Journal of Microbiological Methods 127 (2016) 197–202

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

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

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ARTICLE INFO

Article history:
Received 13 March 2016
Received in revised form 15 June 2016
Accepted 16 June 2016
Available online 16 June 2016

Keywords:
Haemophilus influenzae
Neisseria meningitidis
Streptococcus pneumoniae
Real-time NASBA
IAC
Bacterial meningitis

ABSTRACT

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

1. Introduction

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

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

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http://dx.doi.org/10.1016/j.mimet.2016.06.017
0167-7012/

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

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

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

2. Materials and methods

2.1. Diagnostic target identification

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

2.2. Bacterial strains, culture media and growth conditions

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

2.3. DNA isolation and quantification

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

2.4. Total RNA isolation and quantification

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

2.5. Conventional PCR primer design

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

2.6. Conventional PCR and nucleic acid sequencing

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