TECHNICAL REPORT

External quality assurance scheme for *Haemophilus influenzae*

2009

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ECDC TECHNICAL REPORT

External quality assurance scheme for *Haemophilus influenzae* 2009

As part of the IBD-Labnet surveillance network

The report was commissioned by the European Centre for Disease Prevention and Control (ECDC), coordinated by Lucia Pastore-Celentano and produced by Dr Mary Slack (Health Protection Agency, London, UK), on behalf of the IBD-Labnet consortium (referring to Specific Contract ECD.1027).

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Abbreviations

AMP	Ampicillin
BLNAR	Beta-lactamase-negative ampicillin resistant strain
BSAC	British Society for Antimicrobial Chemotherapy
CA-SFM	Comité de l'ántibiogramme de la Société Française de Microbiologie
CAT	Chloramfenicol acetyl transferase
CIP	Ciprofloxacin
CHLOR	Chloramfenicol
CLAR	Clarithromycin
CLSI	Clinical and Laboratory Standards Institute
COAM	Co-Amoxyclav/Augmentin
CRG	Commissie Richitlijnen Gevoeligheids-bepalingen
CRO	Ceftriaxone
CTX	Cefotaxime
CXM	Cefuroxime
DIN	Deutsches Institut för Normung
EUCAST	European Committee on Antimicrobial Susceptibility Testing
Hinc	Non-capsulated *Haemophilus influenzae*
Hia	Capsule-deficient strain of *H. influenzae* type *a*
Hib	*H. influenzae* type b
Hib$^-$	Capsule deficient strain of *H. influenzae*
Hie	*H influenzae* type e
Hif	*H. influenzae* serotype f
HPA	Health Protection Agency, UK
HRU	*Haemophilus* Reference Unit
I	Intermediate
MIC	Minimum inhibitory concentration
NWGA	Norwegian Working Group on Antibiotics
OMP	Outer membrane protein
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
R	Resistant
RIF	Rifampicin
S	Susceptible
SXT	Trimethoprim-sulphamethoxazole
TET	Tetracycline

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 (i.e. viral infections, immunological deficits). Nevertheless, invasive bacterial diseases account for severe clinical features and child mortality.

Therefore, surveillance of *H. influenzae* is of the utmost importance. An integrated surveillance for this pathogen entails epidemiological as well as laboratory surveillance.

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

In January 2009 a collection of six strains of *Haemophilus* spp. (two non-capsulated *H. influenzae*, one *H influenzae* serotype a^-, one *H. influenzae* serotype b^-, one *H influenzae* serotype e, one *H parahaemolyticus*) and one other microorganism (*Aggregatibacter aphrophilus*, which could be misidentified as *Haemophilus* spp.) was sent to 26 participating reference laboratories in the IBD-Labnet surveillance network for quality assurance testing. The laboratories were requested to perform standard laboratory protocols for the methods usually used by the laboratory for: species identification, biotyping and serotyping by serological methods and/or PCR. Antimicrobial susceptibility tests and beta-lactamase testing were also requested for those laboratories that perform antimicrobial susceptibility testing of the isolates on a routine basis.

The EQA performance has shown that European *Haemophilus* Reference Laboratories differ in the level of characterisation of the strains, ranging from simple speciation to full identification and typing. Similarly, some laboratories routinely serotype isolates whereas others do not. Some laboratories (not all) perform PCR-based capsular genotyping; some laboratories routinely perform antimicrobial susceptibility testing whilst others do not.

The EQA scheme identified some problems with the use of slide agglutination for serotyping. The results can be misinterpreted when non-specific agglutination, cross-reactions and auto-agglutination occur.

The results of this EQA exercise suggest that some laboratories that do perform PCR-based capsular typing only use b primers and do not test for other capsular types.

The antimicrobial susceptibility testing proved difficult to interpret since, in many cases, the methodology and breakpoints used were not defined. Some laboratories used MIC values, whereas others gave zone sizes, with or without interpretation of the results.

The reporting of the results by the laboratories was also heterogeneous: some submitted the results electronically, others by fax.

This EQA scheme has pointed out that it should be advisable to use PCR-based genotyping methods to genotype/serotype strains giving inconclusive results on slide agglutination. This is of particular importance when routine Hib immunisation is used in order to detect Hib vaccine failures. In addition, molecular capsular typing can act as a quality control measure to monitor the accuracy of the results of conventional serotyping.

In the future, it would also be appropriate the use of an electronic form completed online for reporting the results.

In conclusion, the results of the EQA distribution for *Haemophilus influenzae* were excellent and compared favourably with the results from the EQA distribution that took place in 2007 under the auspices of EU-IBIS.

Introduction

The European Centre for Disease Prevention and Control (ECDC) is a European Union (EU) agency with a mandate to operate dedicated surveillance networks (DSNs) 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 assurance schemes.' (Article 5.3, EC 851/2004[1]).

External quality assurance (EQA) is part of quality management systems (QMS) and evaluates performance of laboratories, by an outside agency, on material that is supplied specially 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 costs. The aim of the EQA is to identify needs of improvement in laboratory diagnostic capacities relevant to surveillance of disease listed in Decision No 2119/98/EC and to ensure comparability of results in laboratories from all EU/EEA countries. The main purposes of external quality assurance schemes include:

- assessment of the general standard of performance ('state of the art');
- assessment of the effects of analytical procedures (method principle, instruments, reagents, calibration);
- evaluation of individual laboratory performance;
- identification and justification of problem areas;
- provision of continuing education; and
- Identification of needs for training activities.

Haemophilus influenzae is a common cause of serious disease in children worldwide. Pneumonia and meningitis are the most frequent manifestations. However, it can also be responsible for epiglotitis, soft tissues, bones, joints and other body's sites infections. 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 diseases.

Nevertheless, the availability of vaccines requires a more accurate surveillance system. Completeness and accuracy become key objectives of surveillance when vaccines are introduced and the incidence of the infection approaches low levels, as it is in invasive diseases due to *H. influenzae*. Not only epidemiological surveillance but laboratory data, especially biotyping and serotyping are needed to ensure optimal European surveillance for *H. influenzae*.

The European Union Invasive Bacterial Infections Surveillance Network (EU-IBIS) has been a successful dedicated surveillance network for the surveillance of invasive diseases caused by *Neisseria meningitidis* and *Haemophilus influenzae*. The network had epidemiological and laboratory components. The epidemiological activities focused on the collection and analysis of data on *N. meningitidis* and *H. influenza* cases, and the evaluation of the impact that vaccination programmes using conjugate vaccines have on the epidemiology of meningococcal disease. The laboratory activities focused in EQA and were aimed at strengthening the laboratory capacity in Member States for accurately characterising the isolates of *N. meningitidis* and *H. influenzae*. EUIBIS has been coordinated by the Health Protection Agency (HPA) in London, United Kingdom.

Since October 2007, the coordination of the activities of EU-IBIS has been integrated into the activities of ECDC and the epidemiological and the laboratory data collected to date by the EU-IBIS network have been transferred to ECDC.

The implementation of laboratory surveillance activities, namely the External Quality Assurance (EQA) activities and training, have been outsourced by the Framework contract No ECDC/08/008 to a consortium of European experts (the European Monitoring Group on Meningococci – EMGM – and some other experts in *H. influenzae* and *N. meningitidis*), coordinated by Prof Dr Matthias Frosch, from the University of Würzburg, Germany.

The specific objectives of this EQA exercise are:

- the further harmonisation of molecular typing of *H. influenzae*;
- the 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;
- supporting ECDC in linking laboratory surveillance data and epidemiological data.

[1] 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.

1 Material and methods

The samples for the EQA scheme for *H. influenzae* were sent by the Health Protection Agency (HPA) in collaboration with UK NEQAS, by agreement of the University of Würzburg (Germany), as coordinator of the project.

In January 2009 a collection of six strains of *Haemophilus* spp. and one other organism (*Aggregatibacter aphrophilus*), which could be misidentified as *Haemophilus* spp., was sent to participating reference laboratories in the IBD-Labnet surveillance network for quality assurance testing. We requested that all seven strains be tested using standard laboratory protocols for the methods normally used by the laboratory to characterise submitted isolates of *Haemophilus* spp., namely species identification, biotyping and serotyping by serological methods and/or PCR. It was also requested that antimicrobial susceptibility tests and beta-lactamase testing be carried out using normal laboratory procedures in those laboratories where it is standard practice to determine the antimicrobial susceptibility profile of submitted isolates. An electronic template for recording the results was provided. The strains were sent as lyophilised cultures and shipped by courier.

On receipt the strains were viable. One laboratory reported that one of the strains was non-viable and a replacement lyophilised culture was immediately despatched. The strains were processed as requested and the results were returned to the *Haemophilus* Reference Unit by 26 laboratories (Annex 1).

The intended results are shown in Table 1. The strains sent comprised two non-capsulated *H. influenzae,* one *H. influenzae* serotype a^-, one *H. influenzae* serotype b^-, one *H. influenzae* serotype e, one *H. parahaemolyticus,* and one *Aggregatibacter aphrophilus*.

Table 1 Intended results of 2009 EU-IBIS EQA distribution

EQA number	Strain	Biotype	Beta-lactamase present / absent	Antibiotic susceptibility (S) / resistance (R)*
200	*H. parainfluenzae*	Biotype II	Beta-lactamase negative	All S
201	Hinc	Biotype VI	Beta-lactamase negative	All S
202	*Aggregatibacter aphrophilus*		Beta-lactamase negative	All S
203	Hib^-	Biotype I	Beta-lactamase positive	AMP R, CHLOR R, TET R
204	Hie	Biotype I	Beta-lactamase positive	AMP R
205	Hia^-	Biotype I	Beta-lactamase negative	All S
206	Hinc	Biotype I	Beta-lactamase negative	AMP R, COAM R, TET R

**Sensitive to this antibiotic unless otherwise indicated: ampicillin (AMP), chloramphenicol (CHLOR), ciprofloxacin (CIP), clarithromycin (CLAR), co-amoxyclav/augmentin (COAM), ceftriaxone (CRO), cefotaxime (CTX), cefuroxime (CXM), rifampicin (RIF), trimethoprim-sulfamethoxazole (SXT), tetracycline (TET).*
Hinc = non-capsulated Haemophilus influenzae
Hia^- = capsule-deficient strain of H. influenzae *type a*
Hib = H. influenzae *type b*
Hib^- = capsule-deficient strain of H influenzae
Hie = H. influenzae *type e*

Regarding the methods, 12 countries did not biotype any of their strains, and six did not test beta-lactamase status. 24 countries used slide agglutination to determine serotyping. Seventeen utilised PCR for the same purpose, and one country did not serotype at all.

2 Results

A summary of the results for the countries is given in Table 2 and the full results on strain identification are given in Table 4.

Table 2 Results of strain identification

EQA number	Identification	Ratio reporting consensus (%)	Non-consensus results reported
Strain identification			
200	*H. parainfluenzae*	24/26 (92%)	*H. influenzae*, species not identified
201	*H. influenzae*	25/26 (96%)	*H. haemolyticus*
202	*Aggregatibacter aphrophilus*	21/26 (81%)	*H. parasuis*, species not indentified (x4)
203	*H. influenzae*	26/26 (100%)	
204	*H. influenzae*	26/26 (100%)	
205	*H. influenzae*	26/26 (100%)	
206	*H. influenzae*	24/26 (92%)	Sterile, not *Haemophilus*
Biotype identification			
200	Biotype II	8/9 (89%)	Biotype IV
201	Biotype VI	13/14(93%)	Biotype V
202		N/A	N/A
203	Biotype I	12/14(86%)	Biotype II (x2)
204	Biotype I	14/14(100%)	
205	Biotype I	14/14(100%)	
206	Biotype I	12/14(86%)	Sterile, Biotype II
Serotyping by slide agglutination			
200	N/A	N/A	N/A
201	Hinc	22/23 (95%)	Hib
202	N/A	N/A	N/A
203	Hinc/Hib	21/23 (91%)	Hic, Hif
204	Hie	21/22[a] (95%)	Hic
205	Hinc/Hia	19/22[b] (86%)	Hid, Hib, Hif, Hif/Hie
206	Hinc	20/23[c] (87%)	Hif (x3)
Serotyping by PCR[d]			
200	N/A	N/A	N/A
201	Hinc	15[e]/15 (100%)	
202	N/A	N/A	N/A
203	Hib$^-$	14/16 (87%)	Hinc. Did not detect capsular deficiency
204	Hie	15/16 (93%)	
205	Hia$^-$	11[f]/16 (68%)	Hinc (x5)
206	Hinc	16/16 (100%)	Hif

[a] *Two correctly assigned as non-b, so not included; further identification not possible.*
[b] *One correctly assigned as non-b, so not included; further identification not possible.*
[c] *One sample auto-agglutinated, therefore not included.*
[d] *One set of PCR results undetermined, so not included.*
[e] *One correct result but ignored in overall interpretation.*
[f] *Five did not detect capsule deficiency but result deemed correct.*

Table 3 Overview of the number of participating laboratories per method

Method	Participants
Strain identification	26
Biotype identification	14[a]
Serotyping by slide agglutination	23[b]
Serotyping by PCR[c]	16[d]

[a] *Only nine participants for EQA number 200.*
[b] *Only 22 participants for EQA numbers 204, 205.*
[c] *One set of PCR results undtermined, so not included.*
[d] *Only 15 participants for EQA number 201. No participants for EQA numbers 200 and 202.*

Figure 1 Strain identification

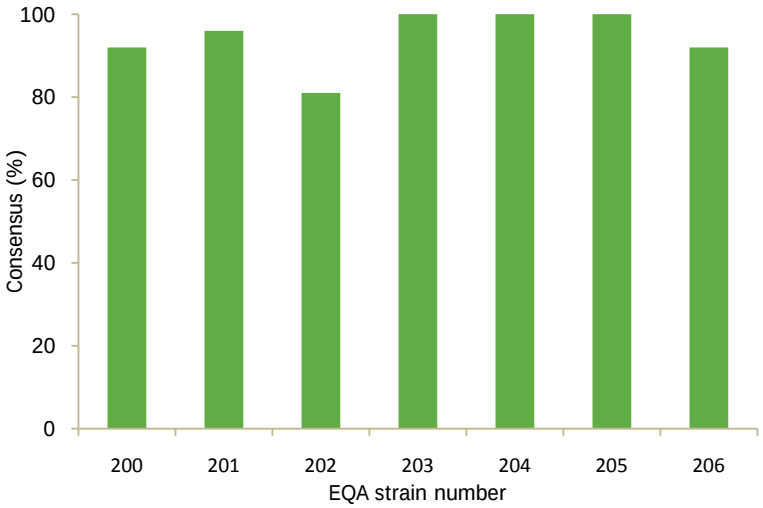


Figure 2 Biotype identification

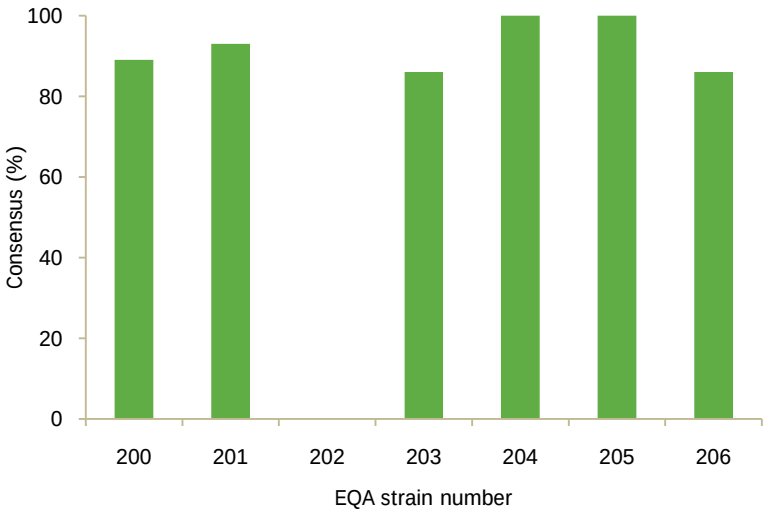


Figure 3 **Serotyping by slide agglutination**

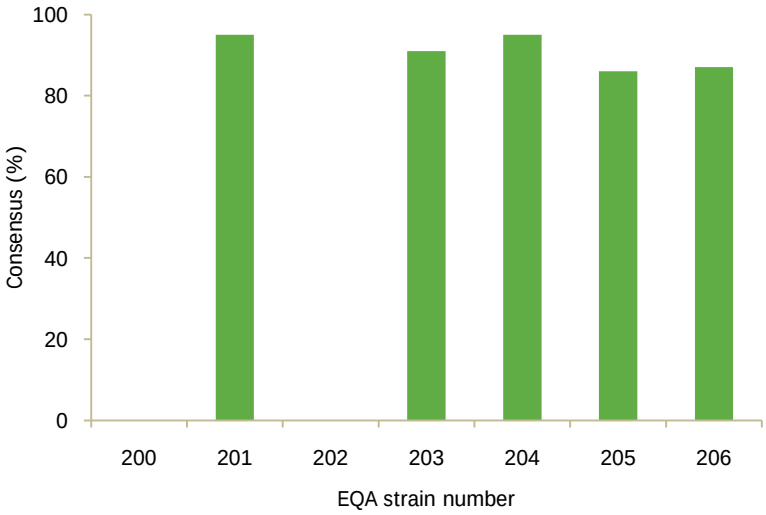


Figure 4 **Serotyping by PCR**

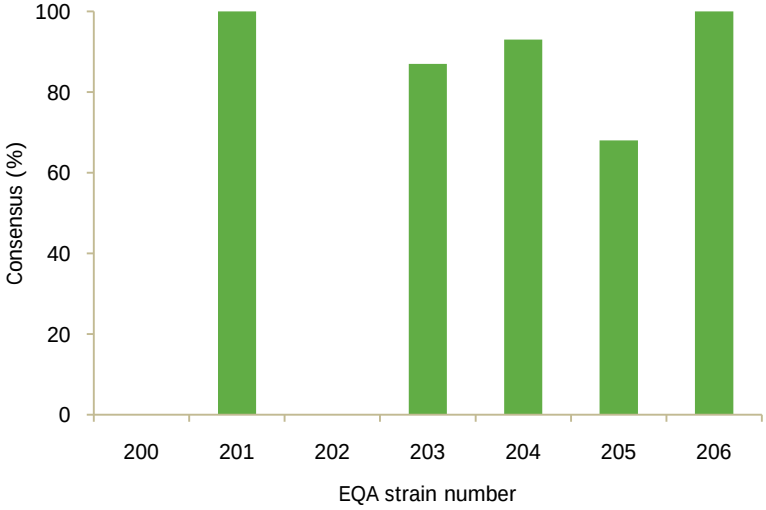


Table 4: Identification results

EQA number		200	201	202	203	204	205	206
Intended result		*H. parahaemolyticus* biotype II	Hinc biotype VI	*A. aphrophilus*	Hib⁻ biotype I	Hie biotype I	Hia⁻ biotype I	Hinc biotype I
Laboratory number	1	*H. parainfluenzae* biotype II	Hinc biotype VI	*A. aphrophilus*	Hib⁻ biotype I	Hie biotype I	Hia⁻ biotype I	Hinc biotype I
	2	*H. parainfluenzae* biotype II	Hinc biotype VI	*H. aphrophilus*	Hib⁻ biotype I	Hie biotype I	Hia biotype I	Hinc biotype I
	3	*H. parahaemolyticus*	Hinc	*H aphrophilus*	Hib⁻	Hie	Hia	Hinc
	4	*H. parainfluenzae*	Hib	not *Haemophilus*	Hib	Hie	Hid	Hif
	5	*H. parainfluenzae*	Hinc	*H. aphrophilus*	Hib⁻	Hie	Hinc	Hinc
	6	*H. parainfluenzae* biotype II	Hinc biotype VI	*H. aphrophilus*	Hinc biotype I	Hie biotype I	Hia biotype I	Hinc biotype I
	7	*H. parahaemolyticus*	Hinc biotype VI	*A. aphrophilus*	Hib⁻ biotype I	Hie biotype I	Hinc biotype I	Hinc biotype I
	8	*H. parahaemolyticus*	Hinc biotype VI	*A. aphrophilus*	Hinc biotype I	Hie biotype I	Hinc biotype I	Hinc biotype I
	9	Not *H. influenzae*	Hinc biotype VI	not *H. influenzae*	Hib⁻ biotype I	Hie biotype I	Hia⁻ biotype I	Hinc biotype I
	10	*H. parahaemolyticus*	Hinc	*A. aphrophilus*	Hib⁻	Hie	Hia⁻	Hinc
	11	*H. parahaemolyticus*	Hinc biotype VI	*H. aphrophilus*	Hinc biotype I	Hie biotype I	Hinc biotype I	Hif
	12	*H. parainfluenzae* biotype II	Hinc biotype VI	*H. aphrophilus*	Hib biotype I	Hie biotype I	Hia biotype I	Hinc biotype I
	13	*H. parainfluenzae*	Hinc	*H. aphrophilus*	Hib	Hie	Hinc	Hinc
	14	Hib biotype IV	Hinc biotype VI	*A. aphrophilus*	Hif biotype II	Hic biotype I	Hib biotype I	Hinc biotype II
	15	*H. parainfluenzae*	*H. influenzae*	*H. paraphrophilus*	*H. influenzae*	*H. influenzae*	*H. influenzae*	*H. influenzae*
	16	*H. parainfluenzae* biotype II	Hinc biotype VI	*H. aphrophilus/ paraphrophilus*	Hib⁻	Hie biotype I	Hinc biotype I	*H. influenzae*
	17	*H. parainfluenzae* biotype II	Hinc biotype VI	*H. aphrophilus*	Hinc biotype I	Hie biotype I	Hif biotype I	Hinc biotype I
	18	*H. parainfluenzae*	*H. haemolyticus*	*H. aphrophilus*	Hib⁻ biotype I	Hie biotype I	Hinc biotype I	Hinc biotype I
	19	*H. parahaemolyticus*	Hinc biotype VI	*A. aphrophilus*	Hib⁻ biotype I	Hie biotype I	Hia⁻ biotype I	Hinc biotype I
	20	*H. parainfluenzae* biotype II	Hinc biotype VI	*H. aphrophilus non-caps*	Hib⁻ biotype I	Hie biotype I	Hinc biotype I	Hinc biotype I
	21	*H. parainfluenzae* biotype II	*H. influenzae nc* biotype VI	*A.aphrophilus*	Hib⁻ biotype II	Hie biotype I	Hia biotype I	Hinc biotype I
	22	*H. parainfluenzae*	*H. influenzae*	*H. parasuis*	Hib⁻	Hinc	Hia	Hinc
	23	*H. parainfluenzae*	Hinc	*A. aphrophilus*	Hib (Hic by slide agglutination)	Hie	Hia	Hinc
	24	*H. parainfluenzae*	Hinc	not *Haemophilus*	Hib⁻	Hie	Hinc	Hinc
	25	*H. parainfluenzae*	Hinc	not *Haemophilus*	Hib	non-b *H. influenzae*	non-b *H. influenzae*	not Haemophilus
	26	*H. parainfluenzae*	Hinc	*H. aphrophilus*	Hinc	Hie	Hif/Hie	Hif

No/incorrect identification
No/incorrect/incomplete serotyping
Incorrect biotype
PCR not done on *H. influenzae*

2.1 Antimicrobial susceptibility

Not all the laboratories determine antimicrobial susceptibilities or beta-lactamase activity of isolates. Table 5 shows the number of laboratories that reported particular antibiotics.

Table 5 Number of laboratories testing different antibiotics

Antibiotic	Number of laboratories
Ampicillin	19
Cefotaxime	13
Ciprofloxacin	13
Tetracycline	13
Chloramfenicol	12
Suxamethoxazole	12
Cefuroxime	10
Co-amoxyclav	10
Rifampicin	10
Azithromycin	7
Ceftriaxone	5
Meropenem	5
Aztreonam	4
Cefaclor	4
Imipenem	4
Clarithromycin	3
Ampicillin+sulbactam	1
Cefixime	1
Cefepime	1
Nalidixic acid	1

Table 6 National and international breakpoints (in mg/L) for *H. influenzae* and selected antibiotics

	EUCAST	BSAC	CA-SFM	SRGA	NWGA	DIN	CRG	CLSI
Date of publication	2009	2009	2009	2009	2009	*	*	2009
Agent								
Ampicillin	1/1	1/1	1/1	1/1	1/1			1/2
Co-amoxyclav	1/1	1/1	1/1	1/1	1/1			4/4
Chloramphenicol	1/2	1/2	1/2	1/2	1/2			2/4
Tetracycline	1/2	1/2	1/2	1/2	1/2			2/4
Rifampicin	0.5/0.5	0.5/0.5	0.5/0.5	0.5/0.5	-			1/2
Cefuroxime	1/2	1/2	-	1/2	1/2			4/8
Ceftriaxone	0.12/0.12	0.12/0.12	0.12/0.12	0.12/0.12	0.12/0.12			2/-

– indicates that no breakpoint is given.
** no recent publication.*
Note: Format is S≤/R>. CLSI uses the format S≤/R≥ in their tables but for comparison these have been converted to European format.

Differences in methodologies for antimicrobial susceptibility testing make it difficult to compare results from different centres. Some laboratories stated that they used ETest; some gave MIC values whilst others reported disc zones sites. Most laboratories did not state the breakpoints used to ascribe strains as sensitive or resistant. In addition, there was variation in the range of antimicrobials tested by different laboratories. For these reasons, the antimicrobial susceptibility testing data are shown, as it was submitted by the participants, together with their own interpretation (where this was made) for a selected subset of antibiotics in Tables 7 to 13. The MIC results obtained by the Antibiotic Resistance Monitoring Reference Laboratory at the HPA Centre for Infections are also shown.

Table 7 Antimicrobial susceptibility testing results and beta-lactamase test results for EQA number 200 (MIC values in µg/mL in green; zone size in blue)

Lab	Beta-lactamase	AMP	CHLOR	CIP	CLAR	COAM	CRO	CTX	CXM	RIF	SXT	TET
Intended	Negative	0.75 (S)	2.00 (S)	0.64 (S)	(S)	1.00 (S)	0.004 (S)	0.023 (S)	(S)	(S)	(S)	(S)
1	Negative	0.38 (I)	0.5	0.064	64	0.38	0.004		0.5	1	0.094	0.5
2	Negative	0.5 (S)		0.047 (S)				0.023 (S)	(S)	1.5 (S)		0.75 (S)
4	Not reported	(S)							(S)		(S)	(I)
5	Negative	0.25										
6	Negative											
7	Not reported											
11	Negative	(S)	(S)	(S)		(S)		(S)	(S)	(S)	(S)	(S)
12	Negative	(S)	(S)	(S)		(S)		(S)		(S)	(S)	
13	Negative	(S)	(S)	(S)	(R)			(S)	(S)		(S)	(S)
14	Negative	0.75	0.5	0.047		0.75	0.016		1.5	0.75	0.047	0.5
15	Not tested	16 (S)	30 (S)	30 (S)	14 (S)	26 (S)		0.023 (S)	28 (S)		23 (S)	
16	Negative	0.38 (S)	35 (S)	36 (S)		0.38 (S)	0.004	0.016 (S)	33 (S)		35 (S)	32 (S)
18	Negative	0.19	0.5	0.5				<0.016				
19	Not reported	0.125		0.16				0.004		1.5	0.094	1
20	Not reported											
21	Not reported	(S)										(R)
22	Not tested	0.19 (S)	2 (S)	0.047 (S)				0.006 (S)		0.75 (S)	0.047 (S)	
23	Not tested											
25	Not tested	(S)	(S)			(S)			(S)			(S)
26	Negative	0.75		0.023			0.016	0.64		1.5		1.5

Table 8 Antimicrobial susceptibility testing results and beta-lactamase test results for EQA number 201 (MIC values in µg/mL in green, zone size in blue)

Lab	Beta-lactamase	AMP	CHLOR	CIP	CLAR	COAM	CRO	CTX	CXM	RIF	SXT	TET
Intended	Negative	0.38 (S)	1.00 (S)	0.230 (S)	(S)	0.75 (S)	0.003 (S)	0.023 (S)	(S)	(S)	(S)	(S)
1	Negative	0.19	0.5	0.023	32	0.5	0.006		0.75	0.38	0.25	0.38
2	Negative	0.19 (S)		0.016 (S)				0.016 (S)	(S)	0.38 (S)		0.5 (S)
4	Not reported	(S)							(S)		(S)	(I)
5	Negative	0.25										
6	Negative											
7	Not reported	0.75	0.5				0.006			0.25	0.032	0.5
11	Negative	(S)	(S)	(S)		(S)		(S)	(S)	(S)	(S)	(S)
12	Negative	(S)	(S)	(S)		(S)		(S)		(S)	(S)	
13	Negative	(S)	(S)	(S)	(S)			(S)	(S)		(S)	(S)
14	Negative	0.19	0.25	0.016		0.75	0.016		1.5	0.5	0.032	0.38
15	Not tested	24 (S)	30 (S)	32 (S)	13 (S)	22 (S)		0.016 (S)	26 (S)		26 (S)	
16	Negative	0.125 (S)	32 (S)	32 (S)		0.25 (S)	0.003	0.008 (S)	28 (S)		28 (S)	30 (S)
18	Negative	0.123 (I)	0.5	0.5				<0.016				
19	Not reported	0.19		0.012				0.008		0.25	0.016	0.25
20	Negative	(S)	(S)	(S)		(S)		(S)	(S)	(S)	(S)	(S)
21	Negative	0.5 (S)										(S)
22	Not tested	0.25 (S)	0.75 (S)	0.016 (S)				0.012 (S)		0.25 (S)	0.023 (S)	
23	Negative	0.19 (S)						0.016 (S)				
25	Not tested	(I)	(S)			(S)			(S)			(S)
26	Negative	1		0.016			0.023	0.125		0.5		2

Table 9 Antimicrobial susceptibility testing results and beta-lactamase test results for EQA number 202 (MIC values in µg/mL in green, zone size in blue)

Lab	Beta-lactamase	AMP	CHLOR	CIP	CLAR	COAM	CRO	CTX	CXM	RIF	SXT	TET
Intended	Negative	0.38 (S)	0.75 (S)	0.016 (S)	(S)	0.25 (S)	0.016 (S)	0.047 (S)	(S)	(S)	(S)	(S)
1	Negative	0.5	0.38	0.012	12	1	0.032		0.5	0.5	0.094	0.38
2	Negative	0.19 (S)		0.032				0.5	28 (S)	0.5		0.19
4	Not tested	(S)							(S)		(S)	(S)
5	Negative	0.5										
6	Negative											
7	Not tested	0.38	0.38				0.012			0.5	0.064	0.38
11	Negative	(S)	(S)	(S)		(S)		(S)	(S)	(S)	(S)	(S)
12	Not tested	(S)	(S)	(S)		(S)		(S)		(S)	(S)	
13	Negative	(R)	(S)	(S)	(S)			(S)	(S)		(S)	(S)
14	Not tested	0.38	0.25	0.016		0.75	0.016		0.75	0.5	0.032	0.25
15	Not tested	24 (S)	32 (S)	34 (S)	16 (S)			0.032 (S)	26 (S)		24 (S)	
16	Negative	0.19 (S)	40 (S)	40 (S)		0.25 (S)	0.008	0.023 (S)	37 (S)		33 (S)	32 (S)
18	Negative	0.25	0.38	0.38				0.016				
19	Not tested	0.19		0.006				0.016		0.19	0.032	0.125
20	Not tested											
21	Negative	(S)										(S)
22	Not tested	0.38 (S)	0.5 (S)	0.016 (S)				0.032 (S)		0.38 (S)	0.032 (S)	
23	Not tested											
25	Not tested											
26	Not tested											

Table 10 Antimicrobial susceptibility testing results and beta-lactamase test results for EQA number 203 (MIC values in µg/mL in green, zone size in blue)

Lab	Beta-lactamase	AMP	CHLOR	CIP	CLAR	COAM	CRO	CTX	CXM	RIF	SXT	TET
Intended	Positive	8.00 (R)	16.00 (R)	0.016 (S)	(S)	0.75 (S)	0.003 (S)	0.023 (S)	(S)	(S)	(S)	(R)
1	Positive	8	8	0.008	4	1	0.008		0.75	0.125	0.19	12
2	Positive	>256 (R)		0.008				0.016	(S)	0.25 (S)		12 (R)
4	Positive	(R)							(S)		(S)	(R)
5	Positive					1.5		0.015				
6	Positive											
7	Positive	1.5	8				0.004			0.125	0.064	12
11	Positive	(R)	(R)	(S)		(S)		(S)	(S)	(S)	(S)	(R)
12	Positive	16 (R)	4 (R)	(S)		(S)		(S)		(S)	(S)	
13	Positive	(R)	(S)	(S)	(S)			(S)	(S)		(S)	(R)
14	Positive	16	6	0.008		1.5	0.016		1.5	0.25	0.064	6
15	Not tested	18 (S)	22 (S)	30 (S)	16 (S)	22 (S)		0.032 (S)			20 (S)	
16	Positive	8 (R)	14 (R)	35 (S)		0.38 (S)	0.003	0.012	28 (S)		26 (S)	17 (R)
18	Positive	12	4	0.008				<0.016				
19	Positive	2		0.004				0.008		0.125	0.047	12
20	Positive	4 (R)	8 (R)									4 (R)
21	Positive	3 (R)				(S)						(R)
22	Not tested	3 (R)	8 (R)	0.006 (S)				0.016 (S)		0.125 (S)	0.064 (S)	
23	Positive	12 (R)						0.023 (S)				
25	Not tested											
26	Positive	24		0.006			0.032	0.125		0.25		16 or 24

Table 11 Antimicrobial susceptibility testing results and beta-lactamase test results for EQA number 204 (MIC values in µg/mL in green, zone size in blue)

Lab	Beta-lactamase	AMP	CHLOR	CIP	CLAR	COAM	CRO	CTX	CXM	RIF	SXT	TET
Intended	Positive	256 (R)	1.00 (S)	0.023 (S)	(S)	1.00 (S)	0.006 (S)	0.047 (S)	(S)	(S)	(S)	(S)
1	Positive	>256	0.38	0.012	12	0.75	0.012		0.75	0.19	0.19	0.25
2	Positive	>256 (R)		0.008				0.016	(S)	0.25 (S)		0.5
4	Positive	(R)							(S)		(S)	(S)
5	Positive					2		0.03				
6	Positive											
7	Positive	4	0.75				0.008			0.19	0.064	0.5
11	Positive	(R)	(S)	(S)		(S)		(S)	(S)	(S)	(S)	(S)
12	Positive	32 (R)	(S)	(S)		(S)		(S)		(S)	(S)	
13	Positive	(R)	(S)	(S)	(S)			(S)	(S)		(S)	(S)
14	Positive	48	0.25	0.016		1.5	0.016		1.5	0.19	0.004	0.38
15	Not tested	14 (R)		30 (S)	16 (S)	22 (S)		0.032 (S)			20 (S)	
16	Positive	>256 (R)	35 (S)	29 (S)		0.5 (S)	0.008	0.012	26 (S)		26 (S)	30 (S)
18	Positive	64	0.38	0.012				< 0.016				
19	Positive	2		0.006				0.016		0.19	0.047	0.25
20	Positive	8 (R)										
21	Positive	8 (R)				(S)						(S)
22	Not tested	>256 (R)	0.75 (S)	0.008 (S)				0.016 (S)		0.19 (S)	0.064 (S)	
23	Positive	>256 (R)						0.023 (S)				
25	Not tested	(R)	(S)			(S)			(S)			(S)
26	Positive	64		0.008			0.023	0.19		0.5		1.5

Table 12 Antimicrobial susceptibility testing results and beta-lactamase test results for EQA number 205 (MIC values in µg/mL in green, zone size in blue)

Lab	Beta-lactamase	AMP	CHLOR	CIP	CLAR	COAM	CRO	CTX	CXM	RIF	SXT	TET
Intended	Negative	0.38 (S)	1.50 (S)	0.012 (S)	(S)	0.75 (S)	0.003 (S)	0.023 (S)	(S)	(S)	(S)	(S)
1	Negative	0.19	0.5	0.008	24	0.5	0.006		0.5	0.19	0.094	0.5
2	Negative	0.064 (S)		0.016 (S)				0.008 (S)	(S)	0.5		0.5
4	Not reported	(S)							(S)		(S)	(S)
5	Negative	0.25										
6	Negative											
7	Not reported	0.125	1				0.003			0.19	0.094	0.38
11	Negative	(S)	(S)	(S)		(S)		(S)	(S)	(S)	(S)	(S)
12	Not reported	(S)	(S)	(S)		(S)		(S)		(S)	(S)	
13	Negative	(S)	(S)	(S)	(S)			(S)	(S)		(S)	(S)
14	Negative	0.094	0.38	0.008		0.25	0.016		0.5	0.25	0.064	0.25
15	Not tested	38 (S)		32 (S)	19 (S)	32 (S)		0.008 (S)	32 (S)		28 (S)	
16	Negative	0.125 (S)	32 (S)	32 (S)		0.25 (S)	0.003	0.006 (S)	29 (S)		27 (S)	32 (S)
18	Negative	2.125	0.75	0.006				<0.016				
19	Not tested	0.125		0.004				0.006		0.19	0.047	0.25
20	Negative	(S)	(S)	(S)				(S)	(S)	(S)	(S)	(S)
21	Negative	0.38 (S)										(I)
22	Not tested	0.19 (S)	0.75 (S)	0.006 (S)				≥0.016 (S)		0.19 (S)	0.047 (S)	
23	Negative	0.19 (S)						0.016 (S)				
25	Not tested	(S)	(S)			(S)			(S)			(I)
26	Negative	0.75		0.008			0.023	0.125		0.5		1.5

Table 13 Antimicrobial susceptibility testing results and beta-lactamase test results for EQA number 206 (MIC values in µg/mL in green, zone size in blue)

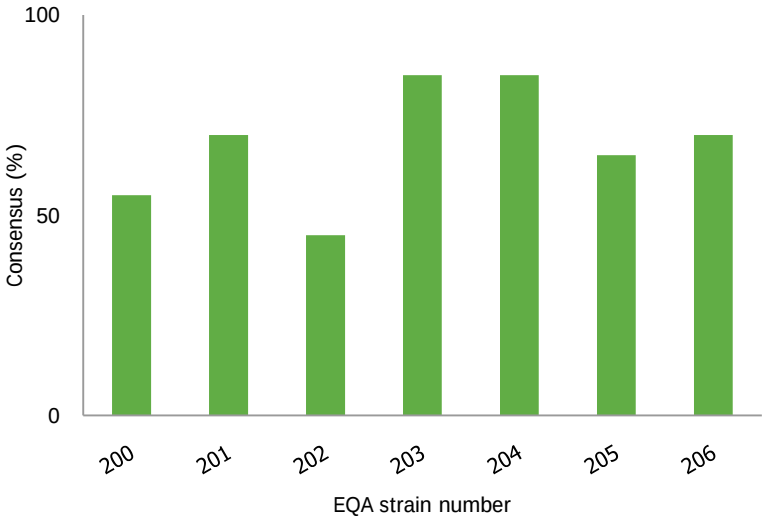
Lab	Beta-lactamase	AMP	CHLOR	CIP	CLAR	COAM	CRO	CTX	CXM	RIF	SXT	TET
Intended	Negative	4.00 (R)	1.50 (S)	0.023 (S)	(S)	8.00 (R)	0.064 (S)	0.025 (S)	(R)	(S)	(S)	(R)
1	Negative	4	0.5	0.016	48	6	0.125		16	0.5	0.19	8
2	Negative	2 (I)		0.016				0.19	(S)	0.5 (S)		8 (R)
4	Negative	3 (I)							16 (R)		(S)	(R)
5	Negative	4				8		0.25				
6	Negative											
7	Not reported	3	0.5				0.19			0.38	0.125	6
11	Negative	(S)	(S)	(S)		(S)		(S)	(S)	(S)	(S)	(R)
12	Negative	4 (R)	(S)	(S)		4 (R)		0.5	4	(S)	(S)	
13	Negative	(R)	(S)	(S)	(S)			(S)	(S)		(S)	(R)
14	Negative	6	0.5	0.016		6	0.19		12	0.25	0.125	6
15	Not tested	22 (S)	34 (S)	24 (S)	14 (S)	21 (S)		0.25 (S)	14 (R)		18 (S)	
16	Sterile											
18	Negative	2	1	0.016				0.125				
19	Negative	2		0.012				0.125		0.25	0.064	6
20	Negative	4 (R)				4/2 (R)		0.25	>8 (R)			>4 (R)
21	Not reported	3 (R)				(R)						(R)
22	Not tested	3 (R)	0.75 (S)	0.012 (S)				0.25 (S)		0.25 (S)	0.125 (S)	
23	Negative	3 (R)						0.025 (S)				
25	Not tested	(S)	(S)			(R)			(R)			(R)
26	Negative	<0.016		0.012			0.5	1		0.75		16 or 24

Table 14 Beta-lactamase testing

EQA number	Intended	Negative	Positive	Not tested	Not reported
200	Negative	**11/20 (55%)**	0/20 (0%)	4/20 (20%)	5/20 (25%)
201	Negative	**14/20 (70%)**	0/20 (0%)	3/20 (15%)	3/20 (25%)
202	Negative	**9/20 (45%)**	0/20 (0%)	11/20 (55%)	0/20 (0%)
203	Positive	0/20 (0%)	**17/20 (85%)**	3/20 (15%)	0/20 (0%)
204	Positive	0/20 (0%)	**17/20 (85%)**	3/20 (15%)	0/20 (0%)
205	Negative	**13/20 (65%)**	0/20 (0%)	4/20 (20%)	3/20 (15%)
206*	Negative	**14/20 (70%)**	0/20 (0%)	3/20 (15%)	2/20 (10%)

**One laboratory reported the sample as 'sterile'.*

Figure 5 Beta-lactamase testing



2.2 Strain 200

This strain was *Haemophilus parainfluenzae,* biotype II, beta-lactamase negative and fully susceptible to ampicillin, chloramphenicol, co-amoxyclav, ciprofloxacin, clarithromycin, rifampicin, sulphamethoxazole and tetracycline.

This strain did exhibit beta-haemolysis on horse blood agar, a characteristic shown by a small number of strains of *H. parainfluenzae.* However, this characteristic means that, by conventional methods, the strain could legitimately be identified as *H. parahaemolyticus.* One method for differentiating the two species is by sequencing the *sodA* gene [1]. Such molecular methods are not available in all reference laboratories and therefore an identification of *H. parainfluenzae* or *H. parahaemolyticus* has been regarded as correct in this EQA.

This strain provides a good example of the challenges posed by the genus *Haemophilus* when using conventional identification methods.

There were no problems with the beta-lactamase testing or the antimicrobial susceptibility tests reported for this strain.

2.3 Strain 201

This strain was non-capsulated strain of *Haemophilus influenzae,* biotype VI, beta-lactamase negative and fully susceptible to ampicillin, chloramphenicol, co-amoxyclav, ciprofloxacin, clarithromycin, rifampicin, sulphamethoxazole and tetracycline.

This strain may give cross-reactions with type b, c and d antisera when tested by slide agglutination. Capsular based PCR typing will confirm the type of strains of *H. influenzae.*

One laboratory identified the strain as *H. influenzae* type b. This laboratory does not currently use PCR to type isolates. One laboratory identified the strain as *H. haemolyticus,* they commented that since it was OMP positive on PCR it should be *H. influenzae*, but as it was haemolytic on horse blood agar they decided it was *H. haemolyticus.* This strain was not haemolytic according to the providers' results.

There were no problems with the beta-lactamase testing or the antimicrobial susceptibility testing.

2.4 Strain 202

This strain was *Aggregatibacter aphrophilus.* The species *Haemophilus aphrophilus* and *H. paraphrophilus* have recently been reclassified as *Aggregatibacter aphrophilus* comb.nov [2]. The majority of laboratories correctly identified this strain, the names *H. aphrophilus, H. paraphrophilus* and *A. aphrophilus* are all considered to be correct. One laboratory incorrectly identified the strain as the porcine pathogen *H. parasuis,* on the basis of 16SrRNA sequencing. Three laboratories said it was 'not haemophilus' and one said it was 'not *H. influenzae'*.

This strain was beta-lactamase negative and fully susceptible to ampicillin, chloramphenicol, coamoxyclav, ciprofloxacin, clarithromycin, rifampicin, sulphamethoxazole and tetracycline.

There were no problems with the antimicrobial susceptibility testing.

2.5 Strain 203

This strain was a capsule-deficient strain of *H. influenzae* type b (so-called Hib$^-$ strain), biotype I, beta-lactamase positive and chloramphenicol acetyl transferase (CAT) positive. This strain was resistant to ampicillin, chloramphenicol and tetracycline.

Capsule production in *H. influenzae* depends on a cluster of genes in the 18Kb *cap* locus. The *bexA* gene within the *cap* locus is essential for the export of capsular polysaccharide to the cell surface. The majority of Hib strains contain a tandem repeat of the *cap b* locus with one complete copy of the *bexA* gene and one truncated copy of the *bexA* gene. Capsule deficient mutants of type b strains (Hib$^-$) have a single copy of the *cap b* locus possessing a deletion in the *bexA* gene and these strains are unable to export capsular polysaccharide to the cell surface. Such strains will often appear non-capsulated by conventional serotyping, but can be detected by PCR.

Fourteen laboratories identified the strain as Hib$^-$, six identified it as Hib and sixidentified it as a non-capsulated strain of *H. influenzae.* Ten laboratories did not perform PCR, six stated that the strain was non-capsulated, three stated that it was Hib and one stated that it was Hif. Without PCR, this strain could appear to be non-capsulated or possibly Hib. The identification of Hif suggests a false positive agglutination reaction.

Eighteen laboratories tested for beta-lactamase and all correctly identified the strain as beta-lactamase positive. Two laboratories that performed antimicrobial susceptibility testing did not perform a beta-lactamase test and one of these laboratories described the strain as susceptible to both ampicillin and chloramphenicol. Ampicillin disc sensitivity testing is unreliable with beta-lactamase-positive strains of *H influenzae* and a beta-lactamase test should be performed.

2.6 Strain 204

This was a strain of *H. influenzae* serotype e, biotype I, beta-lactamase positive, ampicillin resistant, but otherwise susceptible to co-amoxyclav, chloramphenicol, cefuroxime, ceftriaxone, cefotaxime, tertracycline, rifampicin and sulphamethoxazole.

One laboratory, that did not perform PCR, misidentified this strain as Hic, and one laboratory that did perform PCR called it a non-capsulated strain of *H. influenzae.*

All of the antimicrobial susceptibility tests were correct.

2.7 Strain 205

This was a strain of a capsule-deficient strain of *H. influenzae* type a (a so-called Hia$^-$ strain).

This strain was biotype I, beta-lactamase negative and fully susceptible to a wide range of antimicrobials (ampicillin, chloramphenicol, co-amoxyclav, ciprofloxacin, clarithromycin, rifampicin, sulphamethoxazole and tetracycline).

One laboratory misidentified the strain as Hif, one stated it was Hib, one stated it was Hid and one said it was Hif/e. Three of the laboratories giving the incorrect serotype did not perform PCR and the fourth gave PCR results that were difficult to interpret (PCRpositive/negative). The erroneous serotyping results almost certainly reflect problems of cross-reactions with typing antisera, which may occur with strains of *H. influenzae.* These problems can be resolved by the use of PCR-based capsular genotyping.

There were no problems with the biotyping, beta-lactamase testing or antimicrobial susceptibility testing.

2.8 Strain 206

This was a non-capsulated strain of *H. influenzae*, biotype I, beta-lactamase negative. This strain was intrinsically resistant to ampicillin, co-amoxyclav, cefuroxime and tetracycline. This was a beta-lactamase-negative ampicillin resistant (BLNAR) strain. The mechanism of resistance in BLNAR strains involves decreased affinities of penicillin-binding proteins (PBPs) for beta-lactam antibiotics. Mutations in the *ftsI gene* which encodes PBP 3 (which mediates septal peptidoglycan synthesis) results in a decreased affinity of PBPs for beta-lactam antibiotics. The commonest mechanism of ampicillin resistance is mediated by a beta-lactamase (TEM-1 or, more rarely, ROB-1).

There is some evidence that the prevalence of ampicillin resistance of *H. influenzae* in Europe may be decreasing due to a reduction in the number of beta-lactamase-positive ampicillin-resistant strains, whereas the prevalence of BLNAR strains is relatively stable [3]. The level of ampicillin resistance exhibited by BLNAR strains may be low (MIC 0.5–4.0µg/mL) and this may make their detection difficult, particularly if a breakpoint of 1µg/mL is used to define ampicillin susceptibility.

Using PCR and sequencing to detect specific mutations in the *ftsI* gene and associated PBP 3 substitutions, strains can be categorised as low BLNAR or high BLNAR. Low BLNAR usually have ampicillin MICs in the range 0.5 to 2.0µg/mL, and high BLNAR have ampicillin MICs in the range 1.0 to 16.0µg/mL. Garcia-Cobos et al [4] suggest that low BLNAR strains are best detected by broth dilution methods rather than disc susceptibility testing.

BLNAR strains show reduced susceptibility not only to ampicillin but also to other beta-lactam antibiotics, particularly some of the cephalosporins. Livermore et al [5] suggested that cefaclor resistance is a better indicator of a BLNAR strain than ampicillin resistance and James et al [6] used cefuroxime resistance (MIC > 4.0µg/mL) to screen for BLNAR strains. CLSI recommends that BLNAR strains are considered resistant to co-amoxyclav, cefaclor, cefuroxime despite apparent susceptibility of some strains to these antimicrobials.

In this EQA distribution, four laboratories incorrectly considered this strain to be susceptible to ampicillin.

Three laboratories incorrectly called the strain Hif and one laboratory stated that it was 'not haemophilus'. Two of the laboratories giving an incorrect serotype did not use PCR-based capsular genotyping and one used a PCR method that was difficult to interpret (PCR positive/negative).

2.9 Overall comments

The laboratory EQA scheme has shown that the European Haemophilus Reference Laboratories vary in the level to which they characterise strains referred to them, ranging from simple speciation to full identification. Similarly, some laboratories routinely serotype isolates whilst others do not. Some, but not all, laboratories perform PCR-based capsular genotyping; some laboratories routinely perform antimicrobial susceptibility testing whilst others do not.

This EQA distribution scheme identified some problems with the use of slide agglutination for serotyping. The results can be misinterpreted when there are problems such as non-specific agglutination, cross-reactions and auto-agglutination. In a recent study Satola et al [7] found that *H. influenzae* isolates were misidentified by conventional *H. influenzae* serotyping in 17.5% of cases: discrepancies varied by serotype and usually resulted in over-reporting of genotypically non-capsulated *H. influenzae* as encapsulated strains.

The results of this EQA exercise suggest that some laboratories, who do perform PCR-based capsular typing, only use b primers and do not test for other capsular types.

The antimicrobial susceptibility testing results proved very difficult to interpret, as in many instances (but not all) the methodology and the breakpoints used were not defined. Some laboratories gave MIC values, whilst others gave zone sizes, with or without an interpretation of the results.

There are six European National Breakpoint Committees, which until recently set their own MIC breakpoints and defined the standard methodology to be used for antimicrobial susceptibility testing in their country. All of these committees are participating in the EUCAST harmonisation process. However, the harmonisation process was only completed earlier in 2009 so it is possible that some have not yet been implemented in national schemes.

There are major differences between the EUCAST breakpoints and those set by CLSI (formerly NCCLS), as shown in Table 6. It should be noted that, at the time of writing this report, the stated MIC breakpoints for *Haemophilus influenzae* when using ETests are the CLSI breakpoints (EAS 005). This document was last updated in June 2007 and can be viewed at: http://www.abbiodisk.com/pdf/eas/M0000144.pdf.

Some laboratories returned their results electronically and in a clearly legible form. Others faxed the results. In some cases the results were handwritten and in a few cases deciphering the writing was quite challenging.

3 Conclusions

A certain degree of heterogeneity has been detected when characterising *H. influenzae* among the countries. This fact underpins the idea that agreement and consensus in methods for characterising and accurately identifying this pathogen are needed. Moreover, some countries would need support for capacity building regarding this area.

According to the EQA exercise results, the use of PCR-based genotyping methods would provide a serotype/genotype for strains giving inconclusive results on slide agglutination. 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 is used, since it is essential to be able to accurately identify Hib vaccine failures. In addition, molecular capsular typing can act as a quality control measure to monitor the accuracy of the results of conventional serotyping.

The results for antimicrobial susceptibility testing proved difficult to interpret due to the use of different methods and breakpoints and sometimes these two items were not specified by the laboratories. Provided that there are different interpretations according to the breakpoints applied, it would be recommendable in the future that European laboratories will adopt the EUCAST methods of antimicrobial susceptibility testing, which should facilitate the comparison of results from different laboratories (http://www.EUCAST.org).

The use of an electronic form completed online by the laboratories would guarantee an easer reporting and transcription of the results.

Nonetheless, the overall results of the EQA distribution were excellent and compare favourably with the results from the EQA distribution that took place in 2007 under the auspices of EU-IBIS.

References

[1] Cattoir V, Lemenand O, Avril JL ,Gaillot O. The sodA gene as a target for phylogenetic dissection of the genus Haemophilus and accurate identification of human clinical isolates. Int J Med Microbiol 2006; 296:531-40.

[2] Nørskov-Lauritsen N, Kilian M. Reclassification of *Actinobacillus actinomycetemcomitans, Haemophilus aphrophilus, Haemophilus paraphrophilus* and *Haemophilus segnis* as *Aggregatibacter actinomycetemcomitans* gen. nov., comb. nov., *Aggregatibacter aphrophilus* comb. nov. and *Aggregatibacter segnis* comb. nov., and emended description of *Aggregatibacter aphrophilus* to include V factordependent and V factor-independent isolates. Int J Syst Evol Micr 2006;56:2135-46.

[3] Jansen WT, Verel A, Beitsma M, Verhoef J, Milatovic D. Longitudinal European surveillance study of antibiotic resistance of *Haemophilus influenzae.* J Antimicrob Chemoth 2006;58:873-7.

[4] Garcia-Cobos S, Campos J, Lázaro E, Roman F, Cercenado E, García-Rey C et al. Low β-lactamase-negative ampicillin resistant *Haemophilus influenzae* strains are best detected by testing amoxicillin susceptibility by the broth microdilution method. Antimicrob Agents Ch 2008; 52:2407-14.

[5] Livermore DM, Winstanley TG,Shannon KP. Interpretative reading: recognizing the unusual and inferring resistance mechanisms from resistance phenotypes. J Antimicrob Chemoth 2001;48(Suppl. 1):87-102.

[6] James PA, Lewis DA, Jordens J, Cribb J, Dawson SJ, Murray SA. The incidence and epidemiology of betalactam resistance in *Haemophilus influenzae.* J Antimicrob Chemoth 1996;37:737-46.

[7] Satola SW, Collins JT, Napier R, Farley MM. Capsule gene analysis of invasive *Haemophilus influenzae*: accuracy of serotyping and prevalence of IS1016 among nontypeable isolates. J Clin Microbiol 2007;35(10):3230-8.

Annex 1: Participating reference laboratories

Country	Contact person	Institution
Austria	Dr Sigrid Heuberger	National Reference Centre for Meningococci, Pneumococci and *Haemophilus influenzae* Austrian Agency for food and Health Safety Beethovenstraße 6 8010 Graz, Austria
Belgium	Dr Françoise Crokaert	Reference Laboratori Institut Jules Bordet 1 rue Héger Bordet 1000 Bruxelles, Belgium
Czech Republic	Dr Vera Lebedova	National Reference Laboratory for Haemophilus Infections Centre of Public Health Laboratories National Institute of Public Health Srobarova 48 100 42 Prague 10, Czech Republic
Denmark	Lotte Lambertsen	Neisseria and Streptococcus Reference Laboratory Department of Bacteriology, Mycology and Parasitology Statens Serum Institut 5 Artillerivej, building 211/117B 2300 Copenhagen, Denmark
England and Wales	Dr Mary Slack	*Haemophilus* Reference Unit Specialist and Reference Microbiology Divison Health Protection Agency 61 Colindale Avenue London NW9 5HT, UK
Estonia	Dr Unna Jöks	Central Laboratory for Microbiology Health Protectorate Inspectorate Kotka 2 11315 Tallinn, Estonia
Finland	Dr Anni Virolainen-Julkunen	National Institute for Health and Welfare (THL) PO Box 30 00271 Helsinki, Finland
France	Prof Henri Dabernat	Centre National de Référence des *Haemophilus influenzae* Laboratoire de Microbiologie Centre Hospitalier Universitaire de Toulouse Hôpital Purpan Place du docteur Baylec 31059 Toulouse Cedex 9, France
Germany	Prof Dr Matthias Frosch and Prof Dr Ulrich Vogel	Institute for Hygiene and Microbiology University of Würzburg Josef-Schneider-Straße 2 97080 Würzburg, Germany
Greece	Dr Georgina Tzanakaki	National Meningitis Reference Laboratory National School of Public Health 196 Alexandras Avenue 115 21 Athens, Greece
Hungary	Dr Ákos Tóth	Department of Bacteriology Johan Bela National Centre for Epidemiology Gyali ut 2-6 1097 Budapest, Hungary
Iceland	Dr Hjordis Hardardóttoir	Department of Clinical Microbiology Institute of Laboratory Medicine Landspitali University Hospital Baronsstigur, 101 Reykjavik, Iceland
Ireland	Prof Mary Cafferkey	Irish Meningococcal and Meningitis Reference Laboratory Children's University Hospital Temple Street Dublin 1, Ireland
Italy	Dr Marina Cerquetti	Department of Infectious, Parasitic and Immunomediated Diseases Instituto Superiore di Sanitá Viale Regina Elena 299 00161 Rome, Italy

Country	Contact person	Institution
Latvia	Dr Ruta Pabērza	Laboratory of the State Agency Infectology Center of Latvia Bacteriology Department 3 Linezera street Riga, LV 1006, Latvia
Lithuania	Dr Snieguole Dauksiene	Microbiological Department National Public Health Surveillance Laboratory Zolyno str. 36 10210 Vilnius, Lithuania
Luxembourg	Dr Jos Even	Laboratoire National de Santé 42 rue du Laboratoire L-1911 Luxembourg, Luxembourg
Malta	Dr Jackie Maistre Melillo	Disease Surveillance Unit Department of Public Health 37-39 Rue D'Argens Msida MSD 05, Malta
Netherlands	Dr Lodewijk Spanjaard	Netherlands Reference Laboratory for Bacterial Meningitis Department of Medical Microbiology Academic Medical Canter, L-1-Z Meibergdreef 15 1105 AZ Amsterdam, Netherlands
Norway	Dr Arne E Hoiby	Division of Infectious Disease Control Norwegian Institute of Public Health Lovisenberggata 8 0403 Oslo, Norway
Poland	Dr Alicja Kuch/Dr Anna Skoczynska	National Reference Centre for Bacterial Meningitis Department of Epidemiology and Clinical Microbiology National Medicines Institute Chelmska Street 30/34 00-725 Warsaw, Poland
Portugal	Dr Paula Lavado	Departamento de Doenças Infecciosas Laboratório Nacional de Referência de Infecções Respiratórias (agentes bacterianos) Instituto Naional de Saúde Dr Ricardo Jorge Avenida Padre Cruz 1649-016 Lisboa, Portugal
Rumania	Dr Veronica Alecu	Cantacuzino Institute Bacterial Respiratory Infections 102 Splaiul Independentei, Sector 5 C.P.1-525 Bucharest, Romania
Scotland	Dr Edwards Giles	Scottish Meningococcus and Pneumococcus Ref. Lab Stobhill Hospital Balornock Road Glasgow G21 3UW, UK
Slovak Republic	Dr Elena Nováková	National Reference Centre for Haemophilus Infections Regional Public Health Authority RUVZ-NRC HI V Spanyola 27 01171 Žilina, Slovak Republic
Slovenia	Dr Metka Paragi	Head of Laboratory for Immunology and Molecular Diagnostics Institute of Public Health Slovenia Grablovičeva 44 1000 Ljubljana, Slovenia
Spain	Dr José Campos	Centro Nacional de Microbiología Instituto de Salud Carlos III, Ctra Majadahonda-Pozuelo Km 2 28220 Madrid, Spain
Sweden	Prof Birgitta Henriques Normark	Department of Bacteriology Swedish Institute for Infectious Disease Control Nobels väg 18 SE-171 82 Solna, Sweden

Note: Liechtenstein and Malta did not participate in the EQA exercise. The typing of the strains for Malta was done by UK reference laboratory.

Annex 2: Cover letter / Terms and conditions of participation

This letter was sent to all the laboratories participating in the EQA exercise for requesting the agreement of the participants to the terms and conditions of the EQA distribution.

Dear XXXX,

In the next few weeks your institute will be taking part in EQA schemes for *Haemophilus influenzae* and/or *Neisseria meningitidis* as part of the *Laboratory surveillance and External Quality Assurance (EQA) of invasive bacterial diseases in EU* project. The samples for the EQA will be sent to you from the Health Protection Agency (HPA) by agreement of the University of Würzburg.

Before the HPA can send the samples we need you to sign and return the attached conditions of participation agreement. Please obtain signature on behalf of your institute and fax the document back to me on +44 XXXXXX and send the original in the post to:

Business Development Department
Health Protection Agency
Centre for Infections
61 Colindale Avenue
London NW9 5EQ
England

Thank you for your co-operation.

Yours sincerely,

Annex 3: Terms and conditions of participation

Health Protection Agency ("HPA")

N. meningitidis EQA Scheme and or *H.influenzae* EQA Scheme ("Scheme")

1. Samples distributed as part of the Scheme may contain microbiological pathogens of Hazard Groups 1 and 2 as defined by the Advisory Committee on Dangerous Pathogens (The Approved List of Biological Agents , HMSO, 2004) ("Samples"). Participants must ensure and warrant that their laboratory facilities and expertise are adequate to ensure the safe handling of the Samples during their participation in the Scheme and any IQ Use.

2. The Samples shall be used for the purpose of participation in the Scheme only. In addition the Participant may use the Samples or derivatives thereof ("Materials" which expression shall include constructs, strains, derivatives, portions, progeny or improvements obtained from or as a result of the use of the Materials) for other internal quality use by the Participant outside of the Scheme ("IQ Use"). The Materials shall not be passed on to any other party

3. Participants will process the quality assessment Samples in the same way as their routine samples. This is necessary to achieve the primary purpose of the Scheme, which is to allow participants an insight into their levels of performance in routine work.

4. Each participant laboratory will be registered under a unique code number.

5. All reports, and the data they contain, issued by the HPA are Copyright and may not be published in any form without prior permission of the HPA.

6. Participants in the Scheme have entire responsibility for all Samples distributed to them under the Scheme and all activities carried out by them or any third party in relation to the Samples from the time of receipt of the Samples.

7. HPA warrants that all work carried out by it in relation to the Scheme will be carried out using all reasonable care and skill. All conditions, terms and warranties implied by common law, statute or otherwise are, to the extent permitted by law, hereby excluded.

8. The total liability of the HPA to the participant resulting from or in connection with the provision of any or all of the Samples or Materials provided by the HPA to the Participant, or the provision of the Scheme by the HPA to the participant or IQ Use by the Participant shall be for death and personal injury resulting from HPA's negligence or in any other circumstances where liability may not be so limited under any applicable law in England and Wales.

9. HPA shall not be liable in any circumstances for indirect or consequential loss howsoever caused, including, without limitation, loss of anticipated profits, goodwill, reputation, business receipts or contracts, or losses or expenses resulting from third party claims.

10. If the Recipient wishes to submit for publication results from IQ Use of the Materials, the Recipient shall provide HPA with a copy of the final proposed publication at least sixty (60) days prior to its submission. HPA shall within thirty (30) days of receipt provide in writing any reasonable objections it has to the proposed publication and the Recipient shall give due regard to any amendments required by HPA and shall refrain from publication of any information in respect of the Materials which in HPA's reasonable opinion is damaging to its interests

11. The Recipient agrees to inform HPA of any intellectual property or product arising from use of the Materials and, prior to any commercial exploitation of such intellectual property or product, to negotiate with HPA terms properly reflecting the contribution of the Materials.

12. (a) These conditions and any dispute or claim arising out of or in connection with them or their subject matter or formation (including non-contractual disputes or claims) shall be governed by and construed in accordance with the law of England and Wales.

(b) The parties irrevocably agree that the courts of England and Wales shall have exclusive jurisdiction to settle any dispute or claim that arises out of or in connection with these conditions or their subject matter or formation (including non-contractual disputes or claims).

13. The recipient will inform HPA of receipt of the Samples within 5 working days.

14. If you agree to the above conditions, please sign, date and return a copy of these conditions to Business Development Department, HPA Centre for Infections, 61 Colindale Avenue, London NW9 5EQ, England.

We hereby acknowledge receipt and accept the conditions outlined above.

Signed .....

Name .....

For and on behalf of

Name of Recipient Organisation .....

Address .....

.....

.....

Date .....