



TECHNICAL REPORT

External quality assessment scheme for *Haemophilus influenzae*

2014

ECDC TECHNICAL REPORT

External quality assessment scheme for *Haemophilus influenzae* 2014

As part of the IBD-labnet laboratory surveillance network



This report was commissioned by the European Centre of Disease Prevention and Control (ECDC), coordinated by Dr Assimoula Economopoulou and produced by Professor Mary Slack (Institut für Hygiene und Mikrobiologie, Universität Würzburg, Germany, and School of Medicine, Griffith University, Queensland, Australia) and Dr David Litt (Public Health England, UK) on behalf of the IBD-labnet consortium participants (referring to Specific Contract No.4ECD.4857

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Abbreviations

AST	Antimicrobial susceptibility testing
BLNAR	Beta-lactamase-negative ampicillin-resistant
BLPACR	Beta-lactamase-positive amoxicillin-clavulanate-resistant
BLPAR	Beta-lactamase-positive ampicillin-resistant
Cfu	Colony-forming units
CLSI	Clinical and Laboratory Standards Institute
CSF	Cerebrospinal fluid
EEA	European Economic Area
EQA	External quality assessment
EUCAST	European Committee on Antimicrobial Susceptibility Testing
Hib	<i>Haemophilus influenzae</i> serotype b
Hie	<i>Haemophilus influenzae</i> serotype e
I	Intermediate
MIC	Minimum inhibitory concentration
MLST	Multilocus sequence typing
NTHi	Non-typeable (non-capsulated) <i>Haemophilus influenzae</i>
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
PHE	Public Health England
R	Resistant
S	Susceptible

Executive summary

Haemophilus influenzae is a common cause of respiratory tract infections. Most strains of *H. influenzae* are opportunistic pathogens and rarely cause invasive disease unless other factors concur (e.g. viral infections, immunological deficiencies). Despite the effective prevention of infections with invasive *H. influenzae* serotype b (Hib) by the use of conjugated Hib vaccine, infections caused by other capsulated serotypes and non-capsulated strains still occur and are associated with significant morbidity and mortality [1,2]. Surveillance of *H. influenzae* continues to be important, not only to establish the types of *H. influenzae* that cause invasive disease but also to monitor the long-term effectiveness of the Hib immunisation programme. Integrated surveillance for this pathogen entails both epidemiological and laboratory surveillance.

ECDC promotes the performance of external quality assessment (EQA) schemes, in which laboratories are sent simulated clinical specimens or bacterial isolates for testing by routine or reference laboratory methods. EQA schemes, or laboratory proficiency testing, provide information about the accuracy of different characterisation and typing methods as well as antimicrobial susceptibility testing (AST) and the sensitivity of the methods in place to detect a certain pathogen or novel resistance patterns.

In July 2014, a collection of two strains of *Haemophilus* spp. (one non-capsulated *H. influenzae* (NTHi) and one *H. influenzae* serotype b (Hib)) and two simulated samples of cerebrospinal fluid (CSF) (one containing *H. influenzae* serotype b (Hib), one containing *H. influenzae* serotype e (Hie)) was sent to 30 participating reference laboratories in the IBD-labnet surveillance network for quality assessment testing. The laboratories were asked to characterise the viable strains by performing standard laboratory protocols for the methods usually used by the laboratory for species identification, biotyping and serotyping by serological methods and/or PCR. AST and beta-lactamase testing was also requested for those laboratories that perform AST of the isolates on a routine basis. Laboratories were asked to use non-culture methods to attempt detection of *H. influenzae* in the simulated CSF samples.

The results showed that 28 of 30 European *Haemophilus* reference laboratories (93%) routinely serotype the isolates, compared with 26 out of 28 laboratories (93%) in 2012. Twenty-four laboratories (80%) perform PCR-based capsular genotyping, compared with 19 (68%) in 2012. Twenty-seven laboratories (93%) routinely perform AST, compared with 24 (86%) in 2012. The number of laboratories following EUCAST methodological guidelines has increased from 14 out of 27 in 2012 (52%) to 19 out of 27ⁱ in 2014 (70%).

Both strains of *H. influenzae* were correctly identified by all participants. The EQA scheme identified a single error (1/55 results; 1.8%) with slide agglutination for the serotyping, a noticeable improvement from 2012, when there were 10/100 (10%) errors in phenotypic serotyping. There were a few errors or ambiguities in the genotypic capsule typing of the NTHi strain, similar to the results in 2012.

The results of the AST showed that 29 out of 30 (97%) reference laboratories routinely test for beta-lactamase production in strains of *H. influenzae*, and 27 (90%) performed AST against at least one antibiotic in the EQA panel. The AST results were generally excellent for the beta-lactamase-positive ampicillin-resistant (BLPAR) strain. However, the characterisation of the beta-lactamase-negative ampicillin-resistant (BLNAR) strain proved more challenging for several reasons. Low BLNAR strains can have an ampicillin MIC (minimum inhibitory concentration) at or around the breakpoint for this agent and disc diffusion tests or even MIC determinations may fail to identify such strains. The only definitive way of identifying such strains is by partial sequencing of the *ftsI* gene, which is not routinely undertaken by the majority of reference laboratories.

Of those that supplied data, 19 laboratories are using the EUCAST criteria whilst six are still using CLSI guidelines. This makes the comparison of results difficult. It is recommended that all national reference laboratories in the EU/EEA move to using EUCAST guidelines as soon as possible.

Two simulated CSF samples were included in the quality assessment panel to assess methods used for the non-culture detection of *H. influenzae*. The results from 21 laboratories were generally very good, although real-time PCRs against the *ompP2* target had difficulty identifying one of the samples. Eight laboratories attempted further characterisation of the samples with mixed results. Due to limited availability of methods data, it was not possible to evaluate whether participants were reporting results appropriate to the gene targets they were using for their PCRs in all cases.

Overall, the results of this EQA exercise demonstrate an improvement over the previous EQA exercise in 2012. The number of laboratories that routinely serotype isolates has increased by two, and an additional five laboratories now routinely genotype isolates. Three more laboratories routinely perform AST, and the number of laboratories adopting EUCAST guidelines has increased by five. Phenotypic identification and characterisation results were generally very good. The only aspect of the EQA exercise that proved problematic was the identification of the low BLNAR isolate.

ⁱ Two further laboratories stated that they follow EUCAST guidelines but did not submit any AST results in this EQA exercise.

Introduction

The European Centre for Disease Prevention and Control (ECDC) is an EU agency with a mandate to operate dedicated surveillance networks and to identify, assess, and communicate current and emerging threats to human health from communicable diseases. Within its mission, ECDC shall 'foster the development of sufficient capacity within the Community for the diagnosis, detection, identification and characterisation of infectious agents which may threaten public health. The Centre shall maintain and extend such cooperation and support the implementation of quality assessment schemes.' (Article 5.3, Regulation (EC) No 851/2004)ⁱ.

External quality assessment (EQA) is part of a quality management system. Run by an outside agency, it evaluates the performance of laboratories on material that is supplied specifically for the purpose. ECDC's disease-specific networks organise a series of EQA for EU/EEA countries. In some specific networks, non-EU/EEA countries are also involved in the EQA activities organised by ECDC, although at their own cost. The aim of the EQA is to identify needs for improvement in laboratory diagnostic capacities relevant to surveillance of the diseases listed in Decision No 2119/98/ECⁱⁱ and to ensure comparability of results in reference laboratories from all EU/EEA countries. The main purposes of EQA schemes for a given microorganism include:

- The assessment of the general standard of performance.
- The assessment of the effects of analytical procedures (methodological principles, instruments, reagents, calibration).
- The evaluation of individual laboratory performance.
- The identification and description/delineation of problem areas.
- The provision of continuing education.
- The identification of needs for training activities.

Haemophilus influenzae is a common cause of invasive disease in children worldwide. Pneumonia and meningitis are the most frequent manifestations. However, it can also be responsible for epiglottitis, infections of the soft tissues, bones, joints and other body sites. Invasive bacterial diseases are an important cause of morbidity and mortality in neonates and children worldwide. Highly safe and effective protein-polysaccharide conjugate Hib vaccines have been available for almost 20 years and have completely changed the epidemiology of invasive *H. influenzae* infections [1]. Completeness and accuracy become key objectives of surveillance when vaccines are introduced and the incidence of invasive Hib infections approaches low levels, and invasive infections due to NTHi and other capsulated serotypes assume greater importance [2-7].

The specific objectives of this EQA exercise are:

- Further harmonisation of molecular typing of *H. influenzae*.
- Further harmonisation of methods for antimicrobial susceptibility testing of *H. influenzae*.
- Training and dissemination of methods for the laboratory surveillance of invasive bacterial infections.
- Assisting the countries in capacity building, when required.
- Increasing the comparability of microbiological data with respect to *H. influenzae* and thus supporting the surveillance of invasive disease due to *H. influenzae* in the EU.

ⁱ Regulation (EC) no 851/2004 of the European Parliament and of the Council of 21 April 2004 establishing a European centre for disease prevention and control. OJ L 142, 30.4.2004, p. 1.

ⁱⁱ Decision No 2119/98/EC of the European Parliament and of the Council of 24 September 1998 setting up a network for the epidemiological surveillance and control of communicable diseases in the Community. OJ L 268, 3.10.1998, p. 1-7.

1 Materials and methods

The objectives of this exercise were:

- To design an EQA scheme utilising a small panel of material containing viable *H. influenzae* isolates and non-viable simulated clinical samples for phenotypic and genotypic characterisation (where possible) for distribution to all EU Member States and candidate countries with suitable reference facilities.
- To improve the quality of data, assisting in the standardisation of techniques and thereby facilitating consistent epidemiological data for submission to ECDC's surveillance database, the European Surveillance System (TESSy).

1.1 Study design

The design of the project allowed individual reference laboratories to test the material using their routinely available techniques in order to complete some or all of the requested tasks (Table 1) in the allocated time period. The participants were not advised that some tests were considered to be essential and others were included for training purposes or for dissemination of methods. They were merely judged on the tests for which they chose to submit a result. However, phenotypic identification, phenotypic serotyping, genotypic (PCR-based) capsule typing and AST (including beta-lactamase testing) of the bacteria are generally accepted as an essential requirement of a reference laboratory (even if susceptibility testing is outsourced to another laboratory). The ability to genetically confirm the species of isolates and the ability to detect *H. influenzae* by PCR in clinical samples could be seen as desirable, but not essential.

Table 1. Tests requested from the participating laboratories

Procedure	Tests requested	
	Bacterial isolates	Non-culture samples (simulated CSF)
Phenotypic identification	Species	
	Serotype	
	Biotype	
	Antimicrobial susceptibility testing	
	Beta-lactamase production	
Genotypic identification	Species	Detection of <i>H. influenzae</i>
	Capsule type	

An anonymised summary was produced showing the submitted results, the consensus by interpretation and the number of laboratories with each submitted result.

The United Kingdom National External Quality Assessment Service (UK NEQAS) for microbiology undertakes several international EQA schemes for other organisms that also require freeze-drying, distribution, results analysis and web-based reporting. The samples for the EQA scheme were selected by Public Health England (PHE) with the agreement of the University of Würzburg, as coordinator of the IBD-labnet project.

The characterisations (test results) requested of the participating laboratories are shown in Table 1.

1.2 Participants

Thirty European meningococcal reference laboratories participated in the 2014 IBD-labnet EQA distribution.

The participant countries were: Austria, Belgium, Bulgaria, Croatia, Cyprus, the Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, the Netherlands, Norway, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden and the UK.

All participants were contacted prior to the EQA distribution to confirm the address and contact details for despatch of the potentially hazardous material. It was envisaged that the reference laboratories would wish to store the viable cultures and retain any unused material for their own quality processes. It was hoped that the distribution of the well-characterised material would become a resource within and between the reference laboratories.

Participants were strongly encouraged to report their results via the internet into a specially designed web-based report form on the UK NEQAS website (www.ukneqasmicro.org.uk). Each laboratory was given a unique username and password for secure reporting of their results.

1.3 The EQA panel material

The EQA panel comprised two viable bacterial isolates to test participating laboratories' abilities to identify and characterise live cultures, plus two non-viable simulated CSF samples to test their ability to detect *H. influenzae* in clinical specimens using non-culture detection methods.

Bacterial isolates

Two viable isolates of *H. influenzae* were selected for the panel. These were selected to be representative of the major disease-causing serotypes (type b (Hib), and non-capsulated *H. influenzae* (NTHi)), to include a strain demonstrating beta-lactamase production and a strain demonstrating beta-lactamase-negative ampicillin resistance (BLNAR), and to demonstrate a range of MICs to other commonly used antimicrobials. Further details on each strain are included in the results section.

The isolates were selected and pre-screened by staff at the Public Health England Respiratory and Vaccine Preventable Bacteria Reference Unit (RVPBRU) and Antibiotic Resistance Monitoring and Hospital Acquired Infection Reference Laboratory (AMRHAI). The isolates were also sent to the EUCAST Laboratory for Antimicrobial Susceptibility Testing for additional antimicrobial sensitivity testing. They were then grown-up, aliquoted, freeze-dried and distributed at ambient temperature by UK NEQAS for microbiology. The samples were accompanied by instructions for their revival.

Non-culture simulated meningitis samples

The two simulated CSF (non-culture) samples for PCR were prepared from heat-killed suspensions of isolates obtained from the PHE collection of clinical isolates. One sample contained *H. influenzae* type b (Hib) DNA. The other contained *H. influenzae* type e (Hie) DNA.

Suspensions of live bacterial cultures were prepared in phosphate-buffered saline. Viable counts were performed and the cultures were killed by heating to 100°C for 10 minutes. They were then diluted to a concentration equivalent to 100 cfu/μl in simulated CSF solution. The simulated CSF contained 6% sucrose and 1.1% bovine serum albumin. These simulated CSF samples were also distributed by UK NEQAS for microbiology at ambient temperature, with instructions to handle them in the same way as clinical specimens.

2 Results

The strains were processed as requested and the results were returned to NEQAS by 30 laboratories.

A summary of consensus results was released to participants via the UK NEQAS for microbiology website in August 2014. A semi-automated analysis of results from all participants was subsequently generated and released to all participants via the same website in September 2014. Each participant received a customised report containing an analysis of their own results plus a summary of the overall results from all participants. The participation of each laboratory in the various parts of the EQA procedure is shown in Table 2. It must be noted that each laboratory did not necessarily submit a result for all samples for a given test. Hence, the total participants for a given test vary by sample (see Table 4).

2.1 Part 1. Characterisation of viable isolates

The intended results for Part 1 of the analysis are shown in Table 3.

All participants confirmed that the two bacterial isolates were viable following the revival procedure. Not all methods (tests) were performed on the isolates by all laboratories. A summary of the number of laboratories reporting results for each sample, by method, is shown in Table 4. This table shows the ratio of laboratories that successfully reported the intended result for each test. It also lists the results that did not match the intended result. The percentage match varied between 86% and 100%. A detailed description of the results broken down by test is given below.

Table 2. Summary of tests for which each laboratory submitted results

Laboratory identifier	Viable isolates							Non-culture detection
	Phenotypic identification					Genotypic identification		
	Species ID	Serotype	Biotype	Antimicrobial susceptibility	Beta-lactamase production	Species ID	Capsule type	<i>H. influenzae</i> detection
NM09	+	+	+	-	+	-	+	-
NM10	+	+	+	+	+	+	+	+
NM17	+	+	+	+	+	+	+	+
NM20A	+	+	+	+	+	+	+	+
NM22	+	+	+	+	+	-	-	-
NM23	+	+	-	+	+	+	+	+
NM26	+	+	+	+	+	+	+	+
NM27	+	+	-	+	+	+	+	+
NM29	+	+	+	+	+	+	+	-
NM32A	+	+	+	+	+	+	+	+
NM33	+	+	-	-	-	+	+	-
NM34A	+	+	+	+	+	+	+	+
NM35A	+	+	+	-	+	-	-	-
NM36	+	+	-	+	+	-	+	-
NM37A	+	+	-	+	+	-	+*	+
NM39	+	+	+	+	+	+	+	+
NM40	+	+	+	+	+	+	+	+
NM41	+	-	+	+	+	+	+	+
NM43	+	+	+	+	+	+	+	+
NM47	+	-	+	+	+	+	+	+
NM48	+	+	+	+	+	+	+	+
NM49	+	+	-	+	+	+	+	+
NM51	+	+	+	+	+	+	(+)**	+
NM52	+	+	+	+	+	-	-	-
NM53	+	+	+	+	+	-	+	+
NM54	+	+	-	+	+	-	-	-
NM55	+	+	+	+	+	+	+	+
NM56	+	+	+	+	+	+	+	+
NM57	+	+	+	+	+	-	+	-
NM59	+	+	+	+	+	-	-	+
Total	30	28	23	27	29	20	24 (25)	21

^a NM37A reported capsule typing on strain 2507, but not strain 2508.

^{**} NM51 did not officially report capsule typing results, but reported that they had used a *bexA* PCR as part of the species ID test for strain 2507 that confirmed that it was a *Hib*.

Table 3. Intended result for Part 1: Characterisation of viable isolates

EQA Sample	Phenotypic species ID	Phenotypic serotype	Biotype	Genotypic species ID	Genotypic capsule type	Additional typing
2507	<i>H. influenzae</i>	b	IV	<i>H. influenzae</i>	Hib	MLST-6
2508	<i>H. influenzae</i>	NTHi	V	<i>H. influenzae</i>	NTHi	MLST-849

ID: identification; Hib: *Haemophilus influenzae* type b; NTHi: non-typeable (non-capsulated) *Haemophilus influenzae*

Table 4. Results for Part 1: Characterisation of viable isolates

Sample number	Intended result	Proportion of laboratories reporting the intended result (%)	Results not matching intended result (frequency)
Phenotypic species identification			
2507	<i>H. influenzae</i>	30/30 (100%)	NA
2508	<i>H. influenzae</i>	30/30 (100%)	NA
Phenotypic serotyping			
2507	Hib	28/28 (100%)	NA
2508	NTHi	26/27 (96%)	Hib (1)
Biotyping			
2507	IV	19/22 (86%)	II (1), III (2)
2508	V	19/22 (86%)	I (1), VI (1), VII (1)
Genotypic species identification			
2507	<i>H. influenzae</i>	20/20 (100%)	NA
2508	<i>H. influenzae</i>	20/20 (100%)	NA
Genotypic capsular typing			
2507	Hib	24/24 ^(a) (100%)	NA
2508	NTHi	20/23 (87%)	Not Hib (1) ^(b) ; Hib- (1) ^(c) Hib (1)
Multilocus sequence typing (optional)^(d)			
2507	ST-6	5/5 (100%)	NA
2508	ST-849	5/5 (100%)	NA

Hib: *Haemophilus influenzae* type b; NTHi: non-typeable (non-capsulated) *Haemophilus influenzae*; NA: not applicable.

(a) Does not include one laboratory that didn't officially report a result, but commented in their result for genotypic identification that they performed a *bexA* PCR that confirmed it was a Hib.

(b) 'Not Hib' is consistent with the correct result, but insufficiently specific. (c) Interpreted to mean 'Hib minus' (capsule deficient Hib).

(d) Multilocus sequence typing was not a formal requirement of the EQA.

Phenotypic species identification

All thirty laboratories reported phenotypic identification of the viable cultures and correctly identified both cultures as *H. influenzae* (Table 4). Participants were invited to state up to five methods used as part of their phenotypic identification. The results are shown in Table 5 below.

Table 5. Phenotypic tests used in species identification (up to 5 declared)

Laboratory Identifier	Phenotypic tests used in species identification (up to 5 declared)											
	Gram stain	X and V factor dependence	Satellitism	Porphyrin test	Oxidase	Catalase	Biochemical tests/profile	API NH	RapID NH	MALDI-TOF	Vitek	Other (unspecified)
NM09	+			+	+	+						
NM10		+							+			
NM17			+	+	+							
NM20A			+	+			+					
NM22	+		+	+			+					
NM23												+
NM26		+						+				
NM27	+	+	+									
NM29	+	+			+							
NM32A	+				+	+		+				
NM33		+	+									
NM34A								+				
NM35A												
NM36		+										
NM37A							+					
NM39								+				
NM40		+	+						+		+	
NM41		+							+			
NM43	+		+	+			+	+				
NM47		+			+	+		+		+		
NM48	+	+						+			+	
NM49												
NM51	+		+		+	+			+			
NM52	+				+	+					+	
NM53		+								+		
NM54	+		+								+	
NM55	+	+	+				+	+				
NM56												
NM57		+								+		
NM59		+	+			+	+					+
Total	11	14	11	5	7	6	6	8	4	3	4	2

Phenotypic serotyping

Twenty-eight laboratories reported phenotypic serotyping results (Table 4). Participants were asked to state their method for serotyping. The results are summarised in Table 6.

All 28 laboratories identified strain 2507 as Hib. Only 27 reported serotyping of strain 2508 (the 28th lab reported 'not applicable'), and 26 (96%) correctly identified it as a non-capsulated strain of *H. influenzae* (NTHi). One laboratory (using slide agglutination) incorrectly identified strain 2508 as Hib.

Table 6. Phenotypic serotyping methods

Serotyping method	Number of laboratories (%)
Slide agglutination	19 (68%)
Co-agglutination	2 (7%)
Latex agglutination	4 (14%)
Latex and slide agglutination	1 (4%)
Co-agglutination and slide agglutination	1 (4%)
Method not reported	3 (11%)
Total	28

Biotyping

Twenty-two laboratories reported biotyping results for the strains: 8 (36%) generated their results using individual biochemical tests, 10 (45%) used the API NH kit and 4 (18%) used the RapID NH kit.

Nineteen laboratories (86%) correctly identified strain 2507 as biotype IV *H. influenzae* and strain 2508 as biotype V *H. influenzae* (Table 4). Five of the incorrect biotyping results were obtained by laboratories that used API NH and one incorrect result was reported by a laboratory using individual biochemical tests. There was no consistent mistake in the errors with respect to the three biochemical reactions (Tables 4 and 7).

Table 7. Biotyping scheme for *Haemophilus influenzae* [8]

Biotype	Indole	Urea	Ornithine
I	+	+	+
II	+	+	-
III	-	+	-
IV	-	+	+
V	+	-	+
VI	-	-	+
VII	+	-	-
VIII	-	-	-

Genotypic species identification

Twenty laboratories used a PCR-based method to confirm the identity of the strains as *H. influenzae* (Table 8). These comprised either a PCR directed at *ompP2*, *ompP4*, *ompP6*, *hpd* or the 16S rRNA gene, or PCR amplification of part of the 16S rRNA gene followed by DNA sequencing. (One laboratory clarified in their comments that in addition to *ompP6*, they confirmed the result using a triplex comprising *feck*, *hpd* and 'cap' (presumably *bexA*)). The questionnaire did not ask the participants to state whether they were using conventional or real-time PCR. All methods produced the intended result for both cultures (Table 4).

The 20 laboratories used a range of DNA extraction methods, all of which were associated with good results for this, and other, genotypic testing results (Table 8).

Table 8. Number of participants using various combinations of DNA extraction procedure and detection method for genotypic species identification, other genetic typing and capsular typing on viable isolates

Test	Detail	DNA extraction procedure				Total
		Manual procedure + in-house method	Manual procedure + commercial kit	Automated procedure + commercial kit	Other/ not specified*	
PCR for species identification	<i>ompP2</i>	4	6		1	11
	<i>ompP6</i>	1**		1		2
	<i>ompP4 (hel)</i>				1	1
	16S rDNA			1		1
	16S rDNA sequencing	1				1
	<i>hpd</i>		1			1
	Other/not specified*	3				3
Other genetic typing	MLST	3	1		1	5
Capsular typing	Variation of Falla, et al. [7]	7	6	3	3	19
	Other/not specified*	2			3	5

* Other/not specified denotes the selection of 'other' or failure to select an option.

** Participant also stated that they used a triplex PCR against *hpd* + *fucK* + *cap*.

MLST: Multilocus sequence typing

Genotypic capsule typing

Twenty-three laboratories reported PCR capsule typing results for both strains. One additional laboratory reported a result for sample 2507, but not 2508 (Tables 4, 8). DNA extraction methods used by the 24 laboratories are shown in Table 8. Nineteen of them stated that they were carrying out capsule typing using a variation of the PCR method of Falla, et al. [9], one reported 'other' with no clarification and the remaining four did not provide any information. Three laboratories stated in a comment that they were also using a PCR against *bexA* to confirm that isolates were capsulated (results not shown). The reporting scheme did not explicitly ask this question so it was not known how many more participants also did this.

All 24 laboratories correctly identified strain 2507 as a Hib. However, three of the 23 participants reporting a result for 2508 (NTHi) reported an incorrect or incomplete result (Table 4). One reported it as a Hib strain, one reported it as 'not Hib' and one reported 'Hib-'. 'Hib-' was interpreted to mean a capsule-negative Hib strain, containing a single copy of the capsule operon in which a deletion within the *bexA* gene results in failure to export capsular polysaccharide to the cell surface [10]. However, experience from previous EQA distributions has shown that sometimes participants use this term to mean 'not Hib'. No clarification was given in this case.

A 25th laboratory did not officially report any genetic capsule typing results, but did state in a comment field that they had performed a *bexA* PCR that determined that isolate 2507 was a Hib. This is possible if they used a PCR such as that described by Corless, et al. [11].

Other molecular typing

Although not a requirement of the EQA, five laboratories reported multilocus sequence typing (MLST) results for the two strains [12]. The results were all correct (Table 4).

2.2 Part 2. Antimicrobial susceptibility testing

Beta-lactamase activity testing

Twenty-nine laboratories reported beta-lactamase activity results. All of the results were correct for strain 2507, a beta-lactamase-positive strain of *H. influenzae*. Strain 2508 was a beta-lactamase-negative ampicillin-resistant (BLNAR) strain. Twenty-seven of 29 (93%) correctly identified this strain as beta-lactamase negative. However, two laboratories erroneously stated that it was beta-lactamase positive.

Antimicrobial susceptibility testing

The intended results for the antimicrobial susceptibility testing (AST) against ampicillin, amoxicillin-clavulanic acid, ciprofloxacin, ceftriaxone and cefotaxime are shown in Table 9. The results obtained by participating laboratories are shown in Table 10.

Twenty-seven laboratories reported some antimicrobial susceptibility testing results. Of those, 19 adopted EUCAST guidelines, six used CLSI guidelines and two did not state which guidelines they were using. Of the three laboratories that did not report any AST results, two stated that they did use EUCAST guidelines (the third laboratory provided no information).

Analysing the AST results was not straightforward. Some laboratories reported disc zone sizes and their interpretation (but did not determine MICs) and others reported MIC values plus their interpretation. The use of different methodologies, different disc strengths and different breakpoints make it difficult to compare the results from laboratories. Hence, the laboratories were judged on their reported results (susceptible/intermediate/resistant) for each antibiotic, plus (where available) the MIC values they reported. A summary of these results is shown in Table 10. The results from participants using EUCAST guidelines are shown separately from those using other guidelines.

Table 9. Intended results for antimicrobial susceptibility testing of bacterial isolates

Sample number	Beta-lactamase activity	Antimicrobial susceptibility (S)/ resistance (R)
2507	Positive	AMP (R), CO-AM (S), CRO, (S), CTX (S), CIP (S)
2508	Negative	AMP (R), CO-AM (R), CRO (S), CTX (R), CIP (S), BLNAR

AMP: ampicillin; CO-AM: co-amoxycylav (amoxicillin-clavulanic acid); CRO: ceftriaxone; CTX: cefotaxime, CIP: ciprofloxacin; BLNAR: beta-lactamase-negative ampicillin-resistant.

Table 10. Antimicrobial susceptibility testing results submitted by participating laboratories

Antimicrobial agent	Specimen 2507					
	Guidelines	Intended result	Ratio reporting intended result	Deviations from intended result (n)	MIC range, where reported (n)	MIC (mg/L) mode, where reported
Ampicillin	EUCAST	R	17/17	-	>1 – >256 (15)	256
	Non-EUCAST*		8/8	-	1.5 – 256 (3)	none
Amoxicillin-clavulanic acid	EUCAST	S	15/17	R (2)	0.5 – 16 (13)	0.5
	Non-EUCAST*		5/7	R (2)	0.5 – 0.75 (2)	none
Beta-lactamase	NA	Positive	29/29	-	NA	NA
Ciprofloxacin	EUCAST	S	18/18	-	0.004 – 0.032 (16)	0.008
	Non-EUCAST*		7/7	-	0.006 – 0.008 (3)	0.006
Ceftriaxone	EUCAST	S	14/14	-	<0.002 – 0.016 (13)	0.016
	Non-EUCAST*		8/8	-	<0.002 – 0.016 (4)	none
Cefotaxime	EUCAST	S	18/18	-	<0.0016 – 0.12 (17)	0.016
	Non-EUCAST*		7/7	-	<0.016 – 0.023 (3)	none

Antimicrobial agent	Specimen 2508					
	Guidelines	Intended result	Ratio reporting intended result	Deviations from intended result (n)	MIC range, where reported (n)	MIC (mg/L) mode, where reported
Ampicillin	EUCAST	R	18/18	-	1.5 – >256 (18)	2
	Non-EUCAST*		7/8	I (2)	1.5 – 3 (3)	1.5
Amoxicillin-clavulanic acid	EUCAST	R	16/17	S (1)	2 – >256 (15)	4
	Non-EUCAST*		5/7	S (2)	3 (2)	3
Beta-lactamase	NA	Negative	27/29	Positive (2)	NA	NA
Ciprofloxacin	EUCAST	S	18/18	-	0.006 – 0.032 (17)	0.008
	Non-EUCAST*		7/7	-	0.006 – 0.16 (3)	none
Ceftriaxone	EUCAST	S	10/13	R (3)	0.004 – 0.25 (13)	none
	Non-EUCAST*		8/8	-	0.032 – 0.125 (4)	0.094
Cefotaxime	EUCAST	R	13/17	S (4)	0.008 – 0.75 (16)	0.25
	Non-EUCAST*		2/7	S (5)	0.25 – 0.5 (3)	none

n: number of laboratories reporting the relevant result; NA: not applicable; S: sensitive; I: intermediate resistance; R: resistant.
* 'Non-EUCAST guidelines' includes laboratories using CLSI (NCCLS) and undisclosed guidelines.

There were few problems with the AST of strain 2507, a beta-lactamase-positive strain that was only resistant to ampicillin. Four laboratories reported that the strain was resistant to amoxicillin-clavulanic acid. The breakpoint results reported (from three of the participants) showed that this was due to the MIC/breakpoint obtained rather than any reporting error.

Sample 2508 proved more problematic (Table 10). Strain 2508 was beta-lactamase negative, but showed reduced susceptibility to ampicillin, amoxicillin and amoxicillin-clavulanic acid. It was a 'low' BLNAR strain. Reported MICs for sample 2508 ranged between 1.5 and >256 mg/L for ampicillin and between 2 and >256 mg/L for amoxicillin-clavulanic acid (co-amoxyclov). This strain was scored as resistant to ampicillin by 24 out of 26 participants (92%), but 21 out of 24 participants (88%) scored it as resistant to amoxicillin-clavulanic acid. Curiously, the mode MICs reported for the two antibiotics lay between 1.5 and 4 mg/L (Table 10), but one laboratory reported MICs of >256 mg/L for ampicillin and another reported >256 mg/L for amoxicillin-clavulanic acid. Strain 2508 was resistant to cefotaxime, although 9 out of 24 participants reported it to be susceptible. However, the MIC was close to the breakpoint in this strain and the EUCAST reference laboratory confirmed that the results obtained from zone diameters and MICs may conflict in this situation (personal communication. UK NEQAS).

The discrepant results for ampicillin and amoxicillin-clavulanic acid with strain 2508 serve to highlight the different interpretations provided by the EUCAST and CLSI guidelines. According to EUCAST guidelines the breakpoint for ampicillin is 1 mg/L and for amoxicillin-clavulanic acid it is 2 mg/L. The interpretative standards for CLSI state that strains with an ampicillin MIC of ≤ 1 mg/L should be regarded as susceptible, those with an MIC of ≥ 4 mg/L are resistant and an MIC of 2 mg/L indicates intermediate susceptibility. For amoxicillin-clavulanic acid, CLSI guidelines specify that strains with an MIC of $\leq 4/2$ mg/L are susceptible and those with an MIC of $\geq 8/4$ mg/L are resistant (Table 11). All 18 laboratories using EUCAST guidelines reported the sample as ampicillin-resistant and 16 out of 17 laboratories using EUCAST guidelines reported the sample as resistant to amoxicillin-clavulanic acid (Table 10). Two of the six laboratories that stated they were using CLSI guidelines reported the sample as being ampicillin-intermediate (and one of these reported an MIC of 3 mg/L). For amoxicillin-clavulanic acid, five laboratories gave results according to CLSI guidelines; of these, two stated that the strain was susceptible and three found it to be resistant (data not explicitly shown in Table 10). It should be noted that CLSI recommends BLNAR strains should be considered resistant to amoxicillin-clavulanic acid despite apparent in vitro susceptibility of some BLNAR strains. Using EUCAST guidelines will reduce the problem of interpretation of the susceptibility of low BLNAR strains. A comparison of EUCAST and CLSI interpretative standards for MIC determination of a number of antimicrobial agents is shown in Table 11.

Table 11. Comparison of interpretative standards for MIC determinations (mg/L) with *H. influenzae* in EUCAST and CLSI guidelines

Antimicrobial agent	EUCAST MIC breakpoint (mg/L)*		CLSI MIC interpretative standard (mg/L)		
	S	R	S	I	R
Ampicillin	≤ 1	> 1	≤ 1	2	≥ 4
Amoxicillin-clavulanic acid (co-amoxycylav)	≤ 2	> 2	≤ 4/2		≥ 8/4
Ceftriaxone	≤ 0.12	> 0.12	≤ 2		
Cefotaxime	≤ 0.12	> 0.12	≤ 2		
Ciprofloxacin	≤ 0.5	> 0.5	≤ 1		

* In order to simplify the EUCAST tables, the intermediate category is not listed. It is readily interpreted as the values between the S and the R breakpoints. For example, for MIC breakpoints listed as S ≤ 1 mg/L and R > 8 mg/L, the intermediate category is 2-8 (technically >1-8) mg/L.

Information on the BLNAR status of the samples was not explicitly elicited from the participants. However, five laboratories commented that this was a BLNAR strain. Participants were not asked whether they confirmed BLNAR status by partial sequencing of the *ftsI* gene.

Some strains of *H. influenzae* are resistant to aminopenicillins through both mechanisms, that is, they produce a beta-lactamase and have altered penicillin-binding proteins PBP3. Such strains are termed beta-lactamase-positive amoxicillin-clavulanic acid-resistant (BLPACR) strains. Such a strain was not included in the EQA panel since these are rarely encountered in Europe.

2.3 Part 3. Non-culture detection of *H. influenzae*

Two simulated CSF samples (2509 and 2510) were included in the EQA panel to test the participants' ability to extract DNA from clinical samples and assay for the presence of *H. influenzae*. They were also encouraged to offer any additional information that their assay was capable of elucidating about the samples (e.g. capsulation status or capsule type). Sample 2509 contained a strain of *H. influenzae* type e (Hie). Sample 2510 contained a strain of *H. influenzae* type b (Hib). The intended results and breakdown of the submitted data are shown in Table 12. The 21 laboratories used PCR-based methods directed against a range of gene targets to detect *H. influenzae* in the two samples (Table 13).

Eighteen of 21 participants correctly identified *H. influenzae* in sample 2509. (This included one ambiguous result of 'not *H. influenzae* type b' with *ompP2* described as the PCR target.) Two of the remaining laboratories reported a negative result. Both of these had used a real-time PCR against *ompP2*, although one of them also commented that the sample was negative in a 16S rDNA PCR. Both had used commercial kits for DNA extraction, one with a manual protocol and the other with an automated protocol (Table 13). However, other laboratories reporting the same extraction methodologies obtained positive results. The final remaining laboratory reported the sample as negative for *H. influenzae* and positive for *Neisseria meningitidis* DNA. This laboratory also used the *ompP2* PCR target. The reason for the false positive result for *N. meningitidis* is unclear. This EQA panel did not contain any samples of *N. meningitidis*, although an IBD Labnet *N. meningitidis* EQA panel was also circulating and may have been tested at the same time by this laboratory.

The *ompP2* target was used in a real-time PCR by the three laboratories that failed to detect *H. influenzae* in specimen 2509. The fourth laboratory that also used a real-time *ompP2* PCR had added a comment that there seemed to be a very low concentration of DNA detected in their real-time PCR (i.e. late Cq). Furthermore, one additional laboratory that had reported using *hpd* as their gene target also added a comment that this sample had given a negative result in an *ompP2* real-time PCR. Further testing of the sample using the *ompP2* PCR of Maaroufi, et al. [13] by the EQA organisers showed that this sample, but not 2510, failed to give a positive result, although the *hpd#3* PCR of Wang, et al [14] gave similar positive Cq results for both samples (results not shown). None of the four laboratories that used the *ompP2* in a conventional PCR (with gel electrophoresis) appeared to have problems with the assay.

Eight of 21 participants offered further typing results for sample 2509 (Table 12): three correctly identified Hie (although one reported it as negative for *bexA* and, therefore, non-capsulated); one correctly identified it as not Hib; three reported it as positive for Hib (one of whom stated it was a non-capsulated variant) and one reported it as non-capsulated.

Nineteen of 21 participants correctly identified *H. influenzae* in sample 2510 (Table 12). One of the remaining participants reported a negative result, the other reported 'Not *H. influenzae* or non-typeable'. The negative result was obtained using the *ompP6* PCR target in a real-time PCR assay. However, the failure was not consistently linked to the gene target, since the three other laboratories using this target obtained a positive result.

Nine laboratories offered further typing results for sample 2510 (Table 12): six correctly identified it as a Hib; one identified it as Hib/Hic; one identified it as capsulated; and only one erroneously identified it as not Hib.

The DNA extraction methods used by the participants are shown in Table 13. All methods were associated with good results.

Table 12. Intended and submitted results for Part3: Non-culture detection of *H. influenzae*

Sample number	Intended results	Ratio of laboratories reporting the intended result (%)	Results not matching intended result (frequency)	Optional extra typing results offered (frequency)
2509	<i>H. influenzae</i> (optional: serotype e)	18/21 (86%)	Negative (2) <i>N. meningitidis</i> (1)	Hie (2) Hie- (1) Not Hib (1) Hib (2) Hib-(1) Non-capsulated (1) No extra data (10)
2510	<i>H. influenzae</i> (optional: serotype b)	19/21 (90%)*	Negative (1) Not <i>H. influenzae</i> , or non-typeable (1)	Hib (6) Hib/Hic (1) Not Hib (1) Capsulated (1) No extra data (10)

* Includes one laboratory that reported 'not *H. influenzae* type b'.

Table 13. Methods used for preparation and detection of *H. influenzae* DNA in simulated CSF samples

DNA extraction	Detection method	<i>H. influenzae</i> gene target							Other/not specified
		<i>ompP2</i>	<i>ompP6</i>	<i>ompP4</i> (hel)	<i>bexA</i>	16S rDNA	<i>fucK</i>	<i>hpd</i>	
Manual procedure + in-house method	PCR and electrophoresis				1				
Manual procedure + commercial kit	PCR and electrophoresis	4	1						1
	Real-time PCR	3	1					2	
	PCR and sequencing					1			
Automated procedure + commercial kit	PCR and electrophoresis			1					
	Real-time PCR	1*	2			1	1	1**	
	Total	8	4	1	1	2	1	3	1

* This participant also used a PCR against 16S rDNA.

** This participant also used a PCR against *ompP2*.

3 Comments

This laboratory EQA has shown that the majority of European *Haemophilus* reference laboratories are able to identify and phenotypically serotype *H. influenzae*. An increasing number perform PCR-based identification (20/30 laboratories) and capsular genotyping (24/30) compared to the previous distribution (18/28 and 20/28, respectively). Similarly, 29 out of 30 conducted beta-lactamase testing and 27 out of 30 performed further AST (compared with 27/28 and 24/28 in 2012). It is acknowledged that this EQA could not be as rigorous a test of participants' abilities to characterise isolates as previous distributions due to the small number of strains included (two instead of six). In particular, closely related species, such as *H. parainfluenzae*, could not be included to test identification methods more thoroughly.

This EQA distribution only identified a single error in conventional serotyping by slide agglutination. It is known that serotyping sometimes gives rise to erroneous results, particularly reporting non-capsulated *H. influenzae* as capsulated variants [15], and confirmation using PCR-based capsule typing is recommended.

Most laboratories used a variation on the method of Falla, et al. [9] for PCR capsule typing of *H. influenzae* isolates. This includes a PCR against the *bexA* gene, designed to detect all capsulated isolates, and six individual capsule-specific PCRs to detect each of the capsule types. Variations on this method have been described, using multiplex conventional and real-time PCR methodology [13,16,17]. One participant in the EQA did not officially report any genetic capsule typing results, but did state in a comment that they had performed a *bexA* PCR that determined that the isolate was a Hib. The *bexA* gene is present in all capsulated isolates, but a PCR has been designed by Corless, et al. [11] targeted at variants of the gene that are specific to Hib. However, it doesn't show complete specificity for Hib [11] and so would not be recommended for use with cultures as a replacement for standard capsule typing, in which the six type-specific capsule loci are targeted.

Several laboratories had difficulty capsule typing the non-capsulated strain (2508). One reported it as 'non Hib', presumably because the participants only tested for Hib. While this is consistent with the correct result, it doesn't confirm whether the isolate is non-capsulated or a different capsulated type. Furthermore, in countries with high Hib vaccine coverage, Hib isolates now comprise a small percentage of invasive *H. influenzae* isolates [1]. Hence, it is recommended that reference laboratories attempt to fully type all relevant *H. influenzae* isolates. The remaining two participants reported this strain as a Hib and a Hib-isolate respectively. It is not clear whether this was a reporting error or whether the participants generated a false positive in their Hib-specific PCR, either through sample mix-up or contamination problems.

The AST results proved difficult to assess as some laboratories gave MIC values, whilst others gave zone sizes with or without interpretation of the results. Some laboratories are using EUCAST guidelines whilst others are still using CLSI guidelines. There are major differences between the EUCAST and CLSI both in terms of media, and defined clinical breakpoints for a number of antimicrobials. All national reference laboratories in EU/EEA should be moving towards using EUCAST guidelines.

The EQA exercise included testing for beta-lactamase activity in the isolates. The most important mechanism of ampicillin resistance in *H. influenzae* is the production of TEM-1 beta-lactamase [18]. A second beta-lactamase, ROB-1 [19] is less frequently implicated. Although mutations have appeared in the promoter region of these genes, no TEM-type extended-spectrum beta-lactamases (ESBLs) have yet been detected in *H. influenzae*. However, ESBL-type beta-lactamases have been reported in *H. parainfluenzae*, a potential source of DNA for transformation of *H. influenzae* [20]. In this EQA exercise there were no problems with the detection of beta-lactamase production in Specimen 2507 (although there were two false positive results for Specimen 2508, which didn't produce a beta-lactamase).

Specimen 2508 was an example of a *H. influenzae* strain that demonstrates beta-lactamase-independent resistance to beta-lactams, a phenotype termed beta-lactamase-negative ampicillin-resistance (BLNAR). The BLNAR phenotype arises from alterations in bacterial penicillin-binding proteins (PBP) [21], leading to a reduced affinity to penicillins and cephalosporins. *Haemophilus influenzae* has five penicillin-binding proteins (1A, 1B, 2, 3 and 4). PBP 3 is encoded by the *ftsI* gene and mutations in the transpeptidase domain of *ftsI* are correlated with resistance. These mutations can be divided into genotypic groups [22,23]. In Spain an increased prevalence of BLNAR (genotype III-like) strains with characteristics of clonality and increased MICs for cefuroxime (up to 16 µg/mL) and cefotaxime (up to 4 µg/mL) have been reported [24]. There are also reports of an increasing prevalence of BLNAR strains in Poland, France, and Portugal, with genotype III-like strains becoming more common [25-28]. In Sweden, a substantial increase in beta-lactam-resistant invasive *H. influenzae*, mainly caused by beta-lactamase-negative isolates, has been reported from 2007 onwards. One cluster of BLNAR genotype IIb isolates was identified, which included isolates from all geographical areas [29].

In this EQA exercise, the evaluation of beta-lactamase-negative ampicillin-resistance in Specimen 2508 was not straightforward because the consensus MICs for ampicillin, amoxicillin-clavulanic acid and cefotaxime were close to their breakpoints. Some BLNAR strains (high-BLNAR) have ampicillin MICs in the range 8–16 mg/L. High BLNAR

strains have mutations in the *acr* gene, which encodes the AcrAB efflux pump, in addition to mutations in *ftsI* [30]. Low-BLNAR strains usually have ampicillin MICs in the range 0.5 to 2mg/L and such strains may be difficult to identify by conventional susceptibility testing even when low-strength ampicillin (2mg/L) and amoxicillin-clavulanic acid (2+1mg/L) discs are used. García-Cobos, et al. [24] suggest that low-BLNAR strains are best detected by broth dilution methods rather than disc susceptibility testing. Definitive identification of such strains relies on PCR and partial sequencing of the *ftsI* gene, but this is impractical as a routine test. The Nordic countries have agreed on the use of a screening test for detection of such strains (see Figure 1 below). The clinical significance of ampicillin resistance at this low level is, however, far from clear. Nevertheless, if a strain is found to be a low-level BLNAR, it would be prudent to avoid the use of these antimicrobials to treat a serious invasive infection. The level of ampicillin resistance exhibited by BLNAR strains may be low (MIC 0.5–2 µg/ml) and this may make their detection difficult, particularly if a breakpoint of 1µg/ml is used to define ampicillin susceptibility.

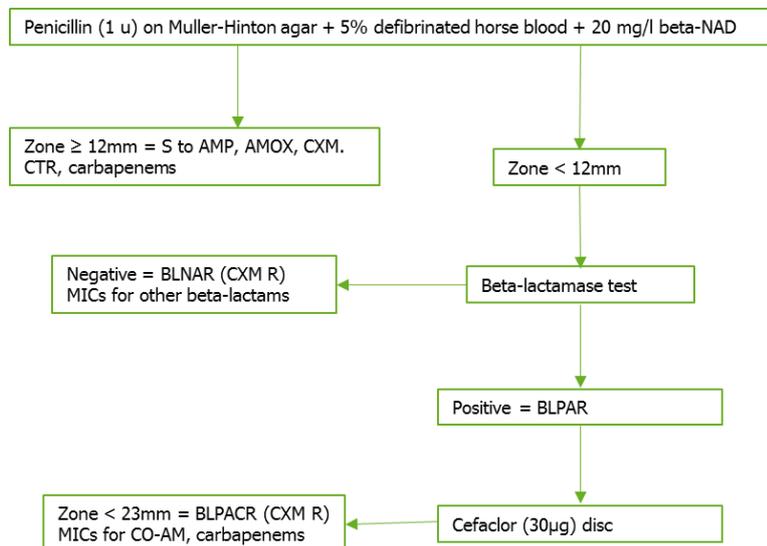
BLNAR strains show reduced susceptibility not only to ampicillin but also to other antibiotics, particularly some of the cephalosporins. Livermore, et al. [31] suggested that cefaclor resistance is a better indicator of a BLNAR strain than ampicillin resistance and James, et al. [32] used cefuroxime resistance (MIC > 4.0 µg/ml) to screen for BLNAR strains. CLSI recommends that BLNAR strains are considered resistant to amoxicillin-clavulanate, cefaclor and cefuroxime despite apparent susceptibility of some strains to these antimicrobials.

Nørskov-Lauritsen, et al. [33] evaluated the efficacy of disc diffusion methods for the detection of low-BLNAR. Forty-seven low-BLNAR strains of *H. influenzae*, identified by partial sequencing of the *ftsI* gene had low-level resistance to ampicillin (MIC ≤ 1 mg/l; MIC₅₀ = 0.5 mg/l) which would be interpreted as susceptible by both EUCAST and CLSI interpretative criteria. The MIC of cefuroxime varied between 1 and 4 mg/l (MIC₅₀ = 2 mg/l) which would be interpreted as resistant by EUCAST but susceptible by CLSI criteria. These authors found that disc diffusion with cefaclor (30 µg discs) on sensitivity test agar + 5% horse blood + NAD was able to discriminate low-BLNAR strains from wild-type strains with 98% sensitivity and 86–99% specificity.

In this EQA exercise, some laboratories used low-strength ampicillin disks (2 µg) as recommended by EUCAST guidelines, whilst others used higher concentration ampicillin disks (10 µg). The use of low-dose ampicillin discs is recommended as it will increase the ability to identify low-BLNAR [33,34]. The screening method outlined by the Nordic Committee on Antimicrobial Susceptibility Testing (as described below) should improve the ability of laboratories to detect low-level BLNAR, as well as beta-lactamase-positive amoxicillin-clavulanate-resistant (BLPACR) strains (that possess a beta-lactamase as well as mutations in PBP3).

The method for screening for BLNAR and BLPACR strains recommended by the Nordic Committee on Antimicrobial Susceptibility Testing (reviewed by Kuch, et al. [35]) is shown in Figure 1.

Figure 1. Disc diffusion screening method for the detection of BLNAR, BLPAR and BLPACR strains of *H. influenzae*



AMP: ampicillin; AMOX: amoxicillin; CO-AM: amoxicillin-clavulanate; CXM: cefuroxime; CTR: ceftriaxone

In this procedure, the strain of *H. influenzae* is plated onto Mueller-Hinton agar supplemented with 5% defibrinated horse blood and 20 mg/L β-NAD. A 1 u penicillin disc is placed on the surface of the plate and the culture is incubated overnight. If the zone of inhibition around the penicillin disc is ≥ 12mm the strain can be assumed to be susceptible to ampicillin, amoxicillin, cefuroxime, ceftriaxone and carbapenems. If the zone of inhibition is < 12 mm a beta-lactamase test should be performed. If the strain is beta-lactamase negative the strain is a BLNAR

and can be assumed to be resistant to cefuroxime. MIC determinations should be carried out to establish susceptibility to other beta-lactams. If the strain is beta-lactamase positive the strain is a BLPAR strain. The strain should then be tested with a 30µg cefaclor disc. If the zone of inhibition around the cefaclor disc < 23mm the strain is both beta-lactamase positive and intrinsically resistant to ampicillin (BLPACR). BLPACR strains can be assumed to be resistant to cefuroxime. MIC determinations should be carried out to determine the susceptibility of BLPACR strain to amoxicillin-clavulanic acid, ceftriaxone and carbapenems.

The simulated CSF samples included in this EQA were designed to contain sufficient numbers of killed *H. influenzae* that they would be reliably detected by PCR following extraction. However, one of the samples was not detected by real-time PCRs directed at *ompP2*. This lack of analytical sensitivity appears to be strain dependent and has been observed in previous studies [12,34]. Until an *ompP2* real-time PCR assay is described with more reliable sensitivity, this target is not recommended for the detection of *H. influenzae* in clinical samples. This problem does not appear to be significant in their use on bacterial cultures, however [13, 36,37], and did not affect the conventional PCRs directed against *ompP2*. Other PCR targets chosen by the participants performed well in this EQA. Nevertheless, one laboratory used *bexA* to detect *H. influenzae*. Although this worked with the samples in this distribution, in general, diagnostic PCRs that are specific for only capsulated *H. influenzae* (e.g. *bexA*) or serotype b (e.g. *bexA* PCR by Corless, et al. [11] or a Hib-specific target) are not recommended as they will fail to detect NTHi or any non-type b strains respectively. If used, care must be taken in reporting PCR-derived results on clinical specimens if the PCR target is not universally present (e.g. *bexA*) and the precise meaning of a positive or negative PCR result must be explained (e.g. whether the test can only detect capsulated *H. influenzae* or only a subset of capsule types). When used in conjunction with a universally present gene target, however, *bexA* or a capsule type-specific PCR provides useful additional information.

Several laboratories attempted to determine the capsulation status of the *H. influenzae* in the simulated CSF samples, with mixed success. Some generated false positive results (e.g. designating the Hie strain as a Hib) and others false negatives (e.g. reporting negatives for the *bexA* PCR). The reason for these discrepancies is not clear, but the false negatives may be caused by a low yield of DNA following extraction. This was not recorded as part of this EQA, but an indication of DNA yield could be solicited in future distributions by asking participants to report Cq values for their real-time PCR results.

With only two samples in the simulated CSF part of the panel it was not possible to test the sensitivity of different methods. Furthermore, in order to test participants' ability to detect both a Hib and a non-Hib isolate, a negative control sample was also omitted. Overcoming these compromises will require a larger number of non-culture samples to be included in a future EQA panel.

4 Conclusions

The level of characterisation of strains of *Haemophilus influenzae* varies between EU countries. This emphasises the need for consensus and agreement in methods for characterising and accurately defining this organism.

The results of this EQA exercise have shown improvements in some areas compared to the results from the 2012 EQA distribution. Twenty-eight European *Haemophilus* reference laboratories (93%) now routinely serotype isolates, compared to 26 in 2012. Twenty-four laboratories (80%) perform PCR-based capsular genotyping, compared to 19 (68%) in 2012. Twenty-seven laboratories routinely perform AST, compared to 24 in 2012, and the number of laboratories following EUCAST guidelines has increased from 14 in 2012 to 19 in 2014.

The EQA exercise has demonstrated that three laboratories still appeared to have problems with PCR-based capsule typing of NTHi (similar to 2012). PCR-based genotyping methods are valuable in providing a serotype/genotype for strains that give inconclusive results on slide agglutination, but the false positive result of Hib for the NTHi strain from three laboratories is concerning. Ideally a genotyping method should be used for all *H. influenzae* isolates in order to confidently identify Hib and capsule deficient Hib strains. This is of particular importance where routine Hib immunisation has been implemented, since it is essential to be able to accurately identify Hib vaccine failures. It is of note that the Hib isolate included in the EQA posed no problems to phenotypic or genotypic capsule typing by any of the participating laboratories. In addition, molecular-based capsular typing can act as a quality control measure to monitor the accuracy of the results of conventional serotyping.

The results of antimicrobial susceptibility testing again proved difficult to interpret due to the use of different methods and breakpoints. It is recommended that all national reference laboratories in the EU/EEA adopt the EUCAST methods for AST which should facilitate better comparison of the results from different laboratories (<http://www.EUCAST.org>). On a positive note, the number of national reference laboratories for *H. influenzae* that do follow the EUCAST guidelines has increased from 14 to 19 since the 2012 EQA distribution. However, this is still lagging behind the adoption of EUCAST methods and breakpoints by clinical microbiology laboratories reporting to EARS-Net [38].

Two simulated clinical samples were again included in the EQA panel to assess non-culture detection methods. Two more laboratories (21) participated in this part of the EQA than in 2012. The results were generally very good, although real-time PCRs against the *ompP2* target had difficulty identifying one of the samples. It was encouraging that almost all participants used PCRs targeted against universally (or almost universally) carried genes and so the serotype e (Hie) isolate did not pose problems for detection. It was also encouraging that some participants also performed additional PCRs and so could determine the capsule type of the *H. influenzae* isolates. The future inclusion of a greater number of non-culture samples would allow the sensitivity of each participant's PCR to be studied.

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