TECHNICAL REPORT

External quality assessment scheme for *Streptococcus pneumoniae*

2014

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ECDC TECHNICAL REPORT

External quality assessment scheme for *Streptococcus pneumoniae* – 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 Assimoula Economopoulou, and produced by Mary Slack (Institut für Hygiene und Mikrobiologie, Universität Würzburg, Germany, and School of Medicine, Griffith University, Queensland, Australia), Dr Carmen Sheppard (Public Health England, UK) and Dr David Litt (Public Health England, UK) on behalf of the IBD-labnet consortium participants (referring to specific contract No.4 ECDC.4857).

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Abbreviations

AMRHAI	Antimicrobial Resistance and Healthcare Associated Infections Reference Unit (Public Health England)
CAP	Community-acquired pneumonia
CLSI	Clinical and Laboratory Standards Institute
CSF	Cerebrospinal fluid
EQA	External quality assessment
eQAD	External Quality Assurance Department of Public Health England
EUCAST	European Committee on Antimicrobial Susceptibility Testing
I	Intermediate
IBD-labnet	Invasive Bacterial Disease laboratory network (funded by ECDC)
IPD	Invasive pneumococcal disease
MIC	Minimum inhibitory concentration
MLST	Multilocus sequence typing
PCR	Polymerase chain reaction
PCV7	7-valent pneumococcal conjugate vaccine
PCV10	10-valent pneumococcal conjugate vaccine
PCV13	13-valent pneumococcal conjugate vaccine
PHE	Public Health England
PPV23	23-valent plain pneumococcal polysaccharide vaccine
R	Resistant
RVPBRU	Respiratory and Vaccine-Preventable Bacteria Reference Unit (Public Health England)
S	Susceptible

Executive summary

Streptococcus pneumoniae (the pneumococcus) is the causative agent of a wide spectrum of diseases ranging from upper respiratory tract infections, including otitis media and sinusitis, to severe invasive disease. *S. pneumoniae* is the most frequently isolated respiratory pathogen in community-acquired pneumonia (CAP). Invasive pneumococcal disease (IPD), which is defined as the isolation of pneumococci or the detection of pneumococcal nucleic acid in normally sterile body fluids (blood, CSF, joint fluid, etc.), may present as meningitis, bacteraemic pneumonia, septic arthritis or peritonitis (Lynch and Zhanel 2009; Lynch and Zhanel 2010).

Almost all strains of the pneumococcus have a polysaccharide capsule, which is a major virulence determinant contributing to evasion of the host immune system (Bentley et al. 2006). It also forms the basis for pneumococcal serotyping. So far, 94 distinct serotypes have been identified.

Prevention of invasive pneumococcal disease can be achieved by vaccination. There are two types of pneumococcal vaccine: a 23-valent plain polysaccharide vaccine and conjugate vaccines which contain an immunogenic non-pneumococcal protein conjugated to the pneumococcal polysaccharides. Currently there are three conjugated pneumococcal vaccines, PCV7, PCV10 and PCV13 which target seven, ten and thirteen pneumococcal serotypes, respectively.

The implementation of infant immunisation with PCV7 resulted in a dramatic decline in IPD caused by vaccine serotypes, both among those targeted for the vaccine (direct effect) but also in older age groups (herd effect) by reducing nasopharyngeal carriage of vaccine serotypes. The nasopharynx acts as a reservoir of pneumococci from which the organisms may be transmitted to other individuals (Simell et al. 2012). Similar indirect protection has been seen for the additional serotypes included in PCV13 in the UK (Waight et al. 2015). However, the use of PCV7 was also accompanied by a significant increase in the circulation of non-vaccine serotypes (serotype replacement), notably serotypes 1, 3, 6A, 6C, 7F and 19A (Weil-Olivier et al. 2012) and an increase in non-vaccine serotype IPD (Weinberger et al. 2011, WHO 2010, Miller et al. 2011). There is also evidence of increasing invasive pneumococcal disease caused by non-vaccine serotypes four years after PCV7 was replaced by PCV13 (Waight et al. 2015), particularly in children aged less than five years of age.

Surveillance of *S. pneumoniae* continues to be of importance, not only to establish the serotypes of *pneumococcus* causing invasive disease and to monitor the impact of the newer pneumococcal conjugate vaccines (PCV10 and PCV13) but also to assess the long-term effectiveness of pneumococcal immunisation programmes. Integrated surveillance for this pathogen entails both epidemiological and laboratory surveillance. Epidemiological surveillance systems for IPD currently vary widely across Europe (Hanquet et al. 2010), making comparison of data difficult.

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 and/or reference laboratory methods) to foster quality and comparability of data reported to The European Surveillance System (TESSy). EQA schemes or laboratory proficiency 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 July 2014 a panel of three strains of *Streptococcus pneumoniae* and two simulated samples of cerebrospinal fluid (CSF) was sent to 30 participating reference laboratories in the IBD-labnet surveillance network for quality assessment testing. The laboratories were asked to perform three tasks:

- Phenotypic characterisation of the viable isolates using their standard laboratory protocols for species identification and serotyping by serological methods. Antimicrobial susceptibility testing was also requested for those laboratories that perform antimicrobial susceptibility testing of the isolates on a routine basis.
- Molecular characterisation of the viable isolates using PCR methods for: species identification and capsule typing, if available. Multilocus sequence typing (MLST) was also requested where performed routinely.
- Molecular detection of *S. pneumoniae* in non-culture simulated CSF samples by PCR species confirmation. PCR-based capsule genotyping was not specifically requested in this assessment.

Twenty-nine laboratories returned results for this EQA exercise. The results have shown that European pneumococcal reference laboratories differ in the level of characterisation of strains, ranging from speciation, serogrouping and serotyping to genotypic characterisation of isolates. Twenty-six laboratories conducted phenotypic serotyping of the viable isolates. Fourteen laboratories performed genotypic species identification and 12 carried out genotypic capsular typing. Ten laboratories reported MLST. Twenty-eight laboratories performed antimicrobial susceptibility testing. Twenty-two laboratories attempted detection of *S. pneumoniae* in the non-viable simulated CSF samples and five of these also reported genotypic capsular typing results.

Overall, out of 242 results submitted, there were only eight errors in phenotypic characterisation of the strains (3.3%). One error was at the species level, where the strain of *S. pneumoniae* was identified as *S. salivarius.* In one case, serotype 15A pneumococcus was incorrectly identified as serotype 8; in one case, serotype 33F

pneumococcus was incorrectly typed as a serotype 4, and in three cases the wrong serotype within a serogroup was identified. Two laboratories reported that at least one of the strains was 'non-typeable'. In the last two EQA distributions in 2010 and 2012, there were 13.1% and 1.7% phenotypic errors, respectively (although the results from these panels are not directly comparable, as this EQA panel included three viable cultures and the previous distributions included five viable cultures). It is acknowledged that both the cultures and simulated CSFs included in this EQA were challenging to type by conventional and genetic methods as they were all members of serogroups containing individual serotypes.

There were no errors in the genotypic species identification of isolates. Out of 33 results for genetic capsule typing, 29 were correct to at least the group level. In one case, the 9N isolate was erroneously typed as 23B. In the other three cases the isolates were reported as 'non-typeable'. There was a single error among the reported MLST results, which was caused by a small mistake in the sequencing of one of the seven MLST loci for one strain.

The antimicrobial susceptibility testing results indicated that the majority of laboratories have little difficulty in identifying susceptible or resistant strains. Twenty laboratories are using the EUCAST criteria while six are still using Clinical and Laboratory Standards Institute (CLSI) guidelines. One laboratory did not state which criteria they were applying. This makes the comparison of results difficult. It is recommended that all European Reference laboratories move to using EUCAST guidelines as soon as possible.

The interpretation of MIC results for β-lactam antibiotics should be based on the source of the isolate (meningitis or non-meningitis) when using EUCAST or CLSI guidelines. All strains included in this EQA distribution were meningitis isolates. A further complication is the discrepancy between the CLSI and EUCAST breakpoints for benzyl penicillin and *S. pneumoniae*. In 2008, CLSI changed the interpretative standard for benzyl penicillin and *S. pneumoniae* (Weinstein et al. 2009), but the discrepancy with EUCAST breakpoints remains.

Two simulated CSF samples were included in the quality assessment panel to assess methods used for the non-culture detection of *S. pneumoniae.* Twenty-two laboratories reported their results for these samples. All 22 (100%) correctly identified *S. pneumoniae* DNA in both samples.

In conclusion, the EQA results show that the majority of European pneumococcal reference laboratories are able to identify and serotype pneumococcal isolates correctly. A few laboratories only reported pheonotypic pneumococcal typing to the serogroup level. The EQA distribution has again indicated that some laboratories lack the necessary reagents to fully serotype isolates and this renders surveillance of IPD difficult. Similarly, some laboratories' attempts to type the isolates by PCR failed due to a limited choice of PCR primer sets chosen. Regular EQA distributions for the European pneumococcal reference laboratories are recommended to ensure that the improved quality of surveillance and epidemiological reports is maintained.

Introduction

The European Centre for Disease Prevention and Control is a European Union 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 assurance schemes.' (Article 5.3, EC 851/2004[1]).

External quality assessment (EQA) is part of quality management systems and evaluates performance of laboratories, by an outside agency, 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 costs. The aim of the EQA is to identify needs for 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 assessment schemes are as follows:

- 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
- identification of needs for training activities.

Streptococcus pneumoniae (pneumococcus) is a common commensal of the upper respiratory tract and is a cause of local and invasive infections. Local infections of the respiratory tract include otitis media, sinusitis and pneumonia. Invasive pneumococcal disease (IPD) may present as a pneumonia, meningitis, septic arthritis or a bacteraemia without obvious focus. Young children, immunocompromised individuals and the elderly are at major risk of developing IPD. The World Health Organization estimates that more than 1.6 million people die of pneumococcal infection every year and half of these deaths are in children aged less than five years of age (Black et al. 2008).

Of the 94 different serotypes of pneumococcus that have to date been identified, only 20–30 are responsible for the majority of pneumococcal infections worldwide (Brueggemann et al. 2004; Isaacman et al. 2010; Weinberger et al. 2011). Among the current 94 serotypes, a limited number of serotypes cause more than 70%–80% of IPD (Hausdorff et al. 2005). Serotypes 1, 4, 5, 7F, 8, 12F, 14, 18C, and 19A are more likely to cause IPD (Hanage et al. 2005; Kronenberg et al. 2006; Sleeman et al. 2006; Yildirim et al. 2010). There is some evidence of an association between serotype and severity of disease. Serotype prevalence varies between geographic regions and may change over time in response to selective vaccine pressure or clonal spread. Furthermore, capsular switching may occur allowing the survival of specific clones and evasion of vaccine-induced immunity. In 2012, 20 785 confirmed cases of IPD were reported by 27 countries, 22 of which run surveillance systems with national coverage (ECDC 2015). The overall reported confirmed case rate was 4.28 per 100 000, comparable with the previous two years. Higher notification rates were observed in Nordic countries than in other countries, with the highest rates reported by Denmark (15.81 per 100 000), Sweden (14.63), Finland (13.92) and Norway (12.56). Luxembourg reported the lowest confirmed case rate, 0.19 per 100 000, followed by Lithuania (0.23), Bulgaria (0.26), Greece (0.39) and Romania (0.39).

A number of pneumococcal vaccines are now available. The first to be introduced was the 23-valent plain pneumococcal polysaccharide vaccine (PPV23). This vaccine is indicated for use in children over the age of two years within risk groups and for the elderly. The first pneumococcal conjugate vaccine (PCV7) was licensed in the United States in 2000 and in Europe in 2001. This vaccine contains purified capsular polysaccharide of seven pneumococcal serotypes. The introduction of PCV7 led to a dramatic fall in the incidence of IPD in young children caused by these seven serotypes. In addition, the vaccination of infants with PCV7 reduced the nasopharyngeal carriage of these serotypes, resulting in a decline in the incidence of IPD to these serotypes in older age groups through a 'herd effect'. However the use of PCV7 was associated with an increase in other serotypes, not included in the vaccine (serotype replacement) notably 19A and 7F. More recently a 10-valent (PCV10) and a 13-valent (PCV13) have been introduced.

Laboratory diagnostics and molecular epidemiology of *S. pneumoniae* are extremely important for the effective surveillance of this organism. Since the introduction of PCV10 and PCV13 in European countries it has become particularly important that disease surveillance monitors the impact of these vaccines to compare the different

[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

vaccine schedules adopted by Member States and to detect and study changes in serotype distribution and any possible serotype replacement due to vaccine pressure.

The implementation of laboratory surveillance activities, namely the external quality assessment (EQA) activities and training, have been outsourced under framework contract ECDC/08/008 to a consortium of European experts (IBD-labnet, coordinated by Prof Dr Matthias Frosch, University of Würzburg, Germany).

The specific objectives of this EQA exercise are:

- Further harmonisation of molecular typing of *S. pneumoniae*
- Further harmonisation of methods for antimicrobial susceptibility testing of *S. pneumoniae*
- Training and dissemination of methods for the laboratory surveillance of invasive bacterial infections
- Assistance to Member States, e.g. capacity building
- Support for ECDC's efforts to link laboratory surveillance data and epidemiological data.

EQA role and aims

In order to support the Member States and further build capacity, ECDC has conducted a series of EQA exercises to ensure that European laboratory surveillance delivers high-quality, standardised results and that training needs can be properly assessed. The ECDC-funded IBD-labnet EQA allows reference laboratories to compare test results so that they can achieve the same level of characterisation for both culture and non-culture-confirmed (PCR only) cases of pneumococcal disease.

This report describes the fourth ECDC-funded EQA. Previous EQA rounds took place in 2009, 2011 and 2012. All reports are available from: http://ecdc.europa.eu/en/publications/.

1 Material and methods

The objectives of this exercise were:

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

1.1 Study design

The samples were selected by C. Sheppard and D. Litt of Public Health England's (PHE) Respiratory and Vaccine-Preventable Bacteria Reference Unit (RVPBRU, PHE, Colindale, London, UK) in collaboration with M. Slack (Institut für Hygiene und Mikrobiologie, Universität Würzburg, Germany, and School of Medicine, Griffith University, Queensland, Australia). The freeze-drying and distribution of samples, collection of results, and production of a semi-automated summary report was carried out by UK NEQAS for Microbiology and facilities in the External Quality Assurance Department (eQAD), PHE, Colindale, London, under the direction of V. James and C. Walton.

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 criteria (Table 1) in the allocated time period. It was requested that all three strains be tested using standard laboratory protocols for the methods normally used by the laboratory to characterise submitted isolates of *S. pneumoniae* – namely species identification, serogrouping and serotyping – by pheonotypic or genotypic methods.

It was also requested that antimicrobial susceptibility testing (penicillin, erythromycin, ceftriaxone and ciprofloxacin) be carried out using normal laboratory procedures. For the antimicrobial susceptibility testing, participants were asked to perform MIC determinations and provide an interpretation of their results – namely whether the strains were susceptible (S), resistant (R), or of intermediate susceptibility (I).

The two simulated CSF samples were to be processed and the presence of *S. pneumoniae* DNA assayed by PCR using standard laboratory protocols.

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

Table 1. Tests requested from the participating laboratories

Procedure	Tests requested	
	Bacterial isolates	Non-culture samples (simulated CSF)
Phenotypic identification	Species	
	Serotype	
	Antimicrobial susceptibility testing	
Genotypic identification	Species	Detection of *S. pneumoniae*
	Capsule type	

This was the first EQA exercise for *Streptococcus pneumonia* organised for IBD-labnet by eQAD using a web-based reporting system via the UK NEQAS website (www.ukneqasmicro.org.uk).

1.2 Participants

Thirty European pneumococcal reference laboratories 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 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. Results were returned by 29 laboratories.

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

1.3 The EQA panel material

The EQA panel comprised three viable bacterial isolates (to test the ability of participating laboratories to identify and characterise live cultures) plus two non-viable simulated CSF samples (to test their ability to detect *S. pneumoniae* in clinical specimens using non-culture detection methods).

1.3.1 Bacterial isolates

Three viable isolates of *S. pneumoniae* were selected for the panel. These were selected to be representative of the major disease-causing serotypes and to include strains demonstrating a range of MICs to other commonly used antimicrobials.

The isolates were selected and pre-screened by staff at the PHE's Respiratory and Vaccine-Preventable Bacteria Reference Unit (RVPBRU) and Antibiotic Resistance Monitoring and Hospital-Acquired Infection Reference Laboratory (AMRHAI). The antimicrobial susceptibilities of the strains were also checked by the EUCAST Laboratory for Antimicrobial Susceptibility Testing, Sweden. 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.

1.3.2 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's collection of UK clinical isolates. Both samples contained *S. pneumoniae* DNA.

Stock solutions of the bacterial cultures were prepared and killed by heating to 100 °C for 10 minutes. These suspensions were then diluted in simulated CSF solution to achieve approximately 10^5 CFU/ml. 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 reported via the NEQAS website by 29 laboratories.

A summary of consensus results was released to participants via the UK NEQAS for Microbiology website in August 2014. An analysis of the results submitted by all of the participants was subsequently generated by UK NEQAS for Microbiology, with input from an advisory panel. This was released to all participants via the UK NEQAS for Microbiology 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 exercise 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 number of participants for a given test varies by sample (see Table 4).

2.1 Part 1: Characterisation of viable isolates

All participants confirmed that the three bacterial isolates were viable following the revival procedure. The intended results for Part 1 of the analysis are shown in Table 3. Table 4 shows the ratio of laboratories who successfully reported the intended result for each test. It also lists the results that did not match the intended result.

The percentage of all participants reporting the intended result for each test is also shown in Figures 1 to 5. In all tests for Part 1 of the study, the consensus of the submitted results matched the intended result. The percentage match varied between 64% 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[a]

Laboratory identifier	Viable isolates						Non-culture detection
	Phenotypic identification		Genotypic identification			AST	Species ID
	Species ID	Serotype	Species ID	Serotype	MLST		
NM05	-	+	-	-	-	+	+
NM06	+	+	+	+	+	+	+
NM08	+	+	+	+	+	+	+
NM09	+	+	-	-	-	-	-
NM10	+	+	-	-	+	+	+
NM11	+	+	-	-	+	+	+
NM12	+	+	-	+	+	+	+
NM14	+	+	-	-	-	+	-
NM17	+	+	+	+	+	+	+
NM20	+	+	+	+	+	+	+
NM22	+	+	-	-	-	+	+
NM23	+	-	+	+	-	+	+
NM26	+	+	+	+	-	+	+
NM33	+	+	-	+	+	+	-
NM34	+	+	-	-	+	+	+
NM36A	+	+	-	-	-	+	-
NM37	-	+	-	-	-	+	-
NM38	+	+	-	-		+	+
NM39A	+	+	+	-	-	+	+
NM40	+	+	-	-	-	+	+
NM41	+	+	+	+	+	+	+
NM43	+	+	+	-	-	+	+
NM45	+	+	+	-	-	+	+
NM47	+	-	+	+	-	+	+
NM48	+	+	+	+	-	+	+
NM49	+	+	+	-	-	+	+
NM51	+	+	+	+	-	+	+
NM52	+	+	-	-	-	+	-
NM54	-	-	-	-		+	-
Total	**26**	**26**	**14**	**12**	**10**	**28**	**22**

[a] *Laboratories did not necessarily submit a result for all samples for a given test.*

Table 3. Intended results for Part 1: characterisation of viable isolates

EQA sample	Species	Serotype	MLST
2511	*S. pneumoniae*	9N	66
2512	*S. pneumoniae*	15A	2613
2513	*S. pneumoniae*	33F	2705

Abbreviations: ID, identification; MLST, multilocus sequence typing

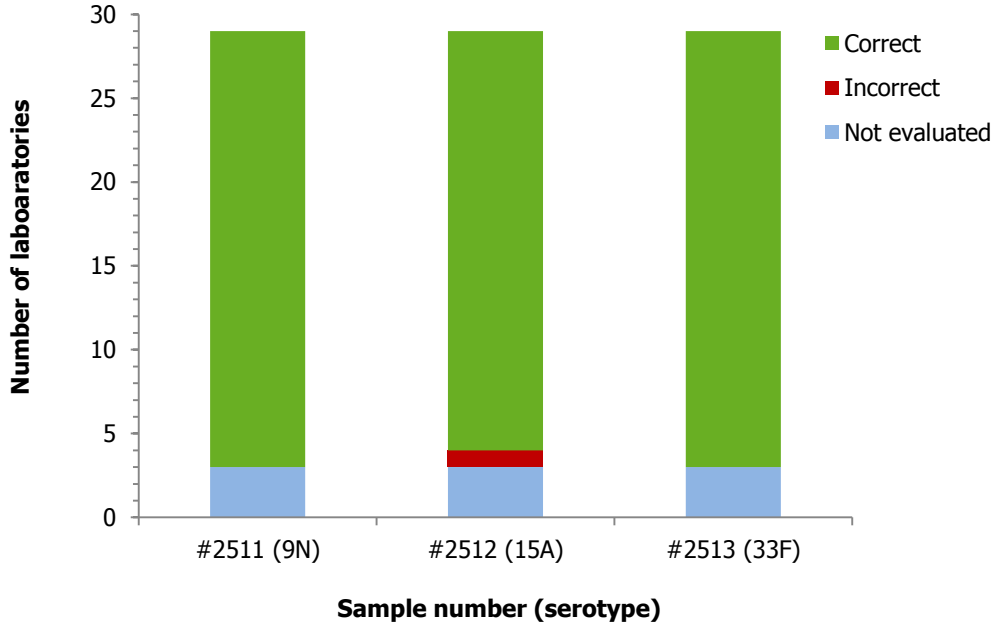
Table 4. Results for Part 1: phenotypic identification of viable isolates

Sample number	Intended result	Ratio of laboratories reporting the intended result (%)	Results not matching intended result (frequency)
Phenotypic species identification			
2511	*S. pneumoniae*	26/26 (100%)	
2512	*S. pneumoniae*	25/26 (96%)	*Strep salivarius* (1)
2513	*S. pneumoniae*	26/26 (100%)	
Phenotypic serotype			
2511	9N	21/26 (81%)	Serogroup 9 (3); 9L (1); 9V (1)
2512	15A	21/26 (81%)	Serogroup 15 (3); 8 (1); NT[d] (1)
2513	33F	17/26 (65%)	Serogroup 33 (5); 33A (2); 4 (1); NT[d] (1)
Genotypic species identification			
2511	*S. pneumoniae*	14/14 (100%)	
2512	*S. pneumoniae*	14/14 (100%)	
2513	*S. pneumoniae*	14/14 (100%)	
Genotypic capsular typing			
2511	9N, 9N/9L[a], 09N-01[c]	8/11(73%)	Group 9 (1); NT[e] (1) 23B (1)
2512	15A, 15A/F[a,b], 15A-01[c]	8/11 (73%)	Group 15 (1); 15A/B/C/F (1); NT[e] (1)
2513	33F, 33A/33F/37[a,b], 33F-03[c]	7/11 (64%)	Group 33 (2); NT[e] (2)
MLST			
2511	66	9/9 (100%)	
2512	2613	10/10 (100%)	
2513	2705	8/9 (89%)	1514 (1)

Abbreviations: NT, non-typeable; MLST, multilocus sequence typing

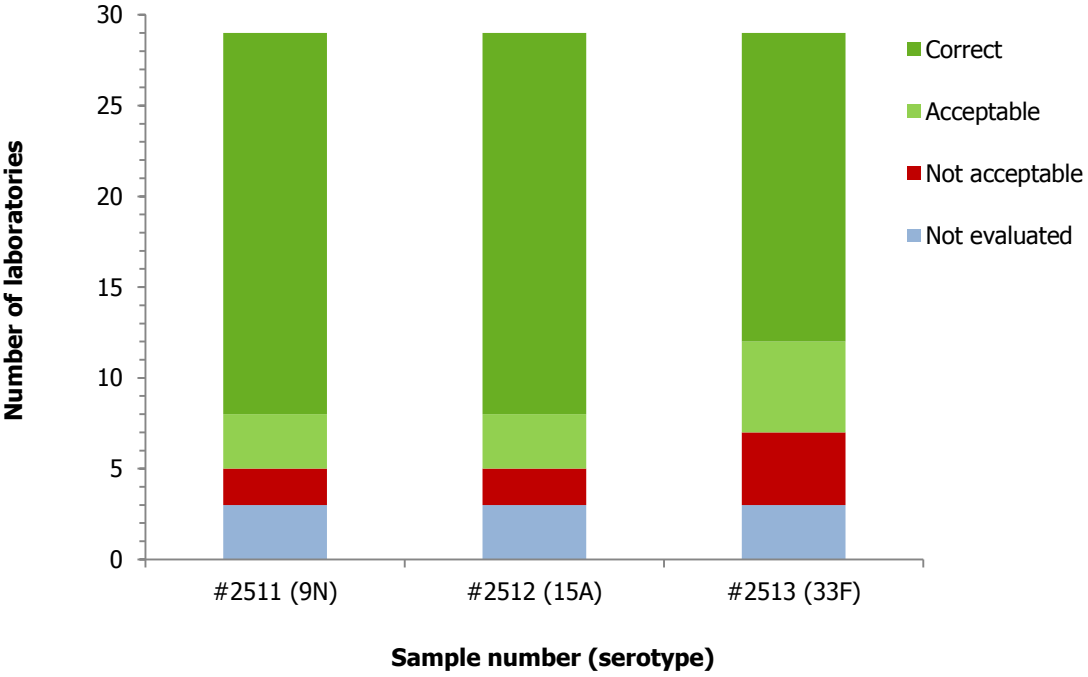
[a] Expected result using CDC conventional PCR for capsule typing (Pai et al. 2006; da Gloria Carvalho et al. 2010; www.cdc.gov/streplab/pcr).
[b] Expected result using CDC real-time PCR for capsule typing (Pimenta et al. 2013; www.cdc.gov/streplab/pcr).
[c] Expected result using the wzh capsular gene typing method of Elberse et al. 2011.
[d] Laboratory used a restricted set of pneumococcal antisera for serotyping
[e] Laboratory used a restricted set of primers for genotyping

Figure 1. Phenotypic species identification of viable cultures



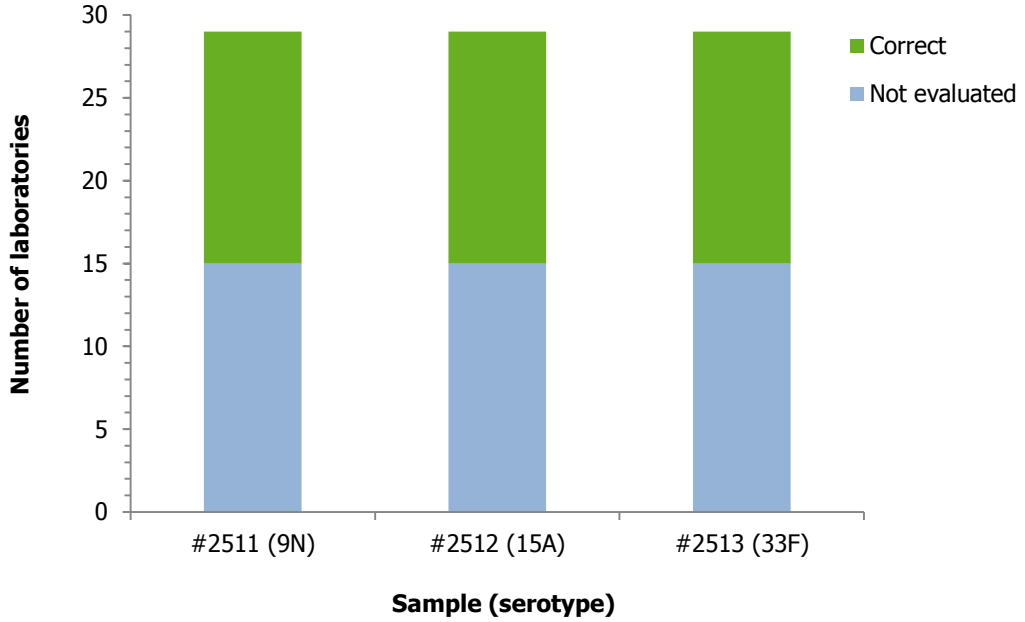
Key: correct = correct serotype; acceptable = correct to serogroup level; not acceptable = incorrect serogroup; not evaluated = no result reported

Figure 2. Phenotypic serotyping of viable cultures



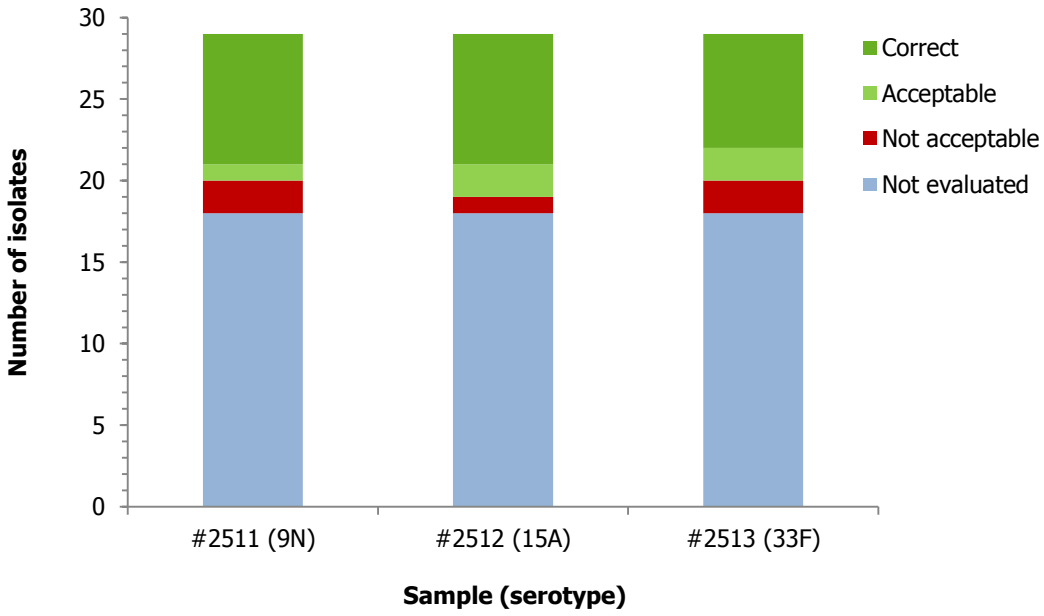
Key: correct = correct serotype; acceptable = correct to serogroup level; not acceptable = incorrect serogroup; not evaluated = no result reported.

Figure 3. Genotypic species identification of viable cultures



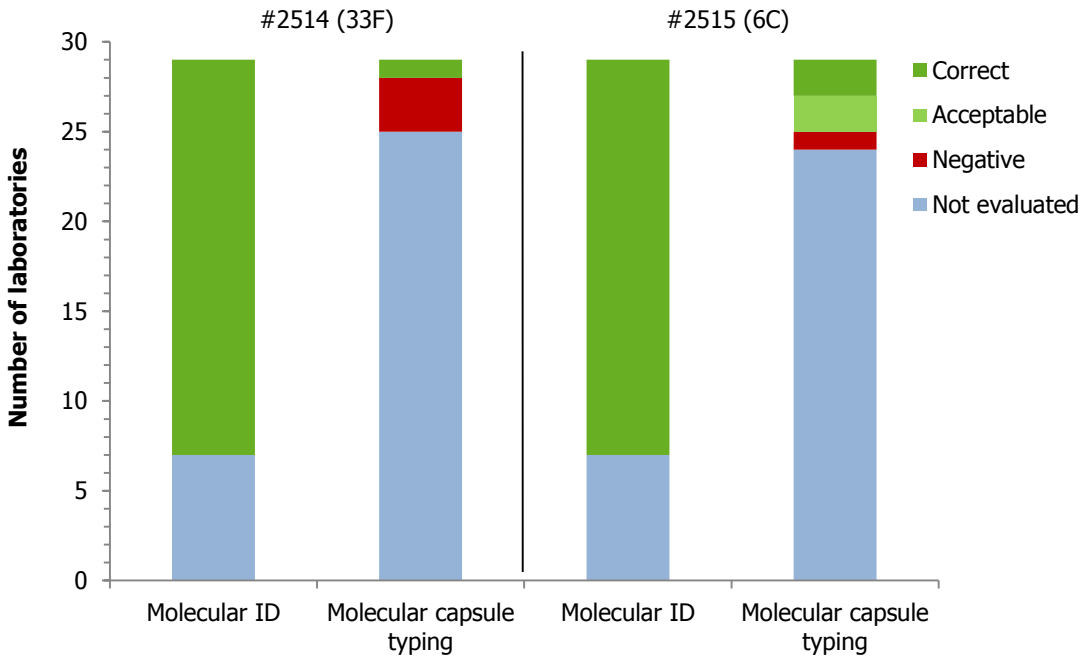
Key: Correct = correct species ID. Not evaluated = no result reported

Figure 4. Molecular capsule typing of viable cultures



Key: correct = correct capsule type combination (Table 4); acceptable = correct to serogroup level; not acceptable = incorrect serogroup or non-typeable; not evaluated = no result reported.

Figure 5. Molecular detection and capsule typing of non-culture samples



Name and serotype (in parentheses) of each sample are shown above the graph.
Key for molecular ID: correct = S. pneumoniae *detected; not evaluated = no result reported.*
Key for molecular capsule typing: correct = correct genotypic capsule type combination (see Table 4); acceptable = correct to serogroup level; negative = typing attempt failed; not evaluated = no result reported.

2.1.1 Phenotypic species identification

Twenty-six laboratories performed phenotypic species identification. Two of the samples were correctly identified as *S. pneumoniae* by all participants. One participant incorrectly identified sample number 2512 as *Streptococcus salivarius* (Table 4).

2.1.2 Phenotypic serotyping

Twenty-six laboratories undertook serotyping. Two laboratories reported erroneous results in serogrouping: one laboratory reported sample number 2511 as serotype 23B rather than 9N, and one laboratory reported sample number 2512 as 8 rather than 15A. Twenty-one laboratories correctly identified sample 2511 as serotype 9N; one laboratory stated that it was 9L, one that it was 9V, and three laboratories stated that it was serogroup 9 but did not report a serotype within that group. Twenty-one laboratories correctly identified # 2512 as serotype 15A; one stated it was non-typeable and three stated that it was serogroup 15. Seventeen laboratories correctly identified sample 2513 as serotype 33F; two stated that it was 33A, one stated that it was non-typeable with the available sera, and five stated that it was serogroup 33 (no subtype reported). In general, 15 participants stated that they used an agglutination typing method (one clarified they used co-agglutination with pool sera, one laboratory stated they used a latex method, one stated that they used the pneumococcus 7-10-13-valent latex kit (Statens Serum Institut, Denmark), and one stated that they used Neufield Quellung as a second method). Ten laboratories stated that they used the Neufield Quellung method and one that they used a gel diffusion technique.

Of the three laboratories that did not submit any phenotypic serotyping results, two did report PCR capsule typing results. The third only reported antimicrobial sensitivity testing in this EQA.

2.1.3 Genotypic species identification

Fourteen laboratories used a PCR-based method to identify the strains as *S. pneumoniae* (Table 2), and all reported the correct result (Table 4). Where reported, DNA extraction methods included boiling/heating (8), DNA capture column with centrifugation (7) and DNA capture with magnetic beads (2).

2.1.4 Genotypic capsule typing

Molecular capsular typing was undertaken by 12 laboratories. Seven used a multiplex PCR method (one of these clarified that they used a CDC method (Pai et al. 2006; da Gloria Carvalho et al. 2010; Pimenta et al. 2013) and another that they used the variation of Siira et al. (2012). Participants were not required to clarify whether they used a conventional or real-time PCR method. These methods would only be expected to identify the capsule type of the three isolates to the resolution of '9N/9L', '15A/15F' and '33A/33F/37', respectively (Table 4). Four of the remaining laboratories used the wzh capsular sequence typing (CST) method of Elberse et al. (2011). For this method the correct results for the three isolates are the alleles 09N-01, 15A-01 and 33F-03, respectively (Table 4). One remaining laboratory stated that they were using a sequencing method (undefined), which was only able to define the capsule types to the group level.

Eleven of the 12 laboratories reported a result for each isolate (not all laboratories reported on all three samples) and most reported the expected result in each case (Table 4). All participants using the CST method reported the correct results. The laboratory that used the unspecified sequencing method and the occasional laboratory using multiplex PCR only reported the result to the serogroup level. Two laboratories reported that at least one of the samples was non-typeable with the available PCR primers (NT in Table 4).

2.1.5 MLST

Ten participants submitted multilocus sequence typing (MLST) results for at least one of the three isolates (Table 2). There was only one error in the reported results; one participant made an error in sequencing one of the seven loci (*recP*) of sample 2513, which resulted in an incorrect sequence type (Table 4).

2.2 Part 2: Antimicrobial susceptibility testing

The intended results for the antimicrobial susceptibility testing are shown in Table 5.

Participants were asked to provide information on the guidelines and MIC methods used to test susceptibilities for specimens 2511–2513. Twenty-seven laboratories reported some antimicrobial susceptibility testing results. The results obtained by participants are shown in Table 6.

EUCAST guidelines were used by 19 laboratories: CLSI guidelines were used by seven participants. One laboratory did not state which guidelines they used.

Table 5. Intended results for antimicrobial susceptibility testing of bacterial isolates

Sample number	Antimicrobial susceptibility (S), intermediate (I), resistance (R)
2511	Ciprofloxacin I[a]
2512	Penicillin R, erythromycin R, ciprofloxacin I[a]
2513	(Penicillin S), erythromycin R, ciprofloxacin I[a]

[a] CLSI does not give an interpretative criterion for ciprofloxacin susceptibility testing of pneumococci. EUCAST gives a breakpoint for ciprofloxacin of S≤ 0.12 R>2 mg/L, but states that wild type S. pneumoniae *are not considered to be susceptible to ciprofloxacin and are therefore categorised as intermediate.*

Ciprofloxacin was specifically requested in the contract for this EQA exercise and was therefore included. However wild-type *S. pneumoniae* strains are not considered to be susceptible to ciprofloxacin and are, therefore, categorised as being of intermediate susceptibility. CLSI guidelines do not specify any interpretative criteria for ciprofloxacin and *S. pneumoniae.* For these reasons the results for ciprofloxacin should be ignored.

Table 6 shows the MIC range, mode consensus interpretation and non-consensus results reported for each specimen and agent combination.

Overall, the antimicrobial susceptibility testing results were good. Most of the discrepancies arose with the use of different interpretative guidelines for the β-lactams (EUCAST and CLSI). There was one striking inconsistency in reporting of the penicillin susceptibility result for sample 2512. This appears to be due to the discrepancy in breakpoint definitions for meningitis and non-meningitis associated isolates under both guidelines (see below). Participants were informed that these isolates were to be assumed to have come from meningitis cases, but some laboratories have reported interpretations based on this not being the case. The MIC values reported would all have generated an interpretation of resistant under EUCAST guidelines for meningitis cases.

Table 6. Antimicrobial susceptibility testing results

Antimicrobial agent	Specimen 2511				
	MIC range (n)	MIC (mg/L) mode	Consensus interpretation	Ratio reporting consensus	Non consensus results (n)
Ceftriaxone	0.006 to 0.32 (27)	<0.016	S	26/26 (100%)	
Ciprofloxacin	0.5 to 4 (21)	1	I	16/21 (76%)	S (2), R (3)
Erythromycin	0.023 to 0.38 (26)	0.032	S	25/26 (96%)	I (1)
Penicillin	0.008 to 0.064 (27)	0.032	S	27/27 (100%)	

Antimicrobial agent	Specimen 2512				
	MIC range (n)	MIC (mg/L) Mode	Consensus interpretation	Ratio reporting consensus	Non consensus results (n)
Ceftriaxone	0.19 to 0.75 (27)	0.5	S	26/27 (96%)	I (1)
Ciprofloxacin	0.5 to 3 (21)	2	I	18/21 (86%)	S (2), R (1)
Erythromycin	128 to >256 (26)	>256	R	25/26 (96%)	S (1)
Penicillin	0.25 to 2 (27)	1	R	13/27 (48%)	S (4) I (10)

Antimicrobial agent	Specimen 2513				
	MIC range (n)	MIC (mg/L) Mode	Consensus interpretation	Ratio reporting consensus	Non consensus results (n)
Ceftriaxone	0.008 to 0.08 (26)	0.016	S	26/26 (100%)	
Ciprofloxacin	0.5 to 1.5 (20)	0.5	I	17/20 (85%)	S (3)
Erythromycin	2 to 32 (26)	6	R	26/26 (100%)	
Penicillin	0.004 to 0.12 (27)	-	S	25/27 (93%)	I (1), R (1)

Abbreviations: n, number of laboratories reporting relevant results. S = susceptible; I = intermediate; R = resistant

The use of different guidelines (EUCAST and CLSI) for interpreting antimicrobial susceptibility makes comparison of results problematic. There are major differences between the EUCAST and CLSI both in terms of media and defined breakpoints for a number of antimicrobials. This is especially true for the β-lactam antimicrobials, but there are also differences for other classes of antimicrobials, including macrolides. Recently, the CLSI interpretative guidelines were modified for benzyl penicillin and pneumococci (Wayne 2011, Weinstein et al. 2009). This has brought the breakpoint for determining penicillin resistance closer to that stated in the EUCAST guidelines (Table 7), but there is still a discrepancy between the level determining resistance for both meningitis (EUCAST >0.06 mg/L; CLSI ≥ 0.12 mg/L) and non-meningitis (EUCAST >2 mg/L; CLSI 8≥ mg/L).

Table 7. Comparison of interpretative standards for MIC determinations (µg/ml) with *S. pneumoniae* in EUCAST and CLSI guidelines

Antimicrobial agent	EUCAST MIC breakpoint (mg/L)		CLSI MIC interpretative standard (mg/L)		
	S≤	R>	S≤	I	R≥
Penicillin parenteral (meningitis)	≤ 0.06	> 0.06	≤ 0.06		≥ 0.12
Penicillin parenteral (non-meningitis)	≤ 0.06	> 2	≤ 0.06	4	≥ 8
Ceftriaxone (meningitis)	≤ 0.5	> 2	≤ 0.5	1	≥ 2
Ceftriaxone (non-meningitis)	≤ 0.5	> 2	≤ 0.5	2	≥ 4
Erythromycin	≤ 0.25	> 0.5	≤ 0.25	0.5	≥ 1
Ciprofloxacin	≤ 0.12	> 2			

2.3 Part 3: Non-culture detection of *S. pneumoniae*

Two simulated CSF samples (2513 and 2514) were included in the EQA panel to test the ability of the participants to extract DNA from the clinical samples and assay for the presence of *S. pneumoniae* DNA. They were also encouraged to offer any further capsule typing information that their assays were capable of elucidating about the samples. Sample 2514 contained *S. pneumoniae* serotype 33F in simulated CSF. Sample number 2515 contained *S. pneumoniae* serotype 6C.

The intended results and breakdown of submitted data are shown in Table 8. Twenty-two laboratories attempted the detection of *S. pneumoniae* in the simulated CSF samples and all were successful. Participants were asked to report the Cq values of their real-time PCRs for the first time in this EQA distribution. Although results cannot be compared directly between different assays and different runs of an individual assay, the results give an indication of the amount of DNA extracted by each participant in conjunction with the sensitivity of each PCR. Thirteen laboratories reported Cq values for their real-time PCR results and they ranged between 24.3–34.2 cycles. (If this was a range within a single run of a PCR, this range would represent a difference of 1000-fold in the amount of target DNA.) The DNA extraction methods and choice of genes target for detecting *S. pneumoniae* are shown in Table 9.

Although not a formal requirement, four laboratories attempted further typing of the DNA in the non-culture samples. Only one was successful with sample 2514. Two of the other laboratories stated this was 'non-typeable' (in one case, this was known to be due to a limitation in the range of primers being tested). The fourth laboratory stated that a PCR for *cpsA* failed (which implies that capsule typing PCRs were not attempted). Three of the four laboratories detected serogroup 6 DNA in sample 2515. The fourth stated that the *cpsA* PCR had failed.

Table 8. Intended and submitted results for non-culture detection of *S. pneumoniae*

Specimen number	Intended result	Ratio of laboratories reporting the intended result (%)	Results not matching intended result (frequency)
Genotypic species identification			
2514	*S. pneumoniae*	22/22 (100%)	
2515	*S. pneumoniae*	22/22 (100%)	
Genotypic capsule typing (optional)			
2514	33F, 33A/33F/37[a,b]	1/4 (25%)	NT (2); *cpsA* not detected (1)
2515	6C, 6C/6D [a,b]	2/4 (50%)	Group 6 (1); *cpsA* not detected (1)

[a] *Expected result using CDC conventional PCR for capsule typing (Pai et al. 2006; da Gloria Carvalho et al. 2010; www.cdc.gov/streplab/pcr)*
[b] *Expected result using CDC real-time PCR for capsule typing (Pimenta et al. 2013; www.cdc.gov/streplab/pcr)*

Table 9. DNA extraction methods, PCR method and gene targets used to detect *S. pneumoniae* in non-culture samples

Extraction method	PCR method	PCR target					
		lytA	*ply*	*lytA + ply*	*pia*	*gyrB*	No data
Boil/heat lysis	Real-time PCR (in-house)	1					
Capture column (no centrifugation)	Real-time PCR (in-house)			1			
Capture column (with centrifugation)	PCR/gel electrophoresis	1		2[b]			1[c]
	Real-time PCR (commercial)	1					
	Real-time PCR (in-house)	5[a]	2				
Magnetic Beads	Real-time PCR (in-house)	3			1		
Automated machine/ commercial kit	PCR/gel electrophoresis		1				
DNA-sorb-AM nucleic acid extraction kit (Amplisens)	PCR/gel electrophoresis					1	
Salt precipitation	PCR/gel electrophoresis					1	
No data	No data	1					
Total		**12**	**3**	**3**	**1**	**2**	**1**

[a] *One laboratory reported* lytA+cpsA*, one laboratory reported* lytA+hpd+sodC
[b] *Both laboratories reported* lytA+ply + cpsA
[c] *Unspecified species-specific PCR target +* cpsA

Overall comments

The laboratory EQA has shown that the European pneumococcal reference laboratories vary in the level to which they characterise strains referred to them, ranging from full speciation and serogrouping to full serotyping and sequence typing.

Serotypes within the serogroup 9, 15 and 33 can be difficult to differentiate, particularly if a molecular method is used for typing.

Overall, out of 242 results submitted, there were only eight errors in phenotypic characterisation of the strains (3.3%). One error was at the species level, where the strain of *S. pneumoniae* was identified as *S. salivarius.* In one case, serotype 15 pneumococcus was incorrectly identified as serotype 8; in one case, serotype 33F pneumococcus was incorrectly typed as a serotype 4; and in three cases, the wrong serotype within a serogroup was identified. Two laboratories reported one of the strains was 'non-typeable'. In the last two EQA distributions in 2010 and 2012 there were 13.1% and 1.7% phenotypic errors, respectively, although it must be acknowledged that the panels are not directly comparable in each distribution, as this EQA panel included three viable cultures instead of five.

If serotyping is performed using conventional Quellung or agglutination methods, it is important to use all of the necessary factor antisera, and to include a positive and a negative control. As an example, four factor antisera are used to serotype pneumococci in serogroup 9 (Table 10), four factor antisera for pneumococci in serogroup 15 (Table 11), and five factor antisera for pneumococci in serogroup 33 (Table 12).

Table 10. Identification of serotypes within serogroup 9 pneumococci[1]

Serotype	Reactions in factor antiserum			
	9b	9d	9e	9g
9A	-	+	-	-
9L	+	-	-	-
9N	+	-	+	-
9V	-	+	-	+

[1] http://www.ssi.dk/~/media/Admin/Diagnostica%20Downloads/Downloads%20UK/Brochures/BrochurePneumococcal%20factor%20antisera%20key%2018058.ashx

Table 11. Identification of serotypes within serogroup 15 pneumococci[1]

Serotype	Reactions in factor antiserum			
	15b	15c	15e	15h
15F	+	+	-	-
15A	-	+	-	-
15B	+	-	+	+
15C	-	-	+	-

[1] http://www.ssi.dk/~/media/Admin/Diagnostica%20Downloads/Downloads%20UK/Brochures/BrochurePneumococcal%20factor%20antisera%20key%2018058.ashx

Table 12. Identification of serotypes within serogroup 33 pneumococci[1]

Serotype	Reactions in factor antiserum				
	33b	33e	33f	6a	20b
33F	+	-	-	-	-
33A	+	-	-	-	+
33B	-	-	+	-	-
33C	-	+	(+)	-	-
33D	-	-	+	+	-

[1] http://www.ssi.dk/~/media/Admin/Diagnostica%20Downloads/Downloads%20UK/Brochures/BrochurePneumococcal%20factor%20antisera%20key%2018058.ashx

The EQA distribution has again indicated that some laboratories lack the necessary reagents to fully serotype isolates, and this renders surveillance of IPD difficult. Comprehensive data on serotype distribution is essential in order to establish the impact of the use of pneumococcal vaccines. Three laboratories did not carry out phenotypic serotyping of isolates. However, two of these did characterise them using genotypic capsule typing. This may reflect a decision to replace conventional serotyping with molecular typing. It must be borne in mind, however, that

the genotypic methods cannot type to the same resolution as conventional serotyping and may not distinguish between some closely related serotypes that are, or are not, included in pneumococcal vaccines.

Genotypic capsule typing gave encouraging results, although some participants were not able to characterise the isolates to the expected resolution. Two laboratories were unable to type at least one of the cultures due to a decision not to test with all the available PCR primer sets in the multiplex PCR method. This may be a reflection on the laborious nature of the multiplex PCR typing method and that these capsule types are rare in these participants' countries. It is acknowledged that the serotypes included in this distribution were challenging to type. The CST sequencing method, in which one gene target within the capsule operon is sequenced and the result compared to a public database (Elberse et al. 2011), worked well with these isolates, apparently typing them to serotype level. The method shows much promise, and may offer increased serotyping resolution over existing multiplex PCR strategies. It must be noted, however, that some CST alleles can be associated with more than one serotype and cannot, therefore, give a definitive result. For example, the 9N-01 allele associated with strain 2511 in this distribution has been found in strains of serotype 9N and 9L, and an alternative allele associated with 33F (33F-02) has also been found in isolates of 33A and 35A (http://www.rivm.nl/mpf/spn/cst#/).

Some laboratories may use solely PCR-based methods to type viable cultures, and some laboratories use a combination of PCR-based typing plus restricted sets of pneumococcal antisera to distinguish the serotypes within serogroups. This can be less laborious or expensive than phenotypic serotyping, but it may result in them being unable to type some strains completely to the serotype level. For example, in relation to this EQA, the CDC real-time PCR does not include primers that can detect 9N (Pimenta et al. 2013). Primers are only included that can detect 9V/9A. The conventional multiplex PCR from CDC can detect 9N/L, but cannot distinguish between the two types (Pai et al. 2006; Carvalho et al. 2010). Since the current molecular protocols cannot completely distinguish the serotypes within the serogroups, further typing using traditional antisera is recommended.

In future EQA distributions it would be useful to request more information regarding the PCR- based typing used by the laboratory, together with details of the types that their PCR protocol can distinguish.

Genetic species identification of cultures by PCR or 16S rDNA sequencing performed well for the 14 laboratories that used it. It would be informative to request more details of these PCR methods in future EQA distributions. Ten laboratories reported molecular typing of cultures by MLST, an increase from the last distribution (6).

The antimicrobial susceptibility testing results indicated that the majority of the laboratories have little difficulty in performing susceptibility testing. The major discrepancy was in the interpretation of the results because of the use of different interpretative guidelines. The majority (70%) of laboratories are now using EUCAST guidelines but 30% are still using CLSI guidelines. There are major differences between the EUCAST and CLSI both in terms of media and defined breakpoints for a number of antimicrobials. All EU reference laboratories should be moving towards using EUCAST guidelines.

An increasing number of laboratories attempted detection of *S. pneumoniae* in simulated CSF samples in this distribution, and there were no errors. The collection of Cq values for real-time PCR results for the first time in this distribution highlighted the wide range of values obtained by participants. Although not directly comparable between laboratories and different assays, the wide range of values suggests large variability in the efficiency of DNA extraction or in the sensitivity of the PCR assays between the different participants. Nevertheless, none reported a false negative result. Due to the invitation to participants to attempt PCR-based capsule typing of the samples, the panel comprised two *S. pneumoniae* samples of different serotypes. However, the small number of samples meant that a negative control was not included, which also meant that participants' abilities to avoid false positives could not be compared to the previous distribution.

Conclusions

The EQA distribution has again indicated that some laboratories lack the necessary reagents to fully serotype isolates, and this renders surveillance of IPD difficult.

A certain degree of heterogeneity exists in the level of characterisation of strains of *S. pneumoniae* among EU countries. This emphasises the need for consensus and agreement in methods for characterising and accurately defining this organism. Some countries still require some capacity building in this area.

It is recommended that all European laboratories adopt the EUCAST methods of antimicrobial susceptibility testing, which should facilitate better comparison of the results from different laboratories (http://www.EUCAST.org) in compliance with the EU case definition for surveillance of antimicrobial resistance.

The inclusion of two simulated clinical samples in the EQA panel to assess non-culture detection methods was, again, very useful. The results were very encouraging, but it would be useful if a larger number of this type of sample should be included in future distributions in order to allow for a more rigorous assessment of the participants' proficiency.

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European Centre for Disease Prevention and Control (ECDC)

Postal address:
Granits väg 8, SE-171 65 Solna, Sweden

Visiting address:
Tomtebodavägen 11A, SE-171 65 Solna, Sweden

Tel. +46 858601000
Fax +46 858601001
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