<table>
<thead>
<tr>
<th><strong>Title</strong></th>
<th>Development of novel nucleic acid diagnostics technologies for the detection of predominant microorganisms associated with bacterial meningitis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Author(s)</strong></td>
<td>Coughlan, Helena</td>
</tr>
<tr>
<td><strong>Publication Date</strong></td>
<td>2017-01-13</td>
</tr>
<tr>
<td><strong>Item record</strong></td>
<td><a href="http://hdl.handle.net/10379/6255">http://hdl.handle.net/10379/6255</a></td>
</tr>
</tbody>
</table>
Development of novel nucleic acid diagnostics technologies for the detection of predominant microorganisms associated with bacterial meningitis

A thesis submitted to the National University of Ireland for the degree of Doctor of Philosophy by:

Helena Coughlan

Nucleic Acid Diagnostics Research Laboratory, Discipline of Microbiology, School of Natural Sciences, College of Science, National University of Ireland, Galway

January 2017
Research Supervisor: Dr. Thomas Barry
Head of Discipline: Dr. Gerard Wall
# Table of Contents

Acknowledgements .................................................................................................................. 7
Abstract .................................................................................................................................. 8

1. Introduction .......................................................................................................................... 12

1.1. Etiological Agents of Bacterial Meningitis ........................................................................ 13
1.1.1. *Haemophilus influenzae* ................................................................................................. 13
1.1.1.1. Closely related species *Haemophilus* species ............................................................... 16
1.1.2. *Neisseria meningitidis* .................................................................................................... 17
1.1.3. *Streptococcus pneumoniae* ............................................................................................ 21

1.2. Bacterial Meningitis Diagnostic Landscape ...................................................................... 23
1.2.1. Conventional Detection Methods .................................................................................... 24
1.2.1.1. CSF Culture .................................................................................................................. 24
1.2.1.2. CSF Cell Count, Glucose and Protein .......................................................................... 24
1.2.1.3. MALDI-TOF MS ......................................................................................................... 25
1.2.1.4. Fluorescent in-situ hybridization (FISH) ...................................................................... 26
1.2.1.5. Other conventional detection methods .......................................................................... 27
1.2.2. Nucleic Acid Based Detection Methods ......................................................................... 28
1.2.2.1. Polymerase Chain Reaction ......................................................................................... 28
1.2.2.2. Real-time Polymerase Chain Reaction ......................................................................... 28
1.2.2.3. Loop Mediated Isothermal Amplification ................................................................... 32
1.2.2.4. Recombinase Polymerase Amplification ..................................................................... 34

1.3. Nucleic Acid Sequence Based Amplification (NASBA) .................................................... 35
1.3.1. General Introduction ....................................................................................................... 35
1.3.2. NASBA Technology ........................................................................................................ 35
Chapter 2: Evaluation of novel diagnostic targets using real-time NASBA for the species specific identification of Haemophilus influenzae, Neisseria meningitidis and Streptococcus pneumoniae. ................................................................. 64

Abstract ......................................................................................................................... 65
2.1. Introduction .................................................................................................................. 65
2.1.1. Nucleic acid diagnostic technologies .................................................................... 66
2.1.2. Nucleic acid diagnostic research laboratory (NADRL) ......................................... 66
2.1.3. NAD targets .......................................................................................................... 67
2.1.3.1. tmRNA .............................................................................................................. 68
2.1.3.2. lepA mRNA ...................................................................................................... 69
2.1.4. Nucleic Acid Sequence Based Amplification ...................................................... 70
2.2. Materials and Methods ............................................................................................ 71
2.2.1. Diagnostic Target Identification ........................................................................... 71
2.2.2. Bacterial strains, Culture media and Growth conditions ....................................... 72
2.2.3. DNA isolation and quantification ......................................................................... 76
2.2.4. Total RNA isolation and quantification ................................................................. 77
2.2.5. Conventional PCR primer design ......................................................................... 77
2.2.6. Conventional PCR and Nucleic Acid Sequencing ............................................ 77
2.2.7. Real-time NASBA primer and molecular beacon probe design .......................... 78
Chapter 3: Comparative genome analysis identifies novel nucleic acid diagnostics targets for use in the specific detection of Haemophilus influenzae

Abstract ..................................................................................................................110

3.1. Introduction ..................................................................................................110

3.2. Materials and Methods .............................................................................112

3.2.1. Diagnostics Target Identification ..............................................................112

3.2.2. Bacterial strains, Culture media and Growth conditions .......................112

3.2.3. DNA isolation and Quantification .............................................................113

3.2.4. Conventional PCR and Nucleic Acid Sequencing ..................................113

3.2.5. PCR primer and probe design ..................................................................113

3.2.6. Real-Time PCR .........................................................................................114

3.2.7. Multiplex PCR assay specificity and LOD .................................................114

3.2.8. Clinical Isolate Evaluation ......................................................................115

3.2.9. fucK Real-Time PCR ................................................................................115

3.3. Results ..........................................................................................................115

3.3.1. Diagnostics Target Identification ..............................................................115
3.3.2. Real-Time PCR assay Specificity .................................................................116
3.3.3. Real-Time PCR assay Efficiency and LOD ..................................................116
3.3.4. fucK real-time PCR assay ........................................................................117
3.3.5. Clinical Isolate Evaluation ........................................................................117
3.4. Discussion ......................................................................................................117
3.5. References .....................................................................................................125

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 ........................................131

Abstract ...............................................................................................................132
4.1. Introduction .....................................................................................................132
4.2. Materials and Methods ..................................................................................134
4.2.1. Diagnostics Target Identification ...............................................................134
4.2.2. Bacterial strains, Culture media and Growth conditions ............................135
4.2.3. DNA isolation and Quantification ...............................................................135
4.2.4. Total RNA isolation and Quantification .....................................................135
4.2.5. Conventional PCR Primer Design .............................................................136
4.2.6. Conventional PCR and Nucleic Acid Sequencing .....................................136
4.2.7. Real-Time NASBA primer and molecular beacon probe design ................137
4.2.8. IAC Development for real-time NASBA ....................................................137
4.2.9. Duplex real-time NASBA Development ....................................................137
4.2.10. Duplex real-time NASBA specificity and LOD .......................................138
4.3. Results ...........................................................................................................138
4.3.1. Diagnostics Target Identification ...............................................................138
4.3.2. Total RNA isolation and quantification .....................................................139
4.3.3. IAC Development ........................................................................................................139
4.3.4. Real-time NASBA assay specificity ..............................................................................139
4.4. Discussion ..........................................................................................................................140
4.5. References ........................................................................................................................151

Chapter 5: Concluding Remarks and Future Directions ....................................................158

5.1. General Conclusions ..........................................................................................................159
5.2. Future Directions ..............................................................................................................163
5.3. References ........................................................................................................................166

Appendix A: Solvent-selective routing for centrifugally automated solid-phase purification of RNA towards a sample-to-answer molecular diagnostic ..................167

Appendix B: Development of on-disc Isothermal In-vitro Amplification and Detection of Bacterial RNA .........................................................................................................................181

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 .................................................................190

Appendix D: Comparative genome analysis identifies novel nucleic acid diagnostics targets for use in the specific detection of *Haemophilus influenzae* ...............198

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 .........................................................204
Acknowledgements

I would like to thank Dr. Thomas Barry for giving me the opportunity to carry out my PhD research in the Nucleic Acid Diagnostics Research Laboratory. It has been a great experience and I would like to thank Tom for his patient guidance, constant support, encouragement, and advice throughout the project. His belief in both my research and my abilities inspired me and gave me the self-assurance to continue when faced with crisis of confidence.

I would like to thank Dr. Eoin Clancy and Dr. Kate Reddington, who acted as mentors throughout this project. Thank you for your constant support, encouragement, guidance, friendship and ability to help me see the woods through the trees throughout the years.

I would also like to thank all members, past and present, of the Nucleic Acid Diagnostics Research Laboratory, the Biomedical Diagnostics Institute, The Tyndall National Institute and the Microbiology Department for all their help, support and friendship over the years.

To my friends and family outside of Microbiology, in particular Mary, thank you for your constant friendship, your willingness to listen and feign interest in what I have doing, and your understanding when sometimes I was just too tired or busy to socialize.

I would like to thank my parents, Bernadette and Pat, and Gareth’s parents, Bridie and Christy, for your love and support during my extensive third level education and for taking such good care of Esme.

To my wonderful and loving husband, Gareth, to whom I dedicate this thesis. Thank you for your continued and unfailing love, support, encouragement and understanding throughout the years that have made completion of this thesis possible. Words could never express how grateful I am to you. And finally, to our little girl Esme, thank you for just being you and for the wonderful kisses and cuddles that made it all worthwhile after a long day of writing up.

“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. haemolyticus*. 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 antimicrobial 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 meningitidis*, *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

<table>
<thead>
<tr>
<th>Age Groups</th>
<th>Causes</th>
</tr>
</thead>
<tbody>
<tr>
<td>New-born</td>
<td>Group B <em>Streptococcus</em>, <em>Streptococcus pneumoniae</em>, <em>Escherichia coli</em>, <em>Listeria monocytogenes</em></td>
</tr>
<tr>
<td>Infants and Children</td>
<td><em>Streptococcus pneumoniae</em>, <em>Neisseria meningitidis</em>, <em>Haemophilus influenzae</em> type b, Group B <em>Streptococcus</em></td>
</tr>
<tr>
<td>Adolescents and Young Adults</td>
<td><em>Streptococcus pneumoniae</em>, <em>Neisseria meningitidis</em></td>
</tr>
<tr>
<td>Older Adults</td>
<td><em>Streptococcus pneumoniae</em>, <em>Neisseria meningitidis</em>, <em>Haemophilus influenzae</em> type b (<em>Hib</em>), Group B <em>Streptococcus</em>, <em>Listeria monocytogenes</em></td>
</tr>
</tbody>
</table>

(Source: [http://www.cdc.gov/meningitis/bacterial.html](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; \textit{H. influenzae}, \textit{Haemophilus aegyptius}, \textit{H. haemolyticus}, \textit{H. parainfluenzae}, \textit{Haemophilus parahaemolyticus}, \textit{Haemophilus paraphrohaemolyticus}, \textit{Haemophilus pittmaniae}, \textit{Haemophilus sputorum} and \textit{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; \textit{Haemophilus felis} (cats), \textit{Haemophilus haemoglobinophilus} (dogs), \textit{Haemophilus paracuniculus} (rabbits), \textit{Haemophilus parasuis} (swine) and \textit{Haemophilus piscium} (fish).

\textit{H. influenzae} was first isolated in 1890 by Richard Pfeiffer [25] and was referred to as ‘Pfeiffer’s Bacillus’ or \textit{Bacillus influenzae} at the time, as it was mistakenly thought to be the etiological agent of the influenza pandemic [21, 26-29]. \textit{H. influenzae} is a member of the \textit{Haemophilus influenzae} group, - sometimes referred to as \textit{Haemophilus sensu stricto} - together with \textit{H. aegyptius} and \textit{H. haemolyticus} [21]. It is the most pathogenic of the eight \textit{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]

\textit{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 \textit{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]. Thjöttà \textit{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 \textit{H. influenzae} from all other \textit{Haemophilus} species with the exception of its most closely related species, \textit{H. aegyptius} and \textit{H. haemolyticus}. Consequently, \textit{H. influenzae} grow optimally on
rich blood media such as chocolate agar (which contains X and V factors) at 35-37°C and growth is further enhanced in a 5% CO₂ 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™), 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].
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 microorganisms. [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
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°C with 5% CO₂ [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 into 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.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Acid Production from:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose</td>
</tr>
<tr>
<td><em>Neisseria meningitidis</em></td>
<td>+</td>
</tr>
<tr>
<td><em>Neisseria lactamica</em></td>
<td>+</td>
</tr>
<tr>
<td><em>Neisseria gonorrhoeae</em></td>
<td>+^2</td>
</tr>
<tr>
<td><em>Neisseria sicca</em></td>
<td>+</td>
</tr>
<tr>
<td><em>Moraxella catarrhalis</em></td>
<td>-</td>
</tr>
</tbody>
</table>

^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](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>
<thead>
<tr>
<th>Trade Name</th>
<th>Type of Vaccine</th>
<th>Meningococcal Serogroup</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bexsero®</td>
<td>Recombinant</td>
<td>B</td>
</tr>
<tr>
<td>Menactra®</td>
<td>Conjugate</td>
<td>A, C, W, Y</td>
</tr>
<tr>
<td>MenHibrix®</td>
<td>Conjugate</td>
<td>C, Y (and <em>Haemophilus influenzae</em> type b [Hib])</td>
</tr>
<tr>
<td>Menomune®</td>
<td>Polysaccharide</td>
<td>A, C, W, Y</td>
</tr>
<tr>
<td>Menveo®</td>
<td>Conjugate</td>
<td>A, C, W, Y</td>
</tr>
<tr>
<td>Trumenba®</td>
<td>Recombinant</td>
<td>B</td>
</tr>
</tbody>
</table>

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 pyogenes* 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. pyogenes* 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% CO₂ 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 cephalosporins, (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 cephalasporin 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 nonspecific 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]. 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 MALDI-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°C. This separates the double stranded DNA into 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°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 deoxyribonucleotides triphosphates (dNTPs), MgCl₂, 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 95°C. (2) Annealing of primers e.g. 55°C. (3) Extension at 72°C (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](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).
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], ltyA [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°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].

![The RPA Cycle](http://www.twistdx.co.uk/our_technology/)

**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
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 fluorophore 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].
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-specialy 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
Chapter 1

Institute at University College Cork and the Biomedical Diagnostics Institute at Dublin City University. The overall project can be broken down into 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 pneumoniae*. 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 pneumoniae*. 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. influenzae*. 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.
1.5. References

1. Meningitis [https://www.cdc.gov/meningitis/]


35. Davis DJ: Food accessory factors (vitamins) in bacterial culture with especial reference to hemophilic bacilli I. *The Journal of Infectious Diseases* 1917:392-703.


40. MacNeil JR, Cohn AC, Farley M, Mair R, Baumbach J, Bennett N, Gershman K, Harrison LH, Lynfield R, Petit S: Current Epidemiology and


virus in clinical samples. *Journal of Medical Microbiology* 2009, 58(9):1168-1172.


64. Note A: Using TaqMan® Endogenous Control Assays to Select an Endogenous Control for Experimental Studies.


77. Genus Neisseria [http://www.bacterio.net/neisseria.html]


87. Wakui D, Nagashima G, Otsuka Y, Takada T, Ueda T, Tanaka Y-i, Hashimoto T: A case of meningitis due to Neisseria subflava after


104. Genus Streptococcus [http://www.bacterio.net/streptococcus.html]


106. Pneumococcal Disease [http://www.cdc.gov/pneumococcal/clinicians/clinical-features.html]


118. Laboratory Diagnosis of Meningitis [http://www.intechopen.com/books/meningitis/laboratory-diagnosis-of-meningitis-]


186. Loens K, Beck T, Goossens H, Ursi D, Overdijk M, Sillekens P, Ieven M: Development of conventional and real-time NASBA (R) for the detection of


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.

![NAD assay development strategy](image)

**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](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, Chlamyphila 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.**

<table>
<thead>
<tr>
<th>Probe/Primer</th>
<th>Gene Target</th>
<th>Function</th>
<th>DNA sequence (5’ – 3’)</th>
<th>Nucleotide position</th>
<th>Genbank accession no.</th>
<th>Final Concentration (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ssrA F1</td>
<td>ssrA</td>
<td>Sequencing forward primer</td>
<td>GGGGCTGATTCTGGATTCGA CGG</td>
<td>1-23</td>
<td>CP00227 7.1</td>
<td>0.4 µM</td>
</tr>
<tr>
<td>ssrA R1</td>
<td>ssrA</td>
<td>Sequencing reverse primer</td>
<td>TGGTGAGCTGGGGGGAGTGAA</td>
<td>343-365</td>
<td>CP00227 7.1</td>
<td>0.4 µM</td>
</tr>
<tr>
<td>lepA F1</td>
<td>lepA</td>
<td>Sequencing forward primer</td>
<td>ATGAAGAATATTCGCAAC</td>
<td>1-18</td>
<td>CP00227 7.1</td>
<td>0.4 µM</td>
</tr>
<tr>
<td>lepA R1</td>
<td>lepA</td>
<td>Sequencing reverse primer</td>
<td>CACTTTAATTTATCGCCT</td>
<td>657-675</td>
<td>CP00227 7.1</td>
<td>0.4 µM</td>
</tr>
<tr>
<td>lepA F2</td>
<td>lepA</td>
<td>Sequencing forward primer</td>
<td>CGTGGTATCTTTATCGCCT</td>
<td>615-637</td>
<td>CP00227 7.1</td>
<td>0.4 µM</td>
</tr>
<tr>
<td>lepA R2</td>
<td>lepA</td>
<td>Sequencing reverse primer</td>
<td>ACCTAAATATTCTCCTGGA C</td>
<td>1228-1248</td>
<td>CP00227 7.1</td>
<td>0.4 µM</td>
</tr>
</tbody>
</table>
### 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*

<table>
<thead>
<tr>
<th>Strain Code</th>
<th>Gene</th>
<th>Species</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Hybridization Probe</th>
<th>Length</th>
<th>Conc. (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>lepA F3</td>
<td>lepA</td>
<td><em>H. influenzae</em></td>
<td>ATGGAAATCATTCAAGAGC</td>
<td>GT</td>
<td></td>
<td>1042-1062</td>
<td>CP00227</td>
</tr>
<tr>
<td>lepA R3</td>
<td>lepA</td>
<td><em>H. influenzae</em></td>
<td>TCTTTTCCTACGTGTAATAAT</td>
<td>TGC</td>
<td></td>
<td>1768-1790</td>
<td>CP00227</td>
</tr>
<tr>
<td>Neisseria F</td>
<td>ssrA</td>
<td><em>N. meningitidis</em></td>
<td>GGCAGACCTTTGATTCGACG</td>
<td></td>
<td></td>
<td>4-22</td>
<td>AE00209</td>
</tr>
<tr>
<td>Neisseria R</td>
<td>ssrA</td>
<td><em>N. meningitidis</em></td>
<td>TCGAAGCCCGTCCGAGAGG</td>
<td></td>
<td></td>
<td>327-345</td>
<td>AE00209</td>
</tr>
<tr>
<td>ssa P1</td>
<td>ssrA</td>
<td><em>H. influenzae</em></td>
<td>AATTTCAATAACGTCTCTATACTCTGAGGTCCTCTCAACGACG</td>
<td></td>
<td></td>
<td>213-230</td>
<td>CP00227</td>
</tr>
<tr>
<td>ssa P2</td>
<td>ssrA</td>
<td><em>H. influenzae</em></td>
<td>GCACGTAAATACGACG</td>
<td></td>
<td></td>
<td>116-130</td>
<td>CP00227</td>
</tr>
<tr>
<td>LepA MB</td>
<td>lepA</td>
<td><em>H. influenzae</em></td>
<td>FAM-CCGAGT-TGCAAAATTGCGCTAC-ACGGC-DB</td>
<td></td>
<td></td>
<td>1161-1178</td>
<td>CP00227</td>
</tr>
<tr>
<td>lepA P1</td>
<td>lepA</td>
<td><em>H. influenzae</em></td>
<td>AATTTCAATAACGTCTCTATACTCTGAGGTCCTCTCAACGACG</td>
<td></td>
<td></td>
<td>1185-1204</td>
<td>CP00227</td>
</tr>
<tr>
<td>lepA P2</td>
<td>lepA</td>
<td><em>H. influenzae</em></td>
<td>GGACAGATCAAGAGC-CTA</td>
<td></td>
<td></td>
<td>1044-1065</td>
<td>CP00227</td>
</tr>
<tr>
<td>Spn P1</td>
<td>lepA</td>
<td><em>S. pneumoniae</em></td>
<td>AATTTCAATAACGTCTCTATACTCTGAGGTCCTCTCAACGACG</td>
<td></td>
<td></td>
<td>1792-1812</td>
<td>AE00731</td>
</tr>
<tr>
<td>Spn P2</td>
<td>lepA</td>
<td><em>S. pneumoniae</em></td>
<td>GCACAGATCAAGAGC-CTA</td>
<td></td>
<td></td>
<td>1628-1654</td>
<td>AE00731</td>
</tr>
<tr>
<td>Spn MB</td>
<td>lepA</td>
<td><em>S. pneumoniae</em></td>
<td>BOX-CGATCGAGGCGGATGAAATCCATCGATCAGTGC-DB</td>
<td></td>
<td></td>
<td>1749-1773</td>
<td>AE00731</td>
</tr>
<tr>
<td>Nmen P1</td>
<td>ssrA</td>
<td><em>N. meningitidis</em></td>
<td>AATTTCAATAACGTCTCTATACTCTGAGGTCCTCTCAACGACG</td>
<td></td>
<td></td>
<td>305-325</td>
<td>AE00209</td>
</tr>
<tr>
<td>Nmen P2</td>
<td>ssrA</td>
<td><em>N. meningitidis</em></td>
<td>CCGTAAACAGTGAATTTGACCATTTA</td>
<td></td>
<td></td>
<td>63-85</td>
<td>AE00209</td>
</tr>
<tr>
<td>Nmen MB</td>
<td>ssrA</td>
<td><em>N. meningitidis</em></td>
<td>HEX-CGATCGAGGCGGATGAAATCCATCGATCAGTGC-DB</td>
<td></td>
<td></td>
<td>236-255</td>
<td>AE00209</td>
</tr>
</tbody>
</table>
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 37°C 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**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Strain</th>
<th>Specificity</th>
<th>ssrA</th>
<th>LepA</th>
<th>LepA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>H. influenzae</em> reference strains</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>H. influenzae</em> type b</td>
<td>DSMZ 23393&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>H. influenzae</em> type b</td>
<td>DSMZ 4690&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>H. influenzae</em> type b</td>
<td>DSMZ 11969&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>H. influenzae</em> type b</td>
<td>DSMZ 11970&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>H. influenzae</em> type b</td>
<td>DSMZ 10001&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>H. influenzae</em> type d</td>
<td>DSMZ 11121&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>H. influenzae</em> type f</td>
<td>DSMZ 10000&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>H. influenzae</em> NTHi</td>
<td>DSMZ 24049&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>H. influenzae</em> biogroup aegyptius</td>
<td>DSMZ 21187&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>H. influenzae</em> clinical isolates</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>H. influenzae</em> NT (Source: Blood)</td>
<td>6 clinical isolates</td>
<td>+</td>
<td>/</td>
<td>/</td>
<td></td>
</tr>
<tr>
<td><em>H. influenzae</em> untyped (Source: Sputum)</td>
<td>24 clinical</td>
<td>+</td>
<td>/</td>
<td>/</td>
<td></td>
</tr>
<tr>
<td><em>H. influenzae</em> untyped (Source: Eye/Ear swab)</td>
<td>8 clinical isolates</td>
<td>+</td>
<td>/</td>
<td>/</td>
<td></td>
</tr>
<tr>
<td>Non-<em>H. influenzae</em> strains</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>H. ducreyi</em></td>
<td>NCTC 11479</td>
<td>-</td>
<td>/</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>H. ducreyi</em></td>
<td>NCTC 10945&lt;sup&gt;b&lt;/sup&gt;</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td></td>
</tr>
<tr>
<td><em>H. felis</em></td>
<td>DSMZ 21192&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
<td>/</td>
<td>/</td>
<td></td>
</tr>
<tr>
<td><em>H. haemoglobinophilus</em></td>
<td>DSMZ 21241</td>
<td>-</td>
<td>/</td>
<td>/</td>
<td></td>
</tr>
<tr>
<td><em>H. haemolyticus</em></td>
<td>NCTC 10839&lt;sup&gt;c&lt;/sup&gt;</td>
<td>+</td>
<td>/</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>H. haemolyticus</em></td>
<td>NCTC 10659&lt;sup&gt;b&lt;/sup&gt;</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td></td>
</tr>
<tr>
<td><em>H. haemolyticus</em></td>
<td>CCUG 24149&lt;sup&gt;c&lt;/sup&gt;</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td></td>
</tr>
<tr>
<td>Organism</td>
<td>Strain</td>
<td>Specificity*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------------------------------</td>
<td>-----------------</td>
<td>--------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ssrA</td>
<td>LepA</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>N. meningitidis</strong> reference strains</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N. meningitidis</td>
<td>NCTC 9392</td>
<td>+</td>
<td>/</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N. meningitidis serogroup a</td>
<td>NCTC 10025</td>
<td>+</td>
<td>/</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N. meningitidis serogroup a</td>
<td>NCTC 3375</td>
<td>+</td>
<td>/</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N. meningitidis serogroup a</td>
<td>NCTC 3372</td>
<td>+</td>
<td>/</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N. meningitidis serogroup a</td>
<td>NCTC 15465d</td>
<td>+</td>
<td>/</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N. meningitidis serogroup c</td>
<td>ATCC 13102</td>
<td>+</td>
<td>/</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N. meningitidis serogroup c</td>
<td>DSMZ 15464d</td>
<td>+</td>
<td>/</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N. meningitidis serogroup x</td>
<td>NCTC 10790</td>
<td>+</td>
<td>/</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N. meningitidis strain reference 73/M6</td>
<td>NCTC 11423</td>
<td>+</td>
<td>/</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Non- N. meningitidis strains</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N. animalis</td>
<td>DSMZ 23392d</td>
<td>-</td>
<td>/</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N. animaloris</td>
<td>DSMZ 21642d</td>
<td>-</td>
<td>/</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N. canis</td>
<td>DSMZ 18000d</td>
<td>-</td>
<td>/</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N. caviae</td>
<td>DSMZ 23336</td>
<td>-</td>
<td>/</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N. dentiae</td>
<td>DSMZ 19151</td>
<td>-</td>
<td>/</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Species</td>
<td>Strain Code</td>
<td>Reference</td>
<td>Source</td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>-------------</td>
<td>-----------</td>
<td>----------</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>N. elongata</em> subsp. <em>elongata</em></td>
<td>DSMZ 17712</td>
<td>d</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>N. elongata</em> subsp. <em>glycolytica</em></td>
<td>DSMZ 23337</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>N. elongata</em> subsp. <em>nitroreducens</em></td>
<td>DSMZ 17632</td>
<td>d</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>N. flavescens</em></td>
<td>DSMZ 17633</td>
<td>d</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>N. gonorrhoeae</em></td>
<td>DSMZ 9189</td>
<td>d</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>N. macciae</em></td>
<td>DSMZ 19175</td>
<td>d</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>N. mucosa</em></td>
<td>DSMZ 17611</td>
<td>d</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>N. ovis</em></td>
<td>DSMZ 218075</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>N. perflava</em></td>
<td>DSMZ 18009</td>
<td>d</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>N. sicca</em></td>
<td>DSMZ 17713</td>
<td>d</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>N. subflava</em></td>
<td>DSMZ 17610</td>
<td>d</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>N. wadsworthii</em></td>
<td>DSMZ 22247</td>
<td>d</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>N. weaveri</em></td>
<td>DSMZ 17688</td>
<td>d</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>N. zoodegmatis</em></td>
<td>DSMZ 21643</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. pneumoniae</em> reference strains</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. pneumoniae</em> serotype 1</td>
<td>DSMZ 20566</td>
<td>/</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. pneumoniae</em> clinical isolates</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. pneumoniae</em> untyped (Source: Blood)</td>
<td>3 isolates</td>
<td>/</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. pneumoniae</em> untyped (Source: Sputum)</td>
<td>7 isolate</td>
<td>/</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. pneumoniae</em> untyped (Source: Eye/Ear)</td>
<td>3 isolates</td>
<td>/</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non- <em>S. pneumoniae</em> strains</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. agalactiae</em></td>
<td>BCCM 15081</td>
<td>/</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. agalactiae</em></td>
<td>BCCM 15082</td>
<td>/</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. agalactiae</em></td>
<td>BCCM 15083</td>
<td>/</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. agalactiae</em></td>
<td>BCCM 15084</td>
<td>/</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. agalactiae</em></td>
<td>BCCM 15085</td>
<td>/</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. agalactiae</em></td>
<td>BCCM 15086</td>
<td>/</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. agalactiae</em></td>
<td>BCCM 15087</td>
<td>/</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. agalactiae</em></td>
<td>BCCM 15094</td>
<td>/</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. agalactiae</em></td>
<td>BCCM 15095</td>
<td>/</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. anginosus</em></td>
<td>DSMZ 20563</td>
<td>/</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. australis</em></td>
<td>DSMZ 15627</td>
<td>/</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. canis</em></td>
<td>DSMZ 20715</td>
<td>/</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. constellatus</em></td>
<td>DSMZ 20575</td>
<td>/</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. cristatus</em></td>
<td>DSMZ 8249</td>
<td>/</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. dysgalactiae</em> subsp. <em>equimilis</em></td>
<td>DSMZ 6176</td>
<td>/</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. equi</em> subsp. <em>Equi</em></td>
<td>DSMZ 20561</td>
<td>/</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. equinis</em></td>
<td>DSMZ 20554</td>
<td>/</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. gordonii</em></td>
<td>DSMZ 6777</td>
<td>/</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. infantis</em></td>
<td>DSMZ 12492</td>
<td>/</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. intermedius</em></td>
<td>DSMZ 20573</td>
<td>/</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>S. mitis</strong></td>
<td>DSMZ 12643</td>
<td>/</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>S. mutans</strong></td>
<td>DSMZ 20523</td>
<td>/</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>S. oralis</strong></td>
<td>DSMZ 20066</td>
<td>/</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>S. oralis</strong></td>
<td>DSMZ 20395</td>
<td>/</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>S. parasanguinis</strong></td>
<td>DSMZ 6778</td>
<td>/</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>S. perosis</strong></td>
<td>DSMZ 12493</td>
<td>/</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>S. porcinus</strong></td>
<td>DSMZ 20725</td>
<td>/</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>S. pseudopneumoniae</strong></td>
<td>DSMZ 18670</td>
<td>/</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>S. pyogenes</strong></td>
<td>DSMZ 20565</td>
<td>/</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>S. pyogenes</strong></td>
<td>DSMZ 2072</td>
<td>/</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>S. salivarius</strong></td>
<td>DSMZ 20560</td>
<td>/</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>S. salivarius</strong></td>
<td>DSMZ 20617</td>
<td>/</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>S. sanguinis</strong></td>
<td>DSMZ 20567</td>
<td>/</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>S. sinensis</strong></td>
<td>DSMZ 14990</td>
<td>/</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>S. suis</strong></td>
<td>DSMZ 9682</td>
<td>/</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>S. uberis</strong></td>
<td>DSMZ 20569</td>
<td>/</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>S. vestibularis</strong></td>
<td>DSMZ 5636</td>
<td>/</td>
<td>-</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) 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 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).

2.2.7. 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 Dabcy (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°C for 5 min to denature the RNA secondary structure followed by 41°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°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 65°C for 5 min followed by 41°C 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 41°C for 60 min with a fluorescent measurement recorded every minute.

2.3. **Results and Discussion**

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

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{amplification_curves.png}
\caption{Amplification curves for real-time NASBA diagnostics assay. Real-time amplification curves for \textit{H. influenzae} (DSM23393) and \textit{H. influenzae} biogroup \textit{aegyptius} (DSM 21187) targeting the tmRNA in the FAM channel (470 -530 nm). No other non-\textit{H. influenzae} species were detected by the assay.}
\end{figure}
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.

![Amplification Curves](image)

**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* 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-\textit{H. influenzae} closely related species, targeting \textit{H. influenzae} \textit{lepA} mRNA, in the FAM channel (470nm-530nm). 2 closely related species \textit{H. haemolyticus} (NCTC 10839) and \textit{Histophilus somnus} (CCUG36257) cross react. 

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


2. Brouwer MC, Tunkel AR, van de Beek D: Epidemiology, Diagnosis, and Antimicrobial Treatment of Acute Bacterial Meningitis. *Clinical Microbiology Reviews* 2010, 23(3):467-+

3. Laboratory Diagnosis of Meningitis [http://www.intechopen.com/books/meningitis/laboratory-diagnosis-of-meningitis-]


2.6. Supplementary Figures

A. Amplification Curves

B. Amplification Curves
**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).
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).
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.
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*
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 37°C 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°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).

### 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 subtilis* 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$_2$O 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 while 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 [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$_2$O. 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 $1 \times 10^6$, $5 \times 10^5$, $1 \times 10^5$, $5 \times 10^4$, $1 \times 10^4$, $5 \times 10^3$, $1 \times 10^3$, $5 \times 10^2$, $1 \times 10^2$ and $5 \times 10^1$ 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 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 10^3, 1 x 10^3, 5 x 10^2, 1 x 10^2, and 5 x 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**

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

*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.

<table>
<thead>
<tr>
<th>Probe/Primer</th>
<th>Function</th>
<th>DNA sequence (5′–3′)</th>
<th>Nucleotide position</th>
<th>Genbank accession no.</th>
<th>Concentration (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pstA F1</td>
<td><em>H. influenzae</em> pstA Sequencing forward</td>
<td>CTAATCAAAAACCTTG CGT</td>
<td>8-24</td>
<td>CP002277.1</td>
<td>0.4 µM</td>
</tr>
<tr>
<td>pstA R1</td>
<td><em>H. influenzae</em> pstA Sequencing reverse primer</td>
<td>TGTTGAAAGAAGAG TCG</td>
<td>823-839</td>
<td>CP002277.1</td>
<td>0.4 µM</td>
</tr>
<tr>
<td>phoB F1</td>
<td><em>H. influenzae</em> phoB Sequencing forward</td>
<td>CTGATAGTTGAAGA TG</td>
<td>16-31</td>
<td>CP002277.1</td>
<td>0.4 µM</td>
</tr>
<tr>
<td>phoB R1</td>
<td><em>H. influenzae</em> phoB Sequencing reverse primer</td>
<td>TCATTGTATCTCG T</td>
<td>681-696</td>
<td>CP002277.1</td>
<td>0.4 µM</td>
</tr>
<tr>
<td>pstA P1</td>
<td><em>H. influenzae</em> pstA forward primer</td>
<td>CGTTTGCACAAATT ACC</td>
<td>310-327</td>
<td>CP002277.1</td>
<td>0.5 µM</td>
</tr>
<tr>
<td>pstA P2</td>
<td><em>H. influenzae</em> pstA reverse primer</td>
<td>GTGCCGACCAGCAT AGG</td>
<td>460-476</td>
<td>CP002277.1</td>
<td>0.5 µM</td>
</tr>
<tr>
<td>pstA probe</td>
<td><em>H. influenzae</em> pstA hydrolysis probe</td>
<td>FAM-CTGGAGCATTCA CGTT-BHQ1</td>
<td>428-448</td>
<td>CP002277.1</td>
<td>0.2 µM</td>
</tr>
<tr>
<td>phoB P1</td>
<td><em>H. influenzae</em> phoB forward primer</td>
<td>TTGGATTGGATGCTA CC</td>
<td>151-167</td>
<td>CP002277.1</td>
<td>0.5 µM</td>
</tr>
<tr>
<td>phoB P2</td>
<td><em>H. influenzae</em> phoB reverse primer</td>
<td>AGTGATGTAGTCAT CAGC</td>
<td>292-309</td>
<td>CP002277.1</td>
<td>0.5 µM</td>
</tr>
<tr>
<td>phoB probe</td>
<td><em>H. influenzae</em> phoB hydrolysis probe</td>
<td>FAM-AGAAAGCTATGCTG CGATTCC-BHQ1</td>
<td>210-230</td>
<td>CP002277.1</td>
<td>0.2 µM</td>
</tr>
<tr>
<td>BSS-1F</td>
<td>IAC forward primer</td>
<td>AACGTAGCATTAGC TGC</td>
<td>111-127</td>
<td>HG519928.1</td>
<td>0.5 µM</td>
</tr>
<tr>
<td>BSS-1R</td>
<td>IAC reverse primer</td>
<td>CTGATCTCTTGCC GC</td>
<td>260-276</td>
<td>HG519928.1</td>
<td>0.5 µM</td>
</tr>
<tr>
<td>BSS-P</td>
<td>IAC – specific hydrolysis probe</td>
<td>Cy5-CATCCACAGTAGG CTACGCT-BHQ2</td>
<td>179-199</td>
<td>HG519928.1</td>
<td>0.2 µM</td>
</tr>
</tbody>
</table>
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.
3.5. References


34. Ridderberg W, Fenger MG, Norskov-Lauritsen N: Haemophilus influenzae may be untypable by the multilocus sequence typing scheme due to a complete deletion of the fucose operon. *Journal of Medical Microbiology* 2010, 59(6):740-742.

35. Shuel ML, Karlowsky KE, Law DKS, Tsang RSW: Nonencapsulated or nontypeable Haemophilus influenzae are more likely than their encapsulated or serotypeable counterparts to have mutations in their fucose operon. *Canadian Journal of Microbiology* 2011, 57(12):982-986.


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

Published by Journal of Microbiological Methods

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 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

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°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 \textit{phoB} gene of \textit{H. influenzae}, and 342 bp of the \textit{ssrA} gene of \textit{N. meningitidis}, to identify optimal diagnostics target regions for NASBA primer and molecular beacon probe design. Due to the extent of publicly available \textit{S. pneumoniae lepA} sequence data, sequencing of in-house \textit{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 \textit{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 \textit{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).
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°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 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 (0.1M 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 $1 \times 10^4$ 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 GufI 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
Chapter 4

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.

<table>
<thead>
<tr>
<th>Probe/Primer</th>
<th>Gene Target</th>
<th>Function</th>
<th>DNA sequence (5’ – 3’)</th>
<th>Nucleotide position</th>
<th>Genbank accession no.</th>
<th>Final Concentration (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>phoB F1</td>
<td>phoB</td>
<td>Sequencing forward primer</td>
<td>CTGATAGTTGAAGATG</td>
<td>16-31</td>
<td>CP002277.1</td>
<td>0.4 µM</td>
</tr>
<tr>
<td>phoB R1</td>
<td>phoB</td>
<td>Sequencing reverse primer</td>
<td>TCATTGTATTCGT</td>
<td>681-696</td>
<td>CP002277.1</td>
<td>0.4 µM</td>
</tr>
<tr>
<td>Neisseria F</td>
<td>ssrA</td>
<td>Sequencing forward primer</td>
<td>GCGGACCTGGTTTCGACG</td>
<td>4-22</td>
<td>AE000209.8</td>
<td>0.3 µM</td>
</tr>
<tr>
<td>Neisseria R</td>
<td>ssrA</td>
<td>Sequencing reverse primer</td>
<td>TCGAACCAGTGCCGAAAG</td>
<td>327-345</td>
<td>AE000209.8</td>
<td>0.3 µM</td>
</tr>
<tr>
<td>Organism</td>
<td>Strain</td>
<td>Specificity&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>--------</td>
<td>------------------------</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>H. influenzae reference strains</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>H. influenzae</em> type a</td>
<td>NCTC 8465</td>
<td>+</td>
<td>/</td>
<td>/</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>N. meningitidis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>S. pneumoniae</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.2: Bacterial species and strains included in this study
<table>
<thead>
<tr>
<th><strong>H. influenzae</strong> type b</th>
<th>DSMZ 4690&lt;sup&gt;b&lt;/sup&gt;</th>
<th>+</th>
<th>/</th>
<th>/</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>H. influenzae</strong> type c</td>
<td>NCTC 8469</td>
<td>+</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td><strong>H. influenzae</strong> type d</td>
<td>DSMZ 11121&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td><strong>H. influenzae</strong> type e</td>
<td>NCTC 8472</td>
<td>+</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td><strong>H. influenzae</strong> type f</td>
<td>DSMZ 10000&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td><strong>H. influenzae</strong> NTHi</td>
<td>CCUG 58365</td>
<td>+</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td><strong>H. influenzae</strong> clinical isolates</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>H. influenzae</strong> NT (Source: Blood)</td>
<td>6 isolates</td>
<td>+</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td><strong>H. influenzae</strong> untyped (Source: Sputum)</td>
<td>6 isolates</td>
<td>+</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td><strong>H. influenzae</strong> untyped (Source: Eye/Ear swab)</td>
<td>4 isolates</td>
<td>+</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td><strong>Non-</strong> <strong>H. influenzae</strong> strains</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>H. haemolyticus</strong></td>
<td>CCUG 24149</td>
<td>-</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td><strong>H. haemolyticus</strong></td>
<td>CCUG 36015</td>
<td>-</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td><strong>H. haemolyticus</strong></td>
<td>CCUG 36016</td>
<td>-</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td><strong>H. haemolyticus</strong></td>
<td>CCUG 15642</td>
<td>-</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td><strong>H. haemolyticus</strong></td>
<td>CCUG 15312</td>
<td>-</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td><strong>H. haemolyticus</strong></td>
<td>NCTC 10839</td>
<td>-</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td><strong>H. haemolyticus</strong></td>
<td>CCUG 12834</td>
<td>-</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td><strong>H. haemolyticus</strong></td>
<td>CDC-M21127</td>
<td>-</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td><strong>H. ducreyi</strong></td>
<td>NCTC11479</td>
<td>-</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td><strong>H. parahaemolyticus</strong></td>
<td>DSMZ 21417</td>
<td>-</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td><strong>H. parainfluenzae</strong></td>
<td>DSMZ 8978</td>
<td>-</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td><strong>H. paraphrohaemolyticus</strong></td>
<td>DSMZ 21451</td>
<td>-</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td><strong>H. pittmaniae</strong></td>
<td>DSMZ 21203</td>
<td>-</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td><strong>Haemophilus sp.</strong></td>
<td>CCUG 34110</td>
<td>-</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td><strong>Actinobacillus suis</strong></td>
<td>DSMZ 22433</td>
<td>-</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td><strong>Actinobacillus</strong></td>
<td>DSMZ 13472</td>
<td>-</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td><strong>Aggregatibacter aphrophilus</strong></td>
<td>NCTC 11096</td>
<td>-</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td><strong>A. aphrophilus</strong></td>
<td>NCTC 10558</td>
<td>-</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td><strong>Aggregatibacter segnis</strong></td>
<td>NCTC 10977</td>
<td>-</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td><strong>N. meningitidis</strong> reference strains</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>N. meningitidis</strong> serogroup a</td>
<td>NCTC 10025&lt;sup&gt;c&lt;/sup&gt;</td>
<td>/</td>
<td>+</td>
<td>/</td>
</tr>
<tr>
<td><strong>N. meningitidis</strong> serogroup a</td>
<td>NCTC 3375</td>
<td>/</td>
<td>+</td>
<td>/</td>
</tr>
<tr>
<td><strong>N. meningitidis</strong> serogroup a</td>
<td>NCTC 3372</td>
<td>/</td>
<td>+</td>
<td>/</td>
</tr>
<tr>
<td><strong>N. meningitidis</strong> serogroup b</td>
<td>ATCC13090</td>
<td>/</td>
<td>+</td>
<td>/</td>
</tr>
<tr>
<td><strong>N. meningitidis</strong> serogroup c</td>
<td>ATCC 13102</td>
<td>/</td>
<td>+</td>
<td>/</td>
</tr>
<tr>
<td><strong>N. meningitidis</strong> serogroup c</td>
<td>DSMZ 15464&lt;sup&gt;c&lt;/sup&gt;</td>
<td>/</td>
<td>+</td>
<td>/</td>
</tr>
<tr>
<td><strong>N. meningitidis</strong> serogroup</td>
<td>NCTC 11203</td>
<td>/</td>
<td>+</td>
<td>/</td>
</tr>
<tr>
<td><strong>N. meningitidis</strong> clinical isolates</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Organism Type</td>
<td>Organism Name</td>
<td>Reference Strain</td>
<td>Blood Isolates</td>
<td>Sputum Isolates</td>
</tr>
<tr>
<td>---------------</td>
<td>---------------</td>
<td>------------------</td>
<td>----------------</td>
<td>----------------</td>
</tr>
<tr>
<td>N. meningitidis serogroup b (Source: Blood)</td>
<td>5 isolates</td>
<td>/</td>
<td>+</td>
<td>/</td>
</tr>
<tr>
<td>N. meningitidis untyped (Source: Sputum)</td>
<td>1 isolates</td>
<td>/</td>
<td>+</td>
<td>/</td>
</tr>
<tr>
<td>N. meningitidis untyped (Source: Eye/Ear swab)</td>
<td>2 isolates</td>
<td>/</td>
<td>+</td>
<td>/</td>
</tr>
<tr>
<td>Non- N. meningitidis strains</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N. animaloris</td>
<td>DSMZ 21642c</td>
<td>/</td>
<td>-</td>
<td>/</td>
</tr>
<tr>
<td>N. bacilliformis</td>
<td>DSMZ 23338c</td>
<td>/</td>
<td>-</td>
<td>/</td>
</tr>
<tr>
<td>N. elongata subsp. elongata</td>
<td>DSMZ 17712c</td>
<td>/</td>
<td>-</td>
<td>/</td>
</tr>
<tr>
<td>N. elongata subsp.</td>
<td>DSMZ 23337c</td>
<td>/</td>
<td>-</td>
<td>/</td>
</tr>
<tr>
<td>N. elongata subsp.</td>
<td>DSMZ 17632c</td>
<td>/</td>
<td>-</td>
<td>/</td>
</tr>
<tr>
<td>N. flavescens</td>
<td>DSMZ 17633c</td>
<td>/</td>
<td>-</td>
<td>/</td>
</tr>
<tr>
<td>N. gonorrhoeae</td>
<td>DSMZ 9189c</td>
<td>/</td>
<td>-</td>
<td>/</td>
</tr>
<tr>
<td>N. lactamica</td>
<td>DSMZ 4691c</td>
<td>/</td>
<td>-</td>
<td>/</td>
</tr>
<tr>
<td>N. mucosa</td>
<td>DSMZ 17611c</td>
<td>/</td>
<td>-</td>
<td>/</td>
</tr>
<tr>
<td>N. perflava</td>
<td>DSMZ 18009c</td>
<td>/</td>
<td>-</td>
<td>/</td>
</tr>
<tr>
<td>N. polysaccharea</td>
<td>DSMZ 22809c</td>
<td>/</td>
<td>-</td>
<td>/</td>
</tr>
<tr>
<td>N. shackegani</td>
<td>DSMZ 22246c</td>
<td>/</td>
<td>-</td>
<td>/</td>
</tr>
<tr>
<td>N. sicca</td>
<td>DSMZ 17713c</td>
<td>/</td>
<td>-</td>
<td>/</td>
</tr>
<tr>
<td>N. subflava</td>
<td>DSMZ 17610c</td>
<td>/</td>
<td>-</td>
<td>/</td>
</tr>
<tr>
<td>N. wadsworthii</td>
<td>DSMZ 22247c</td>
<td>/</td>
<td>-</td>
<td>/</td>
</tr>
<tr>
<td>N. weaveri</td>
<td>DSMZ 17688c</td>
<td>/</td>
<td>-</td>
<td>/</td>
</tr>
<tr>
<td>N. zoodegmatis</td>
<td>DSMZ 21643c</td>
<td>/</td>
<td>-</td>
<td>/</td>
</tr>
<tr>
<td>S. pneumoniae reference strains</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. pneumoniae serotype 1</td>
<td>DSMZ 20566</td>
<td>/</td>
<td>/</td>
<td>+</td>
</tr>
<tr>
<td>S. pneumoniae serotype 9V</td>
<td>DSMZ 11865</td>
<td>/</td>
<td>/</td>
<td>+</td>
</tr>
<tr>
<td>S. pneumoniae serotype 23F</td>
<td>DSMZ 11866</td>
<td>/</td>
<td>/</td>
<td>+</td>
</tr>
<tr>
<td>S. pneumoniae clinical isolates</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. pneumoniae untyped (Source: Blood)</td>
<td>2 isolates</td>
<td>/</td>
<td>/</td>
<td>+</td>
</tr>
<tr>
<td>S. pneumoniae untyped (Source: Sputum)</td>
<td>5 isolate</td>
<td>/</td>
<td>/</td>
<td>+</td>
</tr>
<tr>
<td>S. pneumoniae untyped (Source: Eye/Ear swab)</td>
<td>2 isolates</td>
<td>/</td>
<td>/</td>
<td>+</td>
</tr>
<tr>
<td>Non- S. pneumoniae strains</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. agalactiae</td>
<td>BCCM 15086</td>
<td>/</td>
<td>/</td>
<td>-</td>
</tr>
<tr>
<td>S. anginosus</td>
<td>DSMZ 20563</td>
<td>/</td>
<td>/</td>
<td>-</td>
</tr>
<tr>
<td>S. australis</td>
<td>DSMZ 15627</td>
<td>/</td>
<td>/</td>
<td>-</td>
</tr>
<tr>
<td>S. bovis</td>
<td>DSMZ 20480</td>
<td>/</td>
<td>/</td>
<td>-</td>
</tr>
<tr>
<td>S. canis</td>
<td>DSMZ 20715</td>
<td>/</td>
<td>/</td>
<td>-</td>
</tr>
<tr>
<td>Species</td>
<td>DSMZ Code</td>
<td>/</td>
<td>/</td>
<td>-</td>
</tr>
<tr>
<td>---------------------</td>
<td>-----------</td>
<td>---</td>
<td>---</td>
<td>----</td>
</tr>
<tr>
<td>S. constellatus</td>
<td>DSMZ 20575</td>
<td>/</td>
<td>/</td>
<td>-</td>
</tr>
<tr>
<td>S. cristatus</td>
<td>DSMZ 8249</td>
<td>/</td>
<td>/</td>
<td>-</td>
</tr>
<tr>
<td>S. downei</td>
<td>DSMZ 5635</td>
<td>/</td>
<td>/</td>
<td>-</td>
</tr>
<tr>
<td>S. dysgalactiae subsp.</td>
<td>DSMZ 6176</td>
<td>/</td>
<td>/</td>
<td>-</td>
</tr>
<tr>
<td>S. equi subsp. equi</td>
<td>DSMZ 20561</td>
<td>/</td>
<td>/</td>
<td>-</td>
</tr>
<tr>
<td>S. gordonii</td>
<td>DSMZ 6777</td>
<td>/</td>
<td>/</td>
<td>-</td>
</tr>
<tr>
<td>S. infantis</td>
<td>DSMZ 12492</td>
<td>/</td>
<td>/</td>
<td>-</td>
</tr>
<tr>
<td>S. intermedius</td>
<td>DSMZ 20573</td>
<td>/</td>
<td>/</td>
<td>-</td>
</tr>
<tr>
<td>S. mitis</td>
<td>DSMZ 12643</td>
<td>/</td>
<td>/</td>
<td>-</td>
</tr>
<tr>
<td>S. mutans</td>
<td>DSMZ 20523</td>
<td>/</td>
<td>/</td>
<td>-</td>
</tr>
<tr>
<td>S. oralis</td>
<td>DSMZ 20066</td>
<td>/</td>
<td>/</td>
<td>-</td>
</tr>
<tr>
<td>S. perosis</td>
<td>DSMZ 12493</td>
<td>/</td>
<td>/</td>
<td>-</td>
</tr>
<tr>
<td>S. porcinus</td>
<td>DSMZ 20725</td>
<td>/</td>
<td>/</td>
<td>-</td>
</tr>
<tr>
<td>S. pseudopneumoniae</td>
<td>DSMZ 18670</td>
<td>/</td>
<td>/</td>
<td>-</td>
</tr>
<tr>
<td>S. pyogenes</td>
<td>DSMZ 20565</td>
<td>/</td>
<td>/</td>
<td>-</td>
</tr>
<tr>
<td>S. sanguinis</td>
<td>DSMZ 20567</td>
<td>/</td>
<td>/</td>
<td>-</td>
</tr>
<tr>
<td>S. sinensis</td>
<td>DSMZ 14990</td>
<td>/</td>
<td>/</td>
<td>-</td>
</tr>
<tr>
<td>S. vestibularis</td>
<td>DSMZ 5636</td>
<td>/</td>
<td>/</td>
<td>-</td>
</tr>
</tbody>
</table>

* 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).
4.5. References


virus in clinical samples. *Journal of Medical Microbiology* 2009, 58(9):1168-1172.


63. Note A: Using TaqMan® Endogenous Control Assays to Select an Endogenous Control for Experimental Studies.


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 subtilis* 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. meningitidis* 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


Appendix A:

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

Nikolay Dimov · Eoin Clancy · Jennifer Gaughran · David Boyle · Darren Mc Auley · McAuliffa T. Glynn · Robin M. Dwyer · Helena Conglan · Thomas Barry · Louise M. Barrett · Terry J. Smith · Jens Ducée

Received: 24 May 2014 / Accepted: 28 August 2014 / Published online: 12 September 2014 © Springer-Verlag Berlin Heidelberg 2014

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 collection 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 leukocytes 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

1 Introduction

Microfluidic lab-on-a-chip technologies have been shown to automate and reduce the time-to-result of a wealth of biochemical 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/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; Henehan 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 have the potential to integrate sample preparation with detection to create full-fledged sample-to-answer devices (Madou et al. 2006; Duerre 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 (Ducreé et al. 2007; Zoval and Madou 2004) or siphons (Schembri et al. 1995; Steigert et al. 2007; Nwankwo 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 on-off valve seals. By trapping air between this dissolvable film and the liquid, they implemented centrifugal-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. (2005) 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 by lysed influenza A H3N2 virus using silica, 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 piercable 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, Kinoshita 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 analysers.
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 microfluidically 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 acid 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 Yip 2000).

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 leukocytes 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 outgassing 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 $P_{\text{air}}$ ($V_{\text{air}}/V$) depends on the initial (typically atmospheric) pressure $P_{0}$ and the ratio of the initial and final volumes $V_{\text{air}}$ and $V$, respectively (Fig. 1b). The flow in the lateral channel stops once the pressure in the air pocket equilibrates the centrifugally induced pressure $\rho_0 n^2 \Delta r$ with the liquid density $\rho$, the angular frequency $\omega_0$, the radial length $r$ and the mean position $\Delta r$ 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 DFV 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 $W_{\text{aq}}$ and organic waste $W_{\text{org}}$ and for elute containing the purified RNA ($E_{\text{aq}}$) in the final step of the solid-phase purification.

Within the loading chamber, we placed a needle 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, c) while providing passage of the organic solvents (Fig. 2c, d). These organic phases are both directed to their designated waste $W_{\text{org}}$, 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 creast point at increased rates until they are fully diverted into $W_{\text{aq}}$. 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 $E_{\text{aq}}$ with organic solvents. Following this intermediate, organic routing phase, we intersperse a drying period for the previously primed chambers 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 $E_{\text{aq}}$ 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. 2c, d) are diverted in a binary fashion to the aqueous waste ($W_{\text{aq}}$) and the elution chamber ($E_{\text{aq}}$), respectively. To this end, a normally closed (water) dissolvable film valve (DFV) initially seals the radial outlet to route the liquid into $W_{\text{aq}}$. 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 $E_{\text{aq}}$. Additionally, the hydrophobic membrane (HM) covering the outlet of the aqueous waste ($W_{\text{aq}}$), 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. 2c, d). 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 (Boon et al. 1990).
Fig. 2. Fluidic tests of the router. a Schematic of the device showing the leading 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 50 µL of EtOH from L to W_{org} 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_{org}. The flow distributes 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 (Adano 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 film. EtOH is consecutively loaded that rinses away salts from the beads and the precipitated nucleotides (Fig. 2d), and also residues from the 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
3 Materials and methods

The following materials were used for the study: acid-washed glass beads (≤106 μm), absolute ethanol, isopropanol, phosphate buffered saline (PBS), pressure-sensitive adhesive (PSA, Adhesive Research Inc., Ireland), polyvinyl methyl methacrylate (PMMA) sheets (Evonic 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-bromoninolide (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 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 (d120 mm × d15 mm × 1.5 mm) and two binding PSA layers (Fig. 3). Each disc was cut to size and processed on a CO2 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. Fluids and outlet via-holes 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 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, Chemtillant 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% CO2. 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 3000 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 % CO₂. 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 3000g for 4 min to pellet the cells.

3.5 Haemophilus influenzae 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 × 10⁷ 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 1x PBS and pelleted.

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

Cells were lyzed 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 × 10⁵·7.4 × 10⁵ MCF7 cells. The cells were enumerated by an automated cell counter (Scepter Handheld, Milipore, 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-bromomycinsole 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 (Boon 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 hydroxyxil 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 (EIOH). Finally, the purified RNA was retrieved from the beads with the addition of 100 μL elution buffer (50 mM Tris-HCl, 1 x 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 (E₉₈) 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 electrophrogram such as the Pre region, 28S region, 18S 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 cokaryote 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: AAACTT- AATACGACTCA- TATAGGGAGAAGCTTCGATCCCTAACC; P2: GCACCTTAATACCT and a molecular beacon 5′FAM- CCAGAGGATGATAACGC-GAGGTCAACTCGG 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 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 protocol 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 x 10^5 cells, which was less than the 23.1
Fig. 5 Electrophogram 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 × 10⁵, (yellow) 1.48 × 10⁵, (light blue) 7.4 × 10⁴, (blue) 3.7 × 10⁴ 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 Ead (green, Fig. 2), Wad (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 Wad. 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).

The purification efficiency was further investigated by comparing the RNA content from the purified (Ead) to the unbound (Wad) 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 SPE 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-disc (Hagen et al. 2009) and recently 81.0% on-disc with (TESOS)-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 × 10⁷ 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 $1.48 \times 10^5$ 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 ($D = 2D_t$) 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 behavior 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 $1 \times 10^5$ T47D cells (human ductal breast epithelial tumors cell line) and the leukocyte fraction from 1 mL of human whole blood. The microRNAs, miR-16 and miR-155 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-155 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 leukocytes, 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.

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...
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 mRNAs.

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 Klete 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 Jung 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 improving packing of the solid phase. Based on the developed extraction platform, the fully automated system is planned 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.

Acknowledgments This work was supported by the Science Foundation Ireland under Grant 10/C/R11821. Jennifer Gaughan was funded through BioAnalysis and Therapeutics Structured Ph.D. Programme (Bio-AT) by HiAPRIL V. The authors also thank Prof. Richard O’Kennedy’s group in Dublin City University for allowing us to use their cell culture facilities. We would also like to thank Karsen Holman and her team in Harze PackPro for providing disposable films.

References


Appendix B

Development of on-disc Isothermal In-vitro Amplification and Detection of Bacterial RNA
Development of an on-disc isothermal in vitro amplification and detection of bacterial RNA

Des Brennan, Helena Coughlan, Eoin Clancy, Nikolay Dimov, Thomas Barry, David Kinahan, Jens Ducrée, Terry Smith, Paul Galvin

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 diagnostics. The sensitivity and specificity of NA tests are typically >90%, with multiple 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 LoopT 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 thermocapillary 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
DNA amplification technologies: (i) enabling quantitative detection of microorganism RNA [15-21], (ii) no amplification interference from contaminating background DNA [19], (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 NABSA in microfluidic devices use direct contact heating to maintain reaction temperatures [2,12]. Non-contact heating facilitates LoAD-NABSA amplification, allowing spin without the complexity of contact heating. Approaches to non-contact microfluidic heating include: microwave [23], induction [24] and infrared [29]. Microwave and induction heating require complex electronic components, e.g. high frequency oscillators (26GHz) with high power inputs (20-70W). More recently, non-contact IR heating was applied to the rapid amplification of nucleic acids in microfluidic devices [28] 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 disposable 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 real-time amplification. In this work, non-contact IR heating and fluorescence detection was applied to facilitate NABSA on LoADs. 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 NABSA assays were implemented on LoADs over the range 10^{-10}^{10} H. influenzae CT to determine system detection limits. For comparison, identical qNABSA assays were also carried out on a Roche LightCycler 2.0. The system demonstrated suitability for implementation of the NABSA 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 mRNA over a clinically relevant range.

2. Materials and methods

2.1. Micromachined chamber fabrication

The NASBA reaction chamber was injection moulded using Zeonex™ (160XR, Zeon Chemicals Europe Ltd) on a Babyplast 60/10 machine (Babyplast, Monza, Italy), pellets were dried for sixty minutes at 55°C. The system parameters were set for Zeonex™ [31]: Injection nozzle 195°C, injection chamber 210°C, plastic melt chamber 220°C, with a third injection stay at 60°C. The manufactured components were cleaned as follows: (i) forty minutes at 50°C in an ultrasonic bath (1% methanol) (ii) forty minutes at 50°C in an ultrasonic bath (0.1% TWEEN 20). (iii) twenty minutes at 20°C in an ultrasonic bath (de-ionised water) and (iv) ten minutes ozone clean (ProCleaner™ Bioforce Nanoscience, UT). The microfluidic component was designed using computer-aided design (CAD) soft-

2.2. Hemophilus influenzae culture and RNA extraction

Hemophilus influenzae (DSM22399) 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 (~4 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 RiboFast yeast lysis kit (Ambion, Austin, TX, USA) according to the manufacturer’s 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, Epsom, UK). 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 tRNA transcript of H. influenzae. The primers (5’-3’) P1: CATCCTCATGCACTATAGCG-CAAGC-GGCTCTGCTTCAAAAGC-GT, P2: GGCCCGTAACTT and a molecular beacon 5’FAM CCCACT-CCCCGATACCCGACTA-3’ were designed according to recommended guidelines [33,34]. Primer and molecular beacon probes were supplied by Eurofins MWG Operon (Ebersberg, Germany).

2.4. qNABSA H. influenzae assay

NASBA assays were performed using the NucSSENS EasyQ Basic Kit V2 (Biomérieux, Marcy l’Etoile, France) manufacturer’s instructions. qNABSA 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 probe (0.2 μM and 0.1 μM concentration respectively) mixture. The reaction was incubated on a Kylcer thermocycler (Bio-rad, Hercules, CA) for 65°C for five minutes to denature the DNA 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 NucSSENS EasyQ Basic Kit V2.0 (BioMérieux, 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 0.1 μM enzyme mixture was prepared and used to fill the NASBA chamber. Reagent/KCL, primer 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 washed 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.

183
2.6. System functionality

The instrument implemented denaturation/amplification temperature profiles using a PID controller to modulate heater power. A LaBVIEWSM dock 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 LaDo in discrete 18° steps, while activating the position magnet (Section 2.9). With the LaDo locked in position, the sample chamber is heated to 41°C for 55 min. After amplification, the system rotates chamber 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-DAQ0228) 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 GSK137-1-MA) and modulates power to the heater and cooling fan (20W) via two high voltage relays (MOSFET 100V). A calibrated thermocouple (K type, Chauvin Arnoux TC8000 instrument) was placed in the microfluidic chamber (filled with deionized water) the controller was set to hold the 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 LaDo distance was fixed at 10mm while the sensor to LaDo 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 minimizing overshoot/oscillation around set-points.

2.8. Fluorescence detection system

The detection system (Fig. 1), is controlled via LaBVIEWSM through the NDAQ 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 CPS440) 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 450V with 10 ms integration time, the PMT current was converted to voltage using a transimpedance (x10) amplifier (CF719, Hamamatsu photonics, Japan) and measured using the daq card analogue voltage input (0-10V).

Fig. 1 illustrates the system implementing NASBA heating and detection within a chamber mounted on a Zeonor™ LaDo substrate. The fluorescence signal is recorded after amplification is completed. The LaDo 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. LaDo spin control

The LaDo was mounted on DC motor spindle (2657W024CR, Faulhaber, Switzerland) with a 1000 point line encoder (HEIDENHAIN, Switzerland) the maximum motor speed was set to 6000 rpm. The acceleration/de-acceleration was set below 50 rpm/s. The LaDo 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. Permanet magnets (M004 Magnet Expert Ltd, UK) were attached to the LaDo aligned as the motor rotated the LaDo in short pulses (Fig 3). After each pulse, an external magnet fixed on a linear solenoid (Black Knight™ 122, BLX components Ltd, UK) was actuated when LaDo & external mounted magnets overlapped the LaDo chamber was locked in place. Two magnets mounted on the LaDo 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 (6V). All commands were executed in a LabVIEW™ 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 LaDo 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.
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 Arnoux 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. 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 driver rotation (10 pm per 1 s) of the LoaD motor under LabVIEW™ control.
Fig. 4. The NASHA heating profile was applied for the protocol duration (30 min); the ramp time from room temperature to the denaturation temperature of $65^\circ C$ was 100 s and the cooling time to the incubation temperature of $41^\circ C$ was 31 s. The sample was maintained at $65^\circ C$ for five minutes then cooled and held at $41^\circ C$ for 55 min implementing the complete NASHA heating protocol.

Fig. 5. The thermal profile across the chamber heating with the 1.65 kW 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.

(Rottner LaserTechnik Laser 1LTST800M). 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 bears approximately $3 \text{ mm}^2$. However, the measured temperature fell rapidly from $65^\circ C$ at the centre to $54.5^\circ C$ - 2 mm off centre and $47^\circ C$ - 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 $3.8\text{ W}$ to maintain the reservoir centre at $65^\circ C$. The measured temperature 2 mm and 4 mm above centre was $62^\circ C$ and $54^\circ C$ respectively. Further defocusing wasn’t possible due to the laser power limit. The IR broadband emitter achieved a uniform temperature profile ($65^\circ C$) 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 $10^4$, $10^5$ and $10^6$ CE. The reactions confirmed successful amplification of the $H$. influenzae tmRNA concentrations tested. The qNASBA products were then loaded onto the laser chamber, positioned in the optical fluorescence detector path and the signal recorded (Fig. 6). This established that the fluorescence system could detect $10^5$ CE for end point detection.
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 absorb 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 LoA endpoint NASBA was carried out in the system. Reactions were carried out at 1 x 10⁶, 1 x 10⁷, 5 x 10⁷, and 1 x 10⁸ CEF. A NTC (molecular grade H2O) was also included. For on LoA 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 x 10⁶ to 1 x 10⁷ CEF (Fig. 7). However, at 1 x 10⁸ CEF 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 CEF.

4. Discussion

This work demonstrates end point NASBA amplification of tmRNA transcript (H. influenza) on a LoA system, tmRNA, encoded by the saa gene has previously been shown 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 LoA 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 LoA chamber is rotated between a heating and fluorescence detection locations using LoA 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 Zeromor [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 cause localised diffusion, causing spatial variation in reagent concentration which may impact reaction efficiency [38]. Isothermal amplification does not require rapid thermocycling thus slow heating rates are acceptable (2.5°C/s). The detection system was evaluated with multiplex DNA FAM assays, but it could detect duplex assay e.g. FAM/CYS, CY3/CYS. The system demonstrated fluorescence sensitivity down to 100 CEF with tmRNA (H. influenza) 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 10-10⁶ CEF. The full NASBA heating protocol was implemented on the LoA chamber using the IR heater, the fluorescence signal was recorded before and after incubation. In this work the sample was pipetted onto the LoA which was loaded onto the system for denaturation, upon completion the sample was removed from the LoA by pipette mixed with the enzymes and reloaded onto the system for incubation/amplification.

The LoA motion control system outlined in Section 2.9 was reused to relocate the chamber between heating and detection zones. This demonstrated a system sensitivity of between 100 and 500 CEF. 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 CEF/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 [38]. The heating system generated temperature profiles compatible with the open chamber LoA 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-
surface interaction presents a challenge for microfluidic diagnostics [12]. Thus a published microchip treatment protocol was carried out [11], giving good amplification with comparable results obtained from on-chip and Lightcycler NASBA arrays.

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 10^6 and 10^9 cells. 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 further 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 released 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.

Acknowledgement

This work was supported by Science Foundation Ireland as part of the Biomedical Diagnostics Institute Centre for Science Excellence and Technology (10/CE/B1821).

References


Fig. 7. The fluorescence intensity of H. influenzae RNA amplified and detected on the integrated heating/detection system. The RNA concentrations analyzed were equivalent to 10^6, 10^7, 10^8 and 10^9 cells. The error bars are standard deviation of three repeated measurements per test.
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
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

Kate Reddington,*b* Stefan Schwenk,*b* Nina Tutte,*b* Gareth Platt,*b* Danesh Davar,*b* Helena Coughlan,*b* Yoann Personne,*b* Vanya Gant,*b* Virve I. Enne,*b* Almuddin Zumla,*b* Thomas Barry*ab*

Nucleic Acid Diagnostics Research Laboratory (NADRIL), Microbiology, School of Natural Sciences, National University of Ireland, Galway, Ireland; Centre for Clinical Microbiology, Division of Infection and Immunity, University College London, and Department of Medical Microbiology, University College London Hospitals NHS Foundation Trust,*a* London, United Kingdom

*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 *fack* 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 para influenzae,* 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, non-typeable 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...
TABLE I Oligonucleotide primers and probes developed in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Function</th>
<th>Sequence 5’ to 3’</th>
<th>Accession no. (nucleotide position [bp])</th>
</tr>
</thead>
<tbody>
<tr>
<td>HaemF</td>
<td>Forward smβ real-time PCR assay primer</td>
<td>ATTAATAAAGTCCTAGCACTACCC</td>
<td>NC_00907.1 (213–232)</td>
</tr>
<tr>
<td>HaemR</td>
<td>Reverse smβ real-time PCR assay primer</td>
<td>GATCTTTGGGCGCACCC</td>
<td>NC_00907.1 (356–372)</td>
</tr>
<tr>
<td>H. influenzae smβ PI</td>
<td>smβ real-time PCR probe</td>
<td>FAM-ACGTTTTTATAGTGTACCTTTTGTC-AMQ</td>
<td>NC_00907.1 (317–343)</td>
</tr>
<tr>
<td>IAC, Fw</td>
<td>Forward IAC primer</td>
<td>AAGCTAGCCATGTTGTC</td>
<td>HGSJ9281.1 (111–127)</td>
</tr>
<tr>
<td>IAC, Bv</td>
<td>Reverse IAC primer</td>
<td>CTCTTCATCTTCTCTCTCTCT</td>
<td>HGSJ9281.1 (269–276)</td>
</tr>
<tr>
<td>Bacillus subtilis PI</td>
<td>IAC probe</td>
<td>Cy5-CAACCGGATTCATGCACGCT-AMQ</td>
<td>HGSJ9281.1 (179–199)</td>
</tr>
</tbody>
</table>

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 smβ 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 diagnostic 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 (mRNA) database (http://blastformatics.sandia.gov/mrna/). In silico analysis of each nucleolar 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 IGC standards, Middelbeek, United Kingdom), and the Culture Collection, University of Gothenburg (CCUG, Sweden). All Haemophilus species and strains were cultured on Columbia chocolate agar (Oxoid, Hampshire, United Kingdom) at 37°C with 5% CO2 for 18 to 24 h.

Genomic DNA isolation and quantification. Genomic DNA (gDNA) from Haemophilus isolates and clinical samples were isolated using a modified method involving combined mechanical lysis (IDV lys kit, Section Dickinson, Canada) and purification using a quick gDNA kit (ZymoResearch, Irvine, CA, USA). Briefly, a loop of culture was re-suspended in 250 μl IDV lysis buffer. The suspension was transferred to a GeneOhm lysate tube and bead beaten (Minit-BeadBuster-16Stratech, United Kingdom) for 3 min. After bead beating, 200 μl of the supernatant was transferred to a ZymoSpin 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 instructions. 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 (Eisenberg, Germany). All oligonucleotide primers used in this study were designed to have a melting temperature (Tm) of 58 to 61°C, and all oligonucleotide hydrolysis probes for Tm of 7 to 10°C higher.

For the H. influenzae specific diagnostic assay, PCR primers HaemF and HaemR (Table I) were designed to amplify a 160-bp fragment of the smβ 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, Bv (Table I), were designed to amplify a 206-bp region of the Bacillus subtilis subsp. subsp. strain W3 strain DNA. 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. spizizen strain W3 strain, 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 had 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 using the LightCycler 480 using the LightCycler 480 Probes Master kit (Roche Diagnostics, Basel, Switzerland). The optimized PCR mix contained 2 μl LightCycler 480 Probes Master (6.4 mM MgCl2), 50 μl 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 (dH2O). The B. subtilis internal control DNA was diluted to contain 500 genome equivalents per μl and all other DNA used in this study was diluted to contain ~10^6 genome equivalents per μl.

The cycling parameters consisted of 10 min incubation at 95°C to
activate the Tag, 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 filter 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 = 2; 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, trimethoprim, moxifloxacin, tetracycline, and trimethoprimsulphamethoxazole were determined by Etest, and results were interpreted according to EUCAST guidelines (23). All isolates were tested for beta-lactamase activity using Cefazolin paper disks (Becton Dickinson). The beta-lactamase variant in positive isolates was identified by PCR for blaTEM and blaSHV 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 smbp 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 10−7 in phosphate-buffered saline. A neat sample (50 μl) and each dilution were spread onto Columbia blood agar (CBA), Columbia agar with chocolate horse blood (CHOc), and Brillant 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% CO2 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 smbp 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 srRA 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, smbp, selected and identified by the NADRL, was also evaluated in silico. From this in silico analysis, the srRA and lepA genes were

<table>
<thead>
<tr>
<th>TABLE 2 Clinical isolates used in this study</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of clinical isolates</td>
</tr>
<tr>
<td>--------------------------</td>
</tr>
<tr>
<td>6</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>17</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>1</td>
</tr>
</tbody>
</table>

* SXT, trimethoprim-sulphamethoxazole; AMP, ampicillin; ERY, erythromycin; CRO, ceftriaxone; TET, tetracycline; AMC, amoxicillin-clavulanate; MXF, moxifloxacin.

a – , negative.

<table>
<thead>
<tr>
<th>TABLE 3 Clinical respiratory tract samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of clinical samples</td>
</tr>
<tr>
<td>--------------------------</td>
</tr>
<tr>
<td>25</td>
</tr>
<tr>
<td>7</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>7</td>
</tr>
<tr>
<td>13</td>
</tr>
<tr>
<td>41</td>
</tr>
</tbody>
</table>

* – , 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 × 10^6 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 1.6 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 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 × 10^8 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, beca, smpbP2, and F6 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 smpbP2, they have been found to cross-react with other Haemophilus species resulting in the reporting of false-positive results (9, 16). Also, the fusose 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 ssaA, lepA, and smbp 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 smpbP2 gene demonstrated sufficient in silico nucleotide sequence heterogeneity to allow for the design of an H. influenzae-specific diagnostic assay. The smpbP2 gene, which codes for the RNA binding protein small protein B (SmbP), has been identified in all bacterial species to date (36). It is considered an essential component of quality control in bacteria, as it facilitates the binding of mRNAs 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 mRNA in a bacterial cell, the smpbP2
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 *fucK* 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 *fucK* 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.

**ACKNOWLEDGMENTS**

All authors with the exception of H.C. are supported by EC Grant RFD-RTI FP7-HEALTH-2012-INNOVATION-2 (grant agreement 304865).

We also acknowledge Leonard W. Mayer and Jennifer Dolan Thomas at the Centers for Disease Control and Prevention (CDC), who provided some of the *H. haemolyticus* strains used in this study.
Appendix D

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

Helena Coughlan a,b, Kate Reddington a, Nina Tuite a, Teck Wee Boo c, Martin Cormican d, Louise Barrett e, Terry J. Smith b, Eoin Clancy b, c, Thomas Barry a, b, s

a Nucleic Acid Diagnostics Research Laboratory (NADRL), Microbiology, School of Natural Sciences, National University of Ireland, Galway, Ireland
b Biomedical Diagnostics Institute, Programme, National Centre for Biomedical Engineering Science, National University of Ireland, Galway, Ireland
c Department of Clinical Microbiology, University College Hospital, Galway, Ireland
d School of Medicine, National University of Ireland, Galway, Ireland
s National Centre for Sensor Research, School of Physical Sciences, Biomembrane Diagnostics Institute, Dublin City University, Glasnevin, Dublin 9, Ireland

Molecular Diagnostics Research Group (MDRG), School of Natural Sciences, National University of Ireland, Galway, Ireland

ARTICLE INFO

Article history:
Received 31 March 2015
Received in revised form 3 June 2015
Accepted 7 June 2015
Available online 23 June 2015

Keywords:
H. influenzae
Genome comparison
Real-time PCR
gfb
phd

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 and phylogenetic distance. From this analysis, we identified 2 H. influenzae putative diagnostic targets, gfb and phd, 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 PCR 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.

© 2015 Elsevier Inc. All rights reserved.

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 (NTI) (Pittman, 1931). In the pre-vaccine era, the encapsulated H. influenzae serotype b (Hib) was the primary cause of invasive disease such as meningitis, bacteremia, and pneumonia (van Wessel et al., 2011). Upticks of 95% 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, NTI is now the most common cause of H. influenzae invasive infections (van Wessel et al., 2011; MacNeill et al., 2011; Dworkin et al., 2007; Resman et al., 2011). In addition, NTI have replaced type b strains as the most common bloodstream isolates (Aarli 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 hoxin (X factor) and NAD (V factor) for growth. Phenotypic methods of differentiating H. haemolyticus from NTI 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 may be misidentified as H. influenzae (Murphy et al., 2007). H. haemolyticus was considered a rare pathogen (Aarli and Murphy, 2011); however, more recently, a number of cases of invasive disease originally attributed to NTI have been confirmed as nonhaemolytic H. haemolyticus (Morton et al., 2012; Andersen 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 hbd (Vandeleur et al., 1990; Wroblewski et al., 2013), ompP2(Favarri et al., 2013; Meyler et al., 2012; Wang et al., 2011; Helson et al., 1995),ompF (Vandeleur et al., 1990; Abdelhaim et al., 2009; Heilberg et al., 2009), 16S rRNA (Murphy et al., 2007), gfbA (Meyler et al., 2012), rfbA (Abdelhaim et al., 2009), frdB (Kanthalan et al., 2012), igf (Vitevski et al., 2002) hdp (Wang et al., 2011; Pekering et al., 2014; Hare et al., 2012), and jack (Meyler et al., 2012; Abdelhaim et al., 2013; Meas et al., 2015). Diagnostic assays targeting the frdB and hdp genes have been established.
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 fack (Norskov-Lauritsen, 2009; Fenger et al., 2012; Ridderberg et al., 2010; Stuel et al., 2011) or Hpu genes (Smith-Vaughan et al., 2014). As such, published literature indicates that no one molecular marker can unambiguously 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

Publicly 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 publicly 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, M22139, and H5138), contigs were ordered and assembled using the Maave contig mapper tool in the Maave genome alignment software (http://gelab.wisc.edu/maave/). The European Molecular Biology Open Software Suite tool Emboss union (http://emboss.sourceforge.net/emboss/unix/maave/maave/unix/) was used to concatenate the multiFASTA Maave 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 H5138) and the whole genome sequence of their most closely related H. influenzae strain (R268; 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 microaerophilic conditions in Haemophilus test media broth, chocolate broth, or on Columbia chocolate agar plates at 37°C until sufficient growth was observed.

2.3. Bacterial DNA isolation and quantification

Genomic DNA isolation 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 QuantIt 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

<table>
<thead>
<tr>
<th>Organism</th>
<th>Strain/Source</th>
<th>Assay&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>H. influenzae reference strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H. influenzae type a</td>
<td>NCTC 8665</td>
<td>++</td>
</tr>
<tr>
<td>H. influenzae type b</td>
<td>DSM 23939&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
</tr>
<tr>
<td>H. influenzae type b</td>
<td>DSM 21949&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
</tr>
<tr>
<td>H. influenzae type c</td>
<td>DMS 11970&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
</tr>
<tr>
<td>H. influenzae type d</td>
<td>DMS 20304&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
</tr>
<tr>
<td>H. influenzae type e</td>
<td>DMS 4689&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
</tr>
<tr>
<td>H. influenzae type f</td>
<td>DMS 18466&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
</tr>
<tr>
<td>H. influenzae type g</td>
<td>DMS 11215&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
</tr>
<tr>
<td>H. influenzae type h</td>
<td>DMS 8472&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
</tr>
<tr>
<td>H. influenzae type i</td>
<td>DSM 20000&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
</tr>
<tr>
<td>H. influenzae NTHI</td>
<td>DSM 20009&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
</tr>
<tr>
<td>H. influenzae NTHI</td>
<td>CGM 58165&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
</tr>
<tr>
<td>H. influenzae NTHI</td>
<td>CGM 58165&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
</tr>
<tr>
<td>H. influenzae NTHI</td>
<td>CGM 58165&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
</tr>
<tr>
<td>H. influenzae NTHI</td>
<td>CGM 58165&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
</tr>
<tr>
<td>Clinical isolates (n = 11)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Blood</td>
<td>+</td>
</tr>
<tr>
<td>Clinical isolates (n = 11)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>Clinical isolates (n = 24)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Sputum</td>
<td>+</td>
</tr>
<tr>
<td>Clinical isolates (n = 24)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>++</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> pbA and pbB gene sequence data were generated for each of these H. influenzae strains using primers outlined in Table 2.

<sup>b</sup> pbA and pbB gene sequence data were generated for each of these H. influenzae strains using primers outlined in Table 2.

<sup>c</sup> All clinical isolates were identified as H. influenzae using MALDI-TOF MS.

<sup>d</sup> All clinical isolates were identified as H. influenzae using MALDI-TOF MS.

NCTC = National Collection of Type Cultures, DSM = The German Collection of Microorganisms, CGM = Culture Collection, University of Giessen, Germany, CDC = Center for Disease Control.
Table 2

<table>
<thead>
<tr>
<th>Probe/primer</th>
<th>Product name</th>
<th>Function</th>
<th>DNA sequence (5’→3’)</th>
<th>Nucleotide position</th>
<th>Concentration (µM/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>psAF1</td>
<td>Sequencing forward primer</td>
<td>CAGCATTTTACCTCGG</td>
<td>1-20</td>
<td>C05227.1</td>
<td>0.4 µM/L</td>
</tr>
<tr>
<td>phdF</td>
<td>Sequencing forward primer</td>
<td>CAGCATTTTACCTCGG</td>
<td>1-20</td>
<td>C05227.1</td>
<td>0.4 µM/L</td>
</tr>
<tr>
<td>psAF2</td>
<td>Sequencing reverse primer</td>
<td>TCTTGGTGAAAGGACG</td>
<td>1-20</td>
<td>C05227.1</td>
<td>0.4 µM/L</td>
</tr>
<tr>
<td>phdR1</td>
<td>Sequencing reverse primer</td>
<td>TCTTGGTGAAAGGACG</td>
<td>1-20</td>
<td>C05227.1</td>
<td>0.4 µM/L</td>
</tr>
<tr>
<td>psAF3</td>
<td>40 kbp primer forward primer</td>
<td>CAGCATTTTACCTCGG</td>
<td>1-20</td>
<td>C05227.1</td>
<td>0.4 µM/L</td>
</tr>
<tr>
<td>phdR2</td>
<td>40 kbp primer reverse primer</td>
<td>CAGCATTTTACCTCGG</td>
<td>1-20</td>
<td>C05227.1</td>
<td>0.4 µM/L</td>
</tr>
<tr>
<td>psAFp</td>
<td>40 kbp primer forward primer</td>
<td>CAGCATTTTACCTCGG</td>
<td>1-20</td>
<td>C05227.1</td>
<td>0.4 µM/L</td>
</tr>
<tr>
<td>phdp</td>
<td>40 kbp primer reverse primer</td>
<td>CAGCATTTTACCTCGG</td>
<td>1-20</td>
<td>C05227.1</td>
<td>0.4 µM/L</td>
</tr>
<tr>
<td>psAPprobe</td>
<td>40 kbp primer forward primer</td>
<td>CAGCATTTTACCTCGG</td>
<td>1-20</td>
<td>C05227.1</td>
<td>0.4 µM/L</td>
</tr>
<tr>
<td>phdprobe</td>
<td>40 kbp primer reverse primer</td>
<td>CAGCATTTTACCTCGG</td>
<td>1-20</td>
<td>C05227.1</td>
<td>0.4 µM/L</td>
</tr>
<tr>
<td>psAF2</td>
<td>40 kbp primer forward primer</td>
<td>CAGCATTTTACCTCGG</td>
<td>1-20</td>
<td>C05227.1</td>
<td>0.4 µM/L</td>
</tr>
<tr>
<td>phdR2</td>
<td>40 kbp primer reverse primer</td>
<td>CAGCATTTTACCTCGG</td>
<td>1-20</td>
<td>C05227.1</td>
<td>0.4 µM/L</td>
</tr>
<tr>
<td>psAFp</td>
<td>40 kbp primer forward primer</td>
<td>CAGCATTTTACCTCGG</td>
<td>1-20</td>
<td>C05227.1</td>
<td>0.4 µM/L</td>
</tr>
<tr>
<td>phdp</td>
<td>40 kbp primer reverse primer</td>
<td>CAGCATTTTACCTCGG</td>
<td>1-20</td>
<td>C05227.1</td>
<td>0.4 µM/L</td>
</tr>
</tbody>
</table>

PCR Master Kit (Roche Diagnostics, Basel, Switzerland) according to manufacturer’s instructions. The thermal cycling parameters were set as follows: 95°C for 3 min, followed by 35 cycles at 95°C (50 s), 53°C (20 s), and 72°C (20 s), and a final elongation cycle at 72°C for 5 min. PCR products were purified using the Highpure PCR product purification kit (Roche Diagnostics) and were sequenced externally (Sequenew, 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 program (http://www.clustal.org/). Oligonucleotide primers and probe sequences were manually designed in accordance with general recommendations and guidelines (Teplitskaya, 2006; Robertson and Welsh-Weller, 1998). Species-specific oligonucleotide primers and probes were designed for real-time PCR diagnostic assays. All primers and probes (Table 2) were synthesized in-house.

2.6. Internal amplification control (IAC) development

A noncompetitive IAC targeting the 16S rRNA gene of Haemophilus influenzae was incorporated into the real-time PCR diagnostic assays (Table 2).

2.7. Real-time PCR

Real-time PCR diagnostic assays were optimised and evaluated. The analytical specificity and limit of detection (LOD) were determined. All real-time PCR diagnostic assays were performed on a LightCycler 480 (Roche Diagnostics) using the LightCycler 480 probes master mix. A 20-µL reaction mixture containing 2X probes master mix, forward and reverse primers (0.5 µM/L, final concentration), probes (0.2 µM/L, final concentration), template DNA (2 µL, 10^6 genome equivalents), and IAC DNA (2 µL, 10^7 GE), was added to make up a final volume of 20 µL. A reaction temperature control was performed 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 extension step of 5 s. The LightCycler 480 ramp rate was 4.4°C/s, and a 2°C/s for cooling. To prevent fluorescence signal leakage between detection channels on the LightCycler 480, a colour compensation was generated.

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; Bond, 2010). Purified H. influenzae genomic DNA (DSM4601) was diluted in nucleic-free ddH2O. Twelve replicates of each dilution 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, 10^9, 10^10, 10^11, and 10^12 were tested in triplicate. Standards 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.thermoscientific.com/webtools/gperefficiency).

29. 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 (Falala 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. False real-time PCR

H. influenzae culture-confirmed clinical isolates (n = 42) were tested in triplicate using a published real-time PCR diagnostic assay (Meyers et al., 2012).

3. Results

3.1. Diagnostic target identification

Whole genome sequence comparison of H. influenzae R2866 and 3 H. haemolyticus strains (M9902, M101, and H866) were carried out using WebACT. H. influenzae R2866 was used as a reference genome, as it was recognised as being the closest related strain to H. haemolyticus based on the KAST comparison analysis. A total of 325 unique regions comprising of both genes and intergenic nucleotide sequences were identified, which were present in H. influenzae and absent in H. haemolyticus. Each of these regions was BLAST analyzed 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 (psaS, patC, ptaA, psaB, phoB, and phoR) 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, ptaA 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 4090) was quantified, and serial dilutions were prepared based on H. influenzae genome size (1,830,137 bp) (Fleischmann et al., 1995), which equates to 1.5 fg DNA per cell (http://www.chargenomics.com/genomes.html). Both assays were highly efficient in monoplex (psaB 94.66%, psa4 94.51%). In multiplex, efficiency is comparable in the psaB assay (93.47%) and is slightly reduced in the phoB assay (88.67%). Plots of Ct versus GE were linear over a range of 1 × 10-3, 5 × 10-3, 1 × 10-3, 5 × 10-3, 1 × 10-3, 5 × 10-3, 1 × 10-3, 5 × 10-3, 1 × 10-3, 5 × 10-3 and 5 × 10-3 GE for the phoB and ptaA monoplexes and ptaA 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 ptaA assays was 6.49 and 10.49 GE, respectively.

3.4. fack real-time PCR assay

A fack 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 fack assay.

3.5. Clinical isolate evaluation

All 42 clinical isolates were screened using MALDI-TOF M5. 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 capillary typing methods were confirmed as NTH1.

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 (Godless et al., 2001; Gerber-Kushnir et al., 2012; Nakhiyani 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 (Briggs 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 GUSTAW analysis identified 6 genes, phoB, phoR, psaC, ptaB, psa4, and phoB, 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 phosphonate (phosphate) region and are involved in the transport and assimilation of inorganic phosphate (Pi) (Vershinina and Zamenhanskaia, 2002; Grepin et al., 2011).

Two of the putative diagnostic targets identified, namely, phoB and ptaA, 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 homologous gene in gram-positive bacteria) (Vershinina and Zamenhanskaia, 2002). The ptaA 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 fack, which have LODs of 10 genome copies (95% confidence) (Meyler et al., 2012) and between 5 (5.8 reactions) and 50 (8.8 reactions) genome copies (Abdel-Malik et al., 2013). We also compared our assays to a previously developed fack 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 ptaA assays are more specific for the detection of H. influenzae compared to the fack 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 fack assay. One of the culture collection strains (CCUG 58365) was also not detected using the fack 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 ptaA 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 105 GE in the phoB multiplex PCR assay, revealing that the ptaA 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.

Supplementary data to this article can be found online at [http://dx.doi.org/10.1016/j.diagmicrobio.2015.06.013].

Acknowledgements

This work was supported by Science Foundation Ireland as part of the Biomedical Diagnostics Institute Centre for Science Excellence and Technology (10/CE/1821). We also wish to thank Leonard W. Mayer
and Jennifer De lan Thomasset: for providing some of the H. hemolyticus strains used in this study.

References


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

Eoin Clancy, Helena Coughlan, Owen Higgins, Teck Wee Boo, Martin Cormican, Louise Barrett, Terry J. Smith, Kate Reddington, Thomas Barry

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. (3–40% in children, 20–50% in adults) (Brouwer et al, 2010). Haemophilus influenzae, Neisseria meningitidis and Streptococcus pneumoniae are the most predominant etiological agents of bacterial meningitis, accounting for 75–80% of cases (Wen et al, 2012; Midtlyng et al, 2012; Brouwer et al, 2010). 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 industrialized countries (Brouwer et al, 2010). However, in developing countries, the world health organization estimates vaccine coverage to be as little as 21% (http://www.who.int/immunization/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 PCV23) (Nuorti et al, 2010; Falasca et al, 2013; Bokhua 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; Delcour 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 (Brenderick 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.

http://dx.doi.org/10.1016/j.mimet.2016.04.037
0166-5315/© 2016 Elsevier BV. All rights reserved.
often taking 36 h or more to confirm a specific diagnosis (Collins et al., 2001), has limited sensitivity with slow growing, fastidious or non-culturable microorganisms (Klaucke and Schroder, 2006; 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., 2012; Collins et al., 2001; Klaucke and Schroder, 2006; 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 regimen 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 RNA dependent DNA polymerase to specifically amplify RNA targets (Crompton, 1991). A number of hybridization based end-point detection methods, such as electrochemiluminescence (van Gemert et al., 1994), enzyme-linked gel assay (Ultsch et al., 1994), and fluorescence correlation spectrometry can be used to detect the NASBA in vitro amplified RNA amplicons (Oehlenschlager et al., 1995; 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, 1998). 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 pathogens 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 diagnostic 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 DNA transcript copy number can be greater than DNA copy number in whole 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 instrument requirements which facilitates its potential application on a low cost lab on a chip (LOC) and or point of care (POC) diagnostic devices (Galliksen et al., 2004; Dinov 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, optimization of three novel duplex real-time NASBA diagnostics assays incorporating an endogenous IAC, for use in the detection of the predominant microbial organisms associated with bacterial meningitis.

2. Materials and methods

2.1. Diagnostic target identification

The novel diagnostic targets used in this study were identified bioinformatically using nucleotide sequences retrieved from a number of publicly available databases including the mTmRNA website (http://biotransform.sandia.gov/mrna/), the mRNA database (http://www.ag.arizona.edu/mirror/mtsDB/), 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: Crani Negativ 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 −80°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 manufactures instructions (Ambion, Austin, TX, USA). For human RNA, whole blood was purchased from a commercial provider (Sealabs 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). Concentration 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

Publicly available 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 sequencing primers were manually designed in accordance with general recommendations and guidelines (Robertson and Walsh-Weller, 1998; Doral, 2000) to target conserved regions within the genes. All oligonucleotide primers (Table 1) used in this study were obtained from Eurofins MWG-Operon (Bremen, Germany).

2.6. Conventional PCR and nucleic acid sequencing

Sequencing oligonucleotide primers were designed to amplify 601 bp of the hflA gene of H. influenzae, and 342 bp of the sof 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 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 N. meningitidis 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 (Sequenom, 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 retrieved from publicly 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 amplion structures were checked for secondary structures by using an RNA folding program (Mfold: http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-form23) with the folding temperature parameter adjusted to 41 °C. The molecular beacon probe for the H. influenzae specific assay was labelled with TAMRA 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-Oligonucleotides (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 as the same as outlined for species specific oligonucleotides. The IAC assay (molecular beacon probe) was labelled with Cy5 and Black Hole Quencher 2 (BH2Q2) 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 NuGEN EasyQ Basic Kit V2 (Bonneville, 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 a 10 μl reaction mixture, 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 5 min at 55 °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. 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 (6.1 M final concentration) and that the final concentration of digonucleotide primers and molecular beacon was 1 and 0.5 μM respectively.

2.10. Duplex real-time NASBA specificity and LOQ

In order to evaluate the specificity of the three real-time NASBA diagnostics assays developed, total RNA from H. influenzae and closely related H. parainfluenzae species, N. meningitidis and closely related Neisseria species, and S. pneumoniae and closely related Streptococcus species (Supplementary Table 1), at concentration of 1 × 10⁶ CFU per reaction,
were tested in duplicate in the *H. influenzae*, *N. meningitidis* and *S. pneumoniae* real-time NASBA assays respectively.

To determine the LOD of each duplex assay, purified total RNA (DSMZ 20560, NCIC 10025 and DSMZ 20560) 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 (Bremner and Dennis, 2006). 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

The *B* 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 the *srna* and *lap* RNA transcripts as molecular targets for the identification of *N. meningitidis* and *S. pneumoniae* respectively. This analysis established that the *srna* and *lap* genes contain sufficient nucleotide 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 $1 \times 10^{6}$ CE per reaction for specificity testing. Using the PARQgene method for isolation and purification of RNA from human whole blood, approximately 2.1 μg of total RNA was recovered from a 25 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 (Q5) on the LightCyder 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 TRP mRNA splice variant 1. Subsequently, duplex real-time NASBA assays incorporating the developed IAC were tested with human total RNA spiked in each reaction. For the purposes 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 53.38, 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 (Chandan 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 (Viesfeldt 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 (Caiello 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., 2011; Abdelkrim 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 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; Rohman 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 amplification structure (Lang et al., 2012) which often restricts the ability to incorporate an IAC for diagnostic confidence. Such a 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 advantage over other isothermal technologies (Askelöf and Beæsström, 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 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 mRNA transcript encoded for by the *srna* gene (Ziele, 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, it mRNA has also been established as useful target in real-time NASBA diagnostics assay (O’Grady et al., 2009). Finally, the diagnostic potential of this target was evaluated in the hep means samples for the species identification of P. palmetto. The lea gene, also present in all bacterial sequenced to date, encodes for a highly conserved translational elongation factor protein Lea and is homologous to the G2/F1 gene found in higher organisms (Qin et al., 2008). It has also been previously demonstrated to be useful in nucleic acid diagnostics (Rodríguez-Lázaro et al., 2012; Clancy et al., 2015).

An endogenous non-complementary 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. The ratiocode 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., 2009). In recent years, a number of real-time NASBA assays have been described for (Bezaire et al., 2009) and are currently available in the NASBA reagents which will reduce the possibility of false negative reporting. Furthermore, TBP has previously been described as a 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, hybridization 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 single volume of blood has previously been demonstrated to be suitable for automated on-disc total RNA extraction for infectious disease based diagnostics (Dimonfort et al., 2006). Such an on-disc RNA extraction device has 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 and/or in vitro amplification bias (Craw and Balachandran, 2012). Reduced LOD in multiple NASBA diagnostics assays compared to monoplex NASBA diagnostics assays has been reported (Zhao et al., 2008; Joens et al., 2008; Lau et al., 2010). However, in our study the LQCs achieved were comparable in both monoplex and duplex formats demonstrating the robustness of each of the real-time NASBA diagnostics assays developed.

The study presented here focuses on the development of a 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 onto low cost LOC devices for near patient testing in the future.

Acknowledgements

This work was supported by Science Foundation Ireland as part of the Biomedical Diagnostics Institute Centre for Science Excellence and Technology (FD/05/18121).

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.

References


