Development of novel nucleic acid diagnostics technologies for the detection of predominant microorganisms associated with bacterial meningitis

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"If you can dream it, you can do it"

Walt Disney

Abstract

With an estimated 1.2 million cases annually worldwide, bacterial meningitis is a medical emergency and primary prevention as well as accurate diagnosis and treatment of this often fatal disease is paramount. The most common microorganisms associated with bacterial meningitis are *Haemophilus influenzae, Neisseria meningitidis* and *Streptococcus pneumoniae*.

Several vaccines are available for the prevention of bacterial meningitis caused by these three microorganisms; however, these vaccines offer limited serotype coverage and as a consequence bacterial meningitis cases as a result of non-vaccine serotypes have emerged. Furthermore, limited vaccine availability in resource poor countries in addition to vaccine failures is a major concern in the prevention of bacterial meningitis worldwide. As such, patients with suspected meningitis require immediate medical assessment and accurate diagnosis of the disease in order to provide an optimal therapeutic regime. Moreover, as the incidence of bacterial meningitis varies significantly by age and geographic location, the accurate diagnosis of the causative microorganism would enable unambiguous epidemiological studies to be carried out.

Presently, culture remains the gold standard for the diagnosis of bacterial meningitis, however, this is limited in its ability to rapidly and accurately diagnose and nucleic acid based diagnostic methods are more sensitive and specific for the diagnosis of this fatal disease. The overall aim of this study was to design, develop, optimise and validate robust, internally controlled nucleic acid based *in-vitro* amplification methods for the rapid and accurate identification of *H. influenzae, N. meningitidis* and *S. pneumoniae*. This was achieved using sequential experimental design consisting of three main studies.

In the first study, RNA transcripts encoded for by the *ssrA* (tmRNA) and *lepA* (*lepA* mRNA) genes were evaluated using real-time Nucleic Acid Sequence Based Amplification (NASBA) as potential diagnostic targets for species specific identification of *H. influenzae, N. meningitidis* and *S. pneumoniae.* This established that tmRNA and *lepA* mRNA have potential as diagnostic targets for the species specific identification of *N. meningitidis* and *S. pneumoniae* respectively. However,

neither the tmRNA transcript nor the *lepA* mRNA transcript can be used as a diagnostic target to unequivocally differentiate *H. influenzae* from its most closely related species *Haemophilus haemolyticus*.

In the second study, whole genome comparative analysis of *H. influenzae* and *H. haemolyticus* identified two novel gene targets, *phoB* and *pstA*, present in *H. influenzae* and absent in all other closely related species including *H. haemoylticus*. Subsequently, two internally controlled real-time PCR diagnostic assays were developed targeting both *phoB* and *pstA* and validated against an extensive panel of *H. influenzae* isolates and non-*H. influenzae* closely related species for the specific identification of *H. influenzae.* Analytical sensitivities of the real-time PCR diagnostic assays developed were determined to be comparable to other published diagnostic targets and more superior in terms of specificity than a previously published diagnostics targets for the identification of *H. influenzae*.

In the third study, *phoB* was further validated as a diagnostic target for the species specific detection of *H. influenzae* by targeting the *phoB* mRNA transcript in a duplex real-time NASBA diagnostic assay. In addition, two duplex real-time NASBA diagnostic assays were also developed targeting the RNA transcripts encoded for by the *ssrA* gene and *lepA* gene for the species specific identification of *N. meningitidis* and *S. pneumoniae,* respectively. All three duplex real-time NASBA diagnostic assays were determined to be 100% specific for the target species tested for and analytical sensitivities of less than 60 cell equivalents were determined for each of the diagnostic assays in duplex format. Each real-time NASBA diagnostic assay developed in this study includes an endogenous non-competitive Internal Amplification Control (IAC) to amplify transcript 1 of the *Homo Sapiens TBP* gene from human total RNA. By incorporating an endogenous internal amplification control stably expressed at low levels in human blood, these duplex real-time NASBA diagnostic assays have the potential to be used in a clinical setting for the specific, sensitive and rapid (< 60 mins) detection and identification of the most prominent microorganisms associated with bacterial meningitis in humans.

In summary, nucleic acid based *in-vitro* amplification diagnostic assays described in this study are the first description of internally controlled real-time PCR diagnostic

assays and real-time NASBA diagnostic assay for the accurate, species specific identification of *H. influenzae, N. meningitidis* and *S. pneumoniae.*

Chapter 1:

General Introduction

1. Introduction

Meningitis is an acute inflammation of the meninges, the protective membranes that surround the brain and spinal cord of the central nervous system [1]. It is a life threatening infection that effects all age groups and accounted for up to an estimated 151,000 deaths in children under 5 years in 2013 [2]. Meningitis can be infectious or non-infectious and the severity and incidence rate of the disease depends on the etiological agent as well as geographic location [3, 4]. Most cases of infectious meningitis are caused by viruses and bacteria, however other causes include, fungi and parasites [5-7], while causes of non-infectious meningitis include cancers, systemic lupus erythematous, drugs, head injury and brain injury [8].

Of these meningitis etiological agents, viral meningitis is the most common while bacterial meningitis is the most severe [9, 10]. In the largest reported study the annual incident rate of viral meningitis was 219 cases/100,000 of the population aged <1 year and 27.8 cases/100,000 of the population aged 1-14 years [11]. This number decreases in people aged over 16 years (7.6 cases/100,000 of the population) [12]. However incidence rates may be higher for both adults and children as many cases go unreported due to the self-limiting nature and often benign clinical outcome of the disease [7]. Bacterial meningitis incidence rates vary geographically with the highest incidence in endemic regions (>10 cases/100,000 population) such as the African meningitis belt - a region of sub-Saharan Africa consisting of 26 countries, stretching from Senegal in the west to Ethiopia in the east [13, 14]. While moderate (<10 cases/100,000 population) to low (<2 cases/100,000 population) incidence rates occur in the United States, Europe, Asia and South Africa [13]. In Ireland the pre-vaccine incident rate was 14.3 cases/100,000 of the population, although this has decreased to 2.19 cases/100,000 of the population since the introduction of vaccines targeting predominant etiological bacteria [13]. However, unlike viral meningitis, the clinical outcome for bacterial meningitis is often fatal with significant morbidity and mortality rates worldwide (5-40% in children, 20-50% in adults) in spite of anti-microbial treatment [15]. Whilst in cases of untreated bacterial meningitis, mortality rates approach 100% [16]. Furthermore, survivors are often left with long term sequelae such as seizure disorders, neurologic deficits, hearing/vision loss, loss of limb and impaired cognitive functioning [17, 18].

1.1. Etiological Agents of Bacterial Meningitis

Bacterial meningitis can be caused by almost any bacteria that is pathogenic to humans. Of these, *Haemophilus influenzae, Neisseria meningitis, Streptococcus pneumoniae, Streptococcus agalactiae, Streptococcus suis, Escherichia coli, Mycobacterium tuberculosis* and *Listeria monocytogenes* are the foremost causes of the disease. [15, 16, 19]. The causative microorganism of bacterial meningitis varies across age groups, as listed in Table 1.1, however, the most predominant microorganisms associated with the disease are, *H. influenzae, N. meningitidis* and *S. pneumoniae* [9]. Despite implementation of routine immunization against these 3 microorganisms in recent years, they still account for up to 80% of acute bacterial meningitis cases annually worldwide [15].

Table 1.1. Aetiology of bacterial meningitis according to age groups

Age Groups	**Causes**
New-born	Group B *Streptococcus, Streptococcus pneumoniae, Escherichia coli, Listeria monocytogenes*
Infants and Children	*Streptococcus pneumoniae, Neisseria meningitidis, Haemophilus influenazae* type b, Group B *Streptococcus*
Adolescents and Young Adults	*Streptococcus pneumoniae, Neisseria meningitidis*
Older Adults	*Streptococcus pneumoniae, Neisseria meningitidis, Haemophilus influenzae* type b *(Hib),* Group B *Streptococcus, Listeria monocytogenes*

(**Source :** http://www.cdc.gov/meningitis/bacterial.html)

1.1.1. *Haemophilus influenzae*

H. influenzae is a member of the genus *Haemophilus. Haemophilus* species are small, non-motile, Gram negative bacilli, which are dependent on blood derived

factors for growth, thus giving the genus its name (Haemophilus: derived from the Greek meaning "blood-loving") [20-22]. Presently, there are fourteen species in the genus Haemophilus [23], nine of which are potentially pathogenic to humans; *H. influenzae, Haemophilus aegyptius, H. haemolyticus, H. parainfluenzae, Haemophilus parahaemolyticus, Haemophilus paraphrohaemolyticus, Haemophilus pittmaniae, Haemophilus sputorum* and *H. ducreyi* [21]. The remaining Haemophilus species, recognized by the list of prokaryotic names with standing nomenclature (LPSN)[24], have host specificity for animals and include; *Haemophilus felis* (cats), *Haemophilus haemoglobinophilus* (dogs), *Haemophilus paracuniculus* (rabbits), *Haemophilus parasuis* (swine) and *Haemophilus piscium* (fish) .

H. influenzae was first isolated in 1890 by Richard Pfeiffer [25] and was referred to as 'Pfeiffer's Bacillus' or *Bacillus influenzae* at the time, as it was mistakenly thought to be the etiological agent of the influenza pandemic [21, 26-29]. *H. influenzae* is a member of the *Haemophilus influenzae* group, - sometimes referred to as *Haemophilus sensu stricto* - together with *H. aegyptius* and *H. haemolyticus* [21]. It is the most pathogenic of the eight *Haemophilus* species which reside as commensal organisms in the human respiratory tract. To date, no known animal or environmental isolates have been identified [30, 31]. It is responsible for infections such as otitis media, epiglottitis, and conjunctivitis as well as more invasive infections such as meningitis, sepsis and pneumonia [32, 33]

H. influenzae is a fastidious facultative anaerobe with specific growth requirements. Pfeiffer first demonstrated that haemoglobin is the essential component of blood media that enables *H. influenzae* to grow [29]. This was further validated a few years later and in addition, it was established that a second vitamin like substance, that is destroyed by autoclaving, is also required [34, 35]. *Thjötta et al* went on to coin the terms X factor (hemin), for the heat stable blood associated substance, and V factor (nicotinamide adenine dinucleotide [NAD+]), for the vitamin-like heat labile substance [36]. It is these growth requirements that differentiate *H. influenzae* from all other *Haemophilus* species with the exception of its most closely related species, *H. aegyptius* and *H. haemolyticus*. Consequently, *H. influenzae* grow optimally on

rich blood media such as chocolate agar (which contains X and V factors) at 35-37^{o}C and growth is further enhanced in a 5% CO^2 enriched environment [25].

Typing of *H. influenzae* strains provides valuable information on population biology and genetic structure and is essential to monitoring the changing nature of this organism [31]. Serotyping identifies *H. influenzae* strains as either encapsulated (typeable) or unencapsulated (non-typeable). Encapsulated strains are further divided in to six serotypes, designated a – f (Hia –Hif), based on the antigenic properties of their polysaccharide capsule [21, 37]. Prior to the implementation of the Hib conjugate vaccine worldwide in the 1990s, Hib was the primary cause of invasive disease such as meningitis, bacteraemia and pneumonia [38]. More than a 95% reduction in the number of *H. influenzae* meningitis cases was recorded in 1995 following the implementation of the vaccine [15]. However, the world health organization estimated Hib conjugate vaccine coverage to be only 56% globally in 2014 (Figure 1.1) and Hib invasive disease still remains a concern in developing countries where vaccine availability is limited by cost factors [39]. Whilst, *H. influenzae* as a causative agent of invasive infection has declined as a result of the routine use of the Hib conjugate vaccine in industrialized countries, Non-typeable *H. influenzae* (NTHi) are now the most common cause of *H. influenzae* invasive infections, globally, across all age groups, with a higher incidence among infants and the elderly [38, 40-42]. Furthermore, serotypes a, e and f have also emerged to replace Hib as an important cause of invasive disease [43-45]. The 10-valent pneumococcal polysaccharide protein-D conjugate vaccine (PhiD-CV10; Synflorix^{TM}), approved for use in more than 40 countries worldwide [46], contains ten polysaccharide serotypes of *S. pneumoniae*, of which eight are conjugated to the surface lipoprotein, protein D, of NTHi [47]. However, the absence of the *hpd* gene encoding *Haemophilus* protein D in some NTHi isolates highlights a limitation in the use of the PhiD-CV10 vaccine against NTHi invasive disease [48].

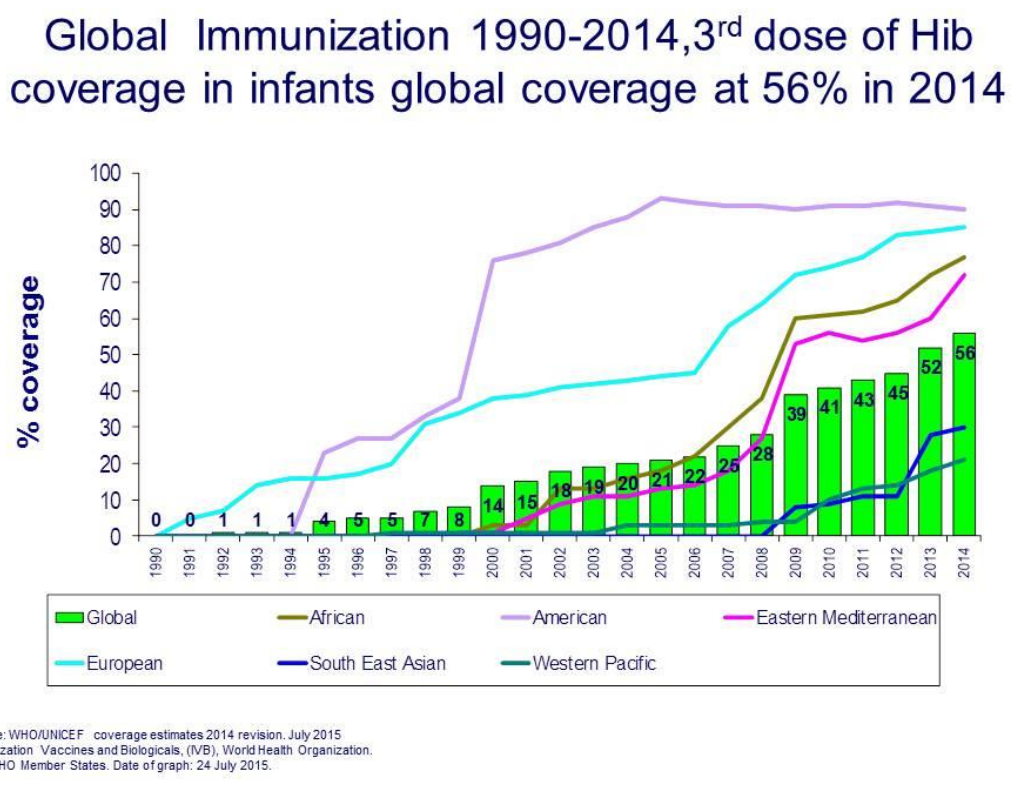

Figure 1.1: Statistics on *H. influenzae* type b conjugate vaccine coverage. (Source: WHO/UNICEF coverage estimates. July 2015)

1.1.1.1. Closely related species Haemophilus species

Haemophilus aegyptius (formally Kochs-Weeks Bacillus), a member of the group *Haemophilus sensu stricto*, was first described as a separate species within the genus Haemophilus in 1950 [49]. It is responsible for Brazilian Purpuric Fever (BPF) in children and is often isolated from patients with purulent conjunctivitis which precedes BPF [50, 51]. *H. aegyptius* was thought to differ from *H. influenzae* by its distinct rod shape, its susceptibility to troleandomycin, its inability to both grow on tryptic Soy Agar (TSA) supplemented with X and V factors and to ferment d-xylose, and its ability to agglutinate human erythrocytes [52-54]. However, these characteristics have never been definitively proven to distinguish the two micro-organisms. [55-58]. Furthermore, DNA relatedness hybridization studies revealed that they are highly similar on a nucleic acid level and should be considered as the same species [59, 60]. Consequently, *Brenner et al* designated *H. aegyptius* as *H. influenzae* biogroup *aegyptius* agreeing with a previous claim by *Casin et al* that *H.*

aegyptius is a different type strain of *H. influenzae.* [61, 62]. However, as of yet, designation of *H. aegyptius* as a separate species or as a biogroup of *H. influenzae* remains uncertain.

Haemophilus haemolyticus, a human commensal bacterium that colonizes the respiratory tract, was first described in *Bergey's Manual of Determinative Bacteriology* in 1923[63]. It is a member of the group *Haemophilus sensu stricto* and is closely related to *H. influenzae.* Similar to *H. influenzae*, *H. haemolyticus* also requires X and V factors for growth, facilitating their differentiation from other *Haemophilus* species [21]. *H. haemolyticus* can be easily distinguished from encapsulated *H. influenzae* as *H. haemolyticus* has not been reported to produce a capsule [64]. However, it's differentiation from NTHi is on the basis of its ability to cause hemolysis on blood agar plates, a characteristic which may be lost after subculture [65-67]. Up until recently, *H. haemolyticus* was considered either non-pathogenic [68] or a rare pathogen [69], and was seldom associated with invasive disease [70, 71]. However, a number of cases of invasive disease originally attributed to NTHi have since been confirmed as non-haemolytic *H. haemolyticus* [71-75]. Since culture methods cannot reliably distinguish NTHi from *H. haemolyticus,* alternative techniques must be used, such as nucleic acid based *in-vitro* amplification and matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS). These are discussed in more detail in section 1.2.2.

1.1.2. *Neisseria meningitidis*

Neisseria meningitidis is a member of the genus *Neisseria* within the family *Neisseriaceae* [76]. There are 29 members of the genus Neisseria as recognized by the LPSN [77]. Of these, the following Neisseria species, in addition to *N. meningitidis,* have been associated with human disease; *Neisseria animaloris* [78], *Neisseria bacilliformis* [79], *Neisseria canis* [80], *Neisseria cinera* [80], *Neisseria elongate* subsp. *elongate* [81], *Neisseria elongata* subsp. *glycolytica* [82], *Neisseria elongata* subsp. *Nitroreducens* [83], *Neisseria flava* [80], *Neisseria gonorrhoeae* [84], *Neisseria sicca* [85], *Neisseria mucosa* [86], *Neisseria oralis* [80], *Neisseria subflava* [87, 88], *Neisseria flavescens* [89], *Neisseria lactamica* [90], *Neisseria polysaccharea* [91], *Neisseria perflava* [92], *Neisseria shayeganii* [93], *Neisseria*

wadsworthii [93], *Neisseria weaveri* [94] and *Neisseria zoodegmatis* [78]. Moreover, *N. gonorrhoeae, N. cineria, N. flavescens, N. perflava, N. sicca,* and *N. subflava* have also been reported to occasionally cause bacterial meningitis [84, 85, 87-89, 92, 95].

N. meningitidis, commonly called meningococcus, was first isolated from the cerebrospinal fluid (CSF) of a patient in 1887 [96]. An exclusive pathogen of humans, it resides as a commensal in the nasopharynx of 5-10% of adults in non-epidemic periods. It is most commonly associated with rapid onset meningitis and sepsis however it is also known to cause septic arthritis, pneumonia, purulent pericarditis, conjunctivitis, otitis, sinusitis, and urethritis [97].

N. meningitidis is a Gram negative fastidious microorganism that grows optimally on blood agar plates or chocolate agar plates at 35-37^{o}C with 5% CO_2 [98] . If growth on blood agar or chocolate agar plates morphologically appear to be *N. meningitidis*, a sequential series of tests are recommended to confirm the identity. Firstly, Kovacs oxidase tested is performed to determine the presence of cytochrome oxidase. An oxidase positive result indicates *N. meningitidis*, however other members of the Neisseria species may also give a positive results [3]. Consequently, a carbohydrate utilization test against four carbohydrates (glucose, maltose, lactose and sucrose) should be carried out to further validate the identification of the strain as *N. meningitidis* [3]. Carbohydrate utilization results for *N. meningitidis* and other closely related species are listed in Table 1.2. Following this, slide agglutination tests should be performed using serogroup-specific antisera to identify the *N. meningitidis* serogroup [3].

N. meningitidis can be encapsulated or unencapsulated [3]. Encapsulated *N. meningitidis* strains are more commonly associated with invasive disease and are classified in to 12 serotypes based on the antigenic properties of their polysaccharide capsule [14]. Of these 12 serogroups, 6 are responsible for most cases of infection in humans, specifically serogroups A, B, C, W135, X and Y [3]. The incidence of bacterial meningitis caused by these serogroups varies widely across the globe (Figure 1.2). Serogroups B and C cause the majority of disease in America, Europe and Australia while serogroup A is more frequent in Africa and Asia [3]. The largest burden of meningococcal meningitis occurs in the meningitis belt of sub-Saharan

Africa, which stretches from Senegal to Ethiopia and is attributed to serogroup A [14]. Although less common, increased outbreaks of serogroup Y in North America and some parts of Europe, and serogroup W-135 in Sudan and Saudi Arabia, in addition to the emergence of serogroup X in the African region have also been reported [13].

Table 1.2: Carbohydrate Utilization by *Neisseria* and *Moraxella* Spp.

	Acid Production from:			
Organism	**Glucose[1]**	**Maltose**	**Lactose**	**Sucrose**
Neisseria meningitidis	+	+	-	-
Neisseria lactamica	+	+	+	-
Neisseria gonorrhoeae	+[2]	-	-	-
Neisseria sicca	+	+	-	+
Moraxella catarrhalis	-	-	-	-

[1] Glucose may also be referred to as Dextrose
[2]Some strains of *N. gonorrhoeae* are weak acid producers and may appear to be glucose negative
(Source: https://www.cdc.gov/meningitis/lab-manual/full-manual.pdf)

Meningococcal polysaccharide and conjugate vaccines have been introduced in developed countries against serogroups A, B, C, W-135 and Y (Table 1.3) resulting in a significant decrease in the number of bacterial meningitis cases caused by these serogroups [99, 100]. However, vaccine availability still remains limited in developing countries and it was not until 2010, that a vaccine against *N. meningitidis* serogroup A was introduced in the countries of the African meningitis belt which has subsequently reduced the incidence of the disease in this region [13, 14]. As a result of routine immunization against serogroups A, C, W-135 and Y since the 1970s, *N. meningitidis* serogroup B is now the most prominent cause of bacterial meningitis in Europe [100, 101]. More recently, in 2014, vaccines against meningococcal B (Table 1.3) were introduced in developed countries, of which, 4cMenB, has already been reported to provide a strong immune response against a representative panel of European serogroup B disease isolates [99, 101].

Despite a decrease in the incidence of bacterial meningitis cases as a result of the implementation of these vaccines, 1.2 million cases of meningococcal meningitis still occur worldwide every year [3]. Similar to the Hib vaccine, vaccines for the

prevention of meningococcal disease offer limited coverage and meningococcal meningitis as a result of non-vaccine serogroup X have been reported [102, 103]. Despite great progress toward increasing worldwide access to vaccines in recent years, a more concerted global effort is required to make more affordable vaccines against serogroups B, C, W-135, and Y available in endemic regions, in addition to the need for new vaccines against emerging serogroup X worldwide.

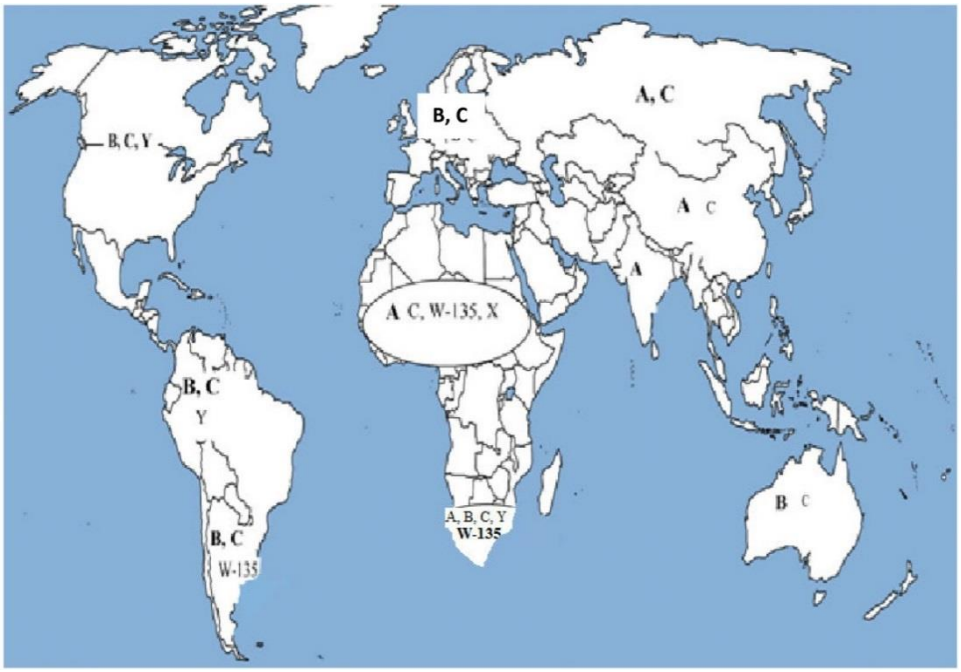

Figure 1.2: Worldwide distribution of *N. meningitidis* serogroups responsible for meningococcal meningitis. (Source: Jafri et al, 2013)

Table 1.3. Meningococcal Vaccines

Trade Name	Type of Vaccine	Meningococcal Serogroup
Bexsero®	Recombinant	B
Menactra®	Conjugate	A, C, W, Y
MenHibrix®	Conjugate	C, Y (and *Haemophilus influenzae* type b [Hib])
Menomune®	Polysaccharide	A, C, W, Y
Menveo®	Conjugate	A, C, W, Y
Trumenba®	Recombinant	B

(Source : http://www.cdc.gov/vaccines/vpd-vac/mening/)

1.1.3. *Streptococcus pneumoniae*

Streptococcus pneumoniae is a member of the genus *Streptococcus.* The LPSN recognizes over 100 species within the genus [104]. While *Streptococci* are important ecologically as part of the normal microbial flora of humans and animals, they are also associated with significant human diseases such as scarlet fever, rheumatic heart disease, glomerulonephritis, bacteraemia, meningitis and pneumococcal pneumonia [105]. *S. pneumoniae* is the leading cause of bacterial meningitis in the United states and Europe accounting for over 50% of cases [15, 106]. Other *Streptococci* reported to cause bacterial meningitis include: *Streptococcus agalactiae* (Group B *Streptococci*), *Streptococcus mitis*, *Streptococcus pyrogenes* and *Streptococcus suis* [105]. However, with the exception of *S. agalactiae*, the incidence of bacterial meningitis as a result of infection with these microorganisms is rare. *S. mitis* has been described in individuals with previous spinal anaesthesia, neurosurgical procedure, malignancy, or neurological complications of endocarditis, while *S. pyrogenes* is often as a direct extension of middle ear if sinus infections [105]. *S. suis* is more commonly associated with meningitis in pigs but has been reported in meat handlers [105].

S. pneumoniae is a Gram positive bacteria which was first isolated from human saliva in 1881 [107]. Similar to *N. meningitidis,* it is an exclusive pathogen of humans and resides as a commensal in the upper respiratory tract of 20-40% of adults [105]. It is a common cause of non-invasive infections such as sinusitis, acute bacterial otitis media, and conjunctivitis as well as more invasive infections such as pneumonia, bacteraemia, meningitis, acute septic arthritis, bone infections in patients with sickle cell disease, peritonitis and endocarditis [105, 108].

It is a fastidious bacterium, growing optimally at 35-37°C with 5% CO_2 on media that contain blood, however it will also grow on a chocolate agar plates [3]. *S. pneumoniae* can be differentiated from other species by their ability to produce alpha-hemolysis on blood agar plates [3]. Once a culture morphologically appears to be *S. pneumoniae*, a specialized series of tests, namely optochin susceptibility and bile solubility tests, can then be performed to further confirm the identity [3]. *S. pneumoniae* demonstrate sensitivity to the chemical optochin unlike other Streptococci [109]. However, a number of *S. pneumoniae* strains have been reported

as optochin resistant [109-111]. In this case a bile solubility test can be carried out to distinguish *S. pneumoniae,* which are bile soluble, from all other alpha-hemolytic Streptococci, which are bile resistant [3, 112]. Once these tests have confirmed *S. pneumoniae*, serological tests can be performed to determine the serotype [3].

S. pneumoniae is an encapsulated bacterium with a large capsular diversity. There are over 90 serotypes identified based on the bacteria's polysaccharide capsule, however only a few cause severe pneumococcal disease globally [3]. The burden of pneumococcal disease varies worldwide but mostly effects children under 5 years of age. In low income countries an estimated 0.7 to 1.0 million deaths occur annually in this age group as a consequence of pneumococcal meningitis [15]. In the United States, it accounts for 61% of bacterial meningitis cases. In order to decrease this burden, various types of vaccines have been introduced. These include, the 13-valent pneumococcal conjugate vaccine (PCV13), which superseded PCV7 and PCV10, and the 23-valent polysaccharide vaccine PPSV23 for the prevention of pneumococcal disease [18, 84]. Initial studies indicated that 74-90% of serotypes recovered from the CSF of patients with pneumococcal meningitis are contained within the PPSV23 vaccine, however overall vaccine efficacy is only estimated at 50% [15]. This may be because polysaccharide vaccines, such as PPSV23, are poorly immunogenic in immunocompromised individuals and children under two years of age [113]. As a consequence, pneumococcal conjugate vaccines were developed. The initial PCV7 vaccine targeting 7 serotypes of *S. pneumoniae* had an overall efficacy rate of 97.4% and resulted in a 33% decrease in pneumococcal meningitis incident rates in children under 2 [15]. However, following it's introduction, multiple studies observed an increase in the number of pneumococcal meningitis cases caused by serotypes not contained within the vaccine. Consequently, PCV10 and PCV13 were developed to provide efficacy against emerging serotypes. However, these vaccines still offer limited coverage and both PCV10 and PCV13 do not include protection against serotypes 22F and 35B while only PCV13 includes serotype 19A [15]. Furthermore, pneumococcal meningitis as a result of non-vaccine serotype 35F has been reported [114]. Presently vaccines are also limited to high income countries where the child mortality rate as a consequence of pneumococcal meningitis is low compared with resource poor countries [15].

1.2.Bacterial Meningitis Diagnostic Landscape

Diagnosis of bacterial meningitis can be difficult due to inconsistencies in clinical findings. Studies have shown that classic symptoms of rash, stiff neck and impaired consciousness do not develop until late in the illness and are often absent in adults. Furthermore, viral meningitis, fungal meningitis and tuberculosis meningitis often present with the same symptoms [5]. Therefore, clinical differentiation between these cases of meningitis is crucial to avoid administration of antibiotics unnecessarily or in the case of tuberculosis meningitis, to ensure the rapid administration of anti-tuberculosis chemotherapy [5]. Once there is a suspicion of bacterial meningitis, antimicrobial therapy must be initiated immediately to improve the overall outcome for the patient. To date, targeted antimicrobial therapy is based on presumptive pathogen identification by CSF Gram stain, while in cases where lumbar puncture is delayed empirical antimicrobial therapy is administered based on the patients age and common bacterial pathogens [115]. At present, third generation cephalasporins, (cefotaxime and ceftriaxone) are the beta-lactam antibiotics of choice for the treatment of meningitis as they provide consistent CSF penetration and potent activity against major pathogens, with the exception of *Listeria monocytogenes* and penicillin resistant *S. pneumoniae* [116]. However, evidence based recommendations for specific microorganisms have been described [115]. Therefore, the accurate identification of the microorganism responsible in bacterial meningitis case would allow for a more targeted antimicrobial treatment regime, particularly in cases of bacterial meningitis caused by multidrug resistant bacteria. For example, vancomycin should be added to the treatment regime for *S. pneumoniae* due to the increase in the number of penicillin or third generation celphalasporin resistant *S. pneumoniae* strains causing bacterial meningitis[116]. Adjunctive therapy with dexamethasone is also beneficial in the treatment of *S. pneumoniae* to help prevent neurological complications. However its beneficial effects are not clear against meningitis caused by other organisms and as such treatment of non-*S. pneumoniae* meningitis cases using dexamethasone is not recommended [16, 116]. The duration of antimicrobial therapy also varies dependant on the isolated microorganism and while 10-14 days is required for most pathogens, 5-7 days is sufficient for the treatment of *N. meningitidis* and as little as 3-4 days is sufficient for the treatment of *L. monocytogenes* [115].

1.2.1. Conventional Detection Methods

1.2.1.1. CSF Culture

The mandatory "gold standard" for diagnosis of bacterial meningitis is CSF culture [5]. However, CSF culture is time consuming, often taking two to three days to identify slow growing, fastidious microorganisms [117]. False negatives may also be reported when viable but non-culturable microorganisms are present in a sample [118-120]. Culture of CSF samples is not very sensitive with a reported limit of detection of 10^2 to 10^3 CFU/ml [121-123]. The predominant causes of bacterial meningitis namely, *H. influenzae*, *N. meningitidis* and *S. pneumoniae* require culture on selective media under anaerobic conditions and can often take up to 24-48 hours to obtain a positive culture, if at all [3, 25, 98]. CSF culture is only positive in 80-90% of patients with acute community acquired bacterial meningitis and a retrospective study of 875 cases of bacterial meningitis established positive CSF culture for only 85% of patients prior to antibiotic treatment [5, 15, 124]. This is a significant concern in the diagnosis of bacterial meningitis as administration of antibiotics prior to sample collection can result in additional diminished capability of culture confirmation [15, 117, 122, 123]. Two large case studies reported decreases in CSF culture yields from 66 to 62% and 88% to 70% of patients as a consequence of prior antimicrobial therapy [124, 125]. Furthermore, in one of these studies, this yield further decreased to 59% 24 hours after treatment [125]. In a third study, CSF culture was negative in children who received antimicrobial treatment prior to CSF examination and sterilization of *N. meningitidis* and *S. pneumoniae* from CSF occurred within 2 hours and 4 hours of antibiotic administration, respectively [119]. Consequently, there is a need for lumbar puncture prior to antibiotic treatment in order to accurately diagnose bacterial meningitis. In addition, CSF culture does not always accurately identify the causative agent of bacterial meningitis, restricting the administration of an appropriate antibiotic treatment regime [5].

1.2.1.2. CSF Cell Count, Glucose and Protein

Bacterial meningitis may also be diagnosed by physical examination of CSF. The normal CSF white blood cell count varies between 0-5 cells/mm^3 [126], while in a patient with untreated bacterial meningitis CSF white blood cell count is 1000-5000

cells/ mm^3 [118]. However, cell count can vary and in rare cases may be lower. This often occurs in immunosuppressed patients or patients with septic shock and systemic complications [15, 118]. In one study, of 258 adults with culture confirmed bacterial meningitis, 19% of patients had CSF white blood cell counts of <1000 cells/mm^3 while CSF examination was normal for 1.7% of patients [127]. Nevertheless, the predominance of polymorphonuclear pleocytosis in CSF is suggestive of bacterial meningitis [118].

CSF glucose levels may be reduced in patients with bacterial meningitis. A CSF glucose level of <0.34g/litre with a ratio of CSF glucose to blood glucose of less than 0.23 is usually indicative of bacterial meningitis infection [15]. However this is only observed in 21% of patients [15]. Furthermore, results are nonspecific, suggesting only a non-viral cause [121].

Raised CSF protein concentrations are observed in 90% of patients with acute community acquired bacterial meningitis and a concentration of >2.2g/litre is associated with the disease [15]. However, similar to CSF glucose levels it is non-specific and a slight increase may also occur in viral and fungal meningitis [128]. Furthermore, pre-treatment with antibiotics can reduce protein and glucose abnormalities within hours of administration [121].

1.2.1.3. MALDI-TOF MS

In recent years, MALDI-TOF MS has emerged as a powerful technique for the accurate identification and speciation of microorganisms such as *Haemophilus* by obtaining a protein mass spectral 'fingerprint' which can be compared with a reference spectra database [21, 102]. Unlike more traditional biochemical and growth factor based detection methods which require a lengthy subculturing step, MALDI-TOF MS can be applied directly to pure and mixed cultures immediately after growth occurs. As a result, mass spectra results can be achieved in less than 10 minutes from a culture positive sample, reducing identification time from ≥48 hr to ≤ 24 hr [102]. However, despite the significant improvement in the time it takes to identify a micro-organism of interest, the requirement for a culture step is problematic when dealing with fastidious microorganisms responsible for bacterial meningitis or in a clinical setting where culture sensitivity is diminished as a result

of antibiotic administration prior to sample collection [129]. Furthermore, the identification of rare microorganisms and those lacking reference spectra within the MALDI-TOF MS database can be challenging [130, 131]. [131, 132]. MALDI-TOF MS has been established as a reliable method for the identification of *H. influenzae*. In particular, the suitability of MALDI-TOF MS for the discrimination of *H. influenzae* from its most closely related species, *H. haemolyticus* using well-defined reference spectra has been described [132-134]. MALDI-TOF MS has also been shown as a useful tool for the species specific identification of *N. meningitidis*. In a one study, 29 strains of *N. meningitidis* were successfully identified and differentiated from closely related species, *N. gonorrhoeae*, and 15 other non-pathogenic *Neisseria* strains [135]. However, so far, MALDI-TOF MS identification of *S. pneumoniae* remains a challenge. This is most likely a result of an incomplete database reference library and as a consequence *S. pneumoniae* is often misidentified as *S. mitis*, *S. oralis* or *S. parasanguinis* and vice versa [136-139]. Consequently, at present, reliable identification of *S. pneumoniae* cannot be performed using MALD-TOF MS [137].

1.2.1.4. Fluorescent in-situ hybridization (FISH)

Fluorescent *in-situ* hybridization (FISH) enables microscopic visualization of bacteria using fluorescence labelled oligonucleotide probes which bind to a complementary target sequence. FISH is a highly valuable and rapid tool for the detection of pathogenic bacteria without the need for culture and while to date only genus specific probes have been described for *Streptococci* [140, 141], FISH with probes specific for *H. influenzae*, *N. meningitidis* and other microorganisms associated with bacterial meningitis have been documented [120, 132, 142]. However, shortcomings in the use of these probes exist. In one study describing a FISH probe set for the rapid diagnosis of bacterial meningitis in CSF, the *N. meningitidis* specific probe only accurately identify 1/2 *N. meningitidis* strains tested [120]. Furthermore, due to the significant 16s rRNA nucleotide sequence similarity within the Haemophilus genus, it was later established that the *H. influenzae* 16S rRNA probe by itself is unable to distinguish *H. influenzae* from closely related species, including *H. haemolyticus* [132]. As a consequence, additional 16S rRNA probes were designed and evaluated for *H. influenzae*, *H. parainfluenzae* and *H.*

haemolyticus. However 15.5% of results remained uninterruptable resulting in 13/84 failed identifications [132].

1.2.1.5. Other conventional detection methods

In the event where lumbar puncture to collect CSF cannot be performed, or a CSF culture negative result is obtained, other conventional methods of diagnosis such as Gram staining, latex agglutination testing, blood culture and skin biopsy are often performed [15]. Both CSF Gram staining and latex slide agglutination are rapid, cost effective, and well validated diagnostic methods for diagnosis of bacterial meningitis, particularly in cases where CSF culture is unable to identify the infection [15, 115]. However reported sensitivities vary between organisms with correct identification as little as 25% and 78%, 69% and 59%, and 30% and 22% for *H. influenzae*, *S. pneumoniae* and *N. meningitidis* Gram staining and latex agglutination tests respectively [15]. Similar to diagnosis using CSF culture, pre-treatment with antibiotic reduces the ability of both Gram stain and latex agglutination to detect bacterial meningitis. In one study for the diagnosis of meningococcal meningitis using latex agglutination test, diagnostic sensitivity was reduced from 60% to 9% following antibiotic treatment [143]. However, the effect of antibiotic pre-treatment on latex agglutination tests is much more significant than observed with Gram staining and only a slight decrease in diagnostic sensitivity is observed with the latter [124, 125, 144]. Conversely, the accuracy of CSF Gram staining results is dependent on operators staining and interpretation, unlike the more reliable latex agglutination test which uses serum containing bacterial antibodies or antisera directed capsular polysaccharide of meningeal bacterial [15, 145]. Comparable to Gram staining and latex agglutination, blood culture and skin biopsy Gram stain are also useful diagnostic tools for the detection of bacterial meningitis in CSF culture negative patients, however their reported sensitivities are variable [15]. Furthermore, although skin biopsy Gram stain is not effected by pre-treatment with antibiotics, it offers extremely limited sensitivity and in a French study of 1,344 children with meningococcal meningitis, only 0.5% of cases were positively identified using this method [146].

1.2.2. Nucleic Acid Based Detection Methods

1.2.2.1. Polymerase Chain Reaction

Since its introduction in 1986, Polymerase Chain Reaction (PCR) has become a widely used technique with significant influence in the area of molecular biology [147]. PCR is an *in vitro* DNA amplification technique based on the ability of a heat stable DNA polymerase, usually derived from *Thermus aquaticus*, to amplify a specific fragment of DNA by exploiting two specific oligonucleotide primers that flank that region [147]. Firstly, template DNA is denatured by heating to 95^{o}C. This separates the double stranded DNA in to two single strands and allows the oligonucleotide primers to anneal to the single stranded DNA upon cooling. The temperature at which the primers anneal to the single stranded DNA is dependent on the primers. Following this, the reaction is heated to 72^{o}C which is the optimum temperature to allow the DNA polymerase to extend the primers. Thus, the primers are extended at this temperature in the presence of deoxyribonucleotide triphosphates (dNTPs), $MgCl_2$, and reaction buffer, using the target DNA as a template. This results in newly synthesized copies of the target DNA which can act as template for subsequent cycles. As a result, PCR is exponential (Figure 1.3). Subsequent detection and visualization of conventional PCR products is usually carried out using agarose gel electrophoresis stained with ethidium bromide under ultraviolet (UV) light [147].

1.2.2.2. Real-time Polymerase Chain Reaction

Real-time PCR technology eliminates the requirement for end-point post *in-vitro* amplification processing of the samples thus reducing the risk of cross contamination [148]. First described in 1992, real-time PCR is a method based on the detection and quantification of PCR products as they accumulate using fluorescent technology [107]. The fluorescent signal emitted increases in direct proportion to the amount of PCR product in the reaction. The point at which the fluorescent signal exceeds a fixed baseline is known as the Cycle Threshold (Ct) value and correlates to the initial amount of target template added to the reaction [148]. For example, if a high concentration of starting template is added to the reaction, an early Ct value will be observed and if a low concentration of target template is added to the reaction, a later

Ct value will be observed. It is this property of real-time PCR which enables quantification of unknown samples when a standard curve of known concentrations is incorporated in to the real-time PCR run. To date there have been a number of fluorescent reporter systems used for monitoring real-time PCR *in-vitro* amplification, these include, non-sequence specific DNA binding agents and species specific Hydrolysis probes [148].

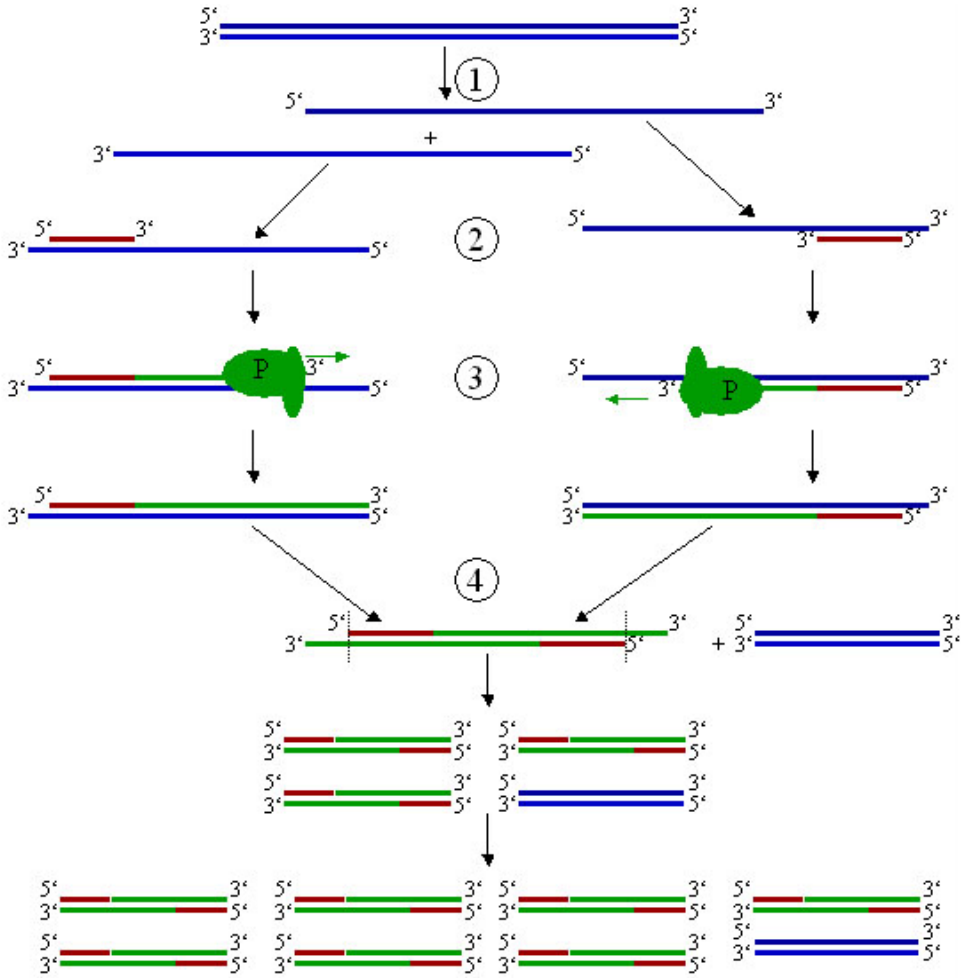

Figure 1.3: Schematic Representation of PCR. (1) Separating the of double stranded DNA at 95ºC. (2) Annealing of primers e.g. 55ºC. (3) Extension at 72ºC (P = DNA polymerase). (4) The first cycle of PCR is complete resulting in two copies of the target DNA. Each additional cycle results in the exponential amplification of the target DNA.

(Source http://serc.carleton.edu/microbelife/research_methods/genomics/pcr.html)

1.2.2.2.1. DNA binding agents

Double stranded DNA binding chemistry detects amplicon production using a non-sequence specific fluorescent intercalating dye such as SYBR green. SYBR green is a fluorogenic minor groove binding dye that exhibits little fluorescence in solution but emits a strong fluorescent signal upon binding to dsDNA (Figure 1.4). Despite being a cheaper alternative to hydrolysis and hybridizing probes, SYBR green real-time PCR requires extensive optimization to reduce non-specific binding which can lead to the reporting of false positives [148].

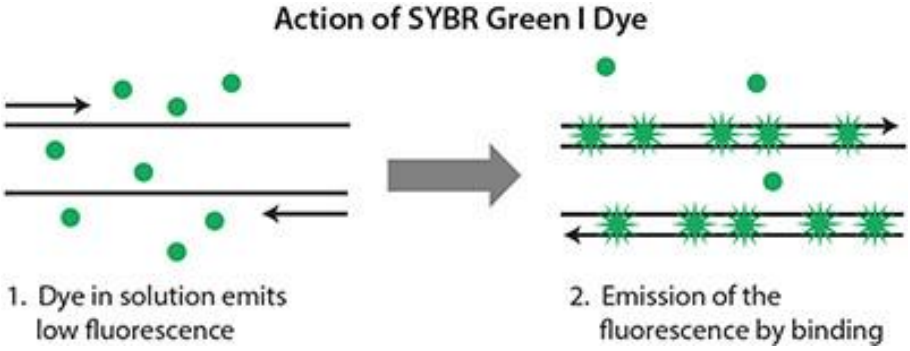

Figure 1.4. Schematic representation of SYBR green technology (Source: http://www.sigmaaldrich.com/)

1.2.2.2.2. Hydrolysis probes

Hydrolysis probes include Taqman probes. Taqman probes are dual labelled oligonucleotides that are specific for a region of interest in the amplified target sequence. They are typically 20-30 bp in length, have a melting temperature of 5-10°C higher than the primers and contain a fluorescent reporter molecule on the 5' end (e.g. FAM, HEX, ROX) and a quencher molecule on the 3' end (e.g. Black Hole Quencher 1 [BHQ1]). Taqman probes utilize 5' exonuclease activity of *Taq* DNA polymerase. Taq polymerase degrades the bound probe releasing the fluorophore from the adjacent quencher. When the fluorophore is released it emits a fluorescent signal [148] (Figure 1.5).

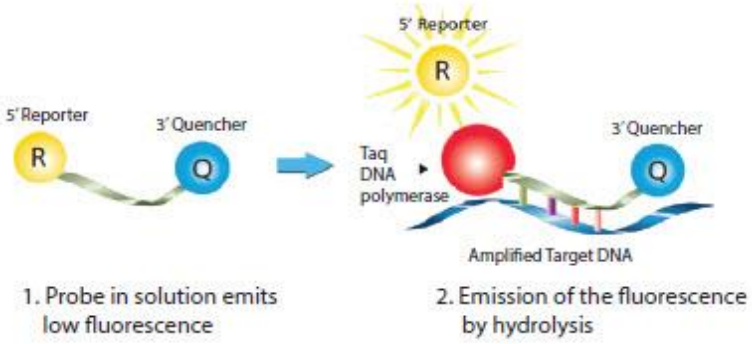

Figure 1.5. Schematic representation of Taqman probe technology (Source: http://www.sigmaaldrich.com/)

Over recent years a number of PCR diagnostic assays have been described targeting various genes including *bexA* [117, 149, 150] *ompP6* [151], *ply* [117, 150], *lytA* [149], *crgA* [149] and *ctrA [117, 150, 151]* for the detection of microorganisms associated with bacterial meningitis. Most notably, *Corless et al* demonstrated the simultaneous real-time PCR *in-vitro* amplification of *H. influenzae*, *S. pneumoniae*, and *N. meningitidis* DNA in CSF from patients with culture confirmed community acquired bacterial meningitis. This real-time PCR diagnostic assay reported sensitivities of 92% (*H. influenzae*), 100% (*S. pneumoniae*) and 88% (*N. meningitidis*) is 100% specific for all three microorganisms [117]. However, primers designed in this study for the identification of *H. influenzae* amplify a capsule type-specific gene, *bexA*. As a consequence, they will only amplify *H. influenzae* serotypes b and c and will not amplify serotypes a, d, e or f or NTHi. In another study, 43%, 27% and 37% of patients with *H. influenzae*, *S. pneumoniae*, and *N. meningitidis* meningitis respectively were diagnosed using only conventional PCR. Whereas, less than 1.1% of patients were diagnosed using only culture and although latex agglutination test provided a higher patient diagnosis rate of 35% for *S. pneumoniae*, diagnosis rates for *H. influenzae* and *N. meningitidis* were only 8.4% and 22% respectively, further validating that PCR sensitivity and specificity is high compared with conventional CSF culture and latex agglutination test methods [149]. Furthermore pre-treatment with antibiotics does not significantly reduce sensitivity, and in one prospective study, 81% of patients were successfully diagnosed with meningococcal meningitis despite antimicrobial therapy [143].

1.2.2.3. Loop Mediated Isothermal Amplification

Loop Mediated Isothermal Amplification (LAMP), first described in 2000 by *Notomi et al* [152], is an isothermal nucleic acid based *in-vitro* amplification technique which utilizes *Bst* DNA polymerase and a set of four specifically designed primers, two inner primers (FIP, BIP) and two outer primers, which recognize six distinct regions of the target DNA. LAMP is highly specific as amplification will only occur when six regions are correctly identified by the four primers. At 65^{o}C, LAMP can amplify up to 10^{9} DNA copies in less than an hour. Two further primers, known as loop primers (LF, LB), are also now commonly utilized to improve the sensitivity of the LAMP reaction and can reduce amplification time to within 30 minutes [153, 154].

In the initial LAMP reaction steps all four primers are used, after which only the inner primers are used in the cycling reaction for strand displacement DNA synthesis. Figure 1.6 illustrates in detail the steps involved in a LAMP reaction. Monitoring techniques for LAMP products include; naked eye visualization of turbidity, fluorescence, or colorimetry, agarose gel electrophoresis, real-time turbidity, real-time fluorescence, electrochemical methods, lateral flow dipstick (LFD) and enzyme-linked immunosorbent assay (ELISA) [155]. Several LAMP diagnostic assays have been described for the detection of common bacterial meningitis pathogens, *H. influenzae*, *S. pneumoniae*, and *N. meningitidis.* LAMP diagnostic assays for the detection of these three microorganisms have been developed targeting well characterized genetic markers, including, *bexA*, *16s* and *ctrA* [156-160]. LAMP has a number of advantages over PCR. It been demonstrated to be more sensitive than previously published PCR diagnostic assays [158] and is more tolerant to biological substances found in clinical samples which might inhibit the reaction [161]. Furthermore, it is more cost effective, therefore, more suitable for use in resource poor settings [156, 158, 160]. However, unlike PCR, LAMP is difficult to multiplex due to its complicated amplicon structure [162] and as a consequence this limits its use for simultaneous detection of more than one DNA target in a single reaction.

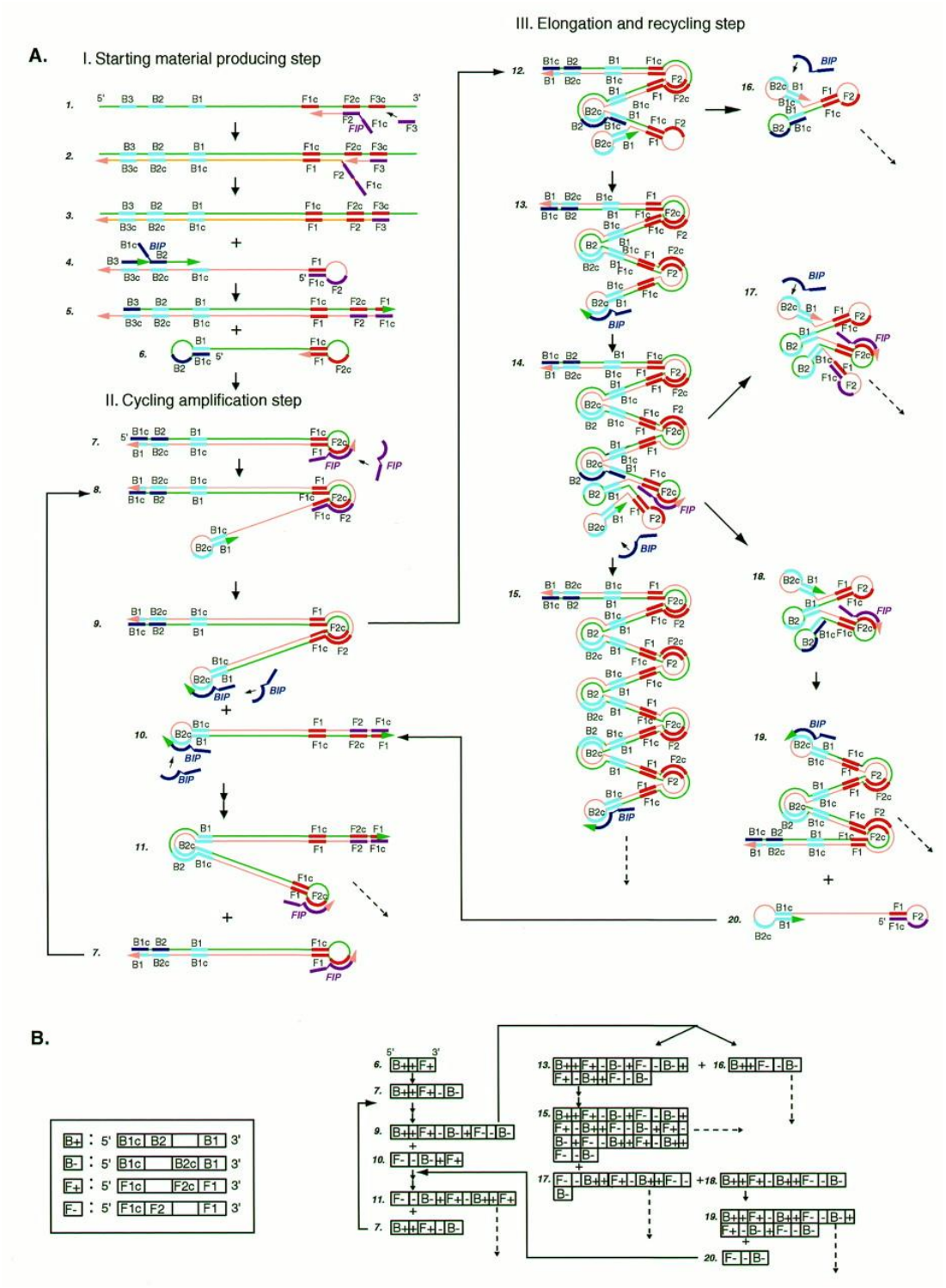

Figure 1.6. Schematic representation of LAMP. (A) Steps in the LAMP reaction. (B) Schematic presentation of the structure of LAMP products in a linearized DNA form.

(Source : http://nar.oxfordjournals.org/content/28/12/e63/F1.large.jpg)

1.2.2.4. Recombinase Polymerase Amplification

Recombinase Polymerase Amplification (RPA) is an isothermal *in-vitro* DNA amplification technique which utilizes a combination of recombinase protein, oligonucleotide primers and a strand displacing polymerase, yielding a result in as little as 10 minutes [163, 164]. A schematic representation of the RPA detection method is illustrated in Figure 1.7. RPA is a rapid, specific and highly sensitive detection method with the capability to operate at low temperatures thus facilitating its application in Point-of-Care (POC) diagnostics [163]. It is less sensitive to inhibitors than PCR and the use of a fluorescent probe allows real-time monitoring of the amplification reaction in addition to multiplexing [50]. Furthermore, the addition of a reverse transcriptase enzyme enables the use of RPA for the *in-vitro* amplification of RNA targets [164]. Despite the significant advantages of RPA, low temperature *in-vitro* amplification also has a disadvantage in that interactions between primers can occur even when well-designed [164].

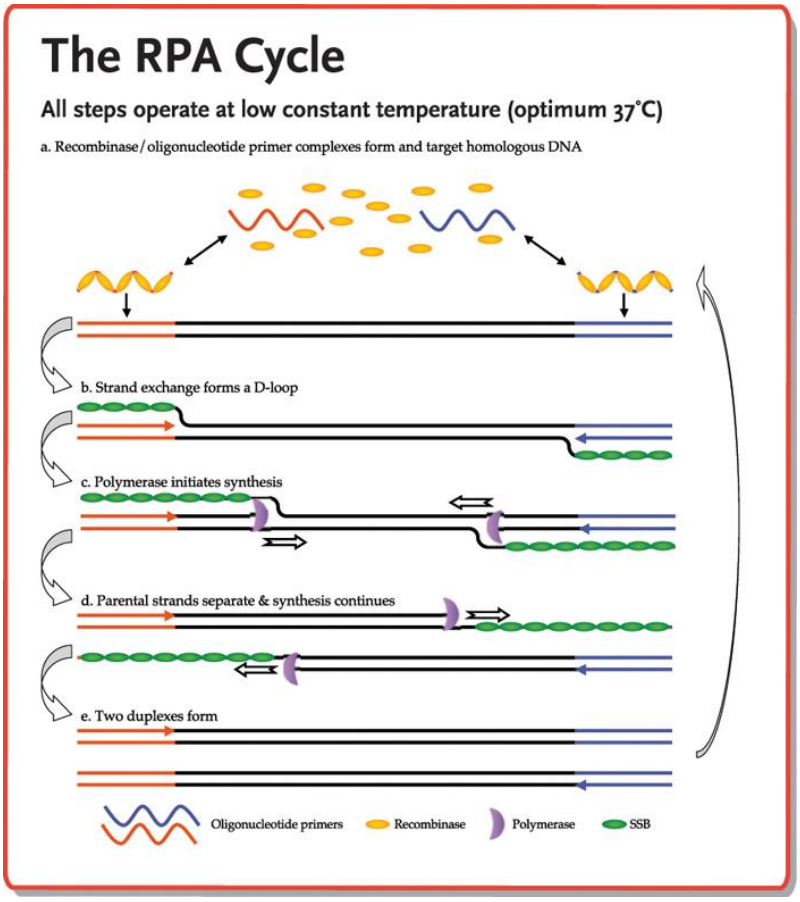

Figure 1.7: Schematic representation of the RPA detection method. (Source : http://www.twistdx.co.uk/our_technology/)

To date, a small number of RPA diagnostic assays have been described for the detection bacterial targets [50, 165, 166]. In particular, a real-time RPA diagnostic assay has been described for the species specific identification of *S. pneumoniae* in whole blood samples [50]. As yet, RPA has not been described for the identification of other predominant microorganisms associated with bacterial meningitis including *H. influenzae* and *N. meningitidis*.

1.3.Nucleic Acid Sequence Based Amplification (NASBA)

1.3.1. General Introduction

Nucleic Acid Sequence Based Amplification (NASBA) is an isothermal, enzymatic, transcription based *in-vitro* amplification system involving specific amplification of RNA sequences. *Guatelli et al* were the first to describe an isothermal nucleic acid sequence based *in-vitro* amplification protocol [167] . This was based on the self-sustained sequence replication (3SR) system characteristic of retroviruses, whereby nucleic acid sequences can be amplified isothermally by the use of three enzymes; avian myeloblastosis virus reverse transcriptase (AMV-RT), *Escherichia coli* ribonuclease H (*E. coli* RNase H) and T7 DNA dependant RNA polymerase (DdRp). NASBA was first described in detail in a review published a year later [168]. It encompasses the same principles as that of the 3SR system. Advantages of NASBA over the original 3SR system is that it is carried out at 41°C instead of 37°C with the addition of dimethyl sulphoxide (DMSO). These adaptions increase reaction specificity as well as the number of RNA molecules produced rendering it an ideal model for the detection of specific microbial species [169]. The following sections describe the characteristics and development of a NASBA assay, NASBA amplicon detection methods, NASBA multiplexing, and NASBA as a miniaturized point of care device.

1.3.2. NASBA Technology

As with the 3SR system, the NASBA reaction exploits three enzymes: T7 DdRp, RNase H and AM-RT. It also utilizes two specific oligonucleotide primers, nucleotide triphosphates; deoxyribonucleoside triphosphates (dNTPs) for the activity of AMV-RT and ribonucleoside triphosphates (NTP) for the activity of T7 DdRp,

and other buffer components [168]. The oligonucleotide primer binding sites should be free from sequence variation and specific for the target nucleic acid. The first oligonucleotide primer (P1) consists of a sequence of about 20-30 bases in length that is complementary to the target RNA and contains a 5' T7 DdRp promoter sequence. The second oligonucleotide primer (P2) encompasses a sequence identical to the target RNA and is located upstream of the primer 1 binding site. The distance between the primer sites is usually between 80-200 bases [169]. NASBA is a two-step process. Although *in-vitro* RNA amplification is carried out isothermally at 41°C, a 65°C denaturation step is required at the start of the reaction to remove secondary structure before the addition of the enzymes. This allows P1, containing a T7 DdRp promoter sequence at the 5' end, to anneal to the target RNA. Following this, the enzymes are added and primer 1 is elongated by AMV reverse transcriptase forming a RNA:DNA hybrid. Simultaneously, the RNA strand of this hybrid is digested by RNase H leaving single stranded (ss) complementary (c) DNA. This enables P2 to anneal to the sscDNA. P2 can then be elongated by the action of AMV reverse transcriptase yielding a double stranded (ds) cDNA copy of the original sequence. The third enzyme, T7 RNA polymerase transcribes multiple RNA copies from the now active double stranded T7 RNA polymerase promoter site that are complimentary to the original target RNA. The process then enters the cyclic phase and can be repeated using the anti-sense RNA as a template and the primers annealing in reverse order (Figure 1.8). In this way the enzymes act continuously under isothermal conditions to achieve approximately a 10^9 fold amplification in as little as 90 minutes [170].

1.3.2.1. NASBA Amplicon Detection

A number of hybridization based methods exist for the detection of the NASBA RNA amplicon: electrochemiluminescence (ECL), enzyme-linked gel assay (ELGA), fluorescence correlation spectroscopy, microarray, and molecular beacon detection [169, 171, 172]. ECL NASBA is a method which employs the use a biotinylated DNA capture probe to immobilize the amplicon by hybridization to streptavidin coated magnetic beads. A ruthenium-labelled probe hybrizes to the immobilized amplicon and a voltage induced oxidation-reduction reaction produces an ECL signal which can be read by a photomultiplier. NASBA ECL has been

used to detect a number of viruses and bacteria such as picornavirus [173], rabies [174], avian influenzae [175-177], astrovirus [178], enterovirus [179], parainfluenza virus [180], flavivirus encephalitis [181], *Salmonella enterica* [182, 183], *Escherichia coli* [184], *Chlamydia trachomatis* and *Neisseria gonorrhoeae* [185], *Legionella* spp. [186], *Plasmodium* spp. [187] and *Cryptosporidum parvum* [188]. ELGA utilizes the hybridization of horseradish peroxidase (HRP) probes to the amplicon and subsequently separation of free and hybridized probes can be analysed by gel electrophoresis. Incubation with HRP substrate then allows visualization.

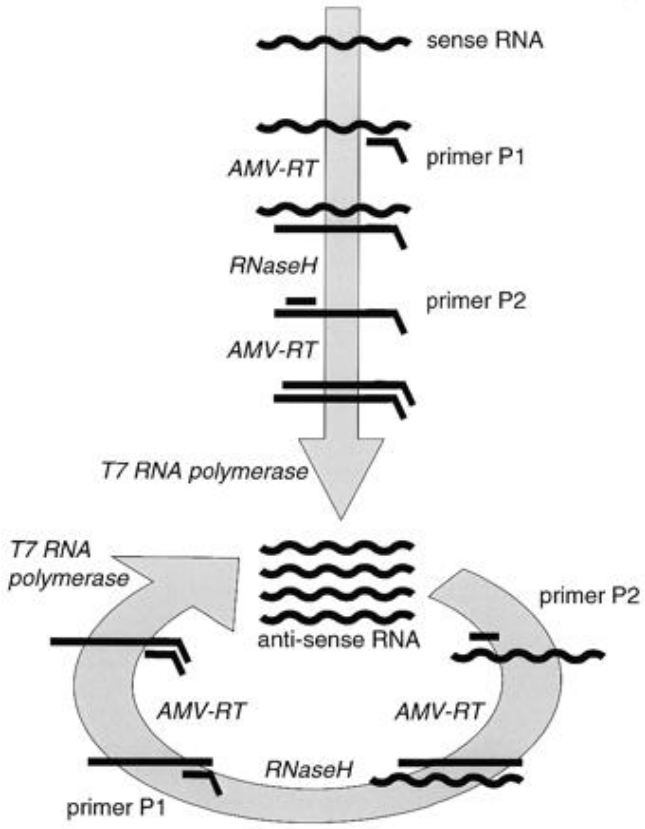

Figure 1.8: Schematic presentation of NASBA technology [189].

Although NASBA- ELGA has been used for the detection of a number of microbial pathogens, it's use has not been recorded in literature recently [190]. Fluorescence correlation spectroscopy (FCS) is a detection method which employs the use of a fluorescent labelled probe. The probe hybridizes to the amplicon and enables online detection of its increased diffusion time. FCS NASBA has been used to detect HIV RNA but similar to ELGA, it has not been recorded in literature as a detection

method for NASBA in recent years [191]. Microarray detection of NASBA products is a method by which the RNA amplicons are labelled with a fluorescent dye and detected by monitoring its intensity signal upon hybridization to a target sequence capture probe on the microarray. NASBA microarray detection has been carried out to detect *S. pneumoniae* tmRNA [172, 192], mRNA encoding ribosomal protein S18, estrogen receptor alpha, human epidermal growth factor receptor 2, caspase 8 D302H, and superoxide dismutase 2 V16A [193].

1.3.2.2. Real-time Molecular Beacon Detection

More recently molecular beacons have become the method of choice for NASBA amplicon detection. Molecular beacons enable the monitoring of NASBA reactions in real time. Molecular beacons were first described by *Tyagi et al* in 1996 [194] and first illustrated for use in NASBA by *Leone et al* two years later [171]. Molecular beacon technology is based on the principle of fluorescence resonance energy transfer (FRET). Molecular beacons are ssDNA molecules which possess a stem-loop structure which is dual labelled with a fluorescent reporter dye at the 5' end and a quencher at the 3' end (Figure 1.10). The loop portion, or probe sequence, is complementary to the target RNA while the stem portion consists of two annealing complementary arm sequences on either side of the probe region which are unrelated to the target sequence. One arm is labelled with a flurophore and the other arm is labelled with a non-fluorescent quencher. Due to its hairpin structure, in the absence of a complementary RNA target sequence the fluorophore is held in close proximity to the quencher and fluorescence is suppressed. Upon encountering a complementary RNA target sequence the stem portion of the molecular beacon opens, separating the fluorophore and the quencher, enabling the loop portion to anneal to the target RNA sequence and produce a fluorescent signal. This allows the assay to be “monitored by a simple photometer or visual observation” [194].

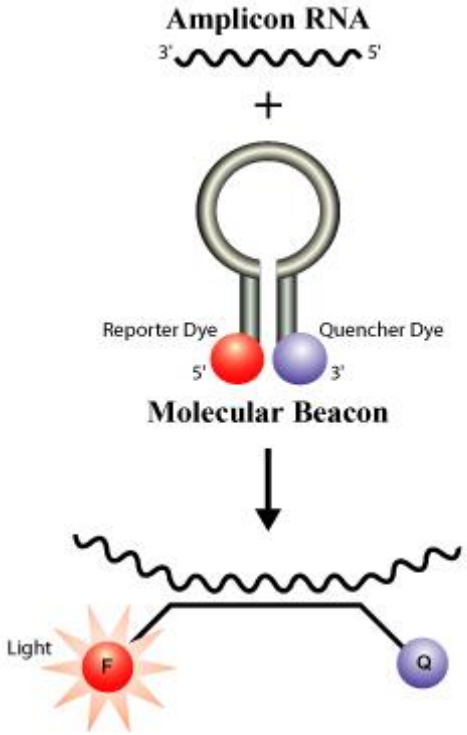

Figure 1.10. Schematic Representation of Molecular Beacon Technology

1.3.2.3. Multiplex real-time NASBA

Labelling molecular beacon probes with fluorophores that emit light at different wavelengths enables the simultaneous amplification and detection of different RNA targets in a single reaction [142]. This provides the opportunity to carry out a multiplex NASBA assay in real time provided there is a known target sequence. The advantage of this technology is the ability to discriminate between specific species or specific serotypes, or the incorporation of a positive control which can determine if the assay is functioning correctly. *Chen et al* developed an assay to determine *agr* activity in *Staphylococcus aureus* [195]. This was carried out by targeting the effector of *agr* response, RNAIII, as well as targeting the housekeeping gene, *gyrase B* (*gyrB),* as a positive internal amplification control (IAC), consequently, two molecular beacon hybridization probes were utilized each labelled with a different fluorophore and quencher. The quencher is determined by ensuring sufficient spectral overlap between its absorption maximum and the fluorophores emission maximum. For example, fluorophores with an emission wavelength of 500 nm to 550 nm work well with quenchers with an absorption spectra of 450 nm to

550 nm [196]. Another recent use of multiplex NASBA incorporating an IAC was described by *Sidoti et al* for the detection of human rhinovirus (HRV) [53]. This assay employed the fluorophores, FAM and HEX, the latter having a maximum emission spectra of 550 nm, and DABCYL, which absorbs maximally at 475 nm, as a quencher. Multiplex NASBA diagnostic assays have not only been described for the simultaneous detection of a target species and assay control but also for the detection of a number of species at the same time. *Zhao et al* illustrated a real-time NASBA diagnostic assay for the detection of pathogens associated with fungal and bacterial bloodstream infections [67]. However, a multiplex real-time NASBA diagnostic assay for predominant microorganisms associated with bacterial meningitis has not been described.

1.3.2.4. Lab on a chip NASBA

Recently, there has been a trend towards the use of miniaturised analytical systems for nucleic acid analysis. The development of these micro-total analysis systems (μTAS) or lab-on-chip (LOC) devices is driven by the requirement for rapid, easy to use, affordable, point of care (POC) diagnostics [197]. Another benefit of these fully enclosed systems is that they involve less manual handling of the sample reducing the possibility of contamination [198]. Like other isothermal *in-vitro* amplification methods, real-time NASBA has the potential to eliminate the need for thermal cycling requirements which facilitates its potential application on a low cost lab on a chip (LOC) and/or point of care (POC) diagnostic device [199, 200]. On-chip real-time NASBA technology was first documented in 2004 with the detection of human papillomavirus (HPV) 16 [201]. Using a 118 bp ssDNA target, *Gulliksen et al* were able to perform an on-chip real-time NASBA *in-vitro* amplification in 10 nl surface treated silicon-glass reaction chambers. This revealed the potential use of microchips to perform NASBA reactions in nanolitre although further work was required to fully integrate the NASBA process to include sample preparation. Subsequently, *Gulliksen et al* described an on-chip NASBA process targeting HPV 16 mRNA on a cyclic olefin co-polymer (COC) microchip [202]. HPV 16 mRNA was amplified simultaneously in 10 reaction chambers at a volume of 80 nl demonstrating the potential to multiplex and detect a number of RNA targets at the same time. This assay also established comparability between conventional and

microchip reader detection limits. Further advances from this research group came with dried enzymes and reagents stored on chip as well as a two chip approach. The first COC polymer chip to contain fully integrated nucleic acid extraction and a second COC polymer chip for NASBA amplification and detection. [203]. The first ever fully integrated NASBA microchip combining nucleic acid isolation was described in 2008 [65]. This polydimethylsiloxane (PDMS) microfluidic system achieves RNA purification in a 0.25 µl silica bead chamber before NASBA amplification of *E. coli* tmRNA in a separate 2 µl volume chamber. The result obtained for the entire analysis of 100 *E. coli* cells, from input to output, was 30 minutes with NASBA detection occurring within 3 minutes [65]. More recently, a fully integrated NASBA microchip has also been developed targeting the *rbcL* gene of *Karenia bravis* [197]. This poly (methyl methacrylate) (PMMA) chip containing a single 16 µl volume chamber with two connecting 0.5 µl channels can detect as little as 10 cells in 2.24 minutes [197]. *Tsalagou et al* state that this microchip could be used as a model for the detection of a number of species with known target sequence and improvements could be made with the addition of an IAC [197]. These on-chip NASBA reactions demonstrate the potential for a rapid POC real-time NASBA diagnostic device for bacterial meningitis. A NASBA lab-on-a-chip POC device that executes laboratory functions such as nucleic acid isolation and *in-vitro* amplification on a single chip of only millimetres to a few square centimetres in size, would be immensely advantageous in the diagnosis of bacterial meningitis as it requires a low fluid volume sample and would enable home or physician based use of a compact, portable, cost effective, rapid diagnostic test by non-specially trained individuals [65]. This would subsequently allow for the more rapid administration of an appropriate pathogen specific antibiotic treatment regime in a patient with suspected meningitis and improve the overall prognosis of the disease.

1.4. Scope of this Thesis

The overall aim of this study was to contribute toward the design of a Point of Care (POC) on chip nucleic acid diagnostic assays for the detection of the predominant microorganisms associated with bacterial meningitis. This is a collaborative, multidisciplinary project being carried out at the Nucleic Acid Diagnostics Research Group (NADRL) at National University of Ireland, Galway, the Tyndall National

Institute at University College Cork and the Biomedical Diagnostics Institute at Dublin City University. The overall project can be broken down in to two main components. The first is to develop an isothermal multiplex nucleic acid based diagnostic assay for the detection of the three main causative bacteria associated with meningitis; *Haemophilus influenzae, Neisseria meningitidis* and *Streptococcus pneumonia*e. The second aim, was to design and fabricate a clinical diagnostic instrumentation platform to detect meningitis specific target molecular markers from whole blood samples.

The main research focus of this thesis has been on the development of isothermal internally controlled real-time nucleic acid sequence based amplification (NASBA) diagnostic assays for the detection of *Haemophilus influenzae, Neisseria meningitidis* and *Streptococcus pneumonia*e. A number of steps were involved in the development of these diagnostic assays. Initially, publicly available and in-house nucleotide sequence information was interrogated using online bioinformatics tools, such as BLAST and ClustalW to determine the suitability of various gene targets for the identification of the micro-organisms of interest. Subsequently, a monoplex real-time NASBA diagnostic assay was developed for each of these three micro-organisms. The real-time NASBA diagnostic assays were then optimized e.g. primer pair combinations, salt concentration titration and subsequently each real-time NASBA diagnostic assay was tested against an extensive inclusivity panel including culture collection strains and clinical isolates as well as an exclusivity panel of the most closely related species.

In **Chapter 2**, RNA transcripts encoded for by the *ssrA* (tmRNA) and *lepA* (*lepA* mRNA) genes were evaluated as potential molecular targets for the species specific identification of *H. influenzae, N. meningitidis* and *S. pneumoniae* using *in-silico* approaches. Potential diagnostics targets were then further validated in real-time NASBA diagnostic assays. This study established both tmRNA and *lepA* mRNA as potential diagnostic targets for the species specific identification of *N. meningitidis* and *S. pneumoniae,* respectively. However, it was established that neither tmRNA or *lepA* mRNA can differentiate *H. influenzae* from its most closely related species and that further study is required to identify a novel diagnostic target for accurate identification of *H. influenzae*.

Chapter 3 describes the study performed during this research to utilise comparative genomic approaches to identify novel diagnostics markers for *H.* i*nfluenzae*. From this analysis 2 *H. influenzae* putative diagnostic targets, *phoB* and *pstA* were identified which are unique to this microorganism. Subsequently, two real-time PCR diagnostic assays using these gene targets were developed and their performance was determined. Both diagnostic assays were determined to be 100% specific for the detection of *H. influenzae* and did not cross react with *H. haemolyticus* or other closely related species. Furthermore, real-time PCR diagnostic assays developed were established as more superior in terms of specificity than a previously published *fucK* real-time PCR diagnostic assay and 7.1% of *H. influenzae* clinical isolates, identified using MALDI-TOF MS and the novel real-time PCR assays developed in this study, were not detected by the *fucK* assay.

Chapter 4 describes the development of three internally controlled duplex real-time NASBA assays for the species specific identification of *H. influenzae, N. meningitidis* and *S. pneumoniae* targeting RNA transcripts encoded for by the *phoB, ssrA* and *lepA* genes respectively. Each real-time NASBA diagnostics assay includes an endogenous non-competitive Internal Amplification Control (IAC) to amplify the splice variant 1 mRNA of the *Homo sapiens TBP* (TATA-box binding protein) gene from human total RNA. The developed assays were determined to be 100% specific for the target species tested for while Limits of Detection (LOD) for the *H. influenzae, N. meningitidis* and *S. pneumoniae* duplex real-time NASBA assays were 50, 10, and 100 Cell Equivalents (CE) respectively.

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Chapter 2

Evaluation of novel diagnostic targets using real-time NASBA for the species specific identification of *Haemophilus influenzae, Neisseria meningitidis* and *Streptococcus pneumoniae.*

Abstract

RNA transcripts encoded for by the *ssrA* (tmRNA) and *lepA* (*lepA* mRNA) genes were evaluated as potential molecular targets for the species specific identification of *H. influenzae, N. meningitidis* and *S. pneumoniae*. Real-time NASBA diagnostic assays were designed targeting both *ssrA* and *lepA* RNA transcripts and tested against an inclusivity panel of culture collection and clinical isolate strains and an exclusivity panel of non-target closely related species. Real-time NASBA diagnostic assays developed for the detection of *N. meningitidis* and *S. pneumoniae* were 100% specific for the target species, demonstrating the potential use of tmRNA and *lepA* mRNA as diagnostic targets for the species specific identification of *N. meningitidis* and *S. pneumoniae*, respectively. The real-time NASBA diagnostic assays designed targeting both tmRNA and *lepA* mRNA were not 100% specific for the detection of *H. influenzae* and cross reacted with closely related *Haemophilus* species. Additional *in-silico* analysis of the *ssrA* and *lepA* gene sequences established insufficient heterogeneity to distinguish *H. influenzae* from its most closely related species, *H. haemolyticus*. Furthermore, the real-time NASBA assays developed and tested in this study, targeting *lepA* mRNA, are not 100% inclusive and amplification efficiency is poor especially at lower RNA concentrations. Comparison of real-time NASBA assays targeting the RNA encoded for by both the *ssrA* and *lepA* genes revealed the *ssrA* gene, and its tmRNA transcript, to be a more suitable diagnostic target for the species specific identification of *H. influenzae*.

2.1. Introduction

Bacterial meningitis is a life threatening disease associated with high mortality and long term disabilities in survivors [1]. Although the epidemiology of bacterial meningitis has evolved since the introduction of conjugate vaccines, *Haemophilus influenzae*, *Neisseria meningitidis* and *Streptococcus pneumoniae* remain the predominant microorganisms associated with the disease, accounting for 80% of cases worldwide [2]. Presently, CSF culture is gold standard for the diagnosis of bacterial meningitis; however positive CSF culture identification can take up to 3 days and negative results are frequently obtained particularly following pre-treatment with antibiotics [3-5]. As a consequence, there is a requirement for a more

rapid, sensitive and specific nucleic acid based *in-vitro* amplification diagnostics method to accurately identify *H. influenzae, N. meningitidis* and *S. pneumoniae.*

2.1.1. Nucleic acid diagnostic technologies

Nucleic acid diagnostic (NAD) technologies identify the presence of microorganisms by either direct detection of *in-vitro* amplification of a specific nucleic acid sequence. The nucleic acid sequence (DNA or RNA) of all microorganisms contains regions that are unique to that strain, species or genus. One of the most commonly used molecular diagnostic targets is 16s ribosomal RNA, which has been proven as a useful target for the species specific identification of a number of microorganisms, including *H. influenzae*, *N. meningitidis* and *S. pneumoniae* [6-8]. However, sequence variation is limited or even absent between closely related species. As a consequence it cannot be used as a molecular target and other genes must be analysed.

2.1.2. Nucleic acid diagnostic research laboratory (NADRL)

NADRL has over 25 years research experience in the discovery of microbial biomarkers and the development of these biomarkers for use in NAD technologies. All designed nucleic acid diagnostic assays are developed to industry standards using a definitive diagnostics assay development strategy and in accordance with MIQE guidelines [9] (Fig 2.1).

Briefly, NADRL assay design strategy involves:

- **Target Bio-discovery:** Novel NAD targets are identified
- **Bioinformatics:** Assembly of nucleotide sequence data from a large panel of relevant species / strains
- **Assay Design:** Specific NAD assays are designed to detect each microorganism of interest
- **Species Specificity panel assembly:** A panel of well characterised species / strains are collected from a wide geographically relevant area

- **Assay performance optimisation and validation**: Evaluation of the assays performance including optimisation and validation. Assays are also multiplex when required.

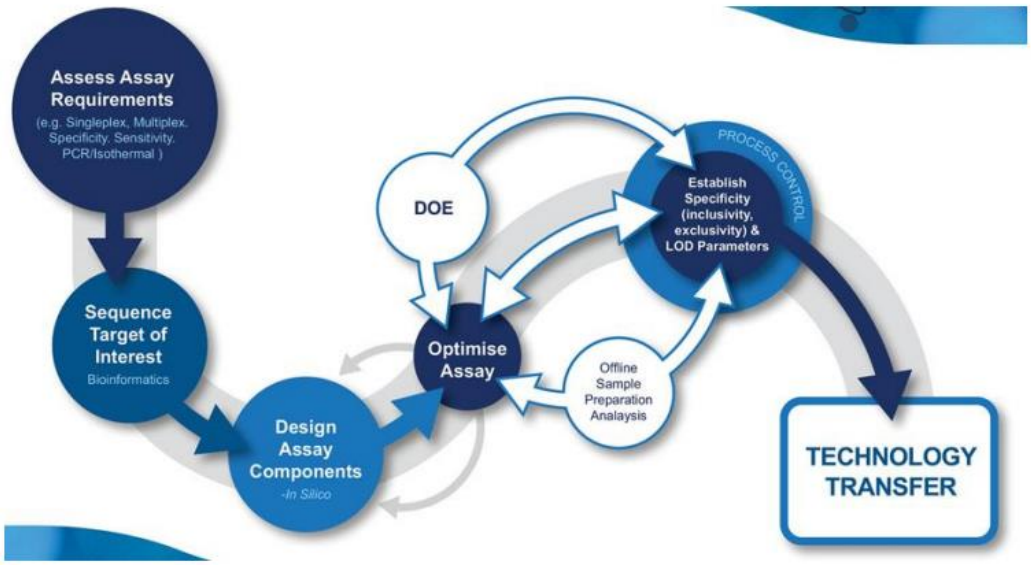


Figure 2.1: NADRL NAD assay development strategy in accordance with MIQE guidelines

2.1.3. NAD targets

An ideal diagnostics target should be both conserved in all species / strains of interest and unique to the microorganism(s) [10]. The diagnostic target should also facilitate the design of specific yet sensitive oligonucleotide sequencing primers and hybridization probes which do not anneal to other regions within the template [11]. In this study, two potential diagnostic targets, the tmRNA transcript (encoded for by the *ssrA* gene) and the leader peptidase A (*LepA*) mRNA transcript were evaluated for the specific detection of *H. influenzae*, *N. meningitidis* and *S. pneumoniae* using real-time Nucleic Acid Sequence Based Amplification (NASBA). Both the *ssrA* gene and the *lepA* gene have previously been demonstrated as suitable and versatile targets for nucleic acid based microbial identification [12-15]. In particular, tmRNA has been established as a useful target in a real-time NASBA assay [13].

2.1.3.1. tmRNA

The *ssrA* gene, found in >99% of bacteria, codes for transfer messenger RNA (tmRNA)[16]. tmRNA, found in all eubacteria and in some eukaryotic organelles, is a bifunctional RNA that has properties of tRNA and mRNA [17]. It is involved in trans-translation, together with smpB, to rescue stalled ribosomes that are unable to finish protein synthesis and clear the cell of unwanted polypeptides and mRNAs [16]. This may occur when a ribosome reads to the 3' end of an mRNA as a result of a missing stop codon or due to mRNA cleavage if translation pauses at an internal codon. tmRNA recognises these stalled ribosomes and acts as tRNA and mRNA to provide an alternative mechanism to complete translation, release the ribosome and mark the nascent polypeptide for degradation [17] (Fig 2.2). tmRNA trans-translation has been shown to be an essential mechanism of *H. influenzae* and saturating mutagenesis experiments established no mutations in the *ssrA* gene of *H. influenzae* [16].

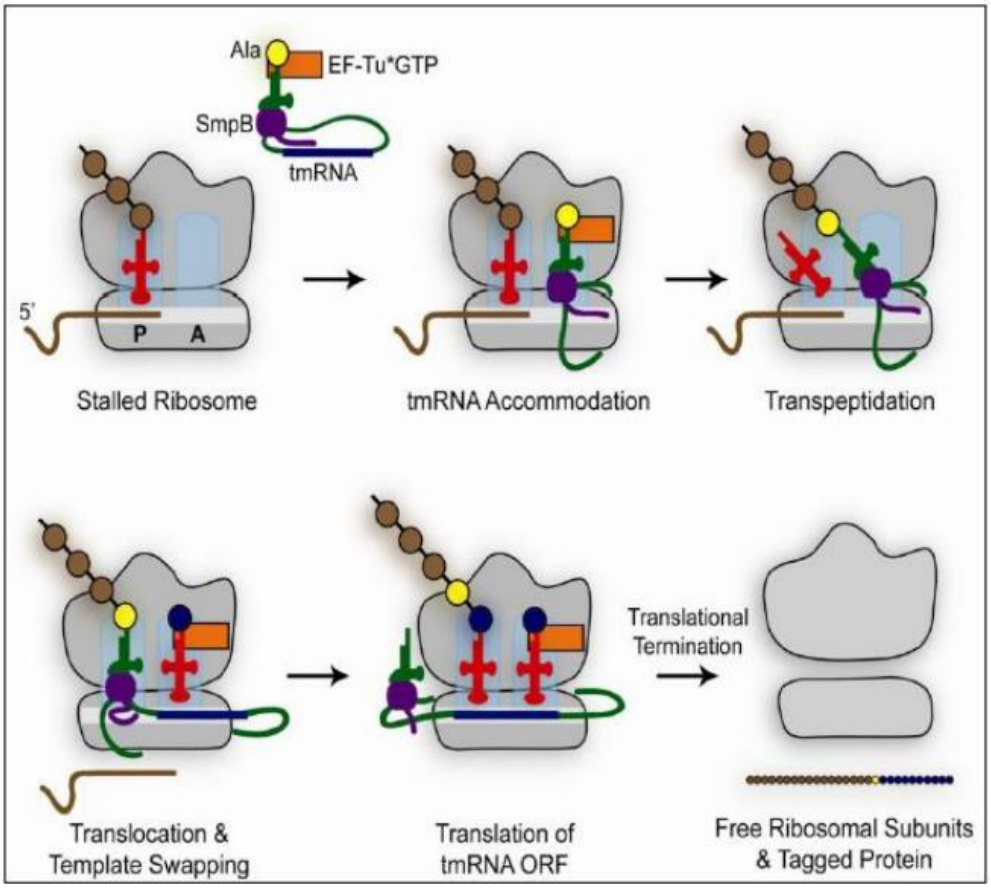


Figure 2.2: Schematic of the mechanism for tmRNA-mediated tagging and ribosome rescue (Source: http://jur.byu.edu/?p=1103)

2.1.3.2. *lepA* mRNA

The Leader Peptidase A (*LepA*) gene codes for a translational elongation factor protein, LepA, found in all bacteria and mitochondria to date [18]. The ribosomal elongation cycle involves a series of reactions to assemble a new polypeptide by the addition of various amino acids corresponding to the mRNA codons [19]. These steps in the protein synthesis process are regulated by multiple elongation factors, of which, lepA is one of them. LepA is involved in recognizing a defective translocation reaction and subsequently initiating back translocation of the mistranslocated tRNAs, resulting in the elongation mechanism moving one codon backwards along the mRNA [20]. This allows the other elongation factors another chance to translocate the tRNA correctly (Figure 2.3). LepA is one of the most highly conserved bacterial proteins (with an amino acid identification of 55% - 68%) and has a homologue, *Guf1*, in higher organisms [18].

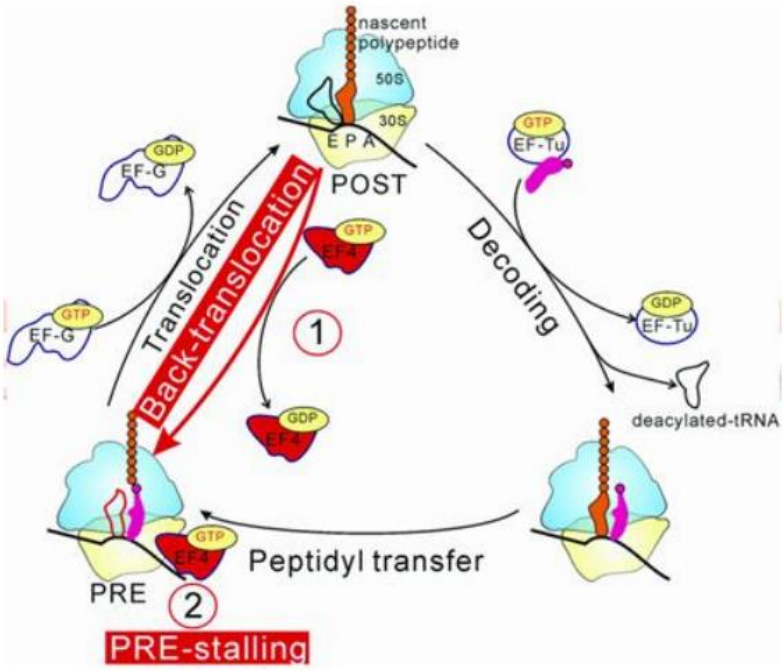


Figure 2.3: Schematic of the elongation cycle of bacterial protein synthesis and the role of EF4 (lepA). (Source: Biochemical Journal 2013 Volume 452, 173-181[20])

2.1.4. Nucleic Acid Sequence Based Amplification

NASBA, first described in detail by *Compton et al* in 1991, is an isothermal, enzymatic, transcription based amplification system involving specific amplification of RNA sequences [21]. A variety of approaches can be taken for the end-point detection of the NASBA RNA amplicon, such as; electrochemiluminescence (ECL) [22], enzyme-linked gel assay (ELGA) [23] and fluorescence correlation spectroscopy [24]. More recently, combining NASBA with molecular beacon detection has enabled the simultaneous *in-vitro* amplification and real time detection of various RNA targets in a single closed tube reaction [25]. Real-time NASBA has a number of advantages over other nucleic acid based diagnostics methods such as real-time PCR. In particular, real-time NASBA can be used to detect viable infectious agents as it will preferentially amplify RNA even in the presence of background DNA [26-28]. Furthermore, as real-time NASBA does not require continuous thermal cycling, it can also be incorporated on to a low cost lab-on-a-chip (LOC) which facilitates its application as a point of care (POC) device to give a rapid diagnostic outcome for the detection of microbial pathogens [28]. A number of real-time NASBA assays have been published for the species specific identification of bacteria such as; *Staphylococcus aureus, Escherichia coli, mycoplasma pneumonia, Chlamydophila pneumonia, Legionella* spp., *Campylobacter jejuni, Campylobacter coli, Vibrio cholera and Listeria monocytogenes* [13, 29-35]. However, to date, a real-time NASBA assay has not been developed for the specific identification of the three predominant microorganisms associated with bacterial meningitis, *H. influenzae, N. meningitidis* and *S. pneumoniae*.

In this study, *in-silico* analysis of both the *ssrA* and *lepA* genes was performed to determine their suitability as diagnostic targets for the accurate identification of *H. influenzae, N. meningitidis* and *S. pneumoniae*. Subsequently, real-time NASBA diagnostic assays were developed targeting the RNA transcribed by each gene and their performance was evaluated.

2.2. Materials and Methods

2.2.1. Diagnostic Target Identification

The *ssrA* gene associated RNA transcript, tmRNA, was analysed as a potential diagnostic target for the species specific identification of *H. influenzae* and *N. meningitidis* by *in-silico* analysis of nucleotide sequence information retrieved from the tmRNA website (http://bioinformatics.sandia.gov/tmrna/), the tmRNA database (http://www.ag.auburn.edu/mirror/tmRDB/) and the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/). The *lepA* gene associated mRNA transcript was analysed as a potential diagnostic target for the species specific identification of *H. influenzae* and *S. pneumoniae* by *in-silico* analysis of nucleotide sequence information retrieved from the NCBI and the Functional Gene Pipeline (FunGene) (http://fungene.cme.msu.edu/).

For each of the diagnostic targets identified, alignments were carried out using ClustalW multiple sequence alignment programme (http://www.ebi.ac.uk/Tools/msa/clustalw2/), from which conventional PCR and real-time NASBA primers and molecular beacon probes were designed (Table 2.1).

Table 2.1: Nucleotide sequences of primers and molecular beacon probes.

Probe/ Primer	Gene Target	Function	DNA sequence (5' – 3')	Nucleotide position	Genbank accession no.	Final Concentration (µM)
ssrA F1	*ssrA*	*Haemophilus ssrA* Sequencing forward primer	GGGGCTGATTCTGGATTCGACGG	1-23	CP002277.1	0.4 µM
ssrA R1	*ssrA*	*Haemophilus ssrA* Sequencing reverse primer	TGGTGGAGCTGGGGGGAGTTGAA	343-365	CP002277.1	0.4 µM
lepA F1	*lepA*	*Haemophilus lepA* Sequencing forward primer	ATGAAGAATATTCGCAAC	1-18	CP002277.1	0.4 µM
lepA R1	*lepA*	*Haemophilus lepA* Sequencing reverse primer	CACTTTAATTTTATCGCCT	657-675	CP002277.1	0.4 µM
lepA F2	*lepA*	*Haemophilus lepA* Sequencing forward primer	CGTGGTATCTTTAGTGCGTATTA	615-637	CP002277.1	0.4 µM
lepA R2	*lepA*	*Haemophilus lepA* Sequencing reverse primer	ACCTAAATATTCTTGCGGCAC	1228-1248	CP002277.1	0.4 µM

lepA F3	*lepA*	*Haemophilus lepA* Sequencing forward primer	ATGGAAATCATTCAAGAGC GT	1042-1062	CP00227 7.1	0.4 µM
lepA R3	*lepA*	*Haemophilus lepA* Sequencing reverse primer	TCTTTTCCTACGTGTAAAAT TGC	1768-1790	CP00227 7.1	0.4 µM
Neisseria F	*ssrA*	*Neisseria ssrA* Sequencing forward primer	GGCGACCTTGGTTTCGACG	4-22	AE00209 8.2	0.3 µM
Neisseria R	*ssrA*	*Neisseria ssrA* Sequencing reverse primer	TCGAACCCCCGTCCGAAAG	327-345	AE00209 8.2	0.3 µM
ssrA P1	*ssrA*	*H. influenzae ssrA* forward primer	AATTCTAATACGACTCACTA TAGGG - AGAAGG - CTTCGATCCTCAAACGGT	213-230	CP00227 7.1	0.2 µM
ssrA P2	*ssrA*	*H. influenzae ssrA* reverse primer	GCAGCTTAATAACCT	116-130	CP00227 7.1	0.2 µM
LepA MB	*lepA*	*H. influenzae lepA* specific hybridization probe	FAM-CCGAGT – TGCAAAATTGCCTCCACT-ACTCGG-DAB	1161-1178	CP00227 7.1	0.1 µM
lepA P1	*lepA*	*H. influenzae lepA* forward primer	AATTCTAATACGACTCACTA TAGGG – AGAAGG – GCTCGCGAATTTCAGCAATA	1185-1204	CP00227 7.1	0.2 µM
lepA P2	*lepA*	*H. influenzae lepA* reverse primer	GGAAATCATTCAAGAGCGT CTA	1044-1065	CP00227 7.1	0.2 µM
Spn P1	*lepA*	*S. pneumoniae* forward primer	AATTCTAATACGACTCACTA TAGGGAGAAGGCATACTCA AGACGCTGAGGAA	1792-1812	AE00731 7.1	0.2 µM
Spn P2	*lepA*	*S. pneumoniae* reverse primer	GACACAAGATTGTCGCTCGT ACTGATA	1628-1654	AE00731 7.1	0.2 µM
Spn MB	*lepA*	*S. pneumoniae lepA* specific hybridization probe	ROX-CGATCGACGCATGAAATCC ATCGGATCAGTTCGATCG - BHQ2	1749-1773	AE00731 7.1	0.1 µM
Nmen P1	*ssrA*	*N. meningitidis* forward primer	AATTCTAATACGACTCACTA TAGGGAGAAGGTCCTCTAC AAAGCGTTCTACA	305-325	AE00209 8.2	0.2 µM
Nmen P2	*ssrA*	*N. meningitidis* reverse primer	CCCGTAAAACACTGAATTCA AAT	63-85	AE00209 8.2	0.2 µM
Nmen MB	*ssrA*	*N. meningitidis ssrA* specific hybridization probe	HEX-CGATCGAACTGGTTTCCAAA AGGCCTCGATCG-DAB	236-255	AE00209 8.2	0.1 µM

2.2.2. Bacterial strains, Culture media and Growth conditions

A panel of culture collection strains of *H. influenzae* (n=8), non-*H. influenzae Haemophilus* species (n=31), *N. meningitidis* (n=9), non-*N. meningitidis Neisseria*

species (n=19), *S. pneumoniae* (n=1) and non-*S. pneumoniae Streptococcus* species (n=37) were obtained from various culture collections (Table 2.2). A collection of recent *H. influenzae* clinical isolates (n=38) and *S. pneumoniae* clinical isolates (n=13) were also obtained from University Hospital Galway to further evaluate the assays. *Haemophilus* strains were cultured in haemophilus test media broth (Oxoid), chocolate broth (Fannin Scientific) or on Columbia chocolate agar plates (Fannin Scientific). *Neisseria* and *Streptococcus* species were cultured in brain heart infusion (BHI) broth (Oxoid), or on Columbia blood agar plates (Fannin Scientific). All bacterial species were cultured under microaerophilic conditions at 37ºC overnight or until sufficient growth was observed, as determined by the degree of turbidity compared to culture negative controls.

Table 2.2: Haemophilus species and strains included in this study

Organism	**Strain** [a]	**Specificity**[e]		
		ssrA	*LepA* (P1.1)	*LepA* (P1.2)
H. influenzae reference strains				
H. influenzae type b	DSMZ 23393[b,c]	+	+	+
H. influenzae type b	DSMZ 4690[b,c]	+	+	+
H. influenzae type b	DSMZ 11969[b,c]	+	+	+
H. influenzae type b	DSMZ 11970[b,c]	+	-	-
H. influenzae type b	DSMZ 10001[b,c]	+	+	+
H. influenzae type d	DSMZ 11121[b,c]	+	+	+
H. influenzae type f	DSMZ 10000[b,c]	+	+	+
H. influenzae NTHi	DSMZ 24049[b,c]	+	+	+
H. influenzae biogroup *aegyptius*	DSMZ 21187[b,c]	+	-	-
H. influenzae clinical isolates				
H. influenzae NT (Source: Blood)	6 clinical isolates	+	/	/
H. influenzae untyped (Source: Sputum)	24 clinical	+	/	/
H. influenzae untyped (Source: Eye/Ear swab)	8 clinical isolates	+	/	/
Non- *H. influenzae* strains				
H. ducreyi	NCTC 11479	-	/	-
H. ducreyi	NCTC 10945[b]	/	/	/
H. felis	DSMZ 21192[b]	-	/	/
H. haemoglobinophilus	DSMZ 21241	-	/	/
H. haemolyticus	NCTC 10839[c]	+	/	+
H. haemolyticus	NCTC 10659[b]	/	/	/
H. haemolyticus	CCUG 24149[c]	/	/	/

H. haemolyticus	CCUG 15312[c]	/	/	/
H. haemolyticus	CCUG 12834[c]	/	/	/
H. haemolyticus	CCUG 36015[c]	/	/	/
H. haemolyticus	CCUG 36016[c]	/	/	/
H. haemolyticus	CCUG 15642[c]	/	/	/
H. paracuniculus	DSMZ 21452[b]	-	/	/
H. parahaemolyticus	DSMZ 21417[b]	-	/	/
H. parainfluenzae	DSMZ 8978[b]	-	/	-
H. paraphrohaemolyticus	DSMZ 21451	-	/	/
H. parasuis	DSMZ 21448[b]	-	/	/
H. pittmaniae	DSMZ 17420[b]	-	/	-
H. pittmaniae	DSMZ 21203[b]	-	/	-
Haemophilus sp.	CCUG 34110[c]	+	/	-
Actinobacillus pleuropneumoniae	DSMZ 13472[b]	-	/	/
Actinobacillus suis	NCTC 6359[b]	/	/	/
Aggregatibacter actinomycetecomitans	DSMZ 8324	-	/	/
Aggregatibacter aphrophilus	NCTC 10558	-	/	/
Aggregatibacter aphrophilus	NCTC 11096[b]	-	/	/
Aggregatibacter segnis	NCTC 10977	-	/	/
Avibacterium avium	DSMZ 18557[b]	-	/	/
Avibacterium paragallinarum	DSMZ 18554	-	/	/
Gardnerella vaginalis	DSMZ 4944	-	/	/
Histophilus somni	CCUG 36157	-	/	+
Organism	**Strain** [a]	**Specificity**[e]		
		ssrA		*LepA*
N. meningitidis reference strains				
N. meningitidis	NCTC 9392	+		/
N. meningitidis serogroup a	NCTC 10025	+		/
N. meningitidis serogroup a	NCTC 3375	+		/
N. meningitidis serogroup a	NCTC 3372	+		/
N. meningitidis serogroup a	NCTC 15465[d]	+		/
N. meningitidis serogroup c	ATCC 13102	+		/
N. meningitidis serogroup c	DSMZ 15464[d]	+		/
N. meningitidis serogroup x	NCTC 10790	+		/
N. meningitidis strain reference 73/M6	NCTC 11423	+		/
Non- *N. meningitidis* strains				
N. animalis	DSMZ 23392[d]	-		/
N. animaloris	DSMZ 21642[d]	-		/
N. canis	DSMZ 18000[d]	-		/
N. caviae	DSMZ 23336	-		/
N. dentiae	DSMZ 19151	-		/

N. elongata subsp. *elongata*	DSMZ 17712[d]	-	/
N. elongata subsp. *glycolytica*	DSMZ 23337	-	/
N. elongata subsp. *nitroreducens*	DSMZ 17632[d]	-	/
N. flavescens	DSMZ 17633[d]	-	/
N. gonorrhoeae	DSMZ 9189[d]	-	/
N. macae	DSMZ 19175[d]	-	/
N. mucosa	DSMZ 17611[d]	-	/
N. ovis	DSMZ 218075	-	/
N. perflava	DSMZ 18009[d]	-	/
N. sicca	DSMZ 17713[d]	-	/
N. subflava	DSMZ 17610[d]	-	/
N. wadsworthii	DSMZ 22247[d]	-	/
N. weaveri	DSMZ 17688[d]	-	/
N. zoodegmatis	DSMZ 21643	-	/
S. pneumoniae reference strains			
S. pneumoniae serotype 1	DSMZ 20566	/	+
S. pneumoniae clinical isolates			
S. pneumoniae untyped (Source: Blood)	3 isolates	/	+
S. pneumoniae untyped (Source: Sputum)	7 isolate	/	+
S. pneumoniae untyped (Source: Eye/Ear	3 isolates	/	+
Non- *S. pneumoniae* strains			
S. agalactiae	BCCM 15081	/	-
S. agalactiae	BCCM 15082	/	-
S. agalactiae	BCCM 15083	/	-
S. agalactiae	BCCM 15084	/	-
S. agalactiae	BCCM 15085	/	-
S. agalactiae	BCCM 15086	/	-
S. agalactiae	BCCM 15087	/	-
S. agalactiae	BCCM 15094	/	-
S. agalactiae	BCCM 15095	/	-
S. anginosus	DSMZ 20563	/	-
S. australis	DSMZ 15627	/	-
S. canis	DSMZ 20715	/	-
S. constellatus	DSMZ 20575	/	-
S. cristatus	DSMZ 8249	/	-
S. dysgalactiae subsp. *equimilis*	DSMZ 6176	/	-
S. equi subsp. Equi	DSMZ 20561	/	-
S. equinis	DSMZ 20554	/	-
S. gordonii	DSMZ 6777	/	-
S. infantis	DSMZ 12492	/	-
S. intermedius	DSMZ 20573	/	-

S. mitis	DSMZ 12643	/	-
S. mutans	DSMZ 20523	/	-
S. oralis	DSMZ 20066	/	-
S. oralis	DSMZ 20395	/	-
S. parasanguinis	DSMZ 6778	/	-
S. perosis	DSMZ 12493	/	-
S. porcinus	DSMZ 20725	/	-
S. pseudopneumoniae	DSMZ 18670	/	-
S. pyogenes	DSMZ 20565	/	-
S. pyogenes	DSMZ 2072	/	-
S. salivarius	DSMZ 20560	/	-
S. salivarius	DSMZ 20617	/	-
S. sanguinis	DSMZ 20567	/	-
S. sinensis	DSMZ 14990	/	-
S. suis	DSMZ 9682	/	-
S. uberis	DSMZ 20569	/	-
S. vestibularis	DSMZ 5636	/	-

[a] NCTC = National Collection of Type Cultures; * DSMZ = The German Collection of Microorganisms; *CCUG = Culture Collection, University of Göteborg, Sweden; *CDC = Centre for Disease Control; *BCCM = Belgian Co-ordinated Collections of Microorganisms

[b] *ssrA* gene sequence data was generated for each of these *H. influenzae* strains using primers outlined in Table 2.1

[c] *lepA* gene sequence data was generated for each of these *H. influenzae* strains using primers outlined in Table 2.1

[d] *ssrA* gene sequence data was generated for each of these *N. meningitidis* strains using primers outlined in Table 2.1

[e] + ve= Positive; - ve= negative; / = not tested for

2.2.3. DNA isolation and quantification

Genomic DNA from a collection of *Haemophilus* and *Neisseria* species was isolated from 1.5 ml of culture using a DNeasy Blood and Tissue Kit as per manufacturers' instructions (Protocol: Gram-Negative Bacteria; Qiagen, Hilden, Germany). DNA integrity was assessed on a 1 % agarose gel and concentrations were determined using the Qubit dsDNA HS Assay and the Qubit 1.0 fluorometer (Life Technologies, Carlsbad, CA). Purified DNA samples were stored at -20°C prior to use.

2.2.4. Total RNA isolation and quantification

Total RNA from all species was isolated from 1.5 ml of culture using a RiboPure Yeast Kit as per manufacturers' instructions (Ambion, Austin, TX, USA). RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.). RNA with a RNA Integrity Number (RIN) above 7.0 was used for real-time NASBA. RNA concentrations were determined using the Qubit RNA BR Assay kit and the Qubit 1.0 fluorometer (Life Technologies, Carlsbad, CA). Purified RNA samples were stored at -80°C prior to use.

2.2.5. Conventional PCR primer design

Publically available nucleotide sequences of the diagnostics targets identified in this study were aligned using ClustalW multiple sequence alignment programme (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Oligonucleotide sequencing primers were manually designed in accordance with general recommendations and guidelines [36, 37] to target conserved regions within the genes. All primers (Table 2.1) used in this study were obtained from Eurofins MWG-Operon (Ebersberg, Germany).

2.2.6. Conventional PCR and Nucleic Acid Sequencing

Sequencing oligonucleotide primers were designed to amplify 365 bp of the *ssrA* gene of *H. influenzae*, and 342 bp of the *ssrA* gene of *N. meningitidis*, to identify optimal diagnostics target regions for NASBA primer and molecular beacon probe design. Due to the extent of publically available *S. pneumoniae lepA* sequence data, sequencing of in-house *S. pneumoniae* culture collection strains was not carried out.

PCR was carried out using the sequencing primers (Table 2.1) on an iCycler iQ thermal cycler (Bio-Rad Laboratories Inc., CA). All reactions were performed using the FastStart PCR Master Kit (Roche Diagnostics, Basel, Switzerland) according to manufacturer's instructions in a final volume of 25 µl. The thermal cycling parameters used for the *H. influenzae* specific primers consisted of a denaturation cycle at 95ºC for 4 min, followed by 35 cycles at 95ºC (30 s), 50ºC (30 s) and 72ºC (30 s), and a final elongation cycle at 72ºC for 7 min. Thermal cycling parameters used for the *Neisseria* specific primers were the same as outlined above with the exception of an annealing temperature of 55ºC instead of 50ºC. The PCR products

were purified using the HighPure PCR product purification kit (Roche Diagnostics) and sequenced externally (Sequiserve, Vaterstetten, Germany).

2.2.7. Real-time NASBA primer and molecular beacon probe design

Species specific oligonucleotide primers and molecular beacon probes were manually designed in accordance with recommended guidelines [26]. The molecular beacon probes were labelled with FAM and Dabcyl (DAB). Primers and molecular beacon probes (Table 2.1) were obtained from Eurofins MWG-Operon (Ebersberg, Germany).

2.2.8. Real-time NASBA

All real-time NASBA diagnostics assays were performed on a LightCycler 2.0 (Roche Diagnostics) using the NucliSENS EasyQ Basic Kit V2 (Biomerieux, Marcy l'Etoile, France), in accordance with manufacturer's instructions. Real-time NASBA reactions were performed in a total volume of 20 µl. 5 µl of target RNA was added to 10 µl reagent/KCL (70 mM final concentration)/primers and molecular beacon probes (0.2 µM and 0.1 µM concentration respectively) mixture. The reaction was incubated for 65^{o}C for 5 min to denature the RNA secondary structure followed by 41^{o}C for 5 min to allow primer and probe annealing. Subsequently, 5 µl of the enzyme mixture containing avian myeloblastosis virus reverse transcriptase, RNAse H and T7 RNA polymerase was added to the reaction. A no template control (NTC) consisting of water instead of RNA was included in each experiment. The reaction was then incubated at 41^{o}C for 60 min with a fluorescent measurement recorded every minute.

2.2.9. Real-time NASBA specificity

In order to evaluate the specificity of the three real-time NASBA diagnostics assays developed, total RNA from *H. influenzae* and closely related *Haemophilus* species, *N. meningitidis* and closely related *Neisseria* species, and *S. pneumoniae* and closely related *Streptococcus* species (Table 2.2) at various RNA concentrations, were tested in the *H. influenzae*, *N. meningitidis* and *S. pneumoniae* real-time NASBA diagnostics assays respectively.

2.2.10. Real-time NASBA with DMSO

In order to evaluate the effect of DMSO on the performance of the real-time NASBA assay targeting *lepA* mRNA for the species specific identification of *H. influenzae*, the most optimum DMSO concentration was determined by titration (0%, 2%, 4%, 6% and 8% DMSO/reaction). Subsequently the real-time NASBA diagnostic assay with optimized DMSO concentration was tested against a panel of *H. influenzae* culture collection strains. The real-time NASBA assay with added DMSO was performed in a total volume of 22 µl and set up as follows: 5 µl of target RNA was added to 10 µl reagent/KCL (70 mM final concentration)/primers and molecular beacon probes (0.2 µM and 0.1 µM concentration respectively) mixture. The reaction was incubated for 65ºC for 5 min followed by 41ºC for 5 min. Subsequently, 5 µl of the enzyme mixture and 2 µl DMSO (at a final concentration of 2%, 4%, 6% and 8%) was added to the reaction. A no template control (NTC) consisting of water instead of RNA was included in each experiment. The reaction was then incubated at 41ºC for 60 min with a fluorescent measurement recorded every minute.

2.3. Results and Discussion

2.3.1. tmRNA diagnsotic target

In-silico analysis established sufficient heterogeneity (at minimum of 1 base difference) between the *H. influenzae ssrA* gene sequence and the *ssrA* gene sequences of its most closely related species. Subsequently, two specific oligonucleotide primers and a molecular beacon were designed for the amplification of the *ssrA* tmRNA transcript in real-time NASBA (Table 2.1.).

The real-time NASBA assay targeting *H. influenzae* tmRNA was 100% inclusive when tested against 8 culture collection strains of *H. influenzae* and 38 clinical isolates of *H. influenzae* (Supplementary S2.1 and Supplementary S2.2). Similar to other published assays, this assay detects *Haemophilus aegyptius* (Figure 2.4), however designation of *H. aegyptius* as a separate species or a biogroup of *H. influenzae* remains uncertain [38-41]. To our knowledge, a nucleic acid based diagnostics method has not been developed that enables the discrimination of *H influenzae* from *H aegyptius*. The real-time NASBA assay was also tested against a

panel of non-*H. influenzae* closely related species (n=21) (Table 2.2). Assay cross reactions were observed with 2 closely related species tested (*H. haemolyticus*; NCTC10839 and an unknown *Haemophilus* sp., CCUG34110 - previously designated *H. haemolyticus*) (Figure 2.5A and 2.5B respectively).

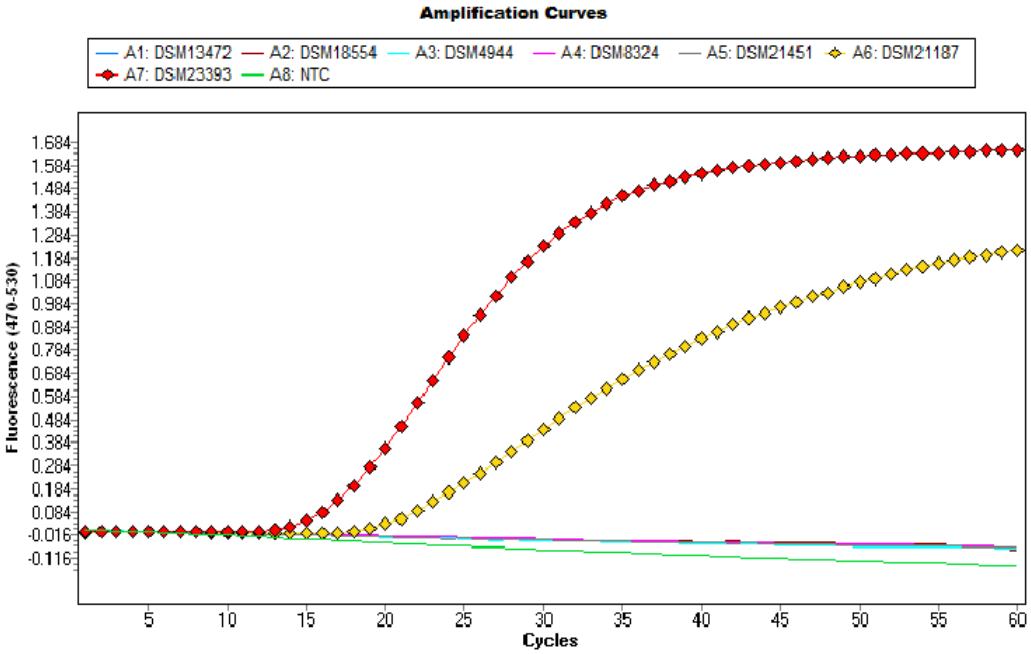
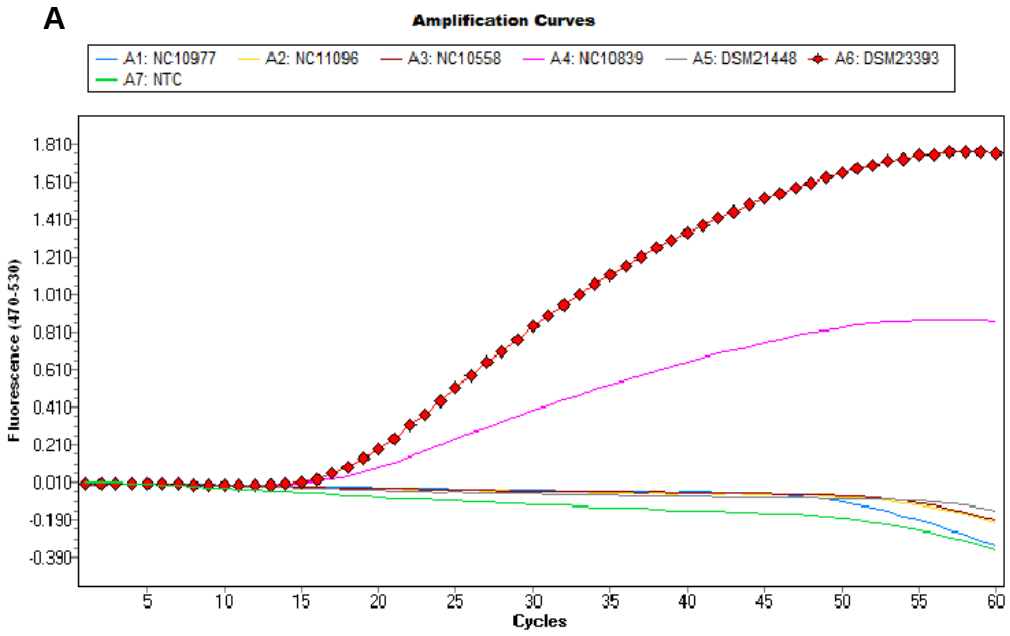


Figure 2.4: Amplification curves for real-time NASBA diagnostics assay. Real-time amplification curves for *H. influenzae* (DSM23393) and *H. influenzae* biogroup *aegyptius* (DSM 21187) targeting the tmRNA in the FAM channel (470 -530 nm). No other non-*H. influenzae* species were detected by the assay.



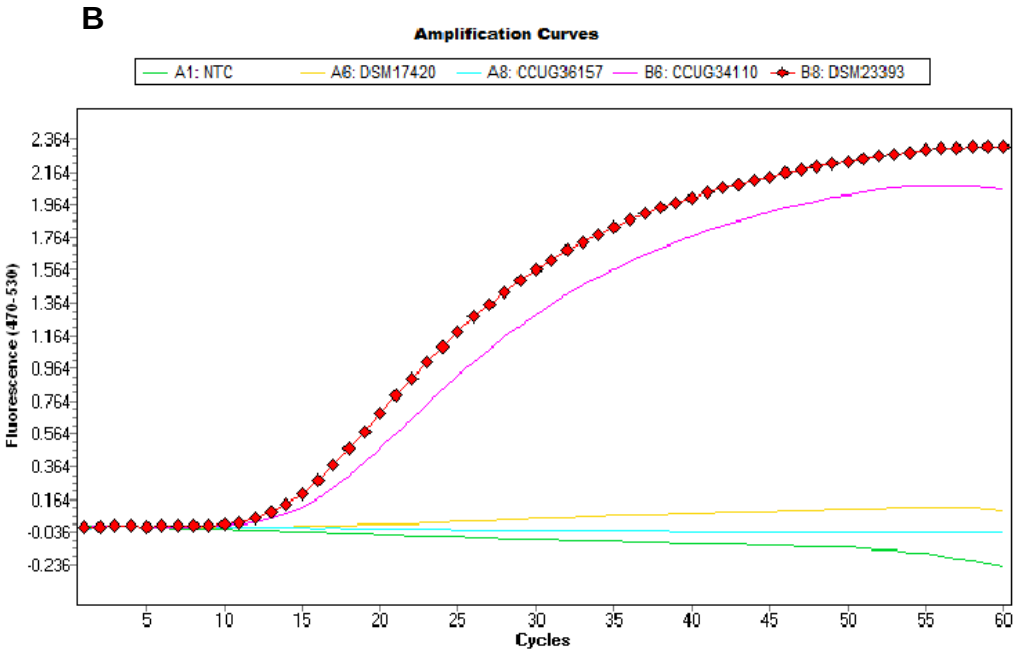


Figure 2.5: Amplification curves for real-time NASBA diagnostics assay. Real-time amplification curves for 1 strain of *H. influenzae* (DSM23393) targeting tmRNA, in the FAM channel (470-530nm). All *H. influenzae* samples detected. *H. haemolyticus* (NCTC 10839) and unknown *Haemophilus* sp. (CCUG34110) cross react (Fig. 2.5A and 2.5B respectively). No other non-*H. influenzae* species detected.

During the course of this study, whole genome sequence data became publically available for 6 *H. haemolyticus* strains (M19501, M19107, M21127, M21621, M21639, and HK386) allowing further *in-silico* analysis to be carried out. This established insufficient heterogeneity within the *ssrA* gene to discriminate *H. influenzae* from *H. haemolyticus* and 5/7 *H. haemolyticus* strains evaluated are identical to the real-time NASBA molecular beacon probe used in this study (Supplementary S2.3).

Initial *in-silico* analysis established sufficient heterogeneity within the *ssrA* gene sequence of *N. meningitidis* and the *ssrA* gene sequence of closely related species to accurately identify *N. meningitidis.* Consequently, a real-time NASBA diagnostic assay was developed targeting the tmRNA transcript encoded for by *ssrA*. The real-time NASBA diagnostic assay developed successfully identified *N. meningitidis* (n=9) and differentiated it from closely related species, *N. gonorrhoeae* and 18 other closely related *Neisseria* species (Figure 2.6, Supplementary S2.4 and S2.5). Further

evaluation of tmRNA as a diagnostic target for the species specific identification of *N. meningitidis* is discussed in Chapter 4.

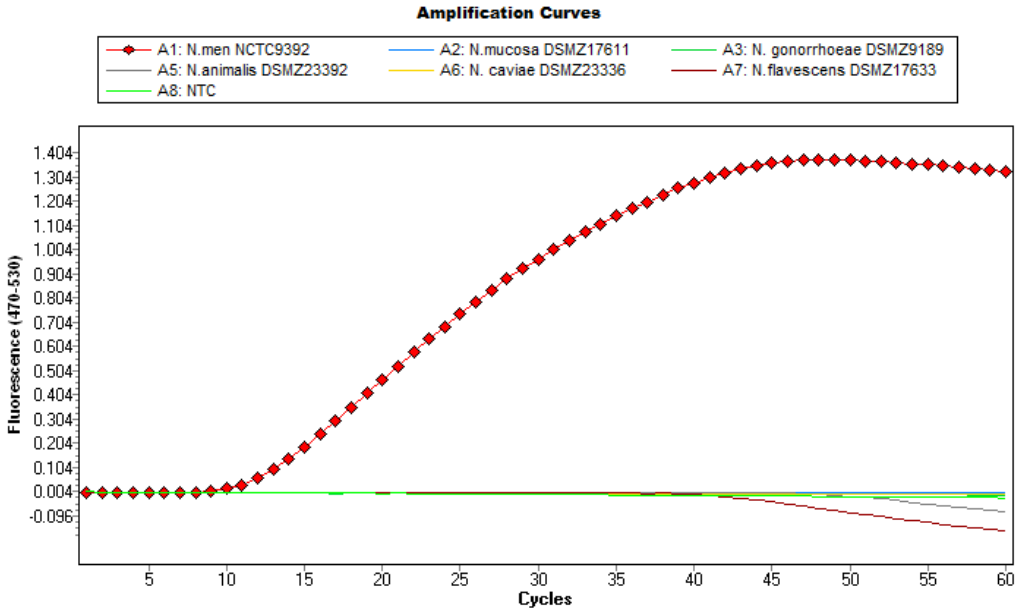


Figure 2.6: Representative Amplification curves for real-time NASBA diagnostics assay. Real-time amplification curves for *N. meningitidis* strains, targeting tmRNA, in the FAM channel (470-530nm). NTC highlighted with triangles. All *N. meningitidis* samples detected.

2.3.2. *lepA* mRNA diagnostic target

lepA mRNA was evaluated as a potential diagnostic target for use in real-time NASBA diagnostic assays for the species specific identification of *H. influenzae* and *S. pneumoniae*.

The *lepA* gene was sequenced for 7 *H. haemolyticus* strains and 1 unknown *Haemophilus* strain, previously designated *H. haemolyticus*, for comparison alongside the 6 publically available *H. haemolyticus lepA* gene sequences. *In-silico* analysis established significant *H. influenzae* inter-strain *lepA* sequence variation which limits real-time NASBA assay design. As a result, there is insufficient heterogeneity to accurately identify all *H. influenzae* and differentiate it from *H. haemolyticus*. Nevertheless, a real-time NASBA assay targeting *lepA* mRNA was designed for the *in-vitro* amplification of *H. influenzae*, for comparison against real-time NASBA *in-vitro* amplification of *H. influenzae* tmRNA (Table 2.1). The molecular beacon probe was chosen where maximum sequence variation exists

between *H. influenzae* and *H. haemolyticus*, without losing specificity for *H. influenzae*. As a result, 1/14 *H. haemolyticus* species analysed is identical to *H. influenzae* in the *lepA* molecular beacon probe region (Supplementary S2.6).

Initially inclusivity testing was carried out using the forward primer lepA P1.1 (Table 2.1) Similar to the real-time NASBA assay targeting tmRNA, specificity testing was carried out at a concentration of 5ng RNA per reaction, which equates to approximately 50,000 cell equivalents (CE) (based on a typical bacterial cell containing 0.1 pg RNA[42]) . At this concentration, only 2/7 *H. influenzae* strains (DSM23393, DSM10000) were detected with certainty (Ct value obtained) (Supplementary S2.7A). At a higher RNA concentration (250ng/reaction [2.5 x 10^6 CE]), all 7 strains were detected in the real-time NASBA assay targeting the *lepA* gene (Supplementary S2.7B). However, further inclusivity testing revealed that the assay does not detect 2 other in-house *H. influenzae* strains, DSM 11970 and the *H. influenzae* biogroup *aegyptius* strain, DSM21187 (Supplementary S2.7C).

Subsequently the real-time NASBA assay P1 forward primer was redesigned to try improve sensitivity and specificity (P1.2; Table 2.1). Inclusivity testing was repeated at the lowest neat stock RNA concentration of 64.5 ng/reaction (6.45 x 10^5 CE). Similar to the previous assay, this assay only detected 7/9 *H. influenzae* culture collection strains and did not detect DSM11970 or DSM21187 (Supplementary S2.7D).

The significant level of inter-strain *lepA* sequence variation lends itself to different NASBA RNA amplicon secondary structures for each *H. influenzae* strain (analysed using mfold RNA folding software; http://unafold.rna.albany.edu/?q=mfold/RNA-Folding-Form2.3). It was hypothesized that the secondary structure of the single stranded RNA amplicons may be impacting assay efficiency and that due to the assays isothermal quality, primers and molecular beacons may not be able to hybridize to the RNA amplicon. Consequently, dimethyl sulfoxide (DMSO) was added to the real-time NASBA reaction to attempt to improve amplification efficiency. DMSO is often added to PCR to increase specificity and efficiency of PCR amplification [43]. Furthermore, it has been shown that DMSO reduces RNA secondary structure, possibly improving reaction efficiency [44]. 4% DMSO concentration was determined empirically as the most optimum concentration to

potentially improve NASBA amplification performance (Supplementary S2.8). Subsequently, in-house *H. influenzae* strains were tested in a real-time NASBA assay with the addition of 4% DMSO (Figure 2.8). However, this did not improve the overall specificity of the assay and DSM 11970 and DSM 21187 are undetected.

The real-time NASBA assay (without the addition of DMSO) was then tested against a panel of most closely related non-*H. influenzae* species as a further comparison to the real-time NASBA assay targeting tmRNA and this revealed the assay cross reacts with *Histophilus somnus* (CCUG36157) and *H. haemolyticus* (NC10839) (Figure 2.9).

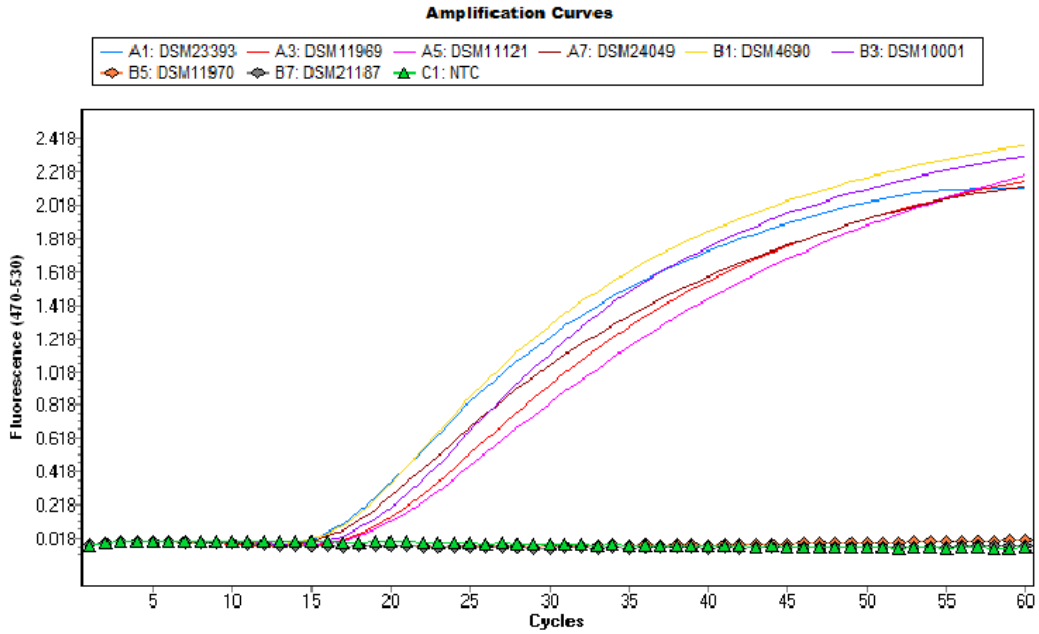


Figure 2.8: Amplification curves for real-time NASBA diagnostics assay. Effects of 4% DMSO on real-time amplification curves for *H. influenzae* culture collection strains, targeting *lepA* mRNA, in the FAM channel (470nm-530nm). 2/8 *H. influenzae* strains not detected (DSM 11970 and DSM 21187; highlighted with diamonds)

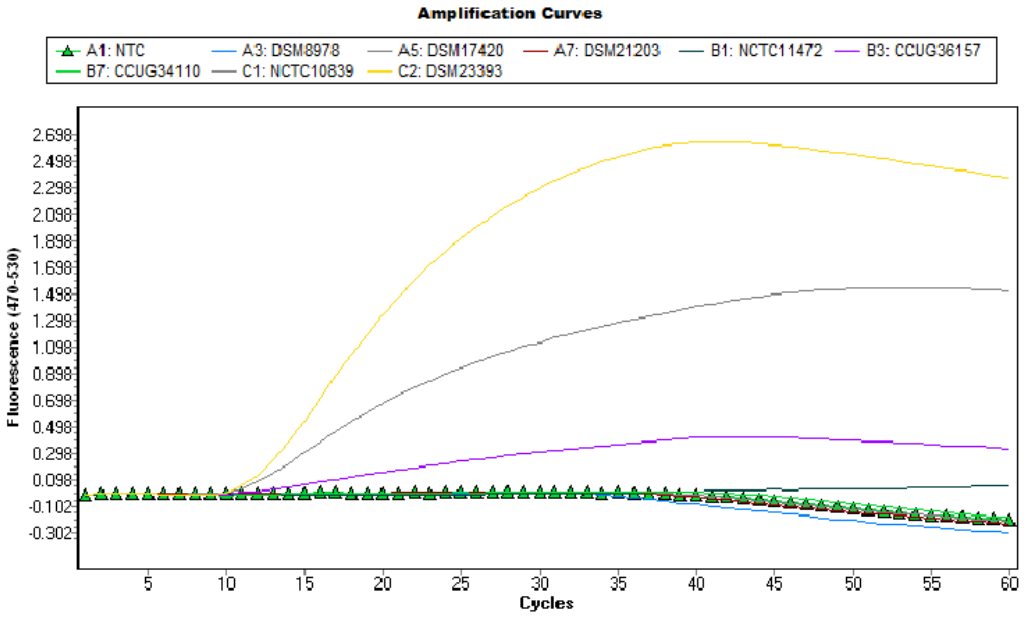


Fig 2.9: Amplification curves for real-time NASBA diagnostics assay. Real-time amplification curves for non- *H. influenzae* closely related species, targeting *H. influenzae lepA* mRNA, in the FAM channel (470nm-530nm). 2 closely related species *H. haemolyticus* (NCTC 10839) and *Histophilus somnus* (CCUG36257) cross react.

In-silico analysis established sufficient heterogeneity within the *lepA* gene sequence of *S. pneumoniae* and the *lepA* gene sequences of closely related species to accurately identify *S. pneumoniae*. Subsequently, a real-time NASBA diagnostic assay was developed targeting the *lepA* mRNA transcript of *S. pneumoniae*. The real-time NASBA diagnostic assay developed was tested against an inclusivity panel of *S. pneumoniae* strains (n=14) and exclusivity panel non-*S. pneumoniae* closely related species (n=37) (Table 2.2) and determined to be 100% specific for the identification of *S. pneumoniae* (Figure 2.10, Supplementary S2.9 and S2.10). Further evaluation of *lepA* mRNA as a diagnostic target for the species specific identification of *S. pneumoniae* is discussed in Chapter 4.

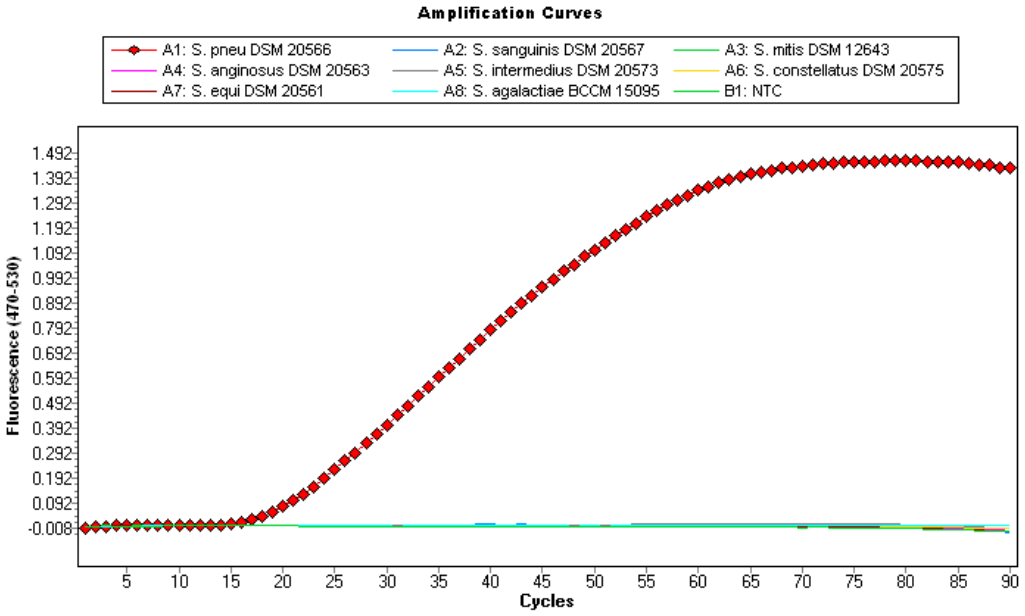


Fig 2.10: Amplification curves for real-time NASBA diagnostics assay. Real-time amplification curves for *S. pneumoniae* (DSMZ 20566) targeting *lepA* mRNA in the FAM channel (470 -530 nm). No non-*S. pneumoniae* species were detected by the assay.

2.4. Conclusion

In this study, both tmRNA and *lepA* mRNA have been established as potential diagnostic targets for the species specific identification of *N. meningitidis* and *S. pneumoniae* in a real-time NASBA assay, respectively. Both real-time NASBA diagnostic assays developed have been determined to be 100% specific for their target species, however, further validation of these real-time NASBA assays is required to conclusively establish the use of these RNA transcripts for the accurate and sensitive identification of *N. meningitidis* and *S. pneumoniae*.

In-silico analysis and testing in real-time NASBA assays established that neither the tmRNA transcript nor the *lepA* mRNA transcript can be used as diagnostic targets to accurately identify *H. influenzae* and differentiate it from its most closely related species. In summary, the real-time NASBA assay targeting *lepA* mRNA is not 100% inclusive and amplification efficiency is poor especially at lower RNA concentrations. This may be due to the significant *H. influenzae* inter-strain variation impacting the NASBA RNA amplicon secondary structure and consequently the ability of the primers and molecular beacons to hybridize. However, the addition of

DMSO does not improve assay inclusivity and further investigation is required to validate this theory. Furthermore, the assay is not 100% exclusive and cross reacts with two closely related non-*H. influenzae* species, *H. haemolyticus* and *H. somnus*. The real-time NASBA assay targeting tmRNA, is 100% inclusive however similar to other published molecular targets, it is unable to differentiate *H. influenzae* from *H. haemolyticus*. As a result, further study is required to establish a target which can unambiguously identify *H. influenzae*. However, based on the comparison of real-time NASBA assays developed in this study, the *ssrA* tmRNA transcript is the more suitable diagnostic target for the detection of *H. influenzae*.

This study highlighted the need to establish a diagnostic target which can unambiguously identify *H. influenzae*. Chapter 3 describes how whole genome comparative analysis using online bioinformatics tools was carried out to identify novel diagnostic targets unique to *H. influenzae*, which were subsequently experimentally validated using real-time PCR. Following on from this, in Chapter 4, a novel diagnostic target identified and experimentally validated in real-time PCR format was transferred to a real-time NASBA assay platform. In addition, real-time NASBA assays developed in this study for the species specific identification of *N. meningitidis* and *S. pneumoniae*, targeting tmRNA and *lepA* mRNA were further validated. In summary, each of the three monoplex NASBA assays for the species specific identification of *H. influenzae*, *N. meningitidis* and *S. pneumoniae* were combined in a duplex format to include a non-competitive internal amplification control and limit of detection (LOD) and specificity established for each of these assays.

2.5. References

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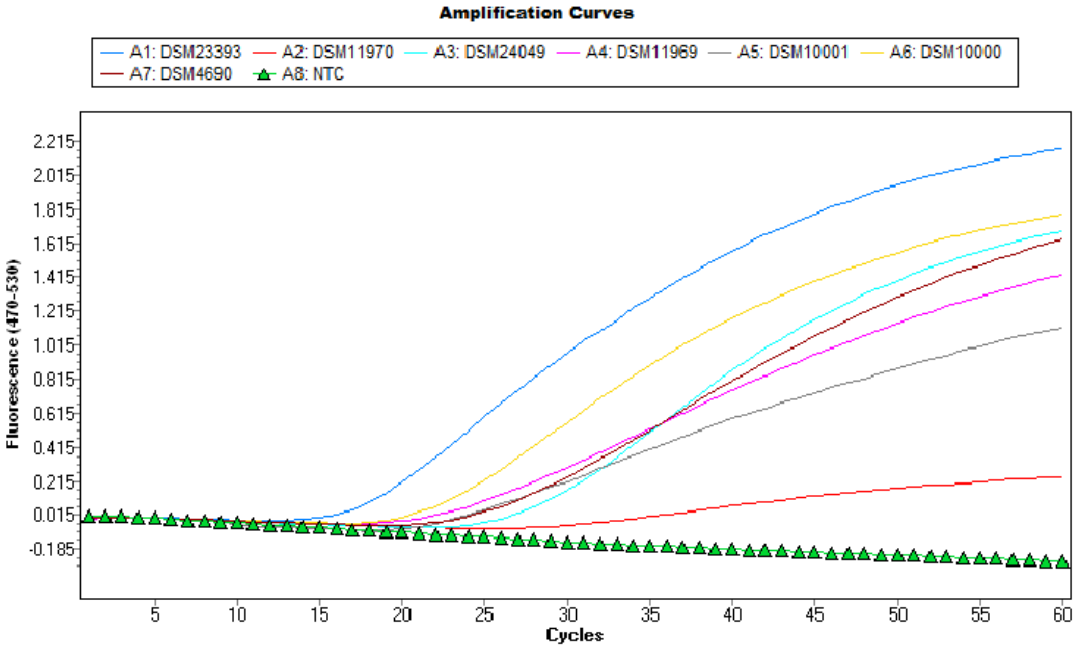
2.6. Supplementary Figures

A



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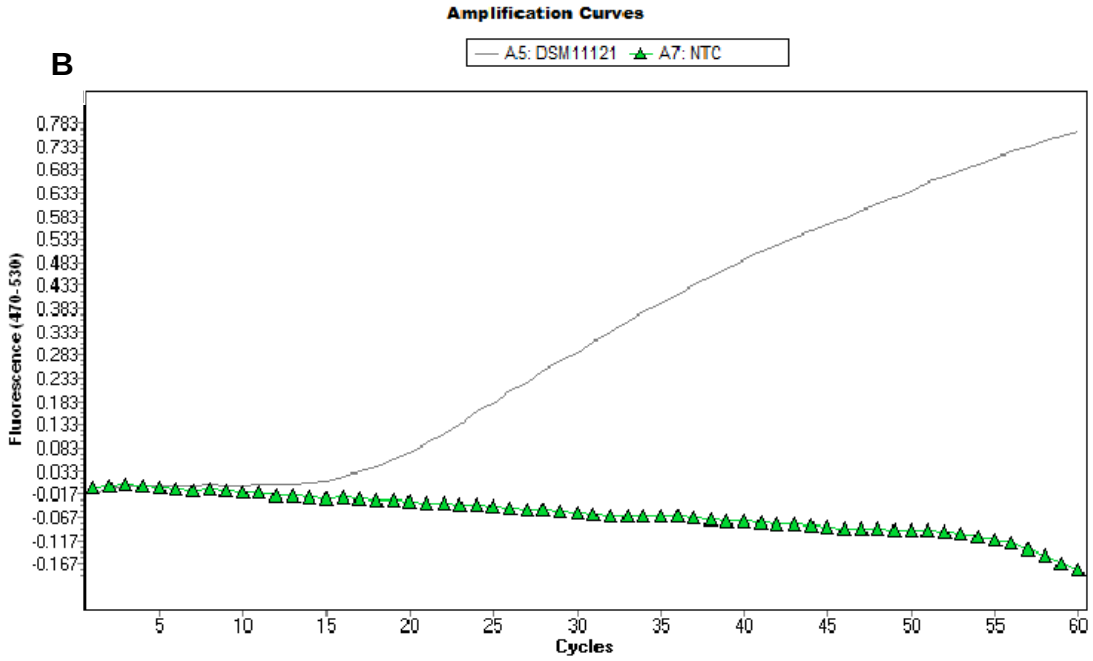

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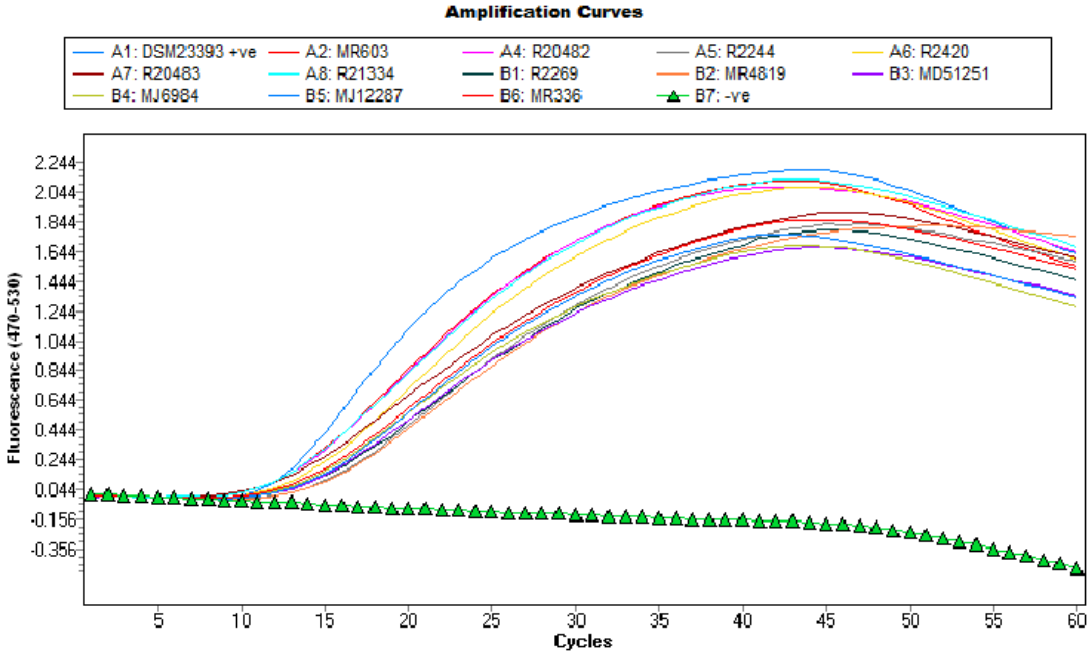
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A2: MR603
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A6: R2420
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B1: R2269
B2: MR4819
B3: MD51251
B4: MJ6984
B5: MJ12287
B6: MR336
B7: -ve
Fluorescence (470-530)
2.244
2.044
1.844
1.644
1.444
1.244
1.044
0.844
0.644
0.444
0.244
0.044
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25
30
35
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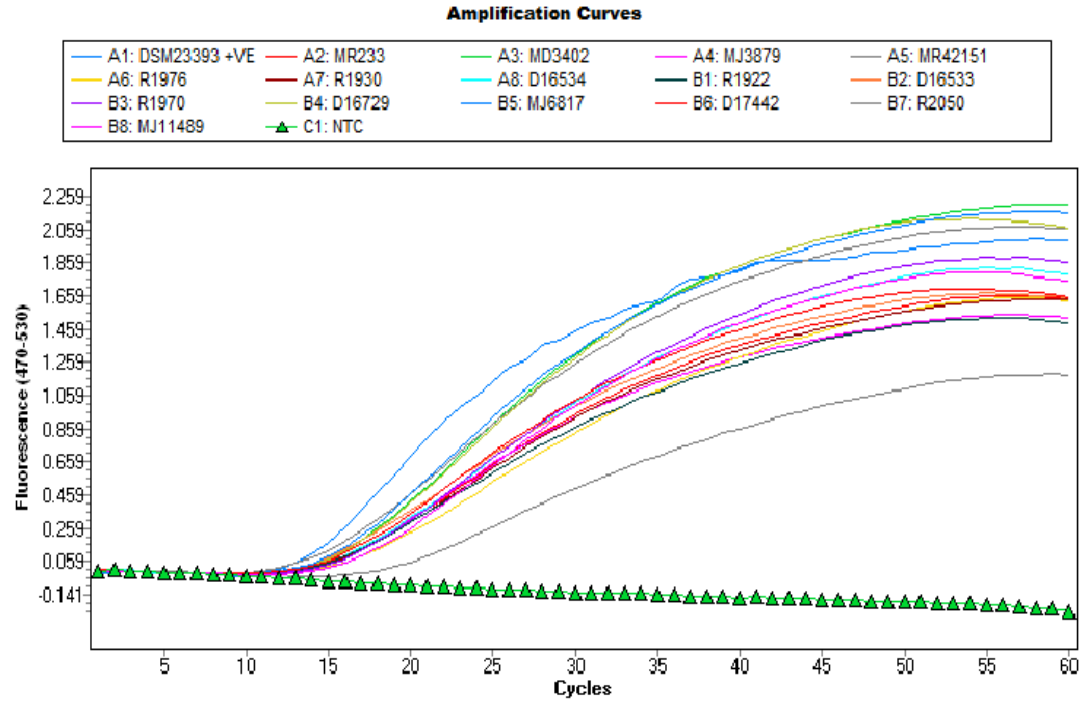
55
60
Cycles

D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A2: MR233
A3: MD3402
A4: MJ3879
A5: MR42151
A6: R1976
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A8: D16534
B1: R1922
B2: D16533
B3: R1970
B4: D16729
B5: MJ6817
B6: D17442
B7: R2050
B8: MJ11489
C1: NTC
Fluorescence (470-530)
2.259
2.059
1.859
1.659
1.459
1.259
1.059
0.859
0.659
0.459
0.259
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Cycles

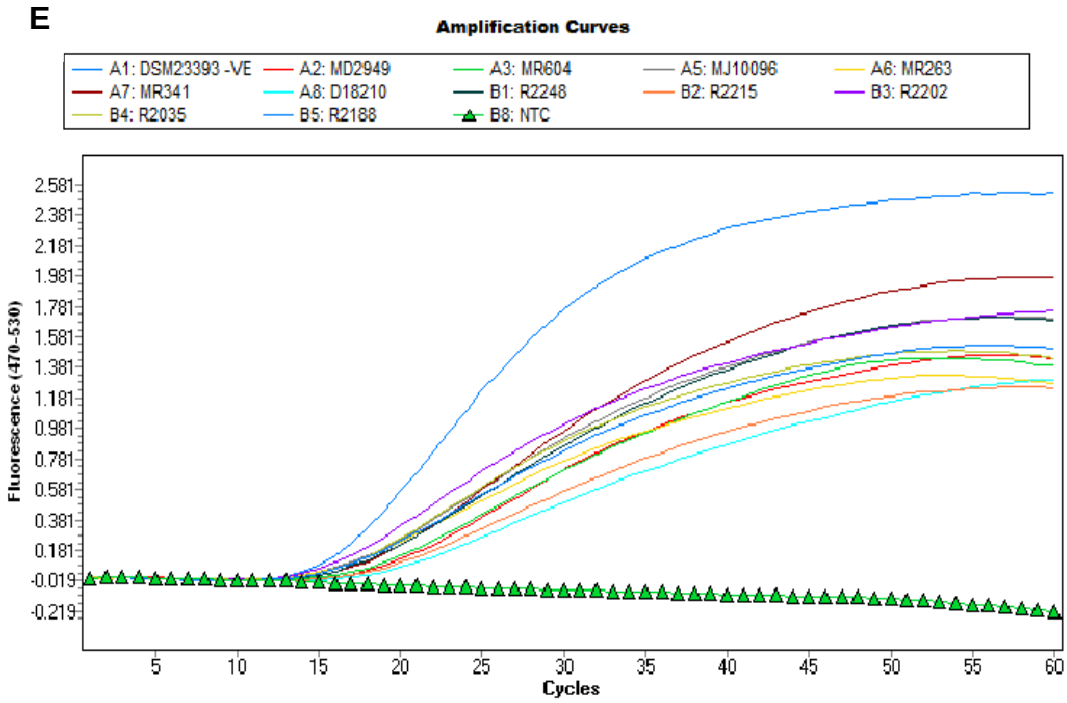


Figure S2.1: Amplification curves for real-time NASBA diagnostics assay. Figure S2.1A and S2.1B: Real-time amplification curves for *H. influenzae* culture collection strains, targeting tmRNA, in the FAM channel (470-530nm). NTC highlighted with triangles. All *H. influenzae* samples detected. Figure S2.1C -E: Real-time amplification curves for *H. influenzae* clinical isolate strains, targeting tmRNA, in the FAM channel (470-530nm). NTC highlighted with triangles. All *H. influenzae* samples detected.

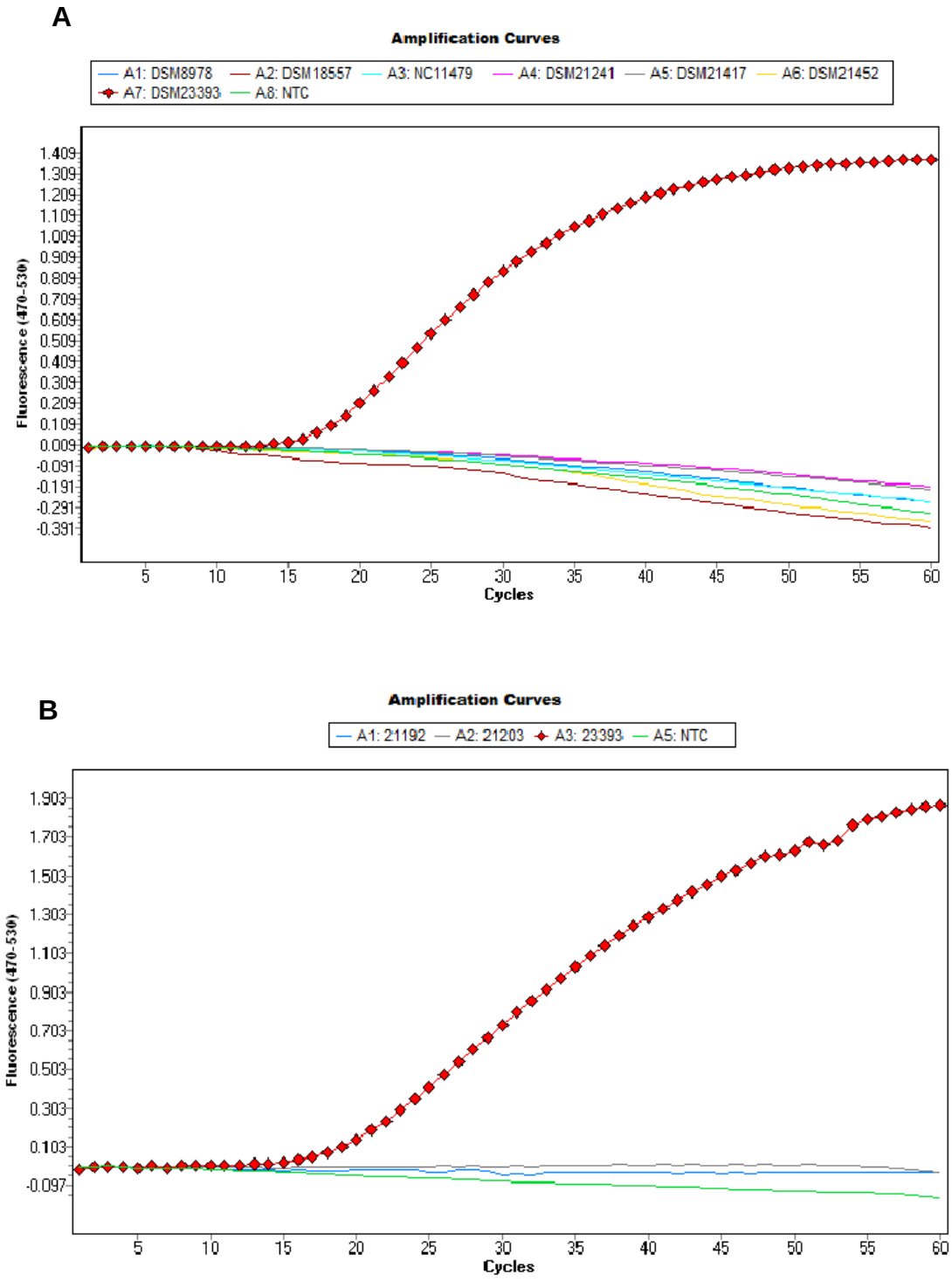


Figure S2.2: Amplification curves for real-time NASBA diagnostics assay. Fig. S2.2A and Fig. S2.2B, Real-time amplification curves for 1 strain of *H. influenzae* (DSM23393) targeting tmRNA, in the FAM channel (470-530nm). All *H. influenzae* samples detected. No non-*H. influenzae* species detected.

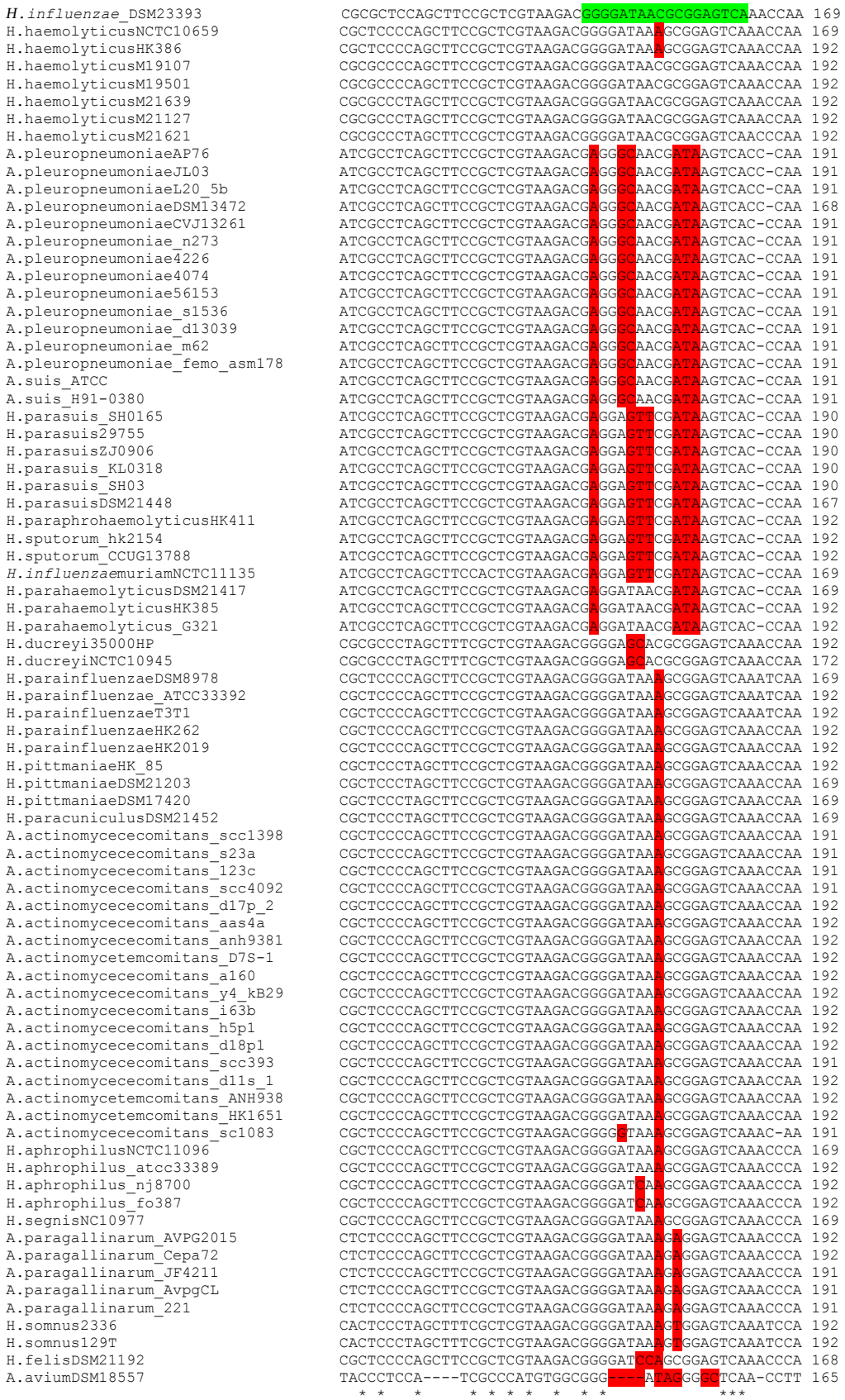
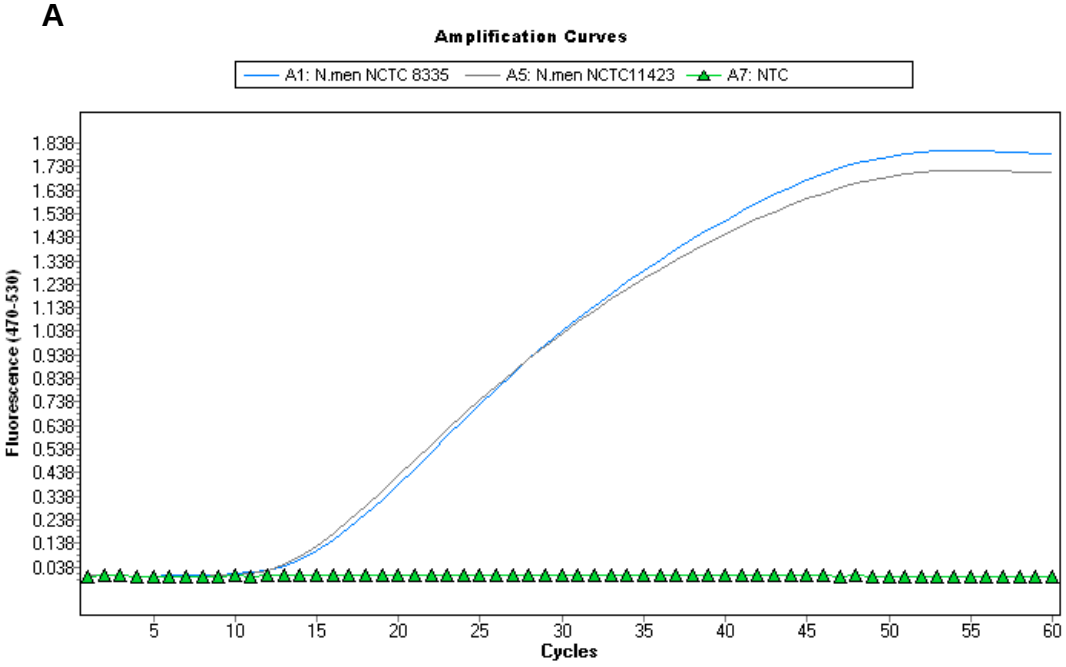
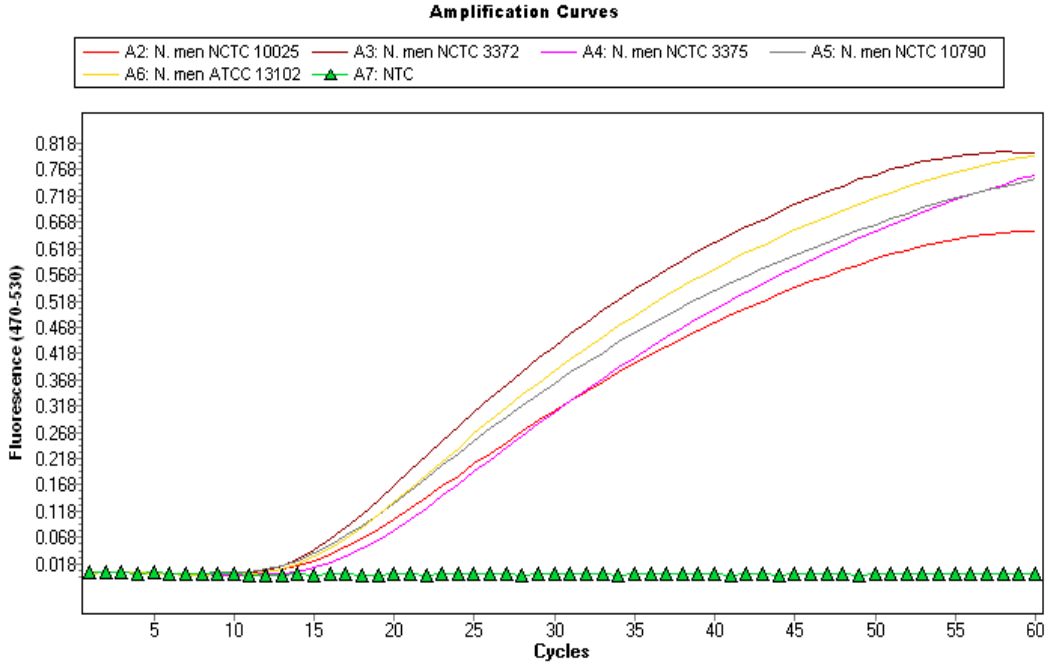


Figure S2.3. ClustalW multiple sequence alignment of the *ssrA* gene of the *Haemophilus* genus highlighting the *H. influenzae* specific real-time NASBA molecular beacon probe (green) and respective base pair mismatches in closely related *Haemophilus* species (red).

A


Amplification Curves
A1: N.men NCTC 8335
A5: N.men NCTC11423
A7: NTC
Fluorescence (470-530)
1.838
1.738
1.638
1.538
1.438
1.338
1.238
1.138
1.038
0.938
0.838
0.738
0.638
0.538
0.438
0.338
0.238
0.138
0.038
5
10
15
20
25
30
35
40
45
50
55
60
Cycles

B


Amplification Curves
A2: N. men NCTC 10025
A3: N. men NCTC 3372
A4: N. men NCTC 3375
A5: N. men NCTC 10790
A6: N. men ATCC 13102
A7: NTC
Fluorescence (470-530)
0.818
0.768
0.718
0.668
0.618
0.568
0.518
0.468
0.418
0.368
0.318
0.268
0.218
0.168
0.118
0.068
0.018
5
10
15
20
25
30
35
40
45
50
55
60
Cycles

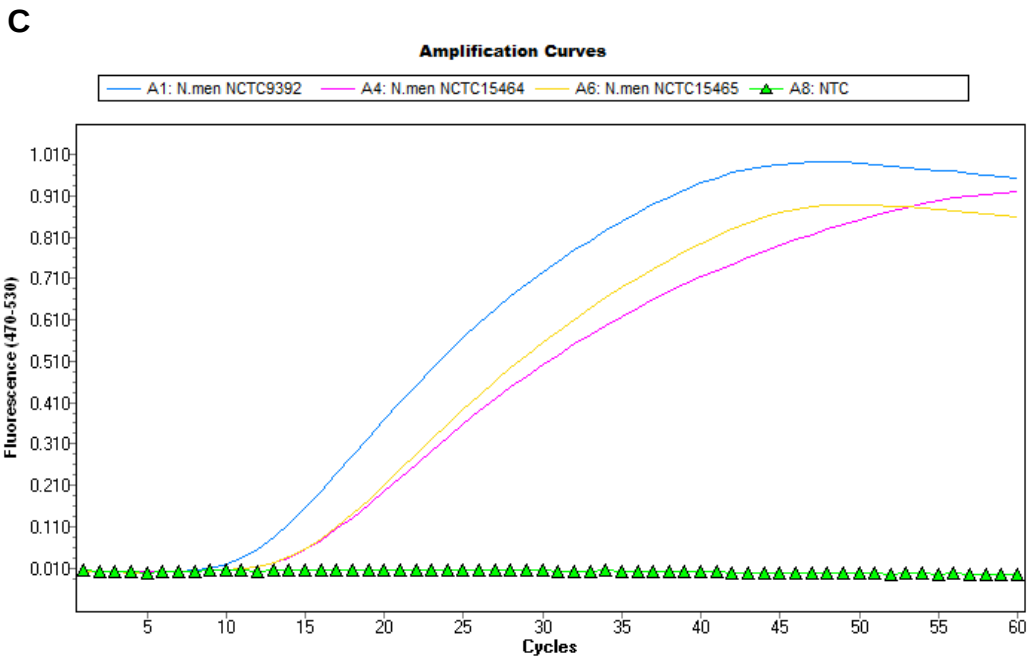
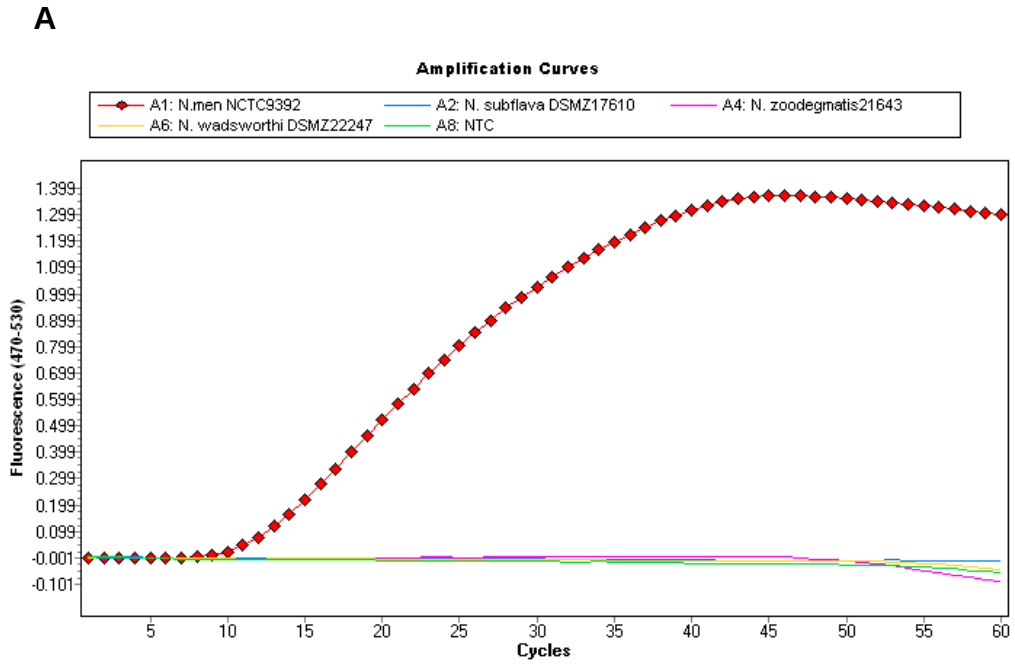


Figure S2.4: Amplification curves for real-time NASBA diagnostics assay. Real-time amplification curves for *N. meningitidis* strains, targeting tmRNA, in the FAM channel (470-530nm). NTC highlighted with triangles. All *N. meningitidis* samples detected.



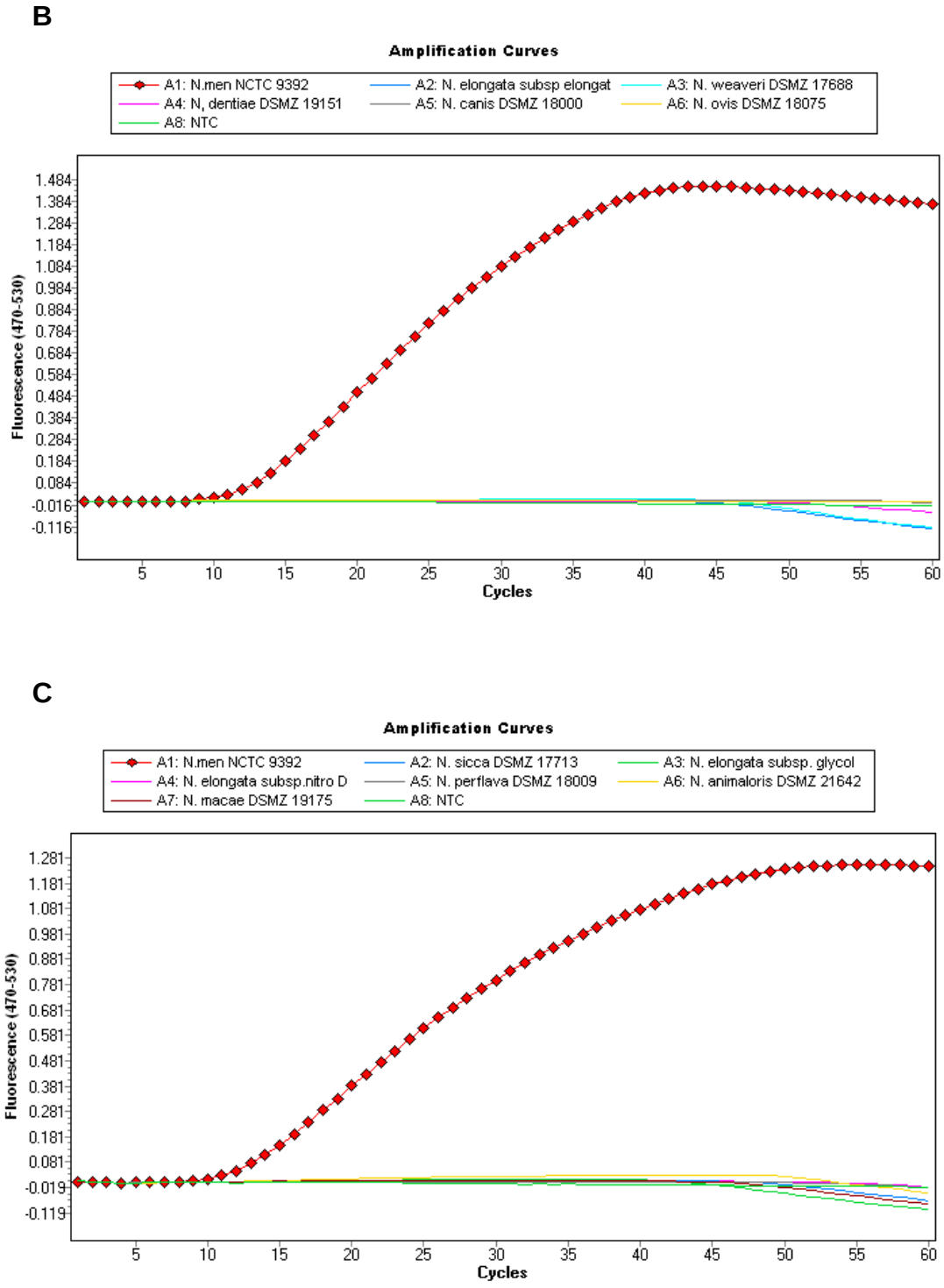


Figure S2.5: Amplification curves for real-time NASBA diagnostics assay. Real-time amplification curves for *N. meningitidis* (NCTC 9392) targeting tmRNA in the FAM channel (470 -530 nm). No non-*N. meningitidis* species were detected by the assay.

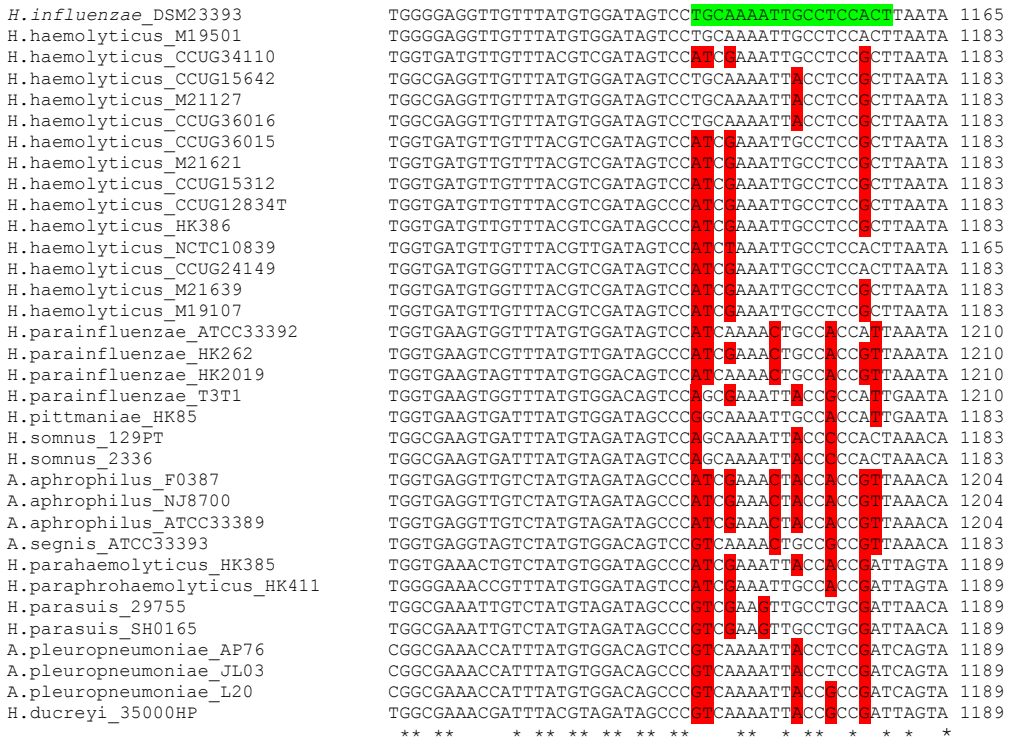
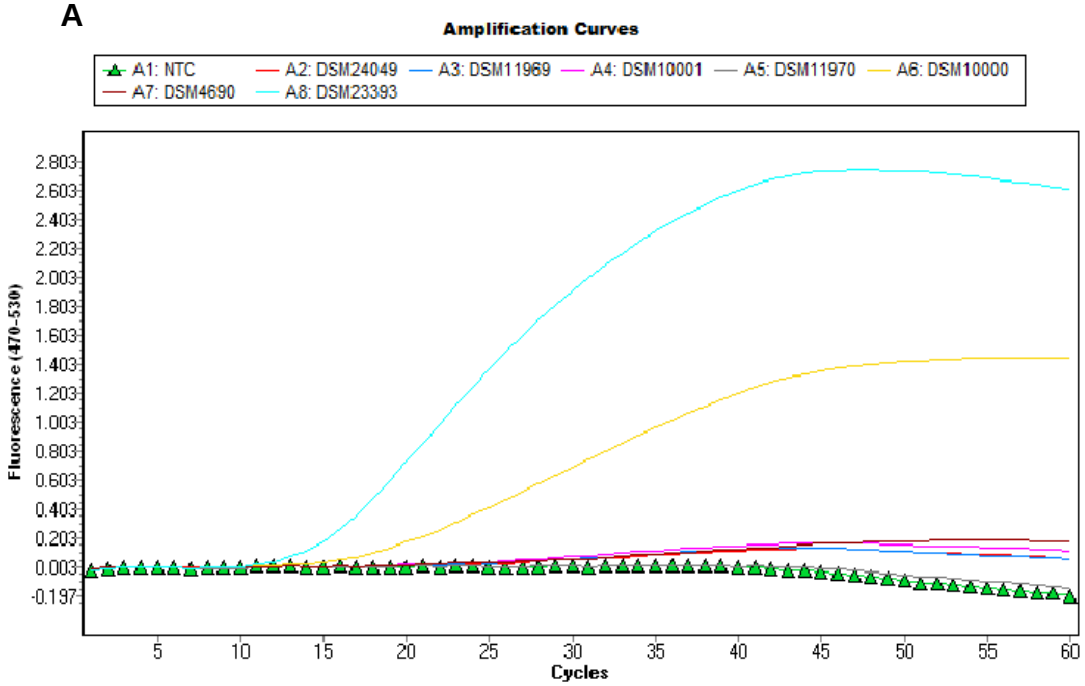
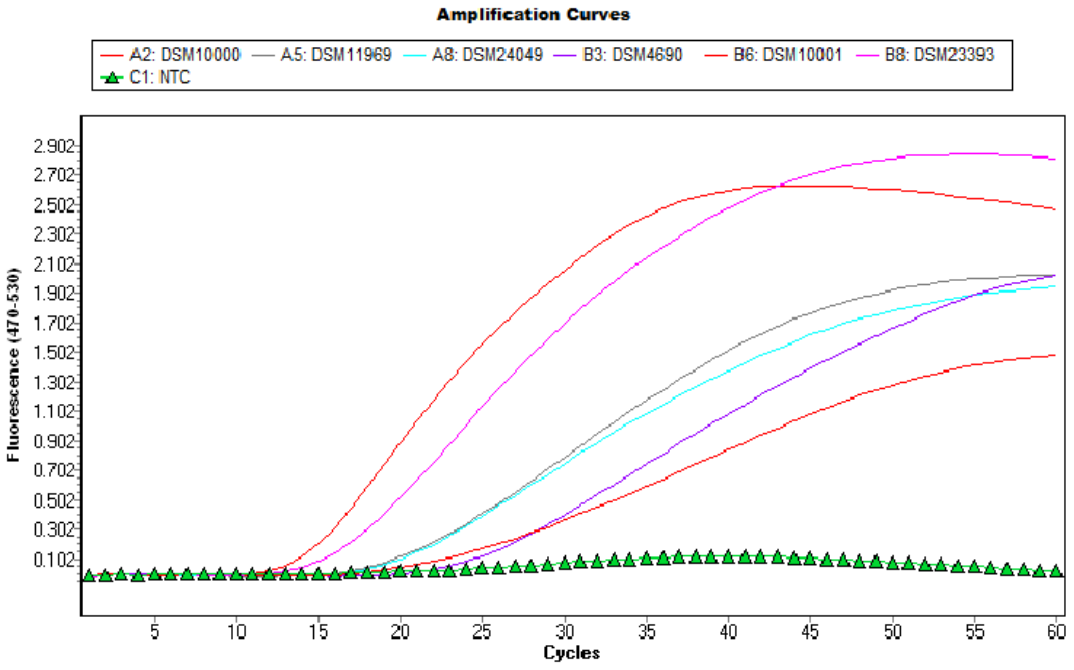


Figure S2.6. ClustalW multiple sequence alignment of the *lepA* gene of the *Haemophilus* genus highlighting the *H. influenzae* specific real-time NASBA molecular beacon probe (green) and respective base pair mismatches in closely related *Haemophilus* species (red).

A


Amplification Curves
A1: NTC
A2: DSM24049
A3: DSM11969
A4: DSM10001
A5: DSM11970
A6: DSM10000
A7: DSM4690
A8: DSM23393
Fluorescence (470-530)
2.803
2.603
2.403
2.203
2.003
1.803
1.603
1.403
1.203
1.003
0.803
0.603
0.403
0.203
0.003
-0.197
5
10
15
20
25
30
35
40
45
50
55
60
Cycles

B


Amplification Curves
A2: DSM10000
A5: DSM11969
A8: DSM24049
B3: DSM4690
B6: DSM10001
B8: DSM23393
C1: NTC
Fluorescence (470-530)
2.902
2.702
2.502
2.302
2.102
1.902
1.702
1.502
1.302
1.102
0.902
0.702
0.502
0.302
0.102
5
10
15
20
25
30
35
40
45
50
55
60
Cycles

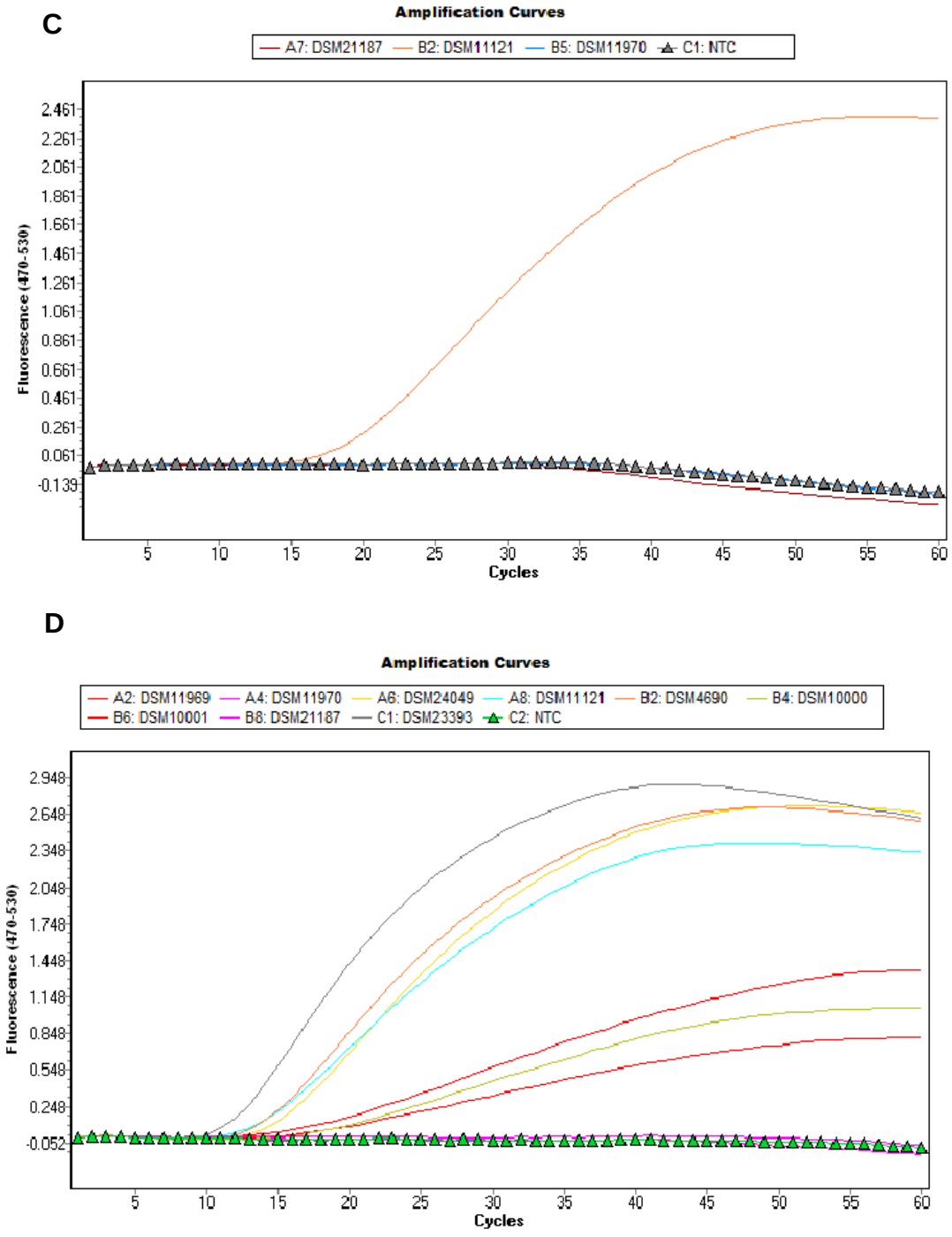


Figure S2.7: Amplification curves for real-time NASBA diagnostics assay. Real-time amplification curves for *H. influenzae* culture collection strains, targeting *lepA* mRNA, in the FAM channel (470-530nm). NTC highlighted with triangles. Fig. S2.6A. 2/7 *H. influenzae* strains detected (DSM23393 and DSM1000). Fig S2.6B- D, 7/9 *H. influenzae* detected. DSM11970 and DSM21187 not detected.

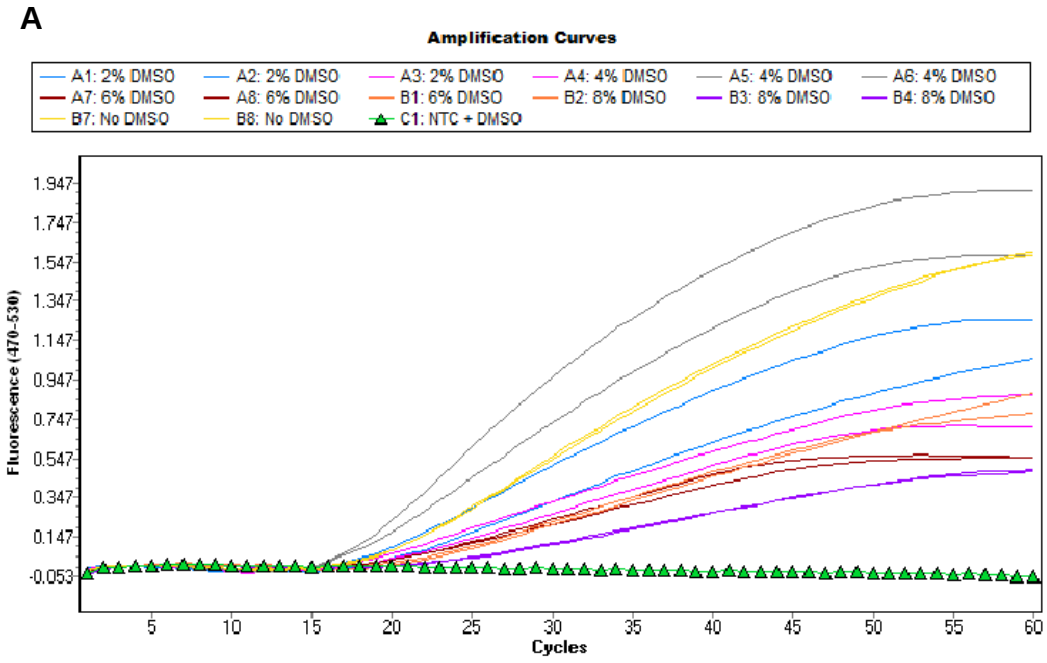
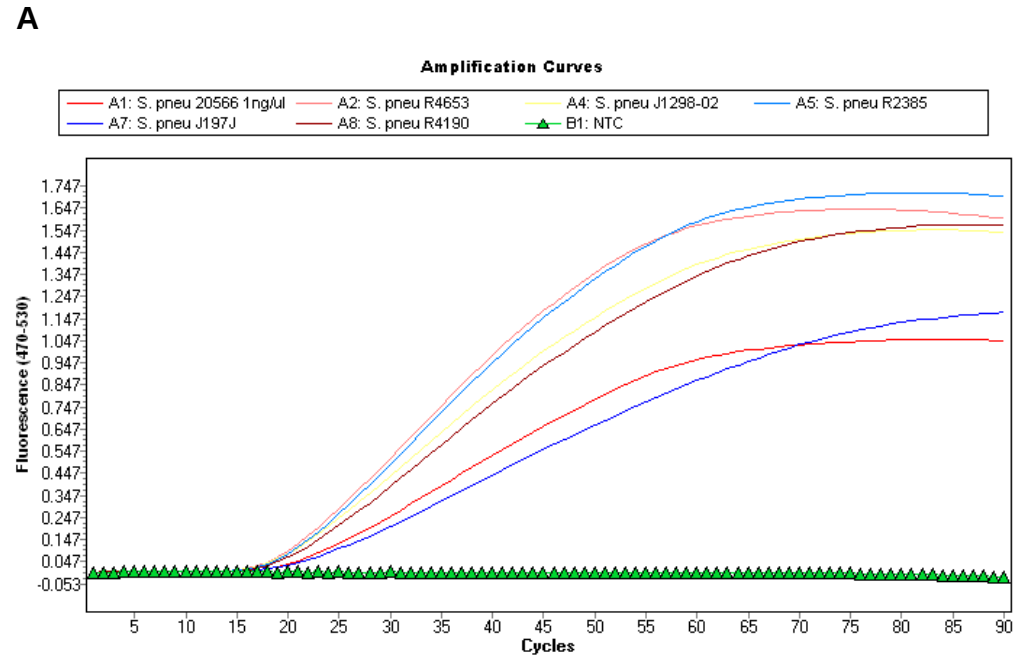


Figure S2.8: Amplification curves for real-time NASBA diagnostics assay. Effects of varying concentrations of DMSO (0-8%) on real-time amplification curves for *H. influenzae* (DSM23393), targeting *lepA* mRNA, in the FAM channel (470nm-530nm).



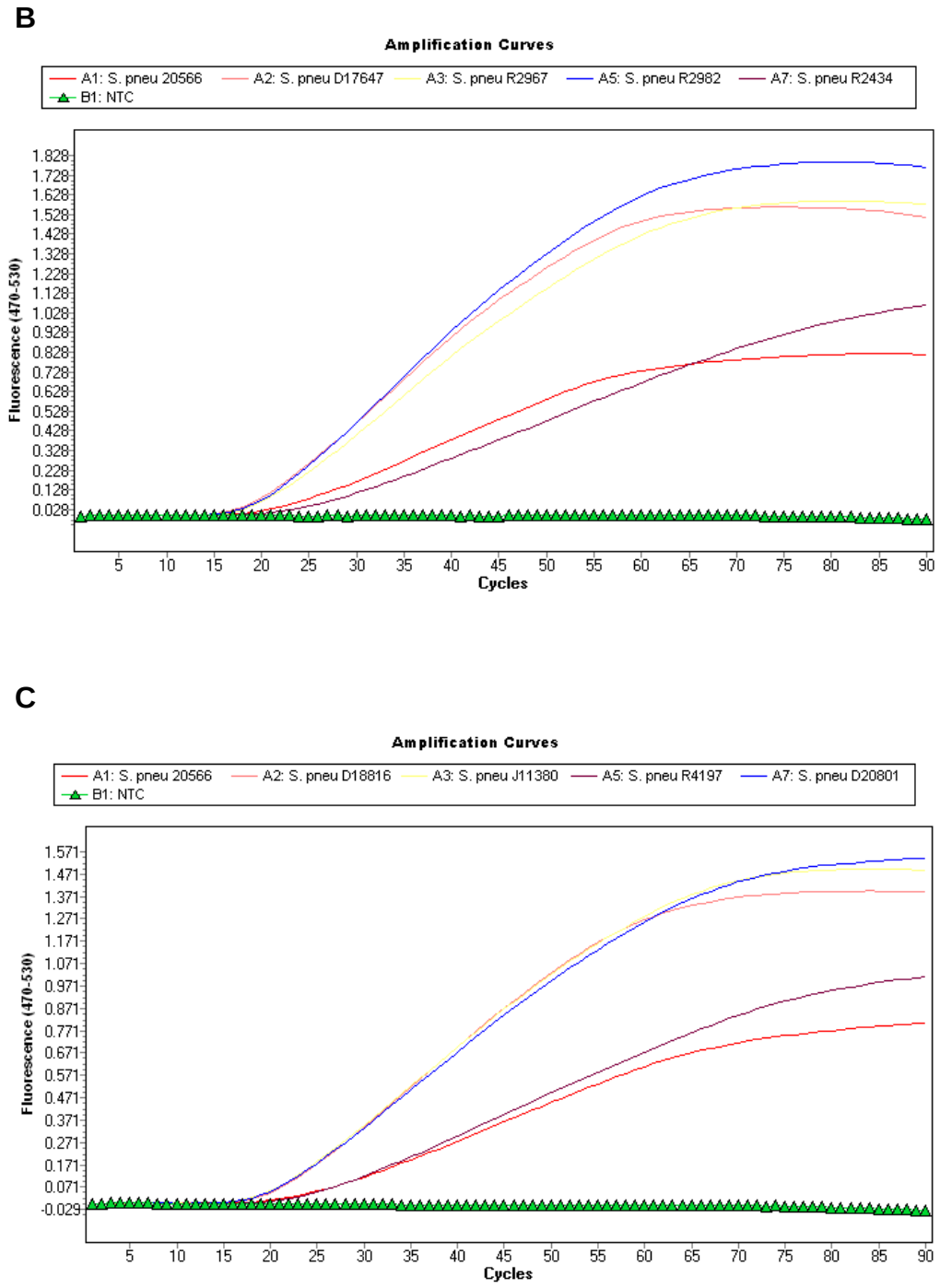
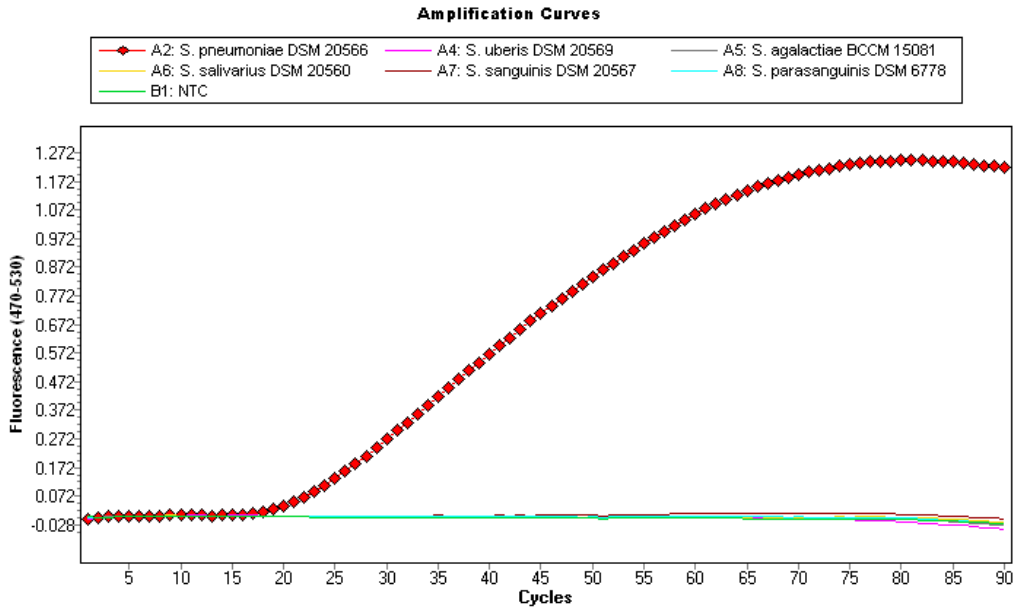
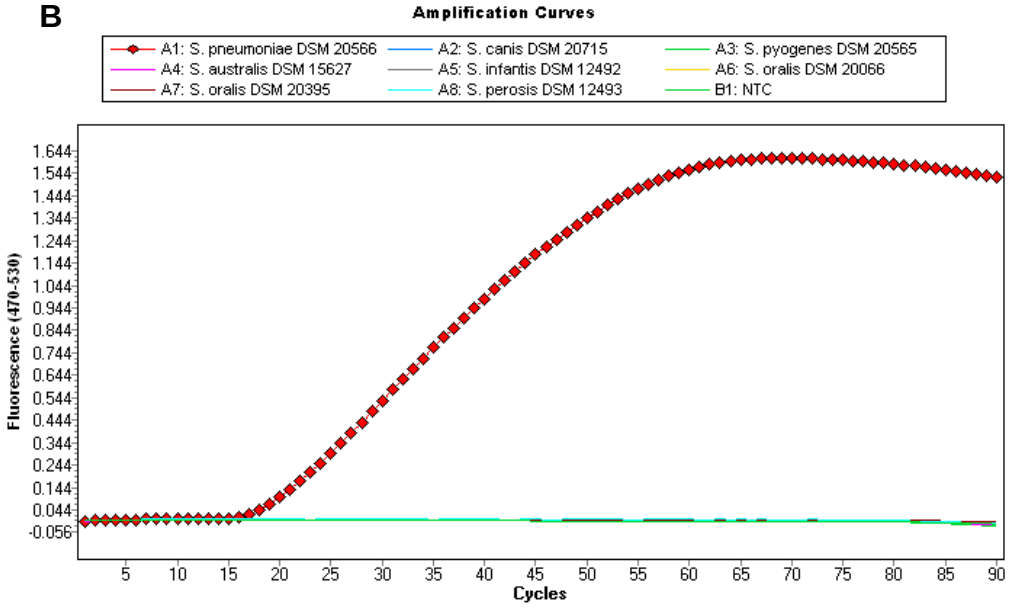


Fig S2.9: Amplification curves for real-time NASBA diagnostics assay. Real-time amplification curves for *S. pneumoniae* strains, targeting *lepA* mRNA, in the FAM channel (470-530nm). NTC highlighted with triangles. All *S. pneumoniae* samples detected.

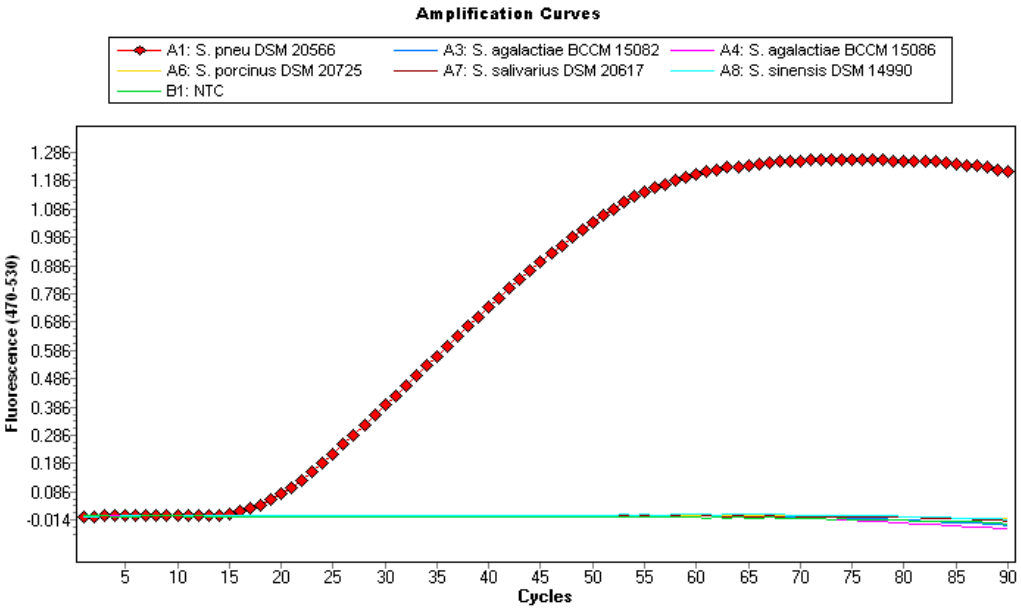
A


Amplification Curves
A2: S. pneumoniae DSM 20566
A4: S. uberis DSM 20569
A5: S. agalactiae BCCM 15081
A6: S. salivarius DSM 20560
A7: S. sanguinis DSM 20567
A8: S. parasanguinis DSM 6778
B1: NTC
Fluorescence (470-530)
1.272
1.172
1.072
0.972
0.872
0.772
0.672
0.572
0.472
0.372
0.272
0.172
0.072
-0.028
5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90
Cycles

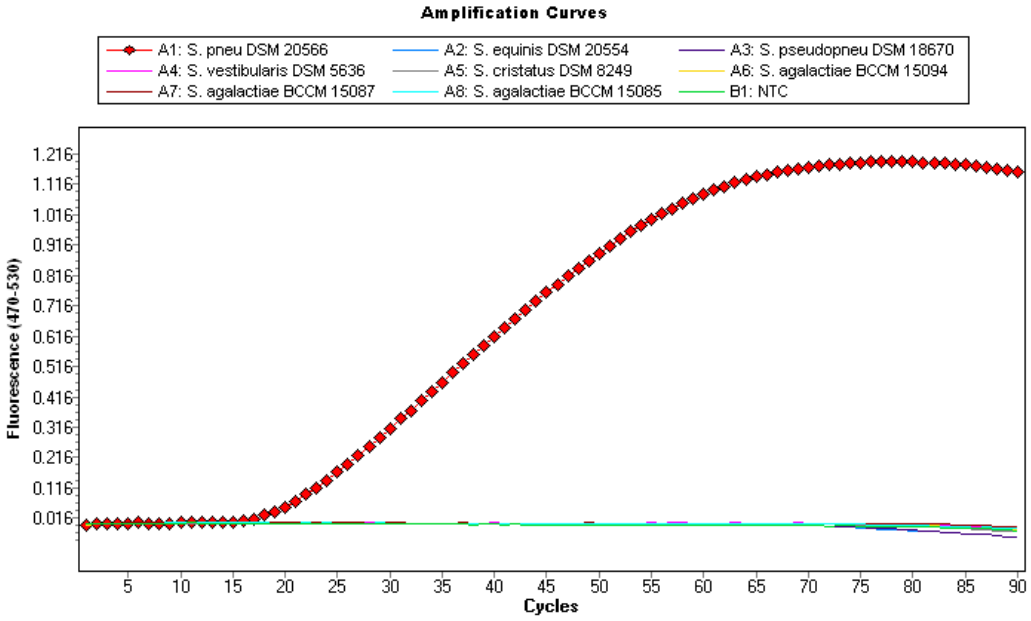
B


Amplification Curves
A1: S. pneumoniae DSM 20566
A2: S. canis DSM 20715
A3: S. pyogenes DSM 20565
A4: S. australis DSM 15627
A5: S. infantis DSM 12492
A6: S. oralis DSM 20066
A7: S. oralis DSM 20395
A8: S. perosis DSM 12493
B1: NTC
Fluorescence (470-530)
1.644
1.544
1.444
1.344
1.244
1.144
1.044
0.944
0.844
0.744
0.644
0.544
0.444
0.344
0.244
0.144
0.044
-0.056
5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90
Cycles

C


Amplification Curves
A1: S. pneu DSM 20566
A3: S. agalactiae BCCM 15082
A4: S. agalactiae BCCM 15086
A6: S. porcinus DSM 20725
A7: S. salivarius DSM 20617
A8: S. sinensis DSM 14990
B1: NTC
Fluorescence (470-530)
1.286
1.186
1.086
0.986
0.886
0.786
0.686
0.586
0.486
0.386
0.286
0.186
0.086
-0.014
5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90
Cycles

D


Amplification Curves
A1: S. pneu DSM 20566
A2: S. equinis DSM 20554
A3: S. pseudopneu DSM 18670
A4: S. vestibularis DSM 5636
A5: S. cristatus DSM 8249
A6: S. agalactiae BCCM 15094
A7: S. agalactiae BCCM 15087
A8: S. agalactiae BCCM 15085
B1: NTC
Fluorescence (470-530)
1.216
1.116
1.016
0.916
0.816
0.716
0.616
0.516
0.416
0.316
0.216
0.116
0.016
5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90
Cycles

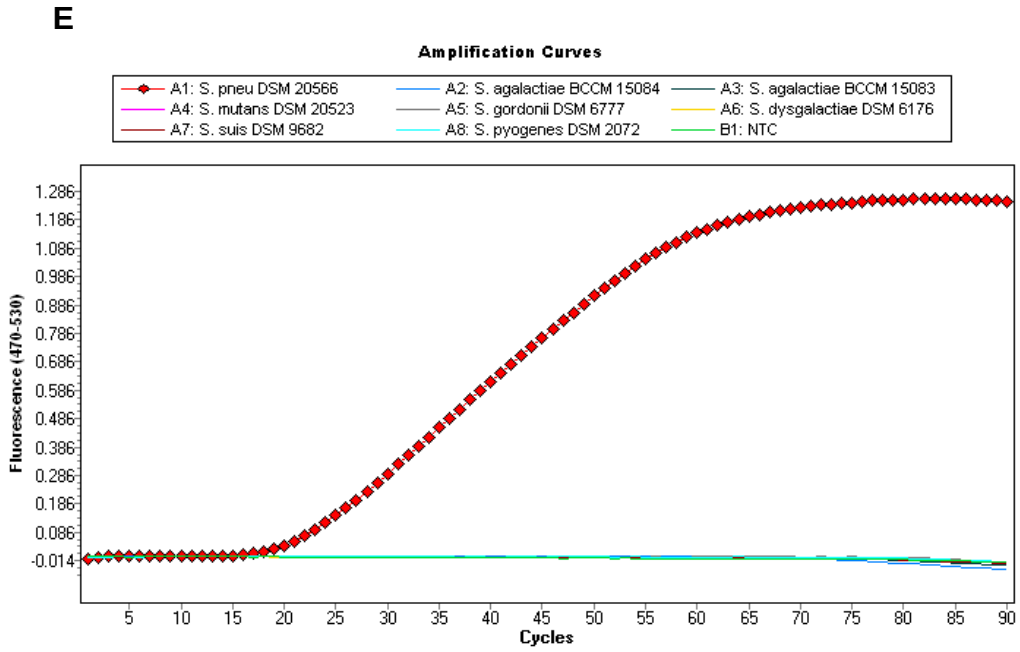


Fig S2.10: Amplification curves for real-time NASBA diagnostics assay. Real-time amplification curves for *S. pneumoniae* (DSMZ 20566) targeting *lepA* mRNA in the FAM channel (470 -530 nm). No non-*S. pneumoniae* species were detected by the assay.

Chapter 3:

Comparative genome analysis identifies novel nucleic acid diagnostics targets for use in the specific detection of *Haemophilus influenzae*

Published by Diagnostic Microbiology and Infectious disease

Coughlan H, Reddington K, Tuite N, Boo TW, Cormican M, Barrett L, Smith TJ, Clancy E, Barry T: Comparative genome analysis identifies novel nucleic acid diagnostic targets for use in the specific detection of Haemophilus influenzae. *Diagnostic Microbiology and Infectious Disease* 2015, 83(2):112-116. (Appendix D)

Abstract

Haemophilus influenzae is recognized as an important human pathogen associated with invasive infections, including blood stream infection and meningitis. Currently used molecular based diagnostics assays lack specificity in correctly detecting and identifying *H. influenzae*. As such, there is a need to develop novel diagnostics assays for the specific identification of *H. influenzae*.

Whole genome comparative analysis was performed to identify putative diagnostics targets which are unique in nucleotide sequence to *H. influenzae*. From this analysis we identified two *H. influenzae* putative diagnostics targets, *phoB* and *pstA*, for use in real-time PCR diagnostics assays. Real-time PCR diagnostics assays using these targets were designed and optimised to specifically detect and identify all 55 *H. influenzae* strains tested. These novel rapid assays can be applied to the specific detection and identification of *H. influenzae,* for use in epidemiological studies and could also enable improved monitoring of invasive disease caused by these bacteria.

3.1. Introduction

Haemophilus influenzae is the most pathogenic of the 8 *Haemophilus* species which reside as commensals in the human respiratory tract [1]. *H. influenzae* strains are categorised based on the presence (encapsulated) or absence (unencapsulated) of a polysaccharide capsule. Encapsulated strains can be further categorised based on their distinct capsular antigens (typeable; serotypes a - f) whilst unencapsulated strains are referred to as Non-Typeable *H. influenzae* (NTHi) [2]. In the pre-vaccine era, the encapsulated *H. influenzae* serotype b (Hib), was the primary cause of invasive disease such as meningitis, bacteraemia and pneumonia [3]. Upwards of 95% reduction in the number of *H. influenzae* meningitis cases have been recorded since the implementation of the Hib conjugate vaccine [4]. Whilst *H. influenzae* invasive infection in developed countries has declined, NTHi are now the most common cause of *H. influenzae* invasive infections [3, 5-7]. In addition, NTHi have replaced type b strains as the most common blood stream isolates [8].

Haemophilus haemolyticus is also a human commensal bacterium that colonizes the respiratory tract and is closely related to *H. influenzae*. Both *H. influenzae* and *H. haemolyticus* require hemin (X factor) and NAD (V factor) for growth. Phenotypic methods of differentiating *H. haemolyticus* from NTHi rely on the ability of *H. haemolyticus* to lyse horse red blood cells [9]. However, this haemolysis may be lost after subculture [10, 11] and as a consequence, non-hemolytic *H. haemolyticus* have been misidentified as *H. influenzae* [12]. *H. haemolyticus* was considered a rare pathogen [8], however, more recently, a number of cases of invasive disease originally attributed to NTHi have been confirmed as non-haemolytic *H. haemolyticus* [13-15]. Phenotypic techniques cannot conclusively differentiate *H. influenzae* from *H. haemolyticus* and other *Haemophilus* species and therefore, alternative molecular techniques must be used to do so [16].

Real-time PCR diagnostics assays for the identification of *H. influenzae* have been developed targeting various genes including *bexA* [17, 18], *ompP2* [19-22], *ompP6* [17, 23, 24], *16S rDNA* [12], *licA* [20], *rnpB* [23], *frdB* [25], *iga* [26] *hpd* [21, 27, 28] and *fucK* [20, 29, 30]. Diagnostics assays targeting the *fucK* and *hpd* genes have been established as superior for the specific detection *of H. influenzae* over other routinely used gene targets [16, 20, 31]. However, some NTHi strains have been reported as lacking the *fucK* [32-35] or *hpd* genes [36]. As such published literature indicates that no one molecular marker can unequivocally differentiate NTHi from *H. haemolyticus* [16, 31].

In this study, we used comparative genome analysis to identify novel diagnostics targets, which could then be used to develop *H. influenzae* specific real-time PCR. Two real-time PCR assays targeting these novel diagnostics targets were developed and their performance determined. To further validate the real-time PCR diagnostics assays developed, a panel of culture positive clinical isolates, that were identified as *H. influenzae* using MALDI-TOF MS, were blindly tested. The results of MALDI TOF MS and the real-time PCR diagnostics assays were 100% concordant.

3.2. Materials and Methods

3.2.1. Diagnostics Target Identification

Publicly available whole genome sequences for *H. influenzae* and *H. haemolyticus* strains were analysed to identify novel diagnostics targets unique to *H. influenzae* and absent from *H. haemolyticus* and other *Haemophilus* species. *H. influenzae* whole genome sequences are publicly available however, *H. haemolyticus* genome sequences (n = 6) are only available as contiguous sequences (contigs) from the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov). For each of the *H. haemolyticus* strains (M19501, M19107, M21127, M21621, M21639 and HK386), contigs were ordered and assembled using the Mauve contig mover tool in the Mauve genome alignment software (http://gel.ahabs.wisc.edu/mauve/). The European Molecular Biology Open Software Suite tool Emboss union (http://emboss.us.es/cgi-bin/emboss/union) was used to concatenate the multifasta Mauve output to create a single FASTA formatted file. Whole genome annotation was then carried out using the online genome annotation service RAST (Rapid Annotation using Subsystem Technology; http://rast.nmpdr.org/) and the resulting data downloaded in EMBL format. Potential diagnostics targets within *H. influenzae* were then identified using WebACT (http://www.webact.org/WebACT/home) by aligning the whole genome sequence of three *H. haemolyticus* strains (M19501, M19107 and HK386) and the whole genome sequence of their most closely related *H. influenzae* strain (R2866: Accession number NC_017451). Potential diagnostic targets were further analysed using microbial nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&BLAST_SPEC=MicrobialGenomes) to search for similarities in closely related species.

3.2.2. Bacterial strains, Culture media and Growth conditions

A panel of *H. influenzae* (n=13), and closely related species (n=33) were obtained from various culture collections (Table 3.1). All strains were cultured under microaerophilic conditions in haemophilus test media broth (Oxoid), chocolate broth (Fannin Scientific) or on Columbia chocolate agar plates (Fannin Scientific) at 37ºC until sufficient growth was observed.

3.2.3. DNA isolation and Quantification

Genomic DNA from all species was isolated from 1.5 ml of culture using a DNeasy Blood and Tissue Kit as per manufacturers' instructions (Qiagen, Hilden, Germany). DNA concentrations were determined using the Qubit dsDNA HS Assay and the Qubit 1.0 fluorometer (Life Technologies, Carlsbad, CA). Purified DNA samples were stored at -20°C prior to use.

3.2.4. Conventional PCR and Nucleic Acid Sequencing

Nucleotide sequence data used for real-time PCR diagnostics assay design was recovered from the National Centre for Biotechnology Information (NCBI-http://www.ncbi.nlm.nih.gov/) or was generated in this study. Sequencing primers were designed to amplify 832 bp of the *pstA* gene and 681 bp of the *phoB* gene of *H. influenzae* to identify optimal diagnostics target regions for primer and probe design.

PCR was carried out using the sequencing primers (Table 3.2) on an iCycler iQ thermal cycler (Bio-Rad Laboratories Inc., CA) to amplify the *pstA* and *phoB* targets in representative culture collection strains of *H. influenzae*. All reactions were performed using the FastStart PCR Master Kit (Roche Diagnostics, Basel, Switzerland) according to manufacturer's instructions. The thermal cycling parameters used consisted of a denaturation cycle at 95^{o}C for 4 min, followed by 35 cycles at 95 ^{o}C (30 s), 50^{o}C (30 s) and 72^{o}C (30 s), and a final elongation cycle at 72 ^{o}C for 7 min. PCR products were purified using the HighPure PCR product purification kit (Roche Diagnostics) and were sequenced externally (Sequiserve, Vaterstetten, Germany).

3.2.5. PCR primer and probe design

Nucleotide sequences of the diagnostics targets identified in this study were aligned using ClustalW multiple sequence alignment programme (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Oligonucleotide primers and hydrolysis probes were manually designed in accordance with general recommendations and guidelines [37, 38]. Species specific oligonucleotide primers and probe were designed for real-time PCR diagnostics assays. All primers and

probe (Table 3.2) used in this study were obtained from Eurofins MWG-Operon (Ebersberg, Germany). IAC Development

A non-competitive internal amplification control (IAC) targeting the *ssrA* gene of *Bacillus subtillis* subsp. *spizizenii* previously developed in-house was incorporated into the real-time PCR diagnostics assays (Table 3.2).

3.2.6. Real-Time PCR

Duplex real-time PCR diagnostics assays were optimised and the analytical specificity and LOD determined. All real-time PCR diagnostics assays were performed on a LightCycler 480 (Roche Diagnostics) using the LightCycler 480 probes master kit (Roche Diagnostics). Real-time PCR reactions were performed in a total volume of 20 µl consisting of 2 x probes master, forward and reverse primers (0.5 µM final concentration), probes (0.2 µM final concentration), template DNA (2 µl, 10^4 genome equivalents GE) and IAC DNA (2 µl, 10^3 GE). Nuclease free H_2O was added to make up final volume. A no template control was included in each experiment. The cycling parameters consisted of a 10 min denaturation step followed by 50 cycles of 95^{o}C for 10 s, 60^{o}C for 30 s and a final cooling step at 40^{o}C for 10 s. The LightCycler 480 ramp rate was 4.4^{o}C/s while heating and 2.2^{o}C/s on cooling. To prevent fluorescence signal leakage between detection channels on the LightCycler 480, a colour compensation was generated [39].

3.2.7. Multiplex PCR assay specificity and LOD

To evaluate the specificity of the diagnostics assays developed, genomic DNA from 55 *H. influenzae* and 33 closely related *Haemophilus* species (Table 3.1) were tested in triplicate. The LOD of each assay in multiplex format was determined using Probit regression analysis in MiniTab (MiniTab Inc., State College, PA) [40]. Purified *H. influenzae* genomic DNA (DSM4690) was diluted in nuclease free dH_2O. Twelve replicates of each dilution 15, 10, 8, 6, 4, 2, 1 and 0.1 GE were evaluated.

To determine the efficiency of each assay in both monoplex and multiplex format, serial dilutions of purified *H. influenzae* genomic DNA (DSM4690) at 1 x 10^6, 5 x 10^5, 1 x 10^5, 5 x 10^4, 1 x 10^4, 5 x 10^3, 1 x 10^3, 5 x 10^2, 1 x 10^2 and 5 x10^1 were tested in triplicate. Standard curves of C_T versus GE were then constructed. Efficiency (%)

was calculated based on the slope of the line using an online qPCR efficiency calculator (http://www.thermoscientificbio.com/webtools/qpcrefficiency/).

3.2.8. Clinical Isolate Evaluation

H. influenzae clinical isolates (n= 42) were cultured on Columbia chocolate agar plates and were subjected to MALDI-TOF MS analysis (MALDI biotyper, Bruker Daltonics) and the spectra were compared to the software Version 3.4. A number of clinical isolates were typed using conventional PCR [41](n = 6, isolated from blood; Table 3.1) and real-time PCR [18] (n = 3, isolated from sputum; Table 3.1).

3.2.9. *fucK* Real-Time PCR

H. influenzae culture confirmed clinical isolates (n = 42), *H. influenzae* culture collection isolates (n = 13), *H. haemolyticus* (n = 10) and 1 *Haemophilus* sp. (previously designated *H. haemolyticus*) were also tested in triplicate using a published *fucK* real-time PCR diagnostics assay [20].

3.3. Results

3.3.1. Diagnostics Target Identification

Whole genome sequence comparison of *H. influenzae* R2866 and three *H. haemolyticus* strains (M19502, M19107 and HK386) was carried out using WebACT. *H. influenzae* R2866 was used as a reference genome as it was recognized as being the most closely related strain to *H. haemolyticus* based on the RAST comparison analysis. 325 unique regions, comprising of both genes and intergenic nucleotide sequence, were identified that were present in *H. influenzae* and absent in *H. haemolyticus*. Each of these regions was BLAST analysed and further evaluated using three criteria to determine their suitability as diagnostics targets. Firstly, regions were refined by ruling out putative diagnostics targets that had significant BLAST hits with *H. haemolyticus.* Secondly, based on *in silico* evaluation, any putative diagnostics targets identified must be present in all 22 publicly available whole and draft genome sequences of *H. influenzae*. Finally, diagnostics targets identified with significant inter-strain sequence variation were then also excluded.

Based on the above criteria, 6 diagnostic targets (*pstS*, *pstC*, *pstA*, *pstB*, *phoR* and *phoB*) were identified as having the greatest potential for the development of a *H. influenzae* specific real-time PCR diagnostics assays. Nucleotide sequence data for each of the potential diagnostics targets was retrieved from NCBI and *in silico* analysis was performed to further assess their suitability for *H. influenzae* specific detection. Of these, *pstA* and *phoB* were found to contain the most sequence variation when compared with other non- *H. influenzae* bacterial species and contain the least *H. influenzae* inter-strain variation, and were therefore selected for further analysis.

3.3.2. Real-Time PCR assay Specificity

The specificity of each real-time PCR diagnostics assay was confirmed in duplex format using the panel of bacteria listed in Table 3.1. All culture collection strains and clinical isolates of *H. influenzae* were detected by both assays. None of the non-*H. influenzae* microorganisms tested were detected by the assays (Figure 3.1A-D).

3.3.3. Real-Time PCR assay Efficiency and LOD

The efficiencies of each assay were evaluated in both monoplex and multiplex real-time PCR format. *H. influenzae* genomic DNA (DSMZ 4690) was quantified and serial dilutions were prepared based on *H. influenzae* genome size (1,830,137 bp) [42] which equates to 1.9 fg DNA per cell (http://www.changbioscience.com/genetics/mw.html). Both assays were highly efficient in monoplex (*phoB* 94.66%, *pstA* 94.51%). In multiplex, efficiency is comparable in the *pstA* assay (93.47%) and is slightly reduced in the *phoB* assay (88.87%). Plots of C_p versus GE were linear over a range of 1×10^6, 5×10^5, 1×10^5, 5×10^4, 1×10^4, 5×10^3, 1×10^3, 5×10^3, 1×10^3, 5×10^2, 1×10^2, and 5×10^1 GEs for the *phoB* and *pstA* monoplex and *pstA* multiplex assays. (Figure 3.2A-C). Linearity is decreased at lower concentrations of *H. influenzae* DNA in the *phoB* multiplex assay (Figure 3.2D).

In multiplex format, the LOD was determined using Probit regression analysis [40]. With 95% confidence, the LOD of the *phoB* and *pstA* assays was 6.49 and 10.49 GE respectively.

3.3.4. *fucK* real-time PCR assay

A *fucK* real-time PCR diagnostics assay was also used to identify all *H. influenzae* culture collection strains and clinical isolates (Table 3.1) used in this study. From this analysis, 51/55 of the tested *H. influenzae* strains were detected by the *fucK* assay.

3.3.5. Clinical Isolate Evaluation

All 42 clinical isolates were screened using MALDI-TOF MS. The MALDI-TOF software identified all strains to species level and confirmed that all clinical isolates are *H. influenzae,* all with scores greater than 2.0. All clinical isolates typed (n=9) using published capsular typing methods were confirmed as NTHi.

3.4. Discussion

H. influenzae is known to cause a wide spectrum of disease, ranging from non-invasive infections including bronchitis and conjunctivitis to more serious infections including meningitis, pneumonia and sepsis. Bacterial culture is the preferred method for the diagnosis of invasive *H. influenzae* [20]. However, culture based methods are time-consuming, often taking up to 36 hr or more [43-45]. Also, in many cases, pre-treatment of the infection with broad spectrum antibiotics may produce false negatives [46]. Evaluation of a number of biomarker genes for differentiating *H. influenzae* from *H. haemolyticus* and other non- *H. influenzae* species suggests that there is no one diagnostic target identified to date that can unambiguously differentiate the two microorganisms [31].

Recently, draft whole genome sequences of six *H. haemolyticus* strains have been published [15]. In the study presented here, comparative analysis between these strains and *H. influenzae* highlighted a number of variable regions between the genomes of the two species. BLAST and ClustalW analysis identified 6 genes, *phoB, phoR, pstS, pstC, pstA*, and *pstB*, as potential diagnostics targets for the discrimination of the two microorganisms. *In silico* nucleotide sequence analysis revealed that these genes are present in *H. influenzae* but are absent in all other recognised *Haemophilus* species. These genes, which have been studied extensively

in *E. coli* , are members of the bacterial *pho* (phosphate) regulon and are involved in the transport and assimilation of inorganic phosphate (Pi) [47, 48].

Two of the putative diagnostic targets identified namely *phoB* and *pstA* were further evaluated for their ability to accurately identify *H. influenzae* and distinguish it from other closely related *Haemophilus* species. *phoB* was chosen as a diagnostics target as it is only present in Gram negative bacteria (with a homolog *phoP* in Gram positive bacteria) [47]. The *pstA* gene was also chosen as a diagnostics target for further evaluation as it was found to have the least inter-strain gene nucleotide sequence heterogeneity and the greatest level of gene nucleotide sequence variation compared with other bacteria (data not shown). Internally controlled real-time duplex PCR assays were developed for each of these selected diagnostics targets. Both assays were determined to be 100% specific for the detection of *H. influenzae*. The established LOD of 6.49 GE and 10.49 GE (95% confidence), for both assays respectively, is comparable to other studies targeting *fucK*, which have LODs of 10 genome copies (95% confidence) [20] and between 5 (5/8 reactions) and 50 (8/8 reactions) genomes copies [29]. We also compared our assays to a previously developed *fucK* real-time PCR assay as this is one of the diagnostics target most commonly used in the literature for the specific detection of *H. influenzae*. This comparative analysis demonstrated that both the *phoB* and *pstA* assays are more specific for the detection of *H. influenzae* compared to the *fucK* assay. Of the 42 clinical isolates identified as *H. influenzae* using MALDI-TOF MS and the novel real-time PCR diagnostics assays developed in this study, 3 (7.1%) were not detected by the *fucK* assay. One of the culture collection strains (CCUG 58365) was also not detected using the *fucK* assay due to a deletion of the fucose operon as previously reported in this strain [34].

When evaluating the two real-time PCR diagnostics assays developed in this study, their performance in terms of specificity and LOD are comparable. However, the *pstA* multiplex real-time PCR assay is more efficient than the *phoB* multiplex real-time PCR assay. In addition, IAC amplification is inhibited at a *H. influenzae* DNA concentration of greater than 10^5 GE in the *phoB* multiplex PCR assay, revealing the *pstA* assay is more robust in terms of overall real-time PCR assay performance.

In this study we set out to identify novel diagnostics targets that could be used in real-time PCR assays to rapidly detect and specifically identify *H. influenzae*. The novel real-time PCR diagnostics assays developed could enable improved monitoring of invasive disease caused by this bacteria while also providing the clinician with valuable information about the optimal therapeutic regimen to initiate patient treatment. We propose that the developed assays now merit extensive multicentre evaluation in clinical settings.

Table 3.1: Bacterial species and strains included in this study

Organism	**Strain** [c]/ Source	**Assay**[d]		
		phoB	*pstA*	*fucK*
H. influenzae reference strains				
H. influenzae type a	NCTC 8465	+	+	+
H. influenzae type b	DSMZ 23393[c]	+	+	+
H. influenzae type b	DSMZ 11969[c]	+	+	+
H. influenzae type b	DSMZ 11970[c]	+	+	+
H. influenzae type b	DSMZ 10001[c]	+	+	+
H. influenzae type b	DSMZ 4690[c]	+	+	+
H. influenzae type c	NCTC 8469	+	+	+
H. influenzae type d	DSMZ 11121[c]	+	+	+
H. influenzae type e	NCTC 8472	+	+	+
H. influenzae type f	DSMZ 10000[c]	+	+	+
H. influenzae NTHi	DSMZ 9999[c]	+	+	+
H. influenzae NTHi	CCUG 58365	+	+	-
H. influenzae biogroup *aegyptius*	DSMZ 21187[c]	+	+	+
Clinical isolates (n=6)[a]	Blood	+	+	+
Clinical isolates (n=12)[a]	Ear/Eye swab	+	+	+
Clinical isolates (n=24)[a]	Sputum	+	+	+ (21)[e]
Non- *H. influenzae* strains				
H. haemolyticus	CDC-M19501	-	-	-
H. haemolyticus	CDC-M21127	-	-	-
H. haemolyticus	CDC--M21621	-	-	-
H. haemolyticus	CCUG 24149	-	-	-
H. haemolyticus	CCUG 36015	-	-	-
H. haemolyticus	CCUG 36016	-	-	-
H. haemolyticus	CCUG 15642	-	-	-
H. haemolyticus	CCUG 15312	-	-	-
H. haemolyticus	NCTC 10839	-	-	-

H. haemolyticus	NCTC 10659	-	-	-
H. ducreyi	DSMZ 8925	-	-	/
H. felis	DSMZ 21192	-	-	/
H. haemoglobinophilus	DSMZ 21241	-	-	/
H. paracuniculus	DSMZ 21452	-	-	/
H. parahaemolyticus	DSMZ 21417	-	-	/
H. parainfluenzae	DSMZ 8978	-	-	/
H. paraphrohaemolyticus	DSMZ 21451	-	-	/
H. parasuis	DSM 21448	-	-	/
H. pittmaniae	DSMZ 21203	-	-	/
H. pittmaniae	DSMZ 17420	-	-	/
Haemophilus sp.	CCUG 34110	-	-	-
Actinobacillus suis	DSMZ 22433	-	-	/
Actinobacillus pleuropneumoniae	DSMZ 13472	-	-	/
Aggregatibacter aphrophilus	NCTC 11096	-	-	/
Aggregatibacter aphrophilus	NCTC 10558	-	-	/
Aggregatibacter actinomycetecomitans	DSMZ 11121	-	-	/
Aggregatibacter actinomycetecomitans	DSMZ 8324	-	-	/
Aggregatibacter segnis	NCTC 10977	-	-	/
Avibacterium avium	DSMZ 18557	-	-	/
Avibacterium paragallinarum	DSMZ 18554	-	-	/
Gardnerella vaginalis	DSMZ 4944	-	-	/
Histophilus somni	CCUG 36157	-	-	/
Taylorella equigenitalis	DSMZ 10668	-	-	/

[a] All Clinical isolates were identified as *H. influenzae* using MALDI-TOF MS

[b] NCTC = National Collection of Type Cultures; * DSMZ = The German Collection of Microorganisms; *CCUG = Culture Collection, University of Göteborg, Sweden; *CDC = Centre for Disease Control;

[c] *pstA* and *phoB* gene sequence data was generated for each of these *H. influenzae* strains using primers outlined in Table 3.2

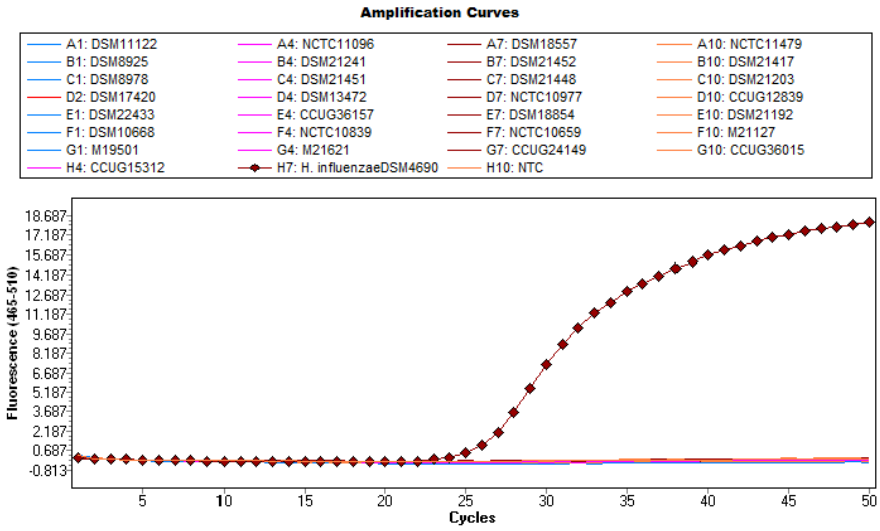
[d] + ve= Positive; - ve= negative; / = not tested for

[e] 3/42 *H. influenzae* clinical isolates not detected by the *fucK* assay

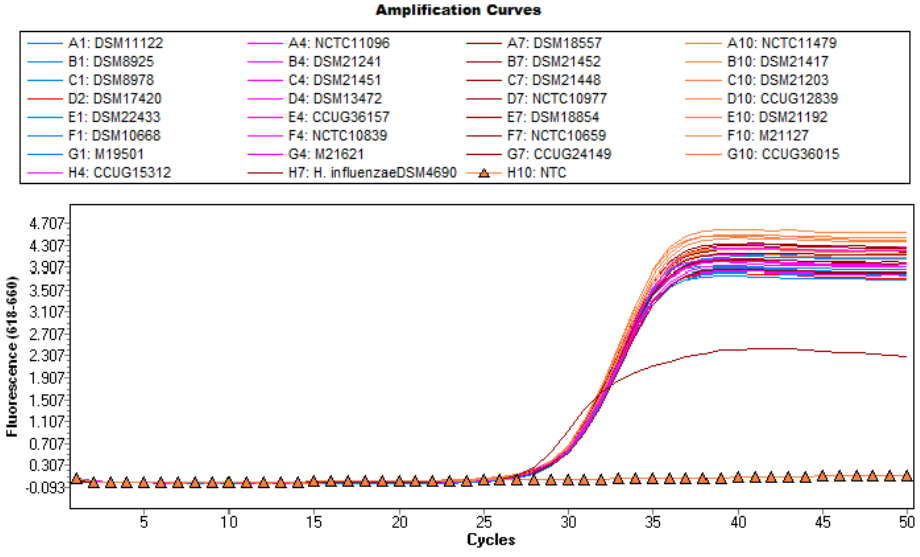
Table 3.2: Nucleotide sequences of primers and probes.

Probe/ Primer	Function	DNA sequence (5' – 3')	Nucleotide position	Genbank accession no.	Concentration (µM)
pstA F1	*H. influenzae pstA* Sequencing forward	CTAATCAAAACTTG CGT	8-24	CP002277.1	0.4 µM
pstA R1	*H. influenzae pstA* Sequencing reverse primer	TGTTGAAAGAAGAG TCG	823-839	CP002277.1	0.4 µM
phoB F1	*H. influenzae phoB* Sequencing forward	CTGATAGTTGAAGA TG	16-31	CP002277.1	0.4 µM
phoB R1	*H. influenzae phoB* Sequencing reverse primer	TCATTGTTTATCTCG T	681-696	CP002277.1	0.4 µM
pstA P1	*H. influenzae pstA* forward primer	CGTTTCGCACAAATT ACC	310-327	CP002277.1	0.5 µM
pstA P2	*H. influenzae pstA* reverse primer	GTGCGTACCACGAT AGG	460-476	CP002277.1	0.5 µM
pstA probe	*H. influenzae pstA* hydrolysis probe	FAM-CTGGAGCATTCGCA TTAGCTT-BHQ1	428-448	CP002277.1	0.2 µM
phoB P1	*H. influenzae phoB* forward primer	TTGGATTGGATGCTA CC	151-167	CP002277.1	0.5 µM
phoB P2	*H. influenzae phoB* reverse primer	AGTGATGTAGTCAT CAGC	292-309	CP002277.1	0.5 µM
phoB probe	*H. influenzae phoB* hydrolysis probe	FAM-AGAAAGCTATGCTG CGATTCC-BHQ1	210-230	CP002277.1	0.2 µM
BSS-1F	IAC forward primer	AACGTAGCATTAGC TGC	111-127	HG519928.1	0.5 µM
BSS-1R	IAC reverse primer	CTCATCTTCTTGCCT GC	260-276	HG519928.1	0.5 µM
BSS-P	IAC – specific hydrolysis probe	Cy5-CACATCCAAGTAGG CTACGCT-BHQ2	179-199	HG519928.1	0.2 µM

A


Amplification Curves
A1: DSM11122
B1: DSM8925
C1: DSM8978
D2: DSM17420
E1: DSM22433
F1: DSM10668
G1: M19501
H4: CCUG15312
A4: NCTC11096
B4: DSM21241
C4: DSM21451
D4: DSM13472
E4: CCUG36157
F4: NCTC10839
G4: M21621
H7: H. influenzaeDSM4690
A7: DSM18557
B7: DSM21452
C7: DSM21448
D7: NCTC10977
E7: DSM18854
F7: NCTC10659
G7: CCUG24149
H10: NTC
A10: NCTC11479
B10: DSM21417
C10: DSM21203
D10: CCUG12839
E10: DSM21192
F10: M21127
G10: CCUG36015
Fluorescence (465-510)
18.687
17.187
15.687
14.187
12.687
11.187
9.687
8.187
6.687
5.187
3.687
2.187
0.687
-0.813
5
10
15
20
25
30
35
40
45
50
Cycles

B


Amplification Curves
A1: DSM11122
B1: DSM8925
C1: DSM8978
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E1: DSM22433
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G1: M19501
H4: CCUG15312
A4: NCTC11096
B4: DSM21241
C4: DSM21451
D4: DSM13472
E4: CCUG36157
F4: NCTC10839
G4: M21621
H7: H. influenzaeDSM4690
A7: DSM18557
B7: DSM21452
C7: DSM21448
D7: NCTC10977
E7: DSM18854
F7: NCTC10659
G7: CCUG24149
H10: NTC
A10: NCTC11479
B10: DSM21417
C10: DSM21203
D10: CCUG12839
E10: DSM21192
F10: M21127
G10: CCUG36015
Fluorescence (618-660)
4.707
4.307
3.907
3.507
3.107
2.707
2.307
1.907
1.507
1.107
0.707
0.307
-0.093
5
10
15
20
25
30
35
40
45
50
Cycles

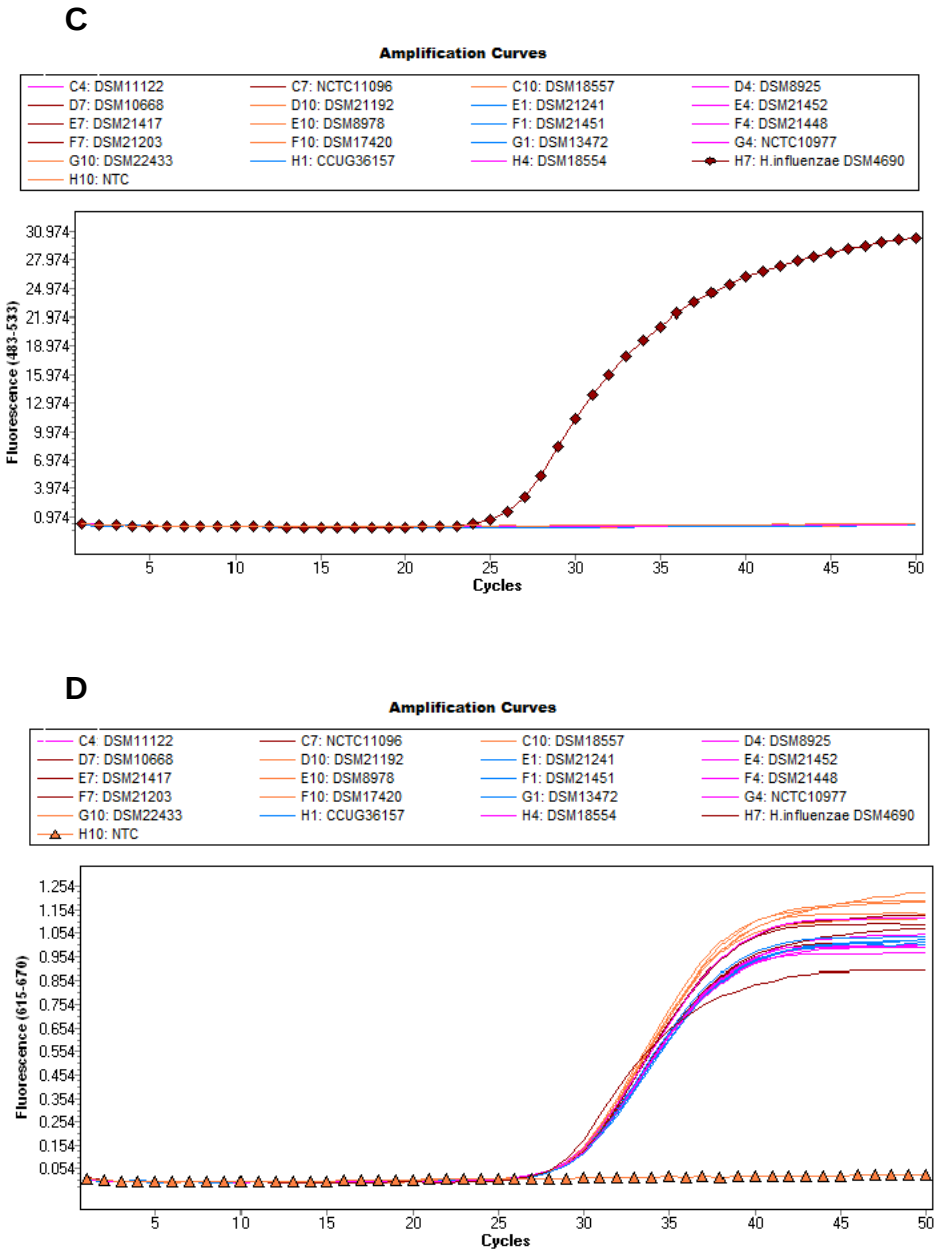

Figure 3.1: Representative amplification curves for multiplex real-time PCR diagnostics assay. Figure 1A: Real-time amplification curves for 1 strain of *H. influenzae* (DSM4690) (Diamonds) targeting the *phoB* gene in the FAM channel (465 -510 nm). No non-*H. influenzae* species were detected by the assay; Figure 1B: Amplification curves for IAC in Cy5 channel (618-660nm) with NTC highlighted with triangles. All samples detected except the no template control (NTC). Figure 1C: Real-time amplification curves for 1 strain of *H. influenzae* (DSM4690) (Diamonds) targeting the *pstA* gene in the FAM channel (483 - 533 nm). No amplification occurred in non-*H. influenzae* species; Figure 1D: Real-time amplification curves for IAC in Cy5 channel (615-670nm) with NTC highlighted with triangles. All samples detected except the no template control (NTC).

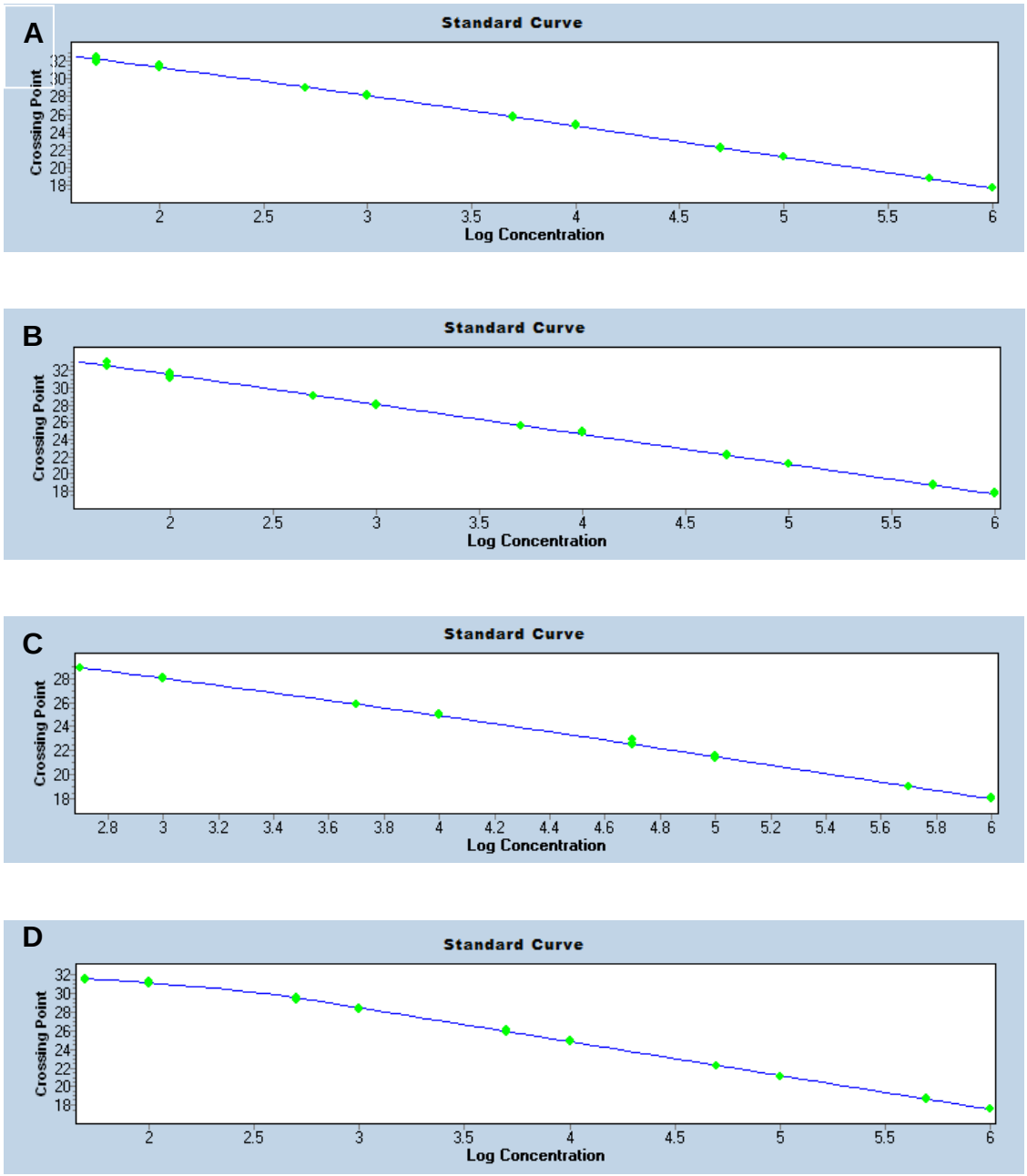

Figure 3.2: Standard curve of Log10 GE *H. influenzae* DSM4690 DNA versus crossing point (Cp) values obtained for monoplex *pstA* (A) and *phoB* (B) assays and multiplex *pstA* (C) and *phoB* (D) assays.

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Chapter 4:

Experimental validation of duplex real-time NASBA diagnostics assays incorporating an endogenous control for the detection of predominant microorganisms associated with bacterial meningitis

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Clancy E, Coughlan H, Higgins O, Boo TW, Cormican M, Barrett L, Smith TJ, Reddington K, Barry T: Development of internally controlled duplex real-time NASBA diagnostics assays for the detection of microorganisms associated with bacterial meningitis. *Journal of Microbiological Methods* 2016, 127:197-202. (Appendix E)

Abstract

Three duplex molecular beacon based real-time Nucleic Acid Sequence Based Amplification (NASBA) assays have been designed and experimentally validated targeting RNA transcripts for the detection and identification of *Haemophilus influenzae, Neisseria meningitidis* and *Streptococcus pneumoniae* respectively. Each real-time NASBA diagnostics assay includes an endogenous non-competitive Internal Amplification Control (IAC) to amplify the splice variant 1 mRNA of the *Homo sapiens* TBP gene from human total RNA. All three duplex real-time NASBA diagnostics assays were determined to be 100% specific for the target species tested for. Also the Limits of Detection (LOD) for the *H. influenzae*, *N. meningitidis* and *S. pneumoniae* duplex real-time NASBA assays were 55.36, 0.99, and 57.24 Cell Equivalents (CE) respectively. These robust duplex real-time NASBA diagnostics assays have the potential to be used in a clinical setting for the rapid (<60 min) specific detection and identification of the most prominent microorganisms associated with bacterial meningitis in humans.

4.1. Introduction

Bacterial meningitis is defined as an inflammation of the meninges surrounding the central nervous system. It is a serious and often fatal infection with high morbidity and mortality rates worldwide (5-40% in children, 20-50% in adults) [1]. *Haemophilus influenzae, Neisseria meningitidis* and *Streptococcus pneumoniae* are the most predominant etiological agents of bacterial meningitis, accounting for 75-80% of cases [2-4]. Since the introduction of the *H. influenzae* type b (Hib) conjugate vaccine in the 1990s, *N. meningitidis* and *S. pneumoniae,* have replaced *H. influenzae* as the leading causes of bacterial meningitis in industrialised countries [4]. However, in developing countries, the World Health Organization estimates vaccine coverage to be as little as 21% (http://www.who.int/mediacentre/factsheets/fs378/en/), *H. influenzae* type b invasive disease still remains a significant concern to human health. Several vaccines have also been developed for the prevention of disease from the most common causes of pneumococcal and meningococcal meningitis (e.g. 4CMenB, MenB-FHbp, MCV4, MPSV4, PCV13 and PPV23) [5-7]. While an overall decrease in the incidence of bacterial meningitis cases has been observed as a result of the implementation of

these vaccines, an estimated 1·2 million cases of bacterial meningitis still occur worldwide every year which resulted in 180 000 deaths in children aged 1–59 months in 2010 [8]. These vaccines are not effective against all strains of these bacteria associated with infection as is illustrated by several cases of pneumococcal and meningococcal meningitis attributed to infection caused by non-vaccine serotypes [9-11]. Finally, while vaccination continues to have a protective effect in humans and is critically important in combating meningococcal disease, vaccine failures are also well described [12].

As such, when a patient presents with suspected bacterial meningitis or with features consistent with meningococcal blood stream infection there is a clinical need to detect all strains of *Haemophilus influenzae, Neisseria meningitidis* and *Streptococcus pneumoniae*. At present, culture of Cerebrospinal Fluid (CSF) and blood remains the gold standard for the diagnosis of bacterial meningitis. However, this is time consuming, often taking 36 hours or more to confirm a specific a diagnosis [13], has limited sensitivity with slow growing, fastidious or non-culturable microorganisms [14, 15] and is problematic in resource poor settings [16]. Administration of antibiotics prior to sample collection further complicates the situation as it diminishes the likelihood of culture confirmation [4, 13-15]. Consequently there is a need for the development of more rapid, sensitive and specific non-culture based diagnostics methods such as nucleic acid *in vitro* amplification technologies. This would enable improved monitoring, allow for the more rapid administration of the appropriate pathogen specific antibiotic treatment regime in a patient with suspected meningitis and improve the overall prognosis of the disease.

Nucleic Acid Sequence Based Amplification (NASBA) represents a suitable technology that could be applied in this setting. NASBA is an isothermal transcription based nucleic acid *in vitro* amplification technique which exploits three enzymes: avian myeloblastosis virus reverse transcriptase, *Escherichia coli* ribonuclease H and T7 DNA dependant RNA polymerase to specifically amplify RNA targets [17]. A number of hybridization based end-point detection methods, such as electrochemiluminescence [18], enzyme-linked gel assay [19], and fluorescence correlation spectroscopy can be used to detect the NASBA *in vitro* amplified RNA amplicons [20, 21]. More recently, molecular beacon probe

technology has enabled the real-time detection of NASBA amplicons [22, 23]. Labelling molecular beacon probes with fluorophores that emit light at different wavelengths enables the simultaneous *in vitro* amplification and real -time detection of different RNA targets in one reaction [24]. An advantage of this technology is the ability to discriminate between specific microbial species in a single closed tube reaction. The ability to detect multiple analytes in one reaction highlights a clear advantage of real-time NASBA over other isothermal amplification methods developed to date. For example this allows for the inclusion of an Internal Amplification Control (IAC) which is deemed an important analytical control in molecular *in vitro* amplification techniques to verify the accuracy of the results obtained and provide a more robust diagnostics assay [25]. Furthermore NASBA is specific for RNA and as such only identifies viable infectious agents [26]. It is also theoretically more sensitive than other *in vitro* amplification technologies that target DNA since target RNA transcript copy number can be greater than DNA copy number in biological cells [27]. Finally, like other isothermal *in vitro* amplification methods, the use of real-time NASBA has the potential to eliminate the need for thermal cycling and ramping instrumentation requirements which facilitates its potential application on a low cost lab on a chip (LOC) and/or point of care (POC) diagnostic devices [28, 29].

To our knowledge there are currently no reported internally controlled NASBA or real-time NASBA diagnostics assays described in the literature for use in the specific detection of *H. influenzae, N. meningitidis* and *S. pneumoniae.* In this study we outline the design, development, optimisation of three novel duplex real-time NASBA diagnostics assays incorporating an endogenous IAC, for use in the detection of the predominant microorganisms associated with bacterial meningitis

4.2. Materials and Methods

4.2.1. Diagnostics Target Identification

The novel diagnostic RNA targets used in this study were identified bioinformatically using nucleotide sequences retrieved from a number of publicly available databases including the tmRNA website (http://bioinformatics.sandia.gov/tmrna/), the tmRNA database (http://www.ag.auburn.edu/mirror/tmRDB/), the National Center for Biotechnology

Information (NCBI) website (http://www.ncbi.nlm.nih.gov/) and the Functional Gene Pipeline (FunGene) website (http://fungene.cme.msu.edu/).

4.2.2. Bacterial strains, Culture media and Growth conditions

A panel of culture collection strains of *H. influenzae* (n=8), non-*H. influenzae Haemophilus* species (n=21), *N. meningitidis* (n=9), non-*N. meningitidis Neisseria* species (n=18), *S. pneumoniae* (n=3) and non-*S. pneumoniae Streptococcus* species (n=26) were obtained from various culture collections (Table 4.2). A collection of recent *H. influenzae* clinical isolates (n=16), *N. meningitidis* clinical isolates (n= 8) and *S. pneumoniae* clinical isolates (n=9) were also obtained from University Hospital Galway to further evaluate the assays. *Haemophilus* strains were cultured in haemophilus test media broth (Oxoid), chocolate broth (Fannin Scientific) or on Columbia chocolate agar plates (Fannin Scientific). *Neisseria* and *Streptococcus* species were cultured in brain heart infusion (BHI) broth (Oxoid), or on Columbia blood agar plates (Fannin Scientific). All bacterial species were cultured under microaerophilic conditions at 37^{o}C overnight or until sufficient growth was observed, as determined by the degree of turbidity compared to culture negative controls.

4.2.3. DNA isolation and Quantification

Genomic DNA from a collection of *Haemophilus* and *Neisseria* species was isolated from 1.5 ml of culture using a DNeasy Blood and Tissue Kit as per manufacturers' instructions (Protocol: Gram-Negative Bacteria; Qiagen, Hilden, Germany). DNA integrity was assessed on a 1 % agarose gel and concentrations were determined using the Qubit dsDNA HS Assay and the Qubit 1.0 fluorometer (Life Technologies, Carlsbad, CA). Purified DNA samples were stored at -20°C prior to use.

4.2.4. Total RNA isolation and Quantification

Total RNA from all bacterial species was isolated and purified from 1.5 ml of culture using a RiboPure Yeast Kit as per manufacturers' instructions (Ambion, Austin, TX, USA). For human RNA, whole blood was purchased from a commercial provider (Seralab, UK). Subsequently total RNA was isolated and purified from 2.5 ml of these blood samples using the PAXgene blood RNA kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. To determine the integrity of the RNA

purified from both the bacterial cultures and human whole blood samples, total RNA was analysed with the Agilent 2100 Bioanalyzer (Agilent Technologies). Concentrations of total RNA were determined using the Qubit RNA BR Assay kit and the Qubit 1.0 fluorometer (Life Technologies, Carlsbad, CA). All purified total RNA samples were diluted and stored at -80°C prior to use.

4.2.5. Conventional PCR Primer Design

Publically available nucleotide sequences of the diagnostics targets identified in this study were aligned using ClustalW multiple sequence alignment programme (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Oligonucleotide sequencing primers were manually designed in accordance with general recommendations and guidelines [21, 30] to target conserved regions within the genes. All oligonucleotide primers (Table 4.1) used in this study were obtained from Eurofins MWG-Operon (Ebersberg, Germany).

4.2.6. Conventional PCR and Nucleic Acid Sequencing

Sequencing oligonucleotide primers were designed to amplify 681 bp of the *phoB* gene of *H. influenzae*, and 342 bp of the *ssrA* gene of *N. meningitidis,* to identify optimal diagnostics target regions for NASBA primer and molecular beacon probe design. Due to the extent of publicly available *S. pneumoniae lepA* sequence data, sequencing of in-house *S. pneumoniae* culture collection strains was not carried out.

PCR was carried out using the sequencing primers (Table 4.1) on an iCycler iQ thermal cycler (Bio-Rad Laboratories Inc., CA). All reactions were performed using the FastStart PCR Master Kit (Roche Diagnostics, Basel, Switzerland) according to manufacturer's instructions in a final volume of 25 µl. The thermal cycling parameters used for the *H. influenzae* specific primers consisted of a denaturation cycle at 95ºC for 4 min, followed by 35 cycles at 95ºC (30 s), 50ºC (30 s) and 72ºC (30 s), and a final elongation cycle at 72ºC for 7 min. Thermal cycling parameters used for the *Neisseria* specific primers were the same as outlined above with the exception of an annealing temperature of 55ºC instead of 50ºC. The PCR products were purified using the HighPure PCR product purification kit (Roche Diagnostics) and sequenced externally (Sequiserve, Vaterstetten, Germany).

4.2.7. Real-Time NASBA primer and molecular beacon probe design

Nucleotide sequence data used for real-time NASBA diagnostics assay design was either recovered from publically available sequence databases or were generated in this study. Species specific oligonucleotide primers and molecular beacon probes were manually designed in accordance with recommended guidelines [31]. Primer, molecular beacon, and NASBA amplicon structure were checked for secondary structures by using an RNA folding program (MFold: http://unafold.rna.albany.edu/?q=mfold/RNA-Folding-Form2.3) with the folding temperature parameter adjusted to 41^{o}C. The molecular beacon probe for the *H. influenzae* specific assay was labelled with FAM and Black Hole Quencher (BHQ) 1. The molecular beacon probe for the *N. meningitidis* specific assay was labelled with HEX and Dabcyl (DAB). The molecular beacon probe for the *S. pneumoniae* specific assay was labelled with ROX and BHQ2. All primers and molecular beacon probes (Table 4.1) were obtained from Eurofins MWG-Operon (Ebersberg, Germany).

4.2.8. IAC Development for real-time NASBA

A non-competitive IAC was developed for the real-time NASBA assays targeting splice variant 1 of the *Homo sapiens* TBP (TATA-box binding protein) mRNA transcript. IAC oligonucleotide primers and molecular beacon probes were designed and obtained the same as outlined for species specific oligonucleotides. The IAC assay molecular beacon probe was labelled with Cy5 and Black Hole Quencher 2 (BHQ2) to facilitate multiplexing of the diagnostic assays.

4.2.9. Duplex real-time NASBA Development

Duplex real-time NASBA diagnostics assays were optimised and the analytical specificity and sensitivity determined. All real-time NASBA diagnostics assays were performed on a LightCycler 480 (Roche Diagnostics) using the NucliSENS EasyQ Basic Kit V2 (Biomerieux, Marcy l'Etoile, France), in accordance with manufacturer's instructions. *H influenzae* and *N. meningitidis* duplex real-time NASBA reactions were performed in a total volume of 20 µl. Target RNA and IAC RNA (2.5 µl each) were added to 10 µl reagent/KCL (70 mM final concentration), oligonucleotide primers and molecular beacon probes (0.2 µM and 0.1 µM final

concentration respectively) mixture. The reaction was incubated for 65ºC for 5 min to denature the RNA secondary structure followed by 41ºC for 5 min to allow primer annealing. Subsequently, 5 µl of the enzyme mixture was added to the reaction. A no template control (NTC) reaction consisting of water instead of RNA was included in each experiment. The reaction was then incubated at 41ºC for 60 min in a LightCycler 480 thermocycler (Roche Diagnostics) with a fluorescent measurement recorded every minute. The *S. pneumoniae* duplex real-time NASBA assay was performed as above with the exception that this assay was supplemented with betaine (0.1M final concentration) and that the final concentration of oligonucleotide primers and molecular beacon was 1 and 0.5µM respectively.

4.2.10. Duplex real-time NASBA specificity and LOD

In order to evaluate the specificity of the three real-time NASBA diagnostics assays developed, total RNA from *H. influenzae* and closely related *Haemophilus* species, *N. meningitidis* and closely related *Neisseria* species, and *S. pneumoniae* and closely related *Streptococcus* species (Table 4.2), at concentration of 1 x 10^4 CE per reaction, were tested in duplicate in the *H. influenzae*, *N. meningitidis* and *S. pneumoniae* real-time NASBA diagnostics assays respectively.

To determine the LOD of each duplex assay, purified total RNA (DSMZ 4690, NCTC 10025 and DSMZ 20566) was two-fold serially diluted from 250 to 1.95 CE (*H. influenzae and S. pneumoniae*) and from 50 to 0.39 CE (*N. meningitidis*) and tested in replicates of ten. Dilutions were prepared, based on a calculation that a typical bacterial cell contains 0.1 pg RNA [32]. The LOD for each duplex real-time NASBA assay was subsequently determined using Probit regression analysis with 95% probability (Minitab Version 17) [33].

4.3. Results

4.3.1. Diagnostics Target Identification

PhoB has recently been described in the literature as species specific target for *H. influenzae* using real-time PCR [34]. Based on our *in silico* evaluation, this diagnostics target demonstrated suitable characteristics for evaluation as a *H. influenzae* species specific mRNA diagnostics target for use in a real-time NASBA assay. Publicly available and in-house sequence information was also interrogated

using online bioinformatics tools to determine the suitability of *ssrA* and *lepA* RNA transcripts as molecular targets for the identification of *N. meningitidis* and *S. pneumoniae* respectively. This analysis established that the *ssrA* and *lepA* genes contain sufficient nucleic acid sequence heterogeneity to allow for the design of highly specific real-time NASBA assays for both *N. meningitidis* and *S. pneumoniae.*

4.3.2. Total RNA isolation and quantification

All total RNA isolated from bacterial cultures and human whole blood samples were found to have an RNA integrity number (RIN) of 7 or greater which demonstrated that the RNA was of sufficient quality for further evaluation. For specificity testing, total RNA isolated from bacterial cultures was diluted to contain approximately 1 x 10^4 CE per reaction for specificity testing. Using the PAXgene method for isolation and purification of RNA from human whole blood, approximately 2.1 µg of total RNA was recovered from a 2.5ml sample.

4.3.3. IAC Development

In order for a result to be considered valid using the real-time NASBA assays developed in this study, a positive signal must also be obtained in the IAC detection channel (Cy5) on the LightCycler 480. If the IAC is not detected the result is considered invalid and must be repeated [25]. In this study, IAC primers and a molecular beacon probe were designed to detect the TBP mRNA splice 1 variant. Subsequently, duplex real-time NASBA assays incorporating the developed IAC were tested with human total RNA spiked in to each reaction. For the purpose of this study, the IAC was spiked into each NASBA reaction at a final concentration of 25 ng to reflect the concentration of human RNA that would be recovered from a 30 µl sample based on the recovery of total RNA above. At this concentration, the IAC was detected in all samples tested (Figure 4.1B, 4.2B, and 4.3B).

4.3.4. Real-time NASBA assay specificity

The specificity of each real-time NASBA diagnostics assay was confirmed in duplex format using the specificity panel listed in Table 4.1. All three assays were specific, detecting only target species (Figure 4.1A, 4.2A and 4.3A). None of the non-*H. influenzae*, non-*N. meningitidis* and non-*S. pneumoniae* closely related microorganisms tested were detected by the assays. The LOD of each duplex assay

was determined using Probit regression analysis. With 95% confidence the LOD of the *H. influenzae, N. meningitidis and S. pneumoniae* duplex assays was determined to be 55.36, 0.99 and 57.24 CE, respectively.

4.4. Discussion

Despite advances in immunization approaches, *H. influenzae, N. meningitidis* and *S. pneumoniae* still account for the majority of bacterial meningitis cases worldwide, particularly in low income countries where vaccine availability is limited. Precise identification of these three microorganisms is essential given the significant mortality and morbidity rates associated with bacterial meningitis, the high risk of substantial long term sequelae in survivors [35], and the differences in therapeutic approach for the different pathogens [36]. Traditional CSF and blood culture methods, are slow and often result in an inaccurate diagnosis [4]. Furthermore, the use of CSF as a sample type is invasive for patients [37].

Molecular techniques have the ability to rapidly identify microorganisms associated with bacterial meningitis. Recent studies have shown that use of technologies which incorporate an *in vitro* amplification step demonstrate increased sensitivity when compared to routine blood culture [38]. The use of such molecular tests in a clinical setting have the ability to improve patient care by reducing time spent in hospitals, while also improving antimicrobial stewardship [39]. Several multiplex real-time PCR diagnostics assays have been described in the literature for the specific identification of *H. influenzae, N. meningitidis* and *S. pneumoniae* directly from patient samples [13, 40]. However, these assays fail to incorporate an IAC which is crucial when working with clinical samples to monitor for PCR inhibition and to reduce reporting of false negative reactions [25]. Furthermore, these real-time PCR assays require expensive instrumentation capable of precise thermal cycling and rapid ramping. Isothermal *in vitro* amplification methods have the potential to eliminate the need for thermal cycling and ramping instrumentation required for real-time PCR. As such these *in vitro* amplification technologies are more suitable for incorporation on to low cost LOC and/or POC diagnostic devices. There are a number of isothermal *in vitro* amplification technologies which could be used for the detection of bacterial pathogens associated with meningitis for example recombinase polymerase

amplification (RPA) or loop-mediated isothermal amplification (LAMP). However these *in vitro* amplification technologies are also limited; for example as RPA has previously been shown to be inhibited when used on nucleic acids purified from whole blood samples, or in the presence of background DNA [41, 42]. Through incorporation of fluorescent fluorophores recent advances in LAMP methodologies facilitate monitoring of a reaction in real-time. However a significant disadvantage of LAMP is that it is difficult to multiplex due to the complicated amplicon structure [43] which often restricts the ability to incorporate an IAC into the diagnostics assay. As such, NASBA with molecular beacon technology enables real-time detection of target nucleic acid sequences and allows for the inclusion of an IAC in a single closed tube reaction offers a significant advantages over other isothermal technologies [44].

In this manuscript, we present three novel duplex real-time NASBA assays, incorporating an IAC, for the detection of the predominant microorganisms associated with bacterial meningitis namely *H. influenzae, N. meningitidis* and *S. pneumoniae*. The *phoB* gene has been previously been described in the literature as a suitable diagnostics assay target for the species specific identification of *H. influenzae* using real-time PCR [34]. In this study we have further validated this target and have successfully demonstrated the suitability of the *phoB* mRNA transcript for use in a duplex real-time NASBA diagnostics assay for the specific detection of *H. influenzae.* The diagnostic target used in this study for the species specific identification of *N. meningitidis* is the tmRNA transcript encoded for by the *ssrA* gene [45]. This diagnostics gene target has previously been demonstrated as suitable for use in nucleic acid based diagnostics for bacterial detection and identification [46-48]. Furthermore, tmRNA, has also been established as a useful target in a real-time NASBA diagnostics assay [46]. Finally the diagnostic mRNA transcript target evaluated is the *lepA* mRNA and enables for the species specific identification of *S. pneumoniae*. The *lepA* gene, also present in all bacteria sequenced to date, encodes for a highly conserved translational elongation factor protein LepA, and is homologous to the *Guf1* gene found in higher organisms [49]. It has also previously been demonstrated to be a useful infectious disease diagnostics target [50, 51].

An endogenous non-competitive IAC targeting transcript 1 mRNA of the *Homo Sapiens* TBP gene was incorporated into each assay. Incorporation of an IAC verifies the accuracy of the real-time NASBA results obtained, that the real-time NASBA reaction is functioning correctly and prevents reporting of false negative results which may occur as a result of problems with the assay reaction mixture, equipment malfunction, operator error, poor enzyme activity or inhibitory substances in the NASBA sample [52].In recent years, a number of real-time NASBA assays have been described for the simultaneous detection of a target microorganism incorporating an IAC [46, 52-62]. However, these internally controlled real-time NASBA assays use *in vitro* synthesized RNAs spiked into the real-time NASBA reaction and some share a common set of primers for both the target species and the IAC which can result in competition for the primary target. In this study, the TBP mRNA transcript was chosen as an IAC target as it is stably expressed at low mRNA transcript levels in humans [63]. The use of a constitutively expressed low copy number endogenous control should ensure that the IAC developed will not out-compete the primary targets being amplified in the real-time NASBA assays which will reduce the possibility of false negative reporting. Furthermore, TBP has previously been described as suitable and robust endogenous control for use with human blood [63]. By incorporating an IAC targeting an endogenous gene transcript present in human whole blood, the assays developed in this study have the potential to be applied directly to whole blood samples facilitating their use in a clinical setting. This would allow the end user to control for each component of the test including sample preparation, amplification, hybridisation and detection. Each duplex real-time NASBA reaction was spiked with 25 ng of total human RNA which was the typical concentration of human RNA recovered from a 30 µl sample of whole blood in this study. Our methodology was optimised to a 30 µl equivalent volume of whole blood as a similar volume of blood has previously been demonstrated to be suitable for automated on-disc total RNA extraction for infectious disease based diagnostics [29]. Such on-disc devices have the potential to be integrated into LOC or POC platforms which will facilitate sample-to-answer diagnostic for RNA detection in whole blood. The rapid duplex real-time NASBA diagnostics assays developed in this study were experimentally validated against a large panel of culture collection isolates and clinical isolates and determined to be 100% specific for the target species. Subsequently, the LOD was

determined for each duplex real-time NASBA diagnostics assay. Multiplex nucleic acid *in vitro* amplification diagnostics assays can be less experimentally sensitive than equivalent monoplex diagnostics assays as a consequence of interactions between primer sets or *in vitro* amplification bias [64]. Reduced LOD in multiplex NASBA diagnostics assays compared to monoplex NASBA diagnostics assays have been reported [65-67]. However, in our study the LODs achieved were comparable in both monoplex and duplex format demonstrating the robustness of each of the real-time NASBA diagnostics assays developed.

The study presented here focuses on the development of highly robust internally controlled real-time NASBA assays for the rapid detection of the predominant etiological agents of bacterial meningitis. To date these have been experimentally validated on a broad range of culture collection strains and blood culture positive clinical isolates with promising results in terms of specificity and LOD. Additional work is now required to demonstrate and evaluate the true clinical utility of the methodologies developed for use directly on human whole blood samples. This would demonstrate the potential advantage of these assays over existing diagnostic methodologies and also determine their applicability for incorporation on to low cost LOC devices for near patient testing in the future.

Table 4.1: Nucleotide sequences of oligonucleotide primers and molecular beacon probes.

Probe/ Primer	Gene Target	Function	DNA sequence (5' – 3')	Nucleotide position	Genbank accession no.	Final Concentration (µM)
phoB F1	*phoB*	*H. influenzae phoB* Sequencing forward primer	CTGATAGTTGAAGATG	16-31	CP002277.1	0.4 µM
phoB R1	*phoB*	*H. influenzae phoB* Sequencing reverse primer	TCATTGTTTATCTCGT	681-696	CP002277.1	0.4 µM
Neisseria F	*ssrA*	*Neisseria ssrA* Sequencing forward primer	GGCGACCTTGGTTTCGACG	4-22	AE002098.2	0.3 µM
Neisseria R	*ssrA*	*Neisseria ssrA* Sequencing reverse primer	TCGAACCCCCGTCCGAAAG	327-345	AE002098.2	0.3 µM

phoB P1	*phoB*	*H. influenzae phoB* forward primer	AATTCTAATACGACTCACTATAGGGAGAAGGGTAGTCATCAGCCCCTGCA	285-303	CP002277.1	0.2 µM
phoB P2	*phoB*	*H. influenzae phoB* reverse primer	GGTCGTTCAGGTATACAA	169-186	CP002277.1	0.2 µM
phoB MB	*phoB*	*H. influenzae phoB specific* hybridization probe	FAM – CCGAGTAAGCTATGCTGCGATTCCACTCGG – BHQ1	213-230	CP002277.1	0.1 µM
Nmen P1	*ssrA*	*N. meningitidis* forward primer	AATTCTAATACGACTCACTATAGGGAGAAGGTCCTCTACAAAGCGTTCTACA	305-325	AE002098.2	0.2 µM
Nmen P2	*ssrA*	*N. meningitidis* reverse primer	CCCGTAAAACACTGAATTCAAAT	63-85	AE002098.2	0.2 µM
Nmen MB	*ssrA*	*N. meningitidis ssrA* specific hybridization probe	HEX-CGATCGAACTGGTTTCCAAAAGGCCTCGATCG-DAB	236-255	AE002098.2	0.1 µM
Spn P1	*lepA*	*S. pneumoniae* forward primer	AATTCTAATACGACTCACTATAGGGAGAAGGCATACTCAAGACGCTGAGGAA	1792-1812	AE007317.1	1 µM
Spn P2	*lepA*	*S. pneumoniae* reverse primer	GACACAAGATTGTCGCTCGTACTGATA	1628-1654	AE007317.1	1 µM
Spn MB	*lepA*	*S. pneumoniae lepA* specific hybridization probe	ROX-CGATCGACGCATGAAATCCATCGGATCAGTTCGATCG -BHQ2	1749-1773	AE007317.1	0.5 µM
TBP P1	*TBP*	IAC - NASBA forward primer	AATTCTAATACGACTCACTATAGGGAGAAGGTGAGCACAAGGCCTTCTA	1088-1105	NC000006.12	0.2 µM
TBP P2	*TBP*	IAC - NASBA reverse primer	AACAGTCCAGACTGGC	962-977	NC000006.12	0.2 µM
TBP MB	*TBP*	IAC – specific hybridization probe	CY5-CTCGAGAGCTGTGATGTGAAGTTTCCCTCGAG-BHQ2	1060-1079	NC000006.12	0.1 µM

Table 4.2: Bacterial species and strains included in this study

Organism	**Strain** [a]	**Specificity**[d]		
		H. influenzae	*N. meningitidis*	*S. pneumoniae*
H. influenzae reference strains				
H. influenzae type a	NCTC 8465	+	/	/

H. influenzae type b	DSMZ 4690[b]	+	/	/
H. influenzae type c	NCTC 8469	+	/	/
H. influenzae type d	DSMZ 11121[b]	+	/	/
H. influenzae type e	NCTC 8472	+	/	/
H. influenzae type f	DSMZ 10000[b]	+	/	/
H. influenzae NTHi	CCUG 58365	+	/	/
H. influenzae clinical isolates				
H. influenzae NT (Source:	6 isolates	+	/	/
H. influenzae untyped (Source: Sputum)	6 isolates	+	/	/
H. influenzae untyped (Source: Eye/Ear swab)	4 isolates	+	/	/
Non- *H. influenzae* strains				
H. haemolyticus	CCUG 24149	-	/	/
H. haemolyticus	CCUG 36015	-	/	/
H. haemolyticus	CCUG 36016	-	/	/
H. haemolyticus	CCUG 15642	-	/	/
H. haemolyticus	CCUG 15312	-	/	/
H. haemolyticus	NCTC 10839	-	/	/
H. haemolyticus	CCUG 12834	-	/	/
H. haemolyticus	CDC-M21127	-	/	/
H. ducreyi	NCTC11479	-	/	/
H. parahaemolyticus	DSMZ 21417	-	/	/
H. parainfluenzae	DSMZ 8978	-	/	/
H. paraphrohaemolyticus	DSMZ 21451	-	/	/
H. pittmaniae	DSMZ 21203	-	/	/
Haemophilus sp.	CCUG 34110	-	/	/
Actinobacillus suis	DSMZ 22433	-	/	/
Actinobacillus	DSMZ 13472	-	/	/
Aggregatibacter aphrophilus	NCTC 11096	-	/	/
A. aphrophilus	NCTC 10558	-	/	/
Aggregatibacter segnis	NCTC 10977	-	/	/
N. meningitidis reference strains				
N. meningitidis serogroup a	NCTC 10025[c]	/	+	/
N. meningitidis serogroup a	NCTC 3375	/	+	/
N. meningitidis serogroup a	NCTC 3372	/	+	/
N. meningitidis serogroup b	ATCC13090	/	+	/
N. meningitidis serogroup c	ATCC 13102	/	+	/
N. meningitidis serogroup c	DSMZ 15464[c]	/	+	/
N. meningitidis serogroup	NCTC 11203	/	+	/
N. meningitidis clinical isolates				

N. meningitidis serogroup b (Source: Blood)	5 isolates	/	+	/
N. meningitidis untyped (Source: Sputum)	1 isolates	/	+	/
N. meningitidis untyped (Source: Eye/Ear swab)	2 isolates	/	+	/
Non- *N. meningitidis* strains				
N. animaloris	DSMZ 21642[c]	/	-	/
N. bacilliformis	DSMZ 23338[c]	/	-	/
N. elongata subsp. *elongata*	DSMZ 17712[c]	/	-	/
N. elongata subsp.	DSMZ 23337[c]	/	-	/
N. elongata subsp.	DSMZ 17632[c]	/	-	/
N. flavescens	DSMZ 17633[c]	/	-	/
N. gonorrhoeae	DSMZ 9189[c]	/	-	/
N. gonorrhoeae	ATCC 19424[c]	/	-	/
N. lactamica	DSMZ 4691[c]	/	-	/
N. mucosa	DSMZ 17611[c]	/	-	/
N. perflava	DSMZ 18009[c]	/	-	/
N. polysaccharea	DSMZ 22809[c]	/	-	/
N. shayeganii	DSMZ 22246[c]	/	-	/
N. sicca	DSMZ 17713[c]	/	-	/
N. subflava	DSMZ 17610[c]	/	-	/
N. wadsworthii	DSMZ 22247[c]	/	-	/
N. weaveri	DSMZ 17688[c]	/	-	/
N. zoodegmatis	DSMZ 21643[c]	/	-	/
S. pneumoniae reference strains				
S. pneumoniae serotype 1	DSMZ 20566	/	/	+
S. pneumoniae serotype 9V	DSMZ 11865	/	/	+
S. pneumoniae serotype 23F	DSMZ 11866	/	/	+
S. pneumoniae clinical isolates				
S. pneumoniae untyped (Source: Blood)	2 isolates	/	/	+
S. pneumoniae untyped (Source: Sputum)	5 isolate	/	/	+
S. pneumoniae untyped (Source: Eye/Ear swab)	2 isolates	/	/	+
Non- *S. pneumoniae strains*				
S. agalactiae	BCCM 15086	/	/	-
S. anginosus	DSMZ 20563	/	/	-
S. australis	DSMZ 15627	/	/	-
S. bovis	DSMZ 20480	/	/	-
S. canis	DSMZ 20715	/	/	-

S. constellatus	DSMZ 20575	/	/	-
S. cristatus	DSMZ 8249	/	/	-
S. downei	DSMZ 5635	/	/	-
S. dysgalactiae subsp.	DSMZ 6176	/	/	-
S. equi subsp. equi	DSMZ 20561	/	/	-
S. gordonii	DSMZ 6777	/	/	-
S. infantis	DSMZ 12492	/	/	-
S. intermedius	DSMZ 20573	/	/	-
S. mitis	DSMZ 12643	/	/	-
S. mutans	DSMZ 20523	/	/	-
S. oralis	DSMZ 20066	/	/	-
S. perosis	DSMZ 12493	/	/	-
S. porcinus	DSMZ 20725	/	/	-
S. pseudopneumoniae	DSMZ 18670	/	/	-
S. pyogenes	DSMZ 20565	/	/	-
S. sanguinis	DSMZ 20567	/	/	-
S. sinensis	DSMZ 14990	/	/	-
S. vestibularis	DSMZ 5636	/	/	-

[a] NCTC = National Collection of Type Cultures; * DSMZ = The German Collection of Microorganisms; *CCUG = Culture Collection, University of Göteborg, Sweden; *CDC = Centre for Disease Control; *BCCM = Belgian Coordinated Collections of Microorganisms

[b] *phoB* gene sequence data was generated for each of these *H. influenzae* strains using primers outlined in Table 4.1

[c] *ssrA* gene sequence data was generated for each of these *N. meningitidis* strains using primers outlined in Table 4.1

[d] + = Positive; - = negative; / = not tested for

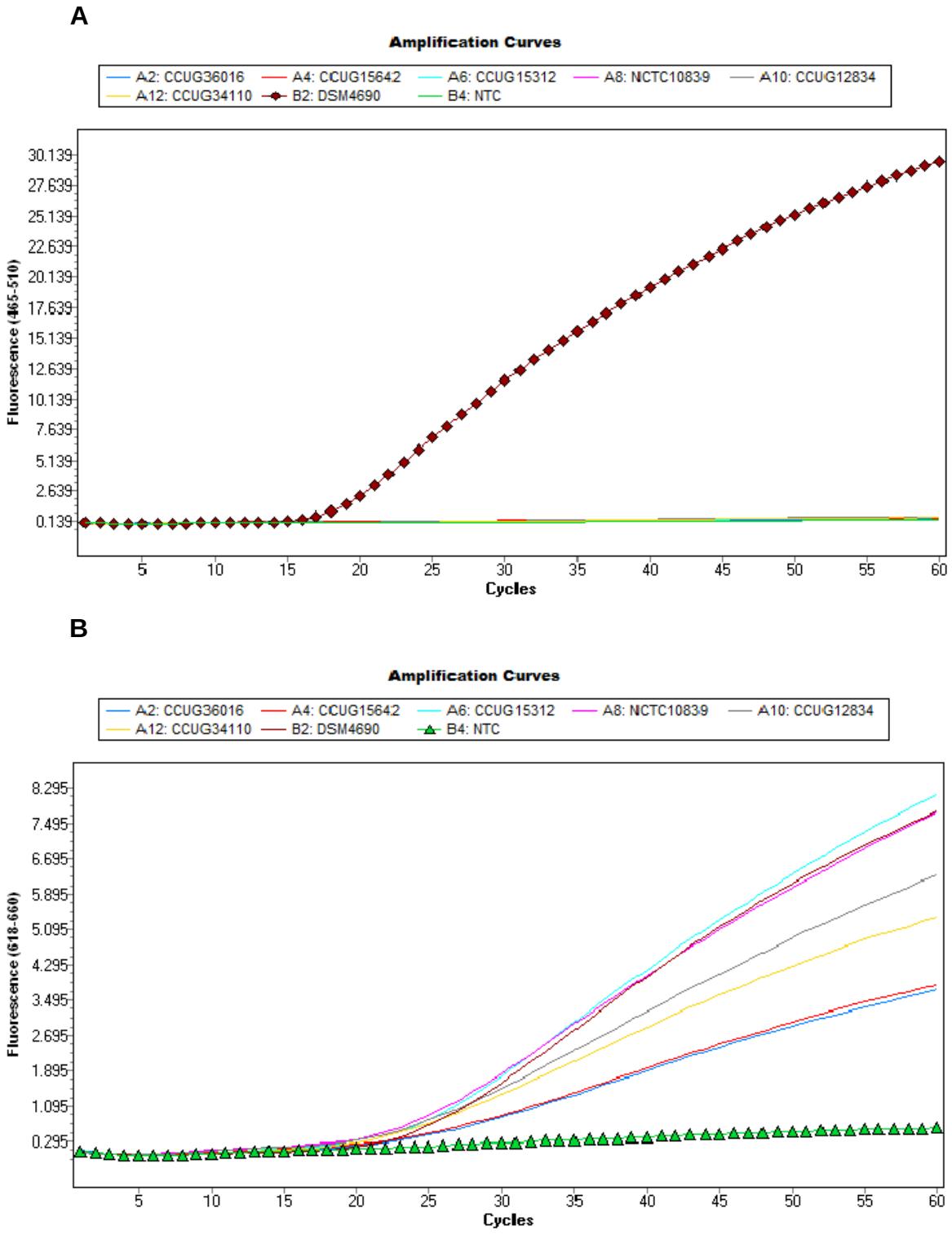

Figure 4.1: Amplification curves for duplex real-time NASBA diagnostics assays. Figure 4.1A: Real-time amplification curves for *H. influenzae* targeting the *phoB* gene in the FAM channel (465-510nm). No non-*H. influenzae* species were detected by the assay. Figure 4.1B: Real-time amplification curves for IAC in the Cy5 channel (618-660nm) with NTC highlighted with triangles. All samples detected except the no template control (NTC).

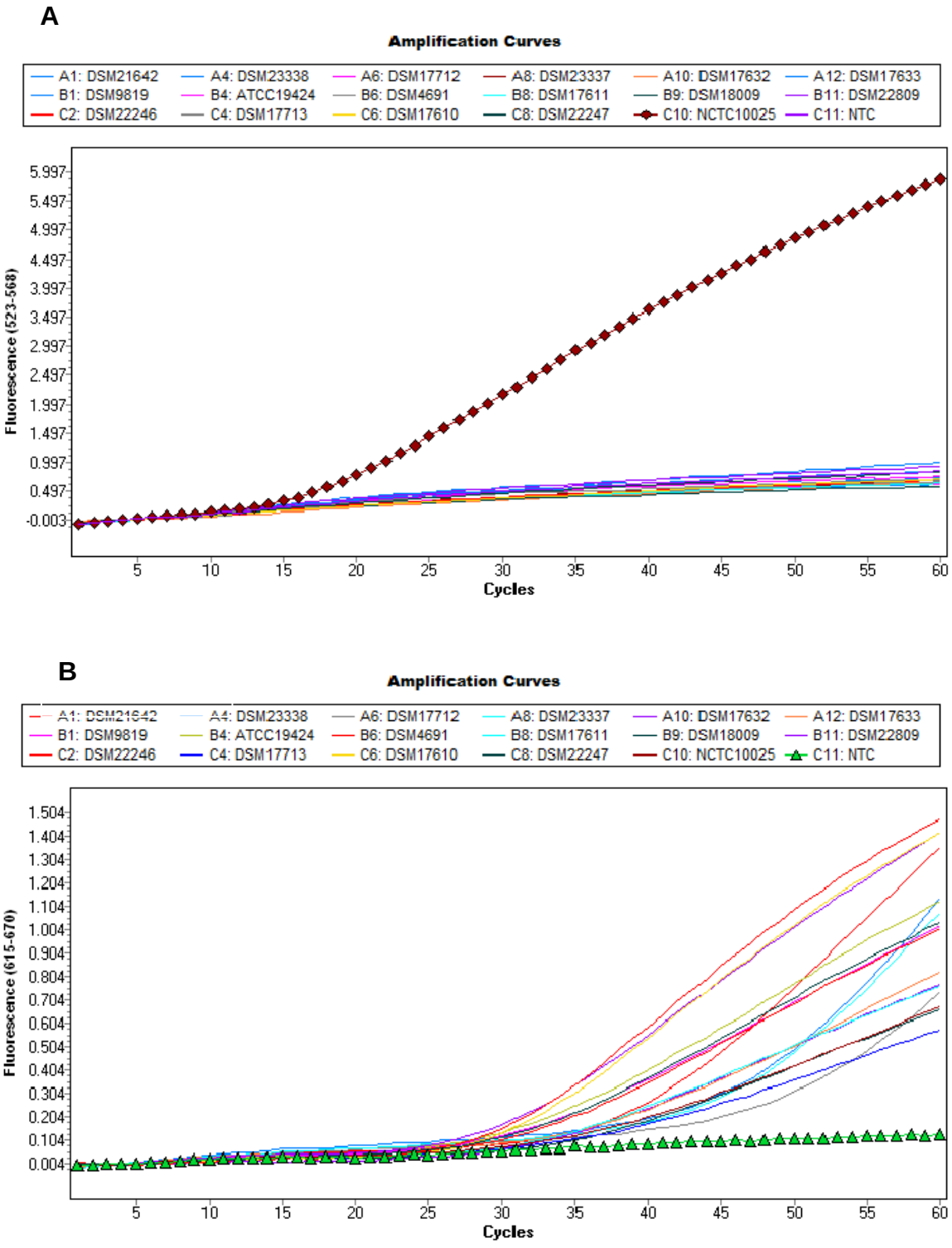

Figure 4.2: Amplification curves for duplex real-time NASBA diagnostics assay. Figure 4.2A: Real-time amplification curves for *N. meningitidis* (Diamonds) targeting the *ssrA* gene in the HEX channel (523-548nm). No non- *N. meningitidis* species were detected by the assay. Figure 4.2B: Real-time amplification curves for IAC in the Cy5 channel (615-670nm) with NTC highlighted with triangles. All samples detected except the no template control (NTC).

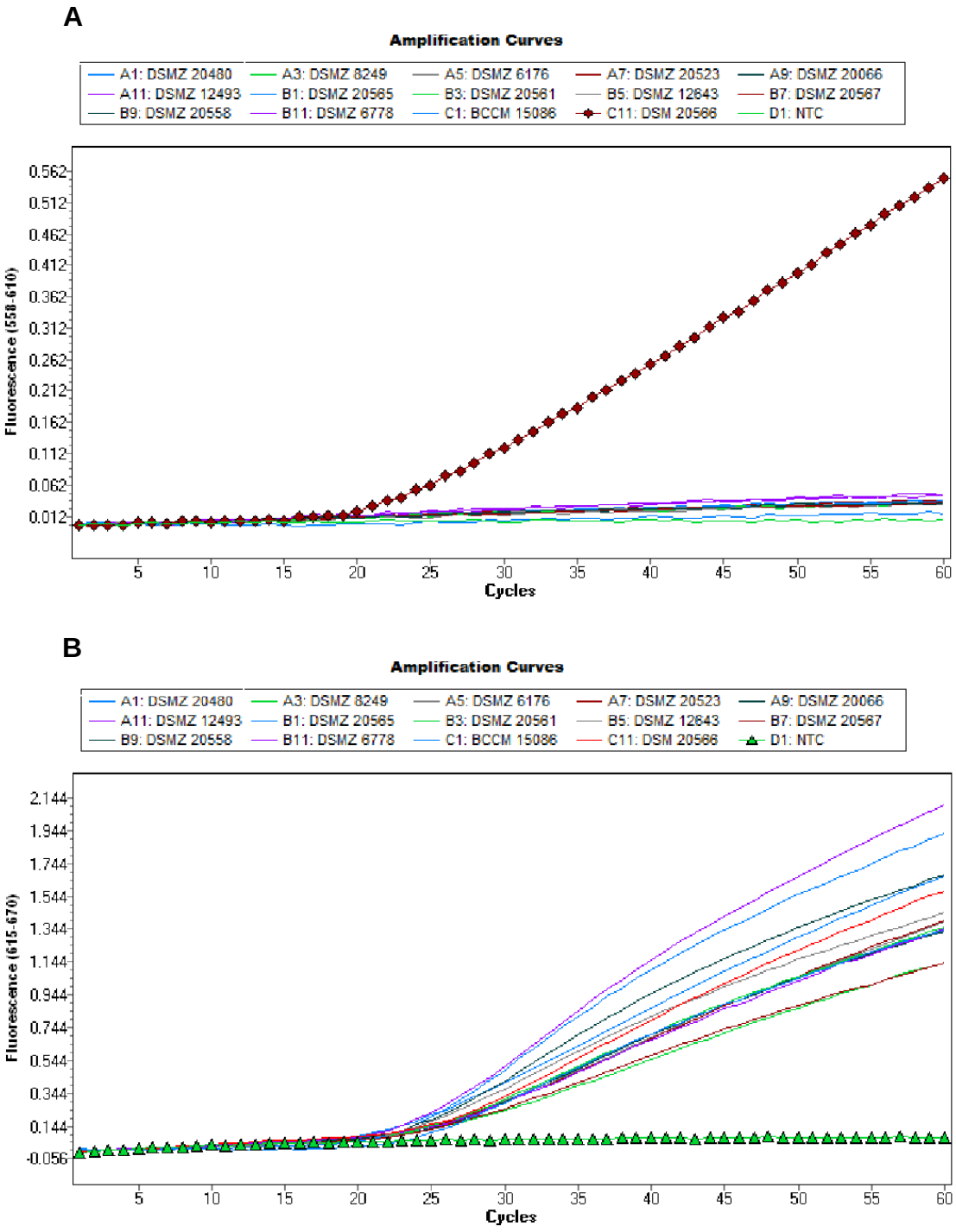

Figure 4.3: Amplification curves for duplex real-time NASBA diagnostics assay. Figure 4.3A: Real-time amplification curves for *S. pneumoniae* (Diamonds) targeting the *lepA* gene in the ROX channel (523-548nm). No non- *S. pneumoniae* species were detected by the assay. Figure 4.3B: Real-time amplification curves for IAC in the Cy5 channel (615-670nm) with NTC highlighted with triangles. All samples detected except the no template control (NTC).

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Chapter 5

Concluding Remarks and Future Directions

5.1. General Conclusions

The overall aim of this thesis was to design, develop, optimize and experimentally validate an internally controlled isothermal real-time nucleic acid *in-vitro* amplification assays for the rapid and species specific detection and identification of predominant microorganisms associated with bacterial meningitis, *Haemophilus influenzae, Neisseria meningitidis* and *Streptococcus pneumoniae*. This was achieved using a sequential experimental approach consisting of 3 studies (**Chapters 2-4**).

In **Chapter 2**, the tmRNA transcript encoded for by the *ssrA* gene and the leader peptidase A (*lepA*) mRNA transcript were evaluated as potential targets for the species specific identification of *H. influenzae, N. meningitidis* and *S. pneumoniae* using real-time NASBA. Both the *ssrA* gene and the *lepA* gene have previously been demonstrated as suitable and versatile diagnostics targets for nucleic acid based identification of microbial pathogens and in particular, *lepA* has been established as a useful target in real-time a real-time NASBA assay.

In summary, an assay targeting a nucleic acid region of the *H. influenzae ssrA* gene associated tmRNA transcript was designed and developed. *In-silico* analysis of publicly available nucleotide sequence data and sequence data generated in this study was carried out using ClustalW to determine regions of variability between the *H. influenzae ssrA* sequence and the *ssrA* sequences of its closely related species. This initial *in-silico* analysis established sufficient heterogeneity to distinguish *H. influenzae* from its most closely related species. Subsequently, a real-time NASBA assay was designed targeting the tmRNA transcript of *H. influenzae*. However, similar to other published assays for the species specific detection of *H. influenzae*, the real-time NASBA assay developed in this study cross reacted with the closely related species, *Haemophilus haemolyticus*. During the course of this study, whole genome sequence data became available for 6 *H. haemolyticus* strains which enabled further *in-silico* nucleotide sequence analysis. This analysis established insufficient heterogeneity within the *ssrA* gene to allow for the design of an oligonucleotide probe that would discriminate *H. influenzae* from *H. haemolyticus*. The *lepA* mRNA transcript was then evaluated as an alternative diagnostic target for this purpose. *In-silico* analysis established insufficient nucleotide sequence heterogeneity within the

lepA gene to accurately identify all *H. influenzae* species and differentiate it from *H. haemolyticus*. Despite this, a real-time NASBA assay was developed targeting *lepA* mRNA for comparison against the real-time NASBA assay targeting *H. influenzae* tmRNA. Testing of the real-time NASBA assay targeting *lepA* mRNA against a panel of non-target closely related species revealed that the assay cross reacts with *H. haemolyticus* in addition to *Histophilus somnus*. Furthermore, this assay is not 100% inclusive, as it is unable to detect 2/9 *H. influenzae* strains tested. We hypothesized that due to the significant inter-strain *lepA* sequence variation impacting NASBA RNA amplicon secondary structure and the isothermal nature of real-time NASBA, primers and molecular beacons may not be able to hybridize to the RNA amplicon, in turn impacting real-time NASBA assay efficiency.

tmRNA and *lepA* mRNA were also evaluated as potential diagnostic nucleotide sequence targets for the species specific identification of *N. meningitidis* and *S. pneumoniae*, respectively. Initial *in-silico* analysis revealed sufficient nucleotide sequence heterogeneity within the *ssrA* and *lepA* gene sequences for both *N. meningitidis* and *S. pneumoniae* and their most closely related species to allow for accurate identification of these microorganisms of interest. Subsequently, real-time NASBA diagnostic assays were developed targeting *N. meningitidis* tmRNA and *S. pneumoniae lepA* mRNA. Both assays were determined to be 100% specific for the target species when tested against a panel of inclusivity strains and did not cross react with non-target closely related species.

These experiments established that tmRNA and *lepA* mRNA have potential as diagnostic targets for the species specific identification of *N. meningitidis* and *S. pneumoniae*, respectively. Further experimental validation of these diagnostic targets is described in **Chapter 4**. However, *in-silico* analysis and testing in real-time NASBA assays established that neither the tmRNA transcript nor the *lepA* mRNA transcript can be used as diagnostic targets to accurately identify *H. influenzae* and differentiate it from its most closely related species. As a result, further study was carried out to identify and establish a suitable diagnostic nucleotide sequence target which can specifically detect and unambiguously identify *H. influenzae* (**Chapter 3**).

In **Chapter 3**, optimised, internally controlled, duplex real-time PCR assays are described for the detection and accurate identification of *H. influenzae* targeting two

novel gene targets identified by whole genome comparative analysis of *H. influenzae* and *H. haemolyticus*.

Publicly available whole genome sequence data for 22 *H. influenzae* and 6 *H. haemolyticus* was analysed to identify novel diagnostic targets unique to *H. influenzae* and which are absent in *H. haemolyticus*. Using online bioinformatics tools such as Mauve, Emboss Union, RAST and WebAct, 6 gene targets (*pstS, pstC, pstA, pstB, phoR* and *phoB*) were identified as having potential to distinguish *H. influenzae* from *H. haemolyticus*. Further *in-silico* analysis using BLAST, established that *pstA* and *phoB* contain the greatest nucleotide sequence variation when compared with other non- *H. influenzae* bacterial species and contain the least observable *H. influenzae* inter-strain variation. Initially, real-time PCR assays were designed and developed targeting, *pstA* and *phoB* and a non-competitive IAC specific for the *ssrA* gene of *Bacillus subtillis* subsp. *spizizenii* was incorporated in to each of the assays designed in order to verify the accuracy of the results and avoid the possible reporting of negative results. This IAC was chosen as it was previously developed in-house and determined as a useful IAC for use with real-time PCR. Furthermore, BLAST analysis established no significant nucleotide sequence homology was present to any member of the *Haemophilus* species.

The duplex real-time PCR assays designed were determined empirically to be 100% specific for *H. influenzae* with LODs of 10.49 and 6.49 GE for real-time PCR assays targeting *pstA* and *phoB* respectively with 95% probability.

Furthermore, both these novel real-time PCR assays were compared to a previously published real-time PCR assay targeting the *fucK* gene of *H. influenzae* [1]. This demonstrated that all three gene targets can distinguish *H. influenzae* from *H. haemolyticus*. However, both the *phoB* and *pstA* assays are more specific for the detection of *H. influenzae* strains compared to the *fucK* assay. Three *H. influenzae* clinical isolates identified by MALDI-TOF MS and the two diagnostic assays developed in this study were undetected by the *fucK* assay. Furthermore, one culture collection *H. influenzae* strain (CCUG 58365) was also not detected by the *fucK* assay due to a deletion of the fucose operon as previously reported in this strain [2].

Chapter 4 describes three duplex real-time NASBA assays, incorporating an endogenous internal amplification control (IAC), for the detection of predominant

microorganisms associated with bacterial meningitis, *H. influenzae, Neisseria meningitidis* and *Streptococcus pneumoniae*.

For all three duplex real-time NASBA assays developed in this study, an endogenous non-competitive IAC was incorporated targeting the stably expressed transcript 1 of the *Homo Sapiens TBP* gene present in whole blood. By targeting the *TBP* RNA transcript, the developed assays have the potential to be used directly on whole blood samples in a clinical setting thus allowing the user to monitor each component of the diagnostic test, to verify the accuracy of the results obtained and to prevent reporting of false negatives.

The *phoB, ssrA* and *lepA* genes were further experimentally validated in this study and the suitability of their respective RNA transcripts for use in a duplex real-time NASBA diagnostics assay was successfully demonstrated for the specific detection of *H. influenzae* (*phoB*), *N. meningitidis* (*ssrA*) and *S. pneumoniae* (*lepA*). Each of these novel diagnostic targets were again experimentally validated against an extensive panel of *H. influenzae, N. meningitis* and *S. pneumoniae* isolates, non-target closely related bacteria and were determined to be 100% specific for the detection of the target species in duplex real-time NASBA assay format. LOD of the *H. influenzae, N. meningitidis and S. pneumoniae* duplex real-time NASBA assays was determined using Probit regression analysis to be 55.36, 0.99 and 57.24 CE, respectively.

In summary, the duplex real-time NASBA assays described in this study are the first description of a multiplex real-time NASBA assays for the rapid detection of *H. influenzae, N. meningitidis* and *S. pneumoniae*, incorporating an independent non-competitive IAC assay amplifying a gene transcript from total RNA.

In addition to the studies carried out in the main body of this thesis, the potential use of real-time NASBA in an on-chip Point-Of-Care (POC) device was demonstrated using the real-time NASBA diagnostics assay targeting *H. influenzae* tmRNA described in chapter 2 (**Appendix A**, **Appendix B**). The study carried out in Appendix A describes total RNA purification from *H. influenzae* cell lysates on a disc-based module for rotationally controlled solid-phase purification of RNA. The integrity of the total RNA purified was then validated using the real-time NASBA targeting *H. influenzae* tmRNA and total RNA purified on-disc and amplified was

established as comparable to bench-top purified RNA. Appendix B describes the NASBA amplification of *H. influenzae* tmRNA on a centrifugal microfluidic "Lab-on-a-Disc" (LoaD) system using non-contact heating and fluorescence end-point detection. To verify the sensitivity of the system, LOD studies were carried out and a current LOD of between 500 and 100 CE on-disc was established.

5.2. Future Directions

Real-time nucleic acid based *in-vitro* amplification assays described in this thesis can accurately and unambiguously identify the predominant microorganisms associated with bacterial meningitis, namely, *H. influenzae*, *N. meningitidis* and *S. pneumoniae.* While real-time nucleic acid based *in-vitro* amplification assays developed in this study offer several advantages over other tests described in literature, further improvements or optimization of the assays can be made in the future.

Each assay in its current state has been experimentally validated against an extensive panel of culture collection and culture confirmed clinical isolates, demonstrating the potential to be useful in a clinical setting. However, there is a need to evaluate the assays developed directly on whole blood clinical samples without the requirement for culture. If these assays can be used directly on blood samples it offers a number of significant advantages over current diagnostic methods which rely on culture. It would allow for the more rapid diagnosis and implementation of an optimal therapeutic regimen in a patient with suspected bacterial meningitis and potentially improve the overall outcome for that patient. Furthermore, it would allow for accurate epidemiology and vaccine efficacy studies to be performed and enable improved monitoring of invasive disease caused by these microorganisms.

In the future, combining the three duplex real-time NASBA assays described in Chapter 4 in to a single multiplex real-time NASBA assay for the detection of all three microorganisms and an IAC would be advantageous. This would allow for only one test to be performed to detect all three pathogens simultaneously, thereby reducing time and costs associated with the test. This involves significant further optimization of the assays developed to ensure specificity and sensitivity are not diminished or reduced, particularly as a decrease in sensitivity in multiplex NASBA assays has been reported previously [3-5].

As part of the overall aim of this study, on-chip NASBA amplification (**Appendix A**) and on-chip RNA purification (**Appendix B**) have been demonstrated and validated using a real-time NASBA assay developed in this study**.** This work demonstrates the potential application of real-time NASBA assays described in this thesis in a POC lab on chip device for rapid diagnosis of bacterial meningitis. However, further advances could also be made to improve on-chip NASBA detection, in order to perform real time heating and detection, in addition to incorporating biological sample preparation, with the aim to progress toward a fully integrated sample in – answer out diagnostics technology. In theory, a rapid fully integrated POC diagnostic test that meets the ASSURED characteristics, i.e. Affordable, Sensitive, Specific, User-friendly, Rapid, Equipment free, and Delivered [6], would enable the determination and identification of a bacterial pathogen in a patient with suspected meningitis, reducing the time spent in hospital and allowing for the more rapid administration of an appropriate pathogen specific antibiotic treatment regime. Furthermore, the application of NASBA on a fully integrated POC, that requires a low fluid volume sample, would potentially enable home or physician based use of a compact, portable, cost effective, rapid diagnostic test by non-specially trained individuals. However, the transition of POC diagnostics from a laboratory setting to a real product for use in the healthcare sector is often difficult, prolonged, and requires significant capital for development. Each year, only one paper is published describing clinical evaluation of POC devices for every 60 papers published describing laboratory testing of devices aimed toward POC diagnostics [7].

In addition to funding requirements for the initial design and fabrication of these fully integrated on-chip diagnostic tests, development of POC technologies face many other challenges including: the requirement for lengthy clinical validation of the product through trials, the need to perform multi-centre studies that demonstrate robustness of the test under different user conditions and the need to change how clinical practice is performed, as well as train personnel on how to use the POC device and interpret the results. Furthermore there is a requirement to comply with complex country specific health authority regulations [8]. For example, in Europe, a diagnostics product can be commercialized with a CE mark, which is obtained through compliance to the Directive of *In-Vitro* Diagnostic Medical Devices,

98/79/EC, without any specific regulations for POC use. While in the U.S, a POC diagnostic device must obtain regulatory approval from the FDA and in some instances require a CLIA waiver for use locations without laboratory-trained personal and equipment [8].

As a consequence, a cost effectiveness analysis needs to be carried out to determine the benefit of these tests to the healthcare sector before embarking on steps toward commercialization. This cost effectiveness analysis should compare costs in monetary terms and in non-monetary terms. In addition it needs to take into account both direct monetary costs e.g. actual cost to perform the test and indirect monetary costs e.g. a delay in the time taken to diagnose and treat a patient may lead to unnecessary hospital admissions and in turn, the need for additional staffing [9]. For example, a definitive POC test that significantly improves the clinical outcome for a patient with a life threatening disease, such as bacterial meningitis; reduces or prevents the need for the expensive, ineffective treatment; and eliminates expensive routine confirmatory testing, would be much more cost effective than a POC test that only marginally improves public health.

5.3. References

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Appendix A:

Solvent-selective routing for centrifugally automated solid-phase purification of RNA towards a sample-to-answer molecular diagnostic

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RESEARCH PAPER

Solvent-selective routing for centrifugally automated solid-phase purification of RNA

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Abstract We present a disc-based module for rotationally controlled solid-phase purification of RNA from cell lysate. To this end, multi-stage routing of a sequence of aqueous and organic liquids into designated waste and elution reservoirs is implemented by a network of strategically placed, solvent-selective composite valves . Using a bead-based stationary phase at the entrance of the router, we show that total RNA is purified with high integrity from cultured MCF7 and T47D cell lines, human leucocytes and *Haemophilus influenzae* cell lysates. Furthermore, we demonstrate the broad applicability of the device through the in vitro amplification of RNA purified on-disc using RT-PCR and NASBA. Our novel router will be at the pivot of a forthcoming, fully integrated and automated sample preparation system for RNA-based analysis .

Keywords Lab-on-a-disc · RNA purification · Solvent-selective valves · Molecular diagnostics

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1 Introduction

Microfluidic lab-on-a-chip technologies have been shown to automate and reduce the time-to-result of a wealth of bioanalytical assays, and ultimately enable their deployment at the point of need. Whilst a range of detection techniques remains implemented on-chip, the integration of sample preparation with detection has been a bottleneck in the development of microfluidic devices for nucleic acid testing (Foudeh et al. 2012; McCalla and Tripathi 2011). In many instances, RNA is a preferable diagnostics target, such as the detection of retroviruses or in the expression analysis of genes. Also, in assays for the detection of pathogens, RNA can be used to obtain information on pathogen viability as RNA is less stable than the DNA. Furthermore, since RNA molecules are typically present in multi-copy (100–1,000 s/per cell), the potential for detection without in vitro enzymatic amplification is possible. One such class of RNA species that have recently received attention for their diagnostic potential is micro-RNAs (miRNAs). miRNAs are small non-coding RNAs that are involved in modulating gene expression at the transcriptional and post-transcriptional level. Their dysregulation has been shown to be associated with a wide variety of human diseases, including cancer (Mitchell et al. 2008; Heneghan et al. 2010), diabetes and cardiovascular diseases. In terms of bacterial RNA diagnostics, ribosomal RNA (rRNA) and in particular 16S

rRNA remains the gold standard. Other functional high-copy number bacterial RNA molecules such as transfer messenger RNA (tmRNA) encoded by the *ssrA* gene has been demonstrated to be a useful marker in bacterial diagnostics (O'Grady et al. 2009; Clancy et al. 2012).

The automation of microfluidic platforms often involves the use of costly peripheral equipment, such as syringe pumps, external valves and pressure controllers. These chip in a laboratory devices require complex, multi-stage off-chip liquid handling steps, thereby severely limiting their widespread adoption in clinical testing. Centrifugal microfluidic Lab-on-a-Disc (LoaD) systems bear the potential to integrate sample preparation with detection to create full-fledged sample-to-answer devices (Madou et al. 2006; Ducrée et al. 2007). A simple rotary microsystem developed by Park et al. (2012a) enabled the purification of RNA from viral lysate with frequency-controlled release of reagents. Using centrifugal microfluidics, Cho et al. presented a device capable of one-step DNA extraction of pathogen-specific DNA from whole blood (Cho et al. 2007). Also multiplexing of immunoassays on-disc (Lee et al. 2009; Park et al. 2012) and parallelized biochemical analysis (Lee et al. 2011) have been demonstrated.

As all liquids resident on the disc are simultaneously exposed to the same centrifugal field, valving technologies are pivotal for establishing a sequence of liquid handling steps. Over the years, the scientific community has pioneered a repertoire of valving schemes, which are commensurate with the rotational nature of the lab-on-a-disc platform. Amongst the central aspects governing the choice of the valving scheme are the upper limit of tolerable spin frequencies (e.g. during *multi-stage* sample prep), the open-state hydrodynamic resistance and vapour barrier properties (e.g. for storage and release of liquid reagents) on behalf of flow control and the complexities involved in fabrication and actuation on the hardware side. Valve actuation on LoaD platforms can be categorized into two schemes: the first type of valve is controlled by the system-innate spindle motor such as capillary burst valves (Ducrée et al. 2007; Zoval and Madou 2004) or siphons (Schembri et al. 1995; Steigert et al. 2007; Nwankire et al. 2013); the alternative, externally actuated schemes often involve the manipulation of a sacrificial material by an external stimulus like heat, for instance, thermally actuated wax valves (Park et al. 2007). Recent reports have utilized such wax valves for the on-disc integration of biochemical assays and immunoassays (Lee et al. 2009, 2011). To avoid the manufacturing complexity of incorporating on-disc heat-induced valves, Mark et al. (2008) introduced a pneumatic microvalve using a thin burstable foil. Zehnle et al. (2012) swiftly balance the pressures on a spinning disc and use the advancing liquid as a valve to achieve inward pumping. In another study, Gorkin et al. III (2012) integrated water-dissolvable films as once-off valve seals. By trapping air between this dissolvable film and the liquid, they implemented centrifugo-pneumatic valving and gating of on-board-stored reagents for a wide range of burst frequencies. We expand upon these dissolvable film valves and develop automated, solvent-selective routing on a LoaD platform.

For solid-phase purification (SPP) of nucleic acids, we here exploit the well-established Boom chemistry (Boom et al. 1990). Fluidic routing to direct flow to a selected output at a bifurcation between a waste and an elution outlet is critical for centrifugally implemented automation. Kim et al. (2008) developed a flow switch by using a capillary valve upstream of an open chamber and a unique 3D junction geometry. A similar router, solely controlled by the rotationally actuated hydrodynamic Coriolis pseudo-force, was reported by Brenner et al. (2005). This virtual routing concept was further sophisticated by Haeberle et al. (2007) who successfully extracted DNA from calf thymus using silica beads by alternating the sense of rotation. Based on a droplet deflection Coriolis-force driven router, they recovered 16 % of the initial nucleic acid. A recent study by Jung reports 81 % capture efficiency of RNA from lysed influenza A H3N2 virus using silane, i.e. TEOS-treated glass beads on-disc (Jung et al. 2013) . Alternatively, 100 % efficient automated extraction of human genomic DNA is demonstrated by Kloke et al. (2014) who implement novel ball-pen pierceable seals to route the sample lysate through an integrated silica membrane in a Lab Tube platform. Whilst these systems have contributed greatly to the development of SPP, to date, reliable, high-efficiency and low-complexity routing of flows (e.g. aqueous or organic flows) on LoaD platforms still remains a challenge.

We report a routing scheme which utilizes solvent-selective valving with unidirectional rotational actuation for SPP of RNA, thereby obviating the need for external actuation other than the system-inherent spinning rates. Previously, Kinahan et al. (2014) successfully achieved solid-phase purification of total RNA from MCF7 cell lysates. Multiple dissolvable film valving strategies were presented, which allowed inbuilt control of reagent release for automated sample preparation and integration of laboratory unit operations on LoaD at constant angular velocity. However, the article briefly touches on the quality of the purified samples and the mechanisms of routing. In contrast, here we investigate solid-phase purification of RNA from various cells, study the suitability of the method for further downstream amplifications, and expand on the selective routing through integrated hydrophobic membrane and dissolvable film valves. Our findings will be at the core of a future, fully automated centrifugal microfluidic nucleic acid analyser.

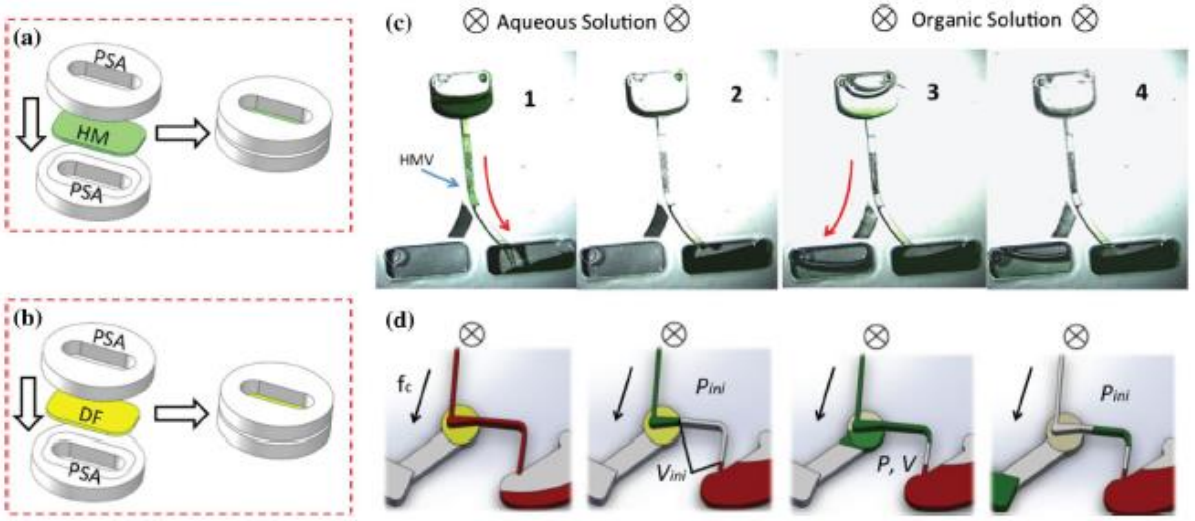

Fig. 1 Routing of flows based on solvent-specific valves in bifurcated microchannels: assembly of composite tabs for hydrophobic membrane (HM, **a**) and dissolvable film (DF, **b**) valves using pressure-sensitive adhesive (PSA). **c** Proof of principle for routing of sequentially loaded aqueous (*middle*) and organic (IPA, *right*) solvents into designated collection chambers using the embedded HM tab (*blue arrow*) in a simple, inverse-Y structure. As the HMV is impermeable to the first aqueous phase, the flow (*red arrow*) deflects through the open channel into the right collection chamber (panels 1 and 2). On the contrary, the second (organic) liquid IPA wets the membrane and flows (*red arrow*) in the left-hand side chamber as the flow resistance of the outlet, governed by the larger cross section and length of the vertical outlet, is significantly smaller than the flow resistance of the tiny channel leading to the alternative right outlet (panel 3 and 4). **d** Schematic of the fluidic capacitance, dissolution of the DF (*yellow*) and routing of the aqueous flows, where P_{ini} is the atmospheric pressure, V_{ini} is the volume of the lateral channel and P with V is their centrifugally compressed counterparts under centrifugal force (f_c), directed from the centre to the periphery of the disc (*black arrows*). The axis of rotation are denoted by ⊗ above each panel (color figure online)

2 Principle of operation

This paper addresses the missing link between upstream, chemically induced cell lysis and 3-phase RNA extraction and downstream nucleic acid analysis techniques which are both well-established bench-top methods that have also been demonstrated microfluidicly using lab-on-a-chip/lab-on-a-disc systems (Linares et al. 2011). The single-step acid guanidinium thiocyanate-phenol-chloroform method, also known as 3-phase RNA extraction, is based on the discovery that RNA (other than DNA and proteins) remains soluble in the acidic aqueous phase (Brenner et al. 2005; Chomczynski and Sacchi 1987). Formulations of acidic guanidinium thiocyanate and phenol are commercially available under various brand names (TRIzol from LifeTechnologies, TRI Reagent from Sigma). In general, the reagent contains phenol that ruptures the cells and guanidinium thiocyanate, a chaotropic salt that strips protein complexes from RNA. The addition of chloroform (or an alternative reagent such as 4-bromoanisole or 1-bromo-3-chloropropane), prior to centrifugation, permits the separation of the non-polar (organic) and polar (aqueous) phases. Due to differences in their solubility, DNA is retained in the organic phase whilst the RNA is concentrated in the aqueous phase, which also contains salt contamination. For accurate downstream analysis of RNA, it is essential that extracted RNA is free of contaminants such as chaotropic salts and phenol (Bustin and Nolan 2004; Tan and Yiap 2009).

Here, we convey the centrifugal microfluidic automation of a 4-stage purification of RNA from the aqueous phase of TRI Reagent-lysed human and bacterial cells. Using human MCF7 breast cancer cells, we validate the device. Subsequently, we demonstrate the applicability of the device for nucleic acid diagnostics with the in vitro enzymatic amplification of miRNAs from cultured T47D cells and human leucocytes using RT-PCR. Furthermore, using nucleic acid based amplification (NASBA) we demonstrate the utility of the device for bacterial molecular diagnostics using *H. influenzae* as a model organism.

First, we demonstrate the underlying principle of solvent-selective routing (Fig. 1) by strategically placing two solvent-selective valve types (Fig. 1a, b) in a simple microfluidic network, inverse-Y bifurcation connecting an inner loading chamber to two outlets (Fig. 1c, d). Within the three-dimensional (3D) disc architecture similar to an earlier reported system (Gorkin et al. III 2012), we placed the two outgoing channels in separate layers. These channels

communicate via a vertical through hole which is initially sealed by a solvent-selective film, in this case a hydrophobic, PTFE-supported membrane valve (HMV) (Fig. 1a, c) which becomes permeable upon exposure to organic solvents. As the HMV is impermeable to aqueous solutions, the first aqueous flow is routed through a narrow channel to the right collection chamber (Fig. 1c, 1–2). However, the HMV is permeable for the subsequent organic solution (Fig. 1c, 3–4), which has thus two options for exiting. The outflow into the left reservoir can be biased by a lower flow resistance, e.g. through a shorter connection channel with a larger cross section compared to the other outlet.

In addition, the centrifugally stabilized liquid volume already resident in the right outlet blocks all vents, thus acting akin to a solid plug to practically create a dead-end channel. The system can be simplified because both liquids, e.g. aqueous solutions, have identical contact angles with the channel walls and the channel has uniform cross section on both sides of the air pocket. For the sake of clarity, we hence neglect capillary and inertial effects, so the flow into the right outlet will hence stop once the pressure in the interspersed air pocket balances the centrifugal pressure exerted by the incoming liquid.

The air pocket thus acts as a fluidic capacitance (Kim et al. 2008) where the final pressure of the air pocket $P_{ini} \cdot (V_{ini}/V)$ depends on the initial (typically atmospheric) pressure P_{ini} and the ratio of the initial and final volumes V_{ini} and V, respectively (Fig. 1d). The flow in the lateral channel stops once the pressure in the air pocket equilibrates the centrifugally induced pressure $\rho\omega^2\bar{r}\Delta r$ with the liquid density ρ, the angular frequency ω, the radial length $\bar{r}$ and the mean position Δr of the incoming liquid plug. So essentially, the here considered routing function is tightly linked to the sequence of liquids and sealing the air pocket formed between the advancing and the stationary liquids.

We integrate a bench-top method starting with (1) loading the aqueous lysate, (2) isopropanol (IPA)-mediated RNA precipitation from the aqueous phase, followed by (3) washing with ethanol (EtOH) and eventual (4) resuspension of the purified RNA in aqueous buffer. A glass bead solid-phase support is utilized to enhance the purification efficiency of the system. To implement the automated routing of two organic solvents between a first and last aqueous phases, we established a sequence of a HMV and a DF valve (with an interspersed siphon) realized by the tab structures outlined in Fig. 1. The valves were assembled into a tab structure using two pieces of pressure-sensitive adhesive (PSA) (Fig. 1a and b). The full router in Fig. 2 displays four chambers for loading of the solid phase, sample and reagents (chamber L), the aqueous (W_{aq}) and organic waste (W_{org}) and for eluate containing the purified RNA (E_{aq}) in the final step of the solid-phase purification. Within the loading chamber, we placed a baffle with laser ablated, 100-μm-wide radial grooves (Fig. 2a) to geometrically retain the glass beads (≤106 μm) under the impact of the centrifugal flow. With its microporous (pore size 0.45 μm) PTFE barrier, the first HMV blocks the aqueous sample and eluate (Fig. 2b, e) while providing passage of the organic solvents (Fig. 2c, d). These organic phases are both directed to their designated waste (W_{org}), which is the hydrodynamically preferred, axial outlet of the open-channel situation on the centrifugal platform.

The hydrophilic siphon, downstream from the loading chamber, lets the aqueous fraction pass at reduced spin rates, while it holds back subsequent IPA and EtOH before the crest point at increased rates until they are fully diverted into W_{org}. Organic solutions are therefore effectively restricted to the loading chamber, solid phase and ascending siphon arm. The siphon thus minimizes the risk of critical cross-contamination of the elution reservoir E_{aq} with organic solvents. Following this intermediate, organic routing phase, we intersperse a drying period for the previously primed channels and membranes, which could be enhanced by centrifugation (Garcia-Cordero et al. 2010). Again the most crucial factor for a subsequent molecular amplification step is that E_{aq} remains free of potential contaminants from preceding solutions.

In the final step, the aqueous elution buffer desorbs the RNA bound to the solid phase. After passing the hydrophilic siphon during slow spinning, the RNA-depleted sample (Fig. 2b) and the elution buffer (Fig. 2e, f) are diverted in a binary fashion to the aqueous waste (W_{aq}) and the elution chamber (E_{aq}), respectively. To this end, a normally closed (water) dissolvable film valve (DFV) initially seals the radial outlet to route the liquid into W_{aq}. Once in contact with the valve, the first liquid initiates the timed dissolution of the thin film so the DFV is opened to direct the subsequent elution buffer to its destination E_{aq}. Additionally, the hydrophobic membrane (HM) covering the outlet of the aqueous waste (W_{aq}), denoted in Fig. 2b, prevents leakage while venting the chamber. Thus, the HM retains the RNA-depleted fraction and maintains the air pocket with the advancing aqueous elution buffer. Contact between the two liquids is averted because of the fluid capacitance, i.e. pressure equilibrium on the two sides of the air pocket (Fig. 2e, f). In total, the router utilizes four phase-selective valves (HMV, hydrophilic siphon, DFV and HM) with different functionalities that allow automated sample purification without exposure of the eluted RNA to preceding flows.

The centrifugal microfluidic SPP with this solvent-selective router is based on the following protocol: first, the crude aqueous extract from a homogenized biological sample is introduced onto the beads inside the loading chamber (Fig. 2b). The RNA from the solution binds to the acid-washed glass beads as a result of charge–charge interactions under chaotropic conditions (Boom et al. 1990).

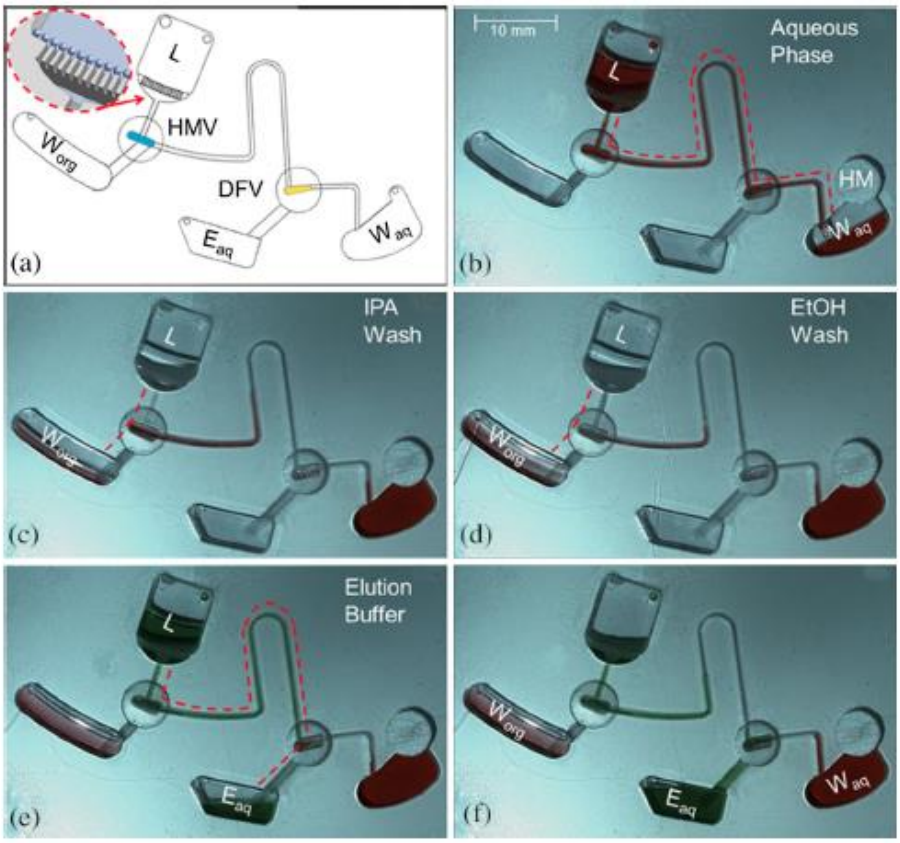

Fig. 2 Fluidic tests of the router. **a** Schematic of the device showing the loading chamber (L), the organic and aqueous waste W_{org} and W_{aq}, respectively, the collection chamber for the eluate E_{aq} containing the purified total RNA and the hydrophobic membrane valve (HMV) and dissolvable film valve (DFV). The inset on the left shows a magnified view of the baffle that holds the glass beads. **b** Flow from L designated to W_{aq}. The initially closed DFV is actuated open by the flow. **c** 60 µL of IPA flow from L into W_{org}, as the HMV is permeable to organic solutions. **d** 60 µL of EtOH from L to W_{org}. **e** and **f** Routing of the elution buffer from L into E_{aq} as the HMV is impermeable to water and DFV is open. Summary of this test is available in ESM 1. Note that W_{aq} and in particular E_{aq} remain free of organic, which are essential for the quality of the extracted RNA

Using a specific spin frequency, the RNA-depleted fraction is delivered through the siphon into W_{aq}. The flow disintegrates the thin film so the DFV is open to route the subsequent elution buffer into E_{aq}. Next, the disc is stopped and IPA is pipetted into the loading chamber (L) to precipitate any remaining unbound RNA (Fig. 2c). A small volume of the RNA-depleted aqueous solution remains trapped in the channel prior to the siphon crest. Both, its flow resistance and the wettability of the PTFE-supported membrane make the HMV impermeable for water, even at high rotational frequencies (75 Hz). As long as the pressure drop across the membrane is lower than the minimum pressure required to drive all of the permeate phase through the membrane (Adamo et al. 2013), the HMV remains closed. Once the IPA is introduced onto disc and is in contact with the HMV pores, it passes through the PTFE-supported membrane. According to the supplier, organic solvents, IPA in this case, changes the membrane permeability and thus gates the RNA-depleted aqueous solution through the pores of the HMV into the organic waste. The high angular velocity (75 Hz) guarantees that IPA does not shoot over the crest of the siphon, which is a low-frequency pass valve. Such HMV permeability facilitates the emptying of the channel, prior to the siphon crest, into the W_{org} and prevents transfer of the high-salt concentration solution to the elution chamber (E_{aq}). The lost volume does not influence the extraction efficiency as the RNA has been retained on the solid phase prior to the treatment with IPA. It takes approximately 3.5 min at spin frequency 75 Hz for the membrane to open as the RNA-depleted solution prevents the IPA from direct contact with the HMV. This is the time required for the IPA to reach the PTFE membrane through the channel at that given spin rate. Using a solvent with higher miscibility would accelerate the processing time. Therefore, the time required to open the HMV is solvent specific, and it also depends on the geometry of the upstream channel, spin rate and intrinsic properties (pore size, contact angle) of the integrated PTFE filter. EtOH is consecutively loaded that rinses away salts from the beads and the precipitated polynucleotides (Fig. 2d), and also residues from the pre-crest channel region. During the drying period, as the EtOH evaporates from the pores of the PTFE-supported membrane, the valve returns to its normally closed state, hence allowing the elution buffer to pass by the HMV undisturbed, at low rotational frequencies over the siphon crest into E_{aq}. Finally, 100 µL of aqueous elution buffer is introduced and driven at lower frequencies (7.5 Hz) through the solid phase where it retrieves the purified RNA, then pass

the HMV over the siphon crest and through the now open DFV and vertical channel into the E_{aq} reservoir (Fig. 2e).

3 Materials and methods

The following materials were used for the study: acid-washed glass beads (≤106 μm), absolute ethanol, isopropanol, phosphase buffered saline (PBS), pressure-sensitive adhesive (PSA, Adhesive Research Inc., Ireland), polymethyl methacrylate (PMMA) sheets (Evonik Industries AG, UK), dissolvable film (FA36, Harke PackPro, Germany), hydrophobic membrane (PTFE membrane circles, 0.45-μm pore diameter, Whatman), DI water (TKA, Germany), nuclease-free water (VWR, UK), RNAse ZAP (Biosciences, Ireland), TRI Reagent, 4-bromoanisole (Bio-Science). All reagents listed above were obtained from Sigma-Aldrich unless otherwise stated.

3.1 Valve assembly

These valves were assembled into a tab structure using two pieces of PSA (Fig. 1a, b). First, the flow-through region in a tab was cut out from the PSA using a precision knife cutter (Graphtec, Yokohama, Japan). The protective foil was peeled off the PSA and either a hydrophobic membrane (HM) or a water-dissolvable film (DF) was stacked on the surface (Fig. 1a, b). The excess material was cut off and removed without penetrating the DF layer. A final cut through the PSA defined the tab size. To improve the mechanical stability of the fluid exposed area, a second PSA layer covered the HM/DF at the top.

3.2 Fabrication and assembly of the disc

The device consisted of three PMMA discs (ϕ120 mm × ϕ 15 mm × 1.5 mm) and two binding PSA layers (Fig. 3). Each disc was cut to size and processed on a CO_2 laser (Epilog Zing, US): the bottom disc had its draining channels ablated; in the middle disc, liquid loading channels and collection chambers were cut out. Additionally in the middle disc connecting channels, siphons and valve grooves were CNC milled (MDX-40 Rolland, UK) into the backside. Inlets and outlet via-wholes were ablated in the top disc. All three discs were sonicated in 2 % aqueous solution Micro 90 (International Products Corp., USA) for 30 min at 50 °C. The discs were then transferred to DI water and sonicated under the same conditions for another 30 min. Finally, the discs were heat-dried at 80 °C for 45 min.

Prior to disc assembly, the work area on the bench was decontaminated using RNAse ZAP and cleaned with 70 % IPA. Valves were placed in their designated grooves in the middle disc. Intermittent layers of PSA with the cut out

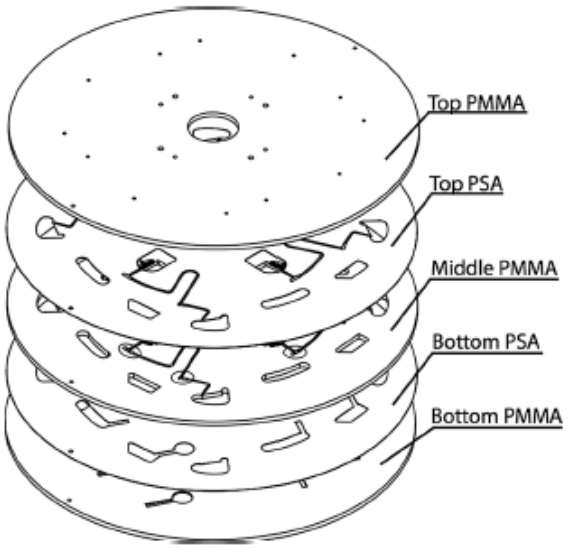

Fig. 3 Exploded view of the disc assembly. The bottom PMMA disc (OD 120 mm × ID 15 mm × 1.5 mm) with laser-ablated draining channels was bonded through a bottom pressure-sensitive adhesive (86 μm, PSA) to the middle disc, which hosted the loading, collection chambers, as well as milled channels and valve beds. The channels had uniform square cross section (0.5 mm × 0.5 mm) and were fabricated by precision milling (MDX40, Roland, UK) on the upper side of the disc, while the valve beds (OD 6 mm, Depth 0.6 mm) were milled from the backside. Top PSA layer with silhouettes of the channels and chambers bonded the stack to the top PMMA disc, which sealed close the chambers and the channels. Two pins and an assembly rig were used to align all the structures

silhouettes (Graphtec, Yokohama, Japan) of all chambers and channels were aligned with their contour parts on the PMMA discs using a bespoke assembly rig. The assembled device was passed multiple times through a roller press (Hot Roll Laminator, Chemsultant Int., USA) to reinforce the bonds of the five-layer structure. Finally, 30 mg of acid-washed, dried glass beads was introduced to the loading chamber *L*.

3.3 MCF7 cell culture

All cell culture reagents were obtained from Sigma-Aldrich (MO, USA) unless otherwise stated, MCF7 cells (DSMZ, Braunschweig, Germany) were cultured in 75 cm flasks in DMEM media, supplemented with 10 % foetal bovine serum (FBS), 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. The cultures were maintained at 37 °C with 5 % CO_2. Cells were harvested by incubation in 5 mL 0.25 % trypsin/0.1 % EDTA at 37 °C for 5 min followed by neutralization with 5 mL culture medium. Cells were centrifuged at 300×*g* for 4 min and resuspended in culture medium. The cells used in this study were collected between their 17 and 25 passages.

3.4 T47D cell culture

The ductal breast cancer (T47D) cells were cultured in RPMI-1640 supplemented with 0.2 U mL^{-1} bovine insulin and FBS to a final concentration of 10 % at 37 °C in 5 % CO_2. Cells were harvested by incubation in trypsin–EDTA solution at 37 °C for 5 min followed by neutralization with 5 mL culture medium. Cells were counted and centrifuged at 300×*g* for 4 min to pellet the cells.

3.5 *Haemophilus influenza* growth conditions

H. influenzae (DSMZ 4690) was cultured overnight in haemophilus test medium (HTM, Oxoid, UK). The following morning, a new culture (10 mL) was inoculated with a 100 µL overnight culture aliquot and allowed to grow to exponential phase (4 hrs). Cells were harvested, and the optical density of the culture was measured at 600 nm and compared to a previously generated growth curve. Cells were diluted in HTM to 1×10^7 cells/mL and pelleted.

3.6 Leucocyte preparation

Human whole blood was obtained from a healthy male volunteer. The leucocyte fraction was isolated by gradient density centrifugation using Ficoll Paque Plus (GE Life Sciences) according to the manufacturers instructions. The cells were washed twice in 1× PBS and pelleted.

3.7 Bench-top cell lysis and RNA extraction with TRI Reagent

Cells were lysed by mixing a 25 µL aliquot of harvested cells in culture media (DMEM, FBS, penicillin and streptomycin) with 80 µL TRI Reagent. The aliquots contained 2.96×10^5–7.4×10^4 MCF7 cells. The cells were enumerated by an automated cell counter (Scepter Handheld, Millipore, USA) and in a haematocrit chamber (depth 0.1 mm, Marienfeld, Germany). For all other cell types (T47D, *H. influenzae* and leucocytes), the cell pellet was resuspended in a mixture containing 80 µL TRI Reagent and 25 µL nuclease-free water. To each sample, 5 µL of 4-bromoanisole was added. The mixture was then vortexed for 1 min and incubated for 9 min at room temperature prior to purification. All samples were then centrifuged at 14,000× *g* for 5 min at room temperature, and the aqueous phase was removed for on-disc purification.

3.8 Measuring the contact angle between PMMA and aqueous cell homogenate

Following lysis, 60 µL of the aqueous phase was collected, and an equal volume of water was added. From this, a 1 µL aliquot was placed on a clean, dry PMMA surface (n = 5) and images of the sitting droplets were acquired. The contact angles were calculated using the tangential method from the built-in software of the goniometer (DataPhysics Instruments GmbH, Germany). The hydrophilic siphon design (Online Resource 2) was calculated based on these data.

3.9 Bead-based total RNA purification on-disc

The use of silica-based substrates in combination with chaotropic salts to purify nucleic acids has been widely implemented (Boom et al. 1990). Under basic and near neutral pH, the silanol groups on glass or silica surfaces are negatively charged (Wen et al. 2008) due to their pKa of 5–7. In spite of the pKa, charge–charge interactions can take place in high-salt concentration solution. Also, strong ionic conditions (NaCl) and lower pH (pH = 5) can mask native hydroxyl negative charges and permit binding of the RNA. In the protocol we implement here, the chaotropic salt from the TRI reagent compensates the anionic charges by removing water molecules from both glass and nucleotides. To strip the beads of the bound RNA, a low ionic strength buffer at near neutral pH 8.1 is used.

On disc, 30 mg of dry acid-washed glass beads were introduced into the main chamber. Next, 120 µL of cell lysate mixed with water (1:1, aqueous phase/water) was loaded. The mixture was incubated for 5 min on a stationary disc to enable RNA binding to the beads. The beads were then subjected to two sequential washing steps with 60 µL of 100 % IPA and 60 µL of 75 % ethanol aqueous solution (EtOH). Finally, the purified RNA was retrieved from the beads with the addition of 100 µL elution buffer (50 mM Tris-HCl, 1 × EDTA at pH 8.1). The liquids were automatically collected in designated chambers (Fig. 2 and ESM-1). The entire volume of the eluted fraction (100 µL) was recovered from the collection chamber (E_{aq}) and analysed.

3.10 Measuring the concentration, purity and integrity of extracted RNA

The concentration and integrity of the purified total RNA was determined by capillary electrophoresis (RNA 6000 Pico kit, Bioanalyzer 2100, Agilent technologies, USA) according to the manufacturers' instructions. The quality of the purified RNA was assessed by the RIN (RNA Integrity Number) provided by a built-in algorithm of the Agilent Expert Software. The RIN algorithm analyses the entire electrophoretic trace originating from an RNA sample. First, to determine whether RIN could be calculated, depending on important elements of the electropherogram such as the Pre region, 5S region, Fast region, Inter

region and Past region are evaluated. If a critical anomaly is detected in any of these regions, the RIN is not computed. Baseline correction and normalization are automatically applied to each electropherogram prior to the feature extraction. Then, based on Bayesian learning technique, the algorithm builds regression models using indicated peak positions, heights, areas, area ratios, S/N ratio, maximum and minimum values, and waviness of the electropherogram trace to assign a 1–10 score (Schroeder et al. 2006). RIN score of 1 means the sample is degraded, and 10 scores for completely intact RNA. According to the description of Agilent, the RIN algorithm is developed to utilize neural networks and adaptive learning in conjunction with a large database of eukaryote total RNA samples, and the RIN score is largely independent of the amount of RNA used and the origin of the sample.

3.11 RT-PCR amplification

The microRNAs, miR-16 and miR-195 were reverse transcribed using miRNA-specific stem loop primers (Life Technologies) and the TaqMan® MicroRNA reverse transcription kit (Life technologies); 1.33 µL of RT product was subsequently amplified by PCR in a 20 µL reaction using the TaqMan® universal PCR master mix II kit and miRNA-specific primers and probes (Life Technologies) on a LightCycler 480 thermocycler (Roche).

3.12 NASBA amplification

The tmRNA transcript in RNA purified from *H. influenzae* was amplified in a real-time NASBA reaction using the NucliSENS EasyQ basic kit version 2 (bioMérieux, Lyon, France) on a LightCycler 2.0 thermocycler (Roche) using the primers (5-3) P1; AATTCT- AATACGACTCACTATAGGGAGAAGGCTTCGATCCTCAAACGGT, P2; GCAGCTTAATAACCT and a molecular beacon 5'FAM-CCGAGTGGGGATAACGC-GGAGTCAACTCGG 3'DAB (MWG Eurofins, Germany). Each sample was amplified in a 20 µL reaction consisting of 10 µL of NASBA reagent-primer mix, 5 µL of RNA template and 5 µL of the enzyme mixture (avian myeloblastosis virus reverse transcriptase, RNAse H, and T7 RNA polymerase).

4 Results and discussion

4.1 Fluidic analysis

Proper operation with liquid volumes, conditions and spin frequencies for each stage of the purification procedure was experimentally validated. The final spin frequency protocol for the purification is outlined in Fig. 4. For total RNA, a

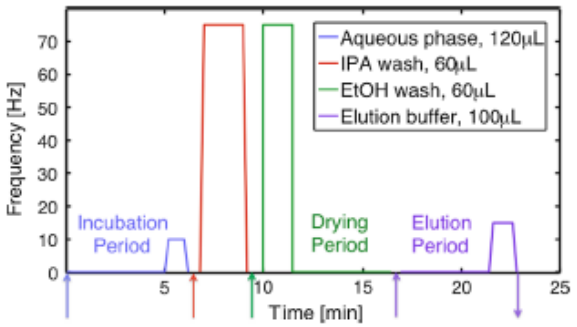

Fig. 4 Spin frequencies of the disc versus time. The *vertical arrows on the horizontal axis* indicate the four sequential loading steps of sample, reagents and collection of the purified RNA. Simultaneous extraction of total RNA from four samples was accomplished in less than 25 min

5-min incubation period was necessary to enable the beads to bind nucleic acids present in the sample. Another 5-min incubation period was required to provide sufficient time for the RNA to elute from the beads into the aqueous elution buffer in the reverse process. These incubation times and elution conditions relate directly to the properties of the solid phase and may vary for different bead materials, e.g. silica (Wen et al. 2008; Duarte et al. 2010), polystyrene (Duarte et al. 2011) or chitosan (Kim et al. 2009b). One advantage of the router presented in this work is that the solid phase inside the loading chamber can be easily substituted, e.g. to reduce the incubation times and/or increase the extraction efficiencies.

It was determined that increasing the period of incubation prior to elution further increased the extraction efficiency. The 5-min drying period (Fig. 4) facilitated the evaporation of any remaining EtOH from the siphon. Drying of the siphon prior to the final elution step stabilized the contact angle, thereby preventing elution buffer from seeping over the siphon crest, thus preventing the elution buffer from mixing with aqueous waste fraction.

4.2 On-disc RNA purification

To validate proper functioning of the on-disc SPP with the solvent-selective router, we performed lysis and phase separation on the bench of MCF7 cells with TRI Reagent, and subsequent on-disc RNA purification from the aqueous phase obtained from the cell lysate. The electropherograms obtained for different numbers of MCF7 cells in Fig. 5 show that high-quality RNA was recovered from the eluted fraction.

For the MCF7 RNA studied (n = 8), RIN values were in the range of 7.2–9.2. An average of 16.8 ng was recovered from the 2.96×10^5 cells, which was less than the 23.1

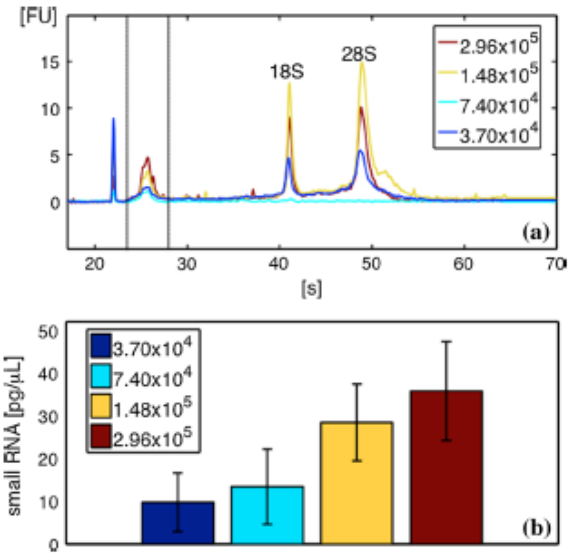

Fig. 5 Electropherogram of the total RNA purified on-disc from four different aliquots of MCF7 cells (**a**). Vertical lines between 24 and 28 s designate the region of small RNA (size range 30–200 nt). **b** Mean values and standard deviations from triplicate samples containing (*red*) 2.96×10^5, (*yellow*) 1.48×10^5, (*light blue*) 7.4×10^4, (*blue*) 3.7×10^4 of MCF7 cells (color figure online)

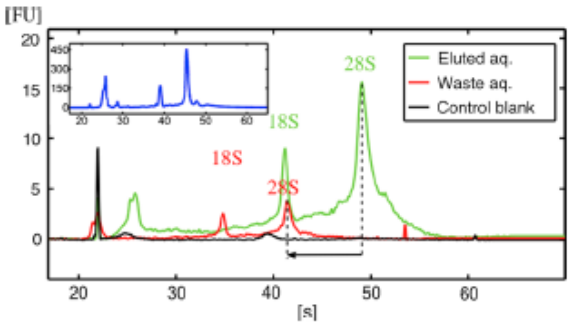

Fig. 6 Electropherograms showing the solid-phase extraction efficiency on-disc. Total RNA content was measured (Agilent Bioanalyzer 2100) in samples collected from E_{aq} (*green*, Fig. 2), W_{aq} (*red*, Fig. 2) and control blank from the growth media (*black*). The spectrum from the waste (*red*) is significantly shifted to the left in relation to purified RNA spectrum (*green*), which is due to the increased salt content (inhibiting subsequent molecular amplification) in the W_{aq}. This shift is denoted with *dashed lines and an arrow* at the ribosomal (28S) peaks. The *inset* displays the bench-top extracted sample using 2-propanol precipitation and the position of the peaks of pure total RNA (*blue*) (color figure online)

ng from 1.48×10^5 MCF7 cells. Evidently, the cell number and the amount of ribosomal RNA (18S and 28S) are not correlated as indicated by the peak height variations in Fig. 5. According to previous studies by Hiorns et al. (2004), random fluctuations of ribosomal RNA are not uncommon; in the authors' opinion, fluctuations in RNA levels can also be due to low recovery after purification. However, the RIN is the criterion used here to evaluate solid-phase extraction and RNA quality on LoaD, which is in the scope of the current article. Based on qualitative analysis of the rRNA, we conclude that the purified RNA has preserved high integrity after on-disc purification with the solvent-specific router.

Further analysis indicates that the quantity of recovered small RNAs is proportional to the number of cells in a starting sample. From the integrated area of the peaks with migration time between 24 and 28 s, the small RNA fraction was quantified for four different cell concentrations. We focused on this region as many potential biomarkers have been identified as small RNAs (Iorio and Croce 2012; Kosaka et al. 2010). Figure 5 compares the purified small RNA concentrations to cell content. In spite of the significant lost of RNA, which is discussed in the following paragraphs, the results suggest that our LoaD platform is applicable for small RNA purification from acid guanidinium isothiocyanate phenol-lysed samples.

The purification efficiency was further investigated by comparing the RNA content from the purified (E_{aq}) to the unbound (W_{aq}) fractions. Our quantitative measurements show that the RNA concentration is higher in the purified fraction in comparison with the non-bound, waste fraction. A concentration of 180 pg μL^{-1} was measured in the purified fraction (100 μL), against 37 pg μL^{-1} (120 μL) in the waste fraction (Fig. 6). These were also compared with the control sample, containing culture media only, where no RNA was detected, demonstrating that the recovered RNA originated from the cells and not from the growth media.

Literature reports on-chip SPP efficiency for DNA of 42.5 % using static silica beads (Duarte et al. 2010). Higher extraction efficiencies of 63.9 % are achieved for DNA using dynamic methods with magnetically induced enhanced mixing (Duarte et al. 2011) and 71.0 % for RNA utilizing chitosan-coated beads on-chip (Hagan et al. 2009) and recently 81.0 % on-disc with (TEOS)-treated glass beads (Jung et al. 2013) to capture RNA from influenza (A H3N2) lysates with known concentrations. The Lab Tube system developed by Kloke et al. (2014) is capable of 100 % automated extraction of human genomic DNA using integrated silica membrane.

As part of our study, bench-top extraction was performed lysing 9.5×10^4 MCF7 cells, using IPA precipitation and two consecutive washes with EtOH. We measured total RNA of 32.3 ng from that sample. The RNA recovered from an identical sample after on-disc extraction resulted in 3.9 ng of total RNA. Assuming that the bench-top extraction was 100 % efficient, our purification efficiency was

12.1 % (weight) of extracted total RNA from an identical sample utilizing a purification protocol without beads. The percentage of purified RNA on-disc varied between samples with different cell counts. For the 1.48×10^5 cells, 43 % of the total RNA was retained on the beads, from which 7.2 % was recovered from the elution fraction. Variations in the packaging of the solid phase would inevitably lead to fluctuations of the amount of recovered total RNA. Even in a tightly packed monolith, diffusion of the RNA molecules would act as a limiting factor. According to the Einstein-Smoluchowski relation for one-dimensional diffusion ($x^2 = 2 \cdot D \cdot t$) during the incubation time (5 min), an RNA molecule with diffusion constant of 10–7 cm2/s, for prokaryotic (16S) ribosomal RNA (Tam et al. 1981), would only travel 7.7 µm. Introducing mixing (Duarte et al. 2011) in the L chamber of the LoaD system can increase the capture efficiency at the loading stage. Further investigation and optimization of the solid-phase extraction protocol should follow in order to further raise both the capture and elution efficiency of total RNA.

A closer look at the electropherogram illustrated in Fig. 6 reveals that the run-time of the sample from W_{aq} is increased in relation to the typical migration times. We attribute this behaviour to residual salts in the W_{aq} chamber (Copois et al. 2007).

4.3 Assessment of on-disc RNA purity

To further investigate the presence of contaminants in the eluted fraction, we sought to determine whether salts and/or other contaminants would inhibit enzymatic amplification of RNA species present in the E_{aq}. Nucleic acid modification enzymes and polymerases are inhibited by organic species such as phenol (Katcher and Schwartz 1994), ethanol (Huggett et al. 2008) and the presence of guanidinium salts. We therefore sought to determine whether on-disc-purified RNA was amplifiable by RT-PCR and NASBA. For RT-PCR, RNA was purified from the lysate of 1×10^5 T47D cells (human ductal breast epithelial tumour cell line) and the leucocyte fraction from 1 mL of human whole blood. The microRNAs, miR-195 and miR-16 were first reverse transcribed using a sequence specific primer and then amplified by real-time PCR. The comparison study between the breast cancer-related miR-195 and the housekeeping miR-16 after RT-PCR is plotted in Fig. 7.

In the RT-PCR, the number of cycles needed for the amplification-associated fluorescence to reach a specific threshold level of detection, called crossing point (Cp) value, is inversely correlated to the amount of nucleic acid found in the original sample (Valasek and Repa 2005). For the T47D cells, Cp values of 30.6 and 29.1 were obtained for miR-16 with on-disc and bench-top purified RNA, respectively. For miR-195, the Cp values were 27.4 and 25.3 for on-disc and bench-top purified RNA, respectively (Fig. 7a). This result indicates an approximately threefold reduction in the quantity of both miRNAs detected from disc-purified RNA compared to bench-top purified RNA. In the case of RNA purified from leucocytes, the difference was even greater. For miR-16, there is a 600-fold reduction in the quantity detected (9.2 cycles) and a ninefold reduction (3.1 cycles) for miR-195. Nonetheless, these results demonstrate that our LoaD is capable of purifying small RNAs that are amplifiable from two different biological sample types.

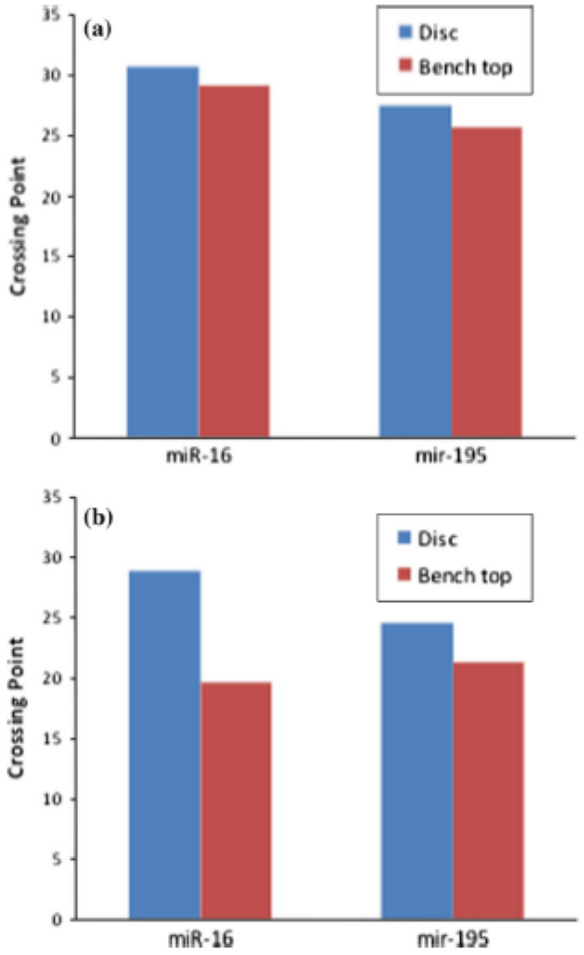

Fig. 7 Comparison study of the RT-PCR amplified miR-16 and miR-195 after on-disc or bench-top purification. **a** The miR-16 and miR-195 purified from 1×10^5 T47D human ductal breast epithelial tumour cells, and in **b**, the leucocyte fraction of 1 mL whole blood is presented

One of the main questions in this study was whether the system was capable of purifying RNA from different organism types (human cells and bacteria) that could be later used in various downstream analysis processes. To further address this question, *H. influenzae* cells were

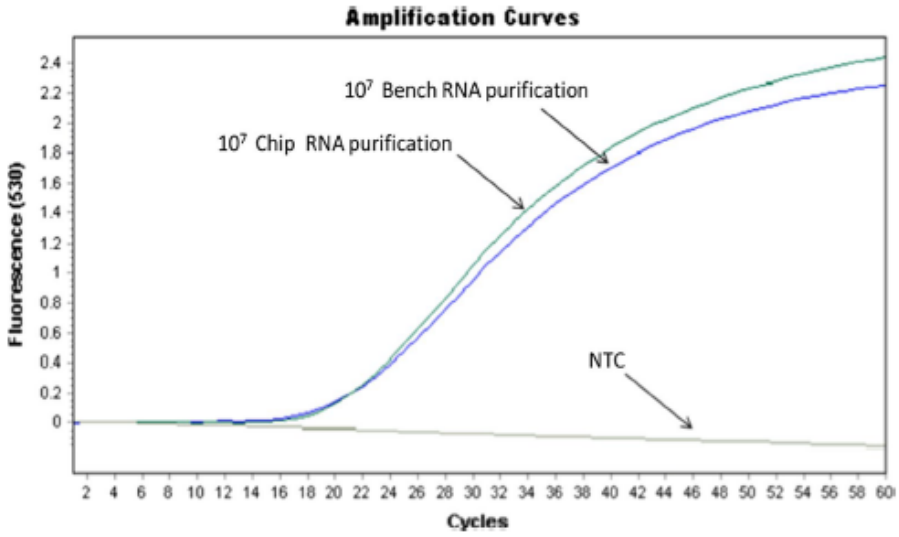

Fig. 8 NASBA amplification of the tmRNA transcript from total RNA purified from *Haemophilus influenzae* (10^7 cells) on-disc and bench-top. Shown in *green* is the disc-purified RNA, *blue* is the bench-top purified RNA and *grey* represents the no template control (NTC) (color figure online)

lysed, RNA purified on disc, and tested for quality using NASBA amplification. The isothermal nature of the reaction lends it to point of care diagnostics. Whilst the reaction is isothermal, it does require the simultaneous action of three enzymes to work. Figure 8 indicates that the amount of RNA purified on-disc and amplified is comparable to the bench-top purified RNA. Decrease of fluorescence signal from NTC (Fig. 8) is attributed to photobleaching of the molecular beacon, FAM/DABSYL as the fluorophore/quencher (Summerer and Marx 2002). It should also be noted that the tmRNA transcript is 360 bases in length, considerably longer than miRNAs.

Preferential retention of tmRNA on disc should correlate to the intrinsic properties of the system and/or the extraction protocol. Prior studies have noted that non-specific adsorption is minimal for RNA or DNA on PMMA (Zhao et al. 2012). Therefore, we believe that it is highly unlikely that non-specific adsorption is a factor in RNA retention within the system. The literature described (Balladur et al. 1997) adsorption as a three-step mechanism starting with (1) diffusion, in our case with convection, from the solution to the surface, (2) lateral diffusion on the surface involving rearrangement of adsorbed entities and (3) adsorption/desorption equilibrium of the molecules at the interface. The observed selectivity during on-disc SPP could be explained in part by the centrifugally induced flux, which forced the liquid through the bead bed in the *L* chamber. At this initial stage of the protocol, time is another factor. Though velocity and contact time contribute to the selective adsorption of tmRNA; in the authors opinion, selectivity was mainly governed by desorption. Our system, however, is limited by the frequencies and accelerations during rotation due to the region of operation of the siphon valve; at low frequency and acceleration, the liquid would pass over the crest (ESM-2, Sup. Fig. 3). For instance, constant angular velocity under similar experimental conditions resulted in higher recovery of total RNA from MCF7 cells (Kinahan et al. 2014). Other known factors are the solid phase and binding conditions, which can significantly influence the efficiency of extraction as demonstrated by Kloke et al. (2014), who used silica membrane, and binding from ethanol for 2 min, for ample capture of DNA. In the context of reversible binding (3), it would be more difficult for larger RNA species (18S and 28S), due to their larger hydrodynamic radii, to remain adsorbed on the solid surface under the shear stress resulting from the disc acceleration (Kim and Hee Jang 2004) during washing with IPA and EtOH. This finding has important implications for developing LoaD systems, which target size-specific purification of molecules by solid-phase purification (SPP).

Despite the limitations in capture and elution during the solid-phase purification of total RNA, sufficient quantities and quality of RNA for downstream analysis are retained and recovered utilizing the solvent-selective LoaD router. These findings further support the feasibility of the router for sample preparations from both prokaryotic and eukaryotic samples.

5 Conclusions and outlook

In summary, we have purified total RNA with high integrity from cell lysates through an automated, merely rotationally actuated flow control strategy involving a network of solvent-selective valves. Purifying mammalian and bacterial RNA, we demonstrated that the applicability of the platform did not depend on the source but on the extraction conditions. Under the studied conditions, the shear stress

induced by acceleration resulted in an overall low recovery of total RNA. In the future, we seek to improve the total RNA extraction efficiency by using alternative solid-phase materials and by improved packing of the solid phase. Based on the developed extraction platform, the fully automated system is planed to integrate pre-storage and release of reagents (Oordt et al. 2013), an upstream chemical cell lysis and the first stage 3-phase liquid–liquid RNA extraction. A downstream microarray will facilitate a sample-to-answer, point-of-care molecular diagnostic for early diagnostics of breast cancer measuring the expression levels of circulating miRNA molecules, including miR-16.

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Appendix B

Development of on-disc Isothermal In-vitro Amplification and Detection of Bacterial RNA

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Development of an on-disc isothermal in vitro amplification and detection of bacterial RNA

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ABSTRACT

We present a centrifugal microfluidic "Lab-on-a-Disc" (LoaD) system capable of implementing nucleic acid *in vitro* amplification using non-contact heating and fluorescence detection. The system functionality is verified by implementing a Nucleic Acid Sequence Based Amplification (NASBA) reaction, targeting the tmRNA transcript of *Haemophilus influenzae*. The NASBA assay incorporates fluorescent molecular beacon probes reporting target tmRNA amplification for endpoint detection. The system implements non-contact IR heating to heat the NASBA reaction to the required target temperatures during denaturation and amplification steps. The LoaD control system facilitates spin speed and chamber positioning for heating and fluorescence detection. The LoaD alignment system uses magnetic fields to locate and lock the chamber in the required position (heating or detection). The NASBA assay was implemented on the system using *Haemophilus influenzae* tmRNA over the range 10^2–10^4 cell equivalent (CE) units. For comparison, identical qNASBA assays were implemented on a Roche LightCycler 2.0 over this concentration range.

1. Introduction

First generation point of care (POC) immunoassay diagnostic tests were relatively complex to perform and typically demonstrate poor sensitivities (25–65%), when compared to established laboratory based nucleic acid (NA) tests [1–3]. Second generation POC tests have adapted NA amplification for rapid disease diagnosis. The sensitivity and specificity of NA tests are typically >90%, with multiplex detection and analytical sensitivities in the range 1–100 cells [4]. Recently a number of commercial PCR platforms have emerged [5,6] for POC infectious disease screening from Cepheid and BiofireDX. Many of these use complex fluidic pump and valve systems to implement biochemical protocols, increasing system cost. The ability to integrate liquid manipulation on the fluidic device has increased interest in centrifugal microfluidics, where centrifugal forces manipulate liquid reagents [7,8]. The LoaD system is an open platform where liquid moves outward towards the disc edge expelling air as it progresses. This facilitates on-disc liquid storage, negating the need for external pumps, valves or reservoirs found in hydrodynamic systems [9]. Furthermore, valving can be implemented and pressure gradients controlled through careful device design and optimized spin protocols [8–10]. At elevated temperatures in open systems, liquid loss by thermo-capillary pumping or evaporation can be an issue, thus on-disc PCR requires robust valving [11].

Isothermal amplification offers advantages over PCR, removing thermal cycling reduces power consumption, eliminates complex electronics [12], and reduces liquid leakage/evaporation. Isothermal amplification methods achieve sensitivities and specificities matching or exceeding that of PCR and are capable of delivering results in <10 min [13]. Nucleic Acid Sequence based Amplification (NASBA), employs the simultaneous action of three different enzymes to amplify sequences from an original single-strand RNA template. The addition of molecular beacon probes to a NASBA reaction enables real-time and multiplex analysis [14]. NASBA has been employed for detecting microorganism RNA from a range of biological samples [15,16]. Since NASBA is an RNA specific amplification technology it offers several advantages over conventional

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DNA amplification technologies; (i) enabling quantitative detection of microorganism RNA [15–17], (ii) no amplification interference from contaminating background DNA [18], (iii) direct detection of RNA transcripts [19], (iv) yields of up to 10^{12} copies [13] and (v) has the potential to only detect viable micro-organisms [20].

Previous reports of NASBA in microfluidic devices use direct contact heating to maintain reaction temperatures [21,22]. Noncontact heating facilitates LoaD NASBA amplification, allowing spin without the complexity of contact heating. Approaches to noncontact microfluidic heating include; microwave [23], induction [24] and infrared [25]. Microwave and induction heating require complex electronic components e.g. high frequency oscillators (26 GHz) with high power inputs (20–70 W). More recently, noncontact IR heating was applied to the rapid amplification of nucleic acids in microfluidic devices [26] and IR heating facilitates rapid heating/cooling of nanolitre sample volumes [27,28].

Previously a LoaD system was demonstrated for solid phase purification of total RNA from MCF7 cell lysates with integrated dissolvable film and hydrophobic membrane valves [29,30]. This highlighted the possibility of integrating complex biochemical protocols on a LoaD system for downstream applications such as realtime amplification. In this work non-contact IR heating and fluorescence detection was applied to endpoint NASBA on LoaD. A software/hardware system implemented:(i)heating, (ii)detection, (iii)spin-control and (iv) chamber alignment for heating/detection. A LabVIEW™ programme controlled system timing, signal acquisition and data analysis. For detection performance endpoint NASBA assays were implemented on LoaD over the range 10–10^4 *H. influenzae* CE to determine system detection limits. For comparison, identical qNASBA assays were also carried out on a Roche LightCycler 2.0. The system demonstrated suitability for implementation of the NASBA heating protocol and optical fluorescence detection. This paper outlines the materials and methods used in: (i) device chamber fabrication/assembly, (ii) biological test samples and (iii) system components to implement heating, detection and LoaD spin control. Results presented detail the system performance for detection of *H. influenzae* tmRNA over a clinically relevant range.

2. Materials and methods

2.1. Microfluidic chamber fabrication

The NASBA reaction chamber was injection moulded using Zeonor™ (1060R, Zeon Chemicals Europe Ltd) on a Babyplast 6/10P machine (Babyplast; Molteno, LC, Italy), pellets were dried for sixty minutes at 55 °C. The system parameters were set for Zeonor™ [31]: Injection nozzle 195 °C, injection chamber 210 °C, plastic melt chamber 220 °C, with three second injection at 60 bar. The manufactured components were cleaned as follows: (i) forty minutes at 50 °C in an ultrasonic bath (10% methanol) (ii) forty minutes at 50 °C in an ultrasonic bath (0.1% TWEEN 20), (iii) forty minutes at 20 °C in an ultrasonic bath (de-ionised water) and (iv) ten minute ozone clean (ProCleaner™ BioForce Nanoscience, UT). The microfluidic component was designed using computer-aided design (CAD) software (Solid Edge; Siemens, Plano, TX) and the injection mould was milled in brass using a Computer Numerical Control (CNC) milling machine (Bridgeport GX 480 VMC;Elmira, NY). The chamber dimensions were 500 μm deep, 4 mm wide and 15 mm long (Fig. 5). The chamber design tapered from 4 mm to 500 μm wide at the inlet/outlet channels minimising air bubble trapping. The reservoir had inlet and outlet ports drilled for pipette sample loading and was mounted on a 4 mm thick LoaD substrate using double sided pressure sensitive adhesive (MH-92712-3, Adhesives Research Ltd, Limerick, Ireland).

2.2. Haemophilus influenzae culture and RNA extraction

Haemophilus influenzae (DSMZ23393) was cultured overnight in liquid haemophilus test medium (HTM) at 37 °C under microaerophilic conditions. Following this the organism was subcultured in HTM broth by transferring 100 μl of overnight culture to 10 ml fresh HTM broth and allowed to grow to exponential phase (~four hours). Once in exponential phase, 1 ml aliquots of culture were harvested and the *H. influenzae* cells were collected by centrifugation for two minutes at 12,000 × *g*. Total RNA was isolated from the cell pellet using the RiboPure yeast kit (Ambion, Austin, TX, USA) according to the manufacturers' instructions. RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA). RNA with an RNA Integrity Number (RIN) above 7.0 was used for NASBA assays. RNA concentrations were determined using the Qubit RNA BR Assay kit and the Qubit 1.0 fluorometer (Life Technologies, Carlsbad, CA) CEs were calculated on the basis of 1 cell containing 100 fg of RNA [32].

2.3. NASBA assay design

A pair of primers and a molecular beacon were designed to specifically amplify and detect the tmRNA transcript of *H. influenzae*. The primers (5′-3′) P1; AATTCTAATACGACTCACTATAGGG-AGAAGG-CTTCGATCCTCAAACGGT, P2; GCAGCTTAACCT and a molecular beacon 5′FAM CCGAGT-GGGGATAACGCGGAGTCA-ACTCGG DAB 3′ were designed according to recommended guidelines [33,34]. Primer and molecular beacon probes were supplied by Eurofins MWG Operon (Ebersberg, Germany).

2.4. qNASBA H. influenzae assay

NASBA assays were performed using the NucliSENS EasyQ Basic Kit V2 (Biomerieux, Marcy l'Etoile, France) to manufacturer's instructions. qNASBA reactions were performed in a total volume of 20 μl. Target RNA (5 μl) was added to 10 μl reagent/KCL (70 mM final concentration)/primers and molecular beacon probes (0.2 μM and 0.1 μM concentration respectively) mixture. The reaction was incubated on an iCycler thermocycler (Bio-rad, Hercules, CA) for 65 °C for five minutes to denature the RNA secondary structure followed by 41 °C for five minutes to allow primer and probe annealing. Subsequently, the enzyme mixture (5 μl) was added to the reaction. The reaction was then incubated on a LightCycler 2.0 (Roche Diagnostics) at 41 °C for 60 min with a fluorescent measurement (530 nm) recorded every minute. A no template control (NTC) consisting of water instead of RNA was included in each experiment.

2.5. On-chip NASBA

NASBA reactions were performed using the NucliSENS EasyQ Basic Kit v2.0 (BioMerieux, Marcy l'Etoile, France). A 40 μl sample volume consisting of 20 μl reagent/KCL (70 mM final concentration)/primers and molecular beacon probes (0.2 μM and 0.1 μM concentration respectively) mixture, 10 μl RNA template, and 10 μl enzyme mix was prepared and used to fill the NASBA chamber. Reagent/KCL/primer mix and RNA template were premixed prior to loading on the chip. The reaction was incubated on the IR heater at 65 °C for five minutes and then cooled to 41 °C for five minutes. Following the 65 °C denaturation step, the enzyme mix was added directly to the chip and placed back on the IR heater at 41 °C for one hour. Endpoint fluorescence on the NASBA product was measured at room temperature using the detection system.

2.6. System functionality

The instrument implemented denaturation/amplification temperature profiles using a PID controller to modulate heater power. A LabVIEW™ clock function held the target chamber at 65 °C for five minutes, it then cooled & held the chamber at 41 °C for five minutes for primer annealing. The motor spin parameters were set to generate centrifugal forces facilitating sample manipulation and chamber repositioning between the heating & detection locations. Chamber repositioning was achieved by rotating the LoaD in discrete 18° steps, while activating the position magnet (Section 2.9). With the LoaD locked in position the sample chamber is heated to 41 °C for 55 min. After amplification, the system rotates chamber rotated to the fluorescence detection and activates the excitation source and acquires the fluorescence signal from the PMT detector (Section 2.8) via the daq card (NI-DAQ6229) analogue input.

2.7. Non-contact IR heating system

The non-contact heating element is a broadband NIR filament emitter from Sci-Tech Instruments (IR-12K) positioned at the inner focus of a parabolic reflector. The PID controller receives a signal from the IR thermal sensor (Omega OSR137-1-MA) and modulates power to the heater and cooling fan (20 W) via two high voltage relays (MOSFET 100 V). A calibrated thermocouple (K type, Chauvin Arnauk TK2000 instrument) was placed in the microfluidic chamber (filled with deionised water) the controller was set to hold the IR heater output fixed for five minutes before the temperature was recorded. For calibration, forty measurements were made over the range 20 °C to 70 °C. The heater to LoaD distance was fixed at 10 mm while the sensor to LoaD distance was fixed at 15 mm, identical materials and chamber designs were used for calibration and test. The PID parameters were tuned to optimise system response minimising overshoot/oscillation around set-points.

2.8. Fluorescence detection system

The detection system (Fig. 1), is controlled via LabVIEW™ through the NI-daq card. The optical setup uses a dual band (FAM/CY5) fluorescence emission filter (Omega optics XF 535-700DBEM). Two laser diodes; (i) 635 nm (Thorlabs CPS182) and (ii) 450 nm (Thorlabs CPS450) excite sample fluorescence for detection. Laser output was defocused illuminating the entire chamber (15 mm x 4 mm), a microscope objective focused light from the chamber onto the detector (PMT H10721-01, Hamamatsu photonics, Japan), via the filter. A 1 mm diameter circular aperture between the filter and detector reduced stray light. The PMT was biased to 450 V with 10 ms integration time, the PMT current was converted to voltage using a transimpedance (×10) amplifier (C7319, Hamamatsu photonics, Japan) and measured using the daq card analogue voltage input (0–10 V).

Fig. 1 illustrates the system implementing NASBA heating and detection within a chamber mounted on a Zeonor™ LoaD substrate. The fluorescence signal is recorded after amplification is completed. The LoaD was mounted in the system and chamber aligned to the detection position for initial measurement and subsequently rotated to the heating position for amplification. Upon NASBA completion the chamber was rotated to the detection position for fluorescent measurement.

2.9. LoaD spin control

The LoaD was mounted on DC motor spindle (2657W024CR, Faulhaber, Switzerland) with a 1000 point line encoder (HEDM5500B, Faulhaber, Switzerland) the maximum motor speed was set to 6000 rpm. The acceleration/de-acceleration was set below 50 rpm/s. The LoaD positioning was automated by activating the motor in timed pulses, relocating the chamber from the heating to the fluorescence detection position.

Fig. 2 outlines system hardware implementing spin control, the programme implements the spin protocol (speed, acceleration, duration etc.) for sample manipulation and chamber repositioning. Permanent magnets (MOD4, Magnet Expert Ltd, UK) attached to the LoaD aligned the chamber as the motor rotated the LoaD in short pulses (Fig. 3). After each pulse, an external magnet fixed on a linear solenoid (Black Knight™ 122, BLP components Ltd, UK) was actuated when LoaD & external mounted magnets overlapped the LoaD/chamber was locked in place. Two magnets mounted on the LoaD in combination with identical solenoid actuated magnets, allowed the chamber to be repositioned between heating and detection points from a random starting location. The motor was controlled via a motion controller (MCDC3006S, Faulhaber, Switzerland) for spin speed, acceleration, de-acceleration etc. The solenoids were controlled via the DAQ card analogue output (6 V). All commands were executed in a LabVIEW™ to interface via an RS232 port & DAQ card. The sequence of timed pulses (Fig. 3) to the external solenoids and timed motor rotation allowed the sample chamber to be repositioned between heating and detection locations. The LoaD rotated by 18° per step, achieved by motor rotation (10 rpm) for one second. The solenoid was activated every 500 ms when the chamber was aligned the magnets overlap and the chamber locks in place.

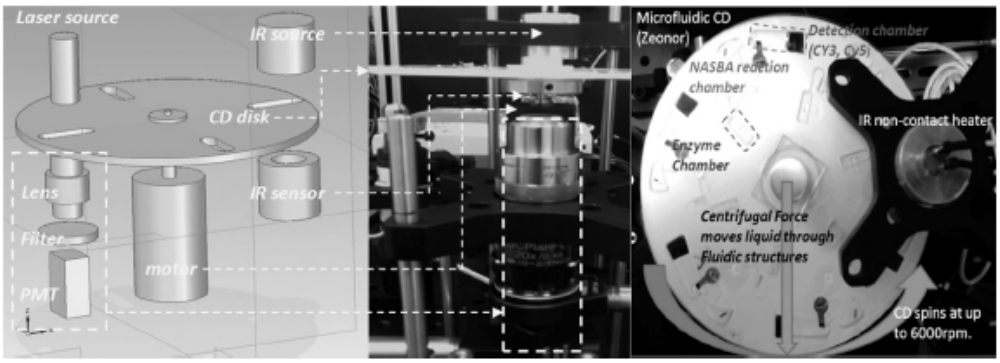

Fig. 1. The system (left & centre) rotates the LoaD counter-clockwise (right) repositioning the NASBA test chamber between the heating and detection locations as outlined in the centre and right images.

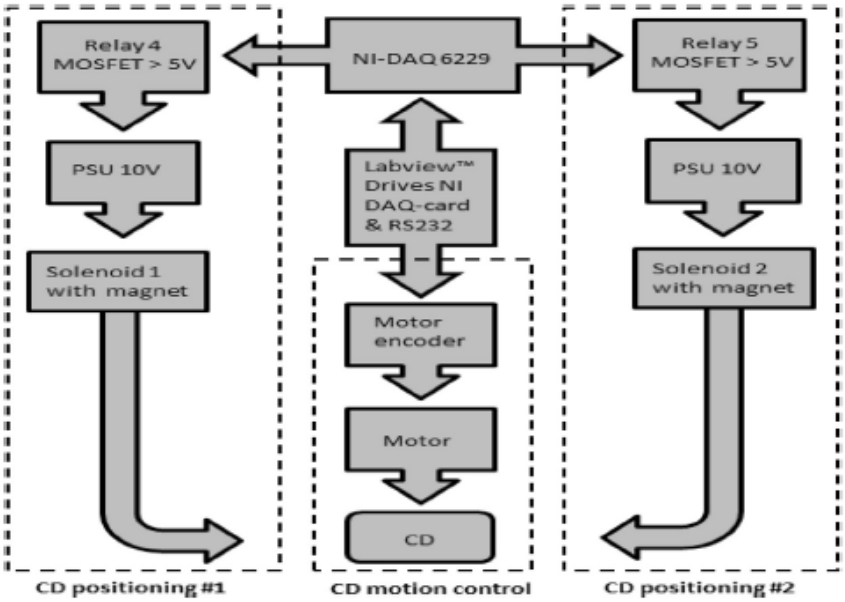

Fig. 2. The LoaD motion control (NI-DAQ 6229) and positioning system (RS232) is driven by LabVIEW™ software to actuate the solenoids/positioning magnets and control the motor e.g. acceleration, deceleration, speed, duration etc.

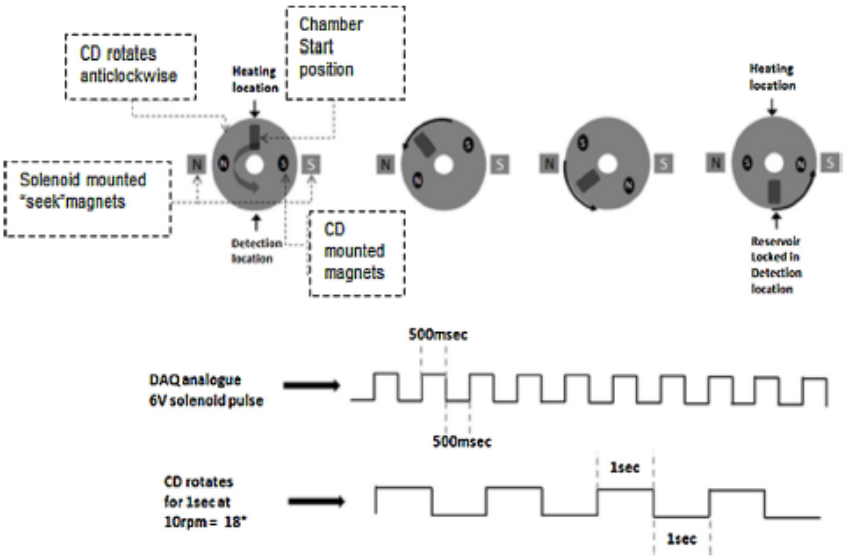

Fig. 3. The rotation control of the LoaD and the position of the sample chamber were implemented by pulsed (500 ms) solenoid actuation of the attached "seek" magnet and the timed rotation (10 rpm for 1 s) of the LoaD motor under LabVIEW™ control.

3. Results

3.1. System heating—temporal profile

The heating control system implemented the NASBA temperature profile within the sample chamber, from room temperature the sample is heated to 65 °C at a rate of 0.45 °C/s as outlined in Fig. 4. The sample was maintained at 65 ± 0.5 °C for five minutes denaturing target RNA and then cooled and held at 41 °C for five minutes. For amplification the sample was maintained at 41 ± 0.5 °C for 55 min. For each power setting the temperature within the microfluidic chamber was measured using a calibrated K-type thermocouple (Chauvin Arnauk TK2000). The power input was slowly adjusted and left to equilibrate for ten minutes before the temperature was recorded.

3.2. System heating—chamber heating uniformity

To confirm uniform heating, calibrated thermocouples were placed at three locations in the microfluidic chamber and temperature measured as illustrated in Fig. 5. The chamber heating profile using the IR heater was compared to that of a 1450 nm laser

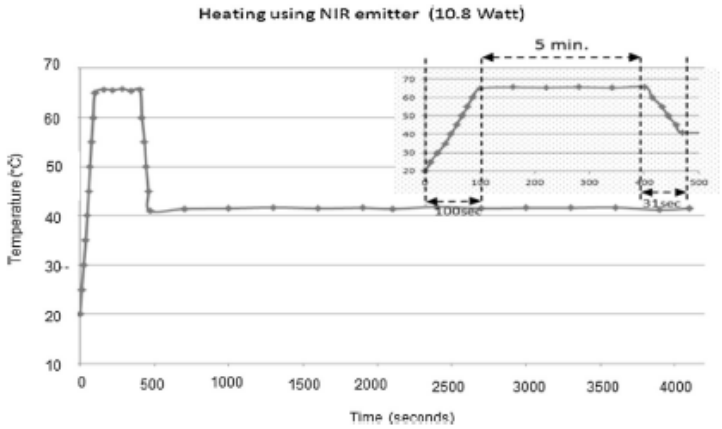

Fig. 4. The NASBA heating profile was applied for the protocol duration (60 min), the ramp time from room temperature to the denature temperature of 65 °C was 100 s and the cooling time to the incubation temperature of 41 °C was 31 s. The sample was maintained at 65 °C for five minutes then cooled and held at 41 °C for 55 min implementing the complete NASBA heating protocol.

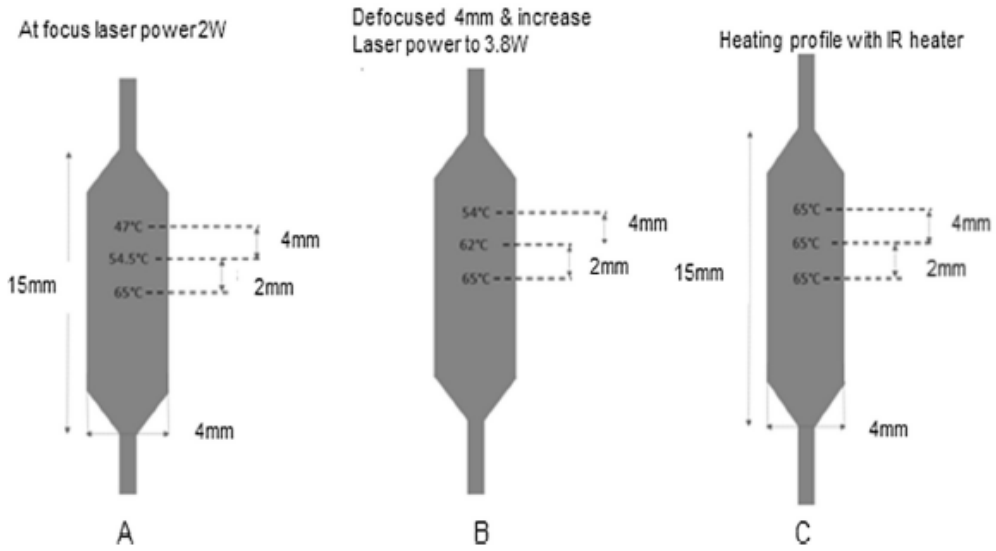

Fig. 5. The thermal profile across the chamber heating with the 1450 nm laser is illustrated. The left profile (A) is from the laser focused on the fluidic chamber (2 mm spot diameter). A significant fall-off in sample temperature is observed off centre of illumination. In (B) defocusing the laser spot on the chamber generates a more uniform profile. However, the sample temperature also decreases off axis. The temperature profile across the chamber using IR emitter (C) achieved a uniform temperature profile across the chamber.

(Roithner LaserTechnik L145T600M). Water has a strong infrared absorption peak at this wavelength making it suitable for microfluidic sample heating. For test, the beam from the laser and IR heater were centred on the chamber (long & short axis). When inverted on the long axis, similar temperature profiles were observed within the chamber.

The focused laser spot size heats approximately 3 mm^2. However, the measured temperature fell rapidly from 65 °C at the centre to 54.5 °C–2 mm off centre and 47 °C – 4 mm off centre (Fig. 5A). Defocusing the laser beam (Fig. 5B) spread the beam over a larger area, resulting in a decrease in the intensity per unit area. Power was increased to 3.8 W to maintaining the reservoir centre at 65 °C. The measured temperature 2 mm and 4 mm above centre was 62 °C and 54 °C respectively. Further defocusing wasn't possible due to the laser power limit. The IR broadband emitter achieved a uniform temperature profile (65 °C) across the chamber (Fig. 5C) when measured with the thermocouple.

3.3. Fluorescence detection

To verify detection sensitivity, qNASBA reactions were carried out on a Roche Lightcycler 2.0 at concentrations of 10^4, 10^3 and 10^2 CE. The reactions confirmed successful amplification of the *H. influenzae* tmRNA concentrations tested. The qNASBA products were then loaded onto the LoaD chamber, positioned in the optical fluorescence detector path and the signal recorded (Fig. 6). This established that the fluorescence system could detect 10^2 CE for end point detection.

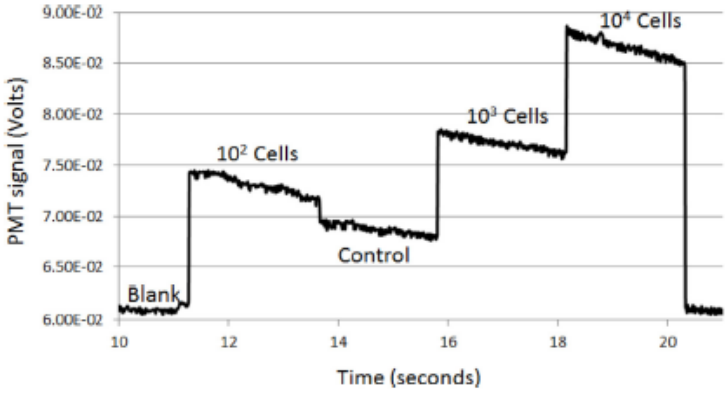

Fig. 6. The fluorescent signal from NASBA amplifications undertaken in a bench-top thermocycler and subsequently analysed on the fluorescence detection system (endpoint).

In Fig. 6 the fluorescence signal increases from the empty chamber (Fig. 6–blank) to the NTC sample (Fig. 6–control) due to molecular beacon characteristics: (i) the quencher may not completely absorbed the reporter dye, (ii) there may be a native fluorescence from the quencher itself and (iii) the possibility some reporter dye molecule detach from the beacon structure. Thus the NTC sample exhibits decaying fluorescence signal during measurement similar to the samples.

3.4. On disc endpoint NASBA using non-contact IR heating

Following independent optimization and validation of the IR heater and fluorescence detection components, on LoaD endpoint NASBA was carried out in the system. Reactions were carried out at 1×10^4, 1×10^3, 5×10^2, and 1×10^2 CE. A NTC (molecular grade H_2O) was also included. For on LoaD tests RNA was extracted from cultured *H. influenzae* cells and amplification was carried out as outlined in Section 2.5. After NASBA incubation the spin control system was used to relocate the sample chamber from the heating position to the detection location. The endpoint fluorescence intensity for each of the on-chip NASBA reactions, including the NTC, was measured. To determine the absolute fluorescence signal from the NASBA product reactions, the NTC fluorescence signal measurement was subtracted.

This established increasing fluorescence signal over the range 1×10^2 to 10^4 CEs (Fig. 7). However, at 10^2 CE no detectable fluorescence signal above the NTC reaction was observed, thus the current detection limit of on-chip NASBA using this system lies between 100 and 500 CEs.

4. Discussion

This work demonstrates end point NASBA amplification of tmRNA transcript (*H. influenzae*) on a LoaD system. tmRNA, encoded by the *ssrA* gene has previously been shown as to be a useful molecular diagnostics target for NASBA [35,36]. It is highly expressed, significantly more stable than mRNA and contains conserved and variable regions making ideal for the sensitive and specific detection of bacterial species [22,36]. The microfluidic LoaD system offers the possibility to integrate fluidic functionality for assay protocol implementation without the need for the external pumps, valves etc., traditionally used in hydrodynamic systems. In this approach the LoaD chamber is rotated between a heating and fluorescence detection locations using LoaD mounted magnets to align the NASBA chamber, eliminating the need for complex position control. A low temperature isothermal microfluidic amplification approach reduces: (i) bubble formation, (ii) liquid evaporation, (iii) high chamber thermodynamic pressure. Cost is a key consideration for disposable diagnostic devices and plastics are preferred over silicon and glass devices. Thus injection moulded Zeonor [37] devices were manufactured in this work, the devices were biocompatible, optically transparent with low auto-fluorescence.

Laser and broad wavelength IR sources were evaluated, while the 1450 nm laser diode achieved the required chamber temperature non-uniform chamber heating was an issue. Thermal gradients within a chamber can create localised diffusion, causing spatial variation in reagent concentration which may impact reaction efficiency [38]. Isothermal amplification doesn't require rapid thermocycling thus slow heating rates are acceptable (2.5 °C/s). The detection system was evaluated with monoplex FAM assays, but it could detect duplex assays e.g. FAM/CY5, CY3/CY5. The system demonstrated fluorescence sensitivity down to 100CE with tmRNA (*H. influenzae*) sample amplified on a LightCycler 2.0 (Roche Diagnostics). To establish the system performance with NASBA heating and detection, a sample set were tested over the range 10–10^4CE. The full NASBA heating protocol was implemented on the LoaD chamber using the IR heater, the fluorescence signal was recorded before and after incubation. In this work the sample was pipetted onto the LoaD which was loaded onto the system for denaturation, upon completion the sample was removed from the LoaD by pipette mixed with the enzymes and reloaded onto the system for incubation/amplification.

The LoaD motion control system outlined in Section 2.9 was used to relocate the chamber between heating and detection zones. This demonstrated a system sensitivity of between 100 and 500 CE. The ideal molecular diagnostics assay should be highly accurate (reproducible, sensitive and specific). One characteristic of a highly accurate assay is its analytical sensitivity. In blood stream infections, bacterial load can very often be less than 100 CE/mL [39]. Low bacterial load may be compounded when sample volumes are limited which is often the case with neonates and children [40]. By further optimising the NASBA assay presented here by for example altering the primer/probe concentration or through the addition of betaine to the reaction cocktail should enable improvement of the analytical sensitivity of the assay [36].The heating system generated temperature profiles compatible with the open chamber LoaD system without air bubble formation, liquid evaporation. Initially on-chamber NASBA tests demonstrated no amplification, a stringent wash protocol prior to test eliminated RNAse chamber contamination delivering successful amplification. Biomolecule-

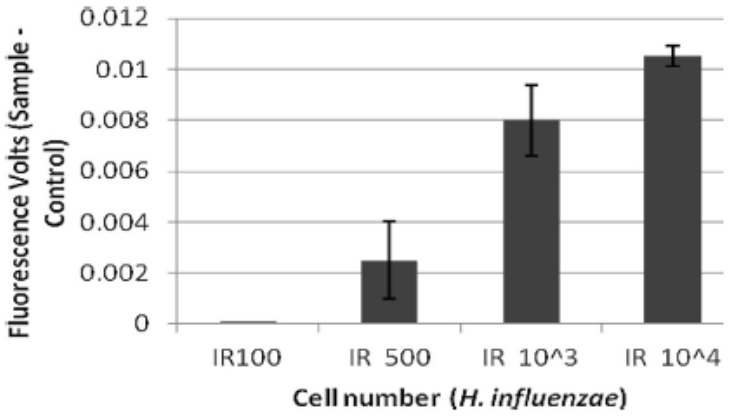

Fig. 7. The fluorescence intensity for *H. influenzae* RNA amplified and detected on the integrated heating/detection system. The RNA concentrations analysed were equivalent to 10^2, 5×10^2, 10^3 and 10^4 cells. The error bars are standard deviation of three repeated measurements per test.

surface interaction presents a challenge for microfluidic diagnostics [12]. Thus a published microchip treatment protocol was carried out [41] giving good amplification with comparable results obtained from on-chip and Lightcycler NASBA assays.

5. Conclusions

A non-contact IR heating approach compatible with fluorescence detection implemented the NASBA amplification protocol within a microfluidic LoaD system for endpoint detection of *H. influenzae* RNA samples between 10^2 and 10^4 CEs. A stringent chamber wash protocol was required to eliminate contamination for amplification. The IR heating approach lends itself to non-contact heating and uniformly heated the sample chamber to the target temperatures suitable for the NASBA assay implementation. Currently the system implements endpoint detection but future work will investigate the possibility of real time heating and detection by rotating the chamber between heating and detection locations. Thus the sample was loaded for denaturation, then extracted for enzyme addition and reloaded onto the chamber for amplification. Future work will implement all functionality on the LoaD. The ultimate goal of such a point-of-care LoaD system is to implement a complete test, incorporating biological sample preparation i.e. extraction, purification and detection. Future work will also focus on achieving a detection limit below 100 target cells.

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Biographies

Des Brennan received his B.Sc. and M.Sc. in Experimental Physics from University College, Dublin in 1995 and 1997, respectively. He worked for a campus based company, Spectral Signatures, in the development of optical sensors for environmental monitoring. He joined the National Microelectronics Research Centre (NMRC)/Tyndall National Institute in 1996 to work on sensors for the food and dairy industries, specifically in the development of Near Infra Red micro-spectrometer systems. In 2001 he joined the Microfluidics group at NMRC/Tyndall National Institute, integrating a micro-HPLC system with an optical waveguide. He is currently a member of the Nanobiotechnology team at Tyndall, where his recent research activities has concentrated in integration of microfluidics with optical detection techniques for on chip molecular diagnostic assay implementation.

Eoin Clancy graduated from the University of Ulster, Coleraine, Northern Ireland in 1995 with a BSc in biotechnology. After working in industry he returned to academia, and in 2004 was awarded a MSc in biomedical nanotechnology from the Institute for Nanoscale Science and Technology (INSAT), University of Newcastle. Here he continued his research and is currently pursuing a PhD focusing on the use of microparticles for molecular extraction and rapid detection. He is currently a Postdoctoral Scientist at the National University of Ireland, Galway where his research focus is development and validation molecular diagnostics assays.

Thomas Barry is the research director of the NADRL in Microbiology at NUI Galway. Research interests are focused on initial concept, biodiscovery, design, development, optimisation, validation (experimental and clinical) of culture independent nucleic acid based diagnostics technologies for the detection and identification of microbial pathogens and contaminants associated with the clinical, environmental, food and pharmaceutical sectors. Dr Barry has over 20 years research experience working with industry where innovation, translation and commercialisation of NADRL nucleic acid diagnostics technologies has been achieved.

Jens Ducrée is a principal investigator for Microfluidic Platforms at the Biomedical Diagnostics Institute (BDI) and holds a Full Professorship of Microsystems in the School of Physical Sciences at Dublin City University (DCU). Dr. Ducrée is also academic member of the National Centre for Sensor Research (NCSR) and the 3U Joint Institute of Global Health (JIGH).The main part of his research is directed towards novel microfluidic platforms and associated actuation, detection, fabrication and instrumentation technologies for the integration, automation, miniaturization and parallelization of sample preparation and detection of bioanalytical assays (e.g. ELISA, nucleic acid testing, general chemistry, cell counting). Typical applications are sample-to-answer systems for biomedical point-of-care and global diagnostics, liquid handling automation for the life sciences (e.g. concentration/purification and amplification of DNA/RNA from a range of biosamples), environmental monitoring and food safety.

Paul Galvin is Head of the ICT for Health Programmes and Head of the Life Sciences Interface Group at the Tyndall National Institute. He has a track record of multidisciplinary research, leveraging nano, photonic and ICT enabled platforms to provide novel solutions for healthcare applications. He was awarded his PhD in 1995 and joined the Tyndall National Institute (then NMRC), in 2000. Research within the ICT for Health Programmes in Tyndall is focussed on exploiting the extensive design, fabrication and characterisation tools available in Tyndall, together with expertise in modeling, embedded software and systems integration for applications related to health. This research capacity underpins the development of new collaborative research programmes with leading medical device companies and clinical experts.

Appendix C

Comparison of Established Diagnostic Methodologies and a Novel Bacterial smpB Real-Time PCR Assay for Specific Detection of *Haemophilus influenzae* Isolates Associated with Respiratory Tract Infections

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Comparison of Established Diagnostic Methodologies and a Novel Bacterial *smpB* Real-Time PCR Assay for Specific Detection of *Haemophilus influenzae* Isolates Associated with Respiratory Tract Infections

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***Haemophilus influenzae* is a significant causative agent of respiratory tract infections (RTI) worldwide. The development of a rapid *H. influenzae* diagnostic assay that would allow for the implementation of infection control measures and also improve antimicrobial stewardship for patients is required. A number of nucleic acid diagnostics approaches that detect *H. influenzae* in RTIs have been described in the literature; however, there are reported specificity and sensitivity limitations for these assays. In this study, a novel real-time PCR diagnostic assay targeting the *smpB* gene was designed to detect all serogroups of *H. influenzae*. The assay was validated using a panel of well-characterized *Haemophilus* spp. Subsequently, 44 *Haemophilus* clinical isolates were collected, and 36 isolates were identified as *H. influenzae* using a gold standard methodology that combined the results of matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) and a *fucK* diagnostic assay. Using the novel *smpB* diagnostic assay, 100% concordance was observed with the gold standard, demonstrating a sensitivity of 100% (95% confidence interval [CI], 90.26% to 100.00%) and a specificity of 100% (95% CI, 63.06% to 100.00%) when used on clinical isolates. To demonstrate the clinical utility of the diagnostic assay presented, a panel of lower RTI samples ($n = 98$) were blindly tested with the gold standard and *smpB* diagnostic assays. The results generated were concordant for 94/98 samples tested, demonstrating a sensitivity of 90.91% (95% CI, 78.33% to 97.47%) and a specificity of 100% (95% CI, 93.40% to 100.00%) for the novel *smpB* assay when used directly on respiratory specimens.**

Haemophilus influenzae is a Gram-negative, coccobacillary, facultatively anaerobic bacterium, which is considered a normal human commensal that frequently colonizes the upper respiratory tract (URT) (1, 2). *H. influenzae* is also an important human pathogen that has been associated with invasive disease and a range of upper and lower respiratory tract infections (LRTIs). Other *Haemophilus* species that are occasionally isolated from the URT include *Haemophilus haemolyticus*, *Haemophilus parainfluenzae*, and *Haemophilus parahaemolyticus*; however, these species are rarely associated with human infections (3).

Typeable *H. influenzae* produces six distinct antigenic capsules (a to f), with type b being historically associated with a significant burden of invasive disease in children prior to the widespread use of the *Haemophilus influenzae* type b (Hib) vaccine (4, 5). Since introduction of the vaccine, nontypeable strains of *H. influenzae* have caused the majority of invasive disease with a lesser incidence of disease caused by other capsule types (6). Nontypeable strains are also commonly associated with noninvasive diseases, such as otitis media, sinusitis, pneumonia, and exacerbations of chronic obstructive pulmonary disease. As such, there is a need to develop rapid diagnostic assays that have the ability to detect all strains of *H. influenzae*, regardless of their antigenic status.

Traditional culture- and phenotypic-based methodologies for the identification of *H. influenzae* are slow and in many cases cannot differentiate *H. influenzae* from the closely related *Haemophilus haemolyticus* or other *Haemophilus* spp. (7–9). Recent studies have demonstrated that phenotypic methods, such as the API NH (bioMérieux, Nürtingen, Germany) or Vitek NH cards (bioMérieux), can fail in 1% to 10% of the analyses (3, 10, 11).

In recent years, a number of novel approaches have been described for the detection of *H. influenzae*, including matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) and nucleic acid-based diagnostic assays (12–15). As mass spectrometers are still relatively expensive and require specialist training, they are not available in all diagnostic and clinical laboratories. More importantly, the requirement for bacterial culture prior to analysis by MALDI-TOF MS takes a minimum of 1 day and often longer. Several real-time PCR diagnostic

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TABLE 1 Oligonucleotide primers and probes developed in this study

Name	Function	Sequence 5′ to 3′	Accession no. (nucleotide position [bp])
HaemF	Forward *smpB* real-time PCR assay primer	ATTAAATGTTGCATCAACGC	NC_000907.1 (213–232)
HaemR	Reverse *smpB* real-time PCR assay primer	GACTTTTGCCCACGCAC	NC_000907.1 (356–372)
H. influenzae smpB P1	*smpB* real-time PCR probe	FAM-ACGRTTTTACCATAGTTGCACTTTCTC-BHQ1	NC_000907.1 (317–343)
IAC_Fw	Forward IAC primer	AACGTAGCATTAGCTGC	HG519928.1 (111–127)
IAC_Rv	Reverse IAC primer	CTCATCTTCTTGCCTGC	HG519928.1 (260–276)
Bacillus subtilis P1	IAC probe	Cy5-CACATCCAAGTAGGCTACGCT-BHQ2	HG519928.1 (179–199)

assays exist for specific detection of *H. influenzae* (14–17). However, many of these diagnostic assays lack specificity, which can pose challenges for the clinical laboratory (15). Accordingly, there is a need for the identification and validation of novel molecular targets that can be used for rapid and specific detection of *H. influenzae*, allowing appropriate targeted antimicrobial therapy to be administered.

This study, which was designed in accordance with the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines (18), reports on the design and performance of an internally controlled real-time PCR diagnostic assay using a novel target sequence to rapidly detect and specifically identify culture-positive isolates of *H. influenzae*. This novel *smpB* real-time PCR diagnostic assay has also been applied to directly detect *H. influenzae* from respiratory samples, including sputum, bronchoalveolar lavage, and endotracheal aspirates.

MATERIALS AND METHODS

Ethics statement. We used anonymized routine specimens surplus to clinical requirements for assay validation, adhering to a governance framework agreed by and with a University College London (UCL) ethics agreement relating to the use of specimens surplus to clinical needs.

***In silico* diagnostics target identification.** We evaluated a number of previously described gene targets. Specifically, publicly available nucleotide sequences for potential diagnostics targets were retrieved from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov), the functional gene pipeline and repository (http://fungene.cme.msu.edu/), and the transfer-messenger RNA (tmRNA) website (http://bioinformatics.sandia.gov/tmrna/). *In silico* analysis of each molecular target was performed following alignments of nucleotide sequences using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/).

Bacterial strains, culture media, and growth conditions. We used a panel of 32 well-characterized *Haemophilus* and closely related species and strains (see Table S1 in the supplemental material) and 30 other bacteria (see Table S2 in the supplemental material). These species and strains were purchased from the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH [DSMZ], Braunschweig, Germany), the National Collection of Type Cultures (NCTC, Public Health England, Salisbury, United Kingdom), the American Type Culture Collection (ATCC, provided by LGC standards, Middlesex, United Kingdom), and the Culture Collection, University of Göteborg (CCUG, Sweden). All *Haemophilus* species and strains were cultured on Columbia chocolate agar (Oxoid, Hampshire, United Kingdom) at 37°C with 5% CO_2 for 18 to 24 h.

Genomic DNA isolation and quantification. Genomic DNA (gDNA) from *Haemophilus* isolates and clinical samples were isolated using a modified procedure combining mechanical lysis (IDI lysis kit; Becton Dickinson, Canada) and purification using a quick gDNA kit (Zymo Research, Irvine, CA, USA). Briefly, a loop of culture was resuspended in 250 μl IDI lysis buffer. The suspension was transferred to a GeneOhm lysis tube and bead beaten (Mini-BeadBeater-16; Stratech, United Kingdom) for 3 min. After bead beating, 200 μl of the supernatant was transferred to a Zymo-Spin column in a collection tube, and steps 2 to 5 of the procedure for purification of total DNA from cell suspensions were followed, according to the manufacturer's instructions. For all other bacterial species tested, DNA was provided from stocks held within this laboratory (Nucleic Acid Diagnostics Research Laboratory [NADRL], Microbiology, National University of Ireland, Galway).

Genomic DNA concentrations for all species and strains used in this study were determined using the Quant-iT dsDNA high-sensitivity assay kit and the Qubit fluorometer (Invitrogen Corporation, CA, USA), as per the manufacturer's instruction. Prior to use, genomic DNA samples were stored at −20°C.

Real-time PCR primers and hydrolysis probe design. Following nucleotide sequence alignments of each of the putative target genes selected for in this study, we adhered to general guidelines and recommendations for diagnostic assay oligonucleotides for the design of primers and hydrolysis probes (19). All oligonucleotides used in this study were supplied from Eurofins genomics (Essenberg, Germany). All oligonucleotide primers used in this study were designed to have a melting temperature (T_m) of 58 to 61°C, and all oligonucleotide hydrolysis probes a T_m of 7 to 10°C higher.

For the *H. influenzae* specific diagnostic assay, PCR primers HaemF and HaemR (Table 1) were designed to amplify a 160-bp fragment of the *smpB* gene, the selected target gene for development. The *H. influenzae* probe was labeled with 6-carboxyfluorescein (FAM) and black hole quencher 1 (BHQ1). The internal amplification control (IAC) PCR primers, IAC_Fw and IAC_Rv (Table 1), were designed to amplify a 206-bp region of the *Bacillus subtilis* subsp. spizizenii strain W23 *ssrA* gene. The IAC probe was labeled with Cy5 and BHQ2.

Development of IAC for real-time PCR. To avoid false-negative results due to PCR inhibition, thermocycler malfunction, and/or reagent problems, a noncompetitive IAC assay targeting the *B. subtilis* subsp. spizizenii strain W23 *ssrA* gene, was incorporated into the real-time PCR diagnostic assays (20). Titration experiments were performed to determine the optimum level of *B. subtilis* DNA to incorporate per reaction to ensure that the IAC was always detected yet have the least impact on diagnostic assay robustness. Five hundred cell equivalents of *B. subtilis* DNA per reaction was determined as the optimum concentration of IAC target DNA to include in the duplex real-time PCR assay.

Development of duplex real-time PCR *H. influenzae* diagnostic assay. To demonstrate the specificity and sensitivity of the Duplex real-time PCR, reactions were carried out on the LightCycler 480 using the LightCycler 480 Probes Master kit (Roche Diagnostics, Basel, Switzerland). The optimized PCR mix contained 2× LightCycler 480 Probes Master (6.4 mM $MgCl_2$), *H. influenzae* forward and reverse primer (0.5 μM final concentration), 6-carboxyfluorescein (FAM)-labeled probe (0.4 μM final concentration), IAC forward and reverse primer (0.25 μM final concentration), Cy5-labeled probe (0.2 μM final concentration), and template DNA (target, 5 μl; IAC, 2 μl) adjusted to a final volume of 20 μl with the addition of nuclease-free distilled water (dH_2O). The *B. subtilis* internal control DNA was diluted to contain 500 genome equivalents per 2 μl and all other DNA used in this study was diluted to contain ~10^4 genome equivalents per 5 μl.

The cycling parameters consisted of 10 min incubation at 95°C to

TABLE 2 Clinical isolates used in this study

No. of clinical isolates	Real-time PCR *fucK* assay	Real-time PCR *smpB* assay	MALDI-TOF identification	Serotype	Antimicrobial resistance(s) detected[a]	Beta-lactamase
6	*H. influenzae*	*H. influenzae*	*H. influenzae*	Nontypeable	SXT	–[b]
2	*H. influenzae*	*H. influenzae*	*H. influenzae*	Nontypeable	AMP, ERY	TEM
1	*H. influenzae*	*H. influenzae*	*H. influenzae*	Nontypeable	AMP, CRO, TET	–
1	*H. influenzae*	*H. influenzae*	*H. influenzae*	Nontypeable	AMP, AMC	–
1	*H. influenzae*	*H. influenzae*	*H. influenzae*	Nontypeable	AMP, AMC, ERY	–
1	–	*H. influenzae*	*H. influenzae*	Nontypeable	AMP, TET	TEM
5	*H. influenzae*	*H. influenzae*	*H. influenzae*	Nontypeable	AMP	TEM
17	*H. influenzae*	*H. influenzae*	*H. influenzae*	Nontypeable	Not detected	–
2	–	*H. influenzae*	*H. influenzae*	Nontypeable	Not detected	–
3	–	–	*H. parainfluenzae*		Not detected	–
1	–	–	*H. parainfluenzae*		CRO, MXF	–
1	–	–	*H. parainfluenzae*		ERY	–
1	–	–	*Haemophilus parahaemolyticus*		Not detected	–
1	–	–	*H. parahaemolyticus*		TET	–
1	–	–	*H. parahaemolyticus*		TET, ERY	–

[a] SXT, trimethoprim-sulfamethoxazole; AMP, ampicillin; ERY, erythromycin; CRO, ceftriaxone; TET, tetracycline; AMC, amoxicillin-clavulanate; MXF, moxifloxacin.
[b] –, negative.

activate the *Taq*, 50 cycles of 95°C for 10 s and 63°C for 30 s, followed by a single cooling step at 40°C for 10 s. The temperature ramp rate on the LightCycler 480 was 4.4°C/s while heating and 2.2°C/s while cooling. A color compensation file was generated to avoid fluorescence leaking from channel to channel prior to experimental analysis on the LightCycler 480, as per the manufacturer's instruction.

Clinical isolate evaluation. To evaluate the performance of the duplex real-time PCR diagnostic assays developed in this study, a panel of 44 recent clinical isolates of the genus *Haemophilus* were collected (Table 2). These isolates were cultured from clinical samples (sputum, $n = 29$; endotracheal aspirate, $n = 2$; nasal swabs, $n = 8$; bronchoalveolar lavage, $n = 2$; eye swabs, $n = 2$; and unknown, $n = 1$) using standard laboratory procedures and identified using MALDI-TOF MS (Bruker Daltonics, Bremen Germany) and MALDI-Biotyper 3.1 software. In accordance with previously published guidelines, only scores of 1.9 or greater were considered reliable for species identification (21, 22).

The antimicrobial susceptibilities of the isolates to ampicillin, amoxicillin-clavulanate, ceftriaxone, erythromycin, imipenem, moxifloxacin, tetracycline, and trimethoprim-sulfamethoxazole were determined by Etest, and results were interpreted according to EUCAST guidelines (23). All isolates were tested for beta-lactamase activity using Cefinase paper disks (Becton Dickinson). The beta-lactamase variant in positive isolates was identified by PCR for bla_{TEM} and bla_{ROB} as described previously (24, 25).

Subsequently, genomic DNA was isolated, as outlined above, from pure isolates of confirmed *Haemophilus* species (Table 2). These DNA samples were then tested blindly in triplicate with an *H. influenzae* real-time PCR diagnostic assay previously described in the literature targeting the *fucK* gene (15). This *fucK* assay was chosen for evaluation of clinical isolates and samples, as it has previously been demonstrated to be highly specific and sensitive for the detection of *H. influenzae* from clinical samples (15). For epidemiological purposes, any isolate that was determined to contain *H. influenzae* using the *smpB* real-time PCR assay was also serotyped using a previously described real-time PCR approach (17).

Direct clinical RTI sample evaluation. To demonstrate the suitability for using the assay developed in this study directly on clinical samples (sputum, $n = 67$; endotracheal aspirates, $n = 19$; bronchoalveolar lavage, $n = 12$), a panel of 98 anonymized surplus specimens (Table 3) from patients with LRTIs was collected. Using 200 μl of sample, 10-fold serial dilutions were carried out down to 10^{-5} in phosphate-buffered saline. A neat sample (50 μl) and each dilution were spread onto Colombia blood agar (CBA), Colombia agar with chocolated horse blood (CHOC), and Brilliance UTI Clarity agar (Oxoid). CBA and urinary tract infection (UTI) plates were incubated at 37°C for 18 h, and CHOC plates were incubated in a 5% CO_2 environment at 37°C for 18 h. Distinct colonies were identified using MALDI-TOF MS (Bruker Microflex LT) using MALDI Biotyper version 3.1 with default settings. From 300 μl of the sample, the total nucleic acid was isolated in accordance with the procedure outlined above (genomic DNA isolation and quantification). Nucleic acids isolated from clinical samples were then blindly tested using the previously described *fucK* real-time PCR diagnostic assay and also the novel *H. influenzae smpB* real-time PCR diagnostic assay.

RESULTS

***In silico* diagnostics target identification.** There is currently no single nucleic acid diagnostics target described in the literature that can unambiguously identify *H. influenzae*. As such, in this study, a number of gene targets, including *ssrA* and *lepA* and genes that have previously been described in the literature as suitable bacterial species specific molecular diagnostics targets, were evaluated *in silico* (26–28). A putative novel diagnostics gene target, *smpB*, selected and identified by the NADRL, was also evaluated *in silico*. From this *in silico* analysis, the *ssrA* and *lepA* genes were

TABLE 3 Clinical respiratory tract samples

No. of clinical samples	*fucK* real-time assay identification[a]	*smpB* real-time assay identification	MALDI-TOF identification
25	*H. influenzae*	*H. influenzae*	*H. influenzae*
7	*H. influenzae*	*H. influenzae*	*Haemophilus* species not detected
1	*H. influenzae*	–	*H. influenzae*
1	–	*H. influenzae*	*H. influenzae*
3	–	–	*H. influenzae*
7	*H. influenzae*	*H. influenzae*	*H. parainfluenzae* and/or *H. parahaemolyticius*
13	–	–	*H. parainfluenzae* and/or *H. parahaemolyticius*
41	–	–	*Haemophilus* species not detected

[a] –, negative.

deemed unsuitable for further use due to the nucleotide sequence similarity observed between *H. influenzae* and other closely related *Haemophilus* species. However, the *smpB* gene demonstrated sufficient intragenic nucleotide sequence variation between closely related *Haemophilus* species to allow for the design of *H. influenzae* specific probes.

Development of IAC. A noncompetitive approach was utilized for the development of the IAC in this study (20). This means that in order for a result to be considered valid using this assay, a positive signal must be obtained in the Cy5 detection channel on the LightCycler 480. For the purposes of this study, *B. subtilis* DNA was spiked into the PCR master mix to act as an internal control target.

Specificity and sensitivity of the diagnostic assays. The specificity of the real-time PCR diagnostic assay developed in this study was confirmed using the specificity panel listed in Tables S1 and S2 in the supplemental material. For inclusivity and exclusivity testing, each sample was tested in triplicate at a concentration of ~1 × 10^4 genome equivalents. The *smpB*-based assay specifically detected all 11 *H. influenzae* isolates; conversely, no other species of the *Haemophilus* genus or other bacteria were detected. The specificity of the IAC assay was also tested against the *Haemophilus* and other bacterial panels and was specific for *B. subtilis* DNA. A typical representation of the amplification curves generated in each of the analysis channels for this duplex assay is provided in Fig. S1A and B in the supplemental material.

The lower limit of detection (LOD) of the assay developed was established using probit regression analysis. With an estimated genome size of 1.8 million base pairs, each *H. influenzae* cell contains approximately 1.96 fg DNA (29, 30). Genomic DNA was quantified, and 12 replicates of each of 15, 10, 7.5, 5, 3.75, 2.5, 1.25, and 0.5 *H. influenzae* genome equivalents were tested. LODs of 6.38 were determined (95% probability). The IAC, at a concentration of 500 genome equivalents per reaction, was included in all samples during sensitivity testing and detected as expected.

Clinical isolate evaluation. For the purpose of this study, it was decided to evaluate the performance of the *smpB* assay against a gold standard that combined results of MALDI-TOF MS and the *fucK* assay. Using this approach, an isolate was identified as *H. influenzae* if a positive result was observed for either the MALDI-TOF or the *fucK* assay. An isolate that was not identified as *H. influenzae* using MALDI-TOF and was also negative with the *fucK* assay was defined as negative for *H. influenzae*.

We collected a panel of 44 recent clinical isolates of *Haemophilus* spp. to evaluate the performance of the duplex real-time diagnostic assays developed in this study (Table 2). Using the gold standard criteria outlined above, 36/44 isolates were identified as *H. influenzae* and 8 isolates were identified as other *Haemophilus* species. Genomic DNA from these isolates was also tested with the novel *smpB* diagnostic assay, and 100% concordance was observed with the gold standard method, identifying the same 36 isolates as *H. influenzae*. This demonstrated a sensitivity of 100% (95% confidence interval [CI], 90.26% to 100.00%) and a specificity of 100% (95% CI, 63.06% to 100.00%) for the *smpB* assay.

For epidemiological purposes, we also determined the capsular serotypes of all *H. influenzae* isolates and determined their antimicrobial susceptibility profiles. Using a previously developed capsular serotyping method, it was determined that all the *H. influenzae* isolates were nontypeable. Furthermore, 21 of the 44 isolates were resistant to one or more antibiotics. The most common resistance phenotypes were ampicillin (25%), trimethoprim-sulfamethoxazole (13.6%), and erythromycin (11.4%). ROB beta-lactamase was not detected in any isolates, but 18.2% of isolates were positive for TEM beta-lactamase.

Direct clinical RTI sample evaluation. We determined that our developed novel *smpB* real-time PCR diagnostic assay may be used directly to test clinical samples for *H. influenzae* by evaluating a panel of 98 respiratory specimens (Table 3). Using traditional culture methods, all of the specimens that contained *Haemophilus* species were determined to contain a minimum of 1 × 10^4 CFU/ml. Using the same criteria as outlined above for a gold standard reference, 44/98 clinical specimens were determined to contain *H. influenzae*, and 54 were negative for *H. influenzae*. Using nucleic acid purified from the above respiratory samples, the novel *smpB* assay identified *H. influenzae* in 40/98 clinical samples, and the remaining 58 samples were negative for *H. influenzae*. This demonstrated a sensitivity of 90.91% (95% CI, 78.33% to 97.47%) and a specificity of 100% (95% CI, 93.40% to 100.00%) for the *smpB* assay when used directly on respiratory specimens.

DISCUSSION

Lower RTIs are among the top four major causes of morbidity and mortality worldwide, causing 3.1 million deaths in 2012 (31). In the context of community-acquired pneumonia, *H. influenzae* has been considered a significant cause of human infection (32, 33). *H. influenzae* is also an important pathogen associated with invasive infection and sepsis, making a rapid and reliable method for its detection highly desirable.

Traditional culture-based methodologies for the detection of *H. influenzae* are slow to perform and lack specificity. Furthermore, false-negative reporting of results can occur if antimicrobial therapy has been administered prior to collection of the clinical samples. In response to this need, several real-time PCR-based diagnostic assays have been described in the literature, including those that target the *fucK*, *hpdA*, *bexA*, *ompP2*, and *P6* genes, respectively (9, 15, 16, 34). However, none of these targets is 100% specific for the detection of *H. influenzae*. For example, in the case of *hpdA* and *ompP2*, they have been found to cross-react with other *Haemophilus* species resulting in the reporting of false-positive results (9, 16). Also, the fucose kinase operon containing the *fucK* gene can be deleted in some *H. influenzae* strains, resulting in the reporting of false-negative results (35). This prompts the need for the identification of novel molecular targets for the specific detection of *H. influenzae*.

In this study, a number of potential diagnostics targets were evaluated *in silico*, including the *ssrA*, *lepA*, and *smpB* genes. These gene targets were chosen, as they are either present in all bacteria sequenced to date and/or have previously been demonstrated to be suitable as bacterial species-specific molecular-based diagnostics targets (26, 28, 36, 37). Of these, only the *smpB* gene demonstrated sufficient *in silico* nucleotide sequence heterogeneity to allow for the design of an *H. influenzae*-specific diagnostic assay. The *smpB* gene, which codes for the RNA binding protein small protein B (SmpB), has been identified in all bacterial species to date (38). It is considered an essential component of quality control in bacteria, as it facilitates the binding of tmRNA to stalled ribosomes, which in turn allows for removal of incomplete polypeptides from the cell (39, 40). As SmpB is considered essential for the correct functioning of tmRNA in a bacterial cell, the *smpB*

gene is considered among a core set of genes necessary to sustain bacterial viability in *Haemophilus* species (41).

A rapid, specific, and sensitive internally controlled real-time PCR diagnostic assay targeting the *H. influenzae smpB* gene was then developed and validated against a panel of well-characterized culture collection species and strains. Subsequently, a panel of culture-positive clinical isolates of various antimicrobial susceptibilities was evaluated with this *smpB* assay. The results obtained were then compared to a gold standard reference method, which combined results from MALDI-TOF MS and a previously described *fucK* real-time PCR assay. A gold standard combining two methods was required due to the fact that currently, no single real-time PCR assay can be used to reliably detect all strains of *H. influenzae*. The results of the *smpB* real-time PCR diagnostic assay demonstrated 100% agreement compared to the gold standard method. Some discordance would have been observed if only the results of the real-time PCR assays were compared, as 3 isolates were determined to be negative with the *fucK* assay but were positive using the *smpB* assay. This may be due to a deletion of the fucose operon in these isolates, which further demonstrates the need for evaluation and validation of novel molecular targets for the specific identification of *H. influenzae*.

For epidemiological purposes, the capsular serotype and mechanisms of antimicrobial resistance for each of these culture-positive isolates that contained *H. influenzae* as determined by MALDI-TOF MS and the *smpB* diagnostic assay were also determined. All isolates were determined to be nontypeable *H. influenzae*, which is consistent with recent findings in Europe (6, 42). The prevalence of antimicrobial resistance was similar to previous reports from the United Kingdom (43, 44). As the sample size here is small and restricted to a single location, no firm conclusions can be drawn; however, it appears prevalence of antimicrobial resistance in this species has stabilized. These results further validate the robustness of the *smpB* assay developed for this study, as they demonstrate that this novel assay can reliably detect the predominant strains of *H. influenzae* that are commonly causing LRTI.

Finally, to further demonstrate the potential clinical utility of this method, a panel of clinical LRTI specimens was evaluated using the gold standard reference mentioned above. Using the gold standard criteria, 44/98 specimens were determined to contain *H. influenzae* whereas the remaining 54 samples were negative for the presence of *H. influenzae*. Using the novel *smpB* assay, 40/98 isolates were determined to contain *H. influenzae* whereas 58 samples were negative for *H. influenzae*.

If considering only the results of the real-time PCR assays, 96/98 samples were concordant. One additional sample was also positive for *H. influenzae* using the *fucK* assay and was not detected using the *smpB* assay. This discordant result is likely due to the sensitivity of the *fucK* assay compared to the *smpB* assay. From the literature, the LOD of the *fucK* assay is ~2.5 cells whereas the LOD of the *smpB* assay presented here is 6.8 cells. One additional specimen was positive for *H. influenzae* using the *smpB* assay but was not detected by the *fucK* assay, which may be a result of a deletion of the fucose operon. As such, if only considering the results of the real-time PCR assays, the *smpB* assay may report false negatives if very low numbers of *H. influenzae* are present in a clinical sample. However, the *smpB* assay has superior specificity than the *fucK* assay and is also internally controlled, which is important when evaluating clinical samples.

When comparing the results of the real-time PCR assays and MALDI-TOF MS, *H. influenzae* was identified in an additional 14 clinical specimens using the *fucK* and *smpB*. The discrepancy in these results may have been due to the *H. influenzae* cells in these specimens being nonviable and therefore not detected by culture. In such instances, if a real-time PCR assay was used, there is a possibility of suboptimal treatment for patients. However, it is also possible that these specimens may contain viable but nonculturable cells or that they were not detected during culture due to being present at low CFU in these specimens. Alternatively, the culture and MALDI-TOF MS approach may have misidentified them, either outright or because these samples contained mixed populations of different *Haemophilus* species. As different *Haemophilus* species demonstrate highly similar colony morphology on agar plates, mixed populations may result in false-negative results when only one colony is analyzed using MALDI-TOF MS. There were 3 remaining samples identified as *H. influenzae* by MALDI-TOF MS that were not detected by the *fucK* or *smpB* real-time PCR diagnostic assays. After real-time PCR on these samples, PCR products were electrophoresed on a 1.5% agarose gel, and no visible PCR products were observed for either the *fucK* or *smpB* or real-time PCR diagnostic assays (data not shown). In the case of the *smpB* real-time PCR diagnostic assay, the discordance observed was not due to PCR inhibition as the IAC was reliably detected, as such a possible explanation for this discordance is that the purified DNA was degraded between extraction and PCR or that *H. influenzae* cells were unevenly spread throughout the sputum samples, resulting in *H. influenzae* DNA not being extracted.

While MALDI-TOF MS is simple to perform, a limiting factor of the technique is the requirement for culture of isolates prior to testing. Methods that depend on conventional bacterial culture are not always successful, particularly if antimicrobial agents have been administered prior to taking patient samples. Furthermore, as may have occurred in this study, false negatives may be reported if low cell counts or mixed cultures of indistinguishable colony morphology are present.

As such, we set out to develop a novel real-time PCR diagnostic assay for the specific detection of *H. influenzae* and to compare it to other platform technologies for performance and robustness. The diagnostic method developed in this study is the first described high-performance internally controlled duplex PCR assay capable of rapidly detecting all serotypes of *H. influenzae* with no cross-reaction observed with other culture collection and cultured clinical isolates of *Haemophilus* species. The method has been validated on a large panel of well-characterized culture collection isolates, culture-positive patient isolates, and directly on patient samples. We believe that this assay's diagnostic performance justifies a further clinical evaluation in a routine clinical microbiology laboratory to confirm the results of this study as well as to investigate its potential for widespread implementation with accompanying analysis of its value—in economic and in terms of clinical relevance.

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Appendix D

Comparative genome analysis identifies novel nucleic acid diagnostics targets for use in the specific detection of *Haemophilus influenzae*

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Comparative genome analysis identifies novel nucleic acid diagnostic targets for use in the specific detection of *Haemophilus influenzae*

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ABSTRACT

Haemophilus influenzae is recognised as an important human pathogen associated with invasive infections, including bloodstream infection and meningitis. Currently used molecular-based diagnostic assays lack specificity in correctly detecting and identifying *H. influenzae*. As such, there is a need to develop novel diagnostic assays for the specific identification of *H. influenzae*. Whole genome comparative analysis was performed to identify putative diagnostic targets, which are unique in nucleotide sequence to *H. influenzae*. From this analysis, we identified 2 *H. influenzae* putative diagnostic targets, *phoB* and *pstA*, for use in real-time PCR diagnostic assays. Real-time PCR diagnostic assays using these targets were designed and optimised to specifically detect and identify all 55 *H. influenzae* strains tested. These novel rapid assays can be applied to the specific detection and identification of *H. influenzae* for use in epidemiological studies and could also enable improved monitoring of invasive disease caused by these bacteria.

1. Introduction

Haemophilus influenzae is the most pathogenic of the 8 *Haemophilus* species, which reside as commensals in the human respiratory tract (McCrea et al., 2008). *H. influenzae* strains are categorised based on the presence (encapsulated) or absence (unencapsulated) of a polysaccharide capsule. Encapsulated strains can be further categorised based on their distinct capsular antigens (typeable; serotypes a–f), whilst unencapsulated strains are referred to as nontypeable *H. influenzae* (NTHi) (Pittman, 1931). In the prevaccine era, the encapsulated *H. influenzae* serotype b (Hib) was the primary cause of invasive disease such as meningitis, bacteraemia, and pneumonia (van Wessel et al., 2011). Upwards of 95% reduction in the number of *H. influenzae* meningitis cases have been recorded since the implementation of the Hib conjugate vaccine (Brouwer et al., 2010). Whilst *H. influenzae* invasive infection in developed countries has declined, NTHi are now the most common cause of *H. influenzae* invasive infections (van Wessel et al., 2011; MacNeil et al., 2011; Dworkin et al., 2007; Resman et al., 2011). In addition, NTHi have replaced type b strains as the most common bloodstream isolates (Aarti and Murphy, 2011).

Haemophilus haemolyticus is also a human commensal bacterium that colonises the respiratory tract and is closely related to *H. influenzae*. Both *H. influenzae* and *H. haemolyticus* require hemin (X factor) and NAD (V factor) for growth. Phenotypic methods of differentiating *H. haemolyticus* from NTHi rely on the ability of *H. haemolyticus* to lyse horse red blood cells (Sandstedt et al., 2008). However, this haemolysis may be lost after subculture (Deepa et al., 2007; Kilian, 1976), and as a consequence, nonhaemolytic *H. haemolyticus* have been misidentified as *H. influenzae* (Murphy et al., 2007). *H. haemolyticus* was considered a rare pathogen (Aarti and Murphy, 2011); however, more recently, a number of cases of invasive disease originally attributed to NTHi have been confirmed as nonhaemolytic *H. haemolyticus* (Morton et al., 2012; Anderson et al., 2012; King et al., 2011). Phenotypic techniques cannot conclusively differentiate *H. influenzae* from *H. haemolyticus* and other *Haemophilus* species, and therefore, alternative molecular techniques must be used to do so (Theodore et al., 2012).

Real-time PCR diagnostic assays for the identification of *H. influenzae* have been developed targeting various genes including *bexA* (Vanketel et al., 1990; Wroblewski et al., 2013), *ompP2* (Favaro et al., 2013; Meyler et al., 2012; Wang et al., 2011; Hobson et al., 1995), *ompP6* (Vanketel et al., 1990; Abdeldaim et al., 2009; Hedberg et al., 2009), *16S rDNA* (Murphy et al., 2007), *licA* (Meyler et al., 2012), *rnpB* (Abdeldaim et al., 2009), *frdB* (Kunthalert et al., 2013), *iga* (Vitovski et al., 2002) *hpd* (Wang et al., 2011; Pickering et al., 2014; Hare et al., 2012), and *fucK* (Meyler et al., 2012; Abdeldaim et al., 2013; Meats et al., 2003). Diagnostic assays targeting the *fucK* and *hpd* genes have been established

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as superior for the specific detection of *H. influenzae* over other routinely used gene targets (Meyler et al., 2012; Binks et al., 2012; Theodore et al., 2012). However, some NTHi strains have been reported as lacking the *fucK* (Norskov-Lauritsen, 2009; Fenger et al., 2012; Ridderberg et al., 2010; Shuel et al., 2011) or *hpd* genes (Smith-Vaughan et al., 2014). As such, published literature indicates that no one molecular marker can unequivocally differentiate NTHi from *H. haemolyticus* (Binks et al., 2012; Theodore et al., 2012).

In this study, we used comparative genome analysis to identify novel diagnostic targets, which could then be used to develop *H. influenzae*–specific real-time PCR. Two real-time PCR assays targeting these novel diagnostic targets were developed, and their performance was determined. To further validate the real-time PCR diagnostic assays developed, a panel of culture-positive clinical isolates that were identified as *H. influenzae* using Matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI-TOF MS) were blindly tested. The results of MALDI-TOF MS and the real-time PCR diagnostic assays were 100% concordant.

2. Materials and methods

2.1. Diagnostic target identification

Publically available whole genome sequences for *H. influenzae* and *H. haemolyticus* strains were analysed to identify novel diagnostic targets unique to *H. influenzae* and absent from *H. haemolyticus* and other *Haemophilus* species. *H. influenzae* whole genome sequences are publically available; however, *H. haemolyticus* genome sequences (n = 6) are only available as contiguous sequences (contigs) from the National Center for Biotechnology Information (NCBI) Web site (http://www.ncbi.nlm.nih.gov). For each of the *H. haemolyticus* strains (M19501, M19107, M21127, M21621, M21639, and HK386), contigs were ordered and assembled using the Mauve contig mover tool in the Mauve genome alignment software (http://gel.ahabs.wisc.edu/mauve/). The European Molecular Biology Open Software Suite tool Emboss union (http://emboss.us.es/cgi-bin/emboss/union) was used to concatenate the multifasta Mauve output to create a single FASTA formatted file. Whole genome annotation was then carried out using the online genome annotation service Rapid Annotation using Subsystem Technology (RAST; http://rast.nmpdr.org/) and the resulting data downloaded in EMBL format. Potential diagnostic targets within *H. influenzae* were then identified using WebACT (http://www.webact.org/WebACT/home) by aligning the whole genome sequence of 3 *H. haemolyticus* strains (M19501, M19107, and HK386) and the whole genome sequence of their most closely related *H. influenzae* strain (R2866: accession number NC_017451). Potential diagnostic targets were further analysed using microbial nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&BLAST_SPEC=MicrobialGenomes) to search for similarities in closely related species.

2.2. Bacterial strains, culture media, and growth conditions

A panel of *H. influenzae* (n = 13) and closely related species (n = 33) were obtained from various culture collections (Table 1). All strains were cultured under microaerophillic conditions in *Haemophilus* test media broth, chocolate broth, or on Columbia chocolate agar plates at 37 °C until sufficient growth was observed.

2.3. DNA isolation and quantification

Genomic DNA from all species was isolated from 1.5 mL of culture using a DNeasy Blood and Tissue Kit as per manufacturers' instructions (Qiagen, Hilden, Germany). DNA concentrations were determined using the Qubit dsDNA HS Assay and the Qubit 1.0 fluorometer (Life Technologies, Carlsbad, CA, USA). Purified DNA samples were stored at −20 °C prior to use.

Table 1
Bacterial species and strains included in this study.

Organism	Strain[a]/source	Assay[b]		
		phoB	*pstA*	*fucK*
H. influenzae reference strains				
H. influenzae type a	NCTC 8465	+	+	+
H. influenzae type b	DSMZ 23393[a]	+	+	+
H. influenzae type b	DSMZ 11969[a]	+	+	+
H. influenzae type b	DSMZ 11970[a]	+	+	+
H. influenzae type b	DSMZ 10001[a]	+	+	+
H. influenzae type b	DSMZ 4690[a]	+	+	+
H. influenzae type c	NCTC 8469	+	+	+
H. influenzae type d	DSMZ 11121[c]	+	+	+
H. influenzae type e	NCTC 8472	+	+	+
H. influenzae type f	DSMZ 10000[a]	+	+	+
H. influenzae NTHi	DSMZ 9999[a]	+	+	+
H. influenzae NTHi	CCUG 58365	+	+	−
H. influenzae biogroup aegyptius	DSMZ 21187[a]	+	+	+
Clinical isolates (n = 6)[c]	Blood	+	+	+
Clinical isolates (n = 12)[c]	Ear/Eye swab	+	+	+
Clinical isolates (n = 24)[c]	Sputum	+	+	+ (21)[d]
Non–*H. influenzae* strains				
H. haemolyticus	CDC-M19501	−	−	−
H. haemolyticus	CDC-M21127	−	−	−
H. haemolyticus	CDC-M21621	−	−	−
H. haemolyticus	CCUG 24149	−	−	−
H. haemolyticus	CCUG 36015	−	−	−
H. haemolyticus	CCUG 36016	−	−	−
H. haemolyticus	CCUG 15642	−	−	−
H. haemolyticus	CCUG 15312	−	−	−
H. haemolyticus	NCTC 10839	−	−	−
H. haemolyticus	NCTC 10659	−	−	−
H. ducreyi	DSMZ 8925	−	−	/
H. felis	DSMZ 21192	−	−	/
H. haemoglobinophilus	DSMZ 21241	−	−	/
H. paracuniculus	DSMZ 21452	−	−	/
H. parahaemolyticus	DSMZ 21417	−	−	/
H. parainfluenzae	DSMZ 8978	−	−	/
H. paraphrohaemolyticus	DSMZ 21451	−	−	/
H. parasuis	DSM 21448	−	−	/
H. pittmaniae	DSMZ 21203	−	−	/
H. pittmaniae	DSMZ 17420	−	−	/
Haemophilus spp.	CCUG 34110	−	−	−
Actinobacillus suis	DSMZ 22433	−	−	/
Actinobacillus pleuropneumoniae	DSMZ 13472	−	−	/
Aggregatibacter aphrophilus	NCTC 11096	−	−	/
Aggregatibacter aphrophilus	NCTC 10558	−	−	/
Aggregatibacter actinomycetecomitans	DSMZ 11121	−	−	/
Aggregatibacter actinomycetecomitans	DSMZ 8324	−	−	/
Aggregatibacter segnis	NCTC 10977	−	−	/
Avibacterium avium	DSMZ 18557	−	−	/
Avibacterium paragallinarum	DSMZ 18554	−	−	/
Gardnerella vaginalis	DSMZ 4944	−	−	/
Histophilus somni	CCUG 36157	−	−	/
Taylorella equigenitalis	DSMZ 10668	−	−	/

NCTC = National Collection of Type Cultures; DSMZ = The German Collection of Microorganisms; CCUG = Culture Collection, University of Göteborg, Sweden; CDC = Centre for Disease Control.

[a] *pstA* and *phoB* gene sequence data were generated for each of these *H. influenzae* strains using primers outlined in Table 2.

[b] + = Positive; − = negative; / = not tested for.

[c] All clinical isolates were identified as *H. influenzae* using MALDI-TOF MS.

[d] 3/24 *H. influenzae* clinical isolates not detected by the *fucK* assay.

2.4. Conventional PCR and nucleic acid sequencing

Nucleotide sequence data used for real-time PCR diagnostic assay design were recovered from the NCBI (http://www.ncbi.nlm.nih.gov/) or was generated in this study. Sequencing primers were designed to amplify 832 bp of the *pstA* gene and 681 bp of the *phoB* gene of *H. influenzae* to identify optimal diagnostic target regions for primer and probe design.

PCR was carried out using the sequencing primers (Table 2) on an iCycler iQ thermal cycler (Bio-Rad Laboratories, Richmond, CA, USA) to amplify the *pstA* and *phoB* targets in representative culture collection strains of *H. influenzae*. All reactions were performed using the FastStart

Table 2
Nucleotide sequences of primers and probes.

Probe/primer	Function	DNA sequence (5′–3′)	Nucleotide position	GenBank accession no.	Concentration (μmol/L)
pstA F1	Sequencing forward primer	CTAATCAAAACTTGCGT	8-24	CP002277.1	0.4 μmol/L
pstA R1	Sequencing reverse primer	TGTTGAAAGAAGAGTCG	823-839	CP002277.1	0.4 μmol/L
phoB F1	Sequencing forward primer	CTGATAGTTGAAGATG	16-31	CP002277.1	0.4 μmol/L
phoB R1	Sequencing reverse primer	TCATTGTTTATCTCGT	681-696	CP002277.1	0.4 μmol/L
pstA P1	*H. influenzae pstA* forward primer	CGTTTCGCACAAATTACC	310-327	CP002277.1	0.5 μmol/L
pstA P2	*H. influenzae pstA* reverse primer	GTGCGTACCACGATAGG	460-476	CP002277.1	0.5 μmol/L
pstA probe	*H. influenzae pstA* hydrolysis probe	FAM-CTGGAGCATTCGCATTAGCTT-BHQ1	428-448	CP002277.1	0.2 μmol/L
phoB P1	*H. influenzae phoB* forward primer	TTGGATTGGATGCTACC	151-167	CP002277.1	0.5 μmol/L
phoB P2	*H. influenzae phoB* reverse primer	AGTGATGTAGTCATCAGC	292-309	CP002277.1	0.5 μmol/L
phoB probe	*H. influenzae phoB* hydrolysis probe	FAM-AGAAAGCTATGCTGCGATTCC-BHQ1	210-230	CP002277.1	0.2 μmol/L
BSS-1 F	IAC forward primer	AACGTAGCATTAGCTGC	111-127	HG519928.1	0.5 μmol/L
BSS-1R	IAC reverse primer	CTCATCTTCTTGCCTGC	260-276	HG519928.1	0.5 μmol/L
BSS-P	IAC – specific hydrolysis probe	Cy5-CACATCCAAGTAGGCTACGCT-BHQ2	179-199	HG519928.1	0.2 μmol/L

PCR Master Kit (Roche Diagnostics, Basel, Switzerland) according to manufacturer's instructions. The thermal cycling parameters used consisted of a denaturation cycle at 95 °C for 4 min, followed by 35 cycles at 95 °C (30 s), 50 °C (30 s), and 72 °C (30 s), and a final elongation cycle at 72 °C for 7 min. PCR products were purified using the HighPure PCR product purification kit (Roche Diagnostics) and were sequenced externally (Sequiserve, Vaterstetten, Germany).

2.5. PCR primer and probe design

Nucleotide sequences of the diagnostic targets identified in this study were aligned using ClustalW multiple sequence alignment programme (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Oligonucleotide primers and hydrolysis probes were manually designed in accordance with general recommendations and guidelines (Tevfik, 2006; Robertson and Walsh-Weller, 1998). Species specific oligonucleotide primers and probe were designed for real-time PCR diagnostic assays. All primers and probe (Table 2) used in this study were obtained from Eurofins MWG-Operon (Ebersberg, Germany).

2.6. Internal amplification control (IAC) development

A noncompetitive IAC targeting the *ssrA* gene of *Bacillus subtilis* subsp. *spizizenii* previously developed in-house was incorporated into the real-time PCR diagnostic assays (Table 2).

2.7. Real-time PCR

Duplex real-time PCR diagnostic assays were optimised, and the analytical specificity and Limit of Detection (LOD) determined. All real-time PCR diagnostic assays were performed on a LightCycler 480 (Roche Diagnostics) using the LightCycler 480 probes master kit (Roche Diagnostics). Real-time PCR reactions were performed in a total volume of 20 μL consisting of 2× probes master, forward and reverse primers (0.5 μmol/L final concentration), probes (0.2 μmol/L final concentration), template DNA (2 μL, 10^4 genome equivalents [GE]) and IAC DNA (2 μL, 10^3 GE). Nuclease-free H_2O was added to make up final volume. A no-template control was included in each experiment. The cycling parameters consisted of a 10-min denaturation step followed by 50 cycles of 95 °C for 10 s, 60 °C for 30 s and a final cooling step at 40 °C for 10 s. The LightCycler 480 ramp rate was 4.4 °C/s whilst heating and 2.2 °C/s on cooling. To prevent fluorescence signal leakage between detection channels on the LightCycler 480, a colour compensation was generated (Anonymous, 2008).

2.8. Multiplex PCR assay specificity and LOD

To evaluate the specificity of the diagnostic assays developed, genomic DNA from 55 *H. influenzae* and 33 closely related *Haemophilus* species (Table 1) were tested in triplicate. The LOD of each assay in multiplex format was determined using probit regression analysis in MiniTab (MiniTab, State College, PA, USA) (Burd, 2010). Purified *H. influenzae* genomic DNA (DSM4690) was diluted in nuclease-free dH_2O. Twelve replicates of each dilution 15, 10, 8, 6, 4, 2, 1, and 0.1 GE were evaluated.

To determine the efficiency of each assay in both monoplex and multiplex format, serial dilutions of purified *H. influenzae* genomic DNA (DSM4690) at 1×10^6, 5×10^5, 1×10^5, 5×10^4, 1×10^4, 5×10^3, 1×10^3, 5×10^2, 1×10^2, and 5×10^1 were tested in triplicate. Standard curves of quantification cycle (Cq) versus GE were then constructed. Efficiency (%) was calculated based on the slope of the line using an online real-time PCR efficiency calculator (http://www.thermoscientificbio.com/webtools/qpcrefficiency/).

2.9. Clinical isolate evaluation

H. influenzae clinical isolates (n = 42) were cultured on Columbia chocolate agar plates and were subjected to MALDI-TOF MS analysis (MALDI biotyper; Bruker Daltonics GmbH, Leipzig, Germany), and the spectra were compared to the software version 3.4. A number of clinical isolates were typed using conventional PCR (Falla et al., 1994) (n = 6, isolated from blood; Table 1) and real-time PCR (Wroblewski et al., 2013) (n = 3, isolated from sputum; Table 1).

2.10. fucK real-time PCR

H. influenzae culture-confirmed clinical isolates (n = 42), *H. influenzae* culture collection isolates (n = 13), *H. haemolyticus* (n = 10), and 1 *Haemophilus* spp. (previously designated *H. haemolyticus*) were also tested in triplicate using a published *fucK* real-time PCR diagnostic assay (Meyler et al., 2012).

3. Results

3.1. Diagnostic target identification

Whole genome sequence comparison of *H. influenzae* R2866 and 3 *H. haemolyticus* strains (M19502, M19107, and HK386) was carried out using WebACT. *H. influenzae* R2866 was used as a reference genome, as it was recognised as being the most closely related strain to *H. haemolyticus* based on the RAST comparison analysis. A total of 325 unique regions, composing of both genes and intergenic nucleotide sequence, were identified, which were present in *H. influenzae* and absent in *H. haemolyticus*. Each of these regions was BLAST analysed and further evaluated using 3 criteria to determine their suitability as diagnostic targets. First, regions were refined by ruling out putative diagnostic targets that had significant BLAST hits with *H. haemolyticus*. Second, based on in silico evaluation, any putative diagnostic targets identified must be present in all 22 publically available whole and draft genome sequences of *H. influenzae*. Finally, diagnostic targets identified with significant interstrain sequence variation were then also excluded.

Based on the above criteria, 6 diagnostic targets (*pstS*, *pstC*, *pstA*, *pstB*, *phoR*, and *phoB*) were identified as having the greatest potential for the development of *H. influenzae*–specific real-time PCR diagnostic assays. Nucleotide sequence data for each of the potential diagnostic targets were retrieved from NCBI, and in silico analysis was performed to further assess their suitability for *H. influenzae* specific detection. Of these, *pstA* and *phoB* were found to contain the most sequence variation when compared with other non–*H. influenzae* bacterial species and contain the least *H. influenzae* interstrain variation and were, therefore, selected for further analysis.

3.2. Real-time PCR assay specificity

The specificity of each real-time PCR diagnostic assay was confirmed in duplex format using the panel of bacteria listed in Table 1. All culture collection strains and clinical isolates of *H. influenzae* were detected by both assays. None of the non–*H. influenzae* microorganisms tested were detected by the assays (Supplementary Fig. 1A–D).

3.3. Real-time PCR assay efficiency and LOD

The efficiencies of each assay were evaluated in both monoplex and multiplex real-time PCR format. *H. influenzae* genomic DNA (DSMZ 4690) was quantified, and serial dilutions were prepared based on *H. influenzae* genome size (1,830,137 bp) (Fleischmann et al., 1995), which equates to 1.9 fg DNA per cell (http://www.changbioscience.com/genetics/mw.html). Both assays were highly efficient in monoplex (*phoB* 94.66%, *pstA* 94.51%). In multiplex, efficiency is comparable in the *pstA* assay (93.47%) and is slightly reduced in the *phoB* assay (88.87%). Plots of C_p versus GE were linear over a range of 1×10^6, 5×10^5, 1×10^5, 5×10^4, 1×10^4, 5×10^3, 1×10^3, 5×10^3, 1×10^3, 5×10^2, 1×10^2, and 5×10^1 GEs for the *phoB* and *pstA* monoplex and *pstA* multiplex assays (Supplementary Fig. 2A–C). Linearity is decreased at lower concentrations of *H. influenzae* DNA in the *phoB* multiplex assay (Supplementary Fig. 2D).

In multiplex format, the LOD was determined using probit regression analysis (Burd, 2010). With 95% confidence, the LOD of the *phoB* and *pstA* assays was 6.49 and 10.49 GE, respectively.

3.4. fucK real-time PCR assay

A *fucK* real-time PCR diagnostic assay was also used to identify all *H. influenzae* culture collection strains and clinical isolates (Table 1) used in this study. From this analysis, 51/55 of the tested *H. influenzae* strains were detected by the *fucK* assay.

3.5. Clinical isolate evaluation

All 42 clinical isolates were screened using MALDI-TOF MS. The MALDI-TOF software identified all strains to species level and confirmed that all clinical isolates are *H. influenzae*, all with scores greater than 2.0. All clinical isolates typed ($n = 9$) using published capsular typing methods were confirmed as NTHi.

4. Discussion

H. influenzae is known to cause a wide spectrum of disease, ranging from noninvasive infections including bronchitis and conjunctivitis to more serious infections including meningitis, pneumonia, and sepsis. Bacterial culture is the preferred method for the diagnosis of invasive *H. influenzae* (Meyler et al., 2012). However, culture-based methods are time consuming, often taking up to 36 h or more (Corless et al., 2001; Greenberg-Kushnir et al., 2012; Nakhjavani et al., 2005). Also, in many cases, pretreatment of the infection with broad spectrum antibiotics may produce false negatives (Dalton and Allison, 1968). Evaluation of a number of biomarker genes for differentiating *H. influenzae* from *H. haemolyticus* and other non–*H. influenzae* species suggests that there is no one diagnostic target identified to date that can unambiguously differentiate the 2 microorganisms (Binks et al., 2012).

Recently, draft whole genome sequences of 6 *H. haemolyticus* strains have been published (King et al., 2011). In the study presented here, comparative analysis between these strains and *H. influenzae* highlighted a number of variable regions between the genomes of the 2 species. BLAST and ClustalW analysis identified 6 genes, *phoB*, *phoR*, *pstS*, *pstC*, *pstA*, and *pstB*, as potential diagnostic targets for the discrimination of the 2 microorganisms. In silico nucleotide sequence analysis revealed that these genes are present in *H. influenzae* but are absent in all other recognised *Haemophilus* species. These genes, which have been studied extensively in *E. coli*, are members of the bacterial *pho* (phosphate) regulon and are involved in the transport and assimilation of inorganic phosphate (Pi) (Vershinina and Znamenskaia, 2002; Crepin et al., 2011).

Two of the putative diagnostic targets identified, namely, *phoB* and *pstA*, were further evaluated for their ability to accurately identify *H. influenzae* and distinguish it from other closely related *Haemophilus* species. *phoB* was chosen as a diagnostic target, as it is only present in gram-negative bacteria (with a homolog *phoP* in gram-positive bacteria) (Vershinina and Znamenskaia, 2002). The *pstA* gene was also chosen as a diagnostic target for further evaluation, as it was found to have the least interstrain gene nucleotide sequence heterogeneity and the greatest level of gene nucleotide sequence variation compared with other bacteria (data not shown). Internally controlled real-time duplex PCR assays were developed for each of these selected diagnostic targets. Both assays were determined to be 100% specific for the detection of *H. influenzae*. The established LOD of 6.49 GE and 10.49 GE (95% confidence) for both assays, respectively, is comparable to other studies targeting *fucK*, which have LODs of 10 genome copies (95% confidence) (Meyler et al., 2012) and between 5 (5/8 reactions) and 50 (8/8 reactions) genomes copies (Abdeldaim et al., 2013). We also compared our assays to a previously developed *fucK* real-time PCR assay, as this is one of the diagnostic target most commonly used in the literature for the specific detection of *H. influenzae*. This comparative analysis demonstrated that both the *phoB* and *pstA* assays are more specific for the detection of *H. influenzae* compared to the *fucK* assay. Of the 42 clinical isolates identified as *H. influenzae* using MALDI-TOF MS and the novel real-time PCR diagnostic assays developed in this study, 3 (7.1%) were not detected by the *fucK* assay. One of the culture collection strains (CCUG 58365) was also not detected using the *fucK* assay due to a deletion of the fucose operon as previously reported in this strain (Ridderberg et al., 2010).

When evaluating the 2 real-time PCR diagnostic assays developed in this study, their performance in terms of specificity and LOD are comparable. However, the *pstA* multiplex real-time PCR assay is more efficient than the *phoB* multiplex real-time PCR assay. In addition, IAC amplification is inhibited at a *H. influenzae* DNA concentration of greater than 10^5 GE in the *phoB* multiplex PCR assay, revealing that the *pstA* assay is more robust in terms of overall real-time PCR assay performance.

In this study, we set out to identify novel diagnostic targets that could be used in real-time PCR assays to rapidly detect and specifically identify *H. influenzae*. The novel real-time PCR diagnostic assays developed could enable improved monitoring of invasive disease caused by these bacteria whilst also providing the clinician with valuable information about the optimal therapeutic regimen to initiate patient treatment. We propose that the developed assays now merit extensive multicentre evaluation in clinical settings.

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Appendix E

Experimental validation of duplex real-time NASBA diagnostics assays incorporating an endogenous control for the detection of predominant microorganisms associated with bacterial meningitis

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Development of internally controlled duplex real-time NASBA diagnostics assays for the detection of microorganisms associated with bacterial meningitis

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ABSTRACT

Three duplex molecular beacon based real-time Nucleic Acid Sequence Based Amplification (NASBA) assays have been designed and experimentally validated targeting RNA transcripts for the detection and identification of *Haemophilus influenzae*, *Neisseria meningitidis* and *Streptococcus pneumoniae* respectively. Each real-time NASBA diagnostics assay includes an endogenous non-competitive Internal Amplification Control (IAC) to amplify the splice variant 1 mRNA of the *Homo sapiens* TBP gene from human total RNA. All three duplex real-time NASBA diagnostics assays were determined to be 100% specific for the target species tested for. Also the Limits of Detection (LODs) for the *H. influenzae*, *N. meningitidis* and *S. pneumoniae* duplex real-time NASBA assays were 55.36, 0.99, and 57.24 Cell Equivalents (CE) respectively. These robust duplex real-time NASBA diagnostics assays have the potential to be used in a clinical setting for the rapid (<60 min) specific detection and identification of the most prominent microorganisms associated with bacterial meningitis in humans.

1. Introduction

Bacterial meningitis is defined as an inflammation of the meninges surrounding the central nervous system. It is a serious and often fatal infection with high morbidity and mortality rates worldwide (5–40% in children, 20–50% in adults) (Brouwer et al., 2010a). *Haemophilus influenzae*, *Neisseria meningitidis* and *Streptococcus pneumoniae* are the most predominant etiological agents of bacterial meningitis, accounting for 75–80% of cases (Won et al., 2012; McIntyre et al., 2012; Brouwer et al., 2010b). Since the introduction of the *H. influenzae* type b (Hib) conjugate vaccine in the 1990s, *N. meningitidis* and *S. pneumoniae*, have replaced *H. influenzae* as the leading causes of bacterial meningitis in industrialised countries (Brouwer et al., 2010b). However, in developing countries, the world health organization estimates vaccine coverage to be as little as 21% (http://www.who.int/mediacentre/factsheets/fs378/en/), *H. influenzae* type b invasive disease still remains a significant concern to human health. Several vaccines have also been developed for the prevention of disease from the most common causes of pneumococcal and meningococcal meningitis (e.g. 4CMenB, MenB-FHbp, MCV4, MPSV4, PCV13 and PPV23) (Nuorti et al., 2010; Folaranmi et al., 2015; Bilukha et al., 2005). While an overall decrease in the incidence of bacterial meningitis cases has been observed as a result of the implementation of these vaccines, an estimated 1·2 million cases of bacterial meningitis still occur worldwide every year which resulted in 180,000 deaths in children aged 1–59 months in 2010 (Liu et al., 2012). These vaccines are not effective against all strains of these bacteria associated with infection as illustrated by several cases of pneumococcal and meningococcal meningitis attributed to infection caused by non-vaccine serotypes (Kara et al., 2014; Xie et al., 2013; Delrieu et al., 2011). Finally, while vaccination continues to have a protective effect in humans and is critically important in combating meningococcal disease, vaccine failures are also well described (Broderick et al., 2012).

As such, when a patient presents with suspected bacterial meningitis or with features consistent with meningococcal blood stream infection there is a clinical need to detect all strains of *Haemophilus influenzae*, *Neisseria meningitidis* and *Streptococcus pneumoniae*. At present, culture of Cerebrospinal Fluid (CSF) and blood remains the gold standard for the diagnosis of bacterial meningitis. However, this is time consuming,

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often taking 36 h or more to confirm a specific a diagnosis (Corless et al., 2001), has limited sensitivity with slow growing, fastidious or non-culturable microorganisms (Klouche and Schroder, 2008; Peters et al., 2004) and is problematic in resource poor settings (Brouwer et al., 2012). Administration of antibiotics prior to sample collection further complicates the situation as it diminishes the likelihood of culture confirmation (Brouwer et al., 2010b; Corless et al., 2001; Klouche and Schroder, 2008; Peters et al., 2004). Consequently there is a need for the development of more rapid, sensitive and specific non-culture based diagnostics methods such as nucleic acid *in vitro* amplification technologies. This would enable improved monitoring, allow for the more rapid administration of the appropriate pathogen specific antibiotic treatment regime in a patient with suspected meningitis and improve the overall prognosis of the disease.

Nucleic Acid Sequence Based Amplification (NASBA) represents a suitable technology that could be applied in this setting. NASBA is an isothermal transcription based nucleic acid *in vitro* amplification technique which exploits three enzymes: avian myeloblastosis virus reverse transcriptase, *Escherichia coli* ribonuclease H and T7 DNA dependant RNA polymerase to specifically amplify RNA targets (Compton, 1991). A number of hybridization based end-point detection methods, such as electrochemiluminescence (van Gemen et al., 1994), enzyme-linked gel assay (Uyttendaele et al., 1994), and fluorescence correlation spectroscopy can be used to detect the NASBA *in vitro* amplified RNA amplicons (Oehlenschlager et al., 1996; Robertson and Walsh-Weller, 1998). More recently, molecular beacon probe technology has enabled the real-time detection of NASBA amplicons (Leone et al., 1998; Tyagi and Kramer, 1996). Labelling molecular beacon probes with fluorophores that emit light at different wavelengths enables the simultaneous *in vitro* amplification and real-time detection of different RNA targets in one reaction (Tyagi et al., 1998). An advantage of this technology is the ability to discriminate between specific microbial species in a single closed tube reaction. The ability to detect multiple analytes in one reaction highlights a clear advantage of real-time NASBA over other isothermal amplification methods developed to date. For example this allows for the inclusion of an Internal Amplification Control (IAC) which is deemed an important analytical control in molecular *in vitro* amplification techniques to verify the accuracy of the results obtained and provide a more robust diagnostics assay (Hoorfar et al., 2004). Furthermore NASBA is specific for RNA and as such only identifies viable infectious agents (Romano et al., 1997). It is also theoretically more sensitive than other *in vitro* amplification technologies that target DNA since target RNA transcript copy number can be greater than DNA copy number in biological cells (van der Meide et al., 2005). Finally, like other isothermal *in vitro* amplification methods, the use of real-time NASBA has the potential to eliminate the need for thermal cycling and ramping instrumentation requirements which facilitates its potential application on a low cost lab on a chip (LOC) and/or point of care (POC) diagnostic devices (Gulliksen et al., 2004; Dimov et al., 2008).

To our knowledge there are currently no reported internally controlled NASBA or real-time NASBA diagnostics assays described in the literature for use in the specific detection of *H. influenzae*, *N. meningitidis* and *S. pneumoniae*. In this study we outline the design, development, optimisation of three novel duplex real-time NASBA diagnostics assays incorporating an endogenous IAC, for use in the detection of the predominant microorganisms associated with bacterial meningitis.

2. Materials and methods

2.1. Diagnostic target identification

The novel diagnostic RNA targets used in this study were identified bioinformatically using nucleotide sequences retrieved from a number of publically available databases including the tmRNA website (http://bioinformatics.sandia.gov/tmrna/), the tmRNA database (http://www.ag.auburn.edu/mirror/tmRDB/), the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/) and the Functional Gene Pipeline (FunGene) website (http://fungene.cme.msu.edu/).

2.2. Bacterial strains, culture media and growth conditions

A panel of culture collection strains of *H. influenzae* (n = 8), non-*H. influenzae Haemophilus* species (n = 21), *N. meningitidis* (n = 9), non-*N. meningitidis Neisseria* species (n = 18), *S. pneumoniae* (n = 3) and non-*S. pneumoniae Streptococcus* species (n = 26) were obtained from various culture collections (Supplementary Table 1). A collection of recent *H. influenzae* clinical isolates (n = 16), *N. meningitidis* clinical isolates (n = 8) and *S. pneumoniae* clinical isolates (n = 9) were also obtained from University Hospital Galway to further evaluate the assays. *Haemophilus* strains were cultured in haemophilus test media broth, chocolate broth or on Columbia chocolate agar plates. *Neisseria* and *Streptococcus* species were cultured in brain heart infusion (BHI) broth, or on Columbia blood agar plates. All bacterial species were cultured under microaerophilic conditions at 37 °C overnight or until sufficient growth was observed, as determined by the degree of turbidity compared to culture negative controls.

2.3. DNA isolation and quantification

Genomic DNA from a collection of *Haemophilus* and *Neisseria* species was isolated from 1.5 ml of culture using a DNeasy Blood and Tissue Kit as per manufacturers' instructions (Protocol: Gram-Negative Bacteria; Qiagen, Hilden, Germany). DNA integrity was assessed on a 1% agarose gel and concentrations were determined using the Qubit dsDNA HS Assay and the Qubit 1.0 fluorometer (Life Technologies, Carlsbad, CA). Purified DNA samples were stored at −20 °C prior to use.

2.4. Total RNA isolation and quantification

Total RNA from all bacterial species was isolated and purified from 1.5 ml of culture using a RiboPure Yeast Kit as per manufacturers' instructions (Ambion, Austin, TX, USA). For human RNA, whole blood was purchased from a commercial provider (Seralab, UK). Subsequently total RNA was isolated and purified from 2.5 ml of these blood samples using the PAXgene blood RNA kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. To determine the integrity of the RNA purified from both the bacterial cultures and human whole blood samples, total RNA was analysed with the Agilent 2100 Bioanalyzer (Agilent Technologies). Concentrations of total RNA were determined using the Qubit RNA BR Assay kit and the Qubit 1.0 fluorometer (Life Technologies, Carlsbad, CA). All purified total RNA samples were diluted and stored at −80 °C prior to use.

2.5. Conventional PCR primer design

Publically available nucleotide sequences of the diagnostics targets identified in this study were aligned using ClustalW multiple sequence alignment programme (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Oligonucleotide sequencing primers were manually designed in accordance with general recommendations and guidelines (Robertson and Walsh-Weller, 1998; Dorak, 2006) to target conserved regions within the genes. All oligonucleotide primers (Table 1) used in this study were obtained from Eurofins MWG-Operon (Ebersberg, Germany).

2.6. Conventional PCR and nucleic acid sequencing

Sequencing oligonucleotide primers were designed to amplify 681 bp of the *phoB* gene of *H. influenzae*, and 342 bp of the *ssrA* gene of *N. meningitidis*, to identify optimal diagnostics target regions for NASBA primer and molecular beacon probe design. Due to the extent

Table 1
Nucleotide sequences of oligonucleotide primers and molecular beacon probes.

Probe/Primer	Gene target	Function	DNA sequence (5′–3′)	Nucleotide position	Genbank accession no.	Final concentration (μM)
PhoB F1	*phoB*	Sequencing forward primer	CTGATAGTTGAAGATG	16–31	CP002277.1	0.4 μM
PhoB R1	*phoB*	Sequencing reverse primer	TCATTGTTTATCTCGT	681–696	CP002277.1	0.4 μM
NeisseriaF	*ssrA*	Sequencing forward primer	GGCGACCTTGGTTTCGACG	4–22	AE002098.2	0.3 μM
NeisseriaR	*ssrA*	Sequencing reverse primer	TCGAACCCCGTCCGAAAG	327–345	AE002098.2	0.3 μM
PhoB P1	*phoB*	*H. influenzae phoB* forward primer	AATTCTAATACGACTCACTATAGGGAGAAGGGTAGTCATCAGCCCCTGCA	285–303	CP002277.1	0.2 μM
PhoB P2	*phoB*	*H. influenzae phoB* reverse primer	GGTCGTTCAGGTATACAA	169–186	CP002277.1	0.2 μM
PhoB MB	*phoB*	*H. influenzae phoB* specific hybridization probe	FAM–CCGAGTAAGCTATGCTGCGATTCCACTCGG–BHQ1	213–230	CP002277.1	0.1 μM
Nmen P1	*ssrA*	*N. meningitidis* forward primer	AATTCTAATACGACTCACTATAGGGAGAAGGTCCTCTACAAAGCGTTCTACA	305–325	AE002098.2	0.2 μM
Nmen P2	*ssrA*	*N. meningitidis* reverse primer	CCCGTAAAACACTGAATTCAAAT	63–85	AE002098.2	0.2 μM
Nmen MB	*ssrA*	*N. meningitidis ssrA* specific hybridization probe	HEX-CGATCGAACTGGTTTCCAAAAGGCCTCGATCG-DAB	236–255	AE002098.2	0.1 μM
Spn P1	*lepA*	*S. pneumoniae* forward primer	AATTCTAATACGACTCACTATAGGGAGAAGGCATACTCAAGACGCTGAGGAA	1792–1812	AE007317.1	1 μM
Spn P2	*lepA*	*S. pneumoniae* reverse primer	GACACAAGATTGTCGCTCGTACTGATA	1628–1654	AE007317.1	1 μM
Spn MB	*lepA*	*S. pneumoniae lepA* specific hybridization probe	ROX-CGATCGACGCATGAAATCCATCGGATCAGTTCGATCG-BHQ2	1749–1773	AE007317.1	0.5 μM
TBP P1	*TBP*	IAC-NASBA forward primer	AATTCTAATACGACTCACTATAGGGAGAAGGTGAGCACAAGGCTTCTA	1088–1105	NC000006.12	0.2 μM
TBP P2	*TBP*	IAC-NASBA reverse primer	AACAGTCCAGACTGGC	962–977	NC000006.12	0.2 μM
TBP MB	*TBP*	IAC-specific hybridization probe	CY5-CTCGAGAGCTGTGATGTGAAGTTTCCCTCGAG-BHQ2	1060–1079	NC000006.12	0.1 μM

of publically available *S. pneumoniae lepA* sequence data, sequencing of in-house *S. pneumoniae* culture collection strains was not carried out.

PCR was carried out using the sequencing primers (Table 1) on an iCycler iQ thermal cycler (Bio-Rad Laboratories Inc., CA). All reactions were performed using the FastStart PCR Master Kit (Roche Diagnostics, Basel, Switzerland) according to manufacturer's instructions in a final volume of 25 μl. The thermal cycling parameters used for the *H. influenzae* specific primers consisted of a denaturation cycle at 95 °C for 4 min, followed by 35 cycles at 95 °C (30 s), 50 °C (30 s) and 72 °C (30 s), and a final elongation cycle at 72 °C for 7 min. Thermal cycling parameters used for the *Neisseria* specific primers were the same as outlined above with the exception of an annealing temperature of 55 °C instead of 50 °C. The PCR products were purified using the HighPure PCR product purification kit (Roche Diagnostics) and sequenced externally (Sequiserve, Vaterstetten, Germany).

2.7. Real-time NASBA primer and molecular beacon probe design

Nucleotide sequence data used for real-time NASBA diagnostics assay design was either recovered from publically available sequence databases or were generated in this study. Species specific oligonucleotide primers and molecular beacon probes were manually designed in accordance with recommended guidelines (Deiman et al., 2002). Primer, molecular beacon, and NASBA amplicon structure were checked for secondary structures by using an RNA folding programme (MFold: http://unafold.rna.albany.edu/?q=mfold/RNA-Folding-Form2.3) with the folding temperature parameter adjusted to 41 °C. The molecular beacon probe for the *H. influenzae* specific assay was labelled with FAM and Black Hole Quencher (BHQ) 1. The molecular beacon probe for the *N. meningitidis* specific assay was labelled with HEX and Dabcyl (DAB). The molecular beacon probe for the *S. pneumoniae* specific assay was labelled with ROX and BHQ2. All primers and molecular beacon probes (Table 1) were obtained from Eurofins MWG-Operon (Ebersberg, Germany).

2.8. IAC development for real-time NASBA

A non-competitive IAC was developed for the real-time NASBA assays targeting splice variant 1 of the *Homo sapiens* TBP (TATA-box binding protein) mRNA transcript. IAC oligonucleotide primers and molecular beacon probes were designed and obtained the same as outlined for species specific oligonucleotides. The IAC assay molecular beacon probe was labelled with Cy5 and Black Hole Quencher 2 (BHQ2) to facilitate multiplexing of the diagnostic assays.

2.9. Duplex real-time NASBA development

Duplex real-time NASBA diagnostics assays were optimised and the analytical specificity and sensitivity determined. All real-time NASBA diagnostics assays were performed on a LightCycler 480 (Roche Diagnostics) using the NucliSENS EasyQ Basic Kit V2 (Biomerieux, Marcy l'Etoile, France), in accordance with manufacturer's instructions. *H influenzae* and *N. meningitidis* duplex real-time NASBA reactions were performed in a total volume of 20 μl. Target RNA and IAC RNA (2.5 μl each) were added to 10 μl reagent/KCL (70 mM final concentration), oligonucleotide primers and molecular beacon probes (0.2 μM and 0.1 μM final concentration respectively) mixture. The reaction was incubated for 65 °C for 5 min to denature the RNA secondary structure followed by 41 °C for 5 min to allow primer annealing. Subsequently, 5 μl of the enzyme mixture was added to the reaction. A no template control (NTC) reaction consisting of water instead of RNA was included in each experiment. The reaction was then incubated at 41 °C for 60 min in a LightCycler 480 thermocycler (Roche Diagnostics) with a fluorescent measurement recorded every minute. The *S. pneumoniae* duplex real-time NASBA assay was performed as above with the exception that this assay was supplemented with betaine (0.1 M final concentration) and that the final concentration of oligonucleotide primers and molecular beacon was 1 and 0.5 μM respectively.

2.10. Duplex real-time NASBA specificity and LOD

In order to evaluate the specificity of the three real-time NASBA diagnostics assays developed, total RNA from *H. influenzae* and closely related *Haemophilus* species, *N. meningitidis* and closely related *Neisseria* species, and *S. pneumoniae* and closely related *Streptococcus* species (Supplementary Table 1), at concentration of 1×10^4 CE per reaction,

were tested in duplicate in the *H. influenzae*, *N. meningitidis* and *S. pneumoniae* real-time NASBA diagnostics assays respectively.

To determine the LOD of each duplex assay, purified total RNA (DSMZ 4690, NCTC 10025 and DSMZ 20566) was two-fold serially diluted from 250 to 1.95 CE (*H. influenzae and S. pneumoniae*) and from 50 to 0.39 CE (*N. meningitidis*) and tested in replicates of ten. Dilutions were prepared, based on a calculation that a typical bacterial cell contains 0.1 pg RNA (Bremer and Dennis, 2008). The LOD for each duplex real-time NASBA assay was subsequently determined using Probit regression analysis with 95% probability (Minitab Version 17) (Burd, 2010).

3. Results

3.1. Diagnostics target identification

PhoB has recently been described in the literature as species specific target for *H. influenzae* using real-time PCR (Coughlan et al., 2015). Based on our *in silico* evaluation, this diagnostics target demonstrated suitable characteristics for evaluation as a *H. influenzae* species specific mRNA diagnostics target for use in a real-time NASBA assay. Publicly available and in-house sequence information was also interrogated using online bioinformatics tools to determine the suitability of *ssrA* and *lepA* RNA transcripts as molecular targets for the identification of *N. meningitidis* and *S. pneumoniae* respectively. This analysis established that the *ssrA* and *lepA* genes contain sufficient nucleic acid sequence heterogeneity to allow for the design of highly specific real-time NASBA assays for both *N. meningitidis* and *S. pneumoniae*.

3.2. Total RNA isolation and quantification

All total RNA isolated from bacterial cultures and human whole blood samples were found to have an RNA integrity number (RIN) of 7 or greater which demonstrated that the RNA was of sufficient quality for further evaluation. For specificity testing, total RNA isolated from bacterial cultures was diluted to contain approximately 1×10^4 CE per reaction for specificity testing. Using the PAXgene method for isolation and purification of RNA from human whole blood, approximately 2.1 μg of total RNA was recovered from a 2.5 ml sample.

3.3. IAC development

In order for a result to be considered valid using the real-time NASBA assays developed in this study, a positive signal must also be obtained in the IAC detection channel (Cy5) on the LightCycler 480. If the IAC is not detected the result is considered invalid and must be repeated (Hoorfar et al., 2004). In this study, IAC primers and a molecular beacon probe were designed to detect the TBP mRNA splice 1 variant. Subsequently, duplex real-time NASBA assays incorporating the developed IAC were tested with human total RNA spiked in to each reaction. For the purpose of this study, the IAC was spiked into each NASBA reaction at a final concentration of 25 ng to reflect the concentration of human RNA that would be recovered from a 30 μl sample based on the recovery of total RNA above. At this concentration, the IAC was detected in all samples tested (Supplementary Figs. S1B, S2B, and S3B).

3.4. Real-time NASBA assay specificity and LOD

The specificity of each real-time NASBA diagnostics assay was confirmed in duplex format using the specificity panel listed in Supplementary Table S1. All three assays were specific, detecting only target species (Supplementary Figs. S1A, S2A and S3A). None of the non-*H. influenzae*, non-*N. meningitidis* and non-*S. pneumoniae* closely related microorganisms tested were detected by the assays. The LOD of each duplex assay was determined using Probit regression analysis. With 95% confidence the LOD of the *H. influenzae*, *N. meningitidis and S. pneumoniae* duplex assays was determined to be 55.36, 0.99 and 57.24 CE, respectively.

4. Discussion

Despite advances in immunization approaches, *H. influenzae*, *N. meningitidis* and *S. pneumoniae* still account for the majority of bacterial meningitis cases worldwide, particularly in low income countries where vaccine availability is limited. Precise identification of these three microorganisms is essential given the significant mortality and morbidity rates associated with bacterial meningitis, the high risk of substantial long term sequelae in survivors (Chandran et al., 2011), and the differences in therapeutic approach for the different pathogens (Tunkel and Scheld, 2002). Traditional CSF and blood culture methods are slow and often result in an inaccurate diagnosis (Brouwer et al., 2010b). Furthermore, the use of CSF as a sample type is invasive for patients (Van de Beek et al., 2006).

Molecular techniques have the ability to rapidly identify microorganisms associated with bacterial meningitis. Recent studies have shown that use of technologies which incorporate an *in vitro* amplification step demonstrate increased sensitivity when compared to routine blood culture (Liesenfeld et al., 2014). The use of such molecular tests in a clinical setting have the ability to improve patient care by reducing time spent in hospitals, while also improving antimicrobial stewardship (Caliendo et al., 2013). Several multiplex real-time PCR diagnostics assays have been described in the literature for the specific identification of *H. influenzae*, *N. meningitidis* and *S. pneumoniae* directly from patient samples (Corless et al., 2001; Abdeldaim et al., 2010). However, these assays fail to incorporate an IAC which is crucial when working with clinical samples to monitor for PCR inhibition and to reduce reporting of false negative reactions (Hoorfar et al., 2004). Furthermore, these real-time PCR assays require expensive instrumentation capable of precise thermal cycling and rapid ramping. Isothermal *in vitro* amplification methods have the potential to eliminate the need for thermal cycling and ramping instrumentation required for real-time PCR. As such these *in vitro* amplification technologies are more suitable for incorporation on to low cost LOC and/or POC diagnostic devices. There are a number of isothermal *in vitro* amplification technologies which could be used for the detection of bacterial pathogens associated with meningitis for example recombinase polymerase amplification (RPA) or loop-mediated isothermal amplification (LAMP). However these *in vitro* amplification technologies are also limited; for example as RPA has previously been shown to be inhibited when used on nucleic acids purified from whole blood samples, or in the presence of background DNA (El Wahed et al., 2013; Rohrman and Richards-Kortum, 2015). Through incorporation of fluorescent fluorophores recent advances in LAMP methodologies facilitate monitoring of a reaction in real-time. However a significant disadvantage of LAMP is that it is difficult to multiplex due to the complicated amplicon structure (Liang et al., 2012) which often restricts the ability to incorporate an IAC into the diagnostics assay. As such, NASBA with molecular beacon technology enables real-time detection of target nucleic acid sequences and allows for the inclusion of an IAC in a single closed tube reaction offers a significant advantages over other isothermal technologies (Asiello and Baeumner, 2011).

In this manuscript, we present three novel duplex real-time NASBA assays, incorporating an IAC, for the detection of the predominant microorganisms associated with bacterial meningitis namely *H. influenzae*, *N. meningitidis* and *S. pneumoniae*. The *phoB* gene has been previously been described in the literature as a suitable diagnostics assay target for the species specific identification of *H. influenzae* using real-time PCR (Coughlan et al., 2015). In this study we have further validated this target and have successfully demonstrated the suitability of the *phoB* mRNA transcript for use in a duplex real-time NASBA diagnostics assay for the specific detection of *H. influenzae*. The diagnostic target used in this study for the species specific identification of *N. meningitidis* is the tmRNA transcript encoded for by the *ssrA* gene (Keiler, 2015). This

diagnostics gene target has previously been demonstrated as suitable for use in nucleic acid based diagnostics for bacterial detection and identification (O'Grady et al., 2009; Wernecke et al., 2009; O'Grady et al., 2008). Furthermore, tmRNA, has also been established as a useful target in a real-time NASBA diagnostics assay (O'Grady et al., 2009). Finally the diagnostic mRNA transcript target evaluated is the *lepA* mRNA and enables for the species specific identification of *S. pneumoniae*. The *lepA* gene, also present in all bacteria sequenced to date, encodes for a highly conserved translational elongation factor protein LepA, and is homologous to the *Guf1* gene found in higher organisms (Qin et al., 2006). It has also previously been demonstrated to be a useful infectious disease diagnostics target (Reddington et al., 2012; Clancy et al., 2015).

An endogenous non-competitive IAC targeting transcript 1 mRNA of the *Homo sapiens* TBP gene was incorporated into each assay. Incorporation of an IAC verifies the accuracy of the real-time NASBA results obtained, that the real-time NASBA reaction is functioning correctly and prevents reporting of false negative results which may occur as a result of problems with the assay reaction mixture, equipment malfunction, operator error, poor enzyme activity or inhibitory substances in the NASBA sample (Rodríguez-Lázaro et al., 2004).In recent years, a number of real-time NASBA assays have been described for the simultaneous detection of a target microorganism incorporating an IAC (O'Grady et al., 2009; Rodríguez-Lázaro et al., 2004; Tsaloglou et al., 2013; Sidoti et al., 2012; Nadal et al., 2007; Rodriguez-Lazaro et al., 2004; Ulrich et al., 2010; Telles et al., 2009; Casper et al., 2005; Patterson et al., 2005; Deiman et al., 2007; Yoo et al., 2008). However, these internally controlled real-time NASBA assays use *in vitro* synthesized RNAs spiked into the real-time NASBA reaction and some share a common set of primers for both the target species and the IAC which can result in competition for the primary target. In this study, the TBP mRNA transcript was chosen as an IAC target as it is stably expressed at low mRNA transcript levels in humans (Note, 2006). The use of a constitutively expressed low copy number endogenous control should ensure that the IAC developed will not out-compete the primary targets being amplified in the real-time NASBA assays which will reduce the possibility of false negative reporting. Furthermore, TBP has previously been described as suitable and robust endogenous control for use with human blood (Note, 2006). By incorporating an IAC targeting an endogenous gene transcript present in human whole blood, the assays developed in this study have the potential to be applied directly to whole blood samples facilitating their use in a clinical setting. This would allow the end user to control for each component of the test including sample preparation, amplification, hybridisation and detection. Each duplex real-time NASBA reaction was spiked with 25 ng of total human RNA which was the typical concentration of human RNA recovered from a 30 μl sample of whole blood in this study. Our methodology was optimised to a 30 μl equivalent volume of whole blood as a similar volume of blood has previously been demonstrated to be suitable for automated on-disc total RNA extraction for infectious disease based diagnostics (Dimov et al., 2008). Such on-disc devices have the potential to be integrated into LOC or POC platforms which will facilitate sample-to-answer diagnostic for RNA detection in whole blood. The rapid duplex real-time NASBA diagnostics assays developed in this study were experimentally validated against a large panel of culture collection isolates and clinical isolates and determined to be 100% specific for the target species. Subsequently, the LOD was determined for each duplex real-time NASBA diagnostics assay. Multiplex nucleic acid *in vitro* amplification diagnostics assays can be less experimentally sensitive than equivalent monoplex diagnostics assays as a consequence of interactions between primer sets or *in vitro* amplification bias (Craw and Balachandran, 2012). Reduced LOD in multiplex NASBA diagnostics assays compared to monoplex NASBA diagnostics assays has been reported (Zhao et al., 2009; Loens et al., 2008; Lau et al., 2010). However, in our study the LODs achieved were comparable in both monoplex and duplex format demonstrating the robustness of each of the real-time NASBA diagnostics assays developed.

The study presented here focuses on the development of highly robust internally controlled real-time NASBA assays for the rapid detection of the predominant etiological agents of bacterial meningitis. To date these have been experimentally validated on a broad range of culture collection strains and blood culture positive clinical isolates with promising results in terms of specificity and LOD. Additional work is now required to demonstrate and evaluate the true clinical utility of the methodologies developed for use directly on human whole blood samples. This would demonstrate the potential advantage of these assays over existing diagnostic methodologies and also determine their applicability for incorporation on to low cost LOC devices for near patient testing in the future.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mimet.2016.06.017.

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