

# Real-time PCR for the diagnosis of invasive *Haemophilus influenzae* bacteraemia and meningitis in children: A diagnostic accuracy study of over 2,000 samples

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## Abstract

**Background:** To compare *Haemophilus influenzae* PCR in blood and cerebrospinal fluid (CSF) to culture results in a paediatric patient cohort and to review discordant results.

**Methods:** A retrospective audit of all blood and CSF samples from a tertiary referral paediatric hospital sent to the Irish Meningitis and Sepsis Reference Laboratory over a four-year period. All PCR tests performed for *H. influenzae* which had a contemporaneous culture performed were included in the analysis.

**Results:** For the blood PCR test, there were 10 positive samples out of 1,367 samples tested. The sensitivity was shown to be 60% (95% CI 14.6–94.73%) and the specificity was 99% (95% CI 98–99.7%). For the CSF assay, the sensitivity was 100% (95% CI 15.8–100%) with a specificity of 99% (95% CI 99.2–99.9%) and there were 5 positive samples by PCR out of the 1,224 samples tested. Ten patients had positive PCR results, with a negative corresponding culture (blood=7, CSF=3). Three of the 10 cases were deemed to be primary *H. influenzae* infections, while seven were deemed to be likely co-infection (respiratory syncytial virus=2, Influenza=2, measles=1, rotavirus=1, *Staphylococcus aureus* pneumonia=1).

**Conclusions:** The incidence of invasive *H. influenzae* disease was low in this population. The sensitivity and specificity of the assay in CSF was excellent, but the sensitivity of the assay in blood was lower at 60%. Most patients with discordant PCR/culture results had viral co-infections. A more rational approach to requesting PCR is required in paediatric patients.

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## Introduction

*Haemophilus influenzae* can cause invasive infections in children such as meningitis and bacteraemia [1, 2]. With the introduction of the *H. influenzae* type b (Hib) vaccine in many countries, there was a decline in the incidence of disease due

to Hib, but some of this disease burden has now been replaced by non-typeable strains [3, 4]. There is increasing interest in developing vaccines for non-typeable *H. influenzae* disease, which could further help to reduce the incidence of invasive disease [5, 6]. Diagnosis of invasive *H. influenzae* bacteraemia and meningitis has predominantly

relied upon traditional culture methods, but this has limitations particularly in patients who have received antimicrobials before sampling occurred. PCR-based methods, including as part of multiplex panels such as the FilmArray (BioMerieux, France), have shown good performance in detecting additional cases that were culture negative. These studies, however, were not focused exclusively on *H. influenzae*, and the incidence of disease was low in the test populations [7-9].

In this retrospective study of patients from a single paediatric centre we aimed to compare a previously described in-house PCR method for *H. influenzae* detection to traditional culture in both blood and cerebrospinal (CSF) samples [10]. The PCR method was the fuculokinase (*fucK*) real-time assay provided by the Irish Meningitis and Sepsis Reference Laboratory for *H. influenzae* detection. The objectives were to look at the sensitivity and specificity of the PCR assay, compared to culture, and to then review the clinical characteristics of patients who were PCR positive but culture negative. Our hypothesis was that with the introduction of the Hib vaccine, the incidence of disease has fallen to a level that no longer justifies routine testing of bacterial PCR samples for *H. influenzae*. The purpose of this clinical review was to determine if patients had likely invasive disease, or if the PCR detection was most likely a false positive result.

## Methods

In this retrospective study, *H. influenzae* Real-Time PCR (Hi-PCR) results were compared to blood culture and CSF culture results for all consecutive patients that met the inclusion criteria. Patients were included if they had a PCR test performed on blood or CSF for *H. influenzae* between 1/3/2011 and 19/8/2015, were aged less than 16 years of age at the time of sampling in Temple Street Hospital. All PCR assays were run in the Irish Meningococcal and Sepsis Reference Laboratory (IMSRL, formerly known as the Irish Meningococcal and Meningitis Reference Laboratory (IMMRL)) and all cultures were investigated in the Clinical Microbiology Laboratory, Children's University Hospital, Temple Street. The Laboratory Information System was

interrogated to identify all PCR samples that were tested for *H. influenzae* during the study period. The computer search of the IMSRL records to identify potentially eligible samples occurred on 27/08/2015 in the IMSRL. The computer search of the Microbiology records occurred on 26/09/2015, in Microbiology, CUH-TS.

The RT-PCR test (index test) used was a Taqman based real-time assay targeting the fuculokinase (*fucK*) housekeeping gene for the detection of *Haemophilus influenzae*. The *fucK* assay, detected typeable and non-typeable *H. influenzae* and has been previously described by Meyler et al. from the same laboratory [10]. The assay did not have false positive results when tested against *Haemophilus spp.* strains but it was not tested against viral DNA/RNA for cross-reactivity. The real-time PCR reaction of *H. influenzae* DNA in both blood and CSF samples was performed on the AB7500 Fast sequence detection System (ThermoFisher, USA) using Applied Biosystems Advance Fast PCR Mastermix. The PCR run had 45 cycles with no cut-off value. A negative template control (NTC) was used in each run, and the run was rejected if the NTC was positive. The reference standard was culture of blood cultures and CSF samples. For the RT-PCR test all samples were extracted in duplicate and a PCR positive result is where *fucK* gene is detected in both extracts on the Hi-PCR assay and the graphs are sigmoidal. A negative PCR result was where *fucK* is not detected on the Hi-PCR assay. A weak PCR positive was defined as *fucK* is detected in both extracts at late cycle threshold values (e.g. >38), but with sigmoidal graphs present. This "weak positive" term was used for such results to identify to clinicians that the result should be interpreted with caution and in line with the clinical situation. An inconclusive result was a sample that only had DNA detected in one of two duplicates and the test could not be repeated due to the small sample volume.

The microbiology laboratory used the BacT/ALERT 3D blood culture system (BioMerieux, France) for the investigation of blood stream infections. Blood cultures are routinely incubated for a maximum of 5 days. When bacterial growth was detected an aliquot is then cultured onto blood, chocolate and MacConkey agars, incubated and identification is

performed using the Vitek-2 (BioMerieux, France). Culture was considered to be the gold standard for the isolation and identification of bacteria, and was thus chosen as the reference standard. Scientists from both laboratories would have been aware of both the PCR and culture results at the time and thus were not blinded. Clinical teams were made aware of results in real-time and the results were used as part of the patients' management.

The diagnostic accuracy, in terms of sensitivity and specificity, was calculated using MedCalc statistical software (version 16.0) ([www.medcalc.org](http://www.medcalc.org), last accessed 8/12/2016). Weak positive samples were considered to be positive while inconclusive results were excluded from the final diagnostic accuracy calculations. Patients that did not have both a culture and PCR result were not included in the study. The sample size was chosen to include all tests from when the test was routinely introduced until there was a change in the requesting criteria for *H. influenzae* PCR. For patients that were PCR positive, but culture negative, their medical records were reviewed by a Consultant Microbiologist to help identify if they were genuine clinical cases or not. Patients were considered to be primary *H. influenzae* infections if they did not have another possible cause identified for their acute illness. For children who had another probable cause for their acute illness, the case was categorised as having likely co-infection but it could also represent a false-positive result. Post-mortem samples were not included as there may have been a delay in taking the samples and the clinical relevance in relation to the cause of death would be uncertain. As this was a retrospective audit, no additional testing was performed. The study was approved by the Ethics Committee of Temple Street Children's University Hospital (Ref-15-058). The paper was written in line with the updated STARD checklist [11].

## Results

In total 1,366 blood samples and 1,223 CSF samples were received from 1,692 individual patients aged less than 16 years of age for RT-PCR for *H. influenzae*. The median age of the 1,692 patients was 2.83 months (Interquartile range 1.4–16.9 months) and 58% were male (n=982/1697). The

inclusion and exclusion of patients is shown in Figures 1 and 2. The number of positive PCR tests were low with only 10 positive blood samples (incidence 0.8%, n=10) and five positive CSF samples (incidence 0.4%, n=5). The number of positive cultures for *H. influenzae* was also low (blood=5, CSF=2). For the blood PCR test the sensitivity was shown to be 60% (95% Confidence interval 14.6-94.7%) and the specificity was 99% (95% CI 98.7-99.7%), while for the CSF assay the sensitivity was 100% (95% CI 15.8-100%) with a specificity if 99% (95% CI 99.2-99.5%) (Table 1) when bacterial culture was considered to be the gold standard comparator.

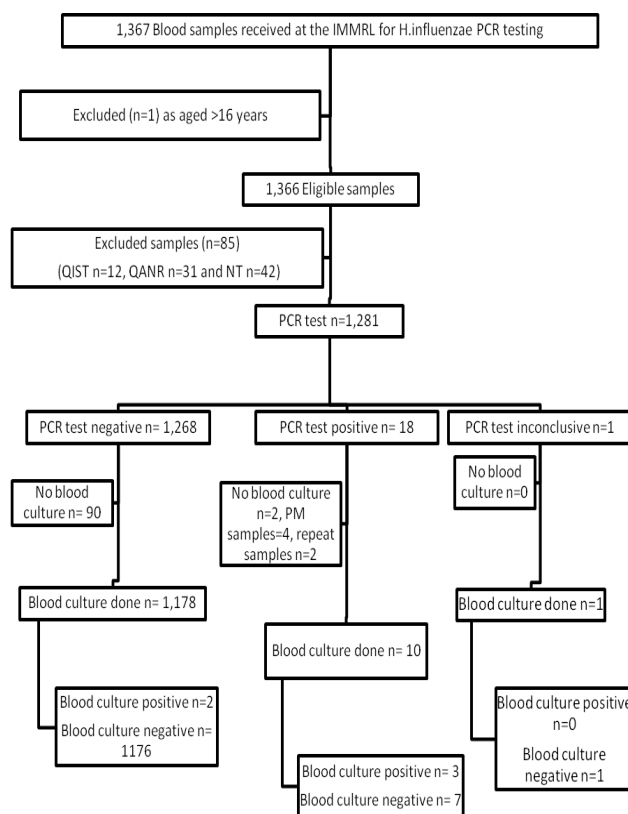
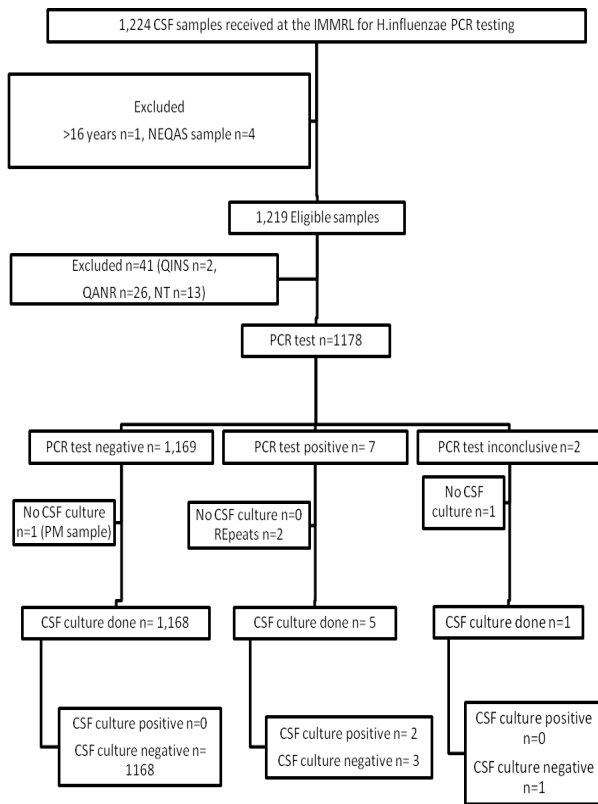


Figure 1. Flow chart for blood samples

This shows the flow of blood samples in the study in line with the STARD criteria for reporting diagnostic accuracy studies.

Abbreviations: CUR = Children's University Hospital, IMMRL = Irish Meningococcal and Meningitis Reference Laboratory, NT= Not tested, PCR= Polymerase chain reaction test, PM= Postmortem , QANR=Analysis not required, QIST= Incorrect specimen type.



**Figure 2.** Flow chart for CSF samples

This shows the flow of cerebrospinal fluid samples in the study in line with the STARD criteria for reporting diagnostic accuracy studies.

Abbreviations: CSF= Cerebrospinal fluid, CUR = Children’s University Hospital, IMMRL = Irish Meningococcal and Meningitis Reference Laboratory, NEQAS= External sample tested for quality assurance, NT= Not tested, PM= Post mortem sample QANR= Analysis not required, QINS= Insufficient sample.

For patients that were PCR positive, but culture negative, their medical records were reviewed and their culture results are shown in table 2. Seven patients had positive blood PCR but negative blood cultures, while three different patients had positive CSF PCR but negative CSF culture. Eight of the 10 patients were male and the median age was 0.68 years, with a range of 0.09-15.9 years. Three of the 10 cases were deemed to be primary *H. influenzae* infections, while seven were deemed to be likely co-infection (RSV=2, Influenza=2, measles=1, rotavirus=1, *Staphylococcus aureus* pneumonia=1).

**Table 1.** Estimate of diagnostic accuracy of blood and CSF PCR for H.influenzae against culture. AUC = , CSF = Cerebrospinal fluid. CI = confidence interval, LR = , NPV = , PPV = .

	PCR Blood		PCR Cerebrospinal fluid	
Blood/CSF	Positive	Negative	Positive	Negative
Culture Positive	3	2	2	0
Culture Negative	7	1176	3	1168
Sensitivity % (95% CI)	60 (14.66–94.73)		100 (15.81–100)	
Specificity % (95% CI)	99.41 (98.78–99.76)		99.74 (99.25–99.95)	
Positive LR (95% CI)	101.4 (36.26–283.60)		390.33 (126.07–1208.53)	
Negative LR (95% CI)	0.40 (0.14–1.18)		0.00	
AUC (95% CI)	0.8 (0.77–0.82)		1.00 (0.99–1.00)	
PPV (95% CI)	30 (6.67–62.25)		40 (5.27–85.34)	
NPV (95% CI)	99.83 (99.39–99.98)		100 (99.68–100)	

## Discussion

This study has shown that the sensitivity of the *H. influenzae* PCR assay is 60% in blood and 100% in CSF, although the specificity is better at 99% in both blood and CSF. However, the incidence of disease remains very low over the study period, with less than 1% of PCR tests being positive which is similar to reports from other countries [12, 13]. Among the 10 PCR positive/culture negative cases, seven were likely due to co-infection raising an issue that when *H. influenzae* is detected by PCR, it may not always be the primary pathogen.

**Table 2.** Clinical details of PCR/culture discordant cases

ID	M/F	Age (yrs)	Abx pre-admission	Abx pre-BC	Abx Pre-LP	CRP (mg/L)	Blood culture	Blood PCR	CSF culture	CSF PCR	CSF WCC (/cm3)	CSF RCC (/cm3)	co-infection	Duration IV antibiotics	Complication	Survived	Interpretation
Blood samples (PCR positive but culture negative)																	
tsh-513-k	f	0.39	n	n	y	17	neg	pos	neg	neg	0	0	none	21	yes (ventilated)	y	Primary <i>H. influenzae</i> infection
tsh-160-m	m	2.39	y	y	y	9	neg	pos	neg	neg	19	0	measles	5	no	y	likely mixed infection/false positive
tsh-322-d	m	0.98	n	y	y	187	neg	pos	not done	not done	not done	not done	PVL-toxin pos MSSA pneumonia	unknown	yes (hypoxic brain damage)	y	likely mixed infection/false positive
tsh-691-m	m	2.38	n	n	y	45	neg	pos	neg	neg	2	1	Influenza A	7	no	y	likely mixed infection/false positive
tsh-901-k	m	1.56	n	n	y	10	neg	pos	not done	not done	not done	not done	none	2	no	y	Primary <i>H. influenzae</i> infection
tsh-805-c	m	0.09	n	n	y	239	neg	pos	not done	not done	n/a	n/a	RSV	10	no	y	likely mixed infection/false positive
tsh-072-d	m	0.10	n	n	y	nd	neg	pos	neg	neg	16	9200	RSV	10	no	y	likely mixed infection/false positive
CSF samples (PCR positive but culture negative)																	
tsh-432-L	m	0.16	n	n	n	15	neg	nd	neg	pos	2	0	rotavirus	3	no	y	likely mixed infection/false positive
tsh-245-g	m	0.36	n	n	n	4	neg	neg	neg	pos	4	0	Influenza A	3	no	y	likely mixed infection/false positive
tsh-338-c	f	15.9	y	y	y	77	neg	neg	neg	pos	562	22	none	10	no	y	Primary <i>H. influenzae</i> infection

Abx = , BC = , CRP= C-reactive protein, CSF= Cerebrospinal fluid, f=female, ID= Study identification number, IV= intravenous, LP = , m=male, MSSA= methicillin susceptible *S. aureus*, n=no, n/a= not applicable, nd= not done, PCR= polymerase chain reaction. pos= positive, PVL= Panton-Valentine Leucocidin toxin, RCC = , RSV=respiratory syncytial virus, WCC = , y=yes.

The sensitivity and specificity of the CSF PCR assay is comparable to results of a previous study on children from Bangladesh with invasive Hib disease, but the sensitivity of the blood PCR assay in this project was lower (60% vs 93%) [14]. Other projects looking at *H. influenzae* PCR testing have been limited by small numbers and it is difficult to accurately calculate sensitivity and specificity [8, 9]. Performing PCR on dried blood spots has been shown in one study to improve the yield of positive test results but this would need further evaluation in areas with a low incidence of invasive *H. influenzae* disease [15].

The two main limitations of this study were that it was single centre, and also that the incidence of disease was low. Since the introduction of the Hib vaccine, the rate of disease has fallen substantially without being completely replaced by non-typeable infections. A bias in the study was that it was retrospective and that the categorisation of PCR positive/culture negative cases was done retrospectively using medical records and laboratory results. It was difficult to determine if the viral infection that was also present was the main cause of the patient's illness or was it a precursor to the main invasive bacterial infection with *H. influenzae*. It was not possible in a retrospective audit to determine the clinical significance of these results. This diagnostic accuracy project could be extrapolated to other paediatric centres with a similar incidence of invasive disease, but differences may be present if other targets are used for the PCR assay.

With the low incidence of invasive *H. influenzae* disease due to the Hib vaccine it is necessary to re-evaluate when PCR tests should be requested as the cost of testing all the samples received for *H. influenzae* infection cannot be justified. This is especially important in the presence of concomitant viral infections such as RSV and influenza. Currently at the IMSRL, *H. influenzae* PCR is only performed on CSF samples that have a high CSF white cell count for age to limit the number of tests and increase the pre-test probability. For blood samples, these are now only performed on children who have been admitted to the paediatric intensive care unit (PICU) with an illness consistent with

invasive *H. influenzae* infection in order to again increase the pre-test probability. In this cohort, two of the three children that were PCR positive/culture negative with primary *Haemophilus influenzae* infection would have been identified (one patient was ventilated, and the other had a high CSF white cell count). This would have meant that only 1 positive sample from over 2,500 samples would have been missed if PCR tests were not routine. By selecting children only from the PICU, it is more likely that the detection of *H. influenzae* by PCR reflects actual bacterial infection, compared to performing it on children in general medical wards where it may be a false positive or reflect a transient bacteraemia. Further studies will be needed to re-evaluate this rationalisation in testing and ensure that non-typeable strains are still detected by the PCR assay in the future.

### Ethical approval

This study was approved by the Ethics Committee of the Children's University Hospital, Temple Street (Approval 15.058).

### Author contribution

All authors contributed to the study design. HOB, MN and RD performed the data analysis. All authors were involved in the drafting and revision of the article, and subsequently approval of the final version for submission.

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