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Evaluation of the World Health Organization Global Invasive Bacterial Vaccine-Preventable Disease (IB-VPD) Surveillance Network's Laboratory External Quality Assessment Programme, 2014–2019

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Abstract

Introduction. In 2009, the World Health Organization (WHO) established the Global Invasive Bacterial Vaccine Preventable Disease (IB-VPD) Surveillance Network (GISN) to monitor the global burden and aetiology of bacterial meningitis, pneumonia and sepsis caused by *Haemophilus influenzae* (Hi), *Neisseria meningitidis* (Nm) and *Streptococcus pneumoniae* (Sp).

Hypothesis/Gap Statement. The GISN established an external quality assessment (EQA) programme for the characterization of Hi, Nm and Sp by culture and diagnostic PCR.

Aim. To assess the performance of sentinel site laboratories (SSLs), national laboratories (NLs) and regional reference laboratories (RRLs) between 2014 and 2019 in the EQA programme.

Methodology. Test samples consisted of bacterial smears for Gram-staining, viable isolates for identification and serotyping or serogrouping (ST/SG), plus simulated cerebrospinal fluid (CSF) samples for species detection and ST/SG by PCR. SSLs and NLs were only required to analyse the slides for Gram staining and identify the species of the live isolates. RRLs, and any SLs and NLs that had the additional laboratory capacity, were also required to ST/SG the viable isolates and analyse the simulated CSF samples.

Results. Across the period, 69–112 SS/NL labs and eight or nine RRLs participated in the EQA exercise. Most participants correctly identified Nm and Sp in Gram-stained smears but were less successful with Hi and other species. SSLs/NLs identified the Hi, Nm and Sp cultures well and also submitted up to 56% of Hi, 62% of Nm and 33% of Sp optional ST/SG results each year. There was an increasing trend in the proportion of correct results submitted over the 6 years for Nm and Sp. Some SSLs/NLs also performed the optional detection and ST/SG of the three organisms by PCR in simulated CSF from 2015 onwards; 89–100% of the CSF samples were correctly identified and 76–93% of Hi-, 90–100% of Nm- and 75–100% of Sp-positive samples were also correctly ST/SG across the distributions. The RRLs performed all parts of the EQA to a very high standard, with very few errors across all aspects of the EQA.

Conclusion. The EQA has been an important tool in maintaining high standards of laboratory testing and building of laboratory capacity in the GISN.

INTRODUCTION

Quality assurance is essential for clinical diagnostic laboratories to ensure the provision of accurate and timely results to support patient care. Ideally, laboratories undertake two separate quality assessment activities: internal quality assessment (IQA), which assesses the reproducibility of tests performed within an individual laboratory on a regular basis; and external quality assessment (EQA), which compares the results obtained by different laboratories when examining a panel of test samples. EQA in diagnostic clinical laboratories not only includes evaluation of laboratory performance but also identifies areas that would benefit from training [1].

In 2009, the World Health Organization (WHO) established the Global Invasive Bacterial Vaccine Preventable Disease (IB-VPD) Surveillance Network (GISN) to monitor the global burden and aetiology of bacterial meningitis, pneumonia and sepsis caused by *Haemophilus influenzae* (Hi), *Neisseria meningitidis* (Nm) and *Streptococcus pneumoniae* (Sp) in children aged <5 years of age [2]. A

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major aim of the GISN was to support pneumococcal conjugate vaccine (PCV) introduction and assess its impact, primarily in low- and middle-income countries (LMICs) with limited surveillance and laboratory capacity [2]. The laboratory network, a key component of GISN, has put efforts into strengthening the diagnosis of the aetiology of bacterial meningitis globally, particularly in detecting and characterizing Hi, Nm and Sp. This will continue to be important in supporting WHO's global strategy for vaccine-preventable disease surveillance [3], its Immunization Agenda 2030 [4] and the Defeating Meningitis by 2030 roadmap [5].

The laboratory network encompasses all WHO Regions: the African Region, Eastern Mediterranean Region, European Region, Region of the Americas, South East Asia Region and the Western Pacific Region. The laboratory network within GISN has a pyramidal structure in which sentinel site laboratories (SSLs) and national laboratories (NLs) are responsible for local diagnosis and surveillance by culturing Hi, Nm and Sp from clinical specimens. They may also serotype or serogroup bacterial isolates, or they refer them to one of nine Regional Reference Laboratories (RRLs) for this purpose. All RRLs, plus selected SSLs and NLs, also perform non-culture PCR detection and serotyping/serogrouping (ST/SG) of Hi, Nm and Sp in clinical specimens such as cerebrospinal fluid (CSF). One Global Reference Laboratory (GRL) oversees the network and coordinates training and quality assurance testing as required. Training has been provided by the GRL or RRLs on behalf of WHO on the collection of appropriate specimens, methods of bacterial culture, and identification and ST/SG of Sp, Nm and Hi [2]. Training in PCR methods for identification, speciation and ST/SG were also provided to laboratories that had the necessary equipment and facilities to undertake molecular testing. A laboratory manual detailing all of the relevant tests was provided to all of the laboratories within the network [6]. Reagents and some essential equipment were provided by WHO to laboratories which had limited capacity.

To support the quality management system of the network, WHO established an annual laboratory EQA programme. From 2011 to 2013, the EQA programme was provided by the National Institute for Communicable Diseases (NICD) in Johannesburg, South Africa. From 2014 to 2019, the EQA scheme was provided by the United Kingdom National External Quality Assessment Services (UK NEQAS) for Microbiology and Public Health England [PHE; PHE was superseded by the United Kingdom Health Security Agency (UKHSA) in October 2021]. Since 2014, the EQA panel of samples has consisted of (1) unstained bacterial smears for Gram staining, (2) viable isolates for identification and ST/SG, and (3) simulated CSF samples for species detection and ST/SG by PCR. In keeping with their role in the laboratory network, SSLs and NLs were only obliged to analyse the slides for Gram staining and identify the species of the live isolates (referred to as testing the partial panel). RRLs, plus any SSLs and NLs that had the necessary additional laboratory capacity, were also assessed on their ability to serotype or serogroup the isolates and to analyse the simulated CSF samples (referred to as testing the full panel). All laboratories were also invited to perform antimicrobial sensitivity testing (AST) as an optional exercise. However, WHO did not provide training on AST and the results of AST are not included here. This report evaluates the global laboratory proficiency from 2014 to 2019 based on the EQA exercise that WHO implemented in collaboration with UK NEQAS and PHE.

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Abbreviations: AST, antimicrobial sensitivity testing; CSF, cerebrospinal fluid; EQA, external quality assessment; GISN, Global Invasive Bacterial Vaccine Preventable Disease Surveillance Network; GRL, Global Reference Laboratory; Hi, *Haemophilus influenzae*; Hib, *Haemophilus influenzae* serotype b; IATA, International Air Transportation Association; IQA, internal quality assessment; LMICs, low- and middle-income countries; MRU, Meningococcal Reference Unit; NCID, National Institute for Communicable Diseases; NL, national laboratory; Nm, *Neisseria meningitidis*; NTHi, non-typable *Haemophilus influenzae*; PCV, pneumococcal conjugate vaccine; PHE, Public Health England; RRL, Regional Reference Laboratory; RVPBRU, Respiratory and Vaccine Preventable Bacteria Reference Unit; Sp, *Streptococcus pneumoniae*; SSL, sentinel site laboratory; ST/SG, Serotyping or serogrouping; UKHSA, United Kingdom Health Security Agency; UK NEAQAS, United Kingdom National External Quality Assessment Services; WHO, World Health Organization.

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Four supplementary figures and six supplementary tables are available with the online version of this article.

Table 1. Assessment and scoring areas for the full and partial panel of EQA samples

Assessment and scoring	Partial panel (all SSL/NLs)	Full panel (RRLs and selected SSLs/NLs)
Three or four bacterial films for Gram staining:		
Gram staining and description	+	+
Seven live cultures:		
Phenotypic and/or genotypic identification	+	+
Phenotypic or genotypic serogrouping/serotyping*	(+) Optional. Not scored	+
Seven non-viable simulated CSFs:		
Genotypic detection	N/A	+
Genotypic grouping/typing	N/A	+

*Serogrouping requested for meningococci; serotyping requested for haemophili and pneumococci.
EQA, external quality assessment; N/A, not applicable; NL, national laboratory; RRL, regional reference laboratory; SSL, sentinel site laboratory.

METHODS

Panel preparation

The EQA panels consisted of specimens derived from Hi, Nm and Sp strains plus other bacterial species that can also cause meningitis. Samples for Gram staining were provided as fixed, but unstained bacterial suspensions (smears) on glass microscope slides. Between 2014 and 2017, all smears were prepared using a relatively low density of bacteria suspended in a simulated CSF medium containing blood buffy coat (white blood cells) in order to simulate a clinical sample. In 2018, only the slide containing Nm was prepared in this way; the other samples contained high densities of bacteria suspended in water. Similarly, in 2019, only the Sp sample was prepared as a simulated CSF and the other samples were made from bacterial suspensions in water. Duplicate slides of each sample were provided to allow participating laboratories to process (stain) them more than once if they so wished.

Viable bacterial isolates of Hi, Nm, Sp and selected other bacterial species were prepared as freeze-dried (lyophilized) pure cultures by UK NEQAS. All samples of viable bacteria were quality controlled to assess their viability, absence of contamination, stability and homogeneity. The quality of the samples before and after freeze-drying was assessed by the PHE reference laboratories for meningococci, pneumococci and Hi.

Non-viable simulated CSF samples (for molecular detection by PCR) were prepared by resuspending heat-killed bacteria in a simulated CSF matrix that contained sucrose suspension, followed by freeze drying. The samples were quality controlled to assess their DNA content and stability. Bacterial cell concentrations in the simulated CSF samples were chosen to ensure sufficiently high concentrations of the target bacterial DNA to enable PCR-based detection and ST/SG. Quality control checks confirmed that all samples remained stable and uncontaminated beyond the official closing date of the EQA exercises.

The full panel in each distribution contained three or four fixed unstained bacterial smears for Gram staining, seven viable cultures and seven non-viable simulated CSF samples. The partial panel omitted the simulated CSF samples. Each sample in the panel was assigned a unique anonymized identifier. Panel contents were selected each year by the PHE's Meningococcal Reference Unit (MRU), Respiratory and Vaccine Preventable Bacteria Reference Unit (RVPBRU) and UK NEQAS, in consultation with WHO and external experts. The serogroups and serotypes of the bacteria were chosen to represent those commonly in circulation and to be typable using the methods the participants were expected to use (particularly in relation to Sp, as described below). The EQA participants were informed that the panels were simulations of samples derived from suspected bacterial meningitis cases and could comprise Hi, Nm, Sp and other species that cause meningitis.

Panel distribution

The full and partial panels for each EQA exercise were distributed to participating laboratories from UK NEQAS in coordination with WHO Headquarters, laboratory coordinators for each WHO Region and WHO Country Offices. Each panel was shipped at ambient temperature in compliance with international packaging and transportation requirements for infectious substances according to the International Air Transportation Association (IATA) Dangerous Goods Regulations UN3373 Category B (www.iata.org). All recipient laboratories had received training in the safe handling of the material. Two external referee laboratories, the GRL for GISN at U.S. Centers for Disease Control and Prevention (Atlanta, GA, USA) and the Scottish

Table 2. Participation in the EQA distributions each year

	Year					
	2014	**2015**	**2016**	**2017**	**2018**	**2019**
Partial panel						
SSLs and NLs: no. of panels distributed	141	137	130	130	137	97
SSLs and NLs: no. returning results	89	109	106	109	112	69
SSLs and NLs: returning rate	63%	80%	82%	84%	82%	71%
Full panel						
SSLs and NLs: no. taking part*	0	16	16	26	22	22
RRLs: no. of panels sent	9	9	9	9	9	9
RRLs: no. returning results	8	9	9	8	8	9
RRLs: returning rate	89%	100%	100%	89%	89%	100%

*This is a subset of the number returning results for the partial panel each year.
EQA, external quality assessment; NL, national laboratory; RRL, regional reference laboratory; SSL, sentinel site laboratory.

Haemophilus, *Legionella*, Meningococcus and Pneumococcus Reference Laboratory (Glasgow, UK), participated annually and received and tested the same samples at the same time as the other participants.

Survey design and the reporting of results by participants

Most SSLs and NLs received the partial panel and were instructed to (1) examine the material on the microscope slides after performing a Gram stain and record the cellular morphology of the bacteria, and (2) to resuspend the lyophilized live bacterial samples, culture them using their routine procedures and identify the species of each culture. They were also invited to conduct appropriate ST/SG on any Hi, Nm and Sp cultures using phenotypic methods, genotypic (PCR) methods or a combination of the two, although this aspect was optional.

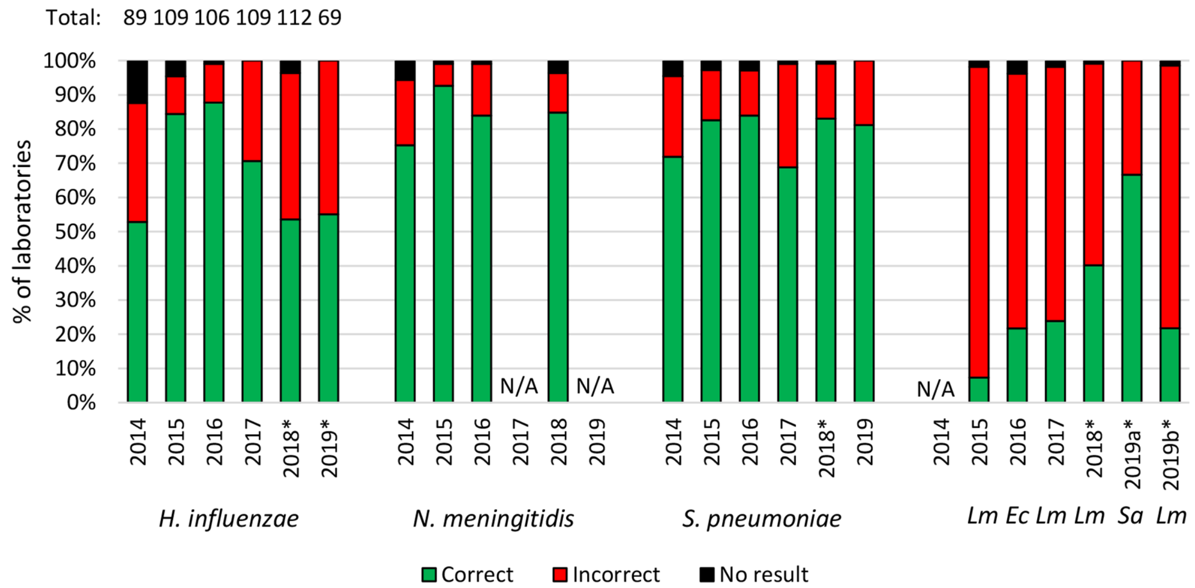
The RRLs, plus selected SSLs and NLs that claimed to have the additional laboratory capacity, received the full panel and were instructed to: (1) examine the material on the microscope slides after performing a Gram stain and record the cellular morphology of the bacteria; (2) resuspend the lyophilized live bacterial samples, culture them using their routine procedures and identify the species of each culture; (3) ST/SG any Hi, Nm and Sp cultures; (4) resuspend the lyophilized simulated CSF samples and detect Sp, Nm and Hi in them using PCR; and (5) ST/SG any Sp-, Nm- or Hi-positive simulated CSF samples using PCR. SSLs and NLs that volunteered to test the full panel were assessed in relation to their analysis of both the partial panel and the full panel. The distribution and assessment of samples in the full and partial panel is summarized in Table 1.

Laboratories were given approximately 1 month to report their test results, but some laboratories were granted an extended deadline if they received the EQA panels late due to shipping delays. The laboratories submitted their test results via an online reporting system managed by UK NEQAS (www.ukneqasmicro.org.uk). When submitting their report, laboratories were given an opportunity to provide comments, provide feedback, ask questions on the EQA exercise, and describe any other issues related to laboratory detection and characterization of invasive bacterial pathogens.

Analysis of results and distribution to participants

After the closing date of the EQA exercise, UK NEQAS revealed the panel contents to all participants and then distributed an individual report to each participating laboratory containing an assessment of their results. This included applying the scoring scheme described in Tables S1–S5 (available with the online version of this article) to the results. In accordance with UK NEQAS accreditation standards (ISO17043), a Gram stain or culture identification sample was excluded from the scoring scheme if fewer than 80% of all participants submitted the correct result. The affected samples are highlighted in Table S6. This exclusion rule was not applied to ST/SG results as it was anticipated that fewer than 80% of participants may be able to ST/SG some of the samples.

In general, it was assumed that participants would use laboratory methods described in WHO's laboratory manual [6], although other methods were acceptable. If they attempted ST/SG of bacterial isolates or simulated CSF samples, they were expected to be able to obtain the correct serogroup for Nm or serotype for Hi; an incomplete result (e.g. containing more than one possible result) was accepted as partially correct. The same rules applied to serotyping of Sp if performed phenotypically; however, for PCR typing, it was assumed that the participants were using the conventional PCR typing method described in the WHO



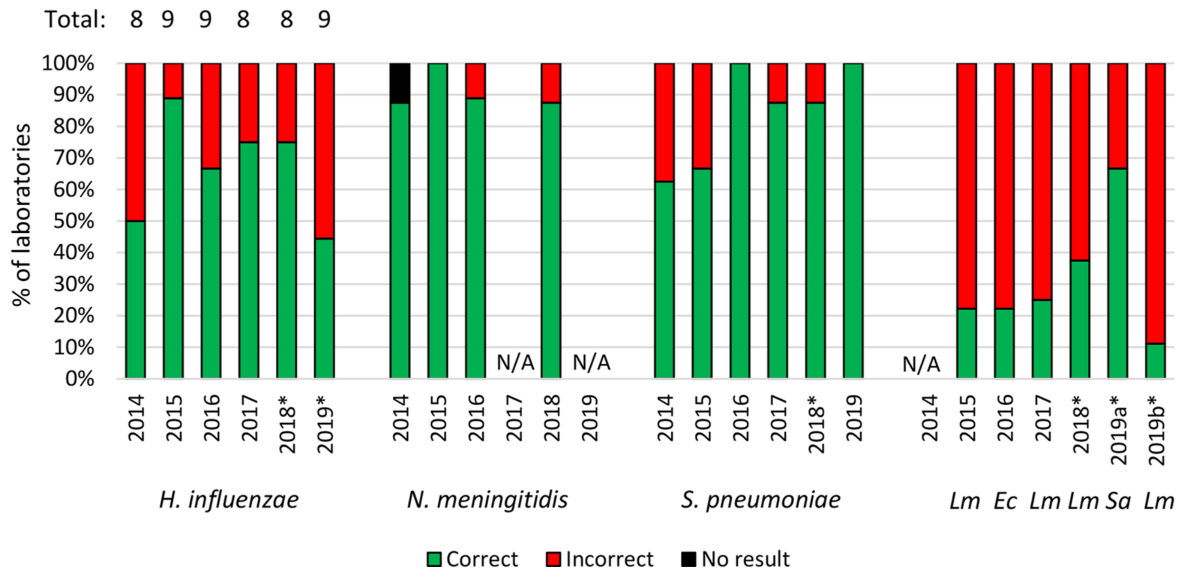


Fig. 1. Gram staining results for bacterial smears. One sample for each species was included in each distribution. *Bacterial smear in water rather than in simulated CSF matrix. *Ec*, *Escherichia coli*; *Lm*, *Listeria monocytogenes*; N/A, not applicable; Sa, *Streptococcus agalactiae*.

laboratory manual or the quantitative PCR equivalent designed by USA CDC [7–10]; these were only able to generate a partially correct result for some serotypes chosen for the panels. These partially correct answers were accepted as fully correct for the purposes of the EQA.

An annual global summary report was prepared after each distribution by PHE, WHO and UK NEQAS, which included results from all participating laboratories and analysis on specific aspects of bacteriology or molecular biology that were highlighted as strengths or weaknesses. WHO distributed this report to the participating laboratories via the GISN for information and to highlight future training needs. Laboratory confidentiality was maintained in all reports by assigning each laboratory a unique ID number that was known only to that laboratory, to UK NEQAS, and to WHO global and regional laboratory coordinators.

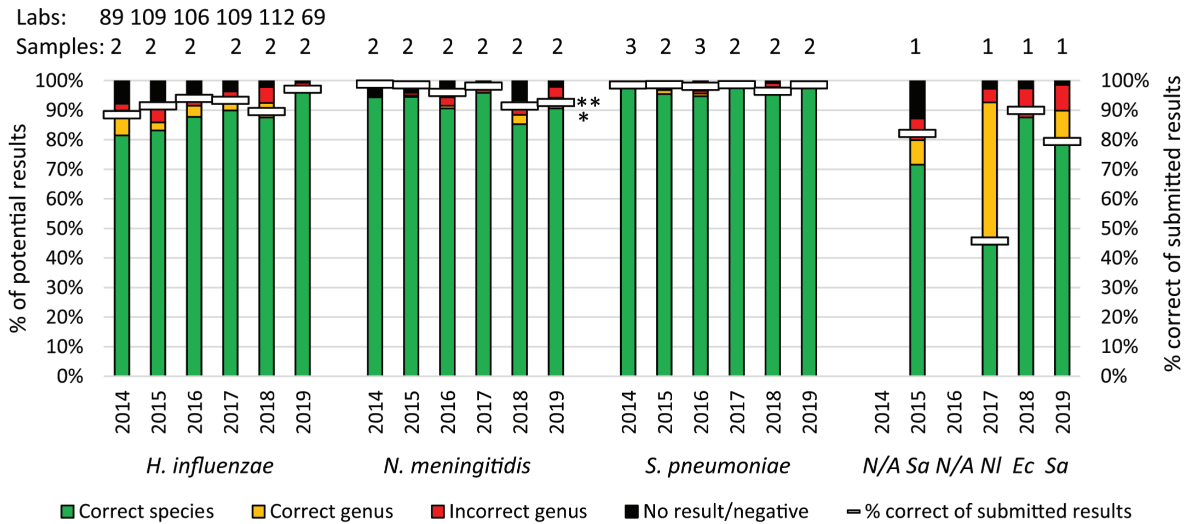


Fig. 2. Culture identification results. Aggregate results for each species are shown (one to three isolates per species each year). *Ec*, *Escherichia coli*; Labs, participating laboratories; *Lm*, *Listeria monocytogenes*; N/A, not applicable; Sa, *Streptococcus agalactiae*. *Significant downward trend in the number of correct results expressed as a proportion of all possible results (including non-returned results; *P*=0.008); **significant downward trend in the number of correct results expressed as a proportion of the submitted results (*P*<0.001).

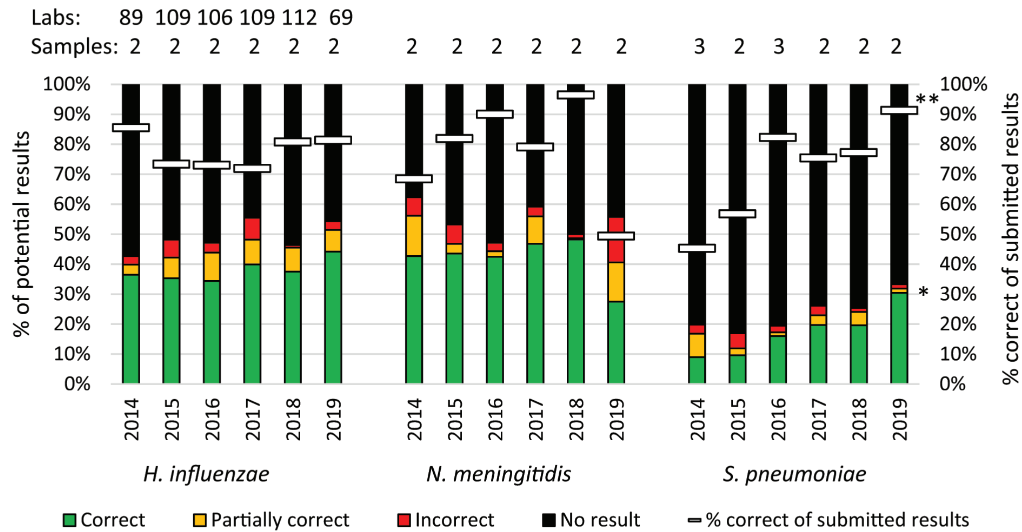


Fig. 3. Serogrouping/serotyping results for cultures. Aggregate results for each species are shown (two or three isolates per species each year). Labs, participating laboratories. *Significant upward trend in the number of correct serotyping results expressed as a proportion of all possible results (including non-returned results; $P<0.001$); **significant upward trend in the number of correct serotyping results expressed as a proportion of the submitted results ($P<0.001$).

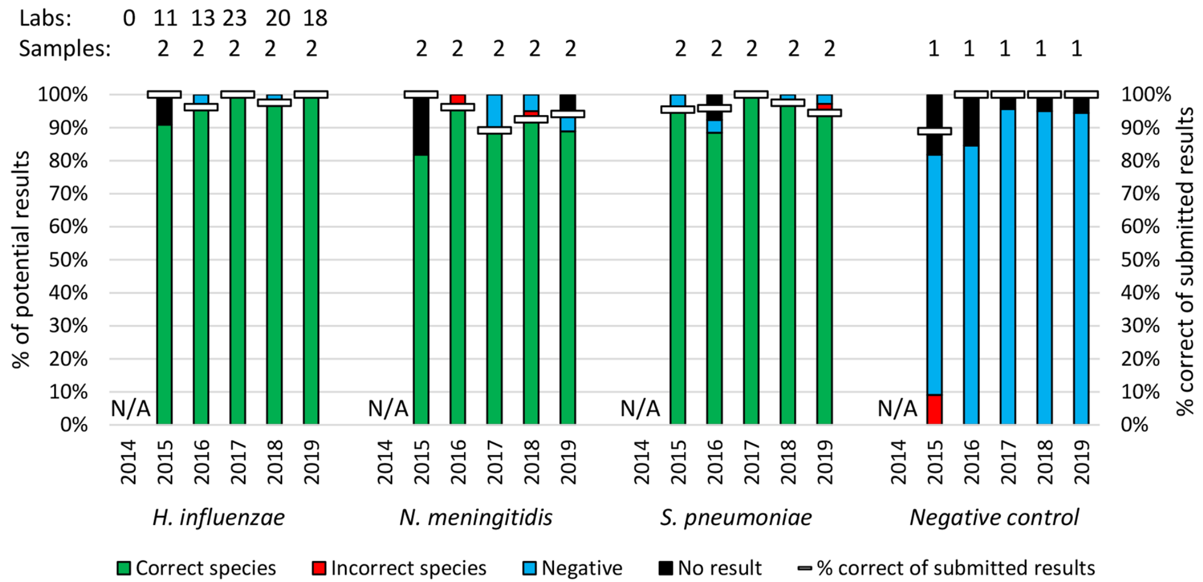
Statistical analysis

Trend analysis by year was performed using logistic regression in Stata 14 (StataCorp) on the proportion with the correct result. A significance level of $P\leq 0.01$ was chosen in order to reduce the risk of false detection arising from multiple testing.

RESULTS

Participation in each EQA distribution

For the purposes of the EQA distributions, SSLs and NLs were treated as a single group (SSL/NLs). The partial EQA panel (only containing slides for Gram staining and viable cultures) was shipped to between 97 and 141 SSL/NLs, depending on the



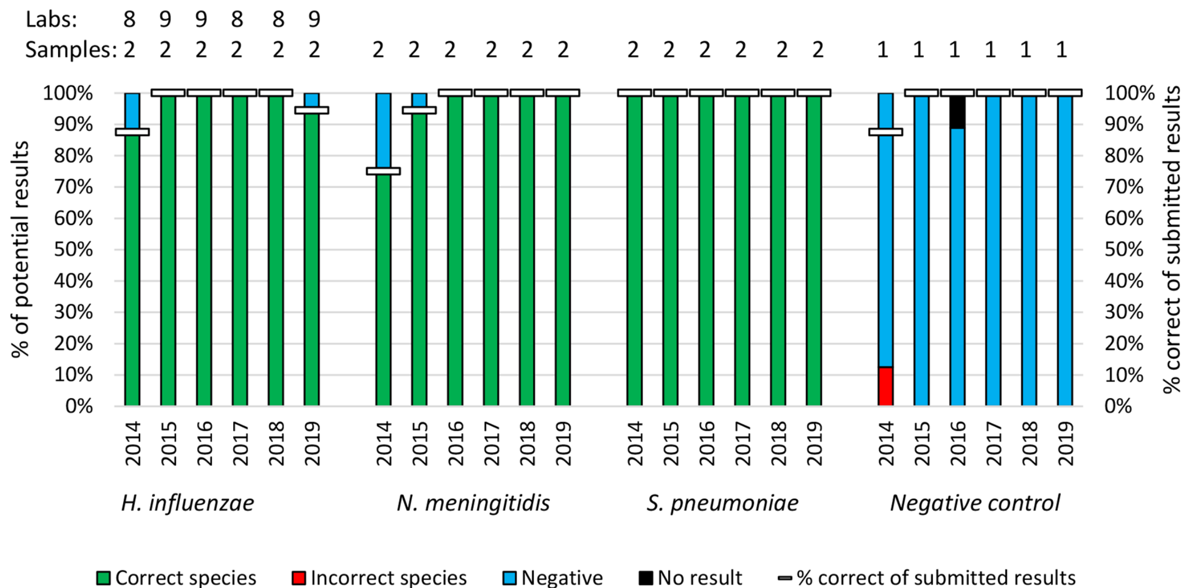
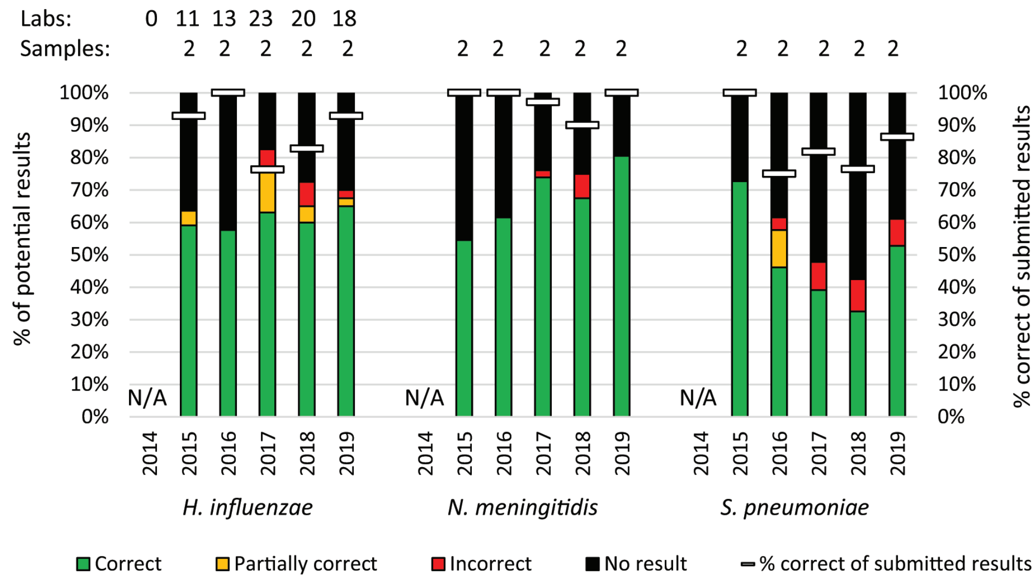


Fig. 4. Results of PCR detection in simulated CSF samples. Aggregate results for samples positive for each species are shown (one or two samples per target each year). Labs, participating laboratories.

year, and 63–84% of the laboratories returned results (Table 2). The overall number of participants (i.e. panels distributed) changed each year as some laboratories declined to take part due to a lack of resources or unforeseen challenges (e.g. an Ebola outbreak in West Africa). The reason that some participants did not return results was not always determined, but, where it was, it was primarily due to problems completing the shipment of the samples to the recipient. Some laboratories that did return results reported that they could not take part in some aspects of the analyses due to the lack of availability of some laboratory reagents (e.g. antisera or PCR reagents for ST/SG).

Between 16 and 26 SSL/NLs, which had capacity for non-culture PCR detection and typing, also requested that they receive the additional simulated CSF samples to complete the full EQA panel each year. They were still assessed as SSL/NLs in their



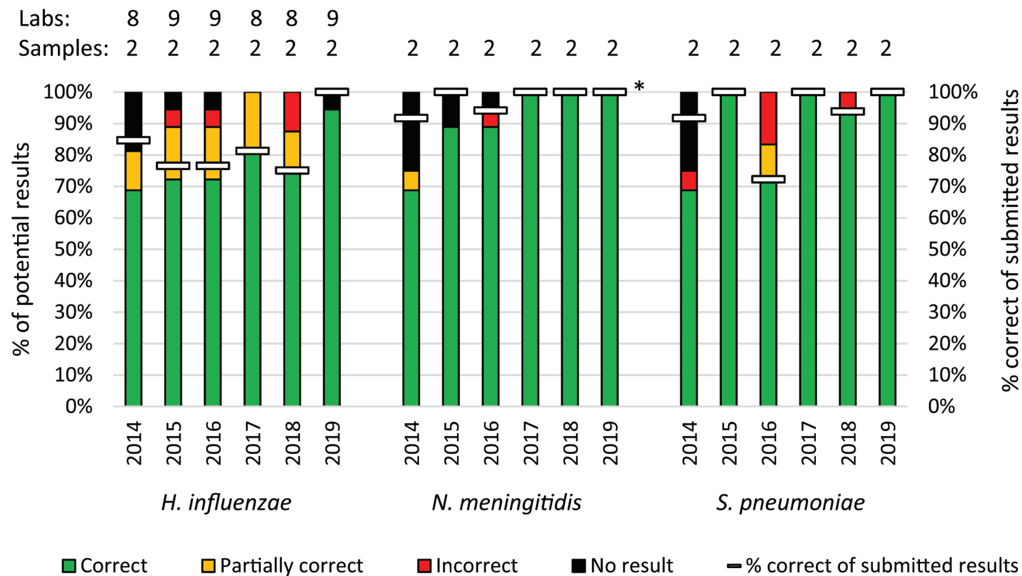


Fig. 5. Results of PCR serotyping/serogrouping in simulated CSF samples. Aggregate results for each species are shown (two samples per target each year). Labs, participating laboratories. *Significant upward trend in the number of correct serogrouping results when expressed as a proportion of all possible results (including non-returned results; P=0.005).

analysis of the partial panel but were also additionally scored alongside the RRLs in their success with the full panel. The full EQA panel was distributed to all nine RRLs each year. On three occasions, one RRL was unable to take part due to problems with delivery of the samples (Table 2).

Gram staining and interpretation of bacterial smears

All laboratories were assessed on their ability to Gram-stain pre-prepared bacterial smears and describe the cellular morphology of the bacteria following microscopic examination (from a set of pre-defined answers). Three samples were

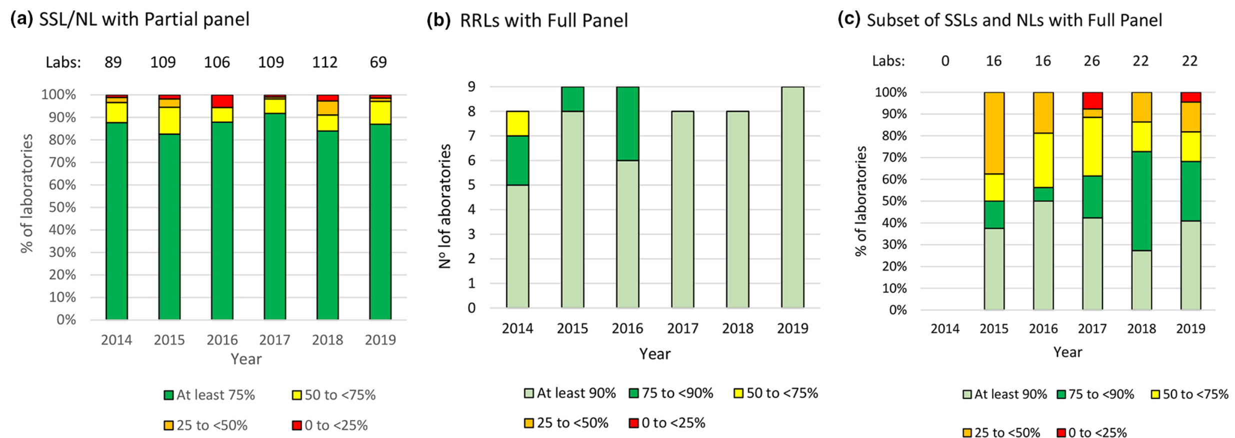


Fig. 6. Summary of scoring results for the partial panel and full panel. Scores were expressed as a percentage of the maximum possible score. SSL, sentinel site laboratory; NL national laboratory; RRL, regional reference laboratory; Labs, participating laboratories.

sent in 2014 and 2017; four were sent in the other years (Fig. 1 and Table S6). The samples included examples of Hi, Nm and Sp plus other bacteria that also caused meningitis (*Listeria monocytogenes*, *Escherichia coli* or *Streptococcus agalactiae*). Both the SSL/NLs and RRLs identified the Nm and Sp smears well, with >80% of participants reporting the correct result in most years. Errors were generally caused by reporting incorrect staining results (Gram-positive vs Gram-negative) rather than an inappropriate cell type (i.e. shape other than coccus or diplococcus). Participants' success at reporting the correct result for Hi (Gram-negative bacillus or cocco-bacillus) was more variable (in the range 53–88% for SSL/NLs and 44–89% for RRLs) across the distributions (Fig. 1); the most common error was reporting the shape as a coccus or diplococcus instead of a bacillus or cocco-bacillus. Many participants did not report an acceptable result for *L. monocytogenes* (Gram-positive bacillus or cocco-bacillus) and *E. coli* (Gram-negative bacillus or cocco-bacillus). The range of incorrect results suggested that, for many, it was because they were only expecting Hi, Nm or Sp, although this was not confirmed. A few participants reported each time that their samples did not contain bacteria or showed high background staining. In 2018 and 2019 some of the bacterial suspensions were prepared in water rather than a simulated CSF matrix in order to assist with identification; however, this did not noticeably improve the proportion of participants reporting the correct result for those species (Fig. 1). At least one Gram staining sample was excluded from the scoring scheme in each year due to the low proportion of participants reporting a correct result (Table S6).

Identification of viable cultures

All participants, whether receiving the partial or full EQA panel, were expected to identify seven live cultures to species level in each distribution. Panels generally contained two Hi, two Nm and two Sp cultures, apart from 2014 and 2016 in which a third Sp was included. In 2015, 2017, 2018 and 2019, a seventh culture of a different bacterial species that can also cause meningitis was included (Fig. 2 and Table S6). SSL/NLs identified Nm and Sp isolates very well, with only a small number of non-returned results, errors or partially correct results (reporting to genus level only); >90% of the submitted results (i.e. not including non-returned or negative results) were correct to species level in every distribution for both species (Fig. 2a). There was a statistically significant small downward trend in the proportion of correct results for Nm [both as a proportion of all potential results (P=0.008) and when calculated only as a proportion of submitted results (P<0.001)]; however, the proportion of correct submitted results remained >90% in all distributions. A greater number of SSL/NLs had difficulty identifying Hi (Fig. 2a). Nevertheless, >88% of the submitted results for Hi were still correct to species level and there was an apparent increasing trend in this success rate, although this did not reach significance (P=0.087). The identification of the additional species was more variable, but generally good; 90% of submitted results correctly identified *E. coli* and ≥79% correctly identified *S. agalactiae* (Fig. 2a). The exception was *Neisseria lactamica*, which was acknowledged to be a particularly challenging species to identify, and only 46% of those that submitted a result reported the correct species; many participants only identified it to genus level and a few misidentified it as *N. meningitidis*. Due to the small proportion of participants reporting the correct result, the *N. lactamica* sample was excluded from the scoring scheme in the 2017 EQA distribution (Table S6). The RRLs generally generated excellent identification results, with only the occasional error (Fig. 2b).

Serotyping and serogrouping of viable cultures

Serotyping Hi and Sp or serogrouping Nm could be performed phenotypically (using antisera) or genotypically (by PCR) by EQA participants. For Hi and Nm, both methods were expected to produce the correct serotype. However, for Sp, a PCR typing

result was accepted as correct even if it was not accurate to within a single serotype (e.g. '15A/15F' for serotype 15A) if it matched the discrimination of the designated multiplex PCR methods [7–10]. For SSL/NL, serotyping Hi and Sp or serogrouping Nm was an optional requirement and the participation rate was much lower than for species identification (Fig. 3a). However, the overall success rate amongst the submitted results was generally >70% overall for all three species (Fig. 3a). Participation in the serotyping was lowest amongst the SSL/NLs for Sp, consistent with it being the most technically demanding (using multiple antisera or several rounds of multiplex PCR). Although the participation rate was lowest for Sp, there was a significant upward trend in the number of correct serotyping results, whether expressed as a proportion of all possible results (including non-returned results; $P<0.001$) or as a proportion of the submitted results ($P<0.001$; Fig. 3a). There was an apparent increase over time in the proportion of correct results amongst those that submitted a serogrouping result for Nm, too, although this was not statistically significant as it fell noticeably in 2019 (Fig. 3a).

If the results were broken down by individual sample in each distribution, the results showed that, for Hi, the SSL/NLs were very good at identifying serotype b (Hib) strains, with 52–62% participation rates and 97–100% of those that submitted an answer reporting a fully correct result (Fig. S1a). However, for the non-b capsulated (non-Hib) strains and non-typable (NTHi) strains, participation was lower (26–49%) and the serotyping success rate poorer (37–62% of submitted results). A result of 'not type b' was accepted as a partially correct result for non-Hib Hi, since it was known that many laboratories only stocked typing reagents for identifying serotype b; when the partially correct and fully correct results were combined, most laboratories reported an acceptable result for the non-Hib Hi (Fig. S1a).

For Nm, when broken down by individual sample, the overall participation rate amongst SSL/NLs in serogrouping remained relatively consistent between samples (44–63%); however, there was a wide range in success at reporting the correct serogroup (between 49 and 98%; Fig. S1b). The highest success rates were obtained with serogroups A, B and C, plus one unusual isolate that was known to cross-react with both serogroup Y and W antisera. Participants were more likely to fail to report a fully correct serogroup for the W isolates due to the use of commercial kits that could only generate a partially correct result of 'Y/W', 'C/W' or 'A/C/Y/W'. However, if these partially correct results were combined with the fully correct results then the success rate was very high (Fig. S1b). The non-groupable isolate included in 2019 was a very challenging strain and many participants incorrectly reported a false positive result. This, plus the fact that the other Nm isolate in 2019 was serogroup W, accounted for the noticeable drop in the proportion of fully correct results observed for Nm serogrouping that year (Fig. 3a).

When the serotyping results from SSL/NLs for Sp isolates were broken down by sample, the participation rate varied between 16 and 33% (Fig. S1c). Similarly, the serotyping success rate varied between 33 and 100%, depending on the serotype. For the serotypes that were members of a serogroup, a result that was accurate to serogroup level was accepted as partially correct and, for the majority of these samples, most of the SSL/NLs did at least identify the correct serogroup (Fig. S1c). One notable exception was the serotype 35B isolates, for which there were several incorrect results each time, particularly reports of serotype 29; this particular error is related to the choice of antisera used and has been described in previous studies [11, 12].

The RRLs generally serotyped or serogrouped all of the Hi, Nm and Sp isolates very well: for Hi, there were only four incomplete reports of 'non-type b' for either an NTHi or a serotype e strain, one incorrect result ('Hib' for an NTHi) and one non-returned result across all six distributions; for Nm, there were only two partially correct reports ('A/C/Y/W') for a serogroup W strain in 2017; for Sp, there were only six 'partially correct' serotyping results (although mostly the wrong serotype within a serogroup) and a single error (35B reported for a 19F sample) across the six distributions (Figs 3b and S2).

PCR detection of Hi, Nm and Sp in simulated CSF samples

Simulated CSF samples were included in the full EQA panel to test participants' abilities to detect Hi, Nm and Sp by PCR and to serotype or serogroup any positive samples (also by PCR). In each of the panels, two positive samples were included for each species, along with a seventh sample that did not contain any bacteria. This was an optional exercise for SSL/NLs. No SSL/NLs analysed the simulated CSF samples in 2014, but between 11 and 23 labs did so in subsequent years (Fig. 4a). In general, the results for detecting the correct species in the samples were very good, with only a few false negatives. From 2016 onwards virtually every participant correctly detected all Hi-positive samples. The results for Nm were almost as good, with only two incorrect results (both Sp) reported across the years. Similarly, there was a high success rate at detecting Sp, with only four false negative results and one incorrect result (of Nm) across the distributions. The negative control sample was included in order to test participants' ability to prevent cross-contamination of samples and, in general, the SSL/NL participants performed well, with only a single false positive result reported (Sp in 2015; Fig. 4a). There were several non-returned results for the negative samples, which may have been due to confusion on how to report a negative result (but this was not investigated).

The RRLs produced a few false negative results and one false positive (Nm) for the simulated CSF samples in 2014, but their results were very good from 2015 onwards (Fig. 4b).

PCR serogrouping and serotyping of positive simulated CSF samples

Between 11 and 23 of the SSL/NLs that opted for detection of Hi, Nm and Sp in simulated CSF samples also attempted PCR-based serotyping or serogrouping and the results were very encouraging; 76–93% of Hi, 90–100% of Nm and 75–100% of Sp submitted results were fully correct (Fig. 5a). The percentage of these laboratories submitting Hi serotyping results fluctuated between 54 and 83% across the six EQA distributions and the percentage of correct results from those that took part varied between 68 and 100% (Fig. S3a). As had been seen with the serotyping of live cultures, participants generally produced excellent results typing Hib-positive CSF samples. The percentage of correct typing results was lower for NTHi and other serotypes, because some laboratories reported 'non-type b' (accepted as partially correct) and a few obtained an incorrect result. The ability of the RRLs to completely serotype Hi in the simulated CSFs was better than SSL/NLs, but there were a few partially correct reports of 'non-type b' for the non-Hib samples and a few errors (Figs 5b and S4a). A breakdown by serotype showed that, in most years, all the RRLs correctly typed the Hib samples but between 50 and 100% fully typed the non-Hib sample (Fig. S4a).

For the Nm-positive samples, an increasing proportion of the SSL/NLs attempted PCR serogrouping each year and the submitted results were generally excellent, with ≥90% of combined results consistently correct each year (Fig. 5a). There was an increasing trend in the proportion of correct results returned (when compared to all possible results), although it did not reach significance (P=0.045). Nevertheless, there were only four individual errors across all distributions (Fig. S3b). The RRLs, too, performed much more consistently with the Nm-positive samples than they had with the Hi samples (Figs 5b and S4b) and the increasing proportion of correct results returned (when compared to all possible results) was significant (P=0.005). There was only one error across all distributions (a serogroup B sample reported as non-groupable), one partially correct result (serogroup Y strain reported as 'not A, B, C or W') and seven non-returned results between 2014 and 2016.

As with the serotyping of cultures, the participation rate amongst SSL/NLs in PCR serotyping of the simulated CSFs was lowest for the Sp-positive samples (Figs 5a and S3c). The number returning results was very variable across the EQA distributions, although the percentage of correct results was consistently ≥75%. The lowest rate of correct results (56%) was for a serotype 6B sample; this was affected by the fact that the recommended PCR typing scheme had a partially correct result of '6A/6B/6C/6D' as well as the correct result of '6A/6B' and some laboratories reported the former result. The participation rate in Sp PCR serotyping amongst the RRLs was 100% from 2015 onwards (Figs 5b and S4c) and there were very few errors (including the 6B-positive sample, which two RRLs reported as '6A/6B/6C/6D' and two as non-typable).

Scoring and pass rate

The EQA exercise included a scoring scheme, with one point awarded for each correct Gram staining result and up to two points for each correct identification or ST/SG result (Tables S1–S6). The SSL/NL labs were scored on their Gram staining and culture identification results with the partial panel and had to achieve a score of ≥75% to pass. RRLs only tested the full EQA panel and so were also scored on the additional criteria of typing the cultures plus identification and typing of the simulated CSF samples (Table 6). They had to achieve a score of ≥90% overall to pass each exercise. Any SSL/NLs that had volunteered to test the full panel were scored alongside the RRLs, but only had to achieve a score of ≥75% to pass.

The scores obtained by the SSL/NLs in relation to the partial EQA panel were generally very good, with between 83 and 92% passing each year (Fig. 6a). There was no evidence of an increasing or decreasing trend in the passing rate. The RRLs also generally performed very well in relation to their performance with the full EQA panel (Fig. 6b); although a few scores were marginally below 90% in the first 3 years, all RRLs passed the EQA exercise from 2017 onwards. The full panel was acknowledged to be a challenge for SSL/NL laboratories that volunteered to test it. However, between 50 and 73% passed (with a score of at least 75%); this demonstrated an overall upward trend, although this was not statistically significant (P=0.145; Fig. 6c). In addition, between 27 and 50% of these SSL/NLs also achieved the 90% target, depending on the year (Fig. 6c).

DISCUSSION

The results from 6 years of the GISN EQA programme show that both the SSL and NL group and the RRLs have maintained high standards in their respective roles within the network. This includes the identification and ST/SG of Hi, Nm and Sp by both culture and molecular methods required to generate high-quality data for meningitis surveillance [2]. One strength of this EQA programme was the inclusion of a very consistent panel of samples each year (typically four Gram stain slides, seven live cultures and seven simulated CSFs, each with a very similar distribution in numbers of Hi, Nm, Sp and other species between years). This supports the comparison of results between years and the application of a scoring scheme to the results. Another advantage of the EQA was that it comprised a partial panel and a full panel of samples. SSLs and NLs were only obliged to test the partial panel and perform species identification, but not ST/SG. However, those that had the additional resources could opt to test the full panel and add ST/SG of cultures plus the non-culture detection and ST/SG of organisms in simulated CSF samples. RRLs were also tested using the same full panel as the SSLs and NLs.

When overall scores were analysed, the SSLs/NLs maintained a consistently high passing rate (requiring a score ≥75%) and those that attempted the more comprehensive testing of the full panel performed increasingly better over the years (Fig. 6). Not only that, but between 27 and 50% of the SSL/NLs attempting the full panel actually performed as well as the RRLs (i.e. passing with a score of ≥90%). The RRLs (that had to achieve a score of 90% with the full panel to pass the EQA) performed consistently very well, with all passing from 2017 onwards (Fig. 6). Apart from the maintenance of high standards, it was encouraging that the SSL/NL group's performance at the optional SG/ST of meningococcal cultures (excluding the particularly challenging samples in 2019) and pneumococcal cultures improved over time.

Smears for Gram staining

When assessing each component of the EQA scheme individually, some areas for improvement were noted. The Gram staining test produced the most variable results over the time period of the programme. All participants reported the correct cellular morphology for Nm and Sp samples well, but their performance with Hi was lower (Fig. 1), probably due to its more pleiomorphic nature (and possibly due to the use of safranin rather than carbol fuchsin as a counter stain [13]). The Gram staining results for species other than Hi, Nm and Sp were quite poor. This may be due to misunderstandings about the terms in the list of predetermined answers from which participants had to select a result or because some participants assumed that only Hi, Nm or Sp would be included (it was not possible to confirm these hypotheses as the EQA did not include a mechanism for the organizers to send follow-up questions to the laboratories). Following initial participant feedback that the bacteria were difficult to identify in the simulated CSF smears, most of the Gram stain samples were subsequently simplified to smears of simple bacterial suspensions in order to assist with identification, although this did not appear to help significantly. This component of the EQA would benefit from further investigation, and discussion with others that have produced similar samples for EQAs [14], before being included in future distributions, to determine the extent to which incorrect results were caused by unanticipated deterioration of the samples, technical difficulties by the participants or communication problems.

Identification and typing of live cultures

The live cultures were generally identified very well by all participants, although success with Hi was slightly lower than Nm and Sp (Fig. 2). A few laboratories reported no growth for Hi. This may have been caused by the use of the wrong agar medium, as Hi is more fastidious than Nm and Sp and requires culture media containing sufficient X factor (haemin) and V factor (NAD) to support its growth (e.g. chocolate agar). It may also be detrimentally influenced by the use of human, rather than sheep or horse, blood to make these media, which is easier to obtain, but can be bactericidal to all three species. The standard operating procedures that are recommended to the GISN laboratories for blood agar plates are to use blood from domesticated sheep and not human blood [6]. Identification of the species other than Hi, Nm and Sp by NL/SSLs was variable; the *N. lactamica* isolate proved particularly difficult to distinguish from Nm for many of them, but it is acknowledged that this was a very challenging sample. A similar problem would be expected in differentiating *Haemophilus haemolyticus* from Hi or *Streptococcus pseudopneumoniae* from Sp if using phenotypic methods alone, although it is rare to encounter these species in invasive disease. The inclusion of a species-specific PCR in culture identification procedures would help to overcome this challenge [15–24].

Serotyping or serogrouping Hi, Nm and Sp cultures was an optional extra for SSLs and NLs. However, it was encouraging that some of these laboratories not only attempted ST/SG, but that >70% of the returned results were fully correct across all three species (Fig. 3). The success rate for serotyping Hi was notably higher for serotype b (Hib) isolates compared to other serotypes for both the NL/SSL group and RRLs (Figs S1 and S2). A greater number of SSLs and NLs attempted Nm serogrouping. Similar to Hi, the success rate was serogroup-dependent. The difficulty in conclusively serogrouping W strains was primarily caused by the limitations of commercial serogrouping kits, which can only generate a partially correct result of 'Y/W', 'C/W' or 'A/C/Y/W'. Serotyping Sp is more complex than Hi and Nm, due to the large number of serotypes, and, understandably, fewer SSLs and NLs attempted this (Figs 3 and S1). There was a wide range in the success rate of typing different serotypes, although it was encouraging that a significant rising trend in the proportion of labs reporting correct Sp serotyping results was observed over time. In order to simplify the procedure, GISN has provided training in multiplex manual and real-time PCR to try and type (or partially type) strains covered by commercially available pneumococcal vaccines [7–10]. In the future, this could be improved by including additional PCR targets to provide more discrimination [25, 26]. It is known that SSLs and NLs may not have access to the full range of typing reagents (antisera or PCR reagents) for Hi, Nm and Sp cultures and so they were given some credit for reporting partially correct results such as 'not type b' for a non-Hib Hi strain, 'Y/W' etc. for a serogroup W Nm strain, and Sp typing results that were correct to serogroup level (or equivalent for the PCR serotyping). It is anticipated that, in time, resources will improve for these laboratories and they can submit a higher proportion of completely correct results, to increase their value to surveillance. This is an important aspiration, as NTHi and serotype a, e and f Hi strains and Nm serogroup W and Y strains do cause a significant amount of invasive disease [27–30]. Serotype replacement following Sp vaccination will also increase the need to broaden Sp serotyping [31].

Detection and typing of bacteria in simulated CSF samples

Detection of Hi, Nm and Sp by PCR in simulated CSFs was also an optional task for SSLs and NLs. Although relatively few attempted this, the proportion doing so broadly increased from 12% in 2015 to 26% in 2019 and they generally performed well (Fig. 4). Not all SSLs and NLs that could detect the three species in clinical specimens also performed PCR SG/ST on the samples, but between 40 and 83% of them did (depending on the year and sample). They were most successful at confirming Nm serogroups (demonstrating a non-significant, but rising trend in the proportion of correct results); for Hi, they were most successful at confirming Hib-positive samples (as some did not test for the other serotypes); and the lowest number attempted Sp serotyping (although with ≥75% typing success rate), consistent with it being the most technically challenging (Fig. 5). This methodology should be a priority for future training as it will considerably enhance the ability of diagnostic laboratories (SSLs and NLs) in the GISN to identify and ST/SG Hi, Nm and Sp as the widespread use of antibiotic treatment before sample collection in LMICs limits the ability of conventional microbiology to isolate the cause of bacterial meningitis [2].

Performance of RRLs

RRLs had to test the full panel of samples and pass with a score of ≥90%, which was a very stringent requirement. Hence, a few RRLs narrowly missed this target in the early years of the EQA (Fig. 6). In general, however, the RRLs performed all tasks in the EQA to a very high standard and this exercise has served to assist them in maintaining the standards required to help manage the GISN network. There were a small number of reports of only partially correct ST/SG results (e.g. 'non-type b' for Hi) that suggested a few RRLs were not attempting to fully type all isolates as recently as 2018; however, all cultures and simulated CSFs were fully typed in 2019.

Limitations

Some limitations of this EQA programme were noted. Due to its size, it was not feasible for the organizers to follow up and discuss any errors with each participant individually and relied on the participants to request further training as required. It also meant that it was not possible to determine the cause of mistakes or reasons for non-return of results each year, although some participants did volunteer information in comment fields in the online reporting form. There was a noticeable drop in the overall number of participants in 2019. This appears to have been influenced by the fact that many countries relied on financial support from Gavi, the Vaccine Alliance, to support their laboratory surveillance networks; as they were phased out of eligibility for Gavi funding, the number of countries taking part in the GISN was known to have decreased [2].

Conclusions

This 6 year review of results from the global GISN EQA exercise has shown that the laboratories in the network have generally maintained a high standard of laboratory testing and, in some cases, increased their capacity for advanced analyses such as SG/ST Hi, Nm and Sp. In future, the value of this EQA could be increased by including collaboration with other laboratory surveillance networks; this has already begun, with laboratories from the MenAfriNet consortium [32] participating in the 2018 and 2019 distributions (results not included here). This global EQA programme is an important component in the surveillance activities of the WHO-coordinated GISN. It helps to assess the quality of the surveillance data that WHO receives from all the participating SSLs, NLs and RRLs. It has also been a driver for increasing laboratory capacity and has provided a platform on which laboratories can compare their results to others in the same country, in their own WHO region or globally. The increasing capacity for molecular testing within the GISN has also had wider benefits to the participants: opening doors for these laboratories to support testing and surveillance for other infectious diseases including diphtheria, pertussis, typhoid and COVID-19 [2].

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Author contributions
F.S., T.N., M.P.E.S., D.L., S.G., C.S., S.S and E.F conceived and planned each EQA distribution. S.S., T.N., E.F., D.L., S.G. and M.P.E.S analysed the results. D.L and M.P.E.S collated the results and prepared the original draft of the manuscript. All authors reviewed and edited the manuscript.

Conflicts of interest
D.L. and C.S.'s laboratory has received funding from pneumococcal vaccine manufacturers to conduct investigator-led research. M.P.E.S. has received personal fees from GlaxoSmithKline, Pfizer, Merck, AstraZeneca and Sanofi Pasteur as a speaker at international meetings and as a member of advisory boards and has undertaken contract work for Pfizer.

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