyhan *et al.,* 2010).

1.3.1. *Streptococcus pneumoniae:*

Streptococcus pneumoniae is a major cause of childhood acute bacterial meningitis in countries where vaccination against *Haemophilus influenzae* type b and/or meningococcal disease has been implemented into the routine immunization schedule (Ceyhan *et al.,* 2010). Numerous conditions have been reported to be associated with an increased risk of pneumococcal meningitis, including hematologic disorders, immunodeficiencies, asplenia,

chronic renal failure, HIV infection, malnutrition, alcoholism, and head injuries (Tebruegge, 2008).

1.3.2. *Neisseria meningitidis.*

Several abnormalities of the immune system have been found to predispose affected individuals to meningitis with *N. meningitidis*, including immunoglobulin deficiencies (Tebruegge, 2008). World Health Organization (WHO) reported that 430 million people at risk of the disease in Sub-Saharan Africa's so-called African Meningitis Belt. (Unemo *et al.*, 2009).

1.3.3. *Haemophilus influenzae.*

As a result of the widespread introduction of Hib vaccines into vaccination programs in many industrialized countries, there has been a dramatic decline in invasive diseases caused by *H. influenzae* over the last two decades (Tebruegge, 2008). *H. influenzae*, like *N. meningitidis and S. pneumoniae* are fastidious organisms that may not survive long transit times or variations in temperature (Gray, 1992).

1.3.4. *Staphylococcus aureus.*

S. aureus is a rare cause of bacterial meningitis. According to the U.S. Bacterial Meningitis Surveillance Study, less than 1% of bacterial meningitis is caused by this bacterium (Tebruegge, 2008).

1.3.5. *Liesteria monocytogenes* and other rare etiological agents:

Listeria monocytogenes was reported relatively infrequently (0.2 cases per 100,000 population) but had the highest fatality rate (22%) (Abasaeed *et al.*, 2009). Other rare bacteria that have been reported to cause meningitis include Acinetobacter, *Bacteroides fragilis, Achromobacterxylosoxidans, Gordona aurantiaca (Rhodococcus aurantiacus), Lactobacillus spp., Corynebacterium aquaticum, Streptococcus mitis, Pasteurella multocida* (Gray, 1992).

In general, the number of cases of bacterial meningitis whose etiological agent is not identified is still significant (Failace *et al.*, 2005).

1.3.6. Bacterial and viral meningitis in Palestine:

A study in Palestine contains additional data from the 2002 are reported in table (1.1) that shows the distribution of etiological agents of bacterial meningitis*; H. influenzae, N. meningitidis, and* other bacteria (as mentioned in the statistical epidemiological record below). These are the leading causes of bacterial meningitis in 15 different cities in Palestine. Another available data about viral meningitis distribution in Palestine (2005-2009) found in table (1.2).The information was obtained by personal communications with the Ministry of health in Palestine.

Table 1.1 Reported cases Bacterial meningitis in Palestine (2001-2002): referring to the Primary Health Care Preventative Medicine Department in the Ministry of Health .Dr I. Arafeh

Daily notified Disease : Meningitis	2001 Cumulative Total	2002 Cumulative Total
Meningococcal meningitis	76	87
Haemophillus influenzae	9	18
Others	443	309

Table1.2: Reported cases of viral meningitis in Palestine (2005-2009): referring to the Primary Health Care Preventative Medicine Department in the Ministry of Health. Dr I. Arafeh

District	Hebron	Bethlehem	Ramallah	Jerusalem	Jericho	Nablus	Tulkarm	Salfeet	Qalqiliah	Jenin	Gaza	Total
2005	42	25	0	10	1	23	32	4	21	11	690	859
2006	18	18	2	6	0	8	7	1	13	3	848	924
2007	120	10	8	6	0	7	17	4	38	7	913	1130
2008	180	12	21	8	2	19	53	3	55	13	192	558
2009	262	11	6	3	0	5	31	9	108	5	343	783

Before 2001 Jerusalem was Include with other Districts(Beithlehem &Ramallah).
Before 2009 Tubas Was Included with Jenin District.

1.4 Diagnosis of bacterial meningitis:

The identification of a bacterial pathogen is often essential to the physician in choosing appropriate antimicrobial therapy and in managing the bacterial infection meningitis (Gray, 1992; Tebruegge, 2008). The current standard for the diagnosis of bacterial meningitis is microscopic examination and subsequent culture of cerebrospinal fluid (CSF) (Rafi *et al.*, 2010; Schuurman *et al.*, 2004). However, this approach might have some disadvantages with regard to the desired rapidity and sensitivity (Schuurman *et al.*, 2004). Results of culture may only be available after 48 to 72 hours which are mostly negative due to prior treatment with antibiotics (Schuurman *et al.*, 2004; Pandit, *et al.*, 2005).

1.4.1. Clinical findings:

Bacterial meningitis can be difficult to diagnose, as the symptoms and signs are often non-specific, especially in young children (Ceyhan *et al.*, 2008; Rafi *et al.*, 2010). Classic clinical signs, which are present in 80% of patients, include headache, fever, and cerebral dysfunction (confusion, delirium, or altered level of consciousness;(Mishal *et al.*, 2008; Saravolatz *et al.*, 2003). However, among neonates and elderly only subtle signs such as lethargy and irritability may herald the onset of meningitis (Saravolatz *et al.*, 2003). In adults, physical findings of nuchal rigidity which is defined as the inability to flex the head forward due to rigidity of the neck muscles with Kernig's and/or Brudzinski's signs are considered to be more

reliable compared to the clinical history alone, in establishing a diagnosis of bacterial meningitis (Mishal *et al.,* 2008; Thomas *et al.,* 2002). Kernig's sign which is defined as positive when the leg is bent at the hip and knee at 90 degree angles, and subsequent further extension in the knee is painful leading to resistance, Brudzinski's sign which is defined as the appearance of involuntary lifting of the legs in meningeal irritation when lifting a patient's head off the examining couch, with the patient lying supine, and nuchal rigidity are 3 bedside diagnostic signs used specifically to assess a patient's risk for meningitis (Thomas *et al.,* 2002). These clinical signs have been used as indicators of meningeal inflammation for almost a century (Mishal *et al.,* 2008; Thomas *et al.,* 2002). However, Kernig's and Brudzinski's signs alone, without nuchal rigidity, have low sensitivity and poor diagnostic accuracy and they should not be used as the sole determinants for further diagnostic testing (Mishal *et al.,* 2008). Because the consequences of delayed diagnosis of bacterial meningitis can be severe, any proposed diagnostic tool must achieve near 100% sensitivity (Dubos *et al.,* 2008).

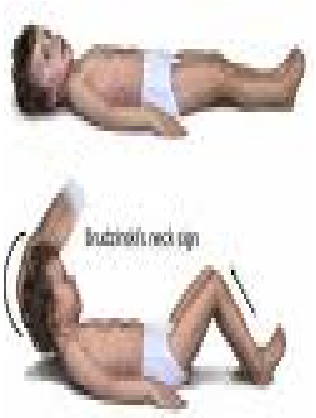

Figure1.2: Bacterial meningitis diagnosis *Brudzinski neck sign:* neck rigidity-passive flexion of both legs and thighs- Adapted from reference No. 66

Figure 1.3 : Bacterial meningitis diagnosis *Kerings sign*: patient supine, with hip flexed 90C,knee can't be fully extended

1.4.2. Laboratory findings:

Bacterial meningitis requires early diagnosis and empirical antimicrobial treatment (Chakrabarti *et al.,* 2009; Kim, 2010). CSF is widely utilized for diagnosis of diseases of the central nervous system (CNS) (Gray, 1992). CSF has important functions; including

cushioning the brain, maintaining a constant intracranial pressure, providing nutrients, and removing toxic metabolites from the (CNS) (Gray, 1992; Yamamoto, 2002). Indirect assessment of brain health status can be obtained from the CSF (Yamamoto, 2002). Since CSF is considered germfree, detection of microbes in CSF, even in low numbers, provides valuable information about possible infection (Yamamoto, 2002). Various laboratory investigations of the CSF have been developed for the rapid diagnosis of acute bacterial meningitis (Pandit *et al.,* 2005; Yamamoto, 2002). Nevertheless, none of these tests alone or in combination are dependable because of their poor sensitivity and specificity (Pandit *et al.,* 2005). Bacterial meningitis is usually suspected on the basis of the clinical presentation of the patient and the finding of purulence in CSF (Kim, 2010; Kotilainen *et al.,* 1998).

1.5 Conventional Methods for processing and culturing CSF:

1.5.1. Concentration:

The probabilities of detecting bacteria by culture and staining techniques are increased by concentrating the bacteria in a CSF specimen (Gray, 1992). The number of bacteria present in a CSF specimen from a patient with meningitis may be as few as 10^3 CFU/ml (Gray, 1992; Kim, 2010). Generally, when less than 0.5 ml of CSF is received by a microbiology laboratory, the specimen should be concentrated by centrifugation for at least 15 min at 1,500 to 2,500 x g (Murray, 1980; Gray, 1992).

1.5.2. Bacterial culture from CSF:

Currently, the diagnosis of meningitis is mostly done by CSF culture (Failace *et al.,* 2005). Culture is considered as the diagnostic test of choice although it is a time-consuming, it requires viable microorganisms for cultivation, and its sensitivity is directly affected by prior antibiotic treatment of the patient (Saravolatz *et al.,* 2003, Failace *et al.,* 2005; Ray *et al.,* 2007). Culture remains the direct way of detecting the etiological agent of CNS bacterial infections (Ray, 2009; Taha, 2004). Difficulties may result due to slow bacterial growth or due to stringent growth requirements or because of prior empirical patient treatment with

antimicrobial agents (Gray, 1992; Kim, 2010; Taha, 2004). CSF sterilization after antibiotic use occurs rapidly, with meningococci and pneumococci disappearing within 2 and 4 hours respectively (Rafi *et al.*, 2010). Furthermore, 24-48 hours are required to grow visible colonies that require further biochemical characterization (Chakrabarti *et al.,* 2009; Rafi *et al.*, 2010). In an extensive study over a 27 year period, it was reported that culture might miss the diagnosis of bacterial meningitis in at least 13% of the cases (Schuurman *et al.,* 2004)**.** The cultures may be also remaining negative if the disease is caused by fastidious and/or slow growing microorganisms (Kotilainen *et al.*, 1998; Drancourt *et al.*, 2000).

1.5.3. Rapid Methods for Detecting Bacteria in CSF (Microscopy):

Due to the fragility of some bacterial species incriminated in CNS infections, CSF should be Gram stained and examined microscopically at bed side, immediately after lumbar puncture (Gray, 1992; Taha , 2004). Because the diagnostic usefulness of staining procedures depends on the concentration of bacteria in the CSF of patients with bacterial meningitis (10 to 10^9 CFU/ml), all CSF specimens of sufficient quantity should be processed to concentrate pathogens prior to microscopic examination and should be cultured as well (Gray, 1992; Harris, 2003). However, in 30% to 40% of the bacterial meningitis cases, the Gram-stained smear shows no bacteria (Ray *et al.*, 2007). Gram stain and culture outcome is affected in many clinical situations or may show negative results due to antibiotic therapy before lumbar puncture (Saravolatz, *et al.,* 2003; Rafi, *et al.,* 2010). Unfortunately, the yield of micro-organism on Gram stain depends on factors like the number of organisms present, prior use of antibiotics, technique of preparing slide and observer's skill (Das *et al.*, 2003). Although CSF Gram stain is quick, it is highly non-specific and often has a low sensitivity (Rafi *et al.*, 2010). Bacterial concentration in the CSF has a profound effect on the results of microscopy (Kim, 2010; Schuurman *et al.,* 2004). Regardless of the type of organism in the CSF, the percentage of positive microscopic results is only 25% with 10^3 CFU/ml and 60% in the range of 10^3 to 10^5 CFU/ml (Schuurman, *et al.*, 2004).

Examination of the CSF (biochemical finding and polymorph nuclear count) may be helpful to differentiate between acute viral and acute bacterial meningitis (Taha, 2004).

1.5.4. Serological tests for CSF analysis

Various other laboratory investigations of CSF have been developed for the rapid and specific diagnosis of acute bacterial meningitis, mainly through detection of bacterial antigen by immunological methods (Gray, 1992; Rafi *et al.,* 2010). These tests alone, or in combination, are limited by low sensitivities and specificities (Rafi *et al.,* 2010). Latex Agglutination Test (LAT) for detection of capsular antigens is a rapid test which is unlikely to be affected by prior antibiotic therapy (Saravolatz *et al.,* 2003; Rafi *et al.,* 2010). Enzyme Immunoassays_(EIAs) have been used also for the detection of bacterial antigens and bacteria components in CSF (Gray, 1992). EIAs have been evaluated for their abilities to detect *H. influenzae* type b, and *N. meningitidis* antigen in CSF (Gray, 1990). The sensitivities and specificities of these tests have been reported to be (84-100) % and (89-100) % respectively (Gray, 1990).

1.6 Molecular identification of Bacterial Meningitis:

Due to the limitations of other techniques (Gram, Cultural, specific-multiplex PCR), a requirement for a robust detection method emerges (Corless *et al.,* 2000). Recently, polymerase chain reaction (PCR) based assays have been considered as accurate diagnostic tools (Chen *et al.,* 2009; Cherian *et al.,* 1998; Corless *et al.,* 2000; Hall *et al.,* 1995; Kotilainen *et al.,* 1998; Lorino *et al.,* 1999; Lu *et al.,* 2000; N. Margall, 2002; Reier-Nilsen *et al.,* 2009; Schuurman *et al.,* 2004). PCR-based assays are the most sensitive of the existing rapid methods to detect microbial pathogens in clinical specimens (Boisier *et al.,* 2009; Chen *et al.,* 1989; Chen *et al.,* 2009; Pandit *et al.,* 2005). In recent years, PCR techniques have been increasingly used to amplify and detect microbial DNA in cerebrospinal fluid for the diagnosis of bacterial meningitis (Chen *et al.,* 2009; Pandit *et al.,* 2005). In particular, when specific pathogens that are difficult to culture in vitro or requires a long cultivation period are expected to be present in specimens, the diagnostic value of PCR is known to be significant (Yamamoto, 2002; Tuyama *et al.,* 2008 ; Kim, 2010; Barghouthi, 2009). With the introduction of molecular techniques, live organisms are not required for detection or identification (Rafi *et*

al., 2010). PCR methods are able to detect small amounts of pathogen including dead ones (Sauer *et al.,* 2005). Recent studies suggest that rapid detection systems can decrease the costs associated with hospitalization and refine application of antibiotic treatment (Peterson, 2004; Sauer *et al.,* 2005). The administration of antibiotics to patients with suspected meningitis before hospital admission and the collection of a cerebrospinal fluid (CSF) sample have become common practice (Boving *et al.,* 2009). This practice may correspondingly impair microbiological diagnosis by culture of the bacteria responsible for the infection (Boving *et al.,* 2009). PCR has the potential for rapid diagnosis of bacterial meningitis while overcoming the poor sensitivity of culture when antibiotic had been already introduced (Chakrabarti *et al.,* 2009) In bacterial meningitis, there is a good concordance between culture-positive and PCR-positive patients (Pada *et al.,* 2009).

1.7 Strategies used for PCR

There are different strategies to PCR amplification of bacterial DNA in clinical samples (Sauer *et al.,* 2005).

1.7.1. Specific Strategies

PCR-Based assays have become available to provide an early and accurate diagnosis of bacterial meningitis; some of these assays are aimed at specific pathogens of bacterial meningitis (Schuurman *et al.,* 2004). This approach based on usage of species-specific primers (Boving *et al.,* 2009; Kim, 2010; Lu *et al.,* 2000; Sauer *et al.,* 2005). Unlike culture, most molecular assays are designed specifically for specific number of organisms (Boving *et al.,* 2009; Elnifro *et al.,* 2000; Harris, 2003; Henegariu *et al.,* 1997; Taha, 2004). This provides high sensitivity and specificity but detects only what you are looking for (Harris, 2003). This method results in one of two possible outcomes; this method does not indicate the presence or absence of other non-target bacterium. Therefore, it lacks the capacity to detect non-target bacteria (Barghouthi, 2009; Lu *et al.,* 2000; McCabe *et al.,* 1999).

1.7.2. Multiplex approach:

Similar to specific primer pairs, only specifically targets a number of pathogens (Boving, *et al.,* 2009). Multiplex is a mixture of primer pairs, each will specifically detect a single pathogen (Henegariu *et al.,* 1997). Similarly, multiplex will not detect any non-target bacterium. The use of standard multiplex PCR has been shown to be useful in identification of infecting pathogens in patients who have previously received antibiotics or in resource-poor settings (Kim, 2010).

1.7.3. Universal Primer strategies:

The other approach uses broad-range bacterial PCR primers (Schuurman *et al.*, 2004; Barghouthi 2009; Boving, *et al*., 2009). PCR has been applied for the identification of *Neisseria meningitidis* (Kristiansen *et al.,* 1991; Ni H. *et al.,* 1992; Seward, 2000), and for simultaneous detection of *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae* as etiological agents of bacterial meningitis (Clarke, 2006; Diggle, 2006; Drakopoulou *et al.,* 2008; Radstrom *et al.,* 1994). However few reports have been published evaluating the use of PCR amplification with universal primers on cerebrospinal fluid samples taken from patients with meningitis (Greisen, *et al.,* 1994; Radstrom, *et al.,* 1994; Hall, *et al.,* 1995; Lu J.J *et al.,* 2000; N. Margall, 2002). This approach involves amplification of sequences found in all bacteria based on universal primers that target sequences common to many bacteria (Barghouthi, 2009; Lu J.J *et al.,* 2000; N. Margall *et al.,* 2002; Sauer *et al.,* 2005; Sontakke *et al.,* 2009). The universal primer approach is a misleading hypothetical concept where there is no one primer pair is capable of detecting all bacteria (Barghouthi 2009). Although the use of universal primes can detect many bacteria and may detect bacteria that are found less frequently (Barghouthi, 2009). Broad-range assays, based on ribosomal genes (rDNA), are designed to overcome species-specific primer limitations (Harris, 2003). Bacterial (rDNA) contains conserved nucleotide sequences that are shared by several bacterial species, interspersed with variable regions that are species-specific (Harris, 2003). 16S (rDNA) gene is widely used for taxonomic purposes (Clarridge, 2004). It is possible to design PCR primers capable of amplifying all eubacteria based on the conservative nature of the 16S ribosomal DNA (Barghouthi, 2009; Greisen *et al.,* 1994; Lu *et al.,* 2000;

Radstrom, *et al.,* 1994; Welinder-Olsson *et al.,* 2007). The Universal Method was designed to detect any bacterium (Barghouthi, 2009).

1.7.4. The Universal Method:

Most recently, a robust method developed by Barghouthi 2009, takes advantage of the multiplex approach, probability, and conservative sequences of the 16S rDNA. A multiplex known as G7 (previously primers mixture prepared by S.Barghouthi) has been tested and is believed to detect any bacterium. The Universal Method offers a number of multiplex mixtures that should yield positive PCR amplification with 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 (Barghouthi, 2009).

PCR can be used as a tool for the rapid detection of bacteria in normal sterile clinical samples and, as such, would be useful in differentiating bacterial from viral infections (Barghouthi, 2009; Corless *et al.,* 2000). This would confirm the necessity for antibiotic treatment and would influence patient management (Corless et al. 2000). Several workers (Barghouthi, 2009; Corless *et al.,* 2000; Harris, 2003; Sauer *et al.,* 2005) have used the 16S rDNA gene as a target for bacterial detection. Strategic application of broad range primers has been found to be superior to conventional techniques for detection of bacteria in sterile body fluids (Chakrabarti *et al.,* 2009). The use of PCR for rapid diagnosis of bacterial meningitis has the potential to overcome the poor sensitivity of culture when antibiotic had been already introduced (Chakrabarti *et al.,* 2009). However, the relative risk of contamination remains the major problem of using general and highly sensitive PCR alone as a specific test to confirm a diagnosis (Chakrabarti *et al.,* 2009). The specificity of broad range universal PCR has been satisfactorily improved by further analysis of the PCR products by different methods (Chakrabarti *et al.,* 2009). The assay is valuable for initial screening to confirm or rule out bacterial meningitis (Barghouthi, 2009; McCabe *et al.,* 1999).

1.7.5. Sequencing of 16S ribosomal DNA:

Since the development discovery DNA sequencing and PCR comparison of DNA sequences loci of bacterial species shown that the 16S rDNA gene is highly suitable for phylogeny of bacterial genera and species (Janda, 2007; Woo *et al.,* 2003). For the previous reason it can be used as the new "gold standard" for identification of bacteria to the species or genus level (Janda and Abbott, 2007; Woo P.C. *et al.,* 2003). Because 16S rDNA gene sequence analysis can discriminate far more finely among bacterial species than is possible with phenotypic methods, it can allow a more precise identification of poorly described, rarely isolated, or phenotypically aberrant strains (Barghouthi, 2009; Clarridge, 2004; Drancourt *et al.,* 2000; Goldenberger *et al.,* 1997). Unlike DNA which is highly stable, phenotypic features of a bacterium fluctuate in response to environmental factors and time of assay (Barghouthi, 2009).

1.7.6. Limitations of molecular methods in the Diagnosis and Characterization of bacteria:

The widespread use of molecular diagnostic tools may be hindered by cost and logistics, particularly in developing countries (Taha, 2004) which is becoming a minor problem in Palestine (Barghouthi, 2009). In universal strategies the risk of finding contamination is high (Harris, 2003; Taha, 2004; Welinder-Olsson *et al.,* 2007). There for using diluted samples may be advantageous since it keeps contaminants below detection limits (Barghouthi, 2009). The main weakness of specific strategies is that investigators can find only what they are looking for (Henegariu, *et al.,* 1997; Boving, *et al.,* 2009; Barghouthi 2009). while missing non-target bacteria (Barghouthi, 2009).

1.8 Treatment of bacterial meningitis:

Accurate diagnosis of bacterial infection leads to appropriate patient management, providing information on prognosis and allowing the use of narrow-spectrum antibiotics (Gray, 1992; Harris, 2003; Kim 2010). Eradication of the infecting organism from the CSF is entirely dependent on antibiotics; bactericidal antibiotics should be administered intravenously at the highest clinically validated doses to patients with suspected bacterial meningitis (Kim, 2010). The antimicrobial agents played a pivotal role in the management and control of bacterial

meningitis (Bergeron, 1998; Kim, 2003; Peterson, 2004). Usefulness of diagnostic tests would be determine whether empirical antimicrobial therapy should or should not be administered and thus potentially eliminate unnecessary administration of antimicrobial therapy to some patients, reduces side-effects for the patient, saves money and may slow the spread of antibiotic resistance (Berzanskyte *et al.,* 2006; Harris, 2003; Saravolatz *et al.,* 2003). The common practice of antibiotic therapy prior to cerebrospinal fluid (CSF) evaluation coupled with inconsistent laboratory support in developing countries hampers diagnosis (Pandit *et al.,* 2005). Because distinguishing between bacterial and viral meningitis in the emergency clinics is difficult, it is recommended that antibiotics be started immediately in children with clinical evidence of acute meningitis and/or cerebrospinal fluid (CSF) pleocytosis and continued at least until bacterial diagnosis is made available (Dubos *et al.,* 2008). These recommendation of empirical and early administration of antibiotics correlates with reduced rate of morbidity and mortality, it is of crucial importance to initiate relevant and targeted prescribing treatment as soon as possible (Levy, 2005; Dubos *et al.,* 2008; Chakrabarti *et al.,* 2009; Sontakke *et al*., 2009). This urgency justifies the administration of broad-spectrum antibiotics to patients with suspected meningitis before the collection of CSF or laboratory results become available (Boving *et al.,* 2009; Failace *et al.,* 2005; Gray, 1992; Lu *et al.,* 2000).

Optimizing diagnostic testing could differentiate true infection with pathogenic microbes which are amenable to specific antimicrobial agent treatment and a clinical symptom complex that does not require antimicrobial agent therapy (Grigoryan *et al.,* 2007; Peterson, 2004). It was found that patients were treated with broad-spectrum systemic antibiotics for suspected bacterial meningitis bacterial infection, however, in only a small subset of treated cases leads to the pathogen identification (Reier-Nilsen *et al.,* 2009). A report from Spain had found that while only 22% of patients seeking medical care were clinically diagnosed as having an infection in general, 67% were actually given antibiotics as a result of clinic visit (Peterson, 2004). The usefulness of any diagnostic test would be to determine whether empirical antimicrobial therapy should or should not be administered and thus potentially eliminate unnecessary administration of antimicrobial therapy to some patients (Saravolatz *et al.,* 2003). Perhaps the first molecular test that was successful in reducing antibiotic use because a specific diagnosis could be rapidly made was the application of PCR to the detection of enteroviral meningoencephalitis (Peterson, 2004; Saravolatz *et al.,* 2003). Since these

technologies do not require organism viability, and thus avoid any adverse effect of longer specimen transport, they can be successfully applied to both the in- and outpatient settings (Peterson, 2004).

Also, the resulting test rapidity theoretically will provide relevant information within a few hours, which would limit any necessary empirical treatment to one or two doses (Peterson, 2004). Precise diagnosis can be the vital element in truly improving anti-infective prescribing in cases of bacterial meningitis (Abasaeed *et al.,* 2009; Boving *et al.,* 2009; Grigoryan *et al.,* 2008; Kim, 2009; Lu J.J *et al.,* 2000).

1.9 Literature review:

Detection and identification of bacterial meningitis using molecular techniques is fairly recent approach which has highlighted the importance of the rapid diagnosis of bacteria in bacterial meningitis (Duan *et al.,* 2009; Tuyama *et al.,* 2008). Currently, the diagnosis of meningitis is mostly done by Microscopy of Gram stained CSF samples and culture (Chakrabarti *et al.,* 2009). Moreover CSF serology for detection of bacterial antigens is not widely practiced because of its lower sensitivity and specificity compared to Gram stain and culture (Chakrabarti *et al.,* 2009).

A series of recent studies by Boving and Pederson have developed a system that uses species-specific multiplex PCR focused on the bacterial and viral microorganisms most frequently found in CSF (Boving *et al.,* 2009). Multiplex real time PCR or broad-range PCR aimed at the 16S ribosomal RNA gene of eubacteria is promising for the detection of pathogens from CSF (Boving *et al.,* 2009; Kim, 2010).

A study by Ben and Kung et al. 2008, identified pathogens by using microarray or biochip; it involves amplification of targeted DNA and hybridization of labeled DNA to specific oliogonucleotide probes immobilized on a microarray (Ben *et al.,* 2008; Kim, 2010). However, its usefulness in clinical practice has been shown to be with low sensitivity as microarray technique uses DNA probes from selected pathogens common to bacterial

meningitis, other pathogens or species (not incorporated on the microarray) cannot be detected (Ben *et al.,* 2008).

Another study carried out by Lu and coworkers designed one set of universal PCR primers and claimed to amplify all bacteria (Lu.J.J *et al.,* 2000). They have found that restriction enzyme digestion patterns of the PCR produced with their universal primers differ from one bacterial species to another. The results of this study, indicate that PCR products followed by restriction digestion is useful for rapid detection and identification of some bacterial pathogens in cerebrospinal fluid, in this study the researchers used { it was one pair , two primers U1 and U2of primers only.(Lu .J.J *et al.,* 2000).

Another recent study concerning rapid diagnosis of sepsis and bacterial meningitis in children carried out by Chen and coworkers 2009 used Real-time Fluorescent quantitative PCR for 16S rRNA gene. they introduced a fluorescent-labeled TaqMan probe into the PCR procedure with a universal probe which aims to determine all of the common bacteria of meningitis (Chen *et al.,* 2009).

A typing approach was tested by Ceyhan and coworkers provides insight into the pathogenesis of pneumococcal meningitis based on that *Streptococcus pneumoniae* as a major cause of childhood acute bacterial meningitis in countries where vaccination *against Haemophilus influenzae type b* and/or meningococcal disease has been implemented into the routine immunization schedule. They performed a single-tube PCR assay for simultaneous identification and targeting the gene *ply* for *S. pneumoniae*, then all positive samples were tested using a multiple antigen detection assay capable of detecting 14 pneumococal serotypes (Ceyhan *et al.,* 2010).

In a study carried by Chiba and coworkers real time PCR was develop that can detect eight pathogens; *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Neisseria meningitidi*s, *Esherichia coli*, *Streptococcus agalactiae*, and *Staphylococcusaureus* which they considered as the major causative pathogens in neonatal meningitis; in addition to *Listeria monocytogenes* and *Mycoplasma pneumoniae*, which are rarely the causative pathogens. They

report an identification system using real-time PCR with pathogen-specific molecular (MB) probes and primers for eight meningitis pathogens (Chiba *et al*., 2009).

1.10. Study objectives:

Two major objectives are the focuses of this study:

1- Evaluation of the capability of the Universal Method (Barghouthi, 2009) for detecting bacteria in clinical samples of CSF as a primary screening test for bacterial meningitis. This general method will allow the detection of any bacterium with the potential for its subsequent identification.

2- Construction of species specific multiplex with primers compatible with the Universal Method thermocycler parameters for the detection of major etiological agents of bacterial meningitis, so that both PCR tests can be conducted simultaneously without any delay

1.11. Statement of the Problem:

Cultural methods used for bacterial detection are slow, insensitive, and inconclusive, whereas molecular methods are fast, sensitive and specific. However, none of the current methods is sufficiently sensitive to detect all bacterial CSF infections. The Universal Method developed by Barghouthi 2009, is a potential candidate to detect any bacterium. This research is designed to test the ability of the Universal Method to detect bacteria in 100-200µl of CSF. In addition, this research will attempt to simultaneously identify the detected bacterial pathogen.

1.12. Hypothesis and Principle:

Bacterial meningitis may not be detected using conventional methods only especially using cultural and specific primer multiplex techniques due to the wide spectrum of bacteria that can cause bacterial meningitis. The detection of bacterial DNA in aseptically obtained clinical samples collected in DNA free sterile tubes is sufficient to continue or stop antibacterial therapy.

The procedure depends on the extraction of the bacterial DNA from the CSF specimen, then applying the Universal Method and in parallel with the newly developed species specific PCR Anchored Multiplex (AM) designed in this study. The amplified product is then used to

identify the bacterial pathogen, directly from AM or after amplicon sequencing and BLAST analysis as in published UM.

Chapter 2: Materials and methods

In the present study, a robust method for detection and identification of bacterial meningitis is developed. The method is a PCR based system that amplifies segments of 16S rDNA bacterial gene allowing both detection and identification of CSF bacterial pathogens. Two methods are applied simultaneously to each sample; The Universal Method golden primer mixture G7 and the anchored species specific PCR multiplex (AM) has been used for simultaneous identification of the most common causative agents of bacterial meningitis: *Streptococcus pneumoniae, Haemophilus influenzae, Neisseria meningitidis,* and *Listeria monocytogenes.* The assembly of general primer mixtures capable of amplifying 16S genes of bacteria represent the key to successful application of the Universal Method (Barghouthi, 2009).

2.1 Patients

Clinical CSF samples were collected from 129 suspected meningitis patients by lumber puncture, (neonates and internal word). All samples were tested by conventional methods (culture). For this study, a duplicate sample was obtained from Al Ahli Hospital (H samples), Alia Hospital (A samples), and Karetas Hospital (K samples) with appropriate permissions between January 2009 and August 2010. Duplicate CSF samples were collected in DNA free screw cap sterile microfuge tubes and stored at – 20 ºC.

2.2 Bacterial Reference Species, Control and Optimization of experiments:

The most common CSF bacteria (*Neisseria meningitidis, Haemophilus influenzae, Streptococcus pneumoniae, and Listeria monocytogenes* were provided by Dr S.Barghouthi, private bacterial collection in the form of pure cultures. DNA was extracted (see below) and used fresh or stored frozen at -65˚C for further utilization.

2.3 DNA Extraction from pure bacterial cultures

To achieve a uniform DNA extraction procedure that is applicable to Gram positive and Gram negative bacteria, the following protocol was performed:

Water-mercaptoethanl mixture (V/V) was prepared (80:1) utilizing sterile distilled water and 2-mercaptoehanol (2ME; Sigma Chemicals). As a reducing agent, 2ME may inhibit or inactivate bacterial enzymes such as proteases and nucleases that may interfere with the quality of DNA or the efficiency of the PCR reaction. A 1µl sterile plastic loop was used to transfer a loop-full of bacteria to 400 µl PBSWater-2ME, PBS is described below. After 5 min of incubation at ambient room temperature, the bacterial cells were collected by centrifugation at 13,000 rpm for 1min, supernatant was aseptically removed (discarded into a biohazard container that was autoclaved before disposition). To the remaining pellet 50 µl of lysis mixture (25 µl of 0.5N NaOH, and 25 µl of 1% Sodium Dodecyl Sulfate; SDS) were added, mixed, and steamed over a boiling water bath for 10 min. The lysates were diluted with 150 µl of sterile pure water, mixed, and centrifuged for 3 min at 13,000 rpm, the supernatant was then used in PCR reactions. The remaining was transferred to a fresh sterile screw cap microfuge and stored at -65˚C for further use.

2.4 Bacterial DNA Extraction from CSF samples

One hundred µl of CSF sample were placed in a labeled sterile screw cap microfuge tube: 0.5 µl of 2-mercaptoethanol (2ME) was added, after incubation at 37˚C for 5 min, centrifuged for 2 min at 13,000 rpm, supernatant was removed and appropriately discarded. The remaining steps were exactly as described above (2.3 DNA Extraction from pure bacterial cultures).

2.4.1. Universal Method and Anchored-Multiplex PCR

The Universal Method was applied as described by Barghouthi 2009. Briefly, the golden mixture multiplex (G7) which amplifies ten target sequences distributed among bacterial 16S rDNA genes, which takes advantage of the probability of any one of those 10 sequence of appearing in any bacterium. It detects all bacteria and identifies many bacterial species (Barghouthi 2009). One of the produced PCR products is then sequenced for bacterial identification through BLAST analysis of available DNA sequences in gene data bases.

The anchored-multiplex (AM) method is a modified multiplex that was developed for this study. A single general reverse primer that represents most bacteria (particularly those causing meningitis; (*Neisseria meningitidis*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Listeria monocytogenes*, *Staphylococcus aureus, and others*) which was utilized by the Universal Method named (QUGP-Rn3) as illustrated with other primers in the shown 16S nucleotide sequences Figure A, Appendix 1 . This primer (QUGP-Rn2 or Rn3) represents the anchor primer that was mixed with four forward species specific primers (Table 2.1) that allowed the amplification of a fixed length region of the target bacterium (Table 2.1).

Table:2.1 General primers mixtures (G7): The sequences of the general primers that is contained in the G7 multiplex.

Table for primers of G7 multiplex mixture	
QUGP- Fn3	5'-CAGGATTAGATACCCTGGTAGTCC-3'
QUGP- F4	5'-CCGCCTGGGGAGTACG-3'
QUGP- Fn5	5'-ACTCCTACGGGAGGCAGCAG-3'
QUGP- Fn6	5'-CCAGCAGCCGCGGTAATAC-3'
QUGP- Rn1	5'-GGCTACCTTGTTACGACTTC-3'
QUGP- Rn2	5'-TGACGGGCGGTGTGTACAAG-3'
QUGP- Rn3	5'-GGCGTGGACTACCAGGGTATC-3'

2.4.2. Specific bacterial PCR identification

Four primers designed to detect bacteria that is known to cause meningitis, a specific region was selected from the 16S rDNA sequence according to the following steps:

- BLAST alignment of one selected rDNA sequence against all bacterial species selected for the study (see primer Table (2.2), sizes and species).

- Complete sequence of 16S ribosomal DNA for the most common bacteria that commonly cause meningitis were downloaded. These included: *Neisseria meningitidis, Streptococcus pneumoniae, Haemophilus influenzae, Listeria monocytogenes.* These sequences were copied as Fasta format. When an oligonucleotide was selected it was subjected to BLAST analysis, if it was unique to the target bacterium it was then searched for in the rDNA string unit to determine its exact location (see Figure A, appendix). The design of each primer took into consideration the PCR product size that should identify that amplicon based on its size.
- The specificity of each band was achieved by designing a primer upstream to the location of the QUGP-Rn2 and QUGP-Rn3 that have been published earlier (Barghouthi 2009) as general 16S PCR primers.
- For Every designed primer it was taken into consideration that Tm must be near 60ºC as determined by the online Tm calculator (http://www.promega.com/biomath/calcll.htm). A complete description for the primers that were used in this study for amplification of conserved and specific regions of bacteria commonly causing meningitis are shown (Table 2.2).

- A mixture containing the four specific forward primers and the QUGP-Rn3 was named Anchored-Multiplex D4, since the QUGP-Rn3 or Rn2 served as the reference point to determine the PCR product size. Species specific primers were also tested individually with one of the general QUGPs primers as shown in Table (2.2).
- The primers designed were from Metabion Co. - Germany.

2.4.3. Anchored-Multiplex D4

A mixture of the four primers was prepared in order to detect specific bacterial pathogens. These were *Neisseria meningitidis, Streptococcus pneumoniae, Haemophilus influenzae, Listeria monocytogenes*, The concept of the anchored –multiplex takes

advantage of the fact that the two reactions (G7 and AM) can be applied simultaneously or sequentially. according to the following procedure:

1- 10 µl (100 pmol) from each forward specific primer were mixed in one microfuge tube
2- 40 µl (400 pmol) of the reverse primer (Rn2) was added
3- 20 µl of DNA- free water, this produced 10 pmol/ µl concentration of each primer, except for the reverse primer which was used at 40 pmol to prevent its depletion in case of the presence of more than one target sequence and to balance the 4 forward primers.
4- This working mixture was diluted 10 times in the final PCR reaction.

2.4.4. PCR Mixture preparation

The PCR mixture was prepared according to the recipe listed below:

- PCR Reaction final volume 25.0 µl
- Master mix (Promega) 12.5 µl
- Forward primer (final quantity) 0.5 µl (5pmoles)
- Reverse primer (final quantity) 0.5 µl (5pmoles)
- Sterilized distilled water to 25.0 µl

Table 2.2: The sequences of the specific primers that were designed in this study and the amplicon size for each

Primer	PCR & Sequensing Primers	Tm ˚C	PCR Product Size with QUGP-Rnn2	PCR Product Size QUGP-Rn3
Listeria monocytogenes *Digestion of (TCCGGA)*	5'-AAGTGTGGCGCATGCCACGCTT-3'	59˚C	1231 bp	631 bp (281+250)
Streptococcus pneumoniae *Digestion of (TCCGGA)**	5'-GTGTGAGAGTGGAAAGTTCACACTG-3'	58˚C	952 bp	351 bp 102+249bp
Neisseria meningitidis	5'-TTTGTCAGGGAAGAAAAGGCTGTTGC-3'	58˚C	969 bp	369 bp Not digested
Haemophilus influenzae	5'-TGAGAGGCCGCATGCCATAGGATGA-3'	59˚C	1219bp	600 bp Not Digested

2.4.5. Optimization of AMD4 Specificity :

The species specific primers which were used for the identification of *Neisseria meningitidis* , *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Listeria monocytogenes* were tested against known isolates preserved as stock cultures in our laboratory. Samples that produce amplicons were shown to produce specific PCR band only when target DNA was provided (Table 2.2). Pure cultures for the four bacterial species were stored and subcultured, DNA was extracted and kept frozen to be used as positive control in optimization experiments.

2.4.6 Optimization of AMD4 Sensitivity:

To test the overall sensitivity of the multiplex,

- Bacterial colony was suspended in 1ml of phosphate saline buffer (PBS) which was incubated for 5 minutes.
  - PBS: Dissolve the following in 800ml distilled H2O.
    - 80g of NaCl
    - 2.0g of KCl
    - 14.4g of Na2HPO4,
    - 2.4g of KH2PO4, Adjust pH to 7.4, adjust volume to 1L with additional distilled H2O, Sterilize by autoclaving.
- Serial dilution were prepared in CSF to presumably (estimated) 10^4 CFU/ml.
- 100µ of 10^4 CFU were used to extract DNA as mentioned in section (2.2).
- In the DNA preparation process the sample was diluted by 2-fold (50µ lysis buffer and 150 µ water) and only 0.5 µ of DNA extract was used per PCR reaction.
- The estimated lower and upper limit of detection for the AMD4 was (12.5-1.25 CFU/ml),

2.5 PCR Thermocycler parameters and gel electrophoresis

A MiniCycler (MJ Research, Inc., Watertown, MA) heated lid thermocycler was used to amplify target DNA. The amplification protocol was as following: Hot start at 95°C for 3minutes, followed by 30 cycles of denaturation at 94°C for 1:30 minutes, annealing at 60°C for 1:30 minutes, extension at 72°C for 1:30°C, followed by a final extension step at 72°C for 3 minutes.

2.5.1. Agarose gel electrophoresis

Agarose gel was prepared as following:

1. 1.5% (w/v) agarose was prepared in 25 ml of 1X Tris-aceteat EDTA (TAE) buffer (4.84 g Tris base, l.14 ml glacial acetic acid, and 2ml of 0.5M EDTA, PH 8.0).
2. The agarose was boiled until dissolved. The agarose solution was cooled to about 50°C and 5 µg of ethidium bromide was added.

3. The gel was casted in a tray 10x5 cm for electrophoresis, with comb in place. After solidifying at room temperature, 1X TEA buffer was added into the electrophoresis tank.
4. A size marker ladder 1Kbp (GeneDirex,USA) or a 100pb-ladder was applied to the gel for DNA sizing and band identification.
5. The gel was allowed to run for ~30 min at 120 volts, and then it was visualized under UV light.
6. The gel was documented by photography using digital camera with sepia filter. The size of DNA bands can be accurately determined.

2.6 Processing of cerebrospinal fluid samples

2.6.1. Identification of PCR product for positive CSF samples

2.6.1.1. Identification using species-specific multiplex PCR

The positive CSF samples that produced PCR by the AM (AMD4 mix) indicated one of the four bacterial species included in the AM.

2.6.1.2. Bacterial identification using PCR product sequencing:

The positive samples that generated PCR product after UM detection but were negative when using AMD4 were subjected to DNA sequencing according to the following procedure: The NucleoSpin DNA and Gel purification kit was from MACHEREY-NAGEL GmbH & Co. KG, Germany. The purification of the band was done according to manufacturer instructions as following:

1. The agarose containing the DNA band to be purified was excised, using a flamed clean scalpel. The slice was cut into several smaller pieces and transferred into to a pre-weighed sterile 1.5 ml microcentrifuge tube, agarose slice was weighted to the nearest 10 mg.

2. The agarose was boiled until dissolved. The agarose solution was cooled to about 50°C and 5 µg of ethidium bromide was added.

3. 0.5 ml of lysis buffer was added to the agarose slice, when dissolved, 0.5 ml of binding buffer and 10 µl of suspension nucleo-trap were added.
4. The tube contents were mixed vigorously, heated at 55˚C allowing dsDNA to bind to the solid phase matrix. (10 minutes).
5. After the double stranded DNA had bound to the binding matrix, it was collected by centrifugation, washed in washing buffer, and eluted in a small volume (20-30 µl) of sterile TE buffer. Checked for, presence, concentration, and purity of purified band.
6. Stored at -20˚C until sequenced, alternatively if the PCR reaction contained a single product, the product was purified directly from the PCR reaction without the need for agarose electrophoresis.

2.7 Blast analysis:

BLAST (Basic local Alignment Search Tool) developed by Altschul (Altschul *et al.*, 1990). The program takes a query sequence and searches it against the database selected by the user. It aligns a query sequence against every subject sequence in the database library by using analysis software (Altschul *et al*., 1990; Clarridge, 2004). There are well-known databases of 16S rDNA gene sequences that can be consulted via the World Wide Web; such as GenBank (http://www.ncbi.nlm.nih.gov/),the Ribosomal Database Project (RDP-II) (http://rdp.cme.msu.edu/html/), the Ribosomal Database Project European Molecular Biology Laboratory (http://www.ebi.ac.uk/embl/), Smart Gene IDNS (http://www.smartgene.ch), and Ribosomal Differentiation of Medical Microorganisms (RIDOM) (http://www.ridom.com/). The proprietary MicroSeq 500 bacterial database (version 1.4.2) contains sequences for 1,434 species or subspecies within 235 genera (Clarridge, 2004). Once a sequence is BLAST aligned, it can be judged if it is related, similar, or identical to any sequence that is deposited in the sequence data base. If the sequence does not match (>97%) usually very little can be said about the query sequence.

Figure:(2.1) summarizes the working plan; it shows the steps of CSF sample processing from DNA extraction to bacterial identification.

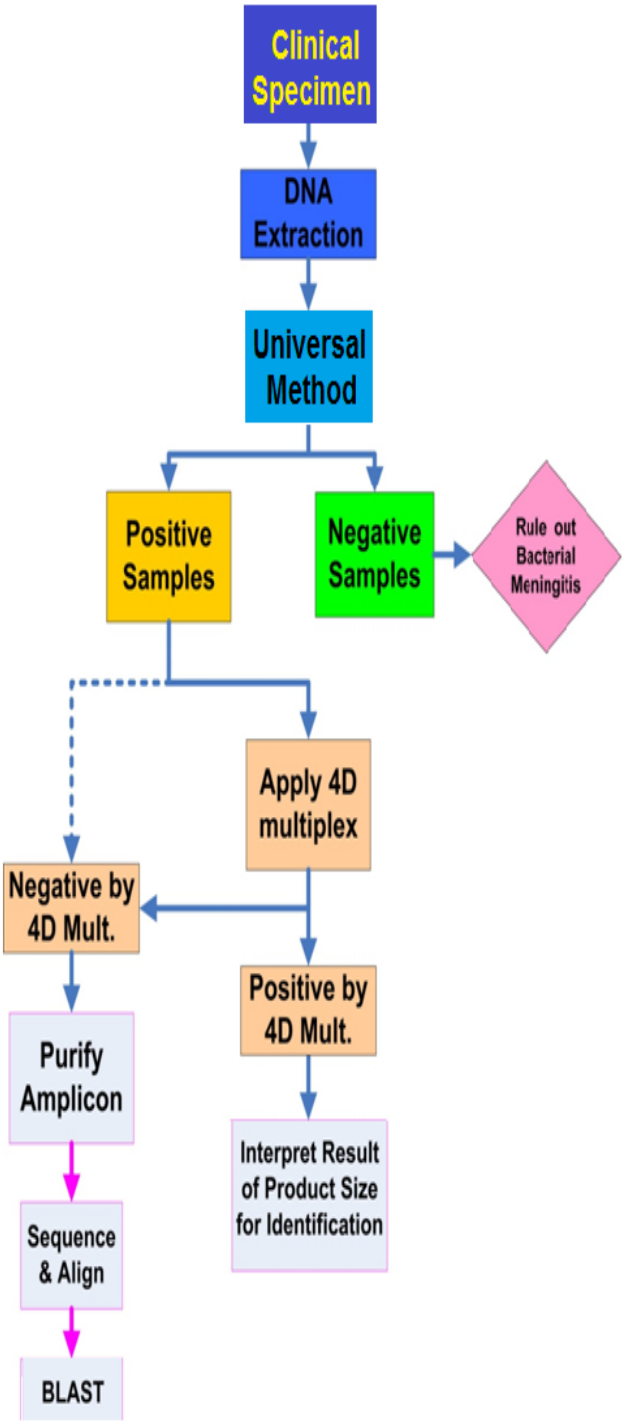

Figure 2.1*:* **A working plan for sample processing steps from DNA extraction to bacterial detection and identification**

Chapter 3: Results

3.1 Assay development

The mixture developed and adopted by the Universal Method; G7 amplify different regions of the 16S rDNA gene. Comparison of these primers sequences with whole bacterial genomes and available nucleotides in the different DNA databases shows that they are complementary to a wide range of different bacterial genera and species. The theoretical broad-range of these primers was validated by successful amplification of 101 different bacterial isolates at Al-Quds University (Barghouthi, 2009).

3.2 Specificity of detection and identification procedures

The designed specific primers underwent a complete optimization for specificity using control bacterial strains were provided by Karetas Hospital and from Barghouthi's bacterial collection (QUBC) by performing careful DNA extraction followed by amplification with species specific primer alone. The results were as expected Fig. (3.1). The figure shows the calculated PCR size products that are depicted in (Table 2.2). *Neisseria meningitidis* showing the specific PCR band at 969bp, *Haemophilus influenzae* 1219bp, *Streptococcus pneumoniae* 925bp, and *Listeria monocytogenes*1231bp.

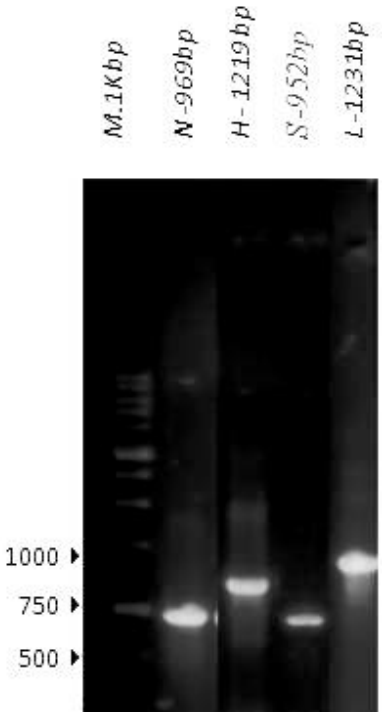

Figure 3.1: PCR products amplified using specific forward primers anchored to a general reverse primer QUGP-Rn2, Optimization for each species specific primer. Different lanes were PCR products from the following control bacteria; Lane 1, *N. Meningitidis;* lane 2, *H. influenzae*; Lane 3, *S. pneumoniae*; lane 4, *L.monocytogenes*. M contained molecular size standards ladder (base pairs). The sizes of the molecular size standards are indicated**.**

To test the performance of all four primers together, the anchored-multiplex D4 (AMD4) was prepared as described in the methods section. AMD4 was applied to each individual bacterium; the results of the PCR amplification are depicted in Fig. (3.2).

The results shown in Fig. (3.2), illustrate that AMD4 produced identical results as when specific primers were applied individually, and that the results in Fig. (3.1) are identical to those shown in Fig.(3.2). This shows high specificity to target DNA and shows no interference between forward primers.

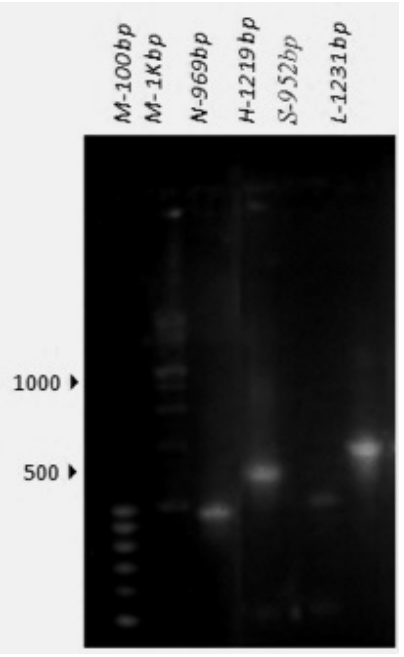

Figure 3.2: PCR products obtained using the AMD4 primer mix. with different bacterial control Results. Lane1, *N. meningitidis; lane 2, H. influenzae; Lane 3; S. pneumoniae; lane 4, L.monocytogenes; M contained molecular size standards ladder (base pairs).*

3.3 Sensitivity of the detection and identification system

To test the sensitivity of detection of bacterial DNA, three negative CSF samples where spiked with different dilutions of bacterial colonies of *Neisseria meningitidis*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, Bacterial DNA extraction was performed according to the procedure mentioned in this study and bacterial DNA (1µ) was detected after (10^4 *)* *Neisseria meningitidis* and *Haemophilus influenzae* and for *Streptococcus pneumoniae* (10^4), when tested with AM4D Multiplex. As shown in Fig.(3.3), higher dilutions were not tested. *L. monocytogenes* was not performed due to negative CSF sample availability.

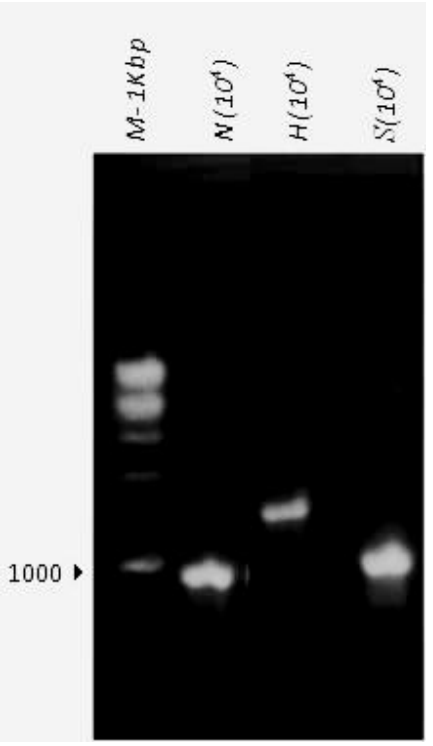

Figure 3.3: Serially diluted bacterial colony, was used to spike negative CSF samples with *Neisseria meningitidis (10^4)*CFU/ml, *Haemophilus influenzae* (10^4) CFU/ml and S*treptococcus pneumoniae (10^4)* CFU/ml , DNA was extracted from CSF then amplified using AM4D.

3.4 Detection of bacteria in clinical CSF samples and identification of positive samples:

A total of 129 CSF clinical samples (see methods), were analyzed by G7 multiplex mixture which produced 126 negative samples and 3 positive samples Fig.(3.4), the other three samples generated a PCR product which gave rise to similar general patterns Fig. (3.4). Performance of these three positive samples with AMD4 resulted in one positive band generated a PCR product of 969bp, according to Fig. (3.5) and Table (2.2) the bacterium was identified as *N. meningitidis*. The other two G7-positive samples (H1 and A35) were negative when tested with AMD4.

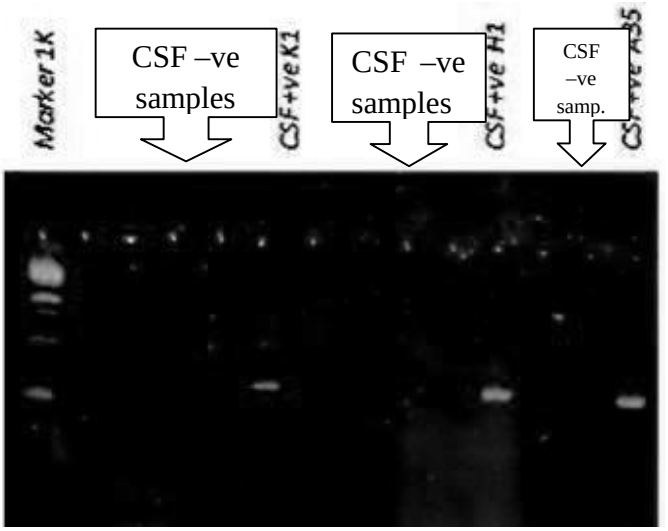

Figure 3.4: Gel electrophoresis for bacterial detection in CSF positive samples: resulted in three general PCR products using universal primers (G7) that indicated bacterial DNA presence in the samples from three different hospitals, the other samples are negative.

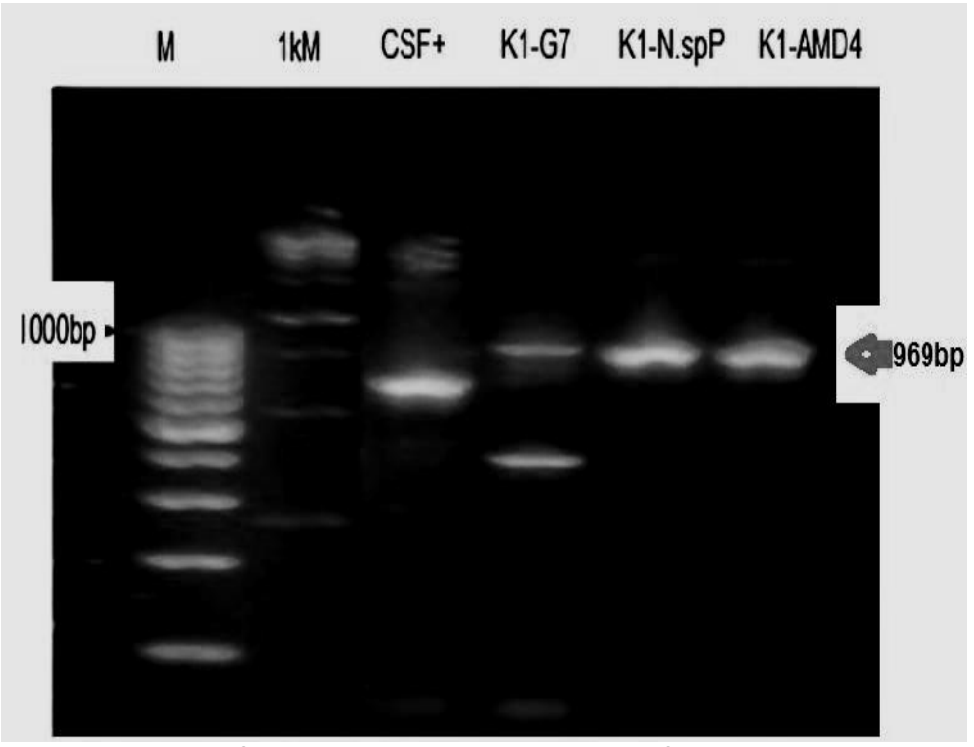

Figure 3.5: Pilot experiment for *N. meningitidis detection and identification*: Lane 1,positive CSF sample, Lane 2, detection of *N. meningitidis* using general primers of UM, Lane 3, identification of *N.meningitidis* by species specific primer, Lane 4, identification of *N.meningitidis* using AM4D Species specific multiplex

3.5 Identity of bacteria in H1 and A35 CSF Samples:

Sequencing of the two positive amplicons obtained from sample A35 and H1 resulted in two rare types of bacteria that are not commonly identified by chemical tests or cultural methods utilized by clinical laboratories. Sequencing of A35 and H1 revealed the sequences shown in Figures (3.6), (3.7),(3.8), and (3.9). As these sequences were analyzed using BLAST alignment for query sequence, they showed no identity which could not be ascertained from the BLAST results most likely due to the absence of these isolates from available bacterial sequences and nucleotide databases. The BLAST alignments are shown in Figure (3.10) and Figure (3.11).

Although there is a high degree of similarity between A35 and H1 sequence Figure (3.12), it should be noted that the two samples originated from two separate hospitals.

Figure 3.6: DNA sequence obtained for CSF sample A35; this sequence using forward primer QUGP- Fn6:

AGGGGCAGCGTTTATTCGGATTCTGGGCGTAAGGCGCGCGTAGGCGGAAATTAAGTCAGATGTGAAATCCCCGGGCTTAACCTGGGAACTGC
ATTTGATACTGGCTGGCTAGAGTGTGGGAGAGGGGAGTGGAATTCCCGGTGTAGCGGTGAAATGCGTAGATATGGGGAGGAACACCAGTGGC
GAAGGCGGCTCCCTGGGCCGATACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAA CAGGATTAGATACCCTGGTAGTCCACGCC

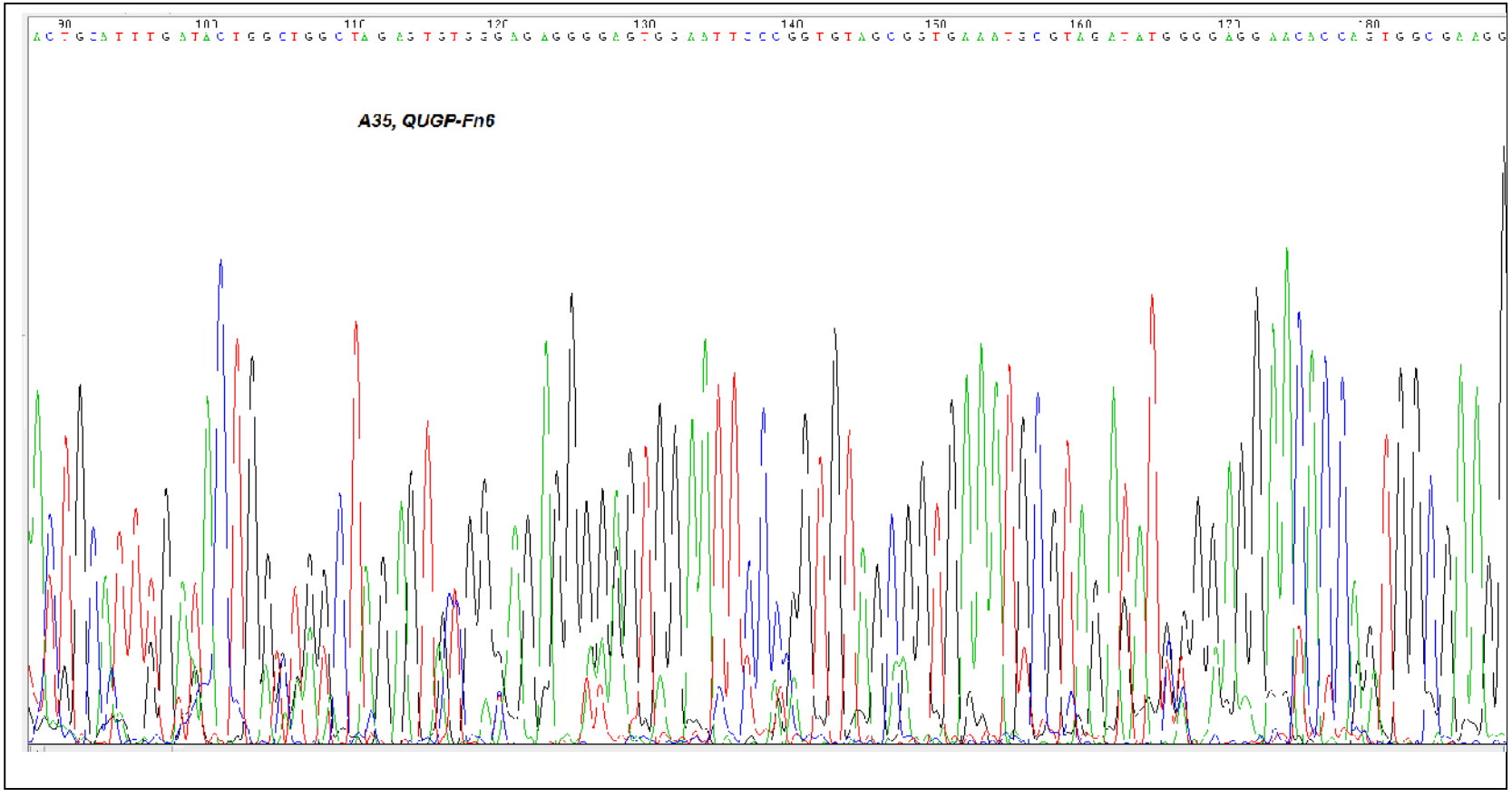

Figure 3.7: DNA sequence obtained for sample A35; this sequence using the reverse primer QUGP-Rn3 *:*

TTTCCTGTTTGCCTACCGCTTTCGCGCCTGCAGCGTCAGTAATGGGAAGGCAGGGAGCCGCTTGCCACTGGTGTTCCTCCCGATATTTACGCAT
TTCACCGCTACACCGGGAATTCCACTCCCCTCTGCCGTACTCTAGCCAGCCAGTTTCAAATGCAGTTCCCAGGTTGAGCCCGGGGATTTCACAT
CTGACTTAACTAACCGCCTACGCGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTTCGTATTACCGCGGCTGCTGGA

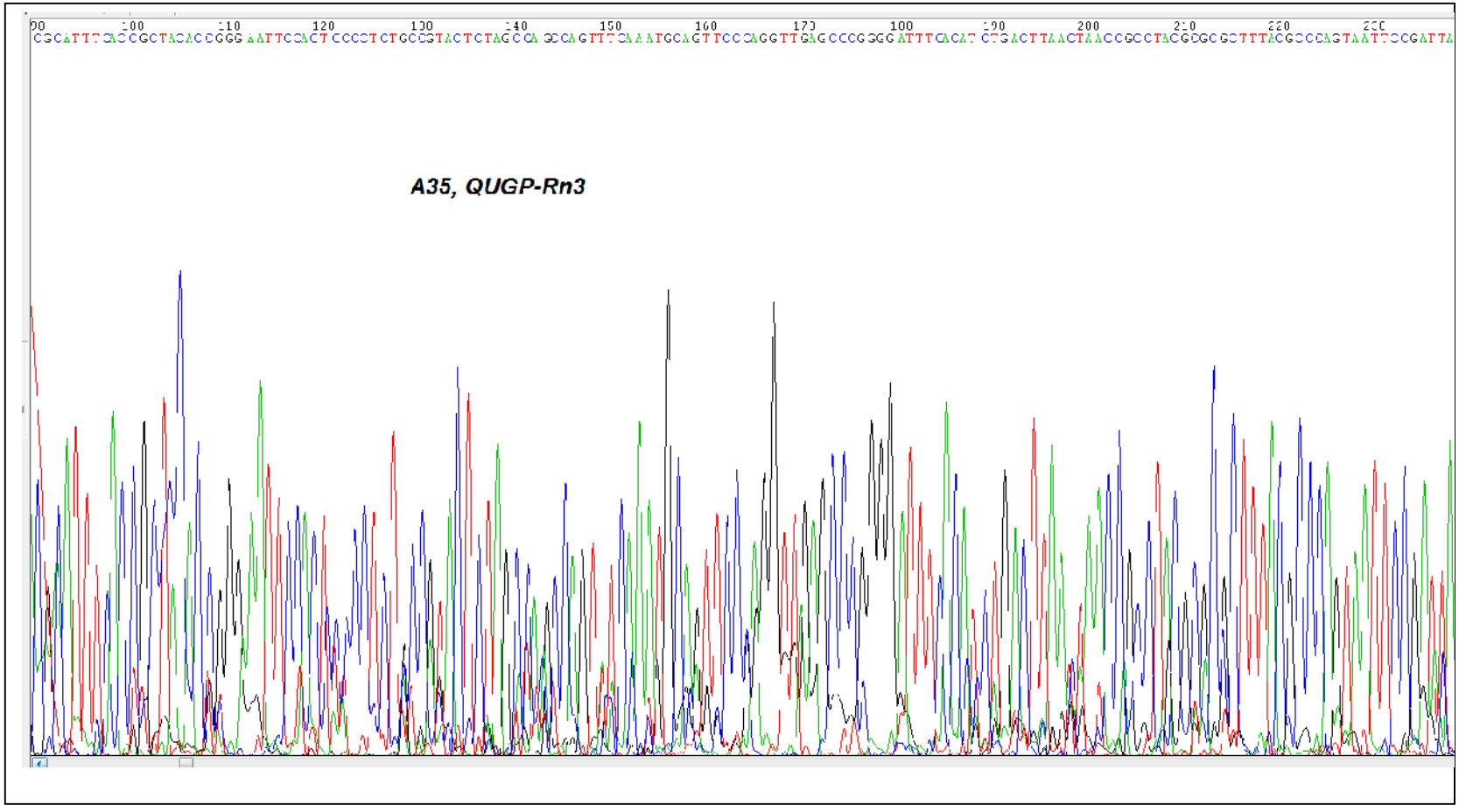

Figure *3.8:* **DNA sequence obtained for H1 CSF sample; this sequence using forward primer QUGP-Fn6:**

GAGTCAGCGTTTTTTCCGAATTCTGGCGTAAGCGCGCGTAGGCGGAAATTAAGTCAGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATT
TGATACTGGCTGGCTAGAGTGTGGGAGAGGGGAGTGGAATTCCCGGTGTAGCGGTGAAATGCGTAGATATCGGGAGGAACACCAGTGGCGA
AGGCGGCTCCCTGGCCCGATACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCC

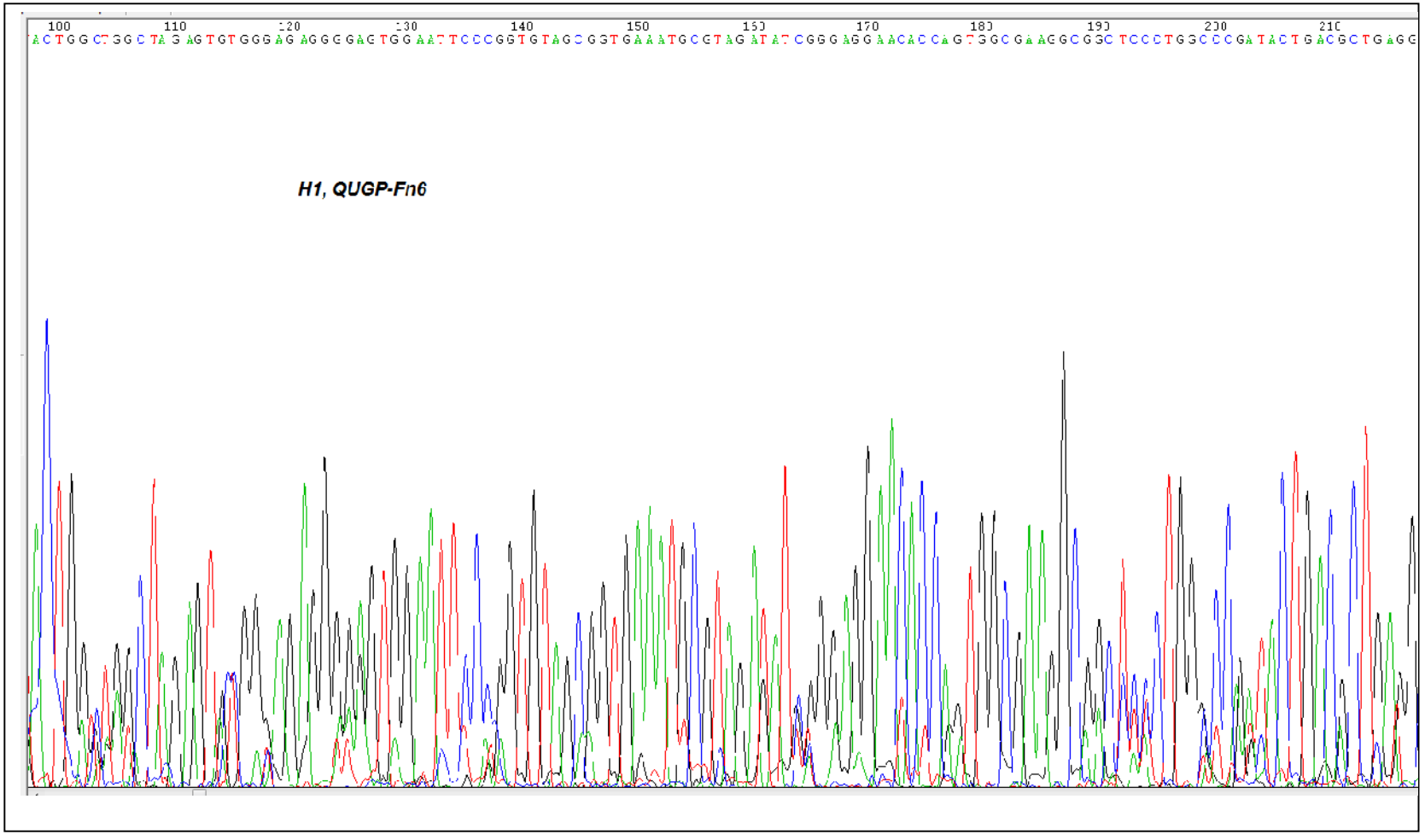

Figure *3.9:* DNA sequence obtained for H1 CSF sample; this sequence using reverse primer QUGP-Rn2:

AACCTGTTTGCTTACAGCTTTCGCGCCTGAGCGTCAGTAACTGGGACCAGGGAGCCGCCTCCGCCACTGGTGTTCCTCCCGATATCTACGCATT
TCACCGCTACACCGGGAATTCCACTCCCCTCTGCCGTACTCTAGCCAGCCAGTATCAAATGCAGTTCCCAGGTTGAGCCCGGGGATTTCACAT
CTGACTTAACTAACCGCCTACGCGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTTCGTATTACCGCGGCTGCTGG.

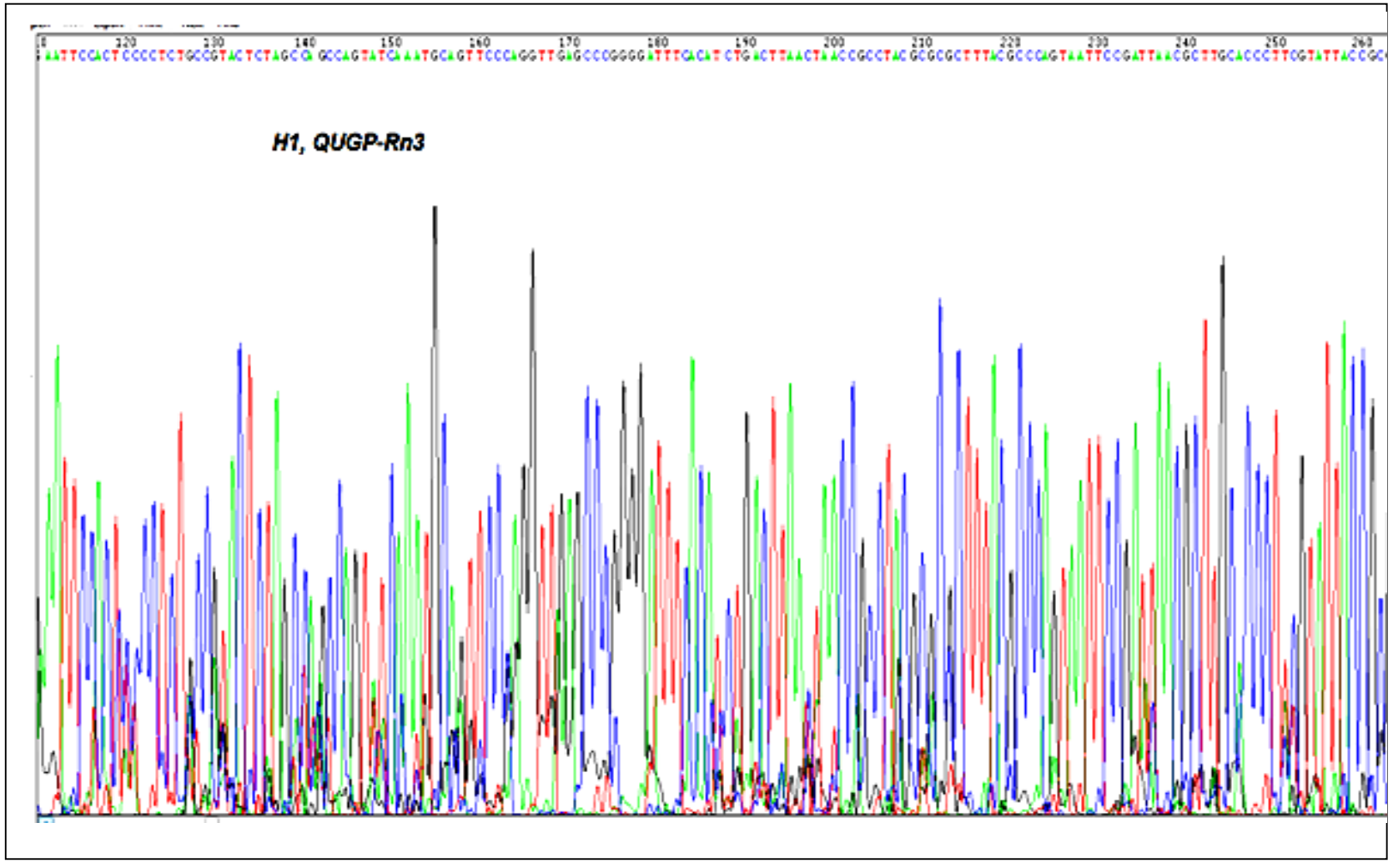

Figure *3.10:* BLAST alignment for PCR product for positive CSF sample A35, using the forward primer QUGP-Fn6 which resulted in *Thioalkalivibrio* sp HL-EbGR7 as the closest match, complete genome.

```
>ref|NC_011901.1|  Thioalkalivibrio sp. HL-EbGR7, complete genome
Length=3464554
 Score =  335 bits (181),  Expect = 3e-89
 Identities = 245/275 (90%), Gaps = 8/275 (2%)
 Strand=Plus/Minus

Query  1        AGGG-GC-AGCGTTTATTCGG-ATT-CTGGGCGTAAGGCGCGCGTAGGCGGAAAT-TAAG  55
                |||| || |||| ||| |||| ||| |||||||||| |||| |||||||||    | ||||
Sbjct  2549250  AGGGTGCAAGCG-TTAATCGGAATTACTGGGCGTAAAGCGCACGTAGGCGGCCTTGTAAG  2549192

Query  56       TCAGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATTTGATACTGGC-TGGCTAGAG  114
                || ||||||||| |||||||||||||||||||| |||||  |||||| ||  ||||||||
Sbjct  2549191  TCGGATGTGAAAGCCCCGGGCTTAACCTGGGAATTGCATCCGATACT-GCAGGGCTAGAG  2549133

Query  115      TGTGGGAGAGGGGAGTGGAATTCCCGGTGTAGCGGTGAAATGCGTAGATATGGGGAGGAA  174
                | ||| ||||| ||||||||||||||||||||||||||||||||||||||||| ||||||||
Sbjct  2549132  TTTGGTAGAGGAGAGTGGAATTCCCGGTGTAGCGGTGAAATGCGTAGATATCGGGAGGAA  2549073

Query  175      CACCAGTGGCGAAGGCGGCTCCCTGGGCCGATACTGACGCTGAGGCGCGAAAGCGTGGGG  234
                |||||||||||||||| ||| |||| || | |||||||||||| ||||||||||||||
Sbjct  2549072  CACCAGTGGCGAAGGCGACTCTCTGGACCAAAACTGACGCTGAGGTGCGAAAGCGTGGGG  2549013

Query  235      AGCAAACAGGATTAGATACCCTGGTAGTCCACGCC  269
                |||||||||||||||||||||||||||||||||||
Sbjct  2549012  AGCAAACAGGATTAGATACCCTGGTAGTCCACGCC  2548978
```

Figure 3.11: BLAST Alignment for query sequence for positive CSF sample H1, using forward primer QUGP- Fn6 which resulted in *Thioalkalivibrio sp*. HL-EbGR7, complete genome

```
>ref|NC_011901.1|  Thioalkalivibrio sp. HL-EbGR7, complete genome
Length=3464554
 Features in this part of subject sequence:
   rRNA-16S ribosomal RNA
 Score =  327 bits (177),  Expect = 4e-87
 Identities = 230/255 (91%), Gaps = 6/255 (2%)
 Strand=Plus/Minus

Query  18       GAATT-CT-GGCGT-AAGCGCGCGTAGGCGGAAAT-TAAGTCAGATGTGAAATCCCCGGG  73
                |||||  ||  |||||  ||||||  |||||||||    |  ||||||  |||||||||  |||||||
Sbjct  2549231  GAATTACTGGGCGTAAAGCGCACGTAGGCGGCCTTGTAAGTCGGATGTGAAAGCCCCGGG  2549172

Query  74       CTTAACCTGGGAACTGCATTTGATACTGGC-TGGCTAGAGTGTGGGAGAGGGGAGTGGAA  132
                |||||||||||||  |||||   ||||||  ||   |||||||||  |||  |||||  ||||||||
Sbjct  2549171  CTTAACCTGGGAATTGCATCCGATACT-GCAGGGCTAGAGTTTGGTAGAGGAGAGTGGAA  2549113

Query  133      TTCCCGGTGTAGCGGTGAAATGCGTAGATATCGGGAGGAACACCAGTGGCGAAGGCGGCT  192
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||  ||
Sbjct  2549112  TTCCCGGTGTAGCGGTGAAATGCGTAGATATCGGGAGGAACACCAGTGGCGAAGGCGACT  2549053

Query  193      CCCTGGCCCGATACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACC  252
                |  ||||  ||  |  ||||||||||||||  ||||||||||||||||||||||||||||||||||
Sbjct  2549052  CTCTGGACCAAAACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACC  2548993

Query  253      CTGGTAGTCCACGCC  267
                |||||||||||||||
Sbjct  2548992  CTGGTAGTCCACGCC  2548978
```

Figure 3.12: The two sequences of A35 appears to be similar to H1 sequence with few differences as indicated below. Reference boxes are indicated, some showing differences between H1 and A35; First Box (GA-TTCTGGGCGTAA) &(GAATTCT-GGCGTAA) indicating different sources of the two bacteria (A35 and H1) or Sequencing errors which is unlikely since both forward and reverse sequencing produced sufficiently good sequences.

3.6 Laboratory findings for CSF samples from conventional procedures

A total of 129 samples were analyzed by both conventional methods (in respective hospital laboratory) and by UM G7 "broad range 16S rDNA PCR and AMD4 multiplex in Al-Quds research laboratory and the results were as following :

3.7 Laboratory findings from broad-range 16S rDNA PCR

A total of 129 CSF samples were analyzed with UM and the specific AMD4 multiplex. Broad range 16S rDNA based PCR could detect organisms among CSF samples. One case of *N. meningitidi*s could be identified by AM4D species specific PCR and the two others were subjected to DNA sequencing, but BLAST analysis was not able to reveal the identities of A35 and H1. The AMD4 were applied on those two samples and the results were negative. The other 126 CSF Samples were negative by UM G7.

3.7.1. Discrepancies between culture and PCR testing

The laboratories reported 128 samples to be negative, whereas UM-G7 reported 126 negative samples. Two samples were found to be positive (A35 and H1). The CSF sample K1 which was identified by the laboratory as *N. meningitidis* which was detected by both PCR methods; UM-G7 and AMD4 put all three methods in complete concordance.

3.7.2. Overall performance of UM and AMD4:

Among the three UM-positive patients, patient (H1) was a healthy 3 year-old female who presented to the hospital with a high fever (up to 39 ºC) and vomiting. After one blood sample for culture was taken, the patient was given 1.5 g of Decort and 80 mg of vancomycin intravenously in addition to Rocephen. The CSF finding was suggestive of bacterial meningitis, but Gram staining revealed no microbes and the bacterial cultures were negative, nonspecific diagnosis which was (No pathogenic growth) have been resulted from the laboratory. The second patient (K1) which was diagnosed as bacterial meningitis caused by *N. meningitidis* by conventional methods; it was in agreement with the results obtained by both AMD4 and by UM G7 PCR. The third sample (A35) was from a 4-year old patient presented with fever, the culture results and gram stain were negative possibly due to treatment that had started prior to lumbar puncture. The CSF analysis indicated bacterial meningitis with high white blood cell (WBC) counts and low sugar level. The UM G7 successfully detected the bacterial presence in this sample (A35) even after antibiotic was introduced. Identification of the bacterium after sequencing resulted in *Thioalkalivibrio* sp. (92% homology) Figure 3.10 and Figure (3.11).

The results suggest that unknown bacteria was present in each of (A35 and H1) CSF samples, since the sequencing results were clean (both forward and reverse sequencing directions, Figures ((3.6) –(3.7) –(3.8) –(3.9)) and the BLAST results (figure 3.12) suggesting strong similarity may exist between A35 an H1 (see discussion).

The two sequenced PCR for the samples (A35 and H1) could not be identified, since the accepted level of homology should be >97%. The identity of these bacteria (A35 and H1) remains obscure mostly because they have not been represented in the gene bank databases yet. The results indicate that the two cases of meningitis (A35 and H1) may have been caused by unusual bacteria that is other than the > 1418 (Dec. 2010) bacteria available for BLAST

analysis (see list at: http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi) and the nucleotide data base at (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome)

Chapter 4: Discussion

The present study illustrates both the strengths and limitations of the broad range bacterial detection and species specific PCR identification for bacterial meningitis.

The validation of broad range bacterial meningitis detection using general primers (Barghouthi 2009), in parallel with the development and validation of bacterial identification using species specific multiplex mixture named as Anchored-Multiplex, AM4D which was designed and prepared manually for this work. In previous study, the extremely robust ability of the Universal Method G7 mixture to amplify 16S rDNA was found to be applicable and useful in detecting bacteria in CSF. This work was in full agreement with the Universal Method. It was also shown that this primers mixture is specific for bacterial DNA and doesn't amplify human DNA. These features would allow The UM to be utilized with clinical CSF samples, and possibly blood samples, where human DNA would certainly be present. The UM had integrated several general primers, PCR amplification, DNA sequencing and bacterial identification in one simple assay.

DNA sequence alignment (using BLAST); a single, simple, inexpensive, and efficient system designed for the detection and identification of bacteria. The capacity and applicability of the UM and primer mixture to detect bacteria was repeatedly demonstrated as described by Barghouthi 2009. The detection and subsequent identification of novel bacterial species should become a simple process especially when all known bacteria have been sequenced and deposited in nucleotide data bases (Barghouthi, 2009).

The strength of the Broad rang detection assays is that it can test for the presence of a broad panel of both bacterial agents and it can provide fast and reliable results, therefore it can be used as a screening test (Barghouthi, 2009; Boving *et al*., 2009). This approach is valuable for initial evaluation of the presence of bacteria when the specific pathogen is unknown, as in “rule out meningitis” and assuming appropriate sensitivity, a negative amplification reaction will indicate that no bacterial DNA is present. However, if a positive amplification is observed then it will indicate the presence of bacterial DNA. In this case of positive results additional

work will be required to identify the organism. The results show that the assay has a high analytical sensitivity and specificity. This study and others (Barghouthi, 2009; Boving *et al.*, 2009; McCabe *et al*., 1999) have shown that, following amplification of target bacterial DNA, the target organism can be identified using species specific primers; as in anchored-multiplex D4, or by sequencing of the amplicon and subsequent nucleotide alignment (BLAST) against available bacterial genomes. The method allows the identification of emerging or reemerging bacterial pathogens, new pathogens, and even new encountered species. After antimicrobial treatment is started, the chances of isolating bacteria from CSF are slim and cultures may remain negative if the disease is caused by fastidious or slow growing microorganisms (Kim, 2010; Kotilainen *et al.*, 1998) .

The morbidity and mortality associated with bacterial meningitis make meningitis a deadfall illness for both physicians and patients. Therefore, fast, accurate, and inexpensive diagnosis of bacterial meningitis will improve health provision while alleviating the worry associated with suspicion of this disease (Kim, 2010; Saravolatz *et al.*, 2003).

Efforts as improved the method of DNA isolation from bacteria including gram-positive bacteria. During the present study it become apparent that the methods for preparing DNA from a diversity of bacterial species, many of which are encapsulated, has a thick wall, or other properties, require further improvement and additional preparation. In previous studies, low analytical sensitivity may have been caused by problems associated with cell wall cracking (Boving *et al.*, 2009; Welinder-Olsson *et al.*, 2007). These problems were solved by combination of physical disruption (bead beating) and silica-guanidiniumthiocyanate (Schuurman *et al.*, 2004), such methods are long, problematic, and may cause contamination, as well as they may require large sample size (which was 100µl of CSF in this study). In this study, uniform DNA extraction was achieved using a modified procedure that entailed pretreatment of bacteria or sample with 2- mercabtoethanol at very low concentrations with subsequent removal. As a reducing agent, 2ME may inhibit or inactivate bacterial enzymes such as proteases and nucleases that may interfere with the quality of DNA or the efficiency of the PCR reaction. The procedure was tested on cultured bacterial species separately, no inhibition of the PCR reaction was observed under tested conditions. Several CSF samples were also tested by this method, the applicability and reliability of the method was established.

It is well known that implementation of molecular methods based on 16S rDNA or other target genes can be hindered by problems with contamination of reagents or even samples to be performed, which may be derived from any bacterial source. In this study, DNA free microfuge tubes were used for the collection of CSF since several false positive results were obtained when performing PCR on CSF samples collected in standard hospital tubes.

A total of 129 CSF samples were collected over one year (2009-2010) were examined, three samples were positive by The UM, each of which generated a 16S PCR product. The significance of the positive results is strengthened by the fact that aliquots of the same sample were used for both the conventional and the molecular biological assay methods and there was a complete opportunity for comparison between the study results and the conventional method results performed by the source hospital laboratory on patients' samples; the negative CSF samples (126 samples) were negative by PCR and culture as well.

One of the three positive samples resulted in a unique PCR product size (969bp) that was obtained with the *N.meningitidis* species specific PCR primers that was applied in the form of anchored-multiplex D4, and it was in accordance with the hospital result of culture and PCR identification. Fig (3.5).

The absence of *Haemophilus influenzae* infection among the tested sample, may be explained by the fact that vaccination of all children against *H.influenzae* type b was introduced, according to Boving *et al.*, 2009, the rate of meningitis caused by this organism has decreased by 97% (Boving *et al.*, 2009). It worth mentioning that *H.influnza*e vaccine was introduce in Palestinian public health care program (MOH) three years ago (2006) which is in agreement with Boving et al. study in Denmark. Unfortunately, the meningococcal meningitis vaccine has not been introduced yet, due to high cost (Referring to Ministry of Health in Palestine, personal communication with Dr. A. Saffen). The meningococcal quadrivalent (A,C,Y, and W-135) conjugate vaccine, Menactra was approved to be used in the gulf states only recently (2010) http://www.ameinfo.com/235947.html.

The DNA sequences obtained for the other two positive samples were aligned, the DNA BLAST analysis results indicated (BLASTN; www.ncbi.nlm.nih.gov). The identification of two positive PCR purified DNA resulted in unique bacterial species that could not be

determined based on available sequences, since 92% identity was observed, which is much lower than the 97% cutoff value for species identity based on ribosomal DNA sequence. The two bacterial sequences could not be identified although they appeared to share high similarity and have identified similar bacteria (~92%) homology; this indicated that the two isolates are not represented by any related bacteria in the DNA data banks. No further conclusions can be made. Whether they represent true bacterial infections or contaminations, or both cannot be ascertained under the obtained data.

Although the results showed 126 negative samples and since the two samples were collected from two different hospitals, both bacterial samples produced good DNA sequences (Figure: (3.6)- (3.8).

Other positive CSF samples from Al Ahli hospital were not subjected to testing and were rejected due to unsuitable collection and storage of CSF samples, those were: two positive *S. pneumonea*, and 27 positive samples diagnosed as (*No pathogenic growth*). 570 CSF samples with no bacterial growth, normal CSF analysis, normal Glucose and protein levels. Referring to the patients files in one of the hospitals (2009- 2010) indicated the low percentage of bacterial infection in CSF even these patient are considered as suspected bacterial meningitis admissions and received antibacterial therapy while waiting for the culture results. In order to do that, the Faculty of Health Professions coordinated with many hospitals in the region in order to obtain as many samples as possible.

There are always common causes for every bacterial disease but usually, exceptions do exist, because the species specific anchored-multiplex is limited to target species. The main weakness of species specific PCR assays, is that investigators can only find the target bacteria(what they are looking for); they will not detect the presence or absence of non-target bacteria (Boving *et al.,* 2009). In a clinical setting, Gram staining and bacterial culture remains the cornerstones of diagnosis. By adopting the Universal Method and the anchored multiplex (D4), patients with negative infections can avoid unduly treatment, waiting, and expenses.

Another minor problem is that the PCR product sizes generated by AMD4 is due to insufficient size difference (see table for PCR sizes of AMD4 products). We suggest that the most suitable solution to this type of problem is the digestion of PCR products which was

sequenced and found to be digested with any of the restriction enzyme (BlfI, Bsp13I, Kpn2I, BspMII, BsiMI, and BseAI) that all recognize and cut the sequence 5'-TCCGGA-3'Such digestion will cut Listeria's PCR product (631bp) into 281+250 that can be easily distinguished from the 600bp PCR product of *Haemophilus influenzae*. The same enzymes can digest the PCR product of *Streptococcus pneumoniae* 351 into (102+249bp), distinguishing it from the resistant PCR of 369 bp *Neisseria meningitidis* which is 369bp in length, see Table (2.1).

Another way to solving this problem is tagging a selected forward primer with a GC clamp that will cause the PCR to change size and allow its unambiguous separation by gel electrophoresis; if a 30bp of GC clamp were added to the 5'- end of the *N. meningitidis* specific primer making The PCR product appear as 399bp instead of 369bp setting it apart from the 351 bp PCR product of *Streptococcus pneumoniae.* Similar primer modification can be done with Listeria to increase its PCR product size to 661 instead of 631bp relative to that of the *Haemophilus influenzae* which is 600bp (Val C. *et al,.* 1989*).*

In this study, it became obvious that species specific multiplex procedure have got limitations in case of bacterial meningitis identification, since in this study two of three samples were missed. The study supports the view that robust general detection methods of bacterial DNA in CSF samples are superior to any other method. Especially in the context of high levels of viral meningitis and absence of double infection (co-infections), see Tables (1.1) and (1.2). The UM is an important tool as an initial screening test leading to identification of positive samples before sequencing and BLAST analysis**.**

The most significant drawback of utilizing molecular and PCR technology in diagnosis is that antibiotic profiling of the infectious agent cannot be assessed by simple techniques. However, identification of the bacterium can improve the development of strategies to determine the therapeutic drug and bacterial culturing techniques, such as creating a culture medium and culturing conditions that are suitable for most pathogenic bacteria of the meninges and CNS.

The basic method to derive a sequence for a noncultured bacterium is to use PCR to amplify the 16S rDNA gene then to sequence the amplicon (Barghouthi, 2009; Chen *et al.,* 1989; Clarridge, 2004; Rivers, 2001).

The sensitivity of the assay should not significantly fluctuate just because the source of DNA has changed as long as good quality DNA is obtained, accordingly, interest in sensitivity should be shifted towards maximizing the utility of the CSF sample and the quality of extracted DNA (Gray, 1992; Kim, 2003).

In this work, the species-specific anchored-multiplex D4 has proven compatible with the G7 of the UM, they both detected the presence of *N. meningitides*. This species is usually present in CSF as low quantity of bacteria may reach to 10^3 CFU/ml(Gray, 1992).

The UM assay can be used as a valuable supplement to the traditional microscopy and culture of CSF specimens in a routine diagnostic settings (Barghouthi, 2009). The anchored-multiplex D4 is useful in eliminating or confirming the most encountered bacteria and should be applied simultaneously with the UM G7 especially since both has compatible thermocycling parameters. Molecular diagnosis of meningitis will eventually improve the patients' management especially when clinical, microscopic and cultural methods are inconclusive. In the present study, the universal method which used a mixture of general primers generating up to 10 PCR products rather than one, as proved in other studies. This have enhanced the ability and reliability of the UM to detect any bacterial DNA that may be found in CSF samples; this study confirmed the compatibility and utility of the UM with CSF samples.

In conclusion, the assays described here, together with the modified DNA extraction protocol, may be a valuable supplement to the traditional microscopy and culture of CSF specimens. The use of this rapid and sensitive method will enable physicians to start treatment with appropriate antimicrobial agents. On the long run, it is expected that new clinical-etiology of meningitis should emerge as this method finds wider application. In this study, identifying bacteria isolated in the clinical laboratory by sequence in combination of phenotype can improve clinical microbiology by better identifying poorly described, rarely isolated, or biochemical aberrant strains. 16S rDNA gene sequences allow bacterial identification in a robust manner, reproducible, and accurate than that obtained by phenotypic testing (Barghouthi, 2009; Boving *et al.*, 2009; Kim, 2010). It was also noticed that species specific multiplex PCR is a limited procedure for bacterial identification even when the mixture contains a high number of primers; especially since bacterial meningitis may result from a multitude of species including *Klebsiella, Clamedia, Acinitobacter, Staphylococcus aureus*

and others (Kim, 2010; Boving *et al.,* 2009; Gray, 1992). This is demonstrated in the present study; the two suspected bacteria of the two CSF samples (A35 and H1), see sequences in the results section figures: (3.6)- (3.9). Hence, the advantages of 16S ribosomal DNA amplification lie in the detection of non-culturable microorganisms or specimens rendered sterile by prior antimicrobial therapy. Potentially, it can rapidly detect rare and fastidious microorganisms (Boving *et al.,* 2009; Kim, 2010; Pada *et al.,* 2009).

Although there is a high degree of similarity between A35 and H1 sequences (Figure: (3.12), the two samples originated from two separate hospitals. Contamination cannot be ruled out even though when UM G7 produced 126 negative samples. Argument against the contamination hypothesis is that the samples were not related; they both produced good DNA sequencing results indicating the purity of the PCR amplicon hence the bacterium in each sample which is usually unlikely when considering contamination. In addition to that, all clinical signs of the two patients (H1 and A35) were indicated bacterial meningitis infection. See section(3.7.2.).

Although the clinical parameters were not always consistent with the diagnosis of bacterial meningitis, it was possible to detect infection using PCR (N. Margall, 2002). Moreover, the use of nonradioactive primers allowed avoiding the danger of handling isotopes without losing sensitivity (N.Margall, 2002).

To the best of our knowledge the present study is one of the first studies that adapt merging between general and specific PCR techniques in order to detect and identify any bacterial DNA in CSF samples. Furthermore, PCR is less affected by prior therapeutic regimen; accordingly it can be useful for ruling out bacterial meningitis within few hours. Clinicians could make informed decisions regarding the continuation or abortion of treatment in patients suspected of having bacterial meningitis.

References:

1. Abasaeed A., Vlcek J., Abuelkhair M., Kubena A. (2009) Self-medication with antibiotics by the community of Abu Dhabi Emirate, United Arab Emirates. J Infect Dev Ctries 3:491-7.

2. Altschul S.F., Gish W., Miller W., Myers E.W., Lipman D.J. (1990) Basic local alignment search tool. J Mol Biol 215:403-10.

3. Barghouthi S. (2009) A Universal Method for the identification of Bacteria Based on general PCR primers. indian J Mic.

4. Ben R.J., Kung S., Chang F.Y., Lu J.J., Feng N.H., Hsieh Y.D. (2008) Rapid diagnosis of bacterial meningitis using a microarray. J Formos Med Assoc 107:448-53.

5. Bergeron M.G., Ouellette M. (1998) Preventing antibiotic resistance through rapid genotypic identification of bacteria and of their antibiotic resistance genes in the clinical microbiology laboratory. J Clin Microbiol 36:2169-72.

6. Berzanskyte A., Valinteliene R., Haaijer-Ruskamp F.M., Gurevicius R., Grigoryan L. (2006) Self-medication with antibiotics in Lithuania. Int J Occup Med Environ Health 19:246-53.

7. Boisier P., Mahamane A.E., Hamidou A.A., Sidikou F., Djibo S., Nato F., Chanteau S. (2009) Field evaluation of rapid diagnostic tests for meningococcal meningitis in Niger. Trop Med Int Health 14:111-7.

8. Boving M.K., Pedersen L.N., Moller J.K. (2009) Eight-plex PCR and liquid-array detection of bacterial and viral pathogens in cerebrospinal fluid from patients with suspected meningitis. J Clin Microbiol 47:908-13.

9. Ceyhan M., Yildirim I., Sheppard C.L., George R.C. (2010) Pneumococcal serotypes causing pediatric meningitis in Turkey: application of a new technology in the investigation of cases negative by conventional culture. Eur J Clin Microbiol Infect Dis 29:289-93.

10. Ceyhan M., Yildirim I., Balmer P., Borrow R., Dikici B., Turgut M., Kurt N., Aydogan A., Ecevit C., Anlar Y., Gulumser O., Tanir G., Salman N., Gurler N., Hatipoglu N., Hacimustafaoglu M., Celebi S., Coskun Y., Alhan E., Celik U., Camcioglu Y., Secmeer G., Gur D., Gray S. (2008) A prospective study of etiology of childhood acute bacterial meningitis, Turkey. Emerg Infect Dis 14:1089-96.

11. Chakrabarti P., Das B.K., Kapil A. (2009) Application of 16S rDNA based seminested PCR for diagnosis of acute bacterial meningitis. Indian J Med Res 129:182-8.

12. Chen K., Neimark H., Rumore P., Steinman C.R. (1989) Broad range DNA probes for detecting and amplifying eubacterial nucleic acids. FEMS Microbiol Lett 48:19-24.

13. Chen L.H., Duan Q.J., Cai M.T., Wu Y.D., Shang S.Q. (2009) Rapid diagnosis of sepsis and bacterial meningitis in children with real-time fluorescent quantitative polymerase chain reaction amplification in the bacterial 16S rRNA gene. Clin Pediatr (Phila) 48:641-7.

14. Cherian T., Lalitha M.K., Manoharan A., Thomas K., Yolken R.H., Steinhoff M.C. (1998) PCR-Enzyme immunoassay for detection of Streptococcus pneumoniae DNA in cerebrospinal fluid samples from patients with culture-negative meningitis. J Clin Microbiol 36:3605-8.

15. Chiba N., Murayama S.Y., Morozumi M., Nakayama E., Okada T., Iwata S., Sunakawa K., Ubukata K. (2009) Rapid detection of eight causative pathogens for the diagnosis of bacterial meningitis by real-time PCR. J Infect Chemother 15:92-8.

16. Clarke S.C. (2006) Detection of Neisseria meningitidis, Streptococcus pneumoniae, and Haemophilus influenzae in blood and cerebrospinal fluid using fluorescence-based PCR. Methods Mol Biol 345:69-77.

17. Clarridge J.E., 3rd. (2004) Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. Clin Microbiol Rev 17:840-62, table of contents.

18. Corless C.E., Guiver M., Borrow R., Edwards-Jones V., Kaczmarski E.B., Fox A.J. (2000) Contamination and sensitivity issues with a real-time universal 16S rRNA PCR. J Clin Microbiol 38:1747-52.

19. Das B.K., Gurubacharya R.L., Mohapatra T.M., Mishra O.P. (2003) Bacterial antigen detection test in meningitis. Indian J Pediatr 70:799-801.

20. Diggle M.A., Clarke S.C. (2006) Molecular methods for the detection and characterization of Neisseria meningitidis. Expert Rev Mol Diagn 6:79-87.

21. Drakopoulou Z., Kesanopoulos K., Sioumala M., Tambaki A., Kremastinou J., Tzanakaki G. (2008) Simultaneous single-tube PCR-based assay for the direct identification of the five most common meningococcal serogroups from clinical samples. FEMS Immunol Med Microbiol 53:178-82.

22. Drancourt M., Bollet C., Carlioz A., Martelin R., Gayral J.P., Raoult D. (2000) 16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. J Clin Microbiol 38:3623-30.

23. Duan Q.J., Shang S.Q., Wu Y.D. (2009) Rapid diagnosis of bacterial meningitis in children with fluorescence quantitative polymerase chain reaction amplification in the bacterial 16S rRNA gene. Eur J Pediatr 168:211-6.

24. Dubos F., De la Rocque F., Levy C., Bingen E., Aujard Y., Cohen R., Breart G., Gendrel D., Chalumeau M. (2008) Sensitivity of the bacterial meningitis score in 889 children with bacterial meningitis. J Pediatr 152:378-82.

25. Elnifro E.M., Ashshi A.M., Cooper R.J., Klapper P.E. (2000) Multiplex PCR: optimization and application in diagnostic virology. Clin Microbiol Rev 13:559-70.

26. Failace L., Wagner M., Chesky M., Scalco R., Jobim L.F. (2005) Simultaneous detection of Neisseria meningitidis, Haemophilus influenzae and Streptococcus sp. by polymerase chain reaction for the diagnosis of bacterial meningitis. Arq Neuropsiquiatr 63:920-4.

27. Goldenberger D., Kunzli A., Vogt P., Zbinden R., Altwegg M. (1997) Molecular diagnosis of bacterial endocarditis by broad-range PCR amplification and direct sequencing. J Clin Microbiol 35:2733-9.

28. Gray L.D., Fedorko D.P. (1992) Laboratory diagnosis of bacterial meningitis. Clin Microbiol Rev 5:130-45.

29. Greisen K., Loeffelholz M., Purohit A., Leong D. (1994) PCR primers and probes for the 16S rRNA gene of most species of pathogenic bacteria, including bacteria found in cerebrospinal fluid. J Clin Microbiol 32:335-51.

30. Grigoryan L., Burgerhof J.G., Degener J.E., Deschepper R., Lundborg C.S., Monnet D.L., Scicluna E.A., Birkin J., Haaijer-Ruskamp F.M. (2008) Determinants of self-medication with antibiotics in Europe: the impact of beliefs, country wealth and the healthcare system. J Antimicrob Chemother 61:1172-9.

31. Grigoryan L., Burgerhof J.G., Haaijer-Ruskamp F.M., Degener J.E., Deschepper R., Monnet D.L., Di Matteo A., Scicluna E.A., Bara A.C., Lundborg C.S., Birkin J. (2007) Is self-medication with antibiotics in Europe driven by prescribed use? J Antimicrob Chemother 59:152-6.

32. Hall L.M., Duke B., Urwin G. (1995) An approach to the identification of the pathogens of bacterial meningitis by the polymerase chain reaction. Eur J Clin Microbiol Infect Dis 14:1090-4.

33. Harris K.A., Hartley J.C. (2003) Development of broad-range 16S rDNA PCR for use in the routine diagnostic clinical microbiology service. J Med Microbiol 52:685-91.

34. Henegariu O., Heerema N.A., Dlouhy S.R., Vance G.H., Vogt P.H. (1997) Multiplex PCR: critical parameters and step-by-step protocol. Biotechniques 23:504-11.

35. Janda J.M., Abbott S.L. (2007) 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. J Clin Microbiol 45:2761-4.

36. Kaijalainen T., Kharit S.M., Kvetnaya A.S., Sirkia K., Herva E., Parkov O.V., Nohynek H. (2008) Invasive infections caused by Neisseria meningitidis, Haemophilus influenzae and Streptococcus pneumoniae among children in St Petersburg, Russia. Clin Microbiol Infect 14:507-10.
37. Kim K.S. (2003) Pathogenesis of bacterial meningitis: from bacteraemia to neuronal injury. Nat Rev Neurosci 4:376-85.

38. Kim K.S. (2003) Emerging molecular targets in the treatment of bacterial meningitis. Expert Opin Ther Targets 7:141-52.

39. Kim K.S. (2009) New diagnostic and therapeutic options in bacterial meningitis in infants and children. Minerva Pediatr 61:531-48.

40. Kim K.S. (2010) Acute bacterial meningitis in infants and children. Lancet Infect Dis 10:32-42.
41. Kotilainen P., Jalava J., Meurman O., Lehtonen O.P., Rintala E., Seppala O.P., Eerola E., Nikkari S. (1998) Diagnosis of meningococcal meningitis by broad-range bacterial PCR with cerebrospinal fluid. J Clin Microbiol 36:2205-9.

42. Kristiansen B.E., Ask E., Jenkins A., Fermer C., Radstrom P., Skold O. (1991) Rapid diagnosis of meningococcal meningitis by polymerase chain reaction. Lancet 337:1568-9.

43. Lorino G., Lilli D., Rivanera D., Guarino P., Angeletti S., Gherardi G., Filadoro F. (1999) Polymerase chain reaction, with sequencing, as a diagnostic tool in culture-negative bacterial meningitis. Clin Microbiol Infect 5:92-96.

44. Lu J.J., Perng C.L., Lee S.Y., Wan C.C. (2000) Use of PCR with universal primers and restriction endonuclease digestions for detection and identification of common bacterial pathogens in cerebrospinal fluid. J Clin Microbiol 38:2076-80.

45. McCabe K.M., Zhang Y.H., Huang B.L., Wagar E.A., McCabe E.R. (1999) Bacterial species identification after DNA amplification with a universal primer pair. Mol Genet Metab 66:205-11.

46. Mishal J., Embon A., Darawshe A., Kidon M., Magen E. (2008) Community acquired acute bacterial meningitis in children and adults: an 11-year survey in a community hospital in Israel. Eur J Intern Med 19:421-6.

47. N. Margall Coscojuela .,M. Majó Moreno.,C. Latorre Otín. D. Fontanals., Aymerich À.D.G.G.P.P. (2002) Use of Universal PCR on Cerebrospinal Fluid to Diagnose Bacterial Meningitis in Culture-Negative Patients. Eur J Clin Microbiol Infect Dis 21.

48. Ni H., Knight A.I., Cartwright K., Palmer W.H., McFadden J. (1992) Polymerase chain reaction for diagnosis of meningococcal meningitis. Lancet 340:1432-4.

49. Pada S., Lye D.C., Leo Y.S., Barkham T. (2009) Utility of 16S ribosomal DNA sequencing in the diagnosis of Staphylococcus lugdunensis native valve infective endocarditis: case report and literature review. Int J Infect Dis 13:e511-3.

50. Pandit L., Kumar S., Karunasagar I. (2005) Diagnosis of partially treated culture-negative bacterial meningitis using 16S rRNA universal primers and restriction endonuclease digestion. J Med Microbiol 54:539-42.

51. Peterson L.R., Dalhoff A. (2004) Towards targeted prescribing: will the cure for antimicrobial resistance be specific, directed therapy through improved diagnostic testing? J Antimicrob Chemother 53:902-5.

52. Radstrom P., Backman A., Qian N., Kragsbjerg P., Pahlson C., Olcen P. (1994) Detection of bacterial DNA in cerebrospinal fluid by an assay for simultaneous detection of Neisseria meningitidis, Haemophilus influenzae, and streptococci using a seminested PCR strategy. J Clin Microbiol 32:2738-44.

53. Rafi W., Chandramuki A., Mani R., Satishchandra P., Shankar S.K. (2010) Rapid diagnosis of acute bacterial meningitis: role of a broad range 16S rRNA polymerase chain reaction. J Emerg Med 38:225-30.

54. Ray B., Rylance G. (2009) QUESTION 1. Normal CSF: does it exclude meningitis? Arch Dis Child 94:988-91.

55. Ray P., Badarou-Acossi G., Viallon A., Boutoille D., Arthaud M., Trystram D., Riou B. (2007) Accuracy of the cerebrospinal fluid results to differentiate bacterial from non bacterial meningitis, in case of negative gram-stained smear. Am J Emerg Med 25:179-84.

56. Reier-Nilsen T., Farstad T., Nakstad B., Lauvrak V., Steinbakk M. (2009) Comparison of broad range 16S rDNA PCR and conventional blood culture for diagnosis of sepsis in the newborn: a case control study. BMC Pediatr 9:5.

57. Rivers B., Steck T.R. (2001) Viable but nonculturable uropathogenic bacteria are present in the mouse urinary tract following urinary tract infection and antibiotic therapy. Urol Res 29:60-6.

58. Saravolatz L.D., Manzor O., VanderVelde N., Pawlak J., Belian B. (2003) Broad-range bacterial polymerase chain reaction for early detection of bacterial meningitis. Clin Infect Dis 36:40-5.

59. Sauer P., Gallo J., Kesselova M., Kolar M., Koukalova D. (2005) Universal primers for detection of common bacterial pathogens causing prosthetic joint infection. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub 149:285-8.

60. Schuurman T., de Boer R.F., Kooistra-Smid A.M., van Zwet A.A. (2004) Prospective study of use of PCR amplification and sequencing of 16S ribosomal DNA from cerebrospinal fluid for diagnosis of bacterial meningitis in a clinical setting. J Clin Microbiol 42:734-40.

61. Seward R.J., Towner K.J. (2000) Evaluation of a PCR-immunoassay technique for detection of Neisseria meningitidis in cerebrospinal fluid and peripheral blood. J Med Microbiol 49:451-6.

62. Sontakke S., Cadenas M.B., Maggi R.G., Diniz P.P., Breitschwerdt E.B. (2009) Use of broad range16S rDNA PCR in clinical microbiology. J Microbiol Methods 76:217-25.

63. Taha M.K., Olcen P. (2004) Molecular genetic methods in diagnosis and direct characterization of acute bacterial central nervous system infections. APMIS 112:753-70.

64. Tamimi W., Al-Kharji N.H., Alanazi M., Felimban N., Al-Khulif A., Alharbi K.K. (2008) Cerebrospinal creatinine kinase level in children with meningitis. Clin Biochem 41:1025-7.

65. Tebruegge M., Curtis N. (2008) Epidemiology, etiology, pathogenesis, and diagnosis of recurrent bacterial meningitis. Clin Microbiol Rev 21:519-37.

66. Thomas K.E., Hasbun R., Jekel J., Quagliarello V.J. (2002) The diagnostic accuracy of Kernig's sign, Brudzinski's sign, and nuchal rigidity in adults with suspected meningitis. Clin Infect Dis 35:46-52.

67. Tunkel A.R., Scheld W.M. (1993) Pathogenesis and pathophysiology of bacterial meningitis. Annu Rev Med 44:103-20.

68. Tuyama M., Boente R.F., Rebelo M.C., Igreja R.P., Barroso D.E. (2008) The utility of the polymerase chain reaction assay for aetiologic definition of unspecified bacterial meningitis cases. Mem Inst Oswaldo Cruz 103:138-42.

69. Tzanakaki G., Tsopanomichalou M., Kesanopoulos K., Matzourani R., Sioumala M., Tabaki A., Kremastinou J. (2005) Simultaneous single-tube PCR assay for the detection of Neisseria meningitidis, Haemophilus influenzae type b and Streptococcus pneumoniae. Clin Microbiol Infect 11:386-90.

70. Unemo M., Fasth O., Fredlund H., Limnios A., Tapsall J. (2009) Phenotypic and genetic characterization of the 2008 WHO Neisseria gonorrhoeae reference strain panel intended for global quality assurance and quality control of gonococcal

antimicrobial resistance surveillance for public health purposes. J Antimicrob Chemother 63:1142-51.

71. Val C .Sheffield, David R. Coxt, Leonard S. Lerman, Richard M. myers (1989) Attachment of a 40-base-pair G+C-rich sequence (GC-clamp) to genomic DNA fragments by the polymerase chain reaction results in improved detection of single-base changes. Genetics86, pp. 232-236

72. Welinder-Olsson C., Dotevall L., Hogevik H., Jungnelius R., Trollfors B., Wahl M., Larsson P. (2007) Comparison of broad-range bacterial PCR and culture of cerebrospinal fluid for diagnosis of community-acquired bacterial meningitis. Clin Microbiol Infect 13:879-86.

73. Woo P.C., Ng K.H., Lau S.K., Yip K.T., Fung A.M., Leung K.W., Tam D.M., Que T.L., Yuen K.Y. (2003) Usefulness of the MicroSeq 500 16S ribosomal DNA-based bacterial identification system for identification of clinically significant bacterial isolates with ambiguous biochemical profiles. J Clin Microbiol 41:1996-2001.

74. Y. N. Sardessai. (2005) Viable but non-culturable bacteria: their impact on public health. CURRENT SCIENCE 89.

75. Yamamoto Y. (2002) PCR in diagnosis of infection: detection of bacteria in cerebrospinal fluids. Clin Diagn Lab Immunol 9:508-14.

References by personal communications:

- Dr. I. Arafeh (Palestine Ministry of Health) -July/2010 - PersonL Comunications.
- Dr. A. Saffen (Palestine Ministry of Health in)- July/2010-personal communications.

الكشف عن البكتيريا في سائل النخاع الشوكي بواسطة تقنية التفاعل التسلسلي للبوليميريز امورث 16S- rDNA

اعداد

ديمة كرم عبدالمنعم الزغير

اشراف

الدكتور سمير البرغوثي

ملخص بالعربية

مرض السحايا البكتيري او التهاب الدماغ الحموي هو التهاب يصيب الغشاء المبطن لسطح الدماغ والحبل الشوكي (ويكون بسبب فيروسي أو بكتيري أو فطري في أحيان أخرى) وان كان يمثل الالتهاب الفيروسي العدد الأعظم من الإصابات. الأعراض تختلف من شخص إلى آخر ولكن توجد بعض الأعراض المشتركة، صداع شديد وتصلب الرقبة. يتميز المرض بأنه ذو بدء فجائي أو تدريجي، حمى، قيء وخمول. يصاحب التهاب السحايا عند الأطفال الصغار انتفاخ اليافوخ وتصلب الرقبة والظهر في الأطفال الأكبر عمرا، وأحيانا تحدث غيبوبة وقد تصاحبها حمى خفيفة لعدة أيام مع ظهور أعراض التهاب الجهاز العصبي المركزي.

التهاب السحايا البكتيري من الخطورة تجعله سبباً سريعاً للوفاة في الأشخاص الأصحاء ذوي المناعة الجيدة ولكن في معظم الأحيان يستمر المرض من ٧ – ١٠ أيام ومن ثم يستعيد المريض صحته. ولكن التهاب السحايا البكتيرية من جهة أخرى لا يمكن شفاءها من غير التدخل العلاجي وقد ينتج عنه تلف اعاقي أو وفاة. السحايا البكتيرية تعتبر اقل انتشارا و لكنها اشد خطورة و البكتيريا الرئيسية المسببة له هي المكورات السحائية (*Neisseria meningitis*). إلا أن هناك مسببات أخرى لا تقل أهمية مثل الهيموفلس بي (*Haemophilus influenzae*) والمكورات الرئوي (*Streptococcus pneumoniae*) بالاضافة الى انواع اخرى من البكتيريا مثل اللستيريا *Liesteria monocytogenes)* وغيرها من البكتيريا التي قد تتمكن من الوصول الى سائل النخاع الشوكي.

يحدث المرض عادة في المجتمعات ذات المستوى الاقتصادي المتدني وفي كل الفصول، ولكن تزداد معدلات الحدوث في فصلي الشتاء والربيع ويحدث في شكل أوبئة في التجمعات المغلقة.

يتم التشخيص بالطرق التقليدية بواسطة عمل مزرعة لعزل البكتيريا من سائل النخاع الشوكي ثم فحص مجهري لشريحة مصبوغة بطريقة غرام (Gram).

وتعتبر الحالة «مشتبهة» لأي طفل دون الخمس سنوات ولديه أعراض الحمى الشوكية، مثل الحمى، الصداع، تصلب الرقبة، انتفاخ اليافوخ. وتعتبر «محتملة» لأي طفل دون الخامسة ولديه أعراض سريرية للحمى الشوكية مع سائل نخاعي شوكي معكر، وارتفاع مكونات البروتين في السائل النخاعي الشوكي(60 mg/100 ml - 15)، ونقص في مستوى السكر في السائل النخاعي (80 mg/100 ml - 50) عن المستوى الطبيعي المذكور، مع ارتفاع نسبة خلايا الدم البيضاء النيتروفيل (Neutrophils).

أما الحالة «المؤكدة» فهي للطفل الذي يقل عمره عن خمس سنوات ولديه أعراض سريرية للحمى الشوكية مع وجود البكتيريا الممرضة في مزرعة سائل النخاع الشوكي أو التعرف على الأجسام المضادة للجرثومة بواسطة اختبار الكرات لاتكس (Latex Agglutination Test) (LAT).

في هذه الدراسة تم التطرق الى الكشف المبدئي عن وجود أي نوع من البكتيريا في سائل السحايا ثم التوصل الى النوع البكتيري المسبب للالتهاب وهذا كان باستخدام تفاعل البوليميريز المتسلسل المتعدد (PCR) polymerase chain reaction وقد تكون الفحص من مرحلتين متوأمتين يستخدم في الاولى مزيج من البادئات العامة (General primers) or (G7 refereeing to The Universal Method)(UM) و التي تعمل على تضخيم قطعة من الجين(16S Ribosomal DNA) و هذه المرحلة تعتبر مهمة جدا لنفي او اثبات وجود الالتهاب البكتيري و الذي يعتبر اقل انتشارا و لشد خطورة.

اما الخطوة الثانية فتعتمد على محلول من البادئات الخاصة بأنواع معينة من البكتيريا(species specific primers) والتي تتسبب عادة بالتهاب السحايا البكتيريا وقد تم تحضير هذا المحلول و اختباره كاملا في مختبر جامعة القدس عن طريق استخدام اداة بحث الاصطفاف الاساسية (BLAST).

تم اختبار المحلول (Control) على الاصناف الاساسية من البكتيريا و التي عادة ما تسبب المرض و قد تم تطوير طريقة استخراج المادة الوراثية البكتيرية (DNA extraction) و اختبارها لعدة مرات للتأكد من قدرتها على استخراج ال (DNA)و خاصة من البكتيريا القابلة للصبغة الموجبة (Gram positive)ز

تم اخضاع ١٢٩ عينة من سائل النخاع الشوكي لمرحلتي التسخيص وقد اعطت ١٢٦ منها نتائج سلبية لمرض السحايا البكتيري خلال ساعات معدودة و هذا بحد ذاته يمثل اهمية هذه الدراسة بحيث انها نفت و جود مرض السحايا البكتيري و بالتالي ادت بالنتيجة الى عدم تعريض المريض لتعاطي المضادات الحيوية الكاملة (Broad spectrum antibiotics) في حال اعتماد الطريقة العادية للقيام بالفحص. و تعاطي المضادات باستمرار و بدون و جود التهاب بكتيري يؤدي الى تشكل مقاومة للمضادات الحيوية (Resistance) لدى البكتيريا و بالتالي فقدان فاعليتها مستقبلا.هذا كله بالاضافة الى تقليل معاناة المرضى و ايام مكوثهم في المستشفى اثناء انتظار نتائج الزراعة في حالة الاشتباه في اصابتهم بالتهاب السحايا البكتيري. اما العينات الثلاثة الباقية والتي اعطت نتائج ايجابية فكانت احداها بسبب المكورات السحائية (*Neisseria meningitidis*) و النتيجتين الاخريين اعطتا نتائج ايجابية ايضا باستخدام الطريقة العادية و تم التعرف على تسلسل القواعد النيتروجينية لهذه القطع المضخمة باستخدام التحليل التسلسلي المباشر (Sequencing) ثم استخدام اداة بحث الاصطفاف الاساسية (BLAST).

تؤكد الدراسة على اهمية استخدام طرق التشخيص و الكشف (Screening test) عن مرض التهاب السحايا البكتيري مما له من سرعة عالية و حساسية (sensitivity) لأقل كميات البكتيريا و هذا سيخفف من معانة المرضى خاصة الاطفال منهم كما سيخفف من حدوث مقاومة لدى البكتيريا (Resistance) للمضادات الحيوية. وسيكون لهذين الامرين اكبر الاثر في ادارة تشخيص و علاج المرض محليا وعالميا.

Appendices

A: Ribosomal DNA sequences and Primer Locations:

First location indicates species specific primers,

Second location is the QUGP-Rn3

Third location is the QUGP-Rn2

A-1 gi|16802048:243556 *Listeria monocytogenes* complete genome

CGAACGGAGGAAGAGCTTGCTCTTCCAATGTTAGTGGCGGACGGGTGAGTAACACGTGGGCAACCT
GCCTGTAAGTTGGGGATAACTCCGGGAAACCGGGGCTAATACCGAATGATAAAGTGTGGCGCATGC
CACGCTTTTGAAAGATGGTTTCGGCTATCGCTTACAGATGGGCCCGCGGTGCATTAGCTAGTTGGTA
GGGTAATGGCCTACCAAGGCAACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGAC
TGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCT
GACGGAGCAACGCCGCGTGTATGAAGAAGGTTTTCGGATCGTAAAGTACTGTTGTTAGAGAAGAAC
AAGGATAAGAGTAACTGCTTGTCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTACGTGCC
AGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGCGCGCAGG
CGGTCTTTTAAGTCTGATGTGAAAGCCCCCGGCTTAACCGGGGAGGGTCATTGGAAACTGGAAGAC
TGGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGATATGTGGAGGAA
CACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAA
ACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGC
CCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTC
AAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGA
ACCTTACCAGGTCTTGACATCCTTTGACCACTCTGGAGACAGAGCTTTCCCTTCGGGGACAAAGTGA
CAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
ACCCTTGATTTTAGTTGCCAGCATTTAGTTGGGCACTCTAAAGTGACTGCCGGTGCAAGCCGGAGGA
AGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATA
GTACAAAGGGTCGCGAAGCCGCGAGGTGGAGCTAATCCCATAAAACTATTCTCAGTTCGGATTGTA
GGCTGCAACTCGCCTACATGAAGCCGGAATCGCTAGTAATCGTGGATCAGCATGCCACGGTGAATA
CGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTAGG
GTAACCTTTATGGAGCCAGCCGCCGAAGGTGGGACAGATAATTGGGGTGAAGTCGTAACAAGGTA
GCCGTATCGGAAGGTGCGGCTGGATCACCTCCTTTCT

A-2 gi|225857809:c1829303-1827846 *Streptococcus pneumoniae* 70585, complete genome

TGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTAGAACGCTGAAGGAGGAGCTTGCT
TCTCTGGATGAGTTGCGAACGGGTGAGTAACGCGTAGGTAACCTGCCTGGTAGCGGGGGATAACTATTGGAAA
CGATAGCTAATACCGCATAAGAGTGGATGTTGCATGACATTTGCTTAAAAGGTGCACTTGCATCACTACCAGAT
GGACCTGCGTTGTATTAGCTAGTTGGTGGGGTAACGGCTCACCAAGGCGACGATACATAGCCGACCTGAGAGG
GTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAAT
GGACGGAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTAAGAGA
AGAACGAGTGTGAGAGTGGAAAGTTCACACTGTGACGGTATCTTACCAGAAAGGGACGGCTAACTACGTGCCA
GCAGCCGCGGTAATACGTAGGTCCCGAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTAGA
TAAGTCTGAAGTTAAAGGCTGTGGCTTAACCATAGTAGGCTTTGGAAACTGTTTAACTTGAGTGCAAGAGGGGA

GAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTG
GCTTGTAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTA
AACGATGAGTGCTAGGTGTTAGACCCTTTCCGGGGTTTAGTGCCGTAGCTAACGCATTAAGCACTCCGCCTGGG
GAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAAT
TCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCCTCTGACCGCTCTAGAGATAGAGTTTTCCTTCGGG
ACAGAGGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCG
CAACCCCTATTGTTAGTTGCCATCATTTAGTTGGGCACTCTAGCGAGACTGCCGGTAATAAACCGGAGGAAGGT
GGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGCTGGTACAACGAGTC
GCAAGCCGGTGACGGCAAGCTAATCTCTTAAAGCCAGTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATG
AAGTCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCG
TCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCGTAAGGAGCCAGCCGC

A-3 gi|254804028:142152-143695 *Neisseria meningitidis*, complete genome

TGAACATAAGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCATGCTTTACACATGCAAGTCGGACGGCAGC
ACAGAGAAGCTTGCTTCTTGGGTGGCGAGTGGCGAACGGGTGAGTAACATATCGGAACGTACCGAGTAGTGGG
GGATAACTGATCGAAAGATCAGCTAATACCGCATACGTCTTGAGAGAGAAAGCAGGGGACCTTTGGGCCTTGC
GCTATTCGAGCGGCCGATATCTGATTAGCTGGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCAGTAGCGG
GTCTGAGAGGATGATCCGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAAT
TTTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGTCTGAAGAAGGCCTTCGGGTTGTAAAGGACTTT
TGTCAGGGAAGAAAAGGCTGTTGCTAATACCAGCGGCTGATGACGGTACCTGAAGAATAAGCACCGGCTAACT
ACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGGGCGCAGA
CGGTTACTTAAGCAGGATGTGAAATCCCCGGGCTCAACCCGGGAACTGCGTTCTGAACTGGGTGACTCGAGTGT
GTCAGAGGGAGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGAGATGTGGAGGAATACCGATGGCGAAGG
CAGCCTCCTGGGACAACACTGACGTTCATGCCCGAAAGCGTGGGTAGCAAACAGGATTAGATACCCTGGTAGT
CCACGCCCTAAACGATGTCAATTAGCTGTTGGGCAACCTGATTGCTTGGTAGCGTAGCTAACGCGTGAAATTGA
CCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGATGATG
TGGATTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATGTACGGAATCCTCCGGAGACGGAGGAGT
GCCTTCGGGAGCCGTAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCG
CAACGAGCGCAACCCTTGTCATTAGTTGCCATCATTCAGTTGGGCACTCTAATGAGACTGCCGGTGACAAGCCG
GAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTATGACCAGGGCTTCACACGTCATACAATGGTCGGT
ACAGAGGGTAGCCAAGCCGCGAGGCGGAGCCAATCTCACAAAACCGATCGTAGTCCGGATTGCACTCTGCAAC
TCGAGTGCATGAAGTCGGAATCGCTAGTAATCGCAGGTCAGCATACTGCGGTGAATACGTTCCCGGGTCTTGTA
CACACCGCCCGTCACACCATGGGAGTGGGGGATACCAGAAGTAGGTAGGATAACCGCAAGGAGTCCGCTTACC
ACGGTATGCTTCATGACTGGGGTGAAGTCGTAACAAGGTAGCCGTAGGGGAACCTGCGGCTGGATCACCTCCTT
TCT

A-4 gi|162960935:c311276-309738 *Haemophilus influenzae* 86-028NP, complete genome

AATTGAAGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCAGGCTTAACACATGCAAGTCGAACGGTAGCAGGAGAAAGC
TTGCTTTCTTGCTGACGAGTGGCGGACGGGTGAGTAATGCTTGGGAATCTGGCTTATGGAGGGGGATAACGACGGGAAACTGT
CGCTAATACCGCGTATTATCGGAAGATGAAAGTGCGGGACTGAGAGGCCGCATGCCATAGGATGAGCCCAAGTGGGATTAGG
TAGTTGGTGGGGTAAATGCCTACCAAGCCTGCGATCTCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACG
GTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCGCAATGGGGGGAACCCTGACGCAGCCATGCCGCGTGAATGAAG
AAGGCCTTCGGGTTGTAAAGTTCTTTCGGTATTGAGGAAGGTTGATGTGATAATAGCACATCAAATTGACGTTAAATACAGAA
GAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATAACTGGGCGTAAAGGGC
ACGCAGGCGGTTATTTAAGTGAGGTGTGAAAGCCCTGGGCTTAACCTAGGAATTGCATTTCAGACTGGGTAACTAGAGTACTT
TAGGGAGGGGTAGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAATACCGAAGGCGAAGGCAGCCCCTTGGG
AATGTACTGACGCTCATGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGCTGTCGAT
TTGGGGGTTGGGGTTTAACTCTGGCGCCCGTAGCTAACGTGATAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACT
CAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGA
CATCCTAAGAAGAGCTCAGAGATGAGCTTGTGCCTTCGGGAACTTAGAGACAGGTGCTGATGGCTGTCGTCAGCTCGTGTTGT
GAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGACTTGGTCGGGAACTCAAAGGAGACTGC
CAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCTCTTACGAGTAGGGCTACACACGTGCTACAATGGC
GTATACAGAGGGAAGCGAAGCTGCGAGGTGGAGCGAATCTCATAAAGTACGTCTAAGTCCGGATTGGAGTCTGCAACTCGAC
TCCATGAAGTCGGAATCGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCA

CACCATGGGAGTGGGTTGTACCAGAAGTAGATAGCTTAACCTTTTGGAGGGCGTTTACCACGGTATGATTCATGACTGGGGTG
AAGTCGTAACAAGGTAACCGTAGGGGAACCTGCGGTTGGATCACCTCCTTA

B: BLAST result with N. meningitidis and Thioalkalivibrio sp. HL-EbGR7

```
>  ref|NC_011901.1|  Thioalkalivibrio sp. HL-EbGR7, complete genome
Length=3464554

 Features in this part of subject sequence:
   rRNA-16S ribosomal RNA

 Score = 1465 bits (793),  Expect = 0.0
 Identities = 1329/1576 (85%), Gaps = 83/1576 (5%)
 Strand=Plus/Minus

Query  8        AAGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCATGCTTTACACATGCAAGTCGGA  67
                |||||||||||||||||||||||||||||||||||||||||||| | ||||||||||||| |
Sbjct  2549776  AAGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCATGCCTAACACATGCAAGTCGAA  2549717

Query  68       CGGCAGCACAGAGAAGCTTGCTTCTTGGGTGGCGAGTGGCGAACGGGTGAGTAACATATC  127
                |||  |  |||| |  |||| |  |   | || ||||||||| ||||||||||||   |
Sbjct  2549716  CGGTA--ACAG-G-GGCTT-CGGC-CCGCTGACGAGTGGCGGACGGGTGAGTAACGCGTA  2549663

Query  128      GGAA-CGTACC-GAGTAGTGGGGGATAACT-GATCGAAAGATC-AGCTAATACCGCATAC  183
                |||| | | || | || |||||||| |||| |   ||||  || ||||||||||||||||
Sbjct  2549662  GGAATC-TGCCTG-GTGGTGGGGGACAACTCG-GGGAAA-CTCGAGCTAATACCGCATAC  2549607

Query  184      GTCTTGA-GAGAGAAAGCAGGGGACCTTTGGGCCTTGCGCTATTCGAG-CG-GCC-GATA  239
                | | | | | | ||||||| ||||| ||| || ||| |||| | || ||  | ||| |
Sbjct  2549606  GCCCT-ACGGGGGAAAGCGGGGGATCTTCGGACCTCGCGCCA-TC-AGATGAGCCTG-CG  2549551

Query  240      TCTGATTAGCTGGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCAGTAGCGGGTCTGA  299
                || |||||||| |||||||||||||| |||||||||||||||||||| ||||| |||||||
Sbjct  2549550  TCCGATTAGCTAGTTGGTGGGGTAATGGCCTACCAAGGCGACGATCGGTAGCTGGTCTGA  2549491

Query  300      GAGGATGATCCGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGT  359
                ||||| |||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2549490  GAGGACGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGT  2549431

Query  360      GGGGAATTTTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGTCTGAAGAAGGC  419
                ||||||| ||||||||||||||| ||||||||||||||| |||||||||| ||||||||||
Sbjct  2549430  GGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCAATGCCGCGTGTGTGAAGAAGGC  2549371

Query  420      CTTCGGGTTGTAAAGGACTTT-TGTCAGGGAAGAAAAGGCTGTTGCTAATACCAG-CGGC  477
                || |||||||||||| |||||  || || ||||||||| |||  | ||||||| | |||
Sbjct  2549370  CTGCGGGTTGTAAAGCACTTTCAGT-AGAGAAGAAAAGCCTGGCGTTAATACC-GTCGGG  2549313

Query  478      TGATGACGGT-ACCTGA-AGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAAT  535
                |  |||| || |||| | |||| ||||||||||||||| ||||||||||||||||||||
Sbjct  2549312  TCTTGAC-GTAACCT-ACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAAT  2549255

Query  536      ACGTAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGGGCGCAGACGGTTAC-T  594
                ||| |||||||| ||||||||||||||||||||||||||||||||  || || |||   | |
Sbjct  2549254  ACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGTAGGCGG--CCTT  2549197

Query  595      -TAAG-CAGGATGTGAAATCCCCGGGCTCAACCCGGGAACTGCGTTCTGA-ACTG--GGT  649
                 |||| | |||||||||| ||||||||| |||| ||||| |||  || || ||||  ||
Sbjct  2549196  GTAAGTC-GGATGTGAAAGCCCCGGGCTTAACCTGGGAATTGC-ATCCGATACTGCAGG-  2549140

Query  650      GACTCGAGTGT-GTCAGAGGGAG-GTAGAATTCCAC-GTGTAGCAGTGAAATGCGTAGAG  706
                | || |||| | || ||| |||| || ||||||| | ||||||| ||||||||||||||
```

```
Sbjct  2549139  G-CTAGAGTTTGGT-AGA-GGAGAGTGGAATTCC-CGGTGTAGCGGTGAAATGCGTAGAT  2549084

Query  707      AT-GTGGAGGAATACCGA-TGGCGAAGGCAGCCTC-CTGGGA-CAACACTGACGTTCATG  762
                || | ||||||| ||| | |||||||||| | ||| || ||| ||| ||||||| | | |
Sbjct  2549083  ATCG-GGAGGAACACC-AGTGGCGAAGGC-GACTCTCT-GGACCAAAACTGACGCTGAGG  2549028

Query  763      CCCGAAAGCGTGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATG  822
                  |||||||||||| |||||||||||||||||||||||||||||||||| |||||||||
Sbjct  2549027  TGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATG  2548968

Query  823      TCAATTAGCTGTT-GG-GCAACCTGATTGCTTGGTAGCGTAGCTAACGCGTGAAATTGAC  880
                  || |||| ||| || | |    ||   | ||| || ||| ||||||||| | || ||  |
Sbjct  2548967  AGAACTAGCCGTTGGGAGGATTATGCCT-CTTAGTGGCGCAGCTAACGCATTAAGTTCTC  2548909

Query  881      CGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGACCCGCACAAG  940
                |||||||||||||||| |||||| || |||||||||||||||||||| |||||||||
Sbjct  2548908  CGCCTGGGGAGTACGGCCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAG  2548849

Query  941      CGGTGGATG-ATGTGGATTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACAT-  998
                ||||||| | |||||| |||||||||||||||||||||||||||||||  ||||||||
Sbjct  2548848  CGGTGGA-GCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGCCCTTGACATC  2548790

Query  999      GTACGGAATCCT-CCGGAGACGGA--GGAGTGCCTTCGGGAGCCGTA-ACACAGGTGCTG  1054
                  | |||||| | |   | ||||  ||    | |||||||||||| ||| |   |||||||||
Sbjct  2548789  CT-CGGAA-CTTGTCAGAGA-TGACTTG-GTGCCTTCGGGAACCG-AGTGACAGGTGCTG  2548735

Query  1055     CATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACC  1114
                |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2548734  CATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACC  2548675

Query  1115     CTTGTCATTAGTTGCCATCA-TTCAGTTGGGCACTCTAATGAGACTGCCGGTGACAAGCC  1173
                ||||||  |||||||||| || ||| | |||| ||||||  |||||||||||||||||| ||
Sbjct  2548674  CTTGTCCCTAGTTGCCAGCACTTCGGGTGGGAACTCTAGGGAGACTGCCGGTGACAAACC  2548615

Query  1174     GGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTATGACCAGGGCTTCACACGT-  1232
                |||||||||||||||||||||||||||| |||||||||||||  |||||||  |||||||
Sbjct  2548614  GGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTATGGGCAGGGCTACACACGTG  2548555

Query  1233     CATACAATGGTCGGTACAGAGGGTAGCCAAGCCGCGAGGCGGAGCCAATCTCACAAAACC  1292
                | |||||||| | |||||||||||| ||||| |||||||| |||||||||| || ||| |
Sbjct  2548554  C-TACAATGGCCAGTACAGAGGGTTGCCAACCCGCGAGGGGGAGCCAATCCCAGAAAGCT  2548496

Query  1293     GATCGTAGTCCGGATTGCACTCTGCAACTCGAGTGCATGAAGTCGGAATCGCTAGTAATC  1352
                | |||||||||||||| | |||||||||||| | ||||||||||||||||||||||||||
Sbjct  2548495  GGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATC  2548436

Query  1353     GCAGG-TCAGCATA-CTGCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACA  1410
                || || ||||||||  | |||||||||||||||||||| ||||||||||||||||||||||
Sbjct  2548435  GC-GGATCAGCATTGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACA  2548377

Query  1411     CCATGGGAGTGGGG-GATACCAGAAGTAGGTAGGATAACCG-CAAGGAGTCCGCTTACCA  1468
                |||||||||| ||  |  |||||||||||| |||  |||||  |  ||||  || ||||||
Sbjct  2548376  CCATGGGAGTTGGCTGC-ACCAGAAGTAGATAGTCTAACCTTCG-GGAGGACGTTTACCA  2548319

Query  1469     CGGTATGCTTCA-TGACTGGGGTGAAGTCGTAACAAGGTAGCCGTAGGGGAACCTGCGGC  1527
                |||| || | || ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2548318  CGGTGTGGT-CAATGACTGGGGTGAAGTCGTAACAAGGTAGCCGTAGGGGAACCTGCGGC  2548260

Query  1528     TGGATCACCTCCTTTC  1543
                ||||||||||||||||
Sbjct  2548259  TGGATCACCTCCTTTC  2548244
```

C: Multiple sequence alignment for A35 vs H1:

```
CLUSTAL 2.0.10 multiple sequence alignment


A35-RN3         TTTCCTGTTTGCCTACCGCTTTCGCGCCTGCAGCGTCAGTAATGGGAAGGCAGGGAGCCG 60
H1-RN3          -AACCTGTTTGCTTACAGCTTTCGCGCCTG-AGCGTCAGTAACTGGGAC-CAGGGAGCCG 57
                   ********* *** ************ ***********  ** *  **********

A35-RN3         CTT--GCCACTGGTGTTCCTCCCGATATTTACGCATTTCACCGCTACACCGGGAATTCCA 118
H1-RN3          CCTCCGCCACTGGTGTTCCTCCCGATATCTACGCATTTCACCGCTACACCGGGAATTCCA 117
                * *  ********************** ******************************

A35-RN3         CTCCCCTCTGCCGTACTCTAGCCAGCCAGTTTCAAATGCAGTTCCCAGGTTGAGCCCGGG 178
H1-RN3          CTCCCCTCTGCCGTACTCTAGCCAGCCAGTATCAAATGCAGTTCCCAGGTTGAGCCCGGG 177
                ****************************** ******************************

A35-RN3         GATTTCACATCTGACTTAACTAACCGCCTACGCGCGCTTTACGCCCAGTAATTCCGATTA 238
H1-RN3          GATTTCACATCTGACTTAACTAACCGCCTACGCGCGCTTTACGCCCAGTAATTCCGATTA 237
                ************************************************************

A35-RN3         ACGCTTGCACCCTTCGTATTACCGCGGCTGCTGGA 273
H1-RN3          ACGCTTGCACCCTTCGTATTACCGCGGCTGCTGG- 271
                ***********************************
```