The use of molecular diagnostics to improve the detection of common bacterial and viral causes of community acquired meningitis in children, South Africa

By

JERMAINE KHUMALO

KHMJER001

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Supervisor(s): Dr Colleen Bamford and Dr Elloise duToit

DECLARATION

I, **Jermaine Khumalo**..., hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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CONTENTS

Abstract

With conventional methods of diagnosis, substantial overlaps are common due to an absence of the expected CSF findings clearly aligning to bacterial or viral infection. Reduced sensitivity is commonly observed chiefly due to empiric antibiotic treatment leading to bacterial culture negative results. This leads to costly hospital admissions and unnecessarily prolonged treatment for the unexplained aetiology, further compounded by routine viral diagnostics not being commonly implemented for meningitis diagnosis. We developed and validated in-house quantitative real-time (qPCR) multiplex assays to test for bacterial causes namely: *Neisseria meningitidis* (*ctrA* gene), *Haemophilus influenzae* (*hpd* gene) and *Streptococcus pneumoniae* (*lytA* gene) and viral causes namely: enterovirus (5‟ UTR), herpes simplex (UL30 gene) and mumps virus (Fusion protein gene). The qPCR assays were carried out on the Biorad CFX 96 real-time instrument. These validated assays were used to screen a cohort of suspected meningitis cases. The retrospective study included 300 paediatric patients aged from 60 days-12 years, over a 1-year period (November1, 2012 to November 30, 2013) with suspected meningitis presented to the outpatient departments of the Red Cross War Memorial Children‟s Hospital (RCCH) in Cape Town. Cerebrospinal fluid with abnormal chemistry and cell counts was selected and total nucleic acid was extracted with the QIAsymphony virus/bacterial DSP kit (QIAGEN, Valencia, CA). The median age of children was 19 months (IQR: 6-65 months). Among the screened 291 CSF samples, 7 (2.4%) cases Gram stain results were obtained along with relatively few cases with positive bacterial culture growth 4/291 (1.4%). Based on bacterial qPCR results, 8 (2.7%), 3 (1%) and 1 (0.3%) were positive for *S. pneumoniae, N. meningitidis and H. influenzae* respectively. A majority of cases were viral positives with enteroviruses being the dominant at 91/291 (31.3%) and mumps *virus* 3/291 (1%). No herpes simplex DNA was detected. The bacterial qPCR showed a sensitivity and specificity of 85.7% and 97.7% respectively when compared against a composite reference standard (CRS). We report an improvement with additional detected causes of bacterial meningitis and highlight the burden of the common viral causes. However, a large proportion of cases (63.6 %) have aetiology still unknown. PCR shows valuable in concluding viral aetiology in routine diagnosis.

ABBREVIATIONS

% : percent

bp : base pairs

CFU : colony forming units

CI : confidence interval

DNA : deoxyribonucleic acid

EDTA : ethylenediaminetetra-acetic acid

EtBr : ethidium bromide

g : gram(s)

gDNA : genomic DNA

GSH : Groote Schuur hospital

IAC : internal amplification control

IMCI: Integrated Management of Childhood Illness

IPTG : isopropyl-β-D-thio-galactosidase

IQR : interquartile quartile ranges

L : litre

LB : Luria Bertani

M : molar

mg : milligram

min : minute(s)

ml : millilitre

mM : millimolar

n : number of samples

ng : nanogram

nm : nanometre

^{o}C: degree(s) Celsius

PCR : polymerase chain reaction

PFU : Plaque forming units

qPCR : quantitative real time PCR

RNA : ribonucleic acid

RXH: Red Cross War Memorial Children‟s Hospital

SDS : sodium dodecyl sulphate

sec : second(s)

SOC :super optimal broth with catabolite repression

Std. Dev : standard deviation

TAE : tris-acetate EDTA

TAE : tris-acetate-EDTA electrophoresis buffer

Taq : Thermus aquaticus

TB : tuberculosis

Tris : tris(hydroxymethyl)aminomethane

v/v : volume per volume

V: volts

WHO : world health organisation

w/v : weight per volume

X-gal : 5-bromo-4chloro-3indolyl-β-D-galactosidase

YT : yeast- tryptone

µg : microgram

µl : microlitre

µM: micromolar

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CHAPTER I

LITERATURE REVIEW

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1.0 Meningitis

Meningitis is an inflammation of the leptomeninges, the membranes that surround the brain and spinal cord due to infection of the central nervous system (CNS)[1]. The syndrome is potentially life threatening, affecting all ages, both adults and children, with significant mortality and morbidity amongst the populations globally. It accounts for approximately 7% of deaths globally in children below the age of 5 years[2]. Incidence rates are highest in endemic regions (>10 cases/100,000 population/year) such as the African Meningitis belt (mostly due to meningococcal infection) stretching from Senegal to Ethiopia with moderate endemic incidence rates (2-10 cases/100,000 population/year) in parts of Europe, Asia and South Africa[3].

Most cases of infectious meningitis are due to bacteria and viruses[4]. However, the aetiology is different in immuno-compromised or immunosuppressed patients where causes of meningitis may include fungi, mycobacteria, parasites, mycoplasma, rickettsia and spirochetes[5,6]. Drug induced aseptic meningitis although rare, along with other causes such as carcinomas and primary tumours are other causes that are generally non-infective[7].

Bacterial meningitis (BM) is the most serious cause of meningitis, with up to one million invasive cases of non-epidemic bacterial meningitis and approximately 170 000 fatalities every year worldwide[8]. Bacterial meningitis ranks among the top ten causes of death in children younger than 14 years in high-income countries[9,10]. A delay in a definitive diagnosis and treatment can lead to severe outcomes including death, with 10 - 20% of survivors experiencing permanent and severe neurological damage [11,12].

Viral meningitis (VM) is usually self-limiting. However, if left untreated, meningitis due to certain viruses (herpes virus, Japanese virus encephalitis), it can result in severe or fatal outcomes[5]. VM is commonly known as aseptic meningitis implying a clinical presentation of meningeal inflammation in the absence of any identifiable bacterial agents in the CSF[13]. This mostly occurs in situations of limited diagnostics or in outbreaks.

1.1 Causes of Meningitis

The identification of a specific causative pathogen is mainly dependent on any underlying comorbidity or predisposing immune state of the individual, geographical area for exposure to prevailing cause,

vaccination state, transmission route of pathogens and age[14]. These factors determine the relative frequency of the different pathogens[15].

1.1.1 Bacterial meningitis

Any pathogenic bacterial organism is able to cause meningitis once in the CNS. However, *Streptococcus pneumoniae* (*S. pneumoniae*), *Neisseria meningitidis* (*N. meningitidis*) and *Haemophilus influenzae* (*H. influenzae*) type b (Hib) are the most common causes of bacterial meningitis in children between the ages of 2 months and early adolescence[8–10]. These species accounted for 75 - 80% of outbreak BM cases globally[16]. However, their relative proportions are reduced by the availability of vaccines. Prevalence varies with geographical location thus populations in areas classically endemic or hyperendemic for any of the pathogens are at greatest risk (the African meningitis belt)[10].

In South Africa in 2013, *S. pneumoniae* and *N. meningitidis* were the commonest causes of bacterial meningitis in children, while *H. influenzae* type b cases occur occasionally. The low incidence of invasive *H. influenzae* type b (Hib) is due to the introduction of Hib vaccination into public immunization programs in 1999 which resulted in a 65% decrease in cases of invasive Hib in children < 1 year[17,18]. *S. pneumoniae* remains the leading vaccine preventable cause of childhood mortality in South Africa[19] even with the introduction of pneumococcal polysaccharide-protein conjugate vaccine (PCV) into public sector immunisation schedules since 2009[18,20]. This persistence is due to non-vaccine targeted serotypes causing infection[21]. However, the PCV vaccine has reduced vaccine serotype-specific invasive pneumococcal disease dramatically, by 65% and 83% in HIV-infected and non HIV- infected children respectively[19].

Other bacterial pathogens can be causative agents of meningitis in some individuals with predisposing conditions. These organisms include aerobic Gram-negative organisms (*Pseudomonas*, *Acinetobacter*, *Salmonella*, *Klebsiella*, *Serratia spp*) and Gram-positives (*Staphylococci spp*, *Enterococcus*)[22,23]. Age also influences the bacterial causes, with infection in neonates and young infants (<2 months) predominantly caused by *Listeria monocytogenes*, *Streptococcus agalactiae* and *Escherichia coli*[4,9] .

1.1.1.1 *Streptococcus pneumoniae*

S. pneumoniae is a Gram-positive coccus which is one of the common causes of meningitis[24]. With more than 90 different serotypes which vary geographically, only a limited number of serotypes cause invasive disease[25]. It is a common coloniser of the nasopharynx and upon infection, subsequent invasion of the CNS, it initiates cellular and tissue damage resulting in inflammation of the leptomeninges[26].

The mortality rates associated with disease caused by this bacterium vary with age, geographical location and serotype of pneumococcus[27]. There is a high case fatality rate (CFR) worldwide of pneumococcal meningitis of 59% (95% CI 27 - 80%) for children less than 5 years of age, with Africa having a CFR of 73 % (95% CI 18 - 94%)[28]. However, vaccines have been developed against pneumococcal infection. Initially polysaccharide vaccines were used but now conjugate vaccines (polysaccharide and protein conjugates) are being employed which elicit better immune response against a wide number of pneumococcal serotypes. The introduction of this vaccine has reduced the burden of disease, and led to the emergence of non-vaccine strains causing invasive cases[29].

1.1.1.2 *Neisseria meningitidis*

N. meningitidis, commonly called meningococcus, is a Gram-negative diplococcus. It is a fastidious bacterium and a commensal of the nasopharynx[30]. This species is comprised of 12 serogroups classified according to the specific polysaccharide antigenic markers on the capsule[31]. Serogroup A, B, C, W135, X and Y account for most infections worldwide[32]. It is considered one of the main causes of community acquired meningitis in children and adults globally[3].

Meningococcal meningitis is a communicable disease largely epidemic in the meningitis belt, with increased incidence owing to Serogroup A, C, X and W135[33,34]. Incidence rates are high in children, teenagers and adults[35]. Meningococcal polysaccharide and conjugate vaccines have been introduced in developed countries against serogroups A and C resulting in a significant decrease in disease incidence[36]. Recently a vaccine against meningococcal B (4cMenB; Bexsero) has been introduced for children in Europe providing an improved efficacy and strong immune response against a wide range of circulating serotype b strains[37–39]. Currently there is no meningococcal vaccine available in public immunisation programs in South Africa.

1.1.1.3 *Haemophilus influenzae*

H. influenzae is a Gram-negative coccobacillus and is a commensal of the upper respiratory tract. Some *H. influenzae* strains have a carbohydrate capsule (polyribosyl ribitol phosphate) which acts as a protective barrier against complement attack during an immune response. This capsule is a recognised virulence factor which allows the bacterium to evade the immune system by being able to resist phagocytosis by polymorphonuclear leukocytes. This occurs as a result of the absence of specific anti-capsular antibodies being formed against it by the immune system[40]. Some strains are non-encapsulated and are thus non typeable[40]. The different strains are classified according to the production of the polysaccharide capsule which is antigenically distinct. The generally recognised encapsulated types include a, b, c, d, e and f. These strains have been implicated in disease with *H. influenzae* type b (Hib) being the most common cause[41].

Paediatric immunisation with the conjugate vaccine has led to near eradication of Hib which was responsible for 99% of *H. influenzae* meningitis cases in high income countries[42]. However, meningitis due to non-Hib has now emerged although the rate of infection is low[17,40,43].

1.1.2 Viral meningitis

Viruses cause inflammation of the meninges and in complicated cases may also cause inflammation of the brain parenchyma or spinal cord leading to meningoencephalitis and meningomyelitis respectively[5,44]. Viral meningitis is usually self-limiting and generally causes lower morbidity and mortality than bacterial meningitis[1,44–46] with the exception of neonatal patients where cases can be severe and fatal[5,47]. However, the course of infection can be significantly prolonged in the case of more severe meningoencephalitis or meningomyelitis[5]. Young children (<5 years) and individuals with predisposed immune states (immunocompromised and immunosuppressed) are most at risk of severe VM[48]. Futhermore a lack of vaccination and crowded facilities (e.g. military camps, day-care centers) increase the risk of transmission from infected individuals[49,50].

Enteroviruses (EVs) are the most common cause of meningitis in both children and adults commonly accounting for 80% of VM cases worldwide[1]. Another important cause of viral meningitis are the herpes viruses, especially herpes simplex virus 1 and 2. Some herpes viruses are commonly identified as causes of aseptic meningitis in immunocompromised patients, for example, cytomegalovirus, varicella zoster virus, epstein barr virus and human herpes virus 6[47,51]. Mumps meningitis is commonly seen in non-

vaccinated individuals (mostly children) with males more susceptible to infection than females[51]. Flaviviruses, which are tick-borne or mosquito-borne viruses, are common causes of viral meningitis in specific geographical areas. The viruses include Japanese encephalitis virus affecting southeast parts of Asia, and West Nile virus affecting the Middle East and Africa[44,51]. Endemicity of the viral causes is generally determined by geographical location and seasonality[47,51]. Other viral causes are extremely rare e.g. lymphocytic choriomeningitis virus which is mostly acquired from house mice[52].

In South Africa, mumps and EVs have been the only documented causes of viral meningitis. This was observed in a report involving 11 360 cases of viral meningitis in children (average population age of 3 years) investigated in Cape Town between 1981 and 1989[53]. The data on the burden of viral meningitis is limited.

1.1.2.1 Enteroviruses

Enteroviruses are non-enveloped single-strand RNA viruses, from the Picornaviridae family composed of 12 species with more than 100 serotypes[54]. They are the leading cause of aseptic meningitis and the most frequent CNS infection worldwide, especially in developing countries[55–57]. Oral-faecal route is the most common transmission route although the respiratory secretions from infected persons can also be involved in transmission. They are ubiquitous viruses common in the intestinal tract, with clinical manifestations including pharyngitis, pleurodynia and pericarditis. Neurotropic serotypes (coxsackievirus A; coxsackievirus B; Echovirus 4, 6, 9, 11, 16, 30, 33) account for 85% of all acute viral meningitis cases with some strongly neurotropic types (e.g. EV 70 and 71) associated with more severe meningoencephalitis, encephalitis, Guillian-Barre syndrome and cerebellar ataxia[5,58]. Hand foot and mouth disease is also commonly associated with coxsackievirus infection with blister formation on the hands, feet and mouth cavity and usually occurs in children <10 years of age[59]. Overall, EV meningitis is more common in children than in adults[60,61].

1.1.2.2 Herpes simplex

Herpes simplex is an enveloped ubiquitous double-stranded DNA virus in the Herpesviridae family with two main subtypes; HSV 1 and HSV 2. HSV is a common cause of encephalitis, but also causes a wide spectrum of clinical manifestations in the CNS with both subtypes causing acute meningitis or meningo-encephalitis[62]. These subtypes can be distinguished by their antigenicity to specific monoclonal antisera. Clinical distinction of the subtypes by the location of lesions (i.e. HSV1-head/face, HSV2-genitalia) is also used although it is not uncommon to encounter each subtype in the reverse location[63,64]. The Herpes

viruses account collectively for 4% of viral meningitis cases, mostly due to HSV 2[5]. HSV1 is the common cause of sporadic encephalitis. Both subtypes result in Mollaret meningitis, a recurrent inflammation which is benign and self-limiting. Following initial infection, the viruses may remain latent in the trigeminal or lumbar ganglia to later cause recurrent disease[63,64].

1.1.2.3 Mumps virus

Mumps virus is an enveloped single-strand RNA virus which belongs to the Paramyxoviridae family. It causes aseptic meningitis and encephalitis, as well as parotitis and orchitis or oophoris, mainly in children. As mumps virus is a vaccine preventable disease[65], vaccination programs have reduced the incidence of mumps infection worldwide. It is responsible for 10 - 20% of meningitis/meningoencephalitis cases in non-vaccine areas (low income countries) but is still a recognised cause of meningitis in some vaccine areas in England and Japan[5,66–68] However, vaccine introduction in developed countries has led to a remarkable decrease in disease incidence[69,70].

Mumps epidemics have re-emerged with rising incidence mostly in undeveloped nations[71–74]. It mostly affects children in populations not routinely receiving mumps vaccination, while sporadic cases in developed countries arise due to vaccination failures or waning immunity to mumps virus[55]. Cases of vaccine strain-induced meningitis can occur as a result of poorly attenuated strains used during immunization programs[66–68]. Also, outbreaks have emerged in the United Kingdom and the American Midwest post vaccination, in young adults, mainly due to the insufficient herd immunity in this high risk exposure setting (college students) which is prone to outbreaks[67]. These occasional outbreaks have increased the interest in mumps infection.

1.2 Clinical presentation

Assessing the clinical presentation is paramount in distinguishing a possible meningitis diagnosis. With signs and symptoms suggestive of meningitis, a lumbar puncture is carried out for further cerebrospinal fluid analysis.

The manifestation of clinical symptoms varies with age. In adults the symptoms are usually, but not always distinctive, whereas in children, particularly infants, they can be subtle, variable and atypical. The classical presentations of meningitis include: fever, nausea with vomiting, severe headache, photophobia,

and an altered mental status[75,76]. Seizures are also common at the onset of disease and with progressively worsening disease[77]. Upon infection, there is immediate and continued mounting of an immune response (inflammation), neck stiffness along with Brudzinski‟s and Kernig‟s signs, observed in both adults and children[6,77]. Any two of the classical symptoms are present in 95% of meningitis cases both in children and adults[6]. Although symptoms of meningitis are common to all causes, some associated features may be specific for certain aetiologies, for example parotitis and orchitis observed with the mumps virus, a purpuric rash is typical with infection by *N. meningitidis*, and history of head trauma/chronically draining ear being associated with pneumococcal meningitis[77]. Some pathogens have typical associated signs but infected infants mostly exhibit atypical symptoms thus analysis of the CSF is necessary for a definitive diagnosis.

1.3 Laboratory diagnosis of meningitis

Cerebrospinal fluid (CSF) plays a crucial role in maintaining the internal environment of the brain and surrounding neuronal environment. Any external interference, mainly from infection, introduces a homeostatic imbalance, altering the normal physiology[78]. Changes in CSF resulting from infection can be assessed by examination and analysis of CSF, involving determination of the total and differential white cell count, as well as performance of a Gram stain and biochemical tests to measure glucose and protein concentration[79].

Normal CSF contains electrolytes, active molecules and catabolites, which are constantly regulated[80]. On performing a lumbar puncture, the expected opening pressure of CSF in both adults and children is 60-200mmH_2O[81]. Any deviation from this is indicative of abnormality. CSF is normally clear, colourless and generally acellular[82]. However, white blood cells can be found with a limit of <6 cells/µl in neonates, <9 cells/µl in children and <5 cells/µl in adults[81]. Red blood cells (RBC) can also be present, but are less common as they are known to lyse in the CSF with prolonged presence (>12 hours)[83]. This leads to xanthochromia, a yellow discoloration of the CSF due to the presence of bilirubin from the haemolysed RBC. If significant numbers of RBCs are found in the CSF, either due to a traumatic spinal tap or to recent haemorrhage in the brain, the CSF white blood cell count should be adjusted to account for the associated leukocytes that could have been introduced[84]. Normal CSF has a protein concentration of 23 to 38 mg/dL in adults and 20 to 170 mg/dL in neonates. The lumbar glucose concentration is measured relative to the serum glucose concentration which is standard at a ratio of 0.6, with a tolerance concentration range of 6 to 18 mg/dL[81].

On infection by a pathogen, these parameters change. White blood cells migrate to the site of infection thus increasing their number. Elevated protein concentrations are common during infection in some cases due to the WBC and inflammatory cells from the immune system along with the replicating infective species (e.g. bacteria) which can be largely composed of protein[85]. Glucose levels can be lowered during CNS infection or remain normal depending on the infecting agent. These changes are different, varying mostly with infecting aetiology. Bacterial infection normally results in an increase in white blood cells, predominantly neutrophils with an accompanying low glucose concentration compared to the serum glucose[6,77]. Aseptic meningitis, commonly attributed to viral infection, normally presents with a mild increase in protein, a predominance in lymphocytes, and normal glucose levels[6,9].

Table 1 Cerebrospinal fluid analysis in central nervous system infection[81]

	Glucose (mg/dL)		**Protein (mg/dL)**		**Total white blood cell count (cells/µL)**		
	<10*	10 to 45$^{\Delta}$	>250$^{\bullet}$	50 to 250$^{\lozenge}$	>1000	100 to 1000	5 to 100
More common	Bacterial meningitis	Bacterial meningitis	Bacterial meningitis	Viral meningitis Nervous system Lyme disease (neuroborreliosis) Neurosyphilis	Bacterial meningitis	Bacterial or viral meningitis TB meningitis	Early Bacterial meningitis Viral meningitis Neurosyphilis TB meningitis
Less common	TB meningitis Fungal meningitis	Neurosyphilis Some viral infections (such as mumps and LCMV)	TB meningitis		Some cases of mumps and LCMV	Encephalitis	Encephalitis

LCMV: *Lymphocyte choriomeningitis virus*; TB: *Tuberculosis*.

* - <0.6 mmol/L

$^{\bullet}$ - >2.5 g/L

$^{\Delta}$ - 0.6 to 2.5 mmol/L

$^{\lozenge}$ - 0.5 to 2.5 g/L

1.4 Microbiological tests for diagnosis of meningitis

Various microbiological tests are used to detect both bacterial and viral causes of meningitis. However, culture of CSF remains the gold standard test for confirming bacterial meningitis with 80-90% of cases resulting in positive bacterial growth in the absence of prior antibiotic treatment[86]. A blood sample cultured in the laboratory has shown to be a confirmatory test in the identification of organisms causing bacterial meningitis along with the antibiotic profile of the organism[87]. Accompanying bacterioscopic methods, mainly Gram staining, are employed to help differentiate the bacterial morphology. Cytospin

centrifugation applied prior to Gram staining, concentrates the bacteria, thus improving the sensitivity (100 to 1000 fold) in clinical samples with low bacterial loads ($<10^4$ cells)[76,88].

Antigen detection tests can be used for determining the aetiology of meningitis. Rapid antigen detection tests including latex agglutination and immunohistochromatographic tests, which use monoclonal or polyclonal antibodies targeting a specific antigen are seldom used[89,90]. These antibody-antigen assays are less likely to be affected by empirical treatment[90]. In addition, counterimmunoelectrophoresis (CIE) has been used for the diagnosis of meningitis due to *H. influenzae* and selected serotypes of *N. meningitidis* (serotypes A, B, C, and W135). CIE detects specific antigens on the polysaccharide capsule common to these bacteria with the use of horse and sheep antisera[89,91] but requires a confirmatory accompanying test due to common false negative results[92].

Another test used although not common involves determining the presence of lactate. This compound accumulates in CSF during infection as a result of bacterial catabolism and has been shown to be a marker for differentiating between bacterial and viral infection[93]. Its high negative likelihood ratio (low probability of disease if the test is negative) makes it reliable in ruling out bacterial meningitis in combination with the other CSF tested characteristics shown in table 1[93].

For viral diagnosis, the gold standard has been cell culture. Recently, cell culture was abandoned for *HSV* and EV due to the availability of more sensitive accredited (FDA and IVD approved) molecular tests[94–96]. The molecular tests not only increased the sensitivity but they reduced the time of analysis and improved the ability of simultaneous multitarget detection (with multiplex assays) thus proving valuable for clinical diagnostics. However, cell culture in research laboratories is still being used due to its ability to characterise and identify viable viral pathogens and even diagnose unexpected or unidentified viruses[96,97].

Serological tests are also possible, e.g. testing for specific antibodies of IgG and IgM in CSF, fluorescent antibody staining/immunofluorescence (IF) and enzyme linked immunosorbent assays (ELISA)[94,98] in the CSF.

1.5 Limitations of traditional detection methods

Traditional detection methods aid in diagnosis of meningitis, but have numerous limitations. Microscopic examination of Gram-stained CSF is highly sensitive with a noted sensitivity of 92% and highly specific in early diagnosis of bacterial meningitis[99]. However, with prior antibiotic treatment, detection by microscopy leads to a reduction in sensitivity, especially in samples with a low bacterial concentration (CFU $<10^4$/ml of CSF)[94,100]. Upon suspicion of bacterial meningitis in children, the attending physician prescribes antibiotics according to the Integrated Management of Childhood Illness (IMCI) program of the WHO to prevent adverse neurological effects with delay in confirmation of diagnosis and treatment. This has seen CSF becoming sterile upon bacterial culturing within 2-6 hours of administration of the antibiotics[101] leading to false negatives observed in 13% - 30% of CSF culture[6,102]. Prior antibiotic administration not only reduces microscopy and culture sensitivity but also leads to unnecessary treatment as fear of possible false negative results leads doctors to treat patients with self-limiting aseptic meningitis with antibiotics[85]. Culturing of peripheral sample sources (e.g. blood) have been used to confirm the aetiology and this has aided in the confirmatory identification in 50% - 80% of cases in children[9].

Prior to the advent of the Polymerase chain reaction (PCR), definitive diagnosis of CNS viral infection was dependent upon either virus isolation from CSF, brain biopsy (a highly invasive procedure), or a positive virus specific intrathecal antibody response[103,104]. Cell culture involves the replication of viral pathogens in suitable host cells for >6 days. Cell culture is not possible for every pathogen due to limited cell lines available for viral cultures and the fastidious nature of certain viruses. It is also labour intensive and requires highly experienced staff. Viral culture compared to nucleic acid testing showed limited benefit in a Finnish cohort of 3231 patients which reported total positive recovery of 1.9% of CSF positive viral culture growth while PCR had a total recovery of 5.1% yielding more diagnoses[105]. Similarly in a review of 22 394 CSF cultures by Polage and Petti, viruses other than enteroviruses or herpes viruses were recovered in <0.1%[106]. Additionally, the overall turnaround time for viral culture, is slow. Thus the results are not clinically relevant, and clinicians are forced to rely on clinical decision making[103,104].

Rapid antigen detection tests are now widely used in routine laboratories for the uncultivable viruses, but have been shown to have poor sensitivity and specificity. Antibody tests are limited by a delayed

intrathecal antibody response from a prolonged primary immune response (4 to 10 days to elicit effective antibody against the antigen), thus leading to false negatives[107].

Given the observed limitations, models to predict diagnosis for either bacterial or viral meningitis have been created. However, the use of these models requires expertise[108,109]. The models seek to combine and assess all the imperfect tests to provide a much more precise diagnosis. However, with no single diagnostic clinical feature, a combination choice of clinical features for an accurate diagnosis is still unclear[75].

1.6 Polymerase chain reaction

Nucleic acid tests (NATs) have demonstrated significantly superior sensitivity, accuracy and a shortened turnaround time compared to culture-based methods for determining the aetiology of meningitis[65]. The most commonly used NAT has been the PCR which amplifies specific target nucleic acid from clinical samples (CSF, sera or petechiae). This identifies specific targeted causative organisms. It allows for improved identification of pathogens that are nonviable, uncultivable, or fastidious[96,106]. PCR was shown to be less affected by reduced bacterial load due to prior antibiotics use compared to bacterial culture[90].

The PCR has become a valuable tool for targeted specific amplification of various genetic materials. An accompanying high accuracy and reliability in detection for the presence or absence of a specific gene and analysis of gene expression has made it the most widely used tool in diagnostics. It involves the exponential amplification of a primer-directed target and subsequent identification of the target amplicon. The primed template is double stranded DNA which is exposed to preset reaction conditions for amplification in a thermocycler[110]. Some genomic target material is RNA based thus a modified PCR method is utilised, Reverse Transcriptase (RT)-PCR. RT-PCR involves the initial conversion of RNA into complementary DNA (cDNA) for polymerisation by reverse transcribing the target RNA. There is then subsequent amplification and detection of the formed cDNA by the PCR primers. Multiple targets can simultaneously be detected with the use of different primer sets.

Commonly, PCR detection of the amplicon is endpoint based, meaning that reliance is on the final accumulated amplicon for confirmation of detection and involves post-PCR processing (e.g. gel electrophoresis). However, endpoint detection reduces the sensitivity of detection as identification is

based on visualising a specific sized target amplicon band. This also introduces subjectivity in differentiating the amplicon band from surrounding artefacts[111]. Other endpoint detection methods involve the transfer of the gel electrophoresed amplicons on to nylon membranes and addition of specific radioactively labelled probes which hybridise with the specific amplicon sequence and fluoresce through autoradiography revealing the specific target amplicon[112]. The additional radioactive material requires careful handling and proper disposal after use. Enzyme linked immunosorbent assays (ELISA) have been used in endpoint detection, with specific non-radioactive digoxygenin (DIG) labels added to the PCR amplicons which are then detected by biotynlated tagged probes specific for the target amplicons giving a colorimetric result[113].These all involve extra post-PCR processing which is labour intensive and standardisation of the procedures is difficult[111,113]. With limited to no multi-panel detection assays available[16] numerous in-house PCR assays are being used which rely on post-PCR processing for endpoint detection[114].

Early detection techniques that use endpoint and competitive quantitative methods have given way to real-time determinations (real-time PCR). Numerous assays have been developed for diagnostic testing, targeting most currently known viruses of clinical importance[115]. Real-time PCR does not have these laborious post-PCR processes and offers rapid a processing time due its improved thermocycling capabilities which include a smaller amplicon target and specific real-time detection[116]. There is a reduced carry-over contamination risk with absence of the varied post-PCR amplification processing[117]. The real-time determination allows for efficient detection of low quantities of template and also allows for quantitation with the introduction of hybridisation, hydrolysis probes, molecular beacons or DNA intercalates for detection[116]. All of these aspects are favourable in a busy diagnostic laboratory and are of clinical relevance for initiation of appropriate therapy.

1.7 The utility of real-time PCR in the diagnosis of meningitis for the common bacterial and viral aetiologies

The culture-independent NATs have the ability to identify the causative agent of meningitis which is vital for determining timeous treatment and efficient hospital management of patients while conserving health care resources[56]. Implementation of molecular diagnostics has shown to shorten hospital stay reducing medical costs and preventing extensive use of unnecessary antibiotics[46,65]. They have also allowed for the initiation and the monitoring of therapy, particularly for severe diseases such as bacterial and viral meningitis (meningoencephalitis)[62]. With timeous detection, initiation of antimicrobial chemoprophylaxis

responses have improved in areas of meningococcal outbreaks in crowded institutions e.g. households, schools and prisons[118].

With early diagnosis essential for urgent chemotherapy, and reduction of BM mortality and morbidity, the IMCI program recommends continued empiric treatment until bacterial culture results are available. There is reduced sensitivity of confirmatory diagnosis leading to culture negative results[119]. These cases remain with unidentified aetiology. Unidentified cases of meningitis lead to an underestimation of the epidemiology of the disease. Mostly, surveillance programs are hindered in developing countries that still experience a high mortality due to the unknown post vaccine prevalence of some vaccine preventable bacterial aetiology. With the lack of reported data from low income nations there is an underestimation of mortality rates and presence of infection worldwide[40]. With regards to this, molecular based diagnostics have been observed to be less affected by prior antibiotic treatment thus have been found more sensitive and potentially advantageous in surveillance programs[90]

In developing countries the use of PCR for clinical diagnostics is gradually being adopted. Specifically for meningitis diagnostics, the standard diagnosis overlaps are common between BM and VM due to an absence of the expected CSF findings clearly aligning to bacterial infection or viral meningitis. This is further compounded by the current lack of viral diagnostics in routine laboratory tests. Misdiagnosis has led to the increased hospital admissions and unnecessarily prolonged antibiotic treatment for suspected meningitis cases [6,101,102,119,120].

In light of the drawbacks in standard tests and their clinical outcomes this study aimed to develop multi-target real-time PCR assays that will simultaneously detect both bacterial and viral meningitis pathogens common to paediatric populations in South Africa. This study will investigate the epidemiology of the common paediatric community acquired meningitis aetiology from increasing culture negative meningitis cases. The targeted pathogens are *S. pneumoniae*, *N. meningitidis* and *H. influenzae* for the bacteria. The viral pathogens are enteroviruses, mumps and herpes simplex virus.

1.8 Efficient recovery of nucleic acid determines the efficiency of the nucleic acid based diagnostic methods

Prior to downstream PCR amplification of the target microorganism, the sample requires pre-treatment to effectively extract high quality nucleic acids for subsequent detection[121,122]. Purification platforms were

traditionally crude chemically based methods. Improved commercial kits optimised for specific sample sources (e.g., blood, tissue and stool) and sample quantities have been introduced for efficient nucleic acid extraction while maintaining the stable moiety and integrity of the nucleic acids[123]. Automated platforms increase the efficiency for high-throughput processing, reducing turnaround time and hands on processing time necessary for clinical laboratories[121,123].

Efficient pre-treatment of CSF removes common amplification inhibitors e.g. hemin, ubiquitous ribonucleases, proteins and competitive inhibitors that might affect the reacting substrates[122,124,125]. The extraction method also introduces exogenous substances (phenol, ethanol, sodium dodecyl sulphate) to the sample that also may hinder the efficiency of subsequent PCR[126]. Thus careful consideration of pre-treatment is required.

Nucleic acid recovery requires optimisation to obtain the most efficient purification process. The purity of the nucleic acid material is scaled measured flourometric or spectrophotometric quantification with the resulting purified sample absorbance (A) at wavelengths of 260nm and 280nm. This assesses the quality of the DNA/RNA relative to protein with a ratio of A260/A280 $\geq$ 1.80 being of satisfactory purity[123]. Alternatively, PCR co-amplification of a quantified specific target in the extracted DNA/RNA can be used to assess the purity and efficiency of the extraction process. The cycle threshold (Cq) values for the amplified specific target by real-time PCR amplification can be compared for evaluation of pre-treatment methods with higher Cq values indicating less efficient pre-treatment while lower Cq values show high efficiency in pre-treatment[127].

Little data is available comparing extraction platforms for the bacterial and viral microorganisms targeted in CSF[121,122,124,125,127,128]. No comparison on extraction platforms was found for the bacterial organisms and mumps virus, however, there were several studies comparing extraction platforms for herpes simplex and enteroviruses. These studies compared various platform efficiencies based on nucleic acid yield and purity, amplification of internal controls (assessing removal of inhibitors) and PCR cycle threshold values for specific targets to suggest an optimal method. There was noted heterogeneity in the evaluation criteria used to compare the performance efficiency for the methods used, but the QIAamp MinElute Kit for DNA and RNA[127,128] extraction kit was shown to be optimal for extraction of both viruses from CSF. The efficiency of the optimal manual method was comparable to that of automated platforms. However, a considerable reduced processing time and improved analytical sensitivity with downstream PCR relative

to compared manual methods made it the more favourite. The only limitation of the automated platforms was the cost of procurement[127,128] .

1.9 Aim

This study aims to use molecular diagnostics to improve the detection of the common bacterial and viral causes of community acquired meningitis in children in South Africa.

1.10 Objectives

1. To optimise nucleic acid extraction protocols from CSF samples for real-time PCR detection of both viral and bacterial meningitis.

2. To develop a rapid multiplex real-time Reverse Transcriptase (RT)-PCR for the detection of enterovirus, herpes simplex and mumps virus

3. To adopt a bacterial multiplex real-time PCR assay for evaluation of infections by bacterial meningitis causing pathogens; *N. meningitidis*, *H. influenzae* and *S. pneumoniae*.

4. To verify and validate the developed assays;

    - Evaluate the analytical sensitivity of the viral and bacterial assays by standardization against a tenfold serial dilution of targeted positive controls to obtain the lowest limit of detection.
    - Evaluate the diagnostic accuracy of the viral and bacterial assay by screening clinical CSF samples to ascertain their clinical sensitivity and specificity.

5. Define the aetiology of suspected acute cases of meningitis in children relative to the common pathogens.

CHAPTER II

Assay Development and Validation

Contents

2.0 Summary

Simultaneous multitarget detection of microbial pathogens is advantageous in screening cerebrospinal fluid (CSF) where sample volumes are small and resources are limited. In addition, in a clinical laboratory, it is also necessary where a rapid diagnostic is required to determine the appropriate treatment. Multiplex assays require optimisation for parallel target detection to ensure there is no interference or cross-reactivity, and validation to ensure efficient sample preparation and downstream processing. In this study a previously published bacterial multiplex and separate viral singleplex assays were adopted, multiplexed and validated. The viral multiplex assay showed minimal interference between the targets on PCR amplification compared to the singleplex assay. In order to monitor possible PCR inhibition during amplification, an internal control was added to the bacterial multiplex and showed little interference upon co-amplification with the bacterial targets. Both assays proved sensitive at low concentration of the targets detecting <3 copies/reaction with high reproducibility. Tested against a panel of related targets, they proved specific thus were valid for sample testing.

2.1 Introduction

The polymerase chain reaction (PCR) has become a valuable tool for amplification of DNA accurately and reliably. It is able to detect the presence or absence of a specific gene, and gene expression analysis has made it the most favoured tool for diagnostics. Conventional PCR is most widely used, utilising endpoint analysis. However, real-time determination not only exponentially amplifies the primer-targeted DNA, it also allows for the simultaneous reporting of the accumulating amplicon by fluorescent based reporters. Real-time PCR is employed in the detection and quantification of target DNA from various sample sources[129–132]. Various detection methods are employed and include SYBR Green (a double stranded DNA intercalating dye), hybridisation probes (FRET probe), hydrolysis probes (TaqMan probes), scorpion probes and molecular beacons[133]. Most commonly used is the "TaqMan" based hydrolysis probes which involve the use of the fluorescence resonance energy transfer (FRET) between two dye molecules (a donor molecule and an acceptor molecule) that are electron excited on a probe oligonucleotide sequence[134]. The Taq polymerase‟s 5‟-3‟ exonuclease activity cleaves the 5‟ excited fluorescent donor molecule during polymerisation thus releasing it from close proximity with the acceptor molecule[134,135]. This prevents the quenching of the fluorescence and the dye florescence is detected (Figure 2.1). Use of different fluorescent dyes with varied excitation spectral wavelengths for the different targets at the 5‟ end allows distinction of multiple targets.

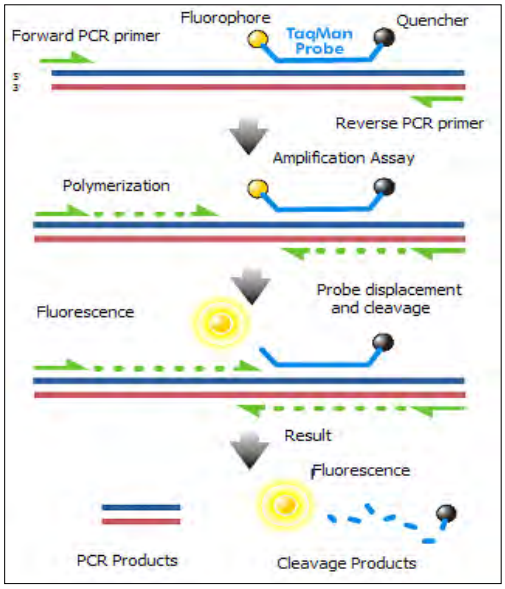


Figure 2.1: Fluorescence resonance energy transfer chemistry of TaqMan (hydrolysis) probe during target detection (adopted from http://en.wikipedia.org)

Gene targets for PCR amplification are critical and consideration should be based on the intended downstream functions either screening assays or typing assays. For screening purposes involving identification of specific targets, highly conserved genes are chosen. For typing purposes, where distinction of the varying subspecies identity is required, variable genetic regions are chosen to allow identification to the subspecies level.

Other key factors to consider when developing a real-time PCR assay are specificity of the primers and probe sequences. This initial validation checks the specificity of the primers and probe targets using a standard web based nucleotide Basic Local Alignment Sequence Tool (nBLAST) search across the National Centre for Biotechnology Information (NCBI) nucleotide database. This nBLAST *in silico* analysis reports the matching sequences with the highest level of sequence identity, also showing potential additional non-specific alignments with other sequences in the database[136]. This allows the researcher to decide if the specificity of the primer pairs or probe sequences is acceptable[137]

The availability of the target for amplification is also crucial and is determined by the sequence structure of the primers and the target template sequence. Formation of any secondary structures (hairpins) is seen with short oligonucleotide sequences reannealing along their length thus reducing their priming/target annealing efficiency, melting and annealing temperatures[137]. The formation of secondary structures is checked *in silico* with the use of software tools such as Mfold (www.mfold.bioinfo.rpi.edu/) which predict the folding tendency and reveals potential optimal and suboptimal structures[137,138]. Formation of any primer dimers of the individual primer sets is one other important parameter to review before empirical optimisation and validation[137].

2.1.1 Assay Validation

Validation of an assay is vital before its use in diagnostics. The analytical sensitivity is the ability to detect low concentrations of the target in a given sample with a 95% hit rate (positive rate of the technical replicates) on all tested samples[139,140]. Analytical sensitivity represents the smallest amount of substance in a sample that can accurately be measured by an assay[140]. This is commonly called the lowest limit of detection and is expressed as a concentration (copies/ml; copies/reaction; copies/µg); thus, the lower the concentration detectable, the greater the sensitivity[139,140]. This is determined empirically in molecular assays by testing serially diluted standards of known concentration with the desired target sequence[141].

The assay also has to maintain high reproducibility. This involves the ability of the assay to reproduce a measurement with repeat of the test on multiple aliquots of a single sample and/or over various test times[140142]. The quantitative measurements of the assay are affected by imprecision (i.e. lack of reproducibility and repeatability) with intra- and inter-assay variation over time due to variations in normal physiologic mechanisms, instrumentation, environment and processing personnel[140,143,144]. From multiple measurements of tested aliquots of sample, the coefficient of variation (CV) is calculated (%CV= (SD/mean) × 100) which is directly related to the level of imprecision in the assay. This implies that the lower the CV, the lower the imprecision (and the higher the reproducibility). Assays reporting a CV magnitude of < 5% are considered to show excellent precision[143,144].

Finally, the assay‟s ability to define a true positive is required. The specificity of the test reveals the ability of the assay to detect only its designed targets from potentially related nucleic acid sources (related organisms) or any related specimen conditions (e.g. xanthochromatic CSF, bloody CSF or clear CSF)[139,140]. This monitors the reporting of false positives mainly by assessing cross-reactivity amongst other potential commensals or pathogens, which could be genetically related. Some cross-reactive species could be found concurrently in the sample i.e. co-infections, or could be the etiological cause of similar disease conditions[139,140]. Interference from these cross-reactive targets and other compounds may lead to false positives[140]. False negatives on the other hand can be due to inhibition of the activity of the reacting species in the PCR master mix especially DNA polymerase or Reverse transcriptase enzyme.

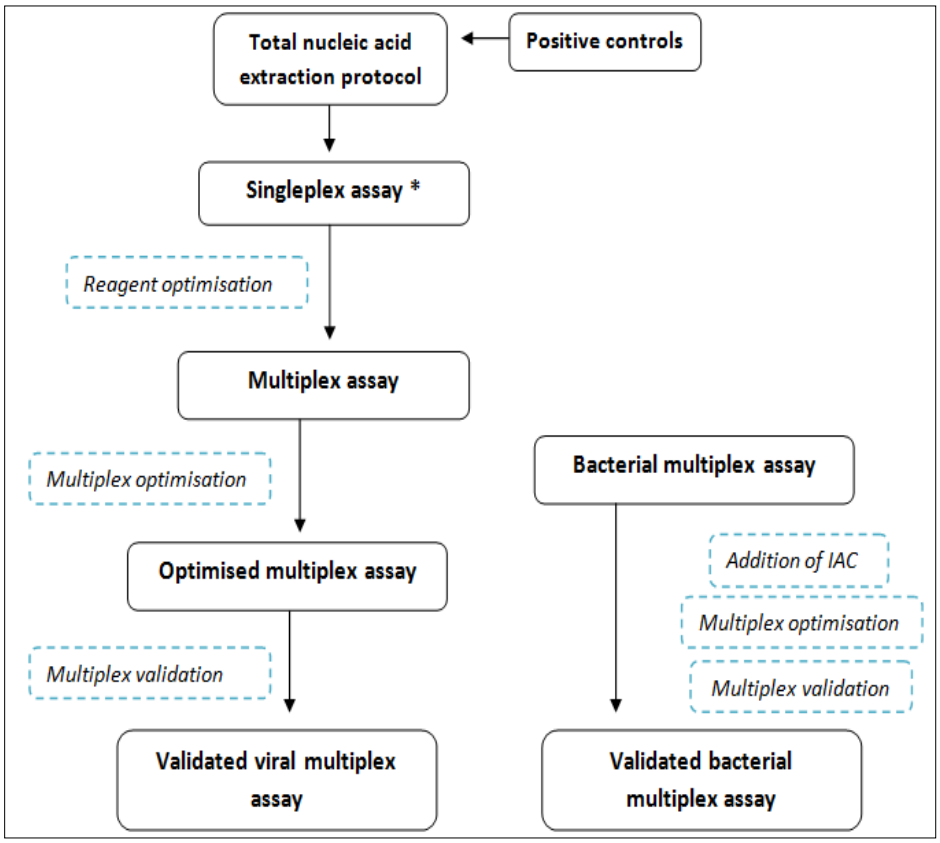
An alternate target can be co-amplified from an exogenous or endogenous DNA target source in the sample to evaluate the level of inhibition and its effect on target determination. These are termed internal controls (IC) and the reactions can be performed in parallel with the desired target amplification. The IC requires a specific set of primers and probe for amplification and detection. Most choices for the internal control are housekeeping genes conserved in the sample source e.g. Glyceraldehyde-3-phosphate dehydrogenase in the human genome (human samples)[145]. Housekeeping genes are referred to as endogenous IC and are able to monitor not only the efficiency of amplification but also the efficiency of extraction of nucleic acid from the sample[140,145]. Foreign synthesised targets can be spiked into samples and used as ICs. However, ICs require optimisation for reliable co-extraction and co-amplification without competition with the desired target amplification[140,145].

Once the assay has been optimised and validated, screening of the samples for the desired targets can occur. We sought to develop and validate a real-time quantitative PCR (qPCR) assay to simultaneously detect enteroviruses, mumps virus and herpes simplex virus in cerebrospinal fluid. We also adopted a published real-time multiplex assay for detection of *Neisseria meningitidis*, *Haemophilus influenzae* and *Streptococcus pneumonia*[146], with incorporation of an synthesised plasmid target IC[147]. The IC monitored any inhibition present in the sample and was exogenously added before extraction.

2.2 Materials and Methodology

2.2.1 Experimental design

The experimental design is outlined in Figure 2.2.



*Each singleplex for each target (enterovirus, mumps and herpes simplex); IAC- Internal amplification control

Figure 2.2: Experimental design for optimisation and validation of viral and bacterial real-time multiplex assays.

2.2.2 Selection and preparation of reference strains and plasmid controls

Bacterial positive controls (Table 2.1) were cultured on 2% chocolate blood agar (Greenpoint Media Laboratory, National Health Laboratory Service, Cape Town, South Africa) aerobically in 5% CO_2 for 24 hrs. A loopful of pure colonies were collected and resuspended in 1ml lysis buffer (ZymoResearch Corp., Irvine, United States of America) and DNA was extracted using the Zymofungal/bacterial Miniprep DNA

kit (ZymoResearch Corp., Irvine, United States of America), following the manufacturers guidelines. The genomic DNA was quantified spectrophotometrically using the NanoDrop® ND-1000 (NanoDrop Technologies Inc., Wilmington, United States of America).

Live resuscitated viral culture isolates (Table 2.1) of mumps virus, enterovirus and herpes simplex were obtained from the National Institute for Communicable Diseases (NICD), Johannesburg, South Africa. The live viral cells (500µl) were heat killed at 57^{o}C for 1 hour. An equal volume of ATL tissue lysis buffer (Qiagen, Hilden, Germany) was added to the heat killed cells in a Zymobead tube (Zymo Research Corp., Irvine, United States of America) for mechanical lysis at 50 Hz for 5 min in a tissue lyser (Qiagen, FRITSCH GmbH, Idar-Oberstein, Germany). The crude lysate was centrifuged 13 000 rpm for 10mins and 400µl of the supernatant was transferred to a Sarstedt tube (Biodex, Edenglen, Germany) for total nucleic acid extraction using the automated QIAsymphony® SP platform (Qiagen, Hilden, Germany) and the QIAsymphony® Virus/Bacteria Mini Kit (Qiagen, Hilden, Germany). The nucleic acid was quantified spectrophotometrically using the NanoDrop® ND-1000 (NanoDrop Technologies Inc., Wilmington, United States of America).

Table 2.1 Bacterial and viral strains used as reference standards

Control strains	Characteristics	Source
Bacterial strains		
Streptococcus pneumoniae ATCC 496190	Type strain of *S. pneumoniae*	NICD
Neisseria meningitidis EMGM6	Type strain of *N. meningitidis*	NICD
Haemophilus influenzae ATCC 49247	Type strain of *H. influenzae*	NICD
Viral strains		
mumps	Clinical isolate	NICD
enterovirus (echovirus 13, 24, 30)	Clinical isolate	NICD
herpes simplex subtype 1, and 2	Clinical isolate	NICD

ATCC: American Type Culture Collection; NICD: National Institute for Communicable Diseases; EMGM: European Monitoring Group on Meningococci.

2.2.2.1 Preparation of plasmid standards

Using the extracted DNA from the reference control strains in Table 2.1, inserts for the targets of interest were generated by PCR using primers listed in Table 2.2. The Go Taq master mix (Promega, United

States of America) was used for amplification of the DNA targets in a final volume of 25µl. The Verso 1Step RT-PCR kit (Thermo scientific) was used for the enterovirus insert and the MyTaq One-step RT-PCR kit (Bioline, London, United Kingdom) was used for the mumps virus insert at a volume of 25µl.

Table 2.2 Primers and PCR assays used for amplification of cloning inserts for the target genes

Target	Primers	Primer sequence (5'---- 3')	Product size (bp)	Source
S. pneumoniae	S. pneu-F:	TTA TTC GTG CAA TAC TCG TGC G		
LytA gene	S. pneu-R:	CAA CCG TAC AGA ATG AAG CGG	319bp	Nagai et al 2001
H. influenzae	H. inf-F:	ACT TTA GCC CTT TCT TTA TTA GCA		
Hpd gene	H. inf-R:	CTT TTA AGA ATT CCA CGC CAG TAT	1072bp	Wang et al 2011
N. meningitidis	N. men-F:	ATG CGG TGG CTG CGG TAG GT		
CtrA gene	N. men-R:	CCG GCG AGA ACA CAA ACG ACA A	533bp	Guiver et al 2000
Herpes simplex virus	HSV-F	AGT ACA TCG GCG TCA TCT GC		
UL30 gene	HSV-R	TAC GGG ATC CGG TCC TTG AT	403bp	This work*
Enterovirus	Ev-F	CCA TGG GAC GCT TCA ATA CTG ACA		
5' UTR region	Ev-R	GGA TGG CCA ATC CAA TAG CTA	250bp	This work*
Mumps	MuV-F	ACA ATG AGG CAG AGA GGC TG		
Fusion protein gene	MuV-R	AAT CCG TCT AGG GAC ACC GT	250bp	This work*

*The Primer-BLAST tool was used for primer design; EV- enterovirus, HSV- herpes simplex virus; MuV- mumps virus H. inf- *Haemophilus influenzae*; S. pneu- *Streptococcus pneumoniae*; N. men- *Neisseria meningitidis*; F- Forward primer; R- Reverse primer; **NB**: The chosen primer sets were synthesized at the Molecular and Cell Biology Department, University of Cape Town.

The cycling conditions for the amplification reactions were optimised for each target insert according to the recommendations of the master mix used (Table 2.3). Gel electrophoresis was carried out on a 1% (w/v) agarose gel at 80 volts for 1hour 30 minutes. The resolved bands were cut out and purified with a MiniElute Gel extraction kit (Qiagen, Hilden, Germany) as per the manufacturer‟s protocol.

Table 2.3 PCR assay conditions for amplification of the cloning inserts for the target genes

Target	Primers and reaction concentration	cDNA synthesis and initial denaturation	PCR Cycle	Final extension
S. pneumoniae LytA gene	S. pneu-F: 400nM S. pneu-R: 400nM	95°C (5mins)	95°C (30s)52°C(30s) 72°C (30s) (X35)	72°C (5mins)
H. influenzae Hpd gene	H. inf-F: 400nM H. inf-R: 400nM	95°C (2mins)	95°C (30s) 55°C(30s)72°C (90s) (X35)	72°C (5mins)
N. meningitidis CtrA gene	N. men-F: 400nM N. men-R: 400nM	95°C (2mins)	95°C (30s) 56°C(30s)72°C (30s) (X35)	72°C (5mins)
Herpes simplex virus UL30 gene	HSV-F 200nM HSV-R 200nM	95°C (2mins)	95°C (20s) 56°C (30) 72°C (60s) (X35)	72°C (5mins)
Enterovirus 5' UTR region	Ev-F 400nM Ev-R 400nM	42°C(15mins) 95°C(2mins)	95°C (20s) 52°C(30s)72°C (60s) (X40)	72°C (5mins)
Mumps Fusion protein gene	MuV-F 400nM MuV-R 400nM	45°C (30s) 95°C (1min)	95°C (10s) 56°C(30s)72°C (30s) (X40)	72°C (5mins)

H. inf- *Haemophilus influenzae*; S. pneu- *Streptococcus pneumoniae*; N. men- *Neisseria meningitidis*; HSV- herpes simplex virus; EV- enterovirus; MuV- mumps; F- Forward primer; R- Reverse primer;

The purified DNA was ligated into the pGEM®-T Easy vector (Promega, United States of America) The ligated DNA was then transformed into JM109 *E. coli* cells (Promega, United States of America) by heat shock according to the manufacturers protocol. The presence of an insert was confirmed by supplemented (Ampicillin, X gal and IPTG) media selection of the recombinant JM109 *E. coli* cells. A single colony was picked and suspended in 15µl of distilled water. To confirm the presence of the insert, a colony PCR was carried out using universal M13 sequencing primers which bind to plasmid DNA positions flanking either side of the insert[148]. The PCR was carried out in a 10µl volume containing 1µl of colony suspension, 0.4µl of each primer (0.4µM final concentration) and 6µl of Go Taq master mix (Promega, United States of America). The reaction conditions include an initial hold at 95°C for 5min, followed by 35 cycles of denaturation at 95°C (1 min), annealing at 55°C (1:50min), and extension at 72°C (1min), with a final extension hold at 72°C for 5min.

The insert was subsequently sequenced commercially (Inqaba Biotechnology, Pretoria, South Africa) with an nBLAST search carried out to confirm the insert sequence. The plasmid DNA extraction was performed using the Qiagen plasmid Miniprep (Qiagen, Germany) and the plasmid preparations were quantified using the Qubit 2.0 flourometer (Invitrogen, USA) employing the double stranded (ds) DNA HS Assay kit (Invitrogen, USA). For use as positive control standards, the copy-number of the plasmids was calculated using the following formula:

(X g/µl DNA / [plasmid length in base pairs x 660]) x 6.022 x 10^{23} = Y molecules/µl[149],

where X g/µl DNA is the plasmid concentration, the entire recombinant plasmid length, 6.022 x10^{23} is the Avogadro"s constant and 660 is the average molecular weight of one base pair[149].

2.2.3 Development, optimisation and validation of the Multiplex Assays

2.2.3.1 Bacterial real-time PCR and viral real-time RT-PCR

A published bacterial real-time multiplex PCR detecting *S. pneumoniae*, *N. meningitidis* and *H. influenzae* was adopted while primers derived from previous publications were used to develop the viral multiplex for detecting enteroviruses, mumps virus and herpes simplex (Table 2.4). Cross-reactivity of the primer and probe sequences, along with the target specificity, was checked using nBLAST on the National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov), accessed 19/05/2014.

Table 2.4 Primers and Probes adopted for the real-time PCR assays

Target	Primers	Primer sequence (5'---- 3')	Gene target and size	Source
S. pneumoniae	F373	ACGCAATCTAGCAGCTGAAGCA	LytA gene 75 bp	Wang et al, 2011[150]
	R424	TCGTGCGTTTTAATTCCAGCT		
	Pb400	TxRd -TGCCGAAAACGC"T"TGATACAGGGAG-BHQ2dT*		
H. influenzae	HPDF729	AGATTGGAAAGAAACACAAGAAAAAGA	Hpd gene 113 bp	Wang et al, 2011[150]
	HPDR819	CACCATCGGCATATTTAACCACT		
	PBR762	Cy5 -AAACATCCAATCG"T"AATTATAGTTTACCCAATAACCCBHQ2dT*		
N. meningitidis	F753	5′-TGTGTTCCGCTATACGCCATT-3′	CtrA gene 114 bp	Wang et al, 2011[150]
	R846	5′-GCCATATTCACACGATATACC-3′		
	Pb820	FAM -AACCTTGAGCAA"T"CCATTTATCCTGACGTTCT-BHQ1dT*		
Herpes simplex	HSV-F	5′-CATCACCGACCCGGAGAGGGAC	UL30 gene 92 bp	Kessler et al, 2000[151]
	HSV-R	5′-GGGCCAGGCGCTTGTTGGTGTA		
	Probe	Cy5 -CCGCCGAACTGAGCAGACACCCGCGC-BHQ2		
Enterovirus	Ev-F	5′-CCTGAATGCGGCTAATCC-3′	5' UTR region 144 bp	Archimbaud et al, 2004 & 2009[152,153]
	Ev-R	5′-ATTGTCACCATAAGCAGCC-3′		
	Probe	TxRd -ACCGACTACTTTGGGTGTCCGTGTTTC- BHQ2		
Mumps	F1073	5′-TCTCACCCATAGCAGGGAGTTATAT	Fusion protein gene 79 bp	Uchida et al, 2005[154]
	R1151	5′-GTTAGACTTCGACAGTTTGCAACAA		
	Probe	FAM -AGGCGATTTGTA GCACTGGATGGAACA –BHQ1		

TxRd- Texas Red; Cy5- Cyanine 5; FAM - 6-Flourescein; BHQ- Black Hole Quencher; *- the quenchers are internally placed at the "T" base region.

For the bacterial multiplex, the 25µl PCR reaction mixture contained 1 × SensiFAST™ Probe No-ROX (Bioline, London, United Kingdom) and 2µl of DNA template. The primer and probe oligonucleotides

used, along with the amplification conditions, are listed in Table 2.5. This was previously optimised and only required verification.

For the development of the viral multiplex, the separate viral singleplex assays were carried out in a 25µl final reaction volume and performed in 1x iScript One Step RT-PCR Kit (Bio-Rad Laboratories Inc., Hercules, CA, United States of America) with 2µl of extracted nucleic acid. The primers and probes for each target together with the amplification conditions are also listed in Table 2.5. All samples were tested in triplicate on the CFX96 Real-Time System (Bio-Rad Laboratories Inc., Hercules, CA, United States of America).

Table 2.5 Reaction conditions for the bacterial multiplex and viral singleplex real-time PCR assays

Targets	Primers and reaction concentration	cDNA synthesis and initial denaturation	PCR Cycles
Multiplex *S. pneumoniae* *H. influenzae* *N. meningitidis*	S. pneu-F: 200nM S. pneu-R: 200nM S. pneu Probe: 200nM H. inf-F: 300nM H. inf-R: 100nM H. inf Probe: 200nM N. men-F:300nM N. men-R: 900nM N. men Probe: 100nM	50°C (2mins)95°C (10mins)	95°C (15s) 60°C(60s) (X40)
Herpes simplex virus Singleplex	HSV-F 500nM HSV-R 500nM HSV Probe: 200nM	50°C (10mins) 95°C (5mins)	95°C (15s) 60°C(30s) (X45)
Enterovirus Singleplex	Ev-F 700nM Ev-R 700nM EV Probe: 200nM	50°C (10mins) 95°C (5mins)	95°C (15s) 60°C(30s) (X45)
Mumps Singleplex	MuV-F 300nM MuV-R 300nM MuV Probe: 200nM	50°C (10mins) 95°C (5mins)	95°C (15s) 60°C(30s) (X45)

H. inf- *Haemophilus influenzae*; S. pneu- *Streptococcus pneumoniae*; N. men- *Neisseria meningitidis*; HSV- herpes simplex virus; EV- enterovirus; MuV- Mumps; F- Forward primer; R- Reverse primer; cDNA – complementary DNA

2.2.3.2 Optimisation of multiplex real-time RT-PCR assay for the viral targets

Once the singleplex RT-qPCR assays for the viral targets had been optimised, the assay was multiplexed adding all the necessary primers and probes to obtain the optimal combination. All primer and probe concentrations were then optimised by testing concentrations in the range of 100nM to 500nM and 100nM to 300nM, respectively, using the primer chess boarding method[155]. This method limited either one of the primers (forward or reverse) while the other primer concentration was held constant to obtain

the optimal concentration for each primer for optimal amplification in the multiplex. The PCR reactions were carried out as in section 2.4.1.

With the optimal primer probe combination obtained, the previous singleplex and current multiplex conditions were compared. This was carried out by adding the primer probe combination and a single template target to the multiplex reaction while simultaneously running the singleplex assay for that specific template on the same plate run. The success of multiplexing was assessed by comparing the Cq values of the specific target in both the singleplex and multiplex assays looking for evidence of any interference or variance. The PCR conditions were carried out as in section 2.4.1 for the multiplex and section 2.3.4.1 for the singleplex assays.

2.2.3.3 Analytical Sensitivity and Precision

After the successful multiplexing of the viral assay and verification of the bacterial assay, the sensitivity and precision of detection was defined. The analytical sensitivity included the initial preparation of a dilution series for each of the prepared plasmid standards (section 2.2.3) ranging from 1000 copies, to 1 copy/reaction to be tested on the multiplex assays for both the viral and bacterial target. This comprised of 7 dilutions of the plasmid standard, each tested in eight replicates. The PCR conditions were carried out as in section 2.4.1 for the viral multiplex and section 2.3.4.1 for the bacterial multiplex assay.

For determination of the precision in both the bacterial and viral qPCR assays, the Cq values from the tested replicates were used to evaluate the intra-assay replication of the qualitative analysis for the bacterial and viral multiplex assay. A single dilution was repeated on three separate days and the Cq values from the tested replicates were used to evaluate the inter-assay variation.

2.2.3.4 Analytical Specificity

To assess any cross reactivity from potentially related species a number of bacterial and viral reference test strains (Table 2.6) were obtained and tested. Crude extractions of the bacteria were performed by adding a loopful of an overnight culture to distilled water and boiling at 100^0C for 10 minutes. The lysate was centrifuged at 10 000 rpm for 1 min, and the clarified supernatant was used as a template for qPCR in the bacterial assay. The viral strains were obtained as extracted nucleic acid. Typically, 10-100ng of DNA or RNA was added as template for both the multiplex assays (section 2.3.4.1 and section 2.4.1.).

Table 2.6 Related bacterial and viral strains from different bacteria to test cross reactivity

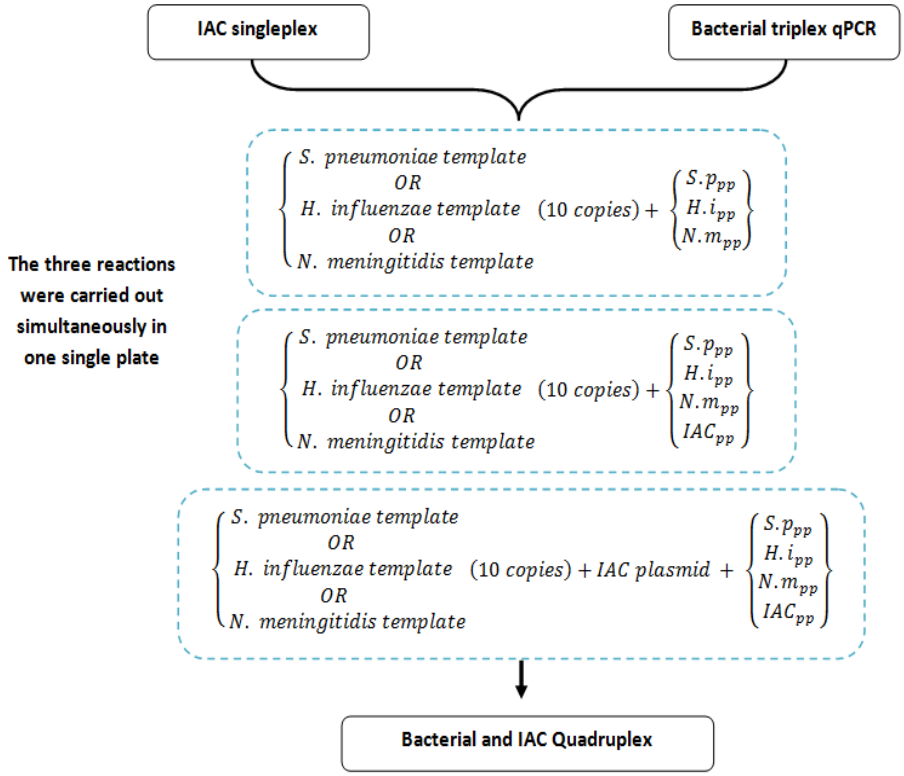
Test organism	Characteristics	Reference
Bacteria		
Acinetobacter baumannii	Clinical isolate	UCT
Enterobacter cloacae	Clinical isolate	UCT
Enterococcus faecalis ATCC 51299	Type strain	UCT
Escherichia coli ATCC 25922	Type strain	UCT
Haemophilus influenzae ATCC 49247	Type strain	NICD
Klebsiella pneumoniae ATCC 1705	Type strain	UCT
Klebsiella pneumoniae ATCC 1706	Type strain	UCT
Klebsiella pneumoniae ATCC 700603	Type strain	UCT
Neisseria meningitidis EMGM6	Type strain	NICD
Pseudomonas aerogenosa ATCC 27853	Type strain	UCT
Pseudomonas aerogenosa ATCC 27853	Type strain	UCT
Staphylococcus scuri	Clinical isolate	UCT
Serratia marcescens	Clinical isolate	UCT
Staphylococcus aureus	Clinical isolate	UCT
Staphylococcus epidermidis	Clinical isolate	UCT
Staphylococcus saprophyticus	Clinical isolate	UCT
Streptococcus anginosus ATCC 2008	Type strain	NICD
Streptococcus bovis ATCC 9809	Type strain	NICD
Streptococcus pneumoniae ATCC 496190	Type strain	NICD/GSH
Streptococcus pyogenes	Clinical isolate	GSH
Streptococcus thermophilus ATCC 192583	Type strain	NICD
Streptococcus viridans	Clinical isolate	GSH
Streptococcus viridans ATCC 7868	Type strain	NICD
Streptococcus pyogenes ATCC19615	Type strain	NICD
Viruses		
Cytomegalovirus	Clinical isolate	GSH
Epstein Barr virus	Clinical isolate	GSH
Varicella zoster virus	Clinical isolate	GSH
Human herpes virus 6	Clinical isolate	GSH
Rhinovirus	Clinical isolate	GSH
Parainfluenza 2	Clinical isolate	GSH
Human respiratory syncytial virus b	Clinical isolate	GSH
Mumps	Clinical isolate	NICD
Enterovirus (echovirus 13, 24, 30)	Clinical isolate	NICD
Herpes simplex subtype 1, and 2	Clinical isolate	NICD

GSH- Groote Schuur Hospital; NICD- National Institute for Communicable Diseases; UCT- University of Cape Town.

2.2.4 Incorporation and optimisation of the IAC into bacterial multiplex assays

The bacterial qualitative real-time RT-PCR described previously[150] was modified to include an exogenous internal amplification control plasmid[147] to assess the quality of CSF sample extraction and the efficiency

of amplification. The optimal spiking concentration was determined by initial spiking of nuclease free water (Qiagen, Germany) with a low bacterial concentration, close to the limit of detection i.e. 10 copies/reaction with an equivalent 10 copies/reaction of IAC plasmid DNA. The experimental design (Figure 2.3) was carried out with stepwise addition of IAC primers, probes and plasmid DNA to each of the bacterial targets in the bacterial qPCR to assess the effect of 10 copies/reaction of the IAC on amplification of low concentration (10 copies/reaction) of each of the targets. All steps were carried out on a single plate and results compared for any interference during co-amplification.



pp- Primers and probe; IAC– Internal Amplification Control, H. inf- *Haemophilus influenzae*; S. pneu- *Streptococcus pneumoniae*; N. men- *Neisseria meningitidis* ; qPCR- real time polymerase chain reaction.

Figure 2.3 The experimental design for the incorporation of the internal amplification control

2.2.5 Spiking of negative CSF with bacterial positive controls and IAC

A pooled preparation of the CSF samples obtained from patients without clinical evidence of meningitis (CSF with normal chemistry excluded from the cohort see section 3.2.2) was initially tested with the developed bacterial assay to confirm the absence of bacterial pathogens. A volume of 350 µl of CSF was

spiked with a specific concentration of both the bacterial plasmid DNA, and IAC (optimised in section 2.3.5.). The spiked CSF was extracted with the QIAsymphony SP platform (Qiagen, Hilden, Germany) using the QIAsymphony® Virus/Bacteria Mini Kit (Qiagen, Hilden, Germany) and qPCR was carried out on 5 replicates for the bacterial targets with the optimised bacterial multiplex as shown in Figure 2.4.

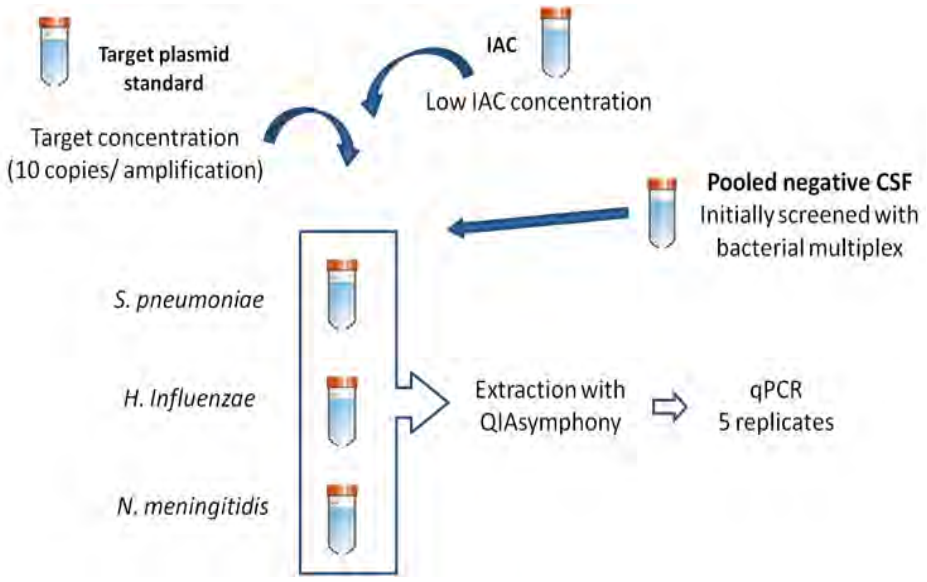


Figure 2.4 Spiking of negative CSF with IAC to analyse the effect of extraction on the efficiency of the quadruplex.

A 25µl PCR reaction mixture contained 1 × SensiFAST™ Probe No-ROX (Bioline, London, United Kingdom) and 2µl of DNA template. The primers and probe concentrations were as listed in table 2.7 along with the reaction conditions. The simultaneous co-amplification Cq values were compared.

Table 2.7 Reaction conditions for the qPCR of negative CSF spiked with IAC and bacterial plasmid DNA

Targets	Primers and probes◊	Reaction concentration	Initial denaturation	PCR Cycles
S. pneumoniae	*S. pneumoniae*-F	200nM	50°C (2mins)95°C (10mins)	95°C (15s) 60°C(60s)* (X40)
H. influenzae	*S. pneumoniae*-R	200nM		
N. meningitidis	*S. pneumoniae* Probe	200nM		
IAC	*H. influenzae*-F	300nM		

H. influenzae-R	100nM
H. influenzae Probe	200nM
N. meningitidis-F	300nM
N. meningitidis-R	900nM
N. meningitidis Probe	100nM
IAC-F	200nM
IAC-R	200nM
IAC Probe	200nM

*- The DNA extension step by the Taq polymerase at 60oC for one minute occurred simultaneously with a plate read for fluorescence emission. ◊- primer and probe sequences for the targets are listed in section 2.3.4.1 and for the IAC[147]; F- forward; R- reverse

2.2.6 Statistical analysis

To assess for any interference in the viral multiplex assay upon multiplexing the singleplex assays, the variance in the mean Cq values for the singleplex and multiplex assays were compared using 95% confidence intervals calculated as follows:

$$(95\%\ CI = mean \pm 1.96 \times SD)$$

To measure the precision of bacterial and viral multiplex assays, the mean Cq, standard deviation (SD) and coefficient of variation (CV) were expressed for each set of 8 replicates. The CV value is calculated as follows:

$$(\%CV = (SD/mean) \times 100)$$

The analytical sensitivity of the bacterial and viral multiplex assays was expressed as the lowest limit of detection (LOD) and was calculated using the probit regression analysis (StatsDirect version 2.02) which reports the lowest concentration detectable in 95% of the 8 replicates tested.

2.3 Results and Discussion

2.3.1 *In silico* analysis of the primer and probe targets

Before use of both the bacterial and viral target primers and probes, the primers and probes were assessed for specificity for their targets. Comparison of the primer and probe sequences to the Genbank database using nBLAST reported 100% specificity to the target organisms with no cross-reactivity to other organisms. With the fear of the adopted assays being relatively outdated, the *in silico* analysis sort to re-check the specificity of the targets also ensuring no cross reactivity with the constantly updated Genbank database.

2.3.2 Multiplex optimisation for the viral targets

The development of the viral qPCR involved the multiplexing of separate adopted viral singleplex assays. With confirmed acceptable specificity, the viral singleplex assays were multiplexed and the success of multiplexing was assessed by comparison of the performance of both the singleplex (S) and multiplex assays (M) simultaneously (Figure 2.5; Table 2.8). The primers and probes of the viral targets showed minimal interference upon multiplexing and the amplification curves for both assays were superimposable (Figure 5). No cross-reactivity was observed for each primer and probe set, as seen by the lack of amplification of the other viral targets.

The enterovirus detection channel in the Biorad CFX96 real-time instrument exhibited spectral overlap with the herpes simplex reporter dye Cy5 thus upon amplification of enteroviral target, the accumulating Texas red fluorescence is slightly cross-detected and appears as Cy5 signal. This overlap was within the expected instrumental spectral overlap for the two dyes thus could be ignored as background noise without affecting the assay‟s specificity (per. comm. R. Holló; BIORAD technician).

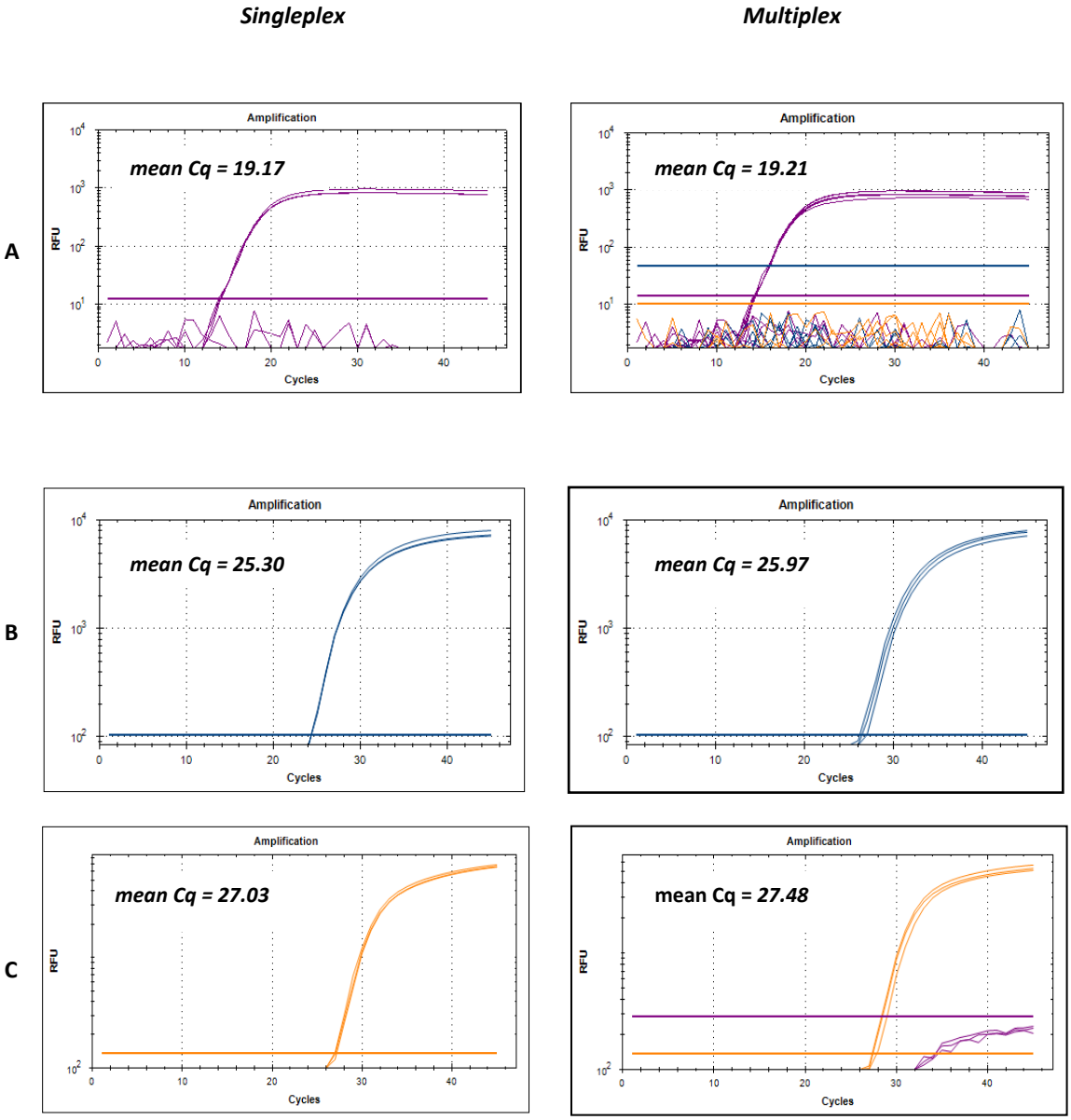


Figure 2.5: Pairwise comparisons of each viral target between singleplex and multiplex assay (A, B, and C). Minimal interference is shown (Δ mean Cq values) between the viral target primers and probes; A- herpes simplex, B- mumps, C- enterovirus. C- Spectral overlap is noted with the enterovirus Texas Red detection channel reporting a Cy5 reporter dye for HSV (this is expected between the two reported dyes)

Table 2.8 The repeatability and reproducibility of the optimised viral multiplex real time PCR assay.

Intra-assay variations			Inter-assay variations		
Mean (Cq value)	Standard deviation (SD)	% Coefficient of variation (CV)	Mean (Cq value)	Standard deviation (SD)	% Coefficient of variation (CV)
Multiplex real time PCR targeting mumps viral RNA (4ng/µl)					
S- 25.30	0.118	0.46	M1-25.94	0.15	0.59
M- 25.97	0.159	0.61	M2- 25.97	0.16	0.61
			M3- 27.15	0.40	1.48
Multiplex real time PCR targeting enterovirus viral RNA (2ng/µl)					
S- 28.32	0.071	0.25	M1-29.07	0.04	0.14
M- 28.20	0.006	0.02	M2- 28.20	0.006	0.02
			M3- 27.48	0.34	1.23
Multiplex real time PCR targeting herpes simplex viral DNA (1.7ng/µl)					
S- 19.21	0.075	0.39	M1- 18.09	0.10	0.58
M- 19.17	0.060	0.31	M2- 19.17	0.06	0.31
			M3- 14.10	0.04	0.33

Note: Mean Cq values mentioned indicates the average of 3 replicates of same run (intra-assay) and between runs (inter-assay); M1-M3, multiplex runs; S, singleplex.

2.3.3 Comparison of the Singleplex assay vs Multiplex assay for the viral targets

With the published singleplex assays for the viral assays and the newly developed viral multiplex, validation of the multiplex was required to confirm successful multiplexing. The mean Cq values for the tested replicates of the viral targets were compared to assess their performance in the singleplex and the multiplex. The tested replicates on the viral multiplex assay showed good reproducibility and repeatability for all three targets with minimal variation observed between mean Cq values of the singleplex and multiplex assays, resulting in low intra-assay CVs of below 2% (Table 2.8). Similarly, the inter-assay CVs for the viral multiplex were also below 2%, specifically 0.59 –1.48%; 0.14% –1.23% and 0.58 – 0.33% observed for mumps, enterovirus and herpes simplex, respectively. This shows that the viral multiplex is reproducible and repeatable.

2.3.4 Validation of bacterial and viral multiplex assays

The bacterial and viral multiplex assays were validated according to Burd (2010), specifically testing for the analytical sensitivity, specificity and precision[140]. This aimed to establish the performance specifications of the developed tests.

The adopted bacterial PCR assay had been previously validated but with use of a different PCR machine and PCR master mix it is prerequisite to re-define the test parameters of the assay adopted. There have been noted differences in reported sensitivities and specificity of adopted assays with different reagents

use, instrumentation even in different testing regions[140,156] . With the viral assay, validation was necessary to define the detection parameters of the individual targets in the multiplex. The validation of the assays ensures adherence of the assay‟s performance to laboratory‟s specification standards (Quality control purposes). This however will require monitoring to evaluate the potential deviations in the assay‟s specificity and sensitivity.

2.3.4.1 Analytical sensitivity

From the prepared plasmid standards for each of the targets, the lowest limit of detection for the bacterial multiplex was 2 copies/reaction for *S. pneumoniae* and 1 copy/reaction for both *H. influenzae* and *N. meningitidis*. The amplification efficiency was close to 100% for all standard curves obtained showing linearity over a span of the different concentrations of the plasmid standards (Figure 2.6). Table 2.9 reports the Cq values obtained for the tested replicates for the bacterial multiplex targets. The cut-off Cq value for the bacterial targets was 35 and corresponded to 10^1 copies of DNA for each of the purified plasmid standards. This threshold determined the cut-off for a positive result during screening of samples.

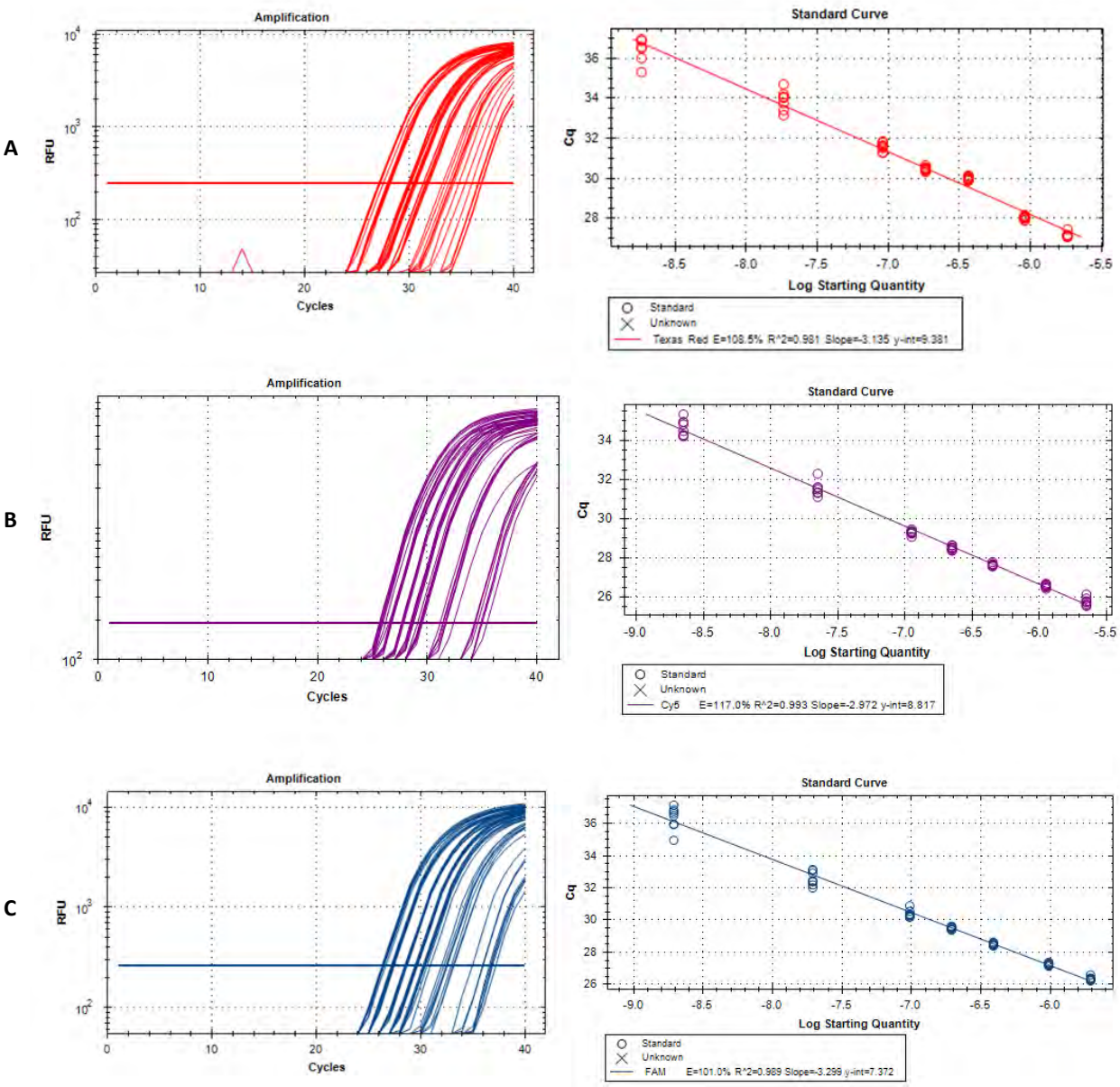


Figure 2.6 Standard curves for the individual bacterial target amplification in the bacterial multiplex real-time PCR assay targets; A- *S. pneumoniae*, B- *H. influenzae*, C- *N. meningitidis*. Left- The amplification curves of the prepared plasmid standard concentrations (1000 copies/reaction -1copy/reaction); Right- the linearity of the tested replicates and efficiency of amplification of the standard.

Table 2.9 Bacterial DNA Limit of detection

	Plasmid copies	No. of replicates	No. positive	% Positive	Cq Mean	Cq Std. Dev	%CV
Streptococcus pneumoniae	1000	8	8	100	27.15	0.122	0.448
	500	8	8	100	28.02	0.093	0.331
	200	8	8	100	30.00	0.097	0.322
	100	8	8	100	30.47	0.102	0.333
	50	8	8	100	31.57	0.204	0.647
	10	8	8	100	33.92	0.480	1.415
	1	8	6	75	36.37	0.618	1.698
	Plasmid copies	**No. of replicates**	**No. positive**	**% Positive**	**Cq Mean**	**Cq Std. Dev**	**%CV**
Neisseria meningitidis	1000	8	8	100	26.91	0.132	0.489
	500	8	8	100	27.76	0.113	0.407
	200	8	8	100	29.05	0.054	0.186
	100	8	8	100	30.07	0.065	0.217
	50	8	8	100	30.91	0.221	0.716
	10	8	8	100	33.05	0.391	1.182
	1	8	8	100	36.74	0.720	1.959
	Plasmid copies	**No. of replicates**	**No. positive**	**% Positive**	**Cq Mean**	**Cq Std. Dev**	**%CV**
Haemophilus influenzae	1000	8	8	100	26.12	0.152	0.581
	500	8	8	100	26.99	0.073	0.272
	200	8	8	100	28.04	0.054	0.191
	100	8	8	100	28.89	0.125	0.431
	50	8	8	100	29.66	0.137	0.463
	10	8	8	100	31.90	0.377	1.182
	1	8	8	100	35.01	0.409	1.170

The viral multiplex was able to detect 2 copies/reaction for both the herpes simplex virus and mumps virus. For enterovirus detection, the LOD was 3 copies/reaction. Table 2.10 describes the Cq values obtained from testing the viral targets‟ dilution series. The cut-off Cq value for the viral targets was 35 and corresponded to 10^1 copies of DNA for each of the purified plasmid standards. This threshold determined the cut-off for a positive result during screening of samples. The regression curve analysis of the multiplex viral assay showed linearity over a span of the different concentrations of the plasmid standards (Figure 2.7).

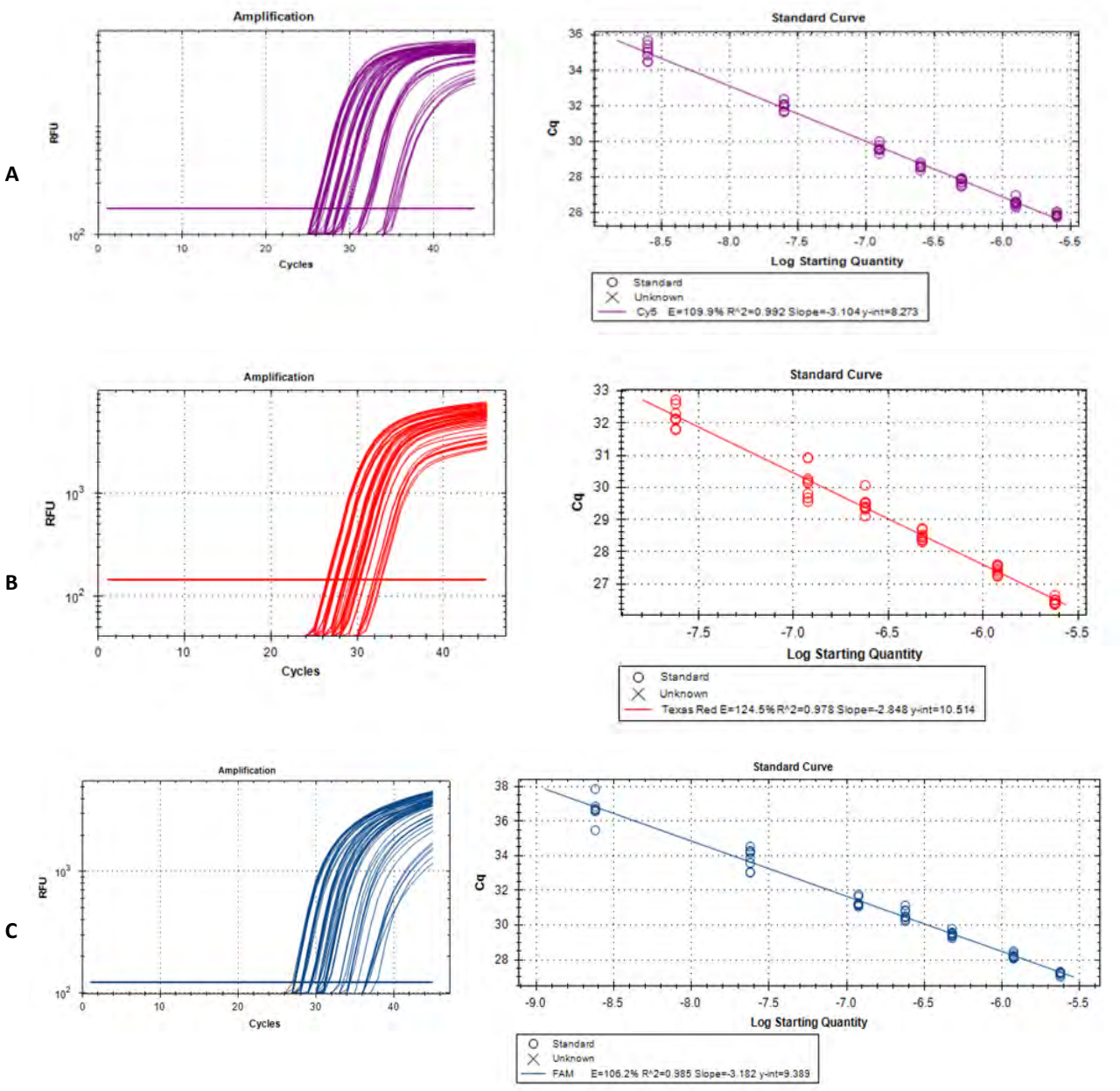


Figure 2.7 Standard curves for the individual viral target amplification in the viral multiplex real-time PCR assay; A- herpes simplex, B- enterovirus, C- mumps. Left- The amplification curves of the prepared plasmid standard concentrations (1000 copies/reaction - 1copy/reaction); Right- the linearity of the tested replicates and efficiency of amplification of the standard.

Table 2.10 Viral DNA Limit of detection

	Plasmid copies	No. of replicates	% Positive	Cq Mean	Cq Std. Dev	%CV
enterovirus	1000	8	100	26.98	0.103	0.381
	500	8	100	27.92	0.159	0.570
	200	8	100	29.02	0.136	0.468
	100	8	100	30.00	0.260	0.867
	50	8	100	30.66	0.479	1.563
	10	8	100	32.66	0.352	1.076
	1	8	50	34.66	0.893	2.578
	Plasmid copies	**No. of replicates**	**% Positive**	**Cq Mean**	**Cq Std. Dev**	**%CV**
mumps	1000	8	100	27.22	0.097	0.356
	500	8	100	28.22	0.143	0.508
	200	8	100	29.48	0.161	0.545
	100	8	100	30.60	0.306	1.000
	50	8	100	31.31	0.250	0.797
	10	8	100	33.85	0.573	1.693
	1	8	87.5	36.68	0.693	1.889
	Plasmid copies	**No. of replicates**	**% Positive**	**Cq Mean**	**Cq Std. Dev**	**%CV**
herpes simplex	1000	8	100	25.69	0.115	0.448
	500	8	100	26.47	0.230	0.868
	200	8	100	27.72	0.226	0.816
	100	8	100	28.61	0.145	0.506
	50	8	100	29.66	0.220	0.742
	10	8	100	32.12	0.276	0.860
	1	8	87.5	35.09	0.505	1.441

The typical quantity of infectious bacterial or viral loads in meningitis patients is reported to be in excess of 10^3-10^5 colony forming units/ml and 10^4 plaque forming units/ml respectively which should be detectable by the developed multiplex assays in the study[157,158]. The limits of detection for both assays are within the range of typical bacterial and viral load detection in infected cases.

There are other ways of determining the LOD besides empirical determination involving preparation and testing serial dilutions of plasmid standards (used in study) or culture standards spiked in cell matrix or statistically. Statistically determining the LOD involves testing blank specimens (without the target species) against low concentration samples to determine the point when a signal can be distinguished from background[140]. However, there should be an expected detection limit and more samples are required for the statistical LOD[140,159].

2.3.5 Analytical Precision

Total imprecision (%CV) values for the different reference standard concentrations in the bacterial multiplex ranged from 0.4% -1.6% for *S. pneumoniae*, 0.4% - 2% for *N. meningitidis* and 0.4% - 1.2% for *H. influenzae*, indicating that the intra-assay variability was increasing (%CV >1%) with lower concentrations (Table 2.9). The inter-assay precision was determined by repeating the multiplex the following day, only using the target organism at a concentration of 10 copies/reaction. The CV values for this were found to be <1% indicating high precision at low concentration (10 copies/reaction) of the bacterial targets. The reported %CV magnitude was <5%, therefore considered to show excellent precision[143,144].

The intra-assay variability for the viral multiplex was reported as 0.38–2.58%; 0.36–1.89% and 0.45–1.44% CV for enterovirus, mumps and herpes simplex virus target DNA, respectively (Table 2.10). The total imprecision values showed the assays to be less precise (%CV >1%) at lower concentrations with expected stochastic variation at low copies of the target[160]. Minimal inter-assay variation was detected with %CV < 1% at low concentration (10 copies/reaction) for each of the viral targets. The intra-assay and inter-assay CV values observed in the study were less than previously reported values for hydrolysis probe based RT-PCR assays using low concentration of standard viral target (2.2%, 5.5%, 3.4% and 6.2%)[158,161,162].

2.3.6 Analytical Specificity

Following an *in silico* analysis of both the bacterial and viral assay primers and probe sequences, the specificity of each bacterial and viral primer probe set was checked empirically in the multiplex by testing of a set of reference strains (Table 2.6). In the bacterial multiplex, extracted DNA from the reference strains tested was not amplified (Cq > 36) thus showing 100% specificity with only the positive controls (*S. pneumoniae*, *N. meningitidis* and *H. influenzae*) showing clear amplification.

The viral assay amplified the following positive controls: echovirus strains, mumps and herpes simplex 1 and 2, with no amplification of other reference strains (Cq value >36). With the limited source of test viral reference isolates, expense for procurement of viral controls and master mix reagents, and limited time, we were constrained to use the readily available isolates from the Virology section at Groote Schuur Hospital (Cape Town, South Africa), but testing with available strains indicates primer and probe specificity.

2.3.7 Incorporation of the IAC into bacterial multiplex assays

Once the bacterial multiplex had been validated, an IAC was added to the assay to control for any amplification inhibition. The initial IAC spiking concentration tested (10 copies/reaction) was too low for reproducible detection by qPCR. The concentration was therefore increased and an initial spiking concentration of 200 copies/reaction was found to be optimal for qPCR amplification efficiency (Figure 2.4). Co-amplification was tested for amplification of 200 copies of IAC and 10 copies of single bacterial target in the newly developed quadruplex assay while comparing the resulting individual Cq values to the original single target separate assays to compare the effect of added IAC (Table 2.11). The Cq values were reported in Table 2.11 along with the 95% CI of the mean Cq for the 5 replicates. The positive IAC signal with co-amplification signal of specific bacterial targets indicated that amplification was sufficient to generate a positive signal from targets present at the limit of detection (i.e. 10 copies) with relatively minimal interference.

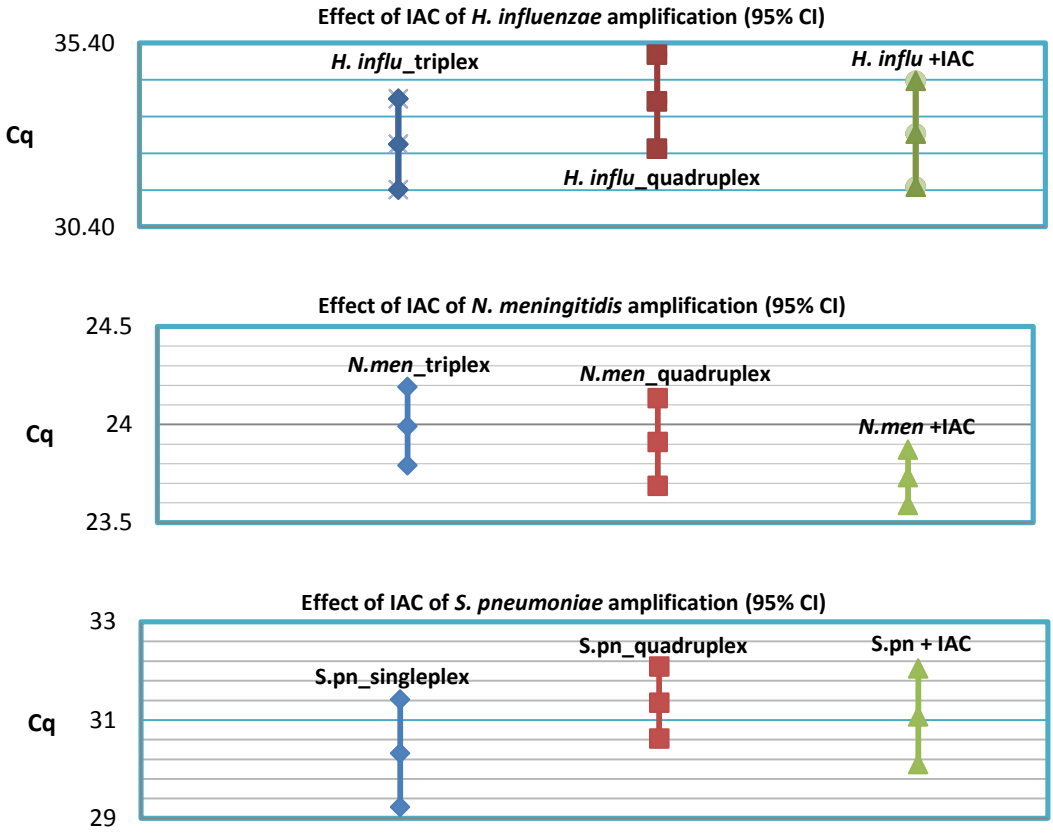
Table 2.11 Optimisation of quadruplex assay and effect of IAC on the bacterial targets

Flourophore	Sample	Cq Mean	Cq Std. Dev	95 %CI
Cy5	*H.influenzae* _triplex	32.65	0.631	(31.41-33.88)
Cy5	*H.influenzae* _quadruplex	33.81	0.653	(32.53-35.09)
Cy5	*H.influenzae* + IAC	32.93	0.735	(31.49-34.37)
VIC	IAC_singleplex	31.52	0.498	(30.54-32.50)
VIC	IAC_quadruplex	31.87	0.495	(30.90-32.84)
VIC	IAC + *H.influenzae*	31.99	0.403	(31.20-32.78)
Texas Red	*S. pnuemoniae*_triplex	31.77	0.448	(29.23-31.41)
Texas Red	*S. pneumoniae*_quadruplex	31.94	0.760	(30.62-32.08)
Texas Red	*S. pneumoniae* + IAC	31.98	0.427	(30.09-32.05)
VIC	IAC_ triplex	30.32	0.558	(29.23-31.41)
VIC	IAC_quadruplex	31.35	0.374	(30.62-32.09)
VIC	IAC + *S. pneumoniae*	31.07	0.498	(30.09-32.05)
FAM	*N. meningitidis*_triplex	23.99	0.102	(23.79-24.19)
FAM	*N. meningitidis*_quadruplex	23.91	0.114	(23.69-24.13)
FAM	*N. meningitidis* + IAC	23.73	0.072	(23.59-23.87)
VIC	IAC_singleplex	32.05	0.707	(30.66-33.44)
VIC	IAC_quadruplex	32.03	0.274	(31.49-32.57)
VIC	IAC + *N. meningitidis*	30.62	0.801	(29.05-32.19)

IAC- internal amplification control; Cy5 - Cyanine 5; FAM - 6-Flourescein; Std. Dev- standard deviation; CI- confidence interval

The addition of the IAC and primers and probes had no significant effect on the Cq of the bacterial target DNA (table 2.11, Figure 2.8). The 95% confidence intervals for each bacterial target with or without the

added IAC were similar, thus showing no effect on the bacterial target detection (Figure 2.8). This showed that the sensitivity of the quadruplex including the IAC for each of the bacterial targets was indistinguishable to that of the bacterial triplex.



S.pn- *S. pneumoniae*; N.men- *N. meningitidis*; H.influ- *H. influenzae*; IAC- Internal amplification control

Figure 2.8 95% Confidence intervals plot for the individual bacterial targets in the multiplex with addition of the internal amplification control

Since the bacterial multiplex was previously optimised and validated for all three targets, the addition of an IAC target would require less optimisation and addition into the viral multiplex with the previously observed spectral overlap, there would be risk of more challenging optimisation during multiplexing. The main assumption is that multiplex assays are carried out simultaneously otherwise upon request/requirement of a viral multiplex PCR test, there is need to also include the IAC target. The IAC in the bacterial multiplex aimed to control for inhibitors in the CSF.

2.3.8 Spiking of negative CSF with positive bacterial controls and IAC

To ensure that the extraction on the automated platform did not affect the downstream amplification, 200 copies of IAC were introduced into a negative CSF sample before extraction. A positive amplification curve for the IAC indicated 200 copies of the IAC spiked into 350µl CSF were sufficient for amplification, giving reproducible Cq values of less than 35.

The spiking of CSF with a single bacterial target in addition to the 200 copies of IAC however, resulted in no amplification of the bacterial target, indicating a reduction in the sensitivity of the bacterial assay. To optimise, bacterial template was added in increasing increments (Table 2.12) prior to extraction. By increasing the spiking quantity of bacterial DNA in the CSF sample the observed sensitivities were: 29 copies/µl, 286 copies/µl and 571 copies/µl of sample for *N. meningitidis*, *H. influenzae* and *S. pneumoniae* respectively (Table 2.12).

With the previously observed low limit of detection of the bacterial multiplex targets diluted in buffer, the observed increase in the LOD with the spiking of negative CSF showed the influence of extraction efficiency on the sensitivity of the assays. This clearly emphasises that the analytical sensitivity is characteristically different from clinical sensitivity. An assay with perfect analytical sensitivity does not equate to exceptional acceptable clinical sensitivity[140]. Various factors come into play, namely: the integrity of the nucleic acid material tested the presence of the target material, efficiency of sample processing and sample collection, and the presence of inhibitory substances in the sample matrix[140,163].

Table 2.12: The limit of detection of spiked CSF with plasmid target DNA

Amount of Target spiked		10 copies	20 copies	50 copies	100 copies	200 copies
% qPCR Positive	*S. pneumoniae*	0	0	0	60%	100%
	H. influenzae	0	40%	80%	100%	-
	N. meningitidis	100%	100%	100%	-	-

The included IAC has the ability to be used for quantitative purposes. Prior inhibition control involved use of the human RNase P as an endogenous target, but upon testing on the control strains of the bacterial targets and some related bacterial species had suspected cross-reactive amplification which proved would be problematic in assessing the presence of inhibitors in the sample. The adopted exogenous control

enabled the bacterial and viral multiplex to have ability to be quantitative in estimating the load of the infecting species thus able to aid in monitoring the infection load and inferring on the severity of high and low loads of aetiology to help decide on prognosis of syndrome[164]. There is a high possibility of a high IAC overshadowing the effect of inhibition in the sample thus the spiking concentration should be carefully optimised[140].

2.4 Conclusion

The developed viral multiplex described showed similar sensitivities to the adopted singleplex PCR for the detection of the viral targets with minimal reported variability. This conserves resources and utilizes small volume samples making it useful in resource limited settings. The addition of the internal control (IC) to the bacterial multiplex demonstrated little interference in the observed reaction kinetics and sensitivities for detection of the bacterial target with IAC co-amplification. With an acceptable performance specification defined for the validated multiplex assays, clinical assessment to validate their diagnostic ability in disease identification in a population is needed.

CHAPTER III

Screening of Samples

Contents

3.0 Summary

With conventional methods of diagnosis, substantial overlaps are common due to an absence of the expected CSF findings clearly aligning to bacterial or viral infection. Their reduced sensitivity is commonly observed mainly due to empiric antibiotic treatment leading to bacterial culture negative results. This leads to costly hospital admissions and unnecessarily prolonged treatment for the unconfirmed aetiology. In addition routine viral diagnostics are not commonly implemented for meningitis diagnosis. Using the developed real-time (qPCR) multiplex assays, we tested for 3 bacterial causes, namely: *Neisseria meningitidis* (*ctrA* gene), *Haemophilus influenzae* (*hpd* gene) and *Streptococcus pneumoniae* (*lytA* gene). In a separate viral multiplex qPCR, the viruses targeted in this study included: enterovirus (5‟ UTR), herpes simplex (UL30 gene) and mumps virus (Fusion protein gene). These validated assays were then used to screen CSF from a paediatric population of suspected meningitis cases. The retrospective study included 292 paediatric patients aged from 60 days to 12 years, (median age 19 months (IQR: 6-65 months)). CSF with abnormal chemistry and cell counts were selected and total nucleic acid extracted with the QIAsymphony virus/bacterial DSP kit (QIAGEN, Valencia, CA). In 2.4% (7) cases Gram stain results were obtained along with a relatively few cases with positive bacterial culture growth 1.4% (4/292). Based on bacterial qPCR results, 2.7% (8), 1% (3) and 0.3% (1) were positive for *S. pneumoniae*, *N. meningitidis* and *H. influenzae* respectively. The majority of cases were viral positives with enteroviruses being the dominant (31.2%) followed by mumps virus positives (1%). No herpes simplex was detected. The bacterial qPCR showed a sensitivity and specificity of 85.7% (95% CI: 48.7-97.4) and 97.9% (95% CI: 95.5-99) respectively when compared against a composite reference standard (CRS) of Gram stain results and bacterial culture. We report an improvement with additional detected cases of bacterial meningitis (BM) and highlight the burden of the common viral causes. These developed multiplex PCRs prove valuable in differentiating between bacterial and viral aetiology in routine diagnosis.

3.1 Introduction

With the use of PCR, an improved molecular based diagnostic test, initial validation is required to establish the analytical performance characteristics of the test. This involves the screening of a population suspected to have a condition or disease in a clinical setting. The diagnostic/clinical performance of the test method can then be analysed against a reference test that defines the condition of interest in the population[139,140]. Regardless of acceptable analytical performance of the test, there is no assurance of acceptable clinical performance, thus careful optimisation of the test is paramount[139,140].

We sought to develop and validate multi-target real-time PCR assays that would detect both bacterial and viral meningitis pathogens common to paediatric populations. The validated tests were then applied to test children with suspected meningitis in a local clinical setting to investigate the burden of some of the common paediatric community acquired meningitis aetiology in the increasing culture negative cases in acute care at the Red Cross Memorial Children‟s hospital.

3.2 Methodology

3.2.1 Ethics statement

The Human Research Ethics Committee at the Faculty of Health Sciences, University of Cape Town, Cape Town, South Africa (HREC REF: 739/2013) approved the surveillance study. No consent was to be obtained as the collection of CSF and screening of samples was carried out retrospectively after routine laboratory processing. Patient demographics, immunization histories, clinical information and laboratory findings were collected by a clinical collaborator using a standardised case record form. No consent was necessary for the record review.

3.2.2 Patient profile

We aimed to recruit children with community acquired meningitis. We therefore identified children who met the following criteria:

- older than 60 days and ≤ 12years
- presented to the acute care or outpatient departments of the Red Cross War Memorial Children‟s Hospital (RCCH) in Cape Town
- had a lumbar puncture performed according to the attending clinician‟s decision, (presumably because of clinical signs and symptoms suggestive of meningitis)
- whose CSF showed an abnormal cell count, defined as any neutrophils or > 5 lymphocytes.

Patients with underlying conditions such as prior head trauma or ventricular peritoneal shunt (VP shunts) were excluded.

3.2.3 Sample collection

CSF samples from patients were collected between 1 November 2012 and 31October 2013. Based on initial CSF result, patients whose CSF showed an abnormal cell count were selected. All CSF samples were stored at 4°C for a minimum of 2 days and a maximum of 5 days to allow for possible requests by clinicians for additional testing. Following routine laboratory processing, residual CSF samples were picked up from the Diagnostic Medical microbiology lab Groote Schuur Laboratory in their sterile CSF tubes and sent to the Research Medical microbiology laboratory. Upon reaching the Medical Microbiology laboratory, these samples were immediately aliquoted into sterile 2ml cryogenic vials for storage at -70^{o}C until total nucleic acid was extracted. For practical reasons we required at least 140µl to be available for testing after completion of routine laboratory tests.

3.2.4 Conventional laboratory microbiology testing

Microscopy and bacterial culture were carried out at Groote Schuur Hospital. The differential white cell counts and biochemical tests for glucose and protein concentration were performed at Red Cross War Memorial Children"s Hospital.

3.2.5 CSF total nucleic acid extraction

Total nucleic acid was extracted from 400µl of CSF with the QIAsymphony virus/bacterial DSP kit (QIAGEN, Valencia, CA) using the QIAsymphony SP (QIAGEN, Valencia, CA) automated platform. Total nucleic acid was eluted in 60µl of elution buffer and stored at -21^{o}C. Specimens with smaller available starting volumes (a minimum volume of 140µl) were topped up with ATL lysis buffer (QIAGEN, Valencia, CA) before extraction. The maximum volume of sample required for extraction was 400µl. An exogenous plasmid control was spiked into each sample at 200 copies/extraction volume as an extraction control. A volume of 400µl, nuclease free water was included as the extraction negative control.

3.2.6 Real-time PCR detection

3.2.6.1 Bacterial detection

A modified real-time multiplex PCR assay (Section 2.3.6) was used to identify patients with bacterial meningitis. The Internal amplification control (IAC) plasmid was co-amplified with the bacterial targets. The PCR reaction mixtures were prepared as described in Chapter 2. The extracted nuclease free water was also included in each plate as a negative or non-template control. The positive controls for all four targets were co-amplified in a single well. A positive result was defined as a cut-off cycle threshold (Cq) value below 36, an inconclusive or negative result as a Cq value ≥36 (Section 2.4.3.1). All inconclusive results were repeated. All reactions were prepared in a 96 well plate that included positive controls, for each of the bacterial target organisms.

3.2.6.2 Viral detection

The in-house multiplex RT-PCR (section 2.4.1) was as described in Chapter 2. A positive result was defined as a cut-off cycle threshold (Cq) value below 35, an inconclusive or negative result as a Cq value

≥35 (Section 2.4.3.1). All inconclusive results were repeated. All reactions were prepared in a 96 well plate that included positive controls, for each of the target organisms.

3.2.7 Data Analysis

The proportions of bacterial and viral pathogen positive tests detected were summarised for all patients. CSF samples that were negative by both bacterial culture and molecular detection methods were classed as „no target detected". The point prevalence for each pathogen target was calculated and changes in proportion were analysed seasonally.

We compared the test parameters (sensitivity and specificity estimates) of the bacterial qPCR assessed against CSF culture reference standard using 2x2 validation analysis (Appendix C). We also evaluated the bacterial qPCR assay against a composite reference standard (CRS) comprising of CSF culture and CSF Gram staining. The CRS aims to validate a test method against multiple index tests when the reference standard test is imperfect, in this case bacterial culture[165,166]. A positive CRS result was defined as a positive CSF culture or Gram stain result while a negative CRS was negative for both the tests. For each sensitivity and specificity parameter resulting from the classical validation and CRS analysis, we calculated its 95% confidence intervals with the Wilson score method[167].

For analysis of the bacterial causes, the included patient cases were classified as suspected cases using the inclusion criteria and further as probable or confirmed bacterial meningitis according to the WHO Coordinated Invasive Bacterial Vaccine Preventable Diseases (IB-VPD) Surveillance Network (Table 3.1). The classification of the cases serves to define the likelihood of patients having meningitis by incorporating various clinical symptoms and diagnostic tests to help define the disease profile. These definitions will evaluate the clinical sensitivity and specificity of the qPCR detected cases to the obtained clinical diagnostic outcome. Additionally, the bacterial qPCR positives were also evaluated against the classified confirmed cases.

For the viral causes, we compared our results to results of other viral tests where these were performed. In both viral and bacterial causes, we also compared our results to the final clinical diagnosis and treatment as recorded in the patient's clinical records, as extracted by our clinical collaborator.

Table 3.1 Case definitions for classification of Bacterial meningitis

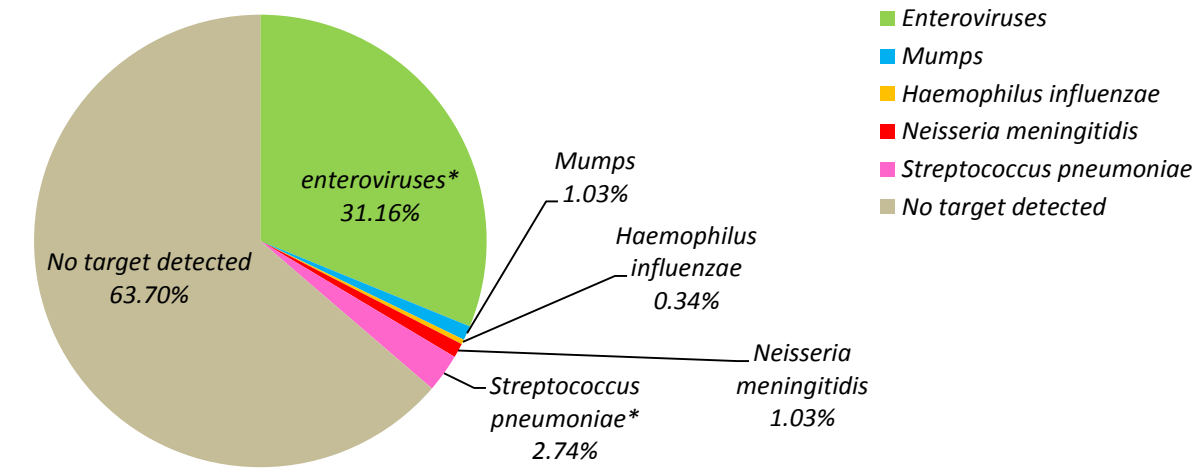
Bacterial meningitis **WHO-recommended standards for surveillance of selected vaccine preventable diseases, 2003**
Suspected meningitis Any child aged 0-59 months admitted to a sentinel hospital conducting surveillance with sudden onset of fever (>38.5°C rectal or 38.0°C axillary) and one of the following signs: neck stiffness, altered consciousness with no other alternative diagnosis, or other meningeal sign **Or** Every patient aged less than 5 years of age hospitalized with a clinical diagnosis of meningitis.
Probable meningitis A suspected meningitis case (as defined above) with CSF examination showing at least one of the following: • Turbid appearance; • Leukocytosis (>100 cells/mm3); • Leukocytosis (10-100 cells/ mm3) AND either an elevated protein (>100 mg/dl) or decreased glucose (< 40 mg/dl) Note: if protein and glucose results are not available, diagnose using the first two conditions (i.e. turbid appearance or leukocytosis >100 cells/mm3)
Confirmed meningitis A suspected meningitis case that is laboratory-confirmed by growing (i.e. culturing) or identifying (i.e. by Gram stain, antigen detection, immunochromotography, PCR or other methods) a bacterial pathogen (Hib, pneumococcus or meningococcus) in the CSF or from the blood in a child with a clinical syndrome consistent with bacterial meningitis

3.3 Results

3.3.1 Screening results

300 CSF samples were screened using the multiplex PCRs, but 8 cases had prior head trauma and/ or missing clinical information and were excluded, leaving 292 cases. Despite the necessary measures taken to avoid contamination, we suspected contamination upon screening the samples with the viral target qPCR due to an unexpectedly high proportion of herpes simplex target positives which were concentrated in a single batch. HSV is a rare cause of meningitis in children being most commonly associated with encephalitis. In addition, some of the non-template PCR controls on the contaminated plates had low signal amplification and there were a couple of mixed positive signals for HSV and EV which is very unlikely to occur. The entire viral qPCR run was repeated and there were no unexpected distributions of the viral targets detected. The results reported are from the second screening. The bacterial qPCR had no obvious contamination problems with no visible amplification signals observed from the added controls and no clustering of positive results.

Using the bacterial multiplex qPCR, 4.1% (12/292) were found to harbour bacterial target DNA, with 2.7% (8/292) having *S. pneumoniae*; 1% (3/292) *N. meningitidis* and 0.3% (1/292) *H. influenzae*. Ninety-four samples tested positive for the target viral DNA with the viral multiplex qPCR. Co-amplification of all the internal amplification controls in the bacterial qPCR revealed the efficient extraction of nucleic acid and no observed inhibition in the samples. The enteroviruses were detected most frequently at 31.2% (91/292) followed by the mumps virus at 1% (3/292). No samples were positive for herpes simplex DNA. A large proportion of cases (63.7%) had no target DNA detected (Figure 3.1).



* *1 case had both S. pneumoniae and* enterovirus *detected and is included in both categories*

Figure 3.1 Distribution of aetiology in suspected meningitis cases occurring among outpatient children at the Red Cross Children"s Hospital in Cape Town, South Africa, November 2012 – October 2013 (n=292).

The enteroviral prevalence showed peaks in the summer period (Figure 3.2). The rest of the targeted etiologic agents occurred throughout the year (Figure 3.2). Pneumococcal meningitis was the most common of the bacterial cases, occurring throughout the year. The other two tested bacterial causes had a more sporadic distribution throughout the sampling timeline.

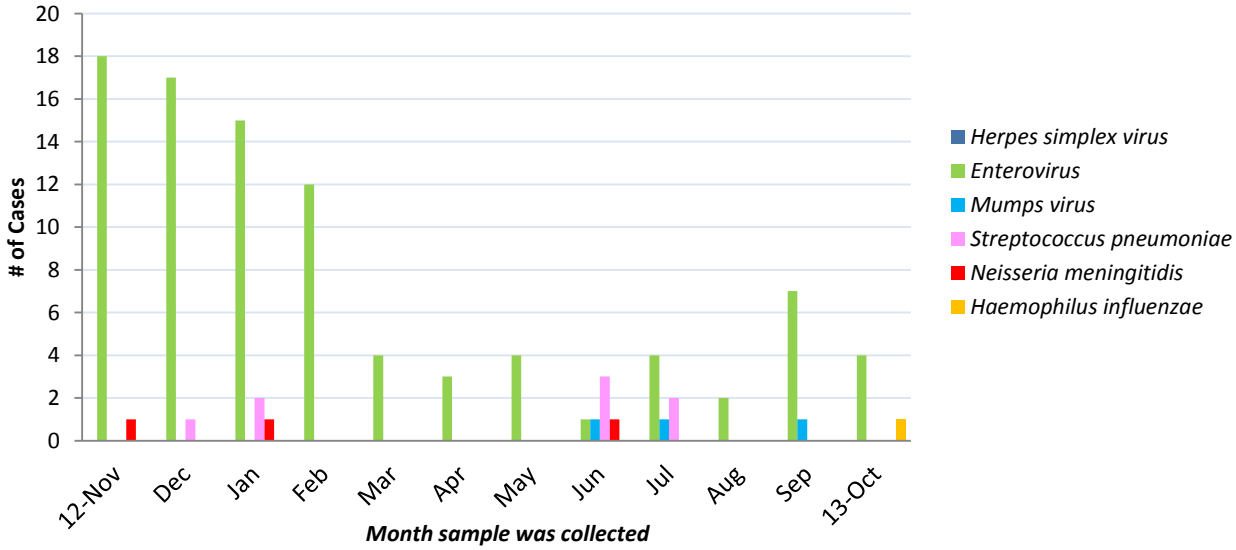
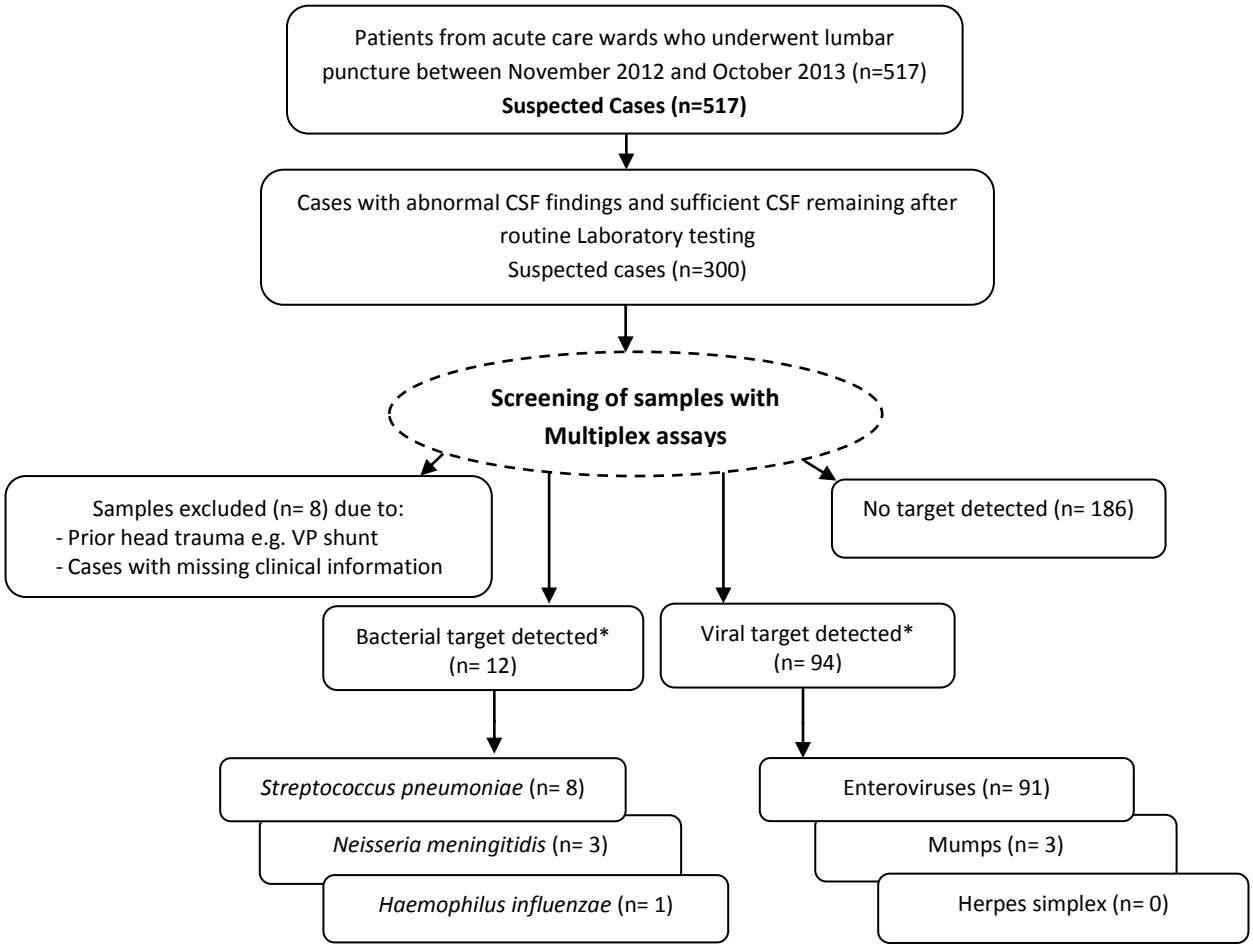


Figure 3.2 Monthly variation of bacterial and viral aetiology of meningitis, occurring among outpatient children at the Red Cross Children"s Hospital in Cape Town, South Africa, 1 November 2012 – 31 October 2013 (n=292).

3.3.2 Study population

The 292 suspected cases were screened and classified according to the WHO case definitions for bacterial meningitis (Table 3.1 and Figure 3.3). The median age of children was 2.94 years (IQR of 60 days to13 years) and a similar male and female proportion of 52.6% and 47.1%, was observed respectively. All the cases were truly outpatients; however some were referrals who had received prior health care before being sent to RXH.



* - One case had both *S. pneumoniae* and enterovirus detected

Figure 3.3 Schematic for bacterial and viral qPCR screening of CSF samples (n=292). No target detected cases were defined as having none of the 3 bacteria or viral targets detected in the samples.

3.3.3 Discharge diagnosis and treatment of the screened population

Table 3.2 summarises the discharge diagnosis of the 292 suspected cases. From these suspected cases, 24% (71/292) had a discharge diagnosis of bacterial meningitis with 80% (57/71) only receiving antibiotic treatment (Table 3.2). 49.3% (35/71) of the BM discharged cases were confirmed viral target positives by the viral multiplex, with 32/35 of the cases being given antibiotic treatment (Table 3.2). Additionally, among the BM diagnosis cases, 12.7% (9/71) were confirmed bacterial qPCR with 6/9 patients not receiving antibiotic treatment (Table 3.2). Of the BM discharge diagnosed patients 22 cases were prescribed antibiotics but they were negative by both the bacterial and viral multiplex.

The discharge diagnosis on the suspected patients showed an increased amount of viral meningitis cases, 33.2% (97/292). Only one case was confirmed for a viral target using the viral multiplex although the patient received antibiotics. Sixteen cases (16/97) positive for the bacterial targets were prescribed antibiotics along with the 22/97 cases, negative by both multiplex PCRs. Fourteen cases had re-admission due to meningitis with 3 cases having initially been confirmed viral target positives by the viral multiplex.

Table 3.2: The discharge diagnosis, treatment and multiplex qPCR results of the screened suspected meningitis patients.

Discharge Diagnosis		**Antibiotics**		**Multiplex qPCR results**			**Re-admission due to meningitis**
		No	*Yes*	Viral	Bacterial	Negative	
Bacterial meningitis	71/292 (24.3%)	14	57	32 3 **35 (49.3 %)**	3 6 **9 (12.7%)**	22 5	2
Viral meningitis	97/292 (33.2%)	58	39	1 28 **29 (29.9%)**	16 - **16 (16.5)**	22 30	7(1 VM)*
Partially treated meningitis	19/292 (6.5%)	3	16	6 1 **7 (36.8%)**	- -	10 2	3 (2 VM)$^{\alpha}$
Other$^{\#}$	105/292 (36%)	50	55	2 5 **7 (6.7%)**	- 2 **2 (0.02%)**	53 43	2

$^{\#}$- the diagnosis is not specified or unknown;*- 1 case was qPCR viral multiplex positive only, $^{\alpha}$ - 2 cases were qPCR viral multiplex positives only

3.3.4 Comparison of microbiological testing to bacterial qPCR test results

For validation of the diagnostic performance of the bacterial qPCR assay, its sensitivity and specificity were estimated against a CSF culture reference standard and a composite reference standard (CRS). The diagnostic performance of Gram staining was also estimated. The 2x2 validation analysis tables are included in Appendix C and the test validation parameters were summarized in Table 3.3. Both the bacterial qPCR and CSF Gram staining showed high sensitivity and specificity when compared against a CSF culture (>95%) although a wide range of CI was observed. The qPCR performance showed a sensitivity of 85.7% and a specificity of 97.9% when compared to the CRS. The positive predictive values for qPCR and Gram‟s stain against detection by culture were generally low at 33.3 % and 50% respectively (Table 3.3). Against the CRS, the qPCR‟s PPV value was also low at 50%.

Table 3.3: Sensitivity and specificity estimates for diagnosis of *S. pneumoniae*, *N. meningitidis*, or *H. influenzae* meningitis

S. pneumoniae, *N. meningitidis*, or *H. influenzae* (n= 292)	CSF bacterial culture				CRS				Confirmed cases			
	Sensitivity (%, 95%CI)	Specificity (%, 95% CI)	**PPV**	**NPV**	Sensitivity (%, 95%CI)	Specificity (%, 95% CI)	**PPV**	**NPV**	Sensitivity (%, 95%CI)	Specificity (%, 95% CI)	**PPV**	**NPV**
Gram's Stain	100 (51-100)	99 (97-99.7)	50%	99.6%	na	na	na	na	na	na	na	na
Bacterial qPCR	100 (51-100)	97.2 (94.6-98.6)	33%	100%	85.7 (48.7-97.4)	97.9 (95.5-99)	50%	99.6%	60 (31.3-83.2)	97.9 (95.4-99)	50%	100%

CRS- composite reference standard; PPV- positive predictive; NPV- negative predictive value; qPCR- real-time PCR; 95% CI- 95% confidence intervals[167] ; na- not applicable

3.3.5 Bacterial meningitis cases: Categorisation of cases and comparison with qPCR results

Using the bacterial qPCR, 12 samples had bacterial target DNA detected. 6/12 met criteria for confirmed meningitis, based on CSF culture in 4, and on CSF Gram stain plus blood culture in 1 and CSF Gram stain alone in 1 (Table 3.4). The remaining 6 cases (4 *S. pneumoniae* and 2 *N. meningitidis*) were not confirmed with conventional testing (Table 3.4). Of these 6 unconfirmed cases, 3 had a discharge diagnosis of bacterial meningitis while 2 of 6 had a CSF cell count >100 and at least 2/6 had documentation of antibiotic administration prior to lumbar puncture.

All the patients were categorised according to the WHO criteria for classification of meningitis. Ten cases were confirmed cases of bacterial meningitis (Table 3.5), and 5 cases were classed as probable meningitis cases (Table 3.6). The remainder (277) were classified as suspected cases.

Six of the confirmed cases (Table 3.5) were qPCR positive and, in each case the organism identified was congruent with the conventional microbiology result. Four of the confirmed cases had negative qPCR results: in 2 patients the confirmation was based on Gram stain alone (Gram positive cocci or Gram positive diplococci), while in the other two patients, confirmation was based on isolation of *S. pneumoniae* from blood culture in a patient with an abnormal CSF cell count. One of the five probable cases was qPCR positive and 4 were negative for all tests (Table 3.6). Of the remaining suspected meningitis cases, 5 cases were qPCR positive.

A 2x2 contingency table was created to compare the sensitivity of bacterial qPCR against the classified confirmed meningitis cases (Table 3.7). A sensitivity of 60% (6/10) was observed with wide confidence intervals and a high specificity of 97% (276/282) (Table 3.7).

Table 3.4 Clinical information and Laboratory results for the bacterial qPCR positive cases

Study number	qPCR Target	Prior antibiotics	Discharge Diagnosis	Discharge Treatment	Leukocytes	Polymorph	Lymphocytes	Erythrocytes	Glucose mg/dl	Protein mg/dl	CSF Gram stain	CSF Culture	BC Gram stain	Blood Culture
Confirmed cases														
20	*S. pneumoniae*	nd	BM	Supplements and Panado	12	10	2	2	37.8	99	GPDC	*S. pneumoniae*	GPC in pairs	*S. pneumoniae*
30	*H. influenzae*	No	BM	Antibiotics	2368	1754	614	26	5.4	112	GNB	*H. influenzae*	GNB	*H. influenzae*
86	*N. meningitidis*	No	BM	Antibiotics, TB treatment	2480	1680	800	400	5.4	102	GNDC	Neg after 3days	GNDC	*N. meningitidis*
112	*S. pneumoniae*	Yes	BM	-	1680	1620	60	25	63	82	GPDC	*S. pneumoniae*	GPC in pairs	*S. pneumoniae*
114	*S. pneumoniae*	Yes	BM	-	2640	2480	160	20	63	48	GPC	Neg after 3days	Negative	Neg after 3days
293	*S. pneumoniae*	No	BM	-	5300	4500	800	0	0	200	GPC in chains	*S. pneumoniae*	Negative	Neg after 3days
Unconfirmed cases														
8	*S. pneumoniae*	nd	Other	Panado	2	1	1	0	70.2	14	Negative	Neg after 3days	Negative	Neg after 3days
99	*S. pneumoniae* & enterovirus	nd	BM	Antibiotics	131	95	36	310	70.2	38	Negative	Neg after 3days	Negative	Neg after 3days
177	*N. meningitidis*	nd	BM	-	251	11	240	25	57.6	71	Negative	Neg after 3days	-	-
228	*S. pneumoniae*	Yes	BM	Supplements	19	18	1	960	115.2	112	Negative	Neg after 3days	Negative	Neg after 3days
250	*N. meningitidis*	Yes	VM	Antibiotics	14	0	14	14	54	18	Negative	Neg after 3days	Negative	Neg after 3days
296	*S. pneumoniae*	No	UTI and GE.	-	10	1	9	390	79.2	22	Negative	Neg after 3days	-	-

CSF- cerebrospinal fluid; qPCR- quantitative real-time PCR; BC- blood culture; GPDC- Gram positive diplococcic; GPC- Gram positive cocci; GNB- Gram negative bacilli; GPB- Gram positive bacilli GNDC- Gram negative diplococcic; nd- not documented; UTI- urinary tract infection; GE- gastroenteritis; Neg- negative

Table 3.5 Confirmed cases of meningitis according to the WHO case definition guidelines

Study number	Glucose mg/dl	Protein mg/dl	Leukocytes	qPCR Result	CSF Gram stain	CSF Culture	BC Gram stain	Blood culture	Extra notes
20	37.8	99	12	*S. pneumoniae*	Negative	*S. pneumoniae*	GPC in pairs	*S. pneumoniae*	BC GPC in pairs. CXR bilateral bibasal patchy lower lobar opacification
30	5.4	112	2368	*H. influenzae*	GNB	*H. influenzae*	GNB	*H. influenzae*	-
78	66.6	12	5	-	GPDC	Neg after 3days	Negative	Neg after 3days	-
86	5.4	102	2480	*N. meningitidis*	GNDC	Neg after 3days	GNDC	*N. meningitidis*	BC was Penicillin sensitive.
98	66.6	133	1035	-	Negative	Neg after 3days	Negative	*S. pneumoniae*	*S. pneumoniae* resistant to penicillin, bactrim, tetracycline. Sensitive to vancomycin and chloramphenicol.
112	63	82	1680	*S. pneumoniae*	GPDC	*S. pneumoniae*	GPC in chains	*S. pneumoniae*	BC *S. pneumoniae* cultured, sensitive to penicillin and ceftriaxone. XR shows bronchovascular markings. Sputum negative for TB after 43 days
114	63	48	2640	*S. pneumoniae*	GPC	Neg after 3days	Negative	Neg after 3days	-
164	72	44	48	-	Negative	Neg after 3days	GPC in chains	*S. pneumoniae*	XR bronchiolitis
277	64.8	25	425	-	GPDC	Neg after 3days	GPB	*Bacillus* species	CSF GPDC. Blood grew *Bacillus* species
293	ND	200	5300	*S. pneumoniae*	GPC in chains	*S. pneumoniae*	Negative	Neg after 3days	CXR right middle lobe atelectasis. Progressive left lower lobe collapse. Bilated basal ganglia infarcts. Diffuse brain swelling. Right subdural hygroma

CSF- cerebrospinal fluid; qPCR- quantitative real-time PCR; BC- blood culture; GPDC- Gram positive diplococcic; GPC- Gram positive cocci; GNB- Gram negative bacilli; GPB- Gram positive bacilli GNDC- Gram negative diplococcic; XR- X-ray; CXR- chest x-ray; Neg- negative

Table 3.6 Probable cases of meningitis according to the WHO case definition guidelines

Study number	Glucose mg/dl	Protein mg/dl	Leukocytes	qPCR Result	CSF Gram stain	CSF Culture	BC Gram stain	Blood culture	Extra notes
58	52.2	100	12	-	Negative	Neg after 3days	Negative	Neg after 3days	Cxr RUL pneumonia CSF grew *Staphylococcus capitis*. Contaminant
228	115.2	112	19	*S. pneumoniae*	Negative	Neg after 3days	Negative	Neg after 3days	-
255	63	332	10	-	Negative	Neg after 3days	Negative	Neg after 3days	-
272	77.4	104	66	-	Negative	Neg after 3days	Negative	Neg after 3days	-
411	153	108	12	-	Negative	Neg after 3days	Negative	Neg after 3days	-

CSF- cerebrospinal fluid; qPCR- quantitative real-time PCR; BC- blood culture; Cxr- chest x-ray; RUL- Right upper lobe; Neg- negative

Table 3.7 2X2 Contingency table for bacterial qPCR results against the confirmed meningitis cases

Bacterial qPCR vs Confirmed meningitis cases		Confirmed meningitis case	Unconfirmed meningitis case	
	qPCR positive	6	6	**12**
	qPCR negative	4	276	**280**
		10	**282**	
Confidence interval:	95%			
Sensitivity:	60.00 %	CI: 0.3127 to 0.8318		
Specificity:	97.87%	CI: 0.9544 to 0.9902		

3.1.1 Viral meningitis cases: Comparison of qPCR results to conventional virology

The viral cases detected by qPCR were summarised in the Appendix A. None of the cases positive for viral target DNA by qPCR had a bacterial cause confirmed on the grounds of CSF Gram stain or culture. Although 4 patients had organisms isolated from their blood culture samples these were considered to be contaminants. Patient 99 had co-infection with both *S. pneumoniae* and enterovirus identified.

Based on clinician request, 9 samples had been referred for in-house viral PCR testing: 6 samples were tested for enteroviruses, 4 for herpes simplex (with 1 sample being tested for both EV and HSV (Appendix B). There were no discrepant results with qPCR testing. Four cases (patient 24, 31, 43 and 191) were confirmed EV positive, 2 (patient 29 and 88) were negative for EV and 4 (patient 56, 88, 109 and 115) were HSV negative. One case (patient 56) tested negative for HSV by in-house PCR, but was positive for mumps by qPCR. Patient 88 was the only sample, referred to another laboratory, with viral culture carried out and was negative for any viral growth.

3.2 Discussion

3.4.1 Bacterial population prevalence

Failure to diagnose the aetiology meningitis using the standard microbiological and biochemical diagnostic methods can be fatal and costly[6,14,101,102,119,120,168]. qPCR has previously been shown to improve the diagnosis of meningitis[89,169,170]. The data obtained from this study reflects not only the burden of disease but also the associated pathogens within the population of suspected meningitis cases in children at Red Cross Children Hospital. However, this is limited to 6 pathogens. The identification of these causative agents is crucial for appropriate therapy to be given to the patient. The main problems highlighted from this study are the challenges involved in the diagnosis of bacterial meningitis with standard laboratory tests being affected by prior antibiotic therapy and the need for viral screening, especially for enteroviruses in routine diagnostics.

The reported proportion of meningitis cases with bacteria identified (4.1%) was consistent with previous predominantly PCR-based, post-vaccine reports of similarly low BM prevalence (4% - 19%) in children worldwide[171–173]. These surveyed acute care emergency (outpatient) departments. The inclusion of neonates in these comparison studies could explain the difference. Previous work reported a seemingly reduced incidence of the targeted bacterial causes due to effective vaccine programs and empiric antibiotic therapy currently employed in South Africa which increase herd immunity and reduce the ability of detection with the available tests respectively[174]. Most of the patient cases admitted at Red Cross children‟s hospital had received prior health care with antibiotic management before referral, even before the LPs were carried out. This explains the low burden observed, and likely underestimation of the true burden of BM cases[174,175].

Even with the low bacterial prevalence seen in these cases, BM still remains a major cause of childhood mortality especially in Africa, mostly owing to the absence of pneumococcal vaccination[176–179]. A low burden of the previously dominant *H. influenzae type b* and *S. pneumoniae* cases showed the effectiveness of the routine paediatric immunisation schedule against the two common bacterial causes with the Hib and PCV vaccines respectively. This was similarly shown with post-vaccine surveillance, PCR based, reporting small numbers of Hib and pneumococcal cases[170,180–185]. Wolzak and colleagues showed similar post Hib vaccine prevalence in Cape Town of 4% (23/557) for *S. pneumoniae* cases, <1% (3/557) *H. influenzae* type b and 1% (6/557) *N. meningitidis* cases[186]. The majority of BM cases were identified in children <5 years emphasizing their substantial high risk to BM infection[9]. Furthermore, *S. pneumoniae*

remains the leading cause of bacterial meningitis[186], even in the face of reported reduction with pneumococcal vaccination[186,187].

qPCR detected additional cases not detected by routine diagnostic tests thus proving the potential value in incorporation into routine clinical diagnostics. The additional confirmed cases were culture negative, proving qPCR to be advantageous in diagnosis and useful for epidemic outbreak surveillance with improved rapid result output[170,188,189]. This is however anticipated with advances in detection technology from culture-based detection to real-time PCR even with viral detection[190]. The limited target coverage of real-time PCR is one limiting factor which has been observed in this study and may account for the high proportion of unidentified cases. The similarly high proportion of unidentified aetiology of meningitis cases reported in other studies[171–173] emphasises a need to improve diagnostics and incorporate broad spectrum target platforms such as commercially available TaqMan Array screening cards or Fast Track Diagnostics multiplex panels (with clinically relevant targets) to better describe the aetiology profile for the syndrome[191]. These also have the ability to expose mixed infection possibilities. Future research using broad range target PCR and massively parallel sequencing platforms to define the microbiome of predominantly culture negative CSF can also be pursued.

Traumatic LPs were common in the screening patients and without any cell count correction, the CSF maintains an abnormal profile[85]. This tends to include non-meningitis cases as probable meningitis cases with abnormal profiles thus increasing the number of unidentified aetiology upon screening.

3.4.2 Analysis of Discrepant results qPCR results and confirmed bacterial meningitis cases

In the study we encountered discrepant results 6/12 possible false positives and 4/10 possible false negatives, resulting in low sensitivity and positive predictive values. Analysis of the clinical information for the inconsistent cases helped to provide possible reasons for these discrepancies.

Abnormal CSF profiles were observed although CSF culture and blood culture growth was negative. These cases were qPCR positive thus could be potential additional cases detected or possible false positives. The observed culture negative/qPCR positives were likely due to the activity of prior antibiotic therapy in CSF, which has been previously described[192]. This was the case for some patients, likely receiving prior care and administration of antibiotics before referral to RXH and prior to the LP being

carried out. Prior antibiotic treatment has been reported as a risk factor for leading to culture negative but qPCR positive results, and even altering the abnormal CSF profiles, leading to sterile blood and CSF culture results[89,193,194](Case 250, 177). For cases 250 and 177 with detected *N. meningitidis* by qPCR, these could have been additionally detected bacterial cases but with prior antibiotics, culture negative results indicated a possible misdiagnosis of BM due to the high susceptibility of the pathogen to short treatments with antibiotics[192].

Contamination of CSF with bacterial DNA is possible if there was bacteraemia at the time of the lumbar puncture and a bloody spinal tap introduced pathogens from the bloodstream into the CSF. In the absence of a contemporary blood culture, it‚s not possible to determine if there was bloodstream infection in 3 such cases (Case 8, 228, 296).

Other possibilities could involve cross contamination in laboratory, either during initial processing or during qPCR, as experienced in the initial round of screening. As for the observed mixed target (*S. pneumoniae* and enteroviruses) detected case with no confirmatory culture growth, there is limited evidence of bacterial-viral co-infection in CSF infections with rare cases seen even in Africa[195–197]. This case could be a reflection of bacterial DNA contamination as the bacterial qPCR results were not repeated. However mixed infections in other syndromes such as pneumonia have been exposed by implementation of molecular techniques thus co-infection could be possible[198,199].

Poor design of the targets in the PCR could possible reduce the specificity of the assay leading to false positive from cross reactive species[140]. With the prior validation of the qPCR‟s cross reactivity to closely related target species by empiric testing and *in silico* analysis of priming targets, we obtained high specificity thus less probable to occur[140,200].

False negative qPCR results reflect the low sensitivity of the assays in detecting the bacterial targets in CSF of suspected meningitis cases. Occurrence of these is possible with expected PCR inhibition in the CSF samples from traumatic spinal taps due to the contaminating blood[201]. The analysed patient clinical information revealed abnormal CSF profiles mainly due to contaminating blood stains from traumatic LPs, thus the accompanying high RBC cells and high leukocyte count. These uncorrected cell counts, if combined with concurrent bacteraemia, could have led to cases being falsely classified as confirmed cases in the absence of meningitis.

With positive microscopy test being part of the criteria, incorrect interpretation of Gram stain results also aided in the inclusion of a test was negative by qPCR. For example, case 277 was classified as confirmed bacterial meningitis based on Gram stain report of Gram positive diplococci, but was positive for enteroviral DNA instead of suspected bacterial target.

Sample collection, storage and even transportation cannot be overlooked as they are other confounders that affect the positivity of conventional microbiology tests and even the qPCR outcome[170].

3.4.3 Viral diagnostics is not routinely done for suspected meningitis cases

PCR has become the preferred test for viral detection in CSF[202]. The rapid diagnosis from detection allows for timeous therapy. The observed high burden of viral meningitis in children is concerning, even with being reported as the most predominant type of meningitis screening is not routine only carried out upon the clinician"s request[173,203,204]. This highlights the advantage of viral aetiology screening[205206]. A ten year surveillance of viral meningo-encephalitis reports in England and Wales showed an increase in cases detected from 311 cases in 2004 to 2168 cases in 2013, mostly owing to adopted molecular based detection[205].

A seasonal variation was observed for enteroviral identification with peaks during the summer months (Table 3.1). The observed enteroviral burden and peaks in the summer months coincided with previously observed seasonal trends in Cape Town[53,207] and also is expected in temperate climate[190].

Limited reports on the burden of viral meningitis in children in Cape Town do exist and the results also vary considerably, although limited to the specific aetiology targeted and sensitivity of method of detection. Based on a study of 3406 cases of confirmed VM in children (mean age 3 years) investigated in 1981-1989 from four teaching hospitals in Cape Town, which used viral culture, enteroviruses accounted for 91% of cases while the proportion of mumps meningitis was 9%[53]. An earlier report during the period of 1981-1982 at another Cape Town hospital also based on viral culture reported a proportion of 75% (81/108) and 25% (27/301) of enteroviruses and mumps virus cases respectively[207].The relatively few

comprehensive studies on viruses in CSF during CNS infection in children, and the limited and out-dated surveillance efforts using viral culture, leads to an underestimation of the viral burden thus masking potential outbreaks, especially during the summer period for enteroviral meningitis.

Further characterisation by genotyping the viral positive PCR samples would be helpful in describing the predominant species infecting the population and whether it‟s a possible outbreak. Outbreak strains can be effectively monitored (e.g. enterovirus D68, EV 71 and Coxsackievirus A6) [208,209].

3.4.4 The overlap in the diagnosis of bacterial and viral meningitis diagnosis leads to meningitis misdiagnosis and unnecessary antibiotic treatment

The data clearly suggests misdiagnosis of both bacterial meningitis at 87.3% (62/71) and viral meningitis at 70.1% (68/97) respectively, including a concerning and costly prescription of unnecessary antibiotics. The emergence of antibiotic resistance (e.g. penicillin- resistant *S. pneumoniae*) has resulted from the increased unnecessary antibiotics prescription on predominantly viral illnesses [210]. The use of species-specific nonculture based diagnosis will guide appropriate treatment by clinician and improve management of the patient (meningitis case ascertainment)[211]. Further follow-up of patients is necessary as readmission will aid in defining whether it is acute or chronic meningitis as a few cases showed recurrent infection.

3.4.5 Diagnostic evaluation of viral qPCR positives

On clinician‟s request a few samples were referred for in-house virology testing and all the tested cases had results congruent with qPCR result. The cases positive by qPCR for the viral targets had a similarly reported predominance of polymorphonuclear cells significant in aseptic meningitis associated with enteroviral seasonal outbreaks (Appendix A)[212]. Additionally, a 60.6% (57/94) of the viral meningitis cases were given antibiotics as discharge diagnosis. These comprised of 70.2% (40/57) of the viral qPCR cases misdiagnosed as bacterial or partially treated meningitis. An improvement of the Hospital‟s management of meningitis patients is evident to reduce occasions of unnecessary antibiotic prescription and over hospitalization of low risk BM children[173]

3.4.6 Analysis of bacterial qPCR diagnostic test parameters

Compared to CSF culture, the bacterial qPCR had high sensitivity and specificity (>95%) along with the commonly implemented Gram staining technique thus proving better performance over the imperfect CSF culture "gold standard". The reduced sensitivity of culture from prior antibiotic use and false positive Gram stain results, tests included in the CRS, tend to increase the qPCR discrepant cases thus leading to the low qPCR"s sensitivity (60%) against the CRS and confirmed cases of meningitis[9,95]. Evidently, the wide CIs reveal the effect of false positives on both the sensitivity and specificity although this can be due to the low sample size.

An overestimation of the sensitivity is observed with comparison to an imperfect gold standard. A previous report of the bacterial qPCR"s test parameters showed similar sensitivity and specificity for the three targeted bacteria ranging at 73.7% - 94.1% and 97.7% - 99% respectively when tested against culture[150]. Of specific note, the biological characteristics of the CSF samples are known to cause differences in test parameters. Furthermore the observed few cases of *S. pneumoniae* and *H. influenzae* cases might be due to prior Hib and PCV vaccination and thus are likely to bias the overall test parameters for the bacterial qPCR. However, the test sensitivity and specificity are not variable as the prevalence of disease changes[90].

Latent class modelling (LCM) is one model used to combine and compare multiple index tests when an imperfect gold standard is the reference test thus addressing any biases introduced use of a single comparator test[150]. Gram staining was shown to be highly accurate in detection of BM compared to the CSF culture. This confirms the necessity and value of Gram stain on culture negative CSF samples, along with its additional benefits such as ease of use, low cost and accessibility in resource poor settings[90].

In this study, we describe the aetiology of meningitis to common pathogens by use of improved real-time PCR in suspected meningitis cases with abnormal chemistry findings and most of which (98.3%) have bacterial culture negative CSF results. The observed qPCR"s increased sensitivity and detection of nucleic acid for identification as compared to isolation of viable bacteria, makes it the most ideal testing tool[213].

3.4.7 Limitations

A few limitations were observed in the study design which should be considered in interpretation of the results. Firstly, the choosing of diagnostic targets for only three common bacterial and three common viral targets limited detection of other possible causative organisms for the clinical syndrome. In order to better define the aetiology of suspected infection cases, a broader spectrum of possible causes should be targeted. Initial focus should be placed on the common causes in the suspected population, thus in the studied area with a high HIV and TB prevalence, cryptococcal meningitis and TB meningitis have been commonly diagnosed[186,214,215]. These two targets were not included in the test panel due to limited funding and testing for these organisms is performed only upon specific request. The proportion of no target DNA cases, reported as 64% in this study could also include potential cases with TB of fungal meningitis thus overestimating the unidentified cases.

Secondly the CSF cell counts were not corrected for white blood cells that could have been introduced with traumatic spinal taps, thus abnormal cell counts forming our inclusion criteria could have included patients with no meningitis at all.

Thirdly, technical problems including sample transportation and storage, sample processing volumes and cross contamination during sample processing could potentially affect the unstable nucleic acid in the sample thus reducing the sensitivity of the test method. The CSF samples were kept at 4^{o}C for more than 2 days before storage at -70^{o}C. Denaturation of the target nucleic acid could occur leading to potential underestimation of detected target, especially RNA based targets. With the use of nucleic acid stabilising buffers or viral transport media to maintain the stability of RNA based viruses this loss in material could be avoided. Immediate prospective processing could be an alternative but has to be optimised to work efficiently with other diagnostic routine tests, even on the limited sample volumes obtained. If prospective processing is carried out, it will reduce the amount of sample handling by the many departments and avoids possible contamination prior to testing.

The sample volumes tested were variable as in some cases the CSF volume that remained after routine laboratory diagnosis was small. In these cases, the samples were topped up with ATL buffer (Qiagen, Hilden, Germany) to the required starting extraction volume for the automated extraction platform. This could have lowered the sensitivity of detection and thus underestimated the burden of disease.

Despite the best efforts to monitor and prevent contamination, we unexpectedly suspected cross contamination during the first viral qPCR screening with a high detection of HSV inconsistently in a single batch. Upon repeating the screening expected profiles were observed along with perfect controls were reported. This is evidence of likely contamination occurring in controlled environments. Contamination may lead to false positives in the PCR results and can be amplified with high throughput handling of samples. When carrying out high throughput sample processing by PCR it is imperative to avoid contamination. With processing on 96 well plates, cross contamination from aerosols of samples with high DNA concentration may occur as it is an "open" plate processing system. This is monitored by placing multiple negative controls across the PCR plate to assess for any cross contamination during target amplification. The positive control should be added last to avoid possible contamination of any neighbouring sample wells. This entire process is carried out in a UV sterilized hood to avoid cross-amplicon contamination. This process however is different when applied to routine qPCR testing which seldom involves high throughput runs on entire 96 well plates. Thus the likelihood of sample cross contamination would be reduced, but, one would still advise the need for repeat testing of any unexpected results.

The case definition needs to be specific enough to clarify on the various presentations of the syndrome as acute meningitis, meningoencephalitis, chronic meningitis, and endemic or outbreak disease. The symptoms overlap thus extra testing including Magnetic Resonance Imaging (MRI) or Computerised Topographic (CT) scanning to investigate any meninges inflammation may exclude meningoencephalitis or confirm meningitis complications. Molecular typing of the aetiology strains to infer on whether outbreak disease while a follow-up of the patient for possible re-admittance through treatment may inform on the severity i.e. chronic or acute. With no clear cut definition and limitation of cross-sectional sampling, this biases the selection and detection to plainly bacterial or viral meningitis.

3.5 Conclusion

We aimed to describe the population prevalence, seasonal trend, and epidemiologic data of the aetiology of suspected meningitis cases during 2011-2012 at the Red Cross Children‟s Hospital, South Africa. In conclusion, a high burden of viral meningitis was observed mainly due to enteroviral infection with relatively low bacterial meningitis prevalence reported. The additional BM case by PCR in this study reflected the effect of empiric antibiotic treatment of CSF sterility on available laboratory tests. The high prevalence of VM emphasises the need for viral pathogen diagnosis in the suspected meningitis paediatric cases. PCR is undeniable as a diagnostic tool, able to detect pathogens with relatively high sensitivity and specificity while preserving limited sample source. The retrospective data presented reflects the prevalence of the common causes of meningitis on culture negative suspected meningitis paediatric patients while explicitly highlighting the value of molecular diagnostics in clinical diagnosis. With duly enhanced simultaneous detection methods, this confirms the need to adopt molecular based methods in diagnosis of suspected meningitis case with abnormal clinical presentation.

CHAPTER IV

Final conclusion

Contents

4 Final conclusion

One major problem in diagnosis of bacterial and viral meningitis is the similar symptoms exhibited. Analysis of CSF is then necessary to distinguish the cause, but a dilemma still arises with standard laboratory diagnostics not clearly defining the cause. Test results are delayed and affected by partial antibiotic treatment. This is further confounded by the absence of routine viral diagnostics thus improvement of diagnosis by use of PCR based detection was the objective of the study. This has proven beneficiary. Additionally, with the consequence of misdiagnosis, consistent collaboration between clinicians and laboratory technicians, and researchers is vital in increasing the efficiency and accuracy of diagnosis.

The power of real-time PCR is undeniable as a diagnostic tool. The implemented simultaneous multi-target detection proved valuable in conserving limited quantities of sample, allowing detection of targeted organisms in single reactions, and reducing the cost and processing time. Concerns do arise on the sensitivity of the individual target being compromised upon multiplexing, but careful optimisation and validation enabled for optimal detection for all targets. The bacterial and viral multiplex had low limits of detection of <3 copies/reaction for the targeted pathogens with minimal reported variability in target detection and high reproducibility. This conserved resources making it useful in resource limited settings. Addition of an internal control (IC) to the bacterial multiplex ensured simultaneous assessment of any inhibition during amplification. With acceptable performance specification defined for the multiplex assays, clinical assessment to validate their diagnostic ability in disease identification in a population is needed.

Screening of a population of suspected meningitis cases with the multiplex assays described the population prevalence, seasonal variation, and epidemiologic data of the targeted aetiology during 2011-2012 at the Red Cross Children‟s Hospital, South Africa. An overall high proportion of VM was observed predominantly due to enteroviral infection mainly peaking during the summer months. There was a low BM prevalence with most detected bacterial target positive cases having been culture negative. This emphasized the effect of empiric antibiotic treatment on CSF sterility and standard routine diagnostics. The additional need for viral diagnostics in the suspected meningitis in paediatric cases was highlighted to reduce the misdiagnosis for BM and unnecessary antibiotic treatment. The retrospective data presented

reflected the prevalence of the common causes of meningitis on culture negative suspected meningitis paediatric patients while explicitly highlighting the potential value of molecular diagnostics in clinical diagnosis.

With duly enhanced detection methods, this confirms the need to adopt molecular based methods in diagnosis of suspected meningitis case with abnormal clinical presentation. The assay would be suited for clinical diagnostics and beneficial in surveillance programs.

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Appendix

Appendix A: Clinical and laboratory information for the Viral qPCR positive target cases (**n=94**)

Study number	qPCR Results	Outcome of child	DC Rx	DC Dx	Polymorphs	Lymphocytes	Erythrocytes	Glucose mg/dl	Protein mg/dl	Gram stain	CSF Culture	BC gram stain	BC results	Extra notes
3	Enterovirus	Transferred	-	PTM	40	56	7	61.2	63	Negative	Neg after 3days	Negative	Neg after 3days	-
5	Enterovirus	Transferred	Antibiotics	VM	63	13	6	50.4	23	Negative	Neg after 3days	Negative	Neg after 3days	-
10	Enterovirus	Transferred	Antibiotics	PTM	105	675	5	59.4	45	Negative	Neg after 3days	Negative	Neg after 3days	-
11	Enterovirus	Discharged	nd	VM	30	38	2	73.8	25	Negative	Neg after 3days	-	-	-
12	Enterovirus	Discharged	nd	VM	59	69	240	54	19	Negative	Neg after 3days	Negative	Neg after 3days	-
15	Enterovirus	Transferred	Antibiotics	BM	175	515	200	41.4	63	Negative	Neg after 3days	Negative	Neg after 3days	-
24	Enterovirus	Transferred	Antibiotics	BM	38	143	14	39.6	77	Negative	Neg after 3days	Negative	Other	CSF Enterovirus positive
31	Enterovirus	Transferred	Antibiotics	BM	52	39	0	70.2	28	Negative	Neg after 3days	Negative	Neg after 3days	CSF Enterovirus positive
35	Enterovirus	Transferred	Antibiotics	BM, PTM	390	55	5	68.4	25	Negative	Neg after 3days	Negative	Neg after 3days	-
36	Enterovirus	Discharged	-	VM	3	16	0	52.2	19	Negative	Neg after 3days	-	-	-
41	Enterovirus	Transferred	Antibiotics	PTM	95	330	45	70.2	36	Negative	Neg after 3days	-	-	-
43	Enterovirus	Transferred	Antibiotics	BM	615	185	20	75.6	42	Negative	Neg after 3days	Negative	Neg after 3days	CSF Enterovirus positive
45	Enterovirus	Discharged	-	VM	2	5	0	59.4	31	Negative	Neg after 3days	-	-	-
47	Enterovirus	Transferred	Antibiotics	BM	80	520	5	61.2	51	Negative	Neg after 3days	Negative	Neg after 3days	-
52	Enterovirus	Transferred	Antibiotics	BM	115	33	0	66.6	32	Negative	Neg after 3days	-	-	-
56	Mumps *virus*	Transferred	Antibiotics	VM	10	675	0	75.6	29	Negative	Neg after 3days	-	-	CSF HSV 1 and 2 negative
60	Enterovirus	Transferred	Antibiotics	BM	200	46	15	68.4	25	Negative	Neg after 3days	Negative	CONS	-
64	Enterovirus	Transferred	Antibiotics	BM	185	60	3360	68.4	39	Negative	Neg after 3days	Negative	Neg after 3days	-

CSF- cerebrospinal fluid; qPCR- quantitative real-time PCR; ND - not documented; VM- viral meningitis; BC- blood culture; BM- bacterial meningitis; PTM- partially treated meningitis; GPDC- Gram positive diplococcic; GPC- Gram positive cocci; GNB- Gram negative bacilli; GNDC- Gram negative diplococcic; CONS- Coagulase Negative *Staphylococcus aureus*; DC- Discharge; DC Rx- Discharge treatment; DC Dx- Discharge diagnosis

Appendix A: continued

Study number	qPCR Results	Outcome of child	DC Rx	DC Dx	Polymorphs	Lymphocytes	Erythrocytes	Glucose mg/dl	Protein mg/dl	Gram stain	CSF Culture	BC gram stain	BC results	Extra notes
97	Enterovirus	Transferred	Antibiotics	BM	141	14	5	70.2	23	Negative	Neg after 3days	-	-	-
99	*S. pneumoniae* & Enterovirus	Transferred	Antibiotics	BM	95	36	310	70.2	38	Negative	Neg after 3days	Negative	Neg after 3days	-
100	Mumps *virus*	Transferred	Antibiotics	Other	5	900	15	57.6	48	Negative	Neg after 3days	Negative	Neg after 3days	-
121	Enterovirus	Transferred	Antibiotics	BM	215	285	5	63	42	Negative	Neg after 3days	Negative	Neg after 3days	-
135	Enterovirus	Discharged	nd	VM	133	407	10	72	0	Negative	Neg after 3days	Negative	Neg after 3days	-
143	Enterovirus	Discharged	nd	nd	9	7	90	64.8	13	Negative	Neg after 3days	-	-	-
150	Enterovirus	Transferred	Antibiotics	BM	83	110	30	63	24	Negative	Neg after 3days	-	-	-
154	Enterovirus	Discharged	nd	VM	116	145	7	70.2	29	Negative	Neg after 3days	-	-	-
165	Enterovirus	Discharged	Other/ URTI DC on panado	Other	2	1	675	68.4	24	Negative	Neg after 3days	-	-	-
176	Enterovirus	Transferred	Antibiotics	VM	240	51	170	68.4	37	Negative	Neg after 3days	Negative	Neg after 3days	-
178	Enterovirus	Discharged	nd	VM	0	28	0	55.8	30	Negative	Neg after 3days	-	-	-
182	Enterovirus	Transferred	Antibiotics	PTM	86	28	260	61.2	27	Negative	Neg after 3days	Negative	Neg after 3days	-
185	Enterovirus	Discharged	Antibiotics	VM	6	105	1	64.8	33	Negative	Neg after 3days	-	-	-
186	Enterovirus	Discharged	Antibiotics	VM	4	6	15	70.2	16	Negative	Neg after 3days	-	-	-
187	Enterovirus	Transferred	Antibiotics	VM	595	35	0	88.2	33	Negative	Neg after 3days	-	-	-
188	Enterovirus	Discharged	DC on panado and brufen	VM	4	22	22	59.4	19	Negative	Neg after 3days	Negative	Neg after 3days	-
191	Enterovirus	Discharged	-	VM	2	3	160	57.6	20	Negative	Neg after 3days	Negative	Neg after 3days	CSF Enterovirus positive
192	Enterovirus	Discharged	Antibiotics	VM	9	88	5	63	35	Negative	Neg after 3days	Negative	Neg after 3days	-

CSF- cerebrospinal fluid; qPCR- quantitative real-time PCR; ND - not documented; VM- viral meningitis; BC- blood culture; BM- bacterial meningitis; PTM- partially treated meningitis; GPDC- Gram positive diplococcic; GPC- Gram positive cocci; GNB- Gram negative bacilli; GNDC- Gram negative diplococcic; CONS- Coagulase Negative *Staphylococcus aureus*; DC- Discharge; DC Rx- Discharge treatment; DC Dx- Discharge diagnosis; URTI- upper respiratory tract infection

Appendix A: continued

Study number	qPCR Results	Outcome of child	DC Rx	DC Dx	Polymorphs	Lymphocytes	Erythrocytes	Glucose mg/dl	Protein mg/dl	Gram stain	CSF Culture	BC gram stain	BC results	Extra notes
195	Enterovirus	Discharged	DC on panado and brufen	BM	1	3	155	72	24	Negative	Neg after 3days	-	-	-
196	Enterovirus	Transferred	Antibiotics	BM	185	685	33	55.8	46	Negative	Neg after 3days	Negative	Neg after 3days	-
198	Enterovirus	Transferred	Antibiotics	PTM	41	48	8	63	25	Negative	Neg after 3days	Negative	Neg after 3days	-
203	Enterovirus	Transferred	Antibiotics	VM	107	15	1	68.4	33	Negative	Neg after 3days	Negative	Neg after 3days	-
204	Enterovirus	Discharged	Antibiotics, DC on panado	VM	394	2880	72	52.2	81	Negative	Neg after 3days	Negative	Neg after 3days	-
206	Enterovirus	Transferred	Antibiotics	VM	140	225	10	88.2	24	Negative	Neg after 3days	Negative	Neg after 3days	-
212	Enterovirus	Transferred	Antibiotics	BM	365	60	0	59.4	26	Negative	Neg after 3days	Negative	Neg after 3days	-
213	Enterovirus	Discharged	Antibiotics	nd	6	14	3440	55.8	28	Negative	Neg after 3days	Negative	Neg after 3days	-
214	Enterovirus	Transferred	Antibiotics	BM	16	5	1	63	24	Negative	Neg after 3days	Negative	Neg after 3days	-
216	Enterovirus	Discharged	DC on panado	VM	30	23	17	66.6	20	Negative	Neg after 3days	Negative	Neg after 3days	-
221	Enterovirus	Discharged	-	BM	9	20	7040	52.2	44	Negative	Neg after 3days	Negative	Neg after 3days	-
222	Enterovirus	Transferred	Antibiotics	PTM	15	53	1	66.6	19	Negative	Neg after 3days	-	-	-
224	Enterovirus	Discharged	Antibiotics	BM	306	149	8	59.4	32	Negative	Neg after 3days	-	-	-
225	Enterovirus	Transferred	Antibiotics	BM	13	109	25	50.4	103	Negative	Neg after 3days	Negative	Neg after 3days	-
232	Enterovirus	Discharged	DC on panado.	VM	6	2	5	73.8	20	Negative	Neg after 3days	Negative	Neg after 3days	-
233	Enterovirus	Transferred	Antibiotics	VM, PTM	3	9	0	73.8	24	Negative	Neg after 3days	-	-	-
234	Enterovirus	Discharged	Antibiotics	VM	15	5	10000	77.4	22	Negative	Neg after 3days	-	-	-
237	Enterovirus	Transferred	-	VM	3	9	1	55.8	32	Negative	Neg after 3days	-	-	-

CSF- cerebrospinal fluid; qPCR- quantitative real-time PCR; nd- not documented; VM- viral meningitis; BC- blood culture; BM- bacterial meningitis; PTM- partially treated meningitis; GPDC- Gram positive diplococcic; GPC- Gram positive cocci; GNB- Gram negative bacilli; GNDC- Gram negative diplococcic; CONS- Coagulase Negative *Staphylococcus aureus*; DC- Discharge; DC Rx- Discharge treatment; DC Dx- Discharge diagnosis

Appendix A: continued

Study number	qPCR Results	Outcome of child	DC Rx	DC Dx	Polymorphs	Lymphocytes	Erythrocytes	Glucose mg/dl	Protein mg/dl	Gram stain	CSF Culture	BC gram stain	BC results	Extra notes
238	Enterovirus	Transferred	Antibiotics	BM	485	10	0	68.4	36	Negative	Neg after 3days	Negative	Neg after 3days	-
241	Enterovirus	Discharged	DC on panado	VM	16	24	4	72	22	Negative	Neg after 3days	-	-	-
244	Enterovirus	Discharged	DC on panado	VM	44	27	5	46.8	28	Negative	Neg after 3days	-	-	-
246	Enterovirus	Transferred	Antibiotics	BM	120	87	64	81	44	Negative	Neg after 3days	-	-	-
247	Enterovirus	Transferred	Antibiotics	BM	7	140	1	54	17	Negative	Neg after 3days	-	-	-
253	Enterovirus	Discharged	DC panado and folate	VM	96	135	42	73.8	27	Negative	Neg after 3days	-	-	-
254	Enterovirus	Transferred	Antibiotics	PTM	13	242	1205	59.4	34	Negative	Neg after 3days	Negative	Neg after 3days	-
256	Enterovirus	Discharged	-	VM	2	8	2	55.8	14	Negative	Neg after 3days	Negative	Neg after 3days	-
257	Enterovirus	Transferred	Antibiotics	BM	42	248	10720	64.8	52	Negative	Neg after 3days	Negative	Neg after 3days	-
260	Enterovirus	Transferred	Antibiotics	BM	490	91	22	63	88	Negative	Neg after 3days	-	-	-
261	Enterovirus	Discharged	DC on panado	VM	3	3	16	61.2	26	Negative	Neg after 3days	-	-	-
262	Enterovirus	Discharged	Antibiotics	VM	12	0	4	59.4	22	Negative	Neg after 3days	Negative	Neg after 3days	-
265	Enterovirus	Discharged	-	VM	29	3	19	64.8	21	Negative	Neg after 3days	-	-	-
267	Enterovirus	Discharged	DC on panado	VM	60	75	150	64.8	25	Negative	Neg after 3days	-	-	-
268	Enterovirus	Transferred	Antibiotics	BM	154	101	10	81	25	Negative	Neg after 3days	GPC in clusters	CONS	-
273	Enterovirus	Discharged	DC on panado	VM	13	63	5	90	24	Negative	Neg after 3days	-	-	-
274	Enterovirus	Transferred	Antibiotics	BM	74	85	7	59.4	20	Negative	Neg after 3days	Negative	Neg after 3days	-

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Appendix A: continued

Study number	qPCR Results	Outcome of child	DC Rx	DC Dx	Polymorphs	Lymphocytes	Erythrocytes	Glucose mg/dl	Protein mg/dl	Gram stain	CSF Culture	BC gram stain	BC results	Extra notes
276	Enterovirus	Discharged	Other*	VM	25	11	1640	68.4	27	Negative	Neg after 3days	Negative	Neg after 3days	-
277	Enterovirus	Transferred	Antibiotics	BM	255	170	20	64.8	25	GPDC	Neg after 3days	GPB	Bacillus species	-
279	Enterovirus	Discharged	Antibiotics	BM	403	29	4	73.8	32	Negative	Neg after 3days	-	-	-
280	Enterovirus	Discharged	DC on panado	VM	32	50	3	59.4	29	Negative	Neg after 3days	-	-	-
286	Enterovirus	Discharged	Antibiotics	VM	10	2	0	73.8	15	Negative	Neg after 3days	Negative	Neg after 3days	-
289	Enterovirus	Discharged	DC on panado	VM	25	100	2	63	24	Negative	Neg after 3days	Negative	Neg after 3days	-
290	Enterovirus	Discharged	Other	nd	18	139	5	64.8	29	Negative	Neg after 3days	-	-	-
291	Enterovirus	Discharged	Antibiotics	VM	119	34	5	70.2	23	Negative	Neg after 3days	Negative	Neg after 3days	-
294	Enterovirus	Transferred	Antibiotics	BM	159	58	21	66.6	26	Negative	Neg after 3days	Negative	Neg after 3days	-
309	Enterovirus	Transferred	Antibiotics	BM	300	205	5	54	99	Negative	Neg after 3days	Negative	Neg after 3days	-
310	Enterovirus	Discharged	Other	BM	32	6	4	73.8	29	Negative	Neg after 3days	-	-	-
312	Enterovirus	Discharged	Other	VM	9	1	597	64.8	15	Negative	Neg after 3days	Negative	Neg after 3days	-
313	Enterovirus	Discharged	Other	VM	76	72	5	54	45	Negative	Neg after 3days	Negative	Neg after 3days	-
315	Enterovirus	Transferred	Antibiotics	BM	47	10	0	63	17	Negative	Neg after 3days	Negative	Neg after 3days	-
319	Enterovirus	Discharged	DC on panado	VM	26	33	48	75.6	36	Negative	Neg after 3days	Negative	Neg after 3days	-
321	Enterovirus	Transferred	Antibiotics	BM	214	33	2	55.8	48	Negative	Neg after 3days	Negative	Neg after 3days	-
324	Enterovirus	Transferred	Antibiotics	BM	0	19	5	52.2	25	Negative	Neg after 3days	-	-	-
326	Enterovirus	Discharged	nd	VM	165	225	350	64.8	42	Negative	Neg after 3days	-	-	

CSF- cerebrospinal fluid; qPCR- quantitative real-time PCR; ND - not documented; VM- viral meningitis; BC- blood culture; BM- bacterial meningitis; GPDC- Gram positive diplococcic; GPC- Gram positive cocci; GNB- Gram negative bacilli; GNDC- Gram negative diplococcic; CONS- Coagulase Negative *Staphylococcus aureus*; DC- Discharge; DC Rx- Discharge treatment; DC Dx- Discharge diagnosis. *- the discharge treatment was not specified.

Appendix A: continued

Study number	qPCR Results	Outcome of child	DC Rx	DC Dx	Polymorphs	Lymphocytes	Erythrocytes	Glucose mg/dl	Protein mg/dl	Gram stain	CSF Culture	BC gram stain	BC results	Extra notes
400	Enterovirus	Discharged	Antibiotics	VM	0	6	1480	84.6	29	Negative	Neg after 3days	Negative	Neg after 3days	-
410	Mumps *virus*	Discharged	Antibiotics	VM	44	139	9	79.2	33	Negative	Neg after 3days	Negative	Neg after 3days	-
413	Enterovirus	Transferred	Antibiotics	BM	0	6	1	63	18	Negative	Neg after 3days	GPC in chains, GPC in clusters	*Streptococcus sanguinis* and *Staphylococcus haemolyticus*	CXR showed signs of LRTI
501	Enterovirus	nd	nd	nd	14	18	6	63	29	Negative	Neg after 3days	nd	nd	-
504	Enterovirus	nd	nd	nd	2	7	3	73.8	22	negative	Neg after 3days	nd	nd	-

CSF- cerebrospinal fluid; qPCR- quantitative real-time PCR; nd - not documented; VM- viral meningitis; BC- blood culture; BM- bacterial meningitis; GPDC- Gram positive diplococcic; GPC- Gram positive cocci; GNB- Gram negative bacilli; GNDC- Gram negative diplococcic; CONS- Coagulase Negative *Staphylococcus aureus*; DC- Discharge; DC Rx- Discharge treatment; DC Dx- Discharge diagnosis

Appendix B: Viral qPCR compared to conventional viral diagnosis

Study number	qPCR result	In-house PCR	viral culture
24	enterovirus	CSF EV positive	-
29	-	CSF EV negative	-
31	enterovirus	CSF EV positive	-
43	enterovirus	EV positive	-
56	mumps virus	CSF HSV 1 and 2 PCR negative	-
65	-	TB PCR positive	-
67	-	TB PCR positive	-
79	-	TB PCR negative	-
88	-	CSF EV PCR and HSV PCR negative	viral culture negative
109	-	CSF HSV PCR negative	-
115	-	HSV PCR negative	-
191	enterovirus	EV PCR positive	-
215	-	TB PCR negative	-
218	-	TB PCR negative	-
226	-	TB PCR negative	-

CSF- cerebrospinal fluid; qPCR- quantitative real-time PCR; TB- Tuberculosis; EV- enteroviruses; HSV- herpes simplex

Appendix C: 2x2 Contingency tables estimating the Confidence Intervals for sensitivity and specificity

qPCR vs CSF culture		CSF culture positive	CSF culture negative	
	qPCR positive	4	8	12
	qPCR negative	0	280	280
		4	288	
Confidence interval	95%			
Sensitivity:	1.0000	CI: 0.5101 to 1		
Specificity:	0.9722	CI: 0.9462 to 0.9859		
Positive predictive value:	4/12			
Negative predictive value:	280/280			

qPCR vs Composite Reference Standard		CRS positive	CRS negative	
	qPCR positive	6	6	12
	qPCR negative	1	279	280
		7	285	
Confidence interval	95%			
Sensitivity:	0.8571	CI: 0.4869 to 0.9743		
Specificity:	0.9789	CI: 0.9548 to 0.9903		
Positive predictive value:	6/12			
Negative predictive value:	279/280			

Gram stain vs CSF culture		CSF culture positive	CSF culture negative	
	Gram Stain positive	4	3	7
	Gram Stain negative	0	285	285
		4	288	
Confidence interval	95%			
Sensitivity:	1.0000	CI: 0.5101 to 1		
Specificity:	0.9896	CI: 0.9698 to 0.9965		
Positive predictive value:	4/7			
Negative predictive value:	285/285			

Bacterial qPCR vs Confirmed meningitis cases		Confirmed meningitis case	Unconfirmed meningitis case	
	qPCR positive	6	6	12
	qPCR negative	4	276	280
		10	282	
Confidence interval:	95%			
Sensitivity:	0.6000	CI: 0.3127 to 0.8318		
Specificity:	0.9787	CI: 0.9544 to 0.9902		
Positive predictive value:	6/12			
Negative predictive value:	276/280			