Title	Comparison of established diagnostic methodologies and a novel bacterial smpB real-time PCR assay for the specific detection of haemophilus influenzae associated with respiratory tract infections
Author(s)	Reddington, Kate; Schwenk, Stefan; Tuite, Nina; Platt, Gareth; Davar, Danesh; Coughlan, Helena; Personne, Yoann; Enne, Virve I.; Zumla, Alimuddin; Barry, Thomas
Publication Date	2015-06-23
Publication Information	Kate Reddington, Stefan Schwenk, Nina Tuite, Gareth Platt, Danesh Davar, Helena Coughlan, Yoann Personne, Vanya Gant, Virve I. Enne, Alimuddin Zumla, and Thomas Barry (2015) 'Comparison of established diagnostic methodologies and a novel bacterial smpB real-time PCR assay for the specific detection of Haemophilus influenzae associated with respiratory tract infections'. Journal Of Clinical Microbiology, .
Publisher	American Society for Microbiology
Link to publisher's version	http://jcm.asm.org/content/53/9/2854.full.pdf+html
Item record	http://hdl.handle.net/10379/5651
DOI	http://dx.doi.org/10.1128/JCM.00777-15

JCM
Journals.ASM.org

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,[a] Stefan Schwenk,[b] Nina Tuite,[a] Gareth Platt,[b] Danesh Davar,[b] Helena Coughlan,[a] Yoann Personne,[b] Vanya Gant,[c] Virve I. Enne,[b] Alimuddin Zumla,[b] Thomas Barry[a]

Nucleic Acid Diagnostics Research Laboratory (NADRL), Microbiology, School of Natural Sciences, National University of Ireland, Galway, Ireland[a]; Centre for Clinical Microbiology, Division of Infection and Immunity, University College London,[b] and Department of Medical Microbiology, University College London Hospitals NHS Foundation Trust,[c] 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 *fucK* diagnostic assay. Using the novel *smpB* diagnostic assay, 100% concordance was observed with the gold standard, demonstrating a sensitivity of 100% (95% confidence interval [CI], 90.26% to 100.00%) and a specificity of 100% (95% CI, 63.06% to 100.00%) when used on clinical isolates. To demonstrate the clinical utility of the diagnostic assay presented, a panel of lower RTI samples ($n = 98$) were blindly tested with the gold standard and *smpB* diagnostic assays. The results generated were concordant for 94/98 samples tested, demonstrating a sensitivity of 90.91% (95% CI, 78.33% to 97.47%) and a specificity of 100% (95% CI, 93.40% to 100.00%) for the novel *smpB* assay when used directly on respiratory specimens.**

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

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

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

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

Received 26 March 2015 **Returned for modification** 24 April 2015
Accepted 17 June 2015

Accepted manuscript posted online 24 June 2015

Citation Reddington K, Schwenk S, Tuite N, Platt G, Davar D, Coughlan H, Personne Y, Gant V, Enne VI, Zumla A, Barry T. 2015. 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. J Clin Microbiol 53:2854–2860. doi:10.1128/JCM.00777-15.

Editor: A. J. McAdam

Address correspondence to Thomas Barry, thomas.barry@nuigalway.ie.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/JCM.00777-15.

doi:10.1128/JCM.00777-15

TABLE 1 Oligonucleotide primers and probes developed in this study

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

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

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

MATERIALS AND METHODS

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

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

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

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

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

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

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

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

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

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

TABLE 2 Clinical isolates used in this study

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

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

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

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

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

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

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

RESULTS

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

TABLE 3 Clinical respiratory tract samples

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

[a] –, negative.

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

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

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

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

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

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

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

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

DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

ACKNOWLEDGMENTS

All authors with the exception of H.C. are supported by EC Grant RiD-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.

REFERENCES

1. **Kilian M.** 1991. *Haemophilus*, p. 463–470. American Society for Microbiology, Washington, DC.
2. **Mukundan D, Ecevit Z, Patel M, Marrs CF, Gilsdorf JR.** 2007. Pharyngeal colonization dynamics of *Haemophilus influenzae* and *Haemophilus haemolyticus* in healthy adult carriers. J Clin Microbiol **45:**3207–3217. http://dx.doi.org/10.1128/JCM.00492-07.
3. **Frickmann H, Podbielski A, Essig A, Schwarz N, Zautner A.** 2014. Difficulties in species identification within the genus *Haemophilus*—a pilot study addressing a significant problem for routine diagnostics. Eur J Microbiol Immunol **4:**99–105. http://dx.doi.org/10.1556/EuJMI.4.2014.2.2.
4. **Pittman M.** 1931. Variation and type specificity in the bacterial species *Hemophilus influenzae*. J Exp Med **53:**471–492. http://dx.doi.org/10.1084/jem.53.4.471.
5. **Peltola H.** 2000. Worldwide *Haemophilus influenzae* type b disease at the beginning of the 21st century: global analysis of the disease burden 25 years after the use of the polysaccharide vaccine and a decade after the advent of conjugates. Clin Microbiol Rev **13:**302–317. http://dx.doi.org/10.1128/CMR.13.2.302-317.2000.
6. **Agrawal A, Murphy TF.** 2011. *Haemophilus influenzae* infections in the *H. influenzae* type b conjugate vaccine era. J Clin Microbiol **49:**3728–3732. http://dx.doi.org/10.1128/JCM.05476-11.
7. **Kilian M.** 1976. A taxonomic study of the genus *Haemophilus*, with the proposal of a new species. J Gen Microbiol **93:**9–62. http://dx.doi.org/10.1099/00221287-93-1-9.
8. **McCrea KW, Xie J, LaCross N, Patel M, Mukundan D, Murphy TF, Marrs CF, Gilsdorf JR.** 2008. Relationships of nontypeable *Haemophilus influenzae* strains to hemolytic and nonhemolytic *Haemophilus haemolyticus* strains. J Clin Microbiol **46:**406–416. http://dx.doi.org/10.1128/JCM.01832-07.
9. **Binks MJ, Temple B, Kirkham LA, Wiertsema SP, Dunne EM, Richmond PC, Marsh RL, Leach AJ, Smith-Vaughan HC.** 2012. Molecular surveillance of true nontypeable *Haemophilus influenzae*: an evaluation of PCR screening assays. PLoS One **7:**e34083. http://dx.doi.org/10.1371/journal.pone.0034083.
10. **Barbé G, Babolat M, Boeufgras JM, Monget D, Freney J.** 1994. Evaluation of API NH, a new 2-hour system for identification of *Neisseria* and *Haemophilus* species and *Moraxella catarrhalis* in a routine clinical laboratory. J Clin Microbiol **32:**187–189.
11. **Munson EL, Doern GV.** 2007. Comparison of three commercial test systems for biotyping *Haemophilus influenzae* and *Haemophilus parainfluenzae*. J Clin Microbiol **45:**4051–4053. http://dx.doi.org/10.1128/JCM.01663-07.
12. **Bruin JP, Kostrzewa M, van der Ende A, Badoux P, Jansen R, Boers SA, Diederen BM.** 2014. Identification of *Haemophilus influenzae* and *Haemophilus haemolyticus* by matrix-assisted laser desorption ionization-time of flight mass spectrometry. Eur J Clin Microbiol Infect Dis **33:**279–284. http://dx.doi.org/10.1007/s10096-013-1958-x.
13. **Zhu B, Xiao D, Zhang H, Zhang Y, Gao Y, Xu L, Lv J, Wang Y, Zhang J, Shao Z.** 2013. MALDI-TOF MS distinctly differentiates nontypable *Haemophilus influenzae* from *Haemophilus haemolyticus*. PLoS One **8:**e56139. http://dx.doi.org/10.1371/journal.pone.0056139.
14. **Theodore MJ, Anderson RD, Wang X, Katz LS, Vuong JT, Bell ME, Juni BA, Lowther SA, Lynfield R, MacNeil JR, Mayer LW.** 2012. Evaluation of new biomarker genes for differentiating *Haemophilus influenzae* from *Haemophilus haemolyticus*. J Clin Microbiol **50:**1422–1424. http://dx.doi.org/10.1128/JCM.06702-11.
15. **Meyler KL, Meehan M, Bennett D, Cunney R, Cafferkey M.** 2012. Development of a diagnostic real-time polymerase chain reaction assay for the detection of invasive *Haemophilus influenzae* in clinical samples. Diagn Microbiol Infect Dis **74:**356–362. http://dx.doi.org/10.1016/j.diagmicrobio.2012.08.018.
16. **Wang X, Mair R, Hatcher C, Theodore MJ, Edmond K, Wu HM, Harcourt BH, Carvalho Mda G, Pimenta F, Nymadawa P, Altantsetseg D, Kirsch M, Satola SW, Cohn A, Messonnier NE, Mayer LW.** 2011. Detection of bacterial pathogens in Mongolia meningitis surveillance with a new real-time PCR assay to detect *Haemophilus influenzae*. Int J Med Microbiol **301:**303–309. http://dx.doi.org/10.1016/j.ijmm.2010.11.004.
17. **Wroblewski D, Halse TA, Hayes J, Kohlerschmidt D, Musser KA.** 2013. Utilization of a real-time PCR approach for *Haemophilus influenzae* serotype determination as an alternative to the slide agglutination test. Mol Cell Probes **27:**86–89. http://dx.doi.org/10.1016/j.mcp.2012.11.003.
18. **Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT.** 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem **55:**611–622. http://dx.doi.org/10.1373/clinchem.2008.112797.
19. **Dorak MT.** 2006. Real-time PCR. http://www.dorak.info/genetics/realtime.html.
20. **Hoorfar J, Malorny B, Abdulmawjood A, Cook N, Wagner M, Fach P.** 2004. Practical considerations in design of internal amplification controls for diagnostic PCR assays. J Clin Microbiol **42:**1863–1868. http://dx.doi.org/10.1128/JCM.42.5.1863-1868.2004.
21. **Couturier MR, Mehinovic E, Croft AC, Fisher MA.** 2011. Identification of HACEK clinical isolates by matrix-assisted laser desorption ionization–time of flight mass spectrometry. J Clin Microbiol **49:**1104–1106. http://dx.doi.org/10.1128/JCM.01777-10.
22. **Seng P, Drancourt M, Gouriet F, La Scola B, Fournier PE, Rolain JM, Raoult D.** 2009. Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Clin Infect Dis **49:**543–551. http://dx.doi.org/10.1086/600885.
23. **EUCAST.** 2014. Breakpoint tables for interpretation of MICs and zone diameters. http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/Breakpoint_table_v_4.0.pdf.
24. **Enne VI, Cassar C, Sprigings K, Woodward MJ, Bennett PM.** 2008. A high prevalence of antimicrobial resistant *Escherichia coli* isolated from pigs and a low prevalence of antimicrobial resistant *E. coli* from cattle and sheep in Great Britain at slaughter. FEMS Microbiol Lett **278:**193–199. http://dx.doi.org/10.1111/j.1574-6968.2007.00991.x.
25. **San Millan A, Escudero JA, Catalan A, Nieto S, Farelo F, Gibert M, Moreno MA, Dominguez L, Gonzalez-Zorn B.** 2007. Beta-lactam resistance in *Haemophilus parasuis* Is mediated by plasmid pB1000 bearing blaROB-1. Antimicrob Agents Chemother **51:**2260–2264. http://dx.doi.org/10.1128/AAC.00242-07.
26. **Reddington K, O'Grady J, Dorai-Raj S, Maher M, van Soolingen D, Barry T.** 2011. Novel multiplex real-time PCR diagnostic assay for identification and differentiation of *Mycobacterium tuberculosis*, *Mycobacterium canettii*, and *Mycobacterium tuberculosis* complex strains. J Clin Microbiol **49:**651–657. http://dx.doi.org/10.1128/JCM.01426-10.
27. **Mignard S, Flandrois JP.** 2008. A seven-gene, multilocus, genus-wide approach to the phylogeny of mycobacteria using supertrees. Int J Syst Evol Microbiol **58:**1432–1441. http://dx.doi.org/10.1099/ijs.0.65658-0.
28. **O'Grady J, Sedano-Balbás S, Maher M, Smith T, Barry T.** 2008. Rapid real-time PCR detection of *Listeria monocytogenes* in enriched food samples based on the ssrA gene, a novel diagnostic target. Food Microbiol **25:**75–84. http://dx.doi.org/10.1016/j.fm.2007.07.007.
29. **Fleischmann RD, Adams MD, White O, Clayton RA, Kirkness EF, Kerlavage AR, Bult CJ, Tomb JF, Dougherty BA, Merrick JM, McKenney K, Sutton G, FitzHugh W, Fields C, Gocayne JD, Scott J, Shirley R, Liu L, Glodek A, Kelley JM, Weidman JF, Phillips CA, Spriggs T, Hedblom E, Cotton MD, Utterback TR, Hanna MC, Nguyen DT, Saudek DM, Brandon RC, Fine LD, Fritchman JL, Fuhrmann JL, Geoghagen NSM, Gnehm CL, McDonald LA, Small KV, Fraser CM, Smith HO, Venter JC.** 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science **269:**496–512. http://dx.doi.org/10.1126/science.7542800.
30. **Yun JJ, Heisler LE, Hwang II, Wilkins O, Lau SK, Hyrcza M, Jayabalasingham B, Jin J, McLaurin J, Tsao MS, Der SD.** 2006. Genomic DNA functions as a universal external standard in quantitative real-time PCR. Nucleic Acids Res **34:**e85. http://dx.doi.org/10.1093/nar/gkl400.
31. **World Health Organization.** 2014. The top 10 causes of death. World Health Organization, Geneva, Switzerland. http://www.who.int/mediacentre/factsheets/fs310/en/.
32. **Saito A, Kohno S, Matsushima T, Watanabe A, Oizumi K, Yamaguchi K, Oda H.** 2006. Prospective multicenter study of the causative organisms of community-acquired pneumonia in adults in Japan. J Infect Chemother **12:**63–69. http://dx.doi.org/10.1007/s10156-005-0425-8.
33. **Johansson N, Kalin M, Tiveljung-Lindell A, Giske CG, Hedlund J.** 2010. Etiology of community-acquired pneumonia: increased microbiological yield with new diagnostic methods. Clin Infect Dis **50:**202–209. http://dx.doi.org/10.1086/648678.
34. **Maaroufi Y, De Bruyne JM, Heymans C, Crokaert F.** 2007. Real-time

PCR for determining capsular serotypes of *Haemophilus influenzae*. J Clin Microbiol **45:**2305–2308. http://dx.doi.org/10.1128/JCM.00102-07.

35. **Ridderberg W, Fenger MG, Nørskov-Lauritsen N.** 2010. *Haemophilus influenzae* may be untypable by the multilocus sequence typing scheme due to a complete deletion of the fucose operon. J Med Microbiol **59:**740–742. http://dx.doi.org/10.1099/jmm.0.018424-0.
36. **Qin Y, Polacek N, Vesper O, Staub E, Einfeldt E, Wilson DN, Nierhaus KH.** 2006. The highly conserved LepA is a ribosomal elongation factor that back-translocates the ribosome. Cell **127:**721–733. http://dx.doi.org/10.1016/j.cell.2006.09.037.
37. **Schonhuber W, Le Bourhis G, Tremblay J, Amann R, Kulakauskas S.** 2001. Utilization of tmRNA sequences for bacterial identification. BMC Microbiol **1:**20. http://dx.doi.org/10.1186/1471-2180-1-20.
38. **Karzai AW, Roche ED, Sauer RT.** 2000. The SsrA-SmpB system for protein tagging, directed degradation and ribosome rescue. Nat Struct Mol Biol **7:**449–455. http://dx.doi.org/10.1038/75843.
39. **Karzai AW, Susskind MM, Sauer RT.** 1999. SmpB, a unique RNA-binding protein essential for the peptide-tagging activity of SsrA (tmRNA). EMBO J **18:**3793–3799. http://dx.doi.org/10.1093/emboj/18.13.3793.
40. **Dulebohn DP, Cho HJ, Karzai AW.** 2006. Role of conserved surface amino acids in binding of SmpB protein to SsrA RNA. J Biol Chem **281:**28536–28545. http://dx.doi.org/10.1074/jbc.M605137200.
41. **Gil R, Silva FJ, Pereto J, Moya A.** 2004. Determination of the core of a minimal bacterial gene set. Microbiol Mol Biol Rev **68:**518–537. http://dx.doi.org/10.1128/MMBR.68.3.518-537.2004.
42. **Puig C, Calatayud L, Martí S, Tubau F, Garcia-Vidal C, Carratalà J, Liñares J, Ardanuy C.** 2013. Molecular epidemiology of nontypeable *Haemophilus influenzae* causing community-acquired pneumonia in adults. PLoS One **8:**e82515. http://dx.doi.org/10.1371/journal.pone.0082515.
43. **Morrissey I, Maher K, Williams L, Shackcloth J, Felmingham D, Reynolds R.** 2008. Non-susceptibility trends among *Haemophilus influenzae* and *Moraxella catarrhalis* from community-acquired respiratory tract infections in the UK and Ireland, 1999-2007. J Antimicrob Chemother **62**(Suppl)**:**97–103.
44. **Blackburn RM, Henderson KL, Lillie M, Sheridan E, George RC, Deas AH, Johnson AP.** 2011. Empirical treatment of influenza-associated pneumonia in primary care: a descriptive study of the antimicrobial susceptibility of lower respiratory tract bacteria (England, Wales and Northern Ireland, January 2007-March 2010). Thorax **66:**389–395. http://dx.doi.org/10.1136/thx.2010.134643.