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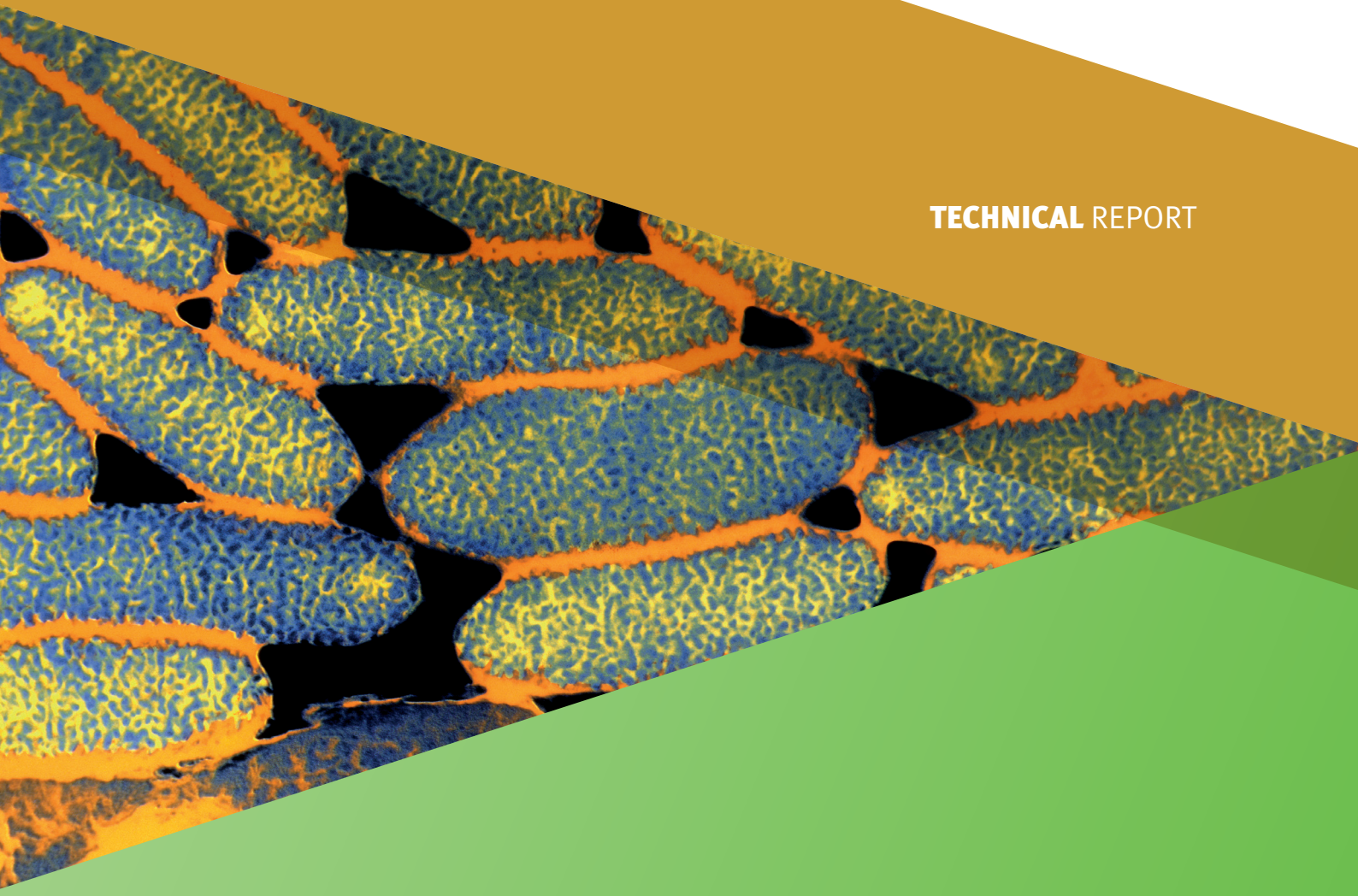

TECHNICAL REPORT

External quality assessment (EQA) schemes to support European surveillance of Legionnaires' disease 2019-2020 - EU/EEA countries

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External quality assessment (EQA) schemes to support European surveillance of Legionnaires' disease 2019–2020

EU/EEA countries

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Contents

Figures

Tables

Abbreviations

BCYE	Buffered charcoal yeast extract
BAL	Broncho-alveolar lavage
CFU	Colony forming units
COVID-19	Disease caused by coronavirus SARS-CoV-2
DFA	Direct fluorescent antibody
ECDC	European Centre for Disease Prevention and Control
EQA	External Quality Assessment
ELDSNet	European Legionnaires' Disease Surveillance Network
EU	European Union
EEA	European Economic Area
ELISA	Enzyme-Linked Immunosorbent Assay
FEPTU	Food and Environmental Proficiency Testing Unit (Public Health England)
GVPC	Glycine vancomycin polymyxin B cycloheximide
GU/L	Genome units per litre
ID	Identification
LUA	*Legionella pneumophila* urinary antigen
MALDI-TOF	Matrix Assisted Laser Desorption Ionisation Time-of-Flight
PCR	Polymerase chain reaction
PFGE	Pulsed Field Gel Electrophoresis
PHE	Public Health England
MLST	Multilocus Sequence Typing
MVLA	Multiple-Locus Variable-Number Tandem-Repeat Analysis
RVPBRU	Respiratory and Vaccine Preventable Bacteria Reference Unit, Public Health England
Sg	Serogroup
SBT	Sequence Base Typing
SNP	Single Nucleotide Polymorphism
ST	Sequence Type
TALD	Travel-associated Legionnaires' disease
UK	United Kingdom
UK NEQAS	United Kingdom National External Quality Assessment Service
WGS	Whole genome sequencing

Executive summary

In 2019, ECDC implemented the start of an EQA scheme for the European Legionnaires' Disease Surveillance network, for the detection, isolation, identification and enumeration of *Legionella* spp. This was organised under a framework contract with the Food and Environmental Proficiency Testing Unit (FEPTU) of Public Health England (PHE) and the United Kingdom National External Quality Assessment Service (UK NEQAS).

This is the first EQA exercise for laboratories participating in surveillance from the ELDSNet network that has been organised by ECDC since 2015 and the EQA format and arrangements have changed. The current EQA scheme uses an outbreak scenario with a package of clinical and environmental samples for the participating laboratories to process, depending on their technical capacity and protocols.

The purpose of the 2019–2020 EQA exercise was to determine the accuracy of *Legionella* testing and results reported by individual laboratories in order to enable comparison of results between laboratories and within countries across Europe. This report presents an analysis of participants' results for the 2019 EQA exercise for the EU/EEA countries.

For each round, up to two nominated laboratories per EU/EEA country were allowed to participate (to cover clinical and/or environmental testing).

Only one round was completed during 2019–2020 due to the impact of COVID-19.

In summary, there was one delivery of EQA samples which was sent on 4 November 2019. This EQA distribution was sent to a maximum of two laboratories per country for a total of 28 EU/EEA countries invited to take part via their national focal point for Legionnaires' disease. Laboratories were selected based on their involvement in the management of public health incidents associated with *Legionella* in their country.

The distribution comprised a total of 20 simulated samples; 10 representing clinical material and 10 representing environmental samples. Strains of *Legionella* was provided by the Respiratory and Vaccine Preventable Bacteria Reference Unit (RVPBRU) and these strains were fully characterised using conventional methods and an analytical profile index system.

Laboratories only needed to report whether the sample/specimen contained a *Legionella* spp., and then provide identification, enumeration, serogroup and sequence type.

Individualised reports were generated for each laboratory that included the results for their individual examinations and the overall results submitted by all laboratories for this exercise.

This report provides an analysis of the EQA performance of laboratories in the 28 EU/EEA countries in relation to the detection/isolation, identification, enumeration and quantification of *Legionella* spp. and the further characterisation of *L. pneumophila*, by serogrouping and sequence-based typing, from both clinical and environmental samples, where applicable. The report is split into two parts - clinical and environmental analysis - as the aim of this first new EQA exercise was to assess the baseline testing.

A separate survey was also organised on methods/kit information and frequency of testing performed for each method/kit by the laboratories.

For this EQA the scenario was a simulation of an outbreak associated with a spa facility. The selected outbreak strain, *Legionella pneumophila,* was serogroup (Sg) 1, sequence type (ST) 47.

Laboratories were given the opportunity to examine samples they would routinely test in their laboratory. For the clinical element, 27 laboratories examined the sputum samples and 24 examined the urine samples. For the environmental element, 26 of the laboratories examined the water samples and 24 examined the swab samples. Where the results reported were not in accordance with the intended exercise, laboratories were advised by contractors to investigate in order to determine the cause.

A total of 27 clinical laboratories from countries within the EU/EEA were sent the clinical distribution (Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Lithuania, Netherlands, Norway, Poland, Portugal, Romania, Slovak Republic, Slovenia, Spain, Sweden and the United Kingdom). All laboratories returned results for this EQA distribution.

For the clinical laboratories, all 27 identified the sample, while 26 identified the serogroup and 14 identified the sequence type.

For the identification of *Legionella pneumophila* in simulated sputum samples, there was excellent concordance with the intended results, with 100% of participants reporting the correct result (specimens 5706, 5710 and 5712). Overall determination of Sg and ST was excellent, with 100% of the participating laboratories achieving the intended result. One specimen (5714) contained *Legionella longbeachae,* for which only 17 out of 27 participants reported the correct identification. A further 6/27 reported either Legionella species, or that the specimen was not *Legionella pneumophila*, both of which were considered correct responses. Overall, 27 participants reported the identification, 26 for Sg and 14 for ST. One specimen (5708) contained commensals only, with no *Legionella* spp.

present. In total, 92.6% of participants reported the correct negative result, however two reported the presence of *Legionella*. All laboratories reported isolation and identification using culture-based methods. A total of 22/27 then went on to use molecular methods to detect *Legionella* and 7/27 performed whole genome sequencing.

The performance for urinary antigen testing was very good, with an overall mean concordance of 95.9% of participating laboratories returning a correct result. However, one country reported an incorrect positive LUA result for specimens 5709 and 5710.

Overall, performance in the identification, serogroup, sequence type and urinary antigen test detection was very good.

A total of 27 environmental laboratories were sent this EQA. The laboratories represented 27 EU/EEA countries (Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Lithuania, Malta, Netherlands, Norway, Poland, Portugal, Romania, Slovak Republic, Slovenia, Sweden and the United Kingdom). All 27 laboratories returned a result.

For *Legionella* isolation, the overall performance was 96%, 27 of the laboratories examined the water samples, with 24 examining the swab samples. Six of the ten simulated samples contained a *Legionella* spp.. The overall performance for correctly identifying the *Legionella* spp. when a sample contained this organism was 99%. Seven of the ten samples were simulated water samples which allowed laboratories to report an enumeration result. The overall performance for the enumeration results correctly reported within the intended range was 86%. The overall performance for reporting a correct serogroup was very good with 98%. For sequence type the overall performance of reporting a correct sequence type was 93% for five of the samples that contained a *L. pneumophila*.

For the environmental laboratories, 27 laboratories reported a result for isolation and identification, with 25 reporting a serogroup, 24 providing an enumeration count and 14 offering a serotype. With regard to molecular methods, eight laboratories analysed the samples for *L. pneumophila* and five looked at those for *Legionella* spp.. The overall isolation performance for culture was 95%, with 27 laboratories reporting a result. The overall performance for molecular methods was very good, with eight laboratories reporting a result for kits detecting *L. pneumophila*. The routine application of molecular methods for water and environmental samples is still being developed in laboratories due to the fact there are currently no guidelines for interpretation of molecular results (GU/L). Therefore, culture remains the preferred method.

The overall performance of the clinical laboratories was very good for the three sets of samples and clearly demonstrated that the fourth paired sample was not associated with the 'outbreak'.

Laboratories have demonstrated that they can undertake testing to an acceptable level of at least 80% concordance with intended results and this is relevant for both clinical and environmental laboratories. This data provides a limited assurance of EU/EEA laboratories' ability to undertake effective public health investigations for *Legionella pneumophila*. Further EQA rounds will provide more data on performance and the robustness of testing.

In conclusion, the performance of laboratories in the EU/EEA countries was very good for culture based/detection methods, which were used by both the clinical (96.2%) and environmental laboratories (95%).

In replies to an ECDC questionnaire, laboratories indicated that this EQA exercise was very useful and overall there was very positive feedback on the new ECDC EQA *Legionella* scheme.

1. Introduction

Background

Legionnaires' disease is a severe and sometimes fatal form of infection caused by the Gram-negative bacteria, *Legionella spp*. These bacteria are found in freshwater and soil worldwide and can contaminate man-made water systems. There are at least 60 species of Legionella and over 20 have been associated with human disease. *Legionella pneumophila* is the most common species isolated both from the environment and from infections. Based on surface antigens, this species can be divided into at least 16 serogroups, of which *L. pneumophila* serogroup 1 is the most common cause of outbreaks. The strains of serogroup 1 most commonly associated with disease share a common epitope, as shown by monoclonal subtyping. It is important to routinely be able to differentiate between *L. pneumophila* and other *Legionella spp*. and to be able to distinguish serogroup 1 from the other serogroups of *L. pneumophila*.

Humans are infected through the inhalation of contaminated aerosols containing *Legionella* bacteria. Legionnaires' disease is classically described as a severe pneumonia that may be accompanied by systemic symptoms and may lead to a fatal outcome. Cases of Legionnaires' disease are mainly reported in older people (>50 years), especially males. Other known risk factors for Legionnaires' disease are smoking, chronic obstructive pulmonary disease, diabetes, immune system compromise and receipt of transplant or chemotherapy. In Europe, most cases (approximately 70%) are community-acquired and sporadic. About 20% of the cases are travel-related and identification of the source of infection often requires international collaboration.

Legionnaires' disease is a statutorily notifiable disease in all EU/EEA countries, but is thought to be under-reported for two reasons:

- it is underdiagnosed by clinicians who may not test patients for Legionnaires' disease before empirically prescribing antibiotics likely to cover *Legionella spp.*;
- some health professionals may fail to notify cases to health authorities. Furthermore, under-ascertainment and differences in laboratory practice may also partly explain the variations in notification rates observed among EU/EEA countries.

Legionnaires' disease surveillance has been carried out at European level since 1987, firstly through a dedicated surveillance network funded by the European Commission and then, since April 2010, through ELDSNet (European Legionnaires' Disease Surveillance Network) coordinated by ECDC. ECDC also coordinates the collation of annual surveillance data on Legionnaires' disease in the EU/EEA with Member States. The resulting surveillance data is available through the European Surveillance Atlas on ECDC's website. A second ELDSNet surveillance system focuses on Travel Associated Legionnaires' Disease (TALD) cases.

The aim of ELDSNet is to detect and communicate on clusters and outbreaks of TALD. The network supports the Member States and other countries involved in sharing information and collaborating on response actions to provide better protection from TALD, both domestically and abroad.

TALD surveillance objectives [1] are:

- to rapidly detect cases and clusters of TALD reported in the EU/EEA and affecting European residents, both in their own countries or abroad;
- to disseminate information on TALD and respond in a coordinated fashion;
- to promote awareness of TALD to support primary preventive action and collaborative investigations;
- to assist in detecting and understanding the extent of common-source outbreaks of Legionnaires' disease worldwide by promptly notifying reported travel-related cases and clusters;
- to reduce the incidence of TALD among EU residents by increasing awareness of active control and prevention measures at accommodation sites.

A laboratory's role during Legionnaires' disease outbreaks includes identifying and characterising the pathogen via clinical and/or environmental samples to support epidemiological investigation, patient treatment/management and source control. Legionnaires' disease cases and environmental findings are reported to the above European surveillance programmes, with cases reported according to agreed case definitions [2].

EQA exercise 2019–2020

In 2019, ECDC organised an EQA exercise for the detection/isolation, identification, enumeration and quantification of *Legionella spp* and the further characterisation of *L. pneumophila* through serogrouping and sequence-based typing from both clinical and environmental samples, where applicable. The EQA was organised in collaboration with the Food and Environmental Proficiency Testing Unit (FEPTU) of Public Health England (PHE) and the United Kingdom National External Quality Assessment Service (UK NEQAS).

This is the first EQA exercise organised by ECDC since 2015 and the EQA format and arrangements have changed. The purpose of this EQA exercise was to determine the accuracy of *Legionella* testing and results reported by

individual laboratories, to allow comparison of results between laboratories and within countries across Europe. This report presents an analysis of participants' results for the 2019 EQA exercise in the EU/EEA countries. The results provided ECDC with information on the laboratories' capabilities of accurately performing *Legionella* testing. This helped to provide confidence in data submitted for surveillance; identify where further support is needed for individual laboratories or countries and allow laboratories to understand their own capabilities, if testing demand were to increase due to an outbreak.

The overall objectives of the 2019–2020 EQA were:

- to provide a baseline understanding of the level of testing undertaken in laboratories in response to routine outbreak scenarios, for both clinical and environmental samples;
- to determine where there were any general performance issues;
- to provide individual technical support to laboratories as a follow up to the exercise, if requested by the countries.

2. Study design and methods

Organisation of EQA

This EQA was organised by FEPTU and UK NEQAS for Microbiology in collaboration RVPBRU, PHE and ECDC as part of an ECDC Framework contract (ECDC/2019/024). The EQA exercise was for laboratories nominated through ECDC National Focal Points for Legionnaires' disease within ELDSNet and up to two nominated laboratories per EU/EEA country (to cover clinical and/or environmental samples) could participate per round. Two rounds were foreseen for 2019–2020, however due to the COVID-19 situation, only one could be implemented.

The laboratories chosen were those involved in the management of public health incidents in their country and/or undertaking expert reference testing for specialised examinations. A unique laboratory identification was created and user name and passwords generated for each one. This allowed the laboratory to return results and view individualised reports through a secure web portal and it meant that the results were anonymised results for ECDC.

Both FEPTU and UK NEQAS are UKAS accredited EQA providers under ISO/IEC 17043:2010 (Conformity assessment- General requirements for proficiency testing) and all these principles and practices was applied to the ECDC EQA scheme.

- The EQA distribution was sent on 4 November 2019 to a maximum of two laboratories per country in a total of 28 EU/EEA countries.
- ECDC invited ECDC National Focal Points for Legionnaires' disease to propose up to two laboratories per country to take part in the EQA exercise, one that undertakes clinical examination of specimens and one that examines environmental samples. One laboratory could also be nominated to participate in both clinical and environmental, if they usually processed both types of sample. Participating laboratories needed to be contributing to national surveillance data or environmental findings that are shared through ELDSNet surveillance activities.
- The EQA organiser sent a letter of invitation to the nominated laboratories informing them of the EQA arrangements and the objectives of the exercise. The letter also provided an opportunity for the laboratories to confirm their interest in participating and that their details in the system were correct.
- The distribution comprised a total of 20 simulated samples; 10 representing clinical material and 10 representing environmental samples. Sample/specimen design and format was agreed in advance with ECDC and PHE *Legionella* experts.
- PHE undertook testing of the samples in accordance with published methods, to replicate where possible testing methods that would be used by the participants. Detection, identification, enumeration, confirmation and further characterisation tests (serogrouping (Sg) and sequence-based typing (SBT)) were also undertaken.
- PHE also ran a separate survey on methods/kit information and frequency of testing performed for each method/kit by the laboratories.
- The distribution exercise simulated an outbreak associated with a spa facility. The outbreak *Legionella pneumophila* strain chosen was serogroup (Sg) 1, sequence type (ST) 47.

A total of 27 environmental and 27 clinical laboratories from 28 EU/EEA countries took part in the EQA, table 1. Each laboratory was provided with a unique laboratory identification. Of those taking part, 20/34 participating countries tested both the clinical specimens and environmental samples.

Table 1. Countries within the EU/EEA that participated in the clinical and environmental EQA

Country	Clinical EQA samples	Environmental EQA samples	Number of participating laboratories per country
Austria	Yes	Yes	1
Belgium	Yes	Yes	1
Bulgaria	Yes	Yes	1
Croatia	Yes	Yes	1
Cyprus	Yes	Yes	2
Czech Republic	Yes	Yes	1
Denmark	Yes	Yes	1
Estonia	Yes	Yes	1
Finland	Yes	Yes	2
France	Yes	Yes	2
Germany	Yes	Yes	1
Greece	Yes	Yes	1
Hungary	Yes	Yes	1
Ireland	Yes	Yes	1
Italy	Yes	Yes	1
Latvia	Yes	Yes	1
Lithuania	Yes	Yes	1
Malta	No	Yes	1
Netherlands	Yes	Yes	1
Norway	Yes	Yes	1
Poland	Yes	Yes	1
Portugal	Yes	Yes	2
Romania	Yes	Yes	1
Slovak Republic	Yes	Yes	2
Slovenia	Yes	Yes	1
Spain	Yes	No	1
Sweden	Yes	Yes	1
United Kingdom	Yes	Yes	2

An EQA protocol was drawn up and sent with the samples which were dispatched in approved United Nations containers. This protocol included information on the sample/specimen details, instructions on how to process samples/specimens, information on the results to be provided, a copy of a method questionnaire (information to be returned electronically) and safety information. All information was also provided electronically to all participants and NFPs for Legionnaires' disease in ELDSNet and was available on the UK NEQAS web portal.

A dedicated web page was available on the UK NEQAS website for participants to enter and submit their results. Participants could access instructions for using the secure web portal and download the protocol describing the process for specimen examination via the web page. Detailed instructions were included on how to access the secure website via a unique user ID and password provided for each participant. The deadline for final submission of results was stated on the paperwork detailing the sample/specimen information. For convenience, there was also a copy of the web reply form available for participants to download so that they could manually record test results before submitting them online. For this first exercise participants were allowed six weeks (42 days) from the date of dispatch of both clinical and environmental samples to examine the EQA specimens/samples and return all their results. The length of time allowed for this exercise was due to the length of time required to isolate the *Legionella* spp. on culture media (minimum 10 days) and undertake the relevant confirmatory testing, which includes the time a reference laboratory may take to provide a result for specialist tests, such as ST.

Six weeks after the date of dispatch (4 November 2019), the web platform was closed for results submission and the intended results were published on the secure website on 20 December 2019 where they could be accessed by participating laboratories. Participants were notified by email that the intended results were available for viewing. Individualised reports were made available ten weeks after the closing date on 6 March 2020.

From 2–23 April 2020, ECDC conducted a short online survey to obtain feedback on the EQA exercise and enable the laboratories to suggest improvement for the next distribution. A summary of this feedback can be found in the discussion section 4.

Certificates of participation were sent electronically to the laboratories on 21 April 2020. A hard copy of the certificate was available on request.

EQA exercise scenario and sample design

The strains selected for the November 2019 exercise were chosen in consultation with PHE *Legionella* experts in clinical and environmental microbiology. Sample/specimen design was also developed in collaboration with the PHE UK NEQAS and ECDC experts and approved by ECDC.

Five outbreak environmental samples were supplied to represent spa pool water, a swab of the biofilm from the spa pool pipeline and water from three cooling towers in the same vicinity as the spa. In addition, five routine monitoring samples were supplied: water and swabs from hot and cold water systems and water from a spa pool.

Clinical samples were taken from six patients with suspected *Legionella* symptoms (sputum and or urine samples.)

The strain of *Legionella pneumophila* serogroup 1, ST47 used for this EQA exercise is considered a leading cause of Legionnaires' disease in north-western Europe, however it is rarely isolated from the environment. The *Legionella* strains were provided, tested and fully characterised (before and after sample/specimen preparation) by the Respiratory and Vaccine Preventable Bacteria Reference Unit (RVPBRU).

Legionella pneumophila serogroup 3, ST2630 was also included, as this is a unique ST with a single documented isolation from a community-acquired clinical case in the UK. Legionnaires' disease attributed to *L. pneumophila* serogroup 3 is less common than detections of serogroup 1 infection. Many commercially available urinary antigen kits for *Legionella pneumophila* are not designed and validated for the detection of non-serogroup 1 type *L. pneumophila* antigens.

Strains of *Legionella* were provided by RVPBRU as fully characterised isolates; commensal/background flora was taken from a bank of organisms held by the EQA organisers and these strains were fully characterised using conventional methods and an analytical profile index system. All isolates were clinical isolates from patients with pneumonia. In 2018, 80% of respiratory samples from patients with *Legionella* were positive for *Legionella pneumophila* serogroup 1, of which ST47 is one of the more commonly isolated strains from patients in the UK. Non-serogroup 1 infections are detected at a much lower frequency*. L bozemanii* is rarely isolated from patients in the UK.

Samples/specimens were prepared and quality-controlled by the EQA organisers and the panels was dispatched as distribution 4680 (clinical) and 4681 (environmental).

All packages with samples were dispatched at ambient temperature, in accordance with the latest International Air Transport Association (IATA) regulations, using an approved airfreight company.

Additional data was collected and analysed through a questionnaire on methodology and the annual number of tests done using this method, materials and EQA participation. This information was captured electronically and findings are shown in the annex of this report.

Provision of individual feedback to the participating laboratories on their results was provided in an environmental EQA report. This included recommendations (where necessary), actions to take and method performance (if applicable.) The PHE *Legionella* expert for clinical and water microbiology provided comments on these reports based on performance and strains used.

The individualised laboratory reports detailed a laboratory's reported results for each examination requested and the intended results for each sample (including the simulated microbiological contents). This included the identification of the *Legionella* species, serogroup, serotype, enumeration results, where applicable. The report also provided an overall performance for each examination based on all the laboratories reported results.

Clinical

A total of 27 sets of specimens were distributed to 27 participating countries. Ten clinical samples were prepared in each set (five simulated sputum samples and five liquid urine samples.) An overview of samples is provided in Table 2.

Participants were asked to provide an organism identification, serogroup and sequence type (simulated sputum samples) and LUA result (urine specimens). Simulated sputum samples were prepared in a lyophilised format. The freeze-dried sample matrix was composed of inositol serum broth with variable concentrations of the pathogen *Legionella pneumophila* or other species. To simulate the specimen close to an authentic clinical material, the freeze-dried vials contained a strain of the pathogen and included commensal flora commonly isolated from lower respiratory tract infections. The serogroups and species of *Legionella* to be used were approved by the commissioned experts at PHE. Participants' results were analysed and considered 'concordant' if the reported categorisation agreed with the PHE reference laboratory (RVPBRU) interpretation. In addition, participants were asked to complete a questionnaire survey to provide further information on methods used, both in general and for this EQA exercise. Additional data was collected and analysed via a questionnaire on methodology and the annual number of tests done for this method, materials and EQA participation.

The yield of the pathogen after reconstitution of the lyophilised vials, ranged between 10^2–10^4 colony-forming units per mL. The yield of the commensal flora following lyophilisation, ranged between 10^2–10^3 colony-forming units per mL.

Two simulated specimens with no pathogens were also included in the set of 10 specimens.

Instructions provided to participants included:

- how to reconstitute lyophilised specimens with 1mL of nutrient broth (the pellet had to be fully dissolved in the liquid media to attain a homogenous suspension);
- how to inoculate the appropriate media with the appropriate incubation conditions to isolate any potential pathogens;
- information on reporting results (absence or presence of *Legionella pneumophila* or other species.)

The simulated sputum samples were examined using the national documents SMI ID18 Identification of *Legionella* species and SMI B 57 Investigation of bronchoalveolar lavage, sputum and associated specimens. This is in accordance with the requirements for clinical laboratories accredited to ISO 15189:2012 (Medical laboratories - Requirements for quality and competence).

Environmental

Ten environmental samples were prepared as LENTICULE discs. This method of preparing samples has been extensively validated and proven to preserve organisms over long periods of time. Samples were tested in the FEPTU laboratory according to the international method ISO 11731:2017 (Water quality - Enumeration of *Legionella*) for water, sludge and swab samples. This is in accordance with water laboratories being accredited to ISO/IEC 17025:2010 (General requirements for the competence of testing and calibration laboratories).

The simulated sample designs included a selection of the following to make the 10 samples required: water taken from various sites such as cooling towers, hot and cold water systems, spa pools and swab samples.

The samples positive for *Legionella* spp. contained bacteria at varying levels from $<10^2 – 10^5$ colony-forming units/L.

Background organisms were included that were relevant to the sample type in order to simulate a real sample, but also to challenge the laboratories' processing techniques, such as acid/heat treatment, and to confirm the performance of the selective agar used. Participants were not asked to report on the background flora included.

Samples were authorised for inclusion in a distribution if:

- they were homogeneous;
- they passed quality control testing prior to the distribution date;
- the sample contents matched those obtained from RVPBRU for identification, serogroup and sequence type.

Samples were quality-controlled as they would have been by the participant. This step involved rehydration and culturing onto Glycine Vancomycin Polymyxin Cycloheximide (GVPC) as neat, following heat and acid treatment. Agar plates were incubated for up to 10 days aerobically at 37°C and read on Day 3, 6 and 10. Any suspected *Legionella* spp. was ascertained by means of confirmatory testing.

Background flora selected for inclusion in the samples were those that would compete with the *Legionella* spp. in the sample. During processing for the isolation of *Legionella*, heat and acid treatment is employed to kill competing organisms - if this is done correctly.

Homogeneity and stability results were analysed using local robust statistics to ensure suitability for use and that defined criteria were met.

Results for environmental samples were analysed according to ISO 13528:2015 (Statistical methods for use in proficiency testing by inter-laboratory comparison). For enumeration values the participants' median was used as the assigned value and the intended range calculated using robust statistics (5 and 95% percentiles).

Data was displayed graphically. Detected/not detected serogroup and sequence type results were analysed against the intended results which were based on RVPBRU confirmation. For molecular examination, the samples were examined according to the procedures in ISO/TS 12869:2019 - Water quality - Detection and quantification of *Legionella* spp. and/or *Legionella pneumophila* by concentration and genic amplification using a quantitative polymerase chain reaction (qPCR).

3. Results

The methods questionnaire sent to participants to gather details on processes and methods was analysed as part of this EQA exercise and findings can be found in Annex 1. Key results are integrated into the separate sections below.

Intended results for the 2019–2020 exercise

Sample contents for the specimens included in the clinical and environmental distributions are described in Tables 2 and 3, including the serogroup and sequence base type when *Legionella pneumophila* was present.

The intended results to be returned by participating laboratories for each specimen are listed in Tables 4–15 for clinical samples and Tables 16–18 for environmental samples.

Table 2. Clinical specimens 5706–5715 provided in the distribution (4 November 2019)

Specimen number	Patient	Sample	Sample type	Sample contents	Sg	SBT	Details
5706	1	1	Sputum	*Legionella pneumophila* *Streptococcus oralis* *Streptococcus salivarius*	1	47	Patient lives in location of cooling tower, has visited spa
5707		2	Urine	*Legionella pneumophila*	1	47	
5708	2	1	Sputum	*Neisseria sicca* *Streptococcus mitis*			Patient lives in location of cooling tower, has visited spa
5709		2	Urine	*Legionella pneumophila*	1	47	
5710	3	1	Sputum	*Legionella pneumophila* *Streptococcus oralis* *Streptococcus salivarius* *Moraxella catarrhalis*	1	47	Patient lives in location of cooling tower, has visited spa
5711		2	Urine	*Legionella pneumophila*	1	47	
5712	4	1	Sputum	*Legionella pneumophila* *Streptococcus mitis* *Streptococcus salivarius*	3	2630	Asthmatic elderly male, lives at home local area, rarely goes out, except to the shops and local public inn
5713		2	Urine	*Legionella pneumophila*	3	2630	
5714	5	5	Sputum	*Legionella longbeachae* *Streptococcus oralis* *Streptococcus salivarius*			Community acquired pneumonia, keen gardener, elderly
5715	6	6	Urine	No organisms			Patient on ECMO, severe pneumonia

Table 3. Environmental samples 5716–5725 provided in the distribution (4 November 2019)

Specimen number	Sample type	Sample contents	Sg	SBT	Comments
5716	Spa water from site 1	*Legionella pneumophila* *Microbacterium luteolum*	1	47	Samples taken as part of one outbreak investigation
5717	Biofilm swab from spa water pipeline (site 1)	*Legionella pneumophila* *Staphylococcus saprophyticus*	1	47	
5718	Cooling tower water (site 2)	*Klebsiella pneumoniae* *Staphylococcus haemolyticus* *Enterococcus faecium*			
5719	Cooling tower water (site 3)	*Legionella pneumophila* *Enterococcus faecalis*	1	47	
5720	Cooling tower water (site 4)	*Klebsiella pneumoniae* *Staphylococcus haemolyticus* *Enterococcus faecium*			
5721	Hot and cold water system	*Legionella pneumophila* *Brevundimonas vesicularis* *Aerococcus viridans*	1	48	Samples taken as part of routine quality monitoring of water
5722	Biofilm swab from hot and cold water system	*Pseudomonas putida* *Staphylococcus epidermidis*			
5723	Hot and cold water system	*Legionella bozemanii* *Acinetobacter junii* *Pseudomonas stutzeri*			
5724	Spa water	*Legionella pneumophila* *Citrobacter braakii*	6	2923	
5725	Biofilm swab from hot and cold water system	*Roseomonas aestuarii* *Pseudomonas aeruginosa*			

Clinical

A total of 27 of participating laboratories from 27 countries reported results for the simulated sputum samples, compared to 24 for urine samples.

From the methods questionnaire, it was determined that a total of 22/27 participants had reported they were clinical diagnostics laboratories, as well as reference laboratories. One laboratory noted that it was only a clinical diagnostics laboratory. The remaining 4/27 were reference laboratories.

A total of 17/27 laboratories participated in a national EQA scheme. However, this was only a mandatory exercise for seven of them. The most commonly tested sample types reported by the 27 laboratories for routine testing were sputum, broncho-alveolar lavage and urine. BCYE (7) and BCYE/BMPA (7) were the most frequently used media for the isolation of *Legionella* spp., together with MALDI-TOF (16) and serology (19) as the confirmatory tests. (See results in Annex 1).

Participants were only requested to report information on *Legionella* spp. and not on the background flora included to simulate a specimen.

Specimen 5706: This specimen contained *Legionella pneumophila* serogroup 1: ST47. An excellent concordance with intended results was achieved for all participating laboratories.

Table 4. *Legionella pneumophila* (5706) - intended results reported by the PHE reference laboratory and overall concordance with participating laboratories

Intended interpretation	Number of laboratories returning correct results/total number reporting a result	Overall concordance (%)
Legionella pneumophila	27/27	100
Serogroup 1	25/25	100
Sequence type 47	14/14	100

Specimen 5707: This specimen was positive for a *Legionella pneumophila* urinary antigen. An excellent concordance with intended results was achieved for all 24 participating laboratories returning a result.

Table 5. *Legionella pneumophila* (5707) - intended results reported by the PHE reference laboratory and overall concordance with participating laboratories

Intended interpretation	Number of laboratories returning correct results/total number reporting a result	Overall concordance (%)
Legionella pneumophila antigen detected	24/24	100

Specimen 5708: This specimen contained *Neisseria sicca* and *Streptococcus mitis* only, no *Legionella* spp. was present. A good concordance with intended results was achieved. Incorrect results reported included the presence of *Legionella* spp. (n=1) and *Legionella pneumophila* (n=1).

Table 6. Negative for *Legionella* (5708) - intended results reported by the PHE reference laboratory and overall concordance with participating laboratories

Intended interpretation	Number of laboratories returning correct results/total number reporting a result	Overall concordance (%)
Negative	25/27	92.6

Specimen 5709: The specimen was positive for *L. pneumophila* urinary antigen. A good concordance with intended results was achieved. Two laboratories incorrectly reported results as 'antigen not detected' and a further three stated that this specimen was 'not examined'.

Table 7. *Legionella pneumophila* (5709) - intended results reported by the PHE reference laboratory and overall concordance with participating laboratories

Intended interpretation	Number of laboratories returning correct results/total number reporting a result	Overall concordance (%)
Legionella pneumophila serogroup 1 antigen detected	22/24	91.7

Specimen 5710: The specimen contained *Legionella pneumophila* serogroup 1: ST47. An excellent concordance with intended results was achieved for all participating laboratories.

Table 8. *Legionella pneumophila* (5710) - intended results reported by the PHE reference laboratory and overall concordance with participating laboratories

Intended interpretation	Number of laboratories returning correct results/total number reporting a result	Overall concordance (%)
Legionella pneumophila	27/27	100
Serogroup 1	26/26	100
Sequence Type 47	14/14	100

Specimen 5711: The specimen was positive for *L. pneumophila* urinary antigen. Two laboratories incorrectly reported the results as 'antigen not detected'. A good concordance with intended results was achieved for the majority of participating laboratories.

Table 9. *Legionella pneumophila* (5711) - intended results reported by the PHE reference laboratory and overall concordance with participating laboratories

Intended interpretation	Number of laboratories returning correct results/total number reporting a result	Overall concordance (%)
Legionella pneumophila antigen detected	22/24	91.7

Specimen 5712: The specimen contained *Legionella pneumophila* serogroup 3: ST2630. An excellent concordance with intended results was achieved for all participating laboratories.

Table 10. *Legionella pneumophila* (5712) - intended results reported by the PHE reference laboratory and overall concordance with participating laboratories

Intended interpretation	Number of laboratories returning correct results/total number reporting a result	Overall concordance (%)
Legionella pneumophila	26/26	100
Serogroup 3	26/26	100
Sequence Type 2630	14/14	100

Specimen 5713: The specimen was negative for *L. pneumophila* urinary antigen. An excellent concordance with intended results was achieved for all participating laboratories.

Table 11. *L. pneumophila* antigen not detected (5713) - intended results reported by the PHE reference laboratory and overall concordance with participating laboratories

Intended interpretation	Number of laboratories returning correct results/total number reporting a result	Overall concordance (%)
Legionella pneumophila antigen not detected	24/24	100

Specimen 5714: The specimen contained *Legionella longbeachae.* Three laboratories reported the result incorrectly as *Legionella pneumophila* and one laboratory did not test the sample. A good concordance with intended results was achieved for the majority of participating laboratories within the EU/EEA.

Table 12. *Legionella longbeachae.* (5714) - intended results reported by the PHE reference laboratory and overall concordance with participating laboratories

Intended interpretation	Number of laboratories returning correct results/total number reporting a result	Overall concordance (%)
Legionella longbeachae	17/27	88.5
Legionella species, not *L. pneumophila*	6/27	

Specimen 5715: The specimen was negative for *L. pneumophila* urinary antigen. A very good concordance with intended results was achieved for the majority of participating laboratories.

Table 13. *L. pneumophila* antigen not detected (5715) - intended results reported by the PHE reference laboratory and overall concordance with participating laboratories

Intended interpretation	Number of laboratories returning correct results/total number reporting a result	Overall concordance (%)
Legionella pneumophila antigen not detected	23/24	95.8

Summary of results by specimen type

Overall determination of Sg and ST was excellent, with 100% of participating laboratories achieving the expected result.

Table 14. Serogroup and sequence type concordance for simulated sputum specimens containing *Legionella* spp.

Specimen number	Serogroup (Sg)	Sequence type (ST)
5706	100 (n=25)	100 (n=14)
5710	100 (n=26)	100 (n=14)
5712	100 (n=26)	100 (n=14)

The performance for urinary antigen testing was very good, with an overall mean concordance of 95.9% of participating laboratories returning a correct result.

Table 15. Urinary antigen result concordance

Specimen number	LUA result *L. pneumophila* antigen	Overall concordance(%)
5707	Detected	100
5709	Detected	91.7
5711	Detected	91.7
5713	Not detected	100
5715	Not detected	95.8

Environmental samples

Ten simulated environmental samples were sent to 27 laboratories in 27 EU/EEA countries. All 27 laboratories returned a result within the six-week timeframe for this EQA exercise.

Sample numbers: 5716–5720 were samples taken as part of one outbreak investigation.

Sample numbers: 5721–5725 were samples were taken as part of routine monitoring.

Sample numbers: 5717, 5722 and 5725 were swab samples.

Sample numbers: 5716, 5718, 5719, 5720, 5721, 5723 and 5724 were water samples.

Performance of the laboratories on these samples were split into culture-based methods (Table 16) and molecular methods (Table 17). Culture-based method analysis included results reported for isolation, identification, enumeration, serogroup and sequence type results. An overall performance assessment column as a percentage has been captured for culture-based method results by sample number and by each examination (including isolation, identification, enumeration, serogroup and serotype). Overall performance by sample was calculated using the mean value across each of the five examinations. For molecular methods and culture results the overall performance is shown by examination only.

Table 18 shows in more detail the enumeration results reported by the laboratories.

Only eight datasets were returned (25 laboratories examined the sample for isolation) with culture-based methods and molecular methods providing molecular results and quantification results. Therefore these were not statistically analysed as the data generated would not be robust.

Table 16. Examinations done on cultured samples

Sample number	Contents	Isolation		Identification		Enumeration		Serogroup		Sequence type		Overall % performance by sample
		N	%	N	%	N	%	N	%	N	%	
5716	*L. pneumophila* sg 1, ST47	26/27	96	26/26	100	23/24	96	25/25	100	12/13	92	97
5717	*L. pneumophila* sg 1, ST47	24/24	100	24/24	100	-	-	23/23	100	13/14	92	98
5718	No Legionella	25/25	100	-	-	-	-	-	-	-	-	100
5719	*L. pneumophila* sg 1, ST47	24/25	96	24/24	100	20/22	91	23/23	100	10/12	83	94
5720	No Legionella	25/25	100	-	-	-	-	-	-	-	-	100
5721	*L. pneumophila* sg 1, ST48	23/27	85	23/23	100	19/24	79	22/22	100	11/11	100	93
5722	No Legionella	24/24	100	-	-	-	-	-	-	-	-	100
5723	*Legionella bozemanii*	24/27	88	23/24	96	19/22	86	-	-	-	-	90
5724	*L. pneumophila* sg 6, ST2923	24/27	88	24/24	100	18/23	78	9/10	90	8/8	100	91
5725	No *Legionella*	24/24	100	-	-	-	-	-	-	-	-	100
Overall performance by examination			95		99		86		98		93	

Table 17. Molecular methods

Sample number	Identification	Intended results for *Legionella pneumophila*	Molecular results *Legionella pneumophila* (%)	Quantification *Legionella pneumophila*	Intended results for *Legionella* spp.	Molecular results *Legionella* spp. (%)	Quantification *Legionella* spp.
5716	*Legionella pneumophila*	Detected	8/8 (100)	2 (154000, 2600000)	Detected	3/4 (75)	2 (139000, 2600000)
5717	*Legionella pneumophila*	Detected	7/7 (100)	-	Detected	2/3 (67)	-
5718	No *Legionella*	Not detected	7/7 (100)	-	Not detected	3/4 (75)	-
5719	*Legionella pneumophila*	Detected	8/8 (100)	1 (177000)	Detected	3/5 (60)	1 (172000)
5720	No *Legionella*	Not detected	6/6 (100)	-	Not detected	3/5 (60)	1 (<608)
5721	*Legionella pneumophila*	Detected	8/8 (100)	2 (9849, 310000)	Detected	4/5 (80)	2 (7852, 480000)
5722	No *Legionella*	Not detected	5/6 (83)	-	Not detected	2/3 (67)	-
5723	*Legionella bozemanii*	Not detected	7/7 (100)	1 (<840)	Detected	5/5 (100)	2 (16300, 850000)
5724	*Legionella pneumophila*	Detected	6/7 (86)	2 (6728, 330000)	Detected	4/4 (100)	2 (6986, 260000)
5725	No *Legionella*	Not detected	5/6 (83)	-	Not detected	2/3 (67)	1 (<608)
Overall performance			95			75	

Table 18. Data on enumeration results

Sample number	Identification	Participants median (cfu/L)	Intended range (cfu/L)	Number of results	Number of outlying counts
5716	*Legionella pneumophila*	$3.1x10^4$	$3.3x10^3 – 6.9x10^4$	24	1 high
5717	*Legionella pneumophila*	-	-	-	-
5718	No *Legionella*	-	-	-	-
5719	*Legionella pneumophila*	$2.2x10^4$	$1.1x10^3 – 8.7x10^4$	22	2 high
5720	No *Legionella*	-	-	7	-
5721	*Legionella pneumophila*	$1.1x10^3$	$98 – 3.9x10^3$	24	3 (1 low, 2 high) and 2 < values
5722	No *Legionella*	-	-	-	-
5723	*Legionella bozemanii*	$5.5x10^3$	$87 – 2.8x10^4$	22	3 (1 low, 2 high)
5724	*Legionella pneumophila*	$1.3x10^3$	$1.1x10^2 – 9.8x10^3$	23	3 (1 low, 2 high) and 2 < values
5725	No *Legionella*	-	-	-	2 < values

Sample 5716: This sample was water from spa (site 1) which contained a *Legionella pneumophila* serogroup 1 sequence type ST47 at levels of approximately 10^5 colony-forming units per litre. The background flora was *Microbacterium luteolum*.

Performance was very good with 26/27 (96%) of participants reporting the correct isolation result, 26/26 (100%) for identification, 23/24 (96%) of the laboratories reporting a count within the intended range, 25/25 (100%) reporting the correct serogroup and 12/13 (92%) of the laboratories reporting the correct sequence type. The overall performance for examinations by culture was 97%.

Eight laboratories examined the sample using a molecular kit which only detects *L. pneumophila* and all eight reported the correct result. In addition, four of these laboratories also examined the sample using a molecular kit that detects *Legionella* spp., with 3/4 reporting a correct result.

Sample 5717: This sample was a swab from the biofilm of a spa water pipeline (site 1) which contained a *Legionella pneumophila* serogroup 1 sequence type ST47 at levels of approximately 10^5 colony-forming units per litre. The background flora was *Staphylococcus saprophyticus*.

Performance was very good, with 24/24 (100%) of participants reporting the correct isolation result, 24/24 (100%) for identification, 23/23 (100%) reporting the correct serogroup and 13/14 (92%) of the laboratories reporting the correct sequence type. The overall performance for examinations by culture was 98%.

Seven laboratories examined the sample using a molecular kit which only detects *L. pneumophila* and all seven reported the correct result. In addition, three of these laboratories also examined the sample using a molecular kit that detects *Legionella* spp., with 2/3 reporting a correct result.

Sample 5718: This sample was water taken from a cooling tower (site 2) which contained no *Legionella* spp.. Background flora were *Klebsiella pneumoniae*, *Staphylococcus haemolyticus* and *Enterococcus faecium.*

Performance was excellent with 25/25 (100%) of the laboratories reporting the correct isolation result. In addition, 8/8 (100%) laboratories reported a low censored value which is considered to be a correct result, although this data is not shown in Table 16. The overall performance for examinations by culture was 100%.

Seven laboratories examined the sample using a molecular kit which only detects *L. pneumophila* and all seven reported the correct result. In addition, four of these laboratories also examined the sample using a molecular kit that detects *Legionella* spp., with 3/4 reporting a correct result.

Sample 5719: This sample was water taken from a cooling tower (site 3) which contained a *Legionella pneumophila* serogroup 1 sequence type ST47 at levels of approximately 10^4 colony-forming units per litre. Background flora was *Enterococcus faecium*.

Performance was very good, with 24/25 (96%) of participants reporting the correct isolation result, 24/24 (100%) for identification, 20/22 (91%) of the laboratories reporting a count within the intended range, 23/23 (100%) reporting the correct serogroup and 10/12 (83%) of the laboratories reporting the correct sequence type. The overall performance for examinations by culture was 94%.

Eight laboratories examined the sample using a molecular kit which only detects *L. pneumophila* and all eight reported the correct result. In addition, five of these laboratories examined the sample using a molecular kit that detects *Legionella* spp., with 3/5 reporting a correct result.

Sample 5720: This sample was water taken from a cooling tower (site 4) which contained no *Legionella* spp.. Background flora were *Klebsiella pneumoniae*, *Staphylococcus haemolyticus* and *Enterococcus faecium.*

Performance was excellent, with 25/25 (100%) of the laboratories reporting the correct isolation result. In addition, 7/7 (100%) laboratories reported a low censored value which is considered to be a correct result, although this data is not shown in Table 16. The overall performance for examinations by culture was 100%.

Six laboratories examined the sample using a molecular kit which only detects *L. pneumophila* and all six reported the correct result. In addition, five of these laboratories examined the sample using a molecular kit that detects *Legionella* spp., with 3/5 reporting a correct result.

Sample 5721: This sample was a water taken from a hot and cold water system which contained a *Legionella pneumophila* serogroup 1 sequence type ST48 at levels of approximately 10^2 colony-forming units per litre. Background flora were *Brevundimonas vesicularis* and *Aerococcus viridans*.

Performance was good, with 23/27 (85%) of participants reporting the correct isolation result, 23/23 (100%) for identification, 19/24 (79%) of the laboratories reporting a count within the intended range, 22/22 (100%) reporting the correct serogroup and 11/11 (100%) of the laboratories reporting the correct sequence type. The overall performance for examinations by culture was 93%.

Eight laboratories examined the sample using a molecular kit which only detects *L. pneumophila* and all eight reported the correct result. In addition, five of these laboratories examined the sample using a molecular kit that detects *Legionella* spp., with 4/5 reporting a correct result.

Sample 5722: This sample was a swab of a biofilm from a hot and cold water system which contained no *Legionella* spp.. Background flora were *Pseudomonas putida* and *Staphylococcus epidermidis*.

Performance was excellent, with 24/24 (100%) of the laboratories reporting the correct result.

Six laboratories examined the sample using a molecular kit which only detects *L. pneumophila* and 5/6 reported the correct negative result for *Legionella*. In addition, three of these laboratories also examined the sample using a molecular kit that only detects *Legionella* spp., with 2/3 reporting the correct negative result for *Legionella*.

Sample 5723: This sample was water taken from a hot and cold water system which contained *Legionella bozemanii* at levels of approximately 10^3 colony-forming units per litre. Background flora were *Acinetobacter junii* and *Pseudomonas stutzeri.*

Performance was good with 24/27 (88%) of participants reporting the correct isolation result, 23/24 (96%) for identification and 19/22 (86%) of the laboratories reporting a count within the intended range. The overall performance for examinations by culture was 90%.

Seven laboratories examined the sample using a molecular kit which only detects *L. pneumophila* and all seven reported the correct result. In addition, five of these laboratories examined the sample using a molecular kit that detects *Legionella* spp. and all five reported a correct result.

Sample 5724: This sample was water from a spa which contained a *Legionella pneumophila* serogroup 6 sequence type ST2923 at levels of approximately 10^3 colony-forming units per litre. Background flora was *Citrobacter braakii*.

Performance was good, with 24/27 (88%) of participants reporting the correct isolation result, 24/24 (100%) for identification, 18/23 (78%) of the laboratories reporting a count within the intended range, 9/10 (90%) reporting the correct serogroup and 8/8 (100%) of the laboratories reporting the correct sequence type. The overall performance for examinations by culture was 91%.

Seven laboratories examined the sample using a molecular kit which only detects *L. pneumophila* and 6/7 reported the correct result. In addition, four of these laboratories also examined the sample using a molecular kit that detects *Legionella* spp. and all reported a correct result.

Sample 5725: This sample was a swab of the biofilm from a hot and cold water system which contained no *Legionella* spp. Background flora were *Roseomonas aestuarii* and *Pseudomonas aeruginosa.*

Performance was excellent, with 24/24 (100%) of the laboratories reporting the correct isolation result.

Six laboratories examined the sample using a molecular kit only which detects *L. pneumophila* and 5/6 reported the correct result. In addition, three of these laboratories examined the sample using a molecular kit that detects *Legionella* spp., with 2/3 reporting a correct result.

Culture methods are considered to be the gold standard and are still the method of choice for many laboratories.

Figures 1 and 2 below illustrate the turnaround time for reporting results via the on-line secure system to PHE to complete the exercise.

Figure 1. Turnaround time for reporting results - clinical laboratories

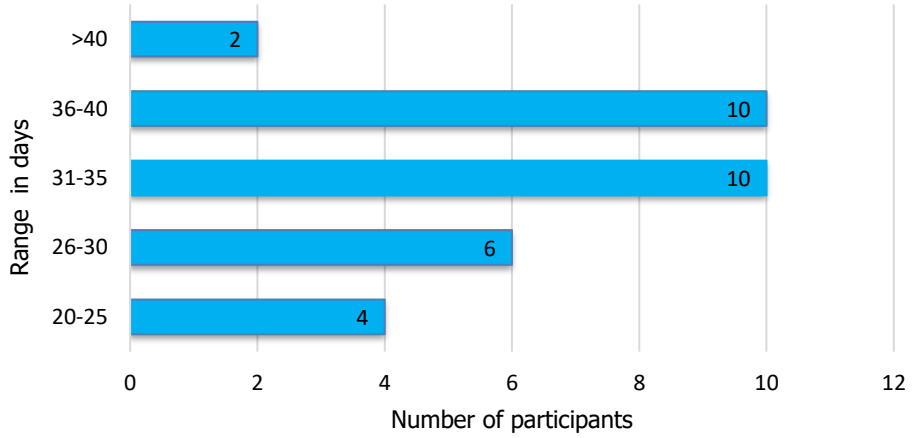
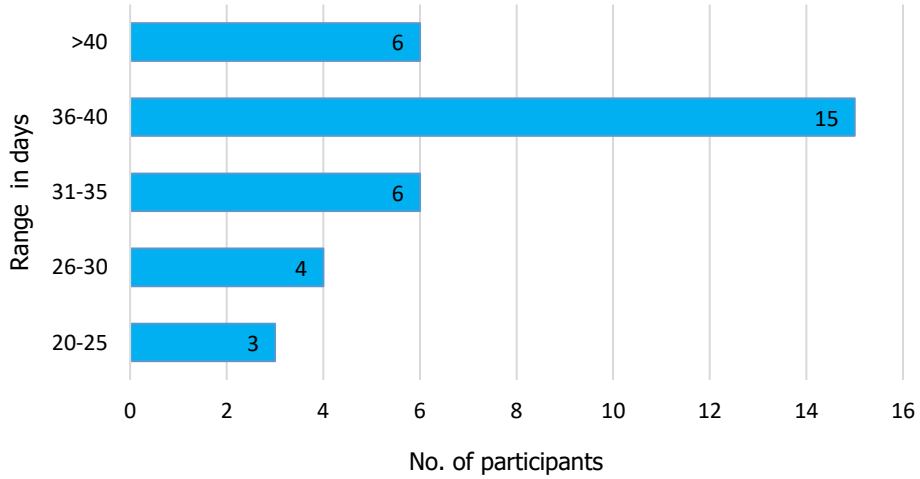


Figure 2. Turnaround times for reporting results - environmental laboratories



4. Discussion

General

Environmental and clinical laboratories play a vital role in protecting the public's health, by helping to ensure public health incidents are effectively detected and managed through the provision of quality results for samples analysed. Laboratories may also be required to report detected cases of Legionnaires' disease to their national surveillance systems if this is a requirement in their country.

External quality assessment provides laboratories with an independent external assessment of their performance. Regular participation in proficiency testing schemes is an important part of laboratory quality procedures and helps to ensure that the results of their tests are accurate. It also ensures high quality for the surveillance data reported.

Overall, the performance of laboratories participating in the 2019–2020 EQA was very good. There were no significant issues arising for species identification, serogroup, enumeration, or sequence type.

The outbreak strain chosen to simulate samples and specimens was *L. pneumophila* Sg 1, ST47. Two clinical specimens (5712 and 5713) contained *Legionella pneumophila* serogroup 3, ST2630. This is a unique ST, with a single documented isolation from a community-acquired clinical case in the UK. Legionnaires' disease attributed to *L. pneumophila* serogroup 3 is less common than serogroup 1 infection and many of the commercially-available urinary antigen kits are not designed or validated for the detection of *L. pneumophila* non-serogroup 1 antigens.

Comparing clinical and environmental isolates using serological and molecular techniques can help identify the source of Legionnaires' disease during potential outbreak investigations. *Legionella* is frequently found in the environment, and examination of clinical isolates can help interpret the findings of an environmental investigation.

There were no issues encountered with the preparation of the simulated specimens/samples. Homogeneity, stability and viability were consistent throughout all the stages of production and distribution. To maintain these parameters, proven technology for preserving organisms/levels of organisms were used, such as lyophilised or LENTICULE® discs. This preservation technique, used to produce simulated EQA samples/specimens, meant that the stability of the organisms would probably be guaranteed during transit to the 28 EU/EEA countries. This was important, given that transit time would probably be longer than that for local or national distribution of samples to designated laboratories.

From the results reported in this EQA it was ascertained that the majority of laboratories for clinical samples identified the pathogen and serogroup, but a significant number did not report the ST (Tables 1, 5 and 7). For environmental samples, the ISO 11731:2017 requires that suspect colonies are identified to at least *L. pneumophila*. Laboratories reported *L. pneumophila* correctly and went further, reporting a serogroup. For the clinical findings, 14 laboratories only examined the sample for ST.

Clinical discussion

The clinical aspect of this EQA was a qualitative exercise designed to assess simulated sputum and urine specimens. The panel of sputum samples were used to ascertain the absence or presence of *L. pneumophila* and when, following isolation of the respiratory pathogen, full identification to species level were requested, with accompanying Sg and ST. Examination to detect the urinary antigen for *Legionella pneumophila* was requested in the simulated urine samples.

Based on published guidance by PHE in the UK, the three most commonly-described sample types analysed were urine and lower respiratory fluids, including sputum and broncho-alveolar lavage (BAL) [4]. Using this information, simulated sputum and urine specimens were designed for distribution as part of the EQA exercise. A survey of methods (Annex 1) was sent out simultaneously with the EQA panel and this confirmed the most common specimen types examined routinely by participating laboratories to be sputum 100% (27/27), urine 92.6% (25/27) and BAL 100% 27/27.

Three paired (sputum/urine) simulated specimens (5707, 5708; 5709, 5710; 5711, 5712) with relevant accompanying clinical details were sent for evaluation. These specimens were designed to mimic an outbreak.

Patient 1

Both the simulated sputum (specimen 5706) and the urine sample (specimen 5707) contained *Legionella pneumophila* Sg1 ST47, one of the most prevalent circulating serogroups for *Legionella pneumophila*.

Of the 27 of laboratories reporting an identification, 100% achieved the correct result confirming the presence of *Legionella pneumophila*. A total of 25/27 laboratories reported the correct serogroup but, interestingly, two laboratories did not report a Sg, one of which was a public health laboratory. Failure to report a serogroup is a concern and may need follow up to understand why the Sg was not reported for the two laboratories in question.

Only 51.9% (14/27) of the participants examined for sequence type. Of those reporting an ST, all 14/14 laboratories achieved the correct result. Only 14/27 reporting a ST result may be a reflection of the level of

services provided by the participating laboratories. Participants were both clinical diagnostic and regional reference laboratories (RRL), and one would expect all RRL to report the ST. The low number of laboratories reporting a ST may be due to the lack of materials available (e.g. sequence base typing) at their facilities. It is currently under consideration as to whether this information should be requested in subsequent EQA exercises, by asking participants to enter the method used to determine the ST in the online reply form.

All 24 laboratories that tested for urinary antigen (specimen 5707) achieved the correct positive result, and can evidently meet the desired quality of service in laboratory reporting for clinical cases of Legionnaires' disease. However, three laboratories did not examine this specimen type.

Patient 2

This reflected a possible scenario in patient screening/or samples taken from a patient presenting with Legionnaires' disease symptoms, but not infected with *Legionella* spp.. Patient 2 sputum specimen 5708 was negative for the presence of the pathogen and 92.6% (25/27) of laboratories correctly reported the absence of the organism. Of the two laboratories reporting an incorrect result, one general hospital laboratory reported *Legionella* spp. and a public health laboratory reported *L. pneumophila.*

In contrast, the urine for patient 2 (specimen 5709) was a simulated urine containing a urinary *Legionella* antigen and 91.7% (22/24) of laboratories reported the correct result. One laboratory that reported a negative result stated that this sample was referred to another hospital for testing. The second was a national reference laboratory for *Legionella* testing. Three laboratories did not examine these specimens. As the corresponding sputum sample tested negative for this patient, it could be that local protocols state that no further testing needs to be performed.

To conclude, the overall intended results for this patient could represent the possibility of an inadequate sputum specimen having been received, and only the positive urine result having been taken into consideration for interpretation of Legionnaires' disease. In this case, a request for a repeat sputum or more sensitive specimen (e.g. BAL) would be appropriate. Further investigations into the two laboratories that achieved an incorrect result and the three laboratories that failed to examine the urine sample are warranted.

Patient 3

With the third set of samples, the sputum specimen (5710) contained a high yield of *L. pneumophila* Sg1, ST47 and a very high level of urinary antigen (specimen 5711) for *L. pneumophila* Sg1, ST47. A 100% concordance was achieved with 27/27 of laboratories reporting correctly to species level. A further 26/27 reported the correct serogroup but only 14 of the 27 reported a ST47. Interestingly, 13 laboratories did not report sequence type and this may warrant further investigation, to find out why they did not report for this test. Of the urinary antigen results, 91.7 % (22/24) of laboratories reported the correct result, detecting the *Legionella* antigen, with only two failing to detect it.

As regards concordance with the intended results, performance was very good to excellent for all three sets of simulated patient samples. This demonstrates the competence level of participating laboratories in isolating and identifying the causative agent and detecting the presence of the circulating antigen.

The methods survey questionnaire was used to obtain information on the various methods applied to identify the pathogen and the most commonly reported were conventional testing (culture) and MALDI-TOF. The most frequently reported confirmatory tests included serology and MALDI-TOF, with some laboratories also reporting use of fluorescent microscopy. A small number also reported using Direct Fluorescent Antibody (DFA) staining.

Patient 4

One set of simulated samples (5712 and 5713) contained *L. pneumophila* Sg 3, ST2630 and this ST was included as a patient suspected of contact with *Legionella*, with indicative accompanying clinical details (asthmatic elderly male, lives at home local area, rarely goes out, except to the shops and local pub). This set of patient samples is not associated with the simulated outbreak, based on the reported Sg and ST and an absence of the antigen for *L. pneumophila* Sg1 being detected in the urine.

A 100% concordance was achieved with 26/26 reporting the correct species, 26/26 reporting the correct Sg and all 14/14 reporting the correct ST.

There are a plethora of testing kits available for use in clinical diagnostic laboratories which are designed and validated for the detection of the surface antigen for Sg 1 in urine (serogroup 1 being the predominant circulating antigen). Results for specimen 5713 concluded that the kits used by the participants do not detect the Sg3 circulating antigen and this was confirmed by all 24/24 laboratories reporting the absence of *Legionella* antigen.

As regards concordance with the intended results, performance was very good to excellent for all four sets of samples, demonstrating the competence level of participating laboratories in isolating and identifying the causative agent and detecting the circulating *Legionella* antigen.

Patient 5

Legionella longbeachae is the second most commonly reported causative agent of Legionnaires' disease. *L. longbeachae* was distributed in a simulated sputum specimen (5714) as an educational objective. This proved a little challenging, with only 88.5% of participants reporting the correct result. Overall, 17 laboratories reported the presence of *L. longbeachae* and six reported the absence of *L. pneumophila*. This illustrates the different testing methods adopted by the reporting laboratories. More details on the methodologies used will be obtained and collated in the next exercise.

Patient 6

EQAs often include a negative sample in the assessment. It is just as important for the participating laboratories to be able to provide a true negative result as it is to determine a positive one. Specimen 5715 was a simulated urine specimen, containing no antigen. Participant performance was excellent with 23/24 (95.8%) of laboratories reporting the absence of a circulating *L. pneumophila* antigen.

The source of infection can be identified by comparing clinical and environmental *L. pneumophila* isolates using various typing methods. A variety of rapid identification and sensitivity methods have been developed for isolates from clinical samples. These include molecular techniques such as Real-time Polymerase Chain Reaction (PCR), Pulsed Field Gel Electrophoresis (PFGE), Multilocus Sequence Typing (MLST), Multiple-Locus Variable-Number Tandem-Repeat Analysis (MVLA), Single Nucleotide Polymorphism (SNP) assays, Whole Genome Sequencing (WGS) and Matrix Assisted Laser Desorption Ionisation Time-of-Flight (MALDI-TOF) Mass Spectrometry. Although these applications enable subtyping of unrelated strains, the accuracy, precision and reproducibility are not comparable. Within this EQA exercise all participants (n=27) reported culturing the simulated sputum specimens. Other methods used included Gram stain (7), UV microscopy (9), serology (19), MALDI-TOF (16), semi-automated methods (1), ELISA (2), direct fluorescent staining (6), monoclonal antibody typing (14), mip sequencing (10) and WGS (7).

Most failures with EQA specimens can be a result of inadequacies in the other components of the quality system, including methodologies used.

Environmental discussion

The environmental aspect of this EQA was a qualitative and quantitative exercise designed to assess simulated environmental water and swabs. The environmental samples were used to ascertain the presence or absence of *Legionella* spp. and, upon isolation/detection of the organism, a full identification to species level. There was also an option to report enumeration/quantification with accompanying Sg and ST, if applicable and as requested.

Legionella spp. are found in cooling towers, hot and cold water systems, air conditioners, spa equipment, fountains, humidifiers and showers, misting devices, decorative fountains and water features, dentistry tools and thermostatic mixing valves (TMVs). The main mode of transmission is through inhalation of airborne droplets contaminated with *Legionella spp.*.

The detection of *Legionella* by culture is the gold standard method for detecting *Legionella* colonies on buffered charcoal yeast extract (BCYE)/glycine vancomycin polymyxin B cycloheximide (GVPC) agar plates. This is a labour-intensive approach which takes ten days to complete. Recovery of *Legionella* bacteria by culture can be challenging as *Legionella* colonies on BCYE agar media can be overgrown or inhibited by competing microbial flora, masking the presence of *Legionella* colonies. Therefore, acid and heat treatment of samples is the key to reducing the amount of background flora [3].

Polymerase chain reaction (PCR) method, is a molecular technique that only takes a few hours to complete and can be a useful method to screen environmental and water samples. The disadvantage of this method is that dirt and debris can have an impact on the test outcome. Molecular testing is not widely used to test water and environmental samples for *Legionella* and only a few commercial laboratories offer this service routinely. Moreover, the detection of DNA from dead *Legionella* cells has limited public health significance. In 2015, the ISO/TS 12869 standard method was published for the detection and quantification of *Legionella* and/or *L. pneumophila* by concentration and genic amplification using real-time polymerase chain reaction (qPCR) in order to standardise this methodology.

A methods survey questionnaire (Annex 1), sent out simultaneously with the EQA panel, confirmed that the most common sample types routinely examined by participating laboratories are water from hot/cold water systems 100% (27/27), cooling towers waters 93% (25/27), water from spas 100% (27/27) and swabs from biofilms 89% (24/27).

The overall performance of the laboratories in the EU/EEA countries was very high. A total of 19 laboratories examining water samples for *Legionella* bacteria indicated that they followed ISO 11731:2017 (Water quality - Enumeration of *Legionella*). All 24 laboratories that returned information responded that they filtered the water sample, 19 cultured the sample untreated, 22 carried out an acid treatment and 20 also used a heat treatment.

- For isolation: the overall performance for isolation of *Legionella* was 96%, with 23–27 of the laboratories reporting a result. Three laboratories did not examine the swab samples. However, performance was lower with sample 5721 (23/27 – 85%) when compared to the other samples containing a *Legionella* spp.. The

low level of *L. pneumophila* in the sample at $9.8x10^2$ colony-forming units per litre may have contributed to this finding. This level of organism content may have been around the lower end of the detection limit for methods used by the laboratories, which is why it is important that they know their detection limits and the potential impact this may have on results. The most common isolation media used was GVPC and/or BCYE. There was variation among laboratories in the use of other culture media and acid and/or heat treatment.

- For identification: six of the ten samples contained a *Legionella* spp.. The overall performance for correctly identifying the *Legionella* spp. was 99%, with 23–26 of the laboratories reporting a result. Sample 5723 contained a *Legionella bozemanii*, one laboratory incorrectly reported the *Legionella* as *Legionella pneumophila*.
- For enumeration: seven of the ten samples were simulated water samples. Of these, five contained a *Legionella* spp.. The overall performance for counts being reported correctly as a censored value or within the intended range was 90%. Performance was lower with three samples, 5721 (79%), 5723 (86%) and 5724 (78%), due to the bacterial load being at the lower end of the detection limit for methods used (10^2-10^3). For swab samples, the number of enumeration results reported was low.
- For serogroup: the overall performance for serogroup confirmation was very good, with 98% of results being reported correctly. One laboratory reported an incorrect serogroup 3 for sample 5724 when the strain was a serogroup 6. According to the data from the questionnaire, a majority of the laboratories used the 'Oxoid Dryspot' *Legionella* latex test.
- For sequence type: the overall performance in reporting a correct sequence type was 93% for five of the samples that contained *L. pneumophila* (5716, 5717, 5719, 5721 and 5724). Between 8–14 laboratories reported the ST results. This may have been due to lack of expertise or linked to the method implemented in the laboratory. Samples 5716 and 5717 contained a *L. pneumophila* sg1 ST47. One laboratory reported the ST as 'other' and did not provide further details. Sample 5719 also contained a *L. pneumophila* sg1 ST47; two laboratories reported the ST as 'other' and did not provide further detail. Further analysis showed that one laboratory reported an incorrect ST for all samples 5716, 5717 and 5719. The most common method used for ST analysis was Sanger sequencing and/or Whole Genome Sequencing (WGS).
- For molecular methods: 5–8 laboratories analysed the samples for *L. pneumophila* using molecular methods and culture methods concurrently. The overall performance with detection/absence of *L. pneumophila* for the 10 samples was 95%. One laboratory reported an incorrect result for samples 5722, 5724 and 5725: samples 5722 and 5725 did not contain *L. pneumophila*. For molecular detection/absence of *Legionella* spp. the overall performance was 75%. A total of 2–5 laboratories examined the samples using a molecular method for *Legionella* spp.. One laboratory reported an incorrect result for samples 5718, 5720 and 5722; this was the same laboratory mentioned above that incorrectly reported the presence of *L. pneumophila*. One laboratory reported correctly that *L. pneumophila* was 'detected' for some samples but 'not detected' for *Legionella* spp.. An analysis of the kits from the method questionnaire did not indicate that one specific molecular test was commonly being used.
- For quantification: one or two laboratories reported a quantification result in terms of genomic copies per litre. Due to the low number of data sets returned, it was impossible to analyse the values. However, where a censored value was reported for samples containing no *Legionella* spp. this can be assumed to be correct.
- The overall performance between the results reported by the clinical laboratories and those reported by environmental laboratories was very good for the three sets of samples and clearly demonstrated that the fourth paired sample was not associated with the 'outbreak'.

The detection and acceptable level of *Legionella* spp. is also an important factor in determining the effectiveness of control measures in an artificial water system. Other types of *Legionella* spp. besides *L. pneumophila* have also been implicated in causing infection, particularly in nosocomial cases. However, the EQA organisers are aware that national guidance documents may only refer to *L. pneumophila* and not necessarily include the requirement to test other species of *Legionella.*

Limitations of this EQA exercise

This EQA was only able to evaluate the analytical and post analytical stages of the total testing process. The pre-analytical stage of the process was not evaluated. The pre-analytical stages would include the demographics of the patient sample, correct sample type, volume of sample, correct tests requested and suitable container, all of which were pre-determined for this EQA panel.

The EQA scheme was only available to a maximum of two selected laboratories per EU/EEA country, therefore the breadth of the cohort was limited to those who received a panel and returned results.

A period of six weeks was given for laboratories to return results. This period of time was allocated to allow sufficient time for the panel to arrive at the laboratories via air freight. The time allowed for the return of results was not meant to reflect the expected turnaround times for clinical or environmental laboratories when investigating and returning results. Nevertheless, the number of days taken to report results from the receipt date by the laboratory was recorded (Figures 1 and 2). Interestingly, the mode for returning results was determined to be 35 days for clinical and 40 days for environmental samples. The turnaround time to report results indicates that the participating laboratories may not have treated the EQA specimens as they would routine samples (having several staff analyse the results prior to reporting online rather than just one member of staff processing and

reporting). For a service provider turnaround times of 35 and 40 days respectively would be unacceptable. However, one limitation to the system used to report results is that it does not allow for the capture of preliminary results, as some laboratories do. There is a need to understand whether the six-week period given to report results truly reflects the way in which laboratories work with genuine samples/specimens.

The clinical samples sent in lyophilised format did not represent the matrix of an authentic liquid purulent sputum which would normally be received by a diagnostic laboratory. However, the specimens distributed for detection of urinary antigens were authentic clinical liquid urine, spiked with species antigen and provided in plastic tubes to resemble a true sample.

For the environmental water samples, once the LENTICULE discs were rehydrated this would constitute one litre of water which would not be representative of the chemical constituents normally be found in real samples. For swabs the laboratory was instructed to rehydrate the sample and then absorb the material onto a swab before suspending the swab into a diluent. This was the most practical way to simulate a swab sample, however this is not representative of how a swab sample would be received in a laboratory for analysis.

For environmental samples, the molecular quantification results could not be assessed due to the low number of data set returned.

An EQA is of limited value without at least some of the other components of a quality system, such as adequate documentation, training of staff and internal quality control (IQC).

EQA benefits

The importance of an EQA is to ascertain and assess the level of competency of the participating laboratories in delivering a service to examine clinical specimens and water samples for presence and detection of *Legionella* spp..

The benefits of participating in this EQA are:

- to provide laboratories with an insight into their performance;
- to help improve local standards;
- to reveal unsuspected areas of difficulty;
- to provide an educational stimulus for improvement;
- to check the efficacy of internal quality control procedures;
- to demonstrate a commitment to quality to colleagues and customers;
- to provide a method performance evaluation
- to provide independent evidence of performance for accreditation bodies;
- to enable the participants to monitor, evaluate and improve their own performance and training needs, since dealing with discrepant EQA results will improve testing performance which, in turn, would directly improve the management of public health incidents and clinical service.

A comprehensive quality assurance system will cover such areas as provision and control of standard operating procedures, education and training, planned maintenance and calibration of equipment and the monitoring of response times. Many laboratories are formally accredited in order to acknowledge compliance with defined objectives and quality standards such as those detailed in ISO 17025:2017 or ISO 15189:2012.

Results of consistently good quality can be expected only when all the components of a quality system are in place.

Participant feedback on this EQA

A short feedback evaluation survey was sent to all participating laboratories by ECDC after the first exercise, with the online survey open from 2–13 April 2020 and then extended to 23 April 2020.

A total of five questions were asked.

Question 1. Regarding any of your analytical test results that did not conform with the intended results, can you specify which corrective action(s), if any, was/were taken (e.g. review and adjust SOPs, verify reagents)?
Question 2. Are the results of this EQA exercise to be used as documentation for accreditation and/or licensing purposes for the method(s) used in your laboratory?
Question 3. Overall, is this EQA exercise important for your laboratory to assure its diagnostic capability?
Question 4. Were you satisfied with the EQA report of results specific to your laboratory?
Question 5. Do you see a benefit in participating in this EQA scheme compared to other (commercial) EQAs for *Legionella*? Please describe why or why not.

Feedback was provided by 18 of the 34 EU/EEA participating laboratories, representing a response rate of 51%.

Among the respondents, 9/18 had participated in both clinical and environmental distributions, 4/18 in the clinical distribution and five in the environmental distribution only.

Four of the EU/EEA respondents indicated that corrective actions were taken based on the EQA distribution results. The types of corrective action were that environmental samples were now sent to a specific environmental laboratory; sample preparation and handling procedures had been extended for different matrices; procedures had been adopted for environmental samples to check for overgrowth (in the event of high background flora) and antigen kits or reagents had been replaced with different types.

In all, 17/18 participating laboratories indicated they would use the results of the EQA exercise as evidence for accreditation and/or licencing purposes for the methods used.

A total of 5/18 laboratories indicated the exercise was important but not essential, 13/18 responded that it was very important. The reasons given for its high importance were:

- use of results as documentation for accreditation;
- lack of other available EQA schemes for the laboratory to participate in (e.g. complex diagnostics for *Legionella*; no other available EQA covering culture/clinical samples; no urine antigen EQA available);
- a means of ensuring the high-quality testing protocols (e.g. good laboratory practice; review of sensitivity and specificity of tests using the lab methods).

Suggested areas of consideration for the next EQA round were:

- a better assessment of quantitative counts for environmental samples;
- inclusion of all data, such as sequence types, allele profile, Dresden typing;
- making the environmental report clearer so that it was easier to see where the method results came from.

Responses from EU/EEA laboratories on the performance of this EQA compared with other providers included:

- the importance of including an outbreak situation in an environmental scheme;
- the fact that this scheme covered all methods at the same time;
- the fact that the scheme was not limited to one restricted matrix assay, having different ranges of microorganisms contained in the sample (also negative samples) which tests the robustness of laboratory work over a short time period;
- the fact that it simulated a real outbreak investigation situation where all the tests required had been evaluated simultaneously which was a good exercise to prepare laboratories for possible outbreaks and the matching of sources.

Overall, there was a very positive feedback on this new ECDC EQA *Legionella* scheme.

Although the comments and feedback represent only half of the laboratories that participated in this distribution, they indicated that offering this EQA scheme to the wider ELDSNet network of EU/EEA countries is beneficial for the surveillance and response to Legionnaires' disease in Europe. Moreover, the format of this EQA scheme as an outbreak simulation comprising environmental samples and clinical specimens was considered an added value when compared to other EQA services available in Europe.

5. Conclusions

The performance of laboratories from the 28 EU/EEA countries in this exercise was very good for culture-based/detection methods used by both the clinical (96.2%) and environmental laboratories (95%).

Both clinical and environmental laboratories demonstrated that they could undertake testing to an acceptable level of at least 80% correctness. Although the data provide some assurance of the laboratories' ability to undertake effective public health investigations for *Legionella pneumophila*, more EQA data is required to determine the actual robustness. If laboratories report accurate data this also ensures that the information provided to surveillance systems is accurate.

Laboratories were provided with the opportunity to examine samples that they would routinely test. For clinical samples, 27 laboratories examined the sputum samples and 24 examined the urine. For environmental samples, 27 of the laboratories examined the water samples and 24 examined the swab samples. Where results reported were not in accordance with those intended, laboratories were advised by contractors to investigate in order to determine the root cause.

For the clinical laboratories, all 27 reported an identification, 26 for serogroup and 14 for sequence type. All laboratories reported isolation and identification using culture-based methods. Isolation in culture remains the gold standard for the diagnosis of infection caused by *Legionella spp.*, due to the low sensitivity and specificity associated with serotyping. MALDI-TOF MS is then frequently used to identify isolates to species level. Differentiation and typing of strains can be achieved using a range of molecular techniques, including SBT and RT-PCR methods. A total of 22/27 participants went on to perform molecular methods for the detection of *Legionella*.

For the environmental laboratories, 27 laboratories reported a result for isolation and identification, 25 reported a serogroup, 24 reported an enumeration count and 14 a serotype. For molecular methods, eight laboratories analysed the samples for *L. pneumophila* and five for *Legionella* spp. The overall isolation performance for culture was 95%, with 27 reporting a result. The overall performance for molecular methods was 95%, with eight laboratories reporting a result for kits detecting *L. pneumophila*. It is known that laboratories are still developing the routine application of molecular methods for water and environmental samples due to the fact there are currently no guidelines for the interpretation of molecular results (GU/L). Therefore, culture remains the preferred method.

The results of a survey carried out by ECDC indicated that laboratories found this EQA exercise to be very useful. Overall, the feedback on this new ECDC EQA *Legionella* scheme was very positive.

6. Recommendations

This exercise will continue to provide a baseline understanding of the level of testing undertaken in the laboratories, determine any performance issues and, where possible, provide support to laboratories/countries who have identified limitations in addressing improvement of their testing capabilities or capacity building.

Main recommendations for future EQA exercises

Sample/specimen design

- To continue providing this EQA exercise and include different *L. pneumophila* serogroups, sequence types (STs) and *Legionella* species. This will allow a better understanding of a laboratory's ability to undertake testing to the level required for successful management of public health incidents.
- To identify through further EQA exercises if there are issues when less commonly encountered species, SG or ST are included. A single EQA distribution cannot confirm this.
- For environmental samples to include levels of *Legionella* spp. that are at the lower end of the detection limit for culture. In addition, to confirm if molecular assessment is of value since, with low numbers of laboratories currently using this method of examination, performance cannot be assessed.
- To continue to include more than one species of *Legionella* spp. within the simulated samples/specimens set. This will help educate and improve knowledge and experience with organisms which are otherwise not frequently encountered.

Methods

- To gather more information on the methods used to report results on the samples/specimens – this will be required when returning results.
- To link the method information more closely to the results reported in order to identify tests that laboratories do routinely but did not report a result for or instances where the organisers did not allow for these examination results to be reported.
- To include the option for laboratories to examine clinical specimens using molecular methods, as the evidence from the methods questionnaire suggests that laboratories do undertake this type of testing.

Scoring

- To determine if performance assessment should be scored and design an appropriate scoring system to make it easier to identify those laboratories that experience significant, ongoing difficulties with their examinations.
- The allocation of scores is provided as a visual management tool to help assess performance.
- Ongoing performance assessment for a laboratory can only be done if the same laboratory takes part in all the EQA distributions.

Future objectives

- To improve awareness of the different *Legionella* spp. that may be isolated from clinical specimens and environmental samples.
- To improve awareness of the confirmatory tests done and their limitations when confirming *Legionella* spp. Isolates in samples.
- To improve awareness of the importance of following standardised methods when managing public health incidents.
- To encourage regular participation in the EQA by the same laboratories in the countries as it is an important element of their quality procedures and helps to ensure that the results of their tests are accurate. Laboratories should participate regularly throughout the year in order to review performance on an ongoing basis. Ongoing performance assessment is designed to identify genuine problems.
- To determine if performance assessment should be scored and design the appropriate scoring system to make it easier to identify those laboratories that experience significant, ongoing difficulties with their examinations. The allocation of scores is provided as a visual management tool to help assess performance.
- To determine if participating in EQA exercises can improve understanding of the link between clinical and environmental laboratories within countries when dealing with outbreaks to make the management of public health incidents more effective.
- To explore participants' feedback from the evaluation survey in greater depth to improve the exercise (e.g. improving information in the individual EQA reports.)
- To update the IT platform so that results on methods used to can be provided.
- To communicate the results of this EQA at future ELDSNET and *Legionella* conference meetings to increase awareness of the importance of EQAs for the quality of *Legionella* detection in laboratories.

References

1. European Legionnaires' Disease Surveillance Network (ELDSNet); 2017. Available at: https://www.ecdc.europa.eu/sites/default/files/documents/ELDSNET_2017-revised_guidelines_2017-web.pdf
2. Publications Office of the European Union. Commission Implementing Decision (EU) 2018/945 of 22 June 2018 on the communicable diseases and related special health issues to be covered by epidemiological surveillance as well as relevant case decisions (text with EEA relevance). Available at: https://op.europa.eu/en/publication-detail/-/publication/57ed0506-80e4-11e8-ac6a-01aa75ed71a1/language-en
3. Control of Biohazards, *Legionella* detection and sampling methods. [cited 1 December 2020.] Available at: https://www.legionella.com/legionella-detection-and-sampling-methods
4. National Infection Service, Reference Laboratories Colindale, Bacteriology Reference Dept., User manual, Public Health England Publications: Gateway Number: GW-604, Version 11. October 2019.

Annex 1. Findings from methods survey questionnaire

As part of the EQA exercise, a questionnaire was sent out on the methods used to analyse the samples/specimens. The data presented below is for all EU/EEA and non-EU/EEA countries.

Methods survey findings for clinical specimens

Distribution 4680, which closed on 16 December 2019, consisted of 10 simulated clinical samples. A questionnaire sent out together with the EQA was completed by all 27 of the participating laboratories in the 28 EU/EEA countries.

General information

A total of 22/27 participants reported that, in addition to being reference laboratories, they were clinical diagnostic laboratories. One laboratory reported that they were only a clinical diagnostic laboratory. The remaining 4/27 were reference laboratories only.

The majority of laboratories (63% - 17/27) participated in a national EQA scheme. Of these, the national scheme was mandatory for only 41.2% (7/17).

Figure A3. Sample types analysed for *Legionella* by participating laboratories in the EU/EEA

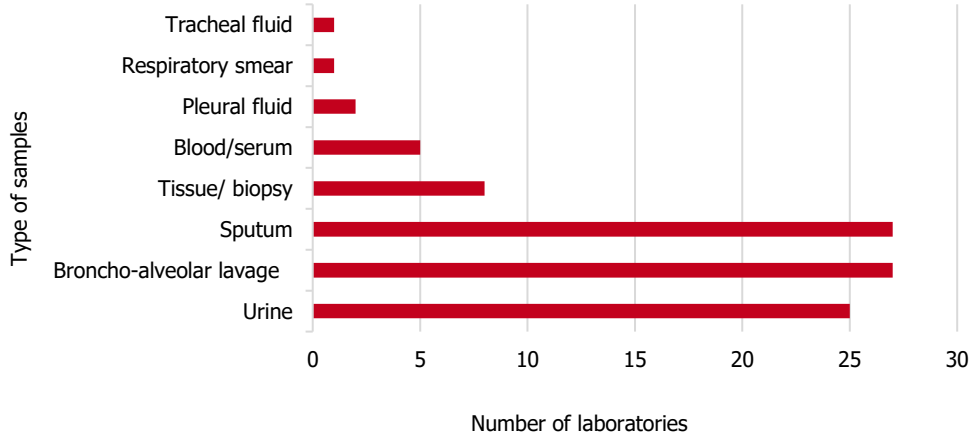
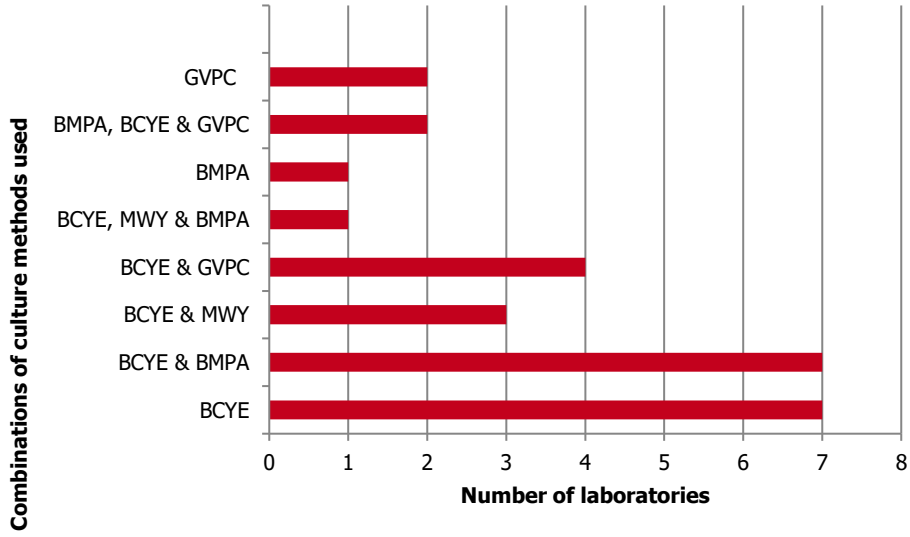


Figure A4. Different types of media used for the detection of *Legionella*



Codes:
BCYE: Buffered charcoal yeast extract agar
GVPC: Glycine vancomycin polymyxin B cycloheximide
MWY: Modified Wadowsky Yee
BMPA: Legionella BMPα selective media with activated charcoal, yeast extract and ACES- buffer

Comments:
All laboratories noted culture prior to confirmatory testing. The majority of laboratories (n=19) used commercial media, while the others (n=8) used in-house media. The manufacturers included Oxoid, Biomerieux, BioMaxima, Liofilchem, Beckton Dickinson, Thermo Fisher scientific, PVL and E&O Laboratories.

Table A19. Incubation period of culture plates (in days)

Incubation period (days)	Number of laboratories
3	1
5	1
7	9
>=10	17

Figure A5. Confirmatory tests used for detection of *Legionella*

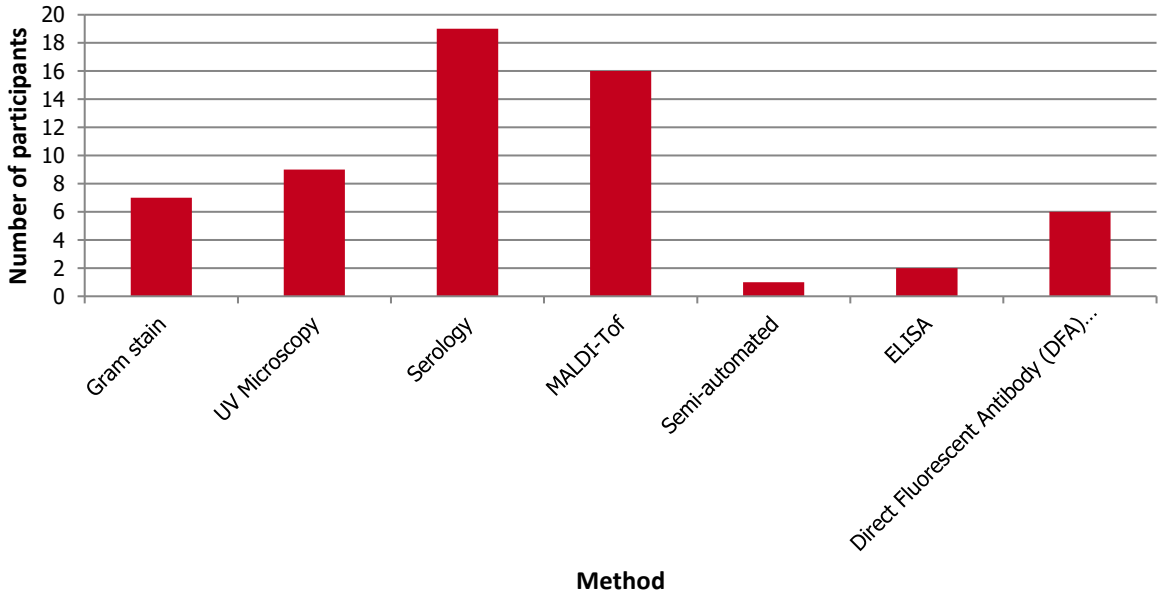
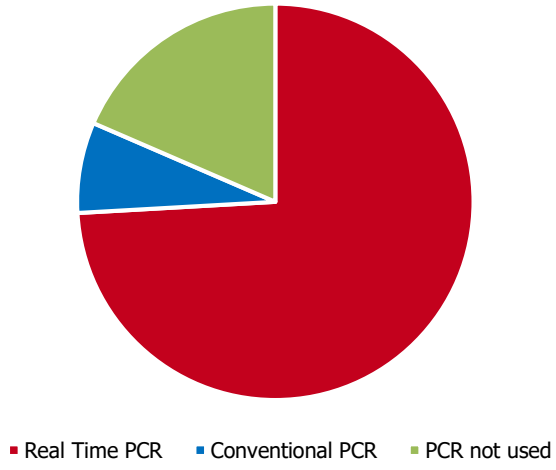
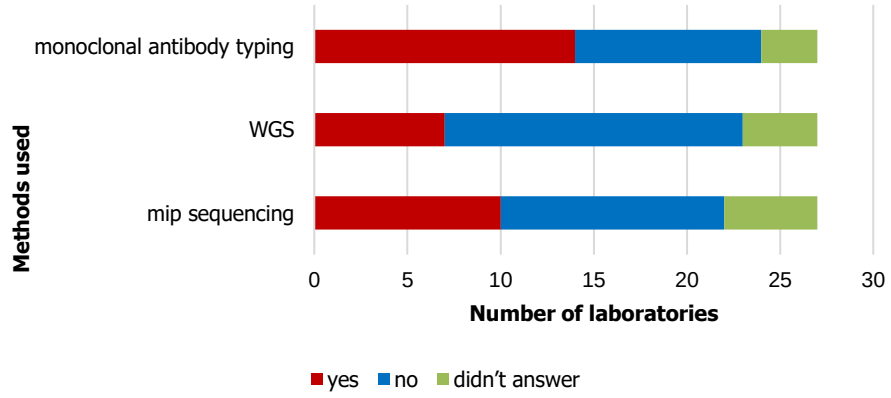


Figure A6. Distribution of reported use of PCR molecular methods by EQA clinical participating laboratories



Comments:
A total of 22/27 laboratories performed molecular testing, the majority of these (n=20) used Real Time PCR. The two other labs used conventional PCR. The manufacturers listed vary, there is no predominant kit or supplier used.

Figure A7. Typing and species identification methods, including phenotypic and genotypic



Comments:
Only five labs outsourced molecular testing

All of the laboratories performing WGS (n=7) were reference laboratories. There appears to have been more laboratories performing sequence-based typing than actually reported here (n=14 reporting results for sequence type.)

Methods survey findings for environmental samples

A questionnaire was sent to all participants who participated in distribution 4681. The objective was to gather information on the method/processes used for this EQA exercise.

In all, 24/27 (88%) of the participating laboratories provided information on their methods/processes. No replies were provided for Italy, Lithuania or Hungary. Of the responders, the total numbers will not always correspond to 24 as some participants did not provide information on all the questions and some questions allowed for more than one option to be selected.

The method data shown is for information only. It does not evaluate or associate the data with a failure in the EQA or method/process used and it does not attempt to compare performance of the various molecular kits/processes.

All data is presented for all EU/EEA laboratories that participated in the EQA November 2019 exercise.

General information

Figure A8 below shows the approximate number of water and environmental samples examined for *Legionella* spp. in a year (n=19).

Figure A8. Number of samples examined per year for *Legionella*

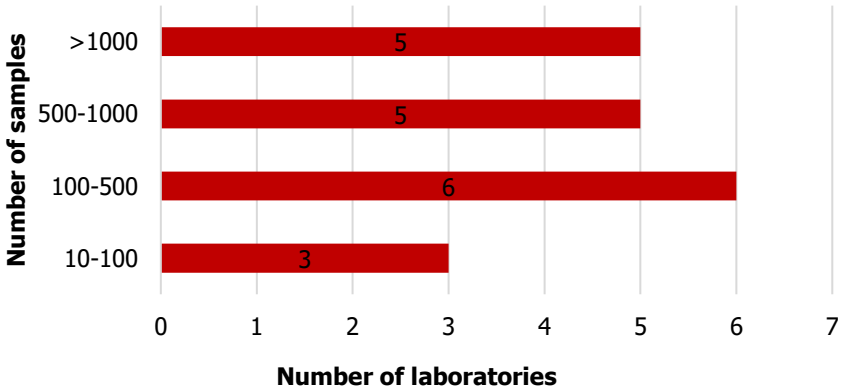


Figure A9. Type of samples analysed by laboratories for *Legionella* (n=27)

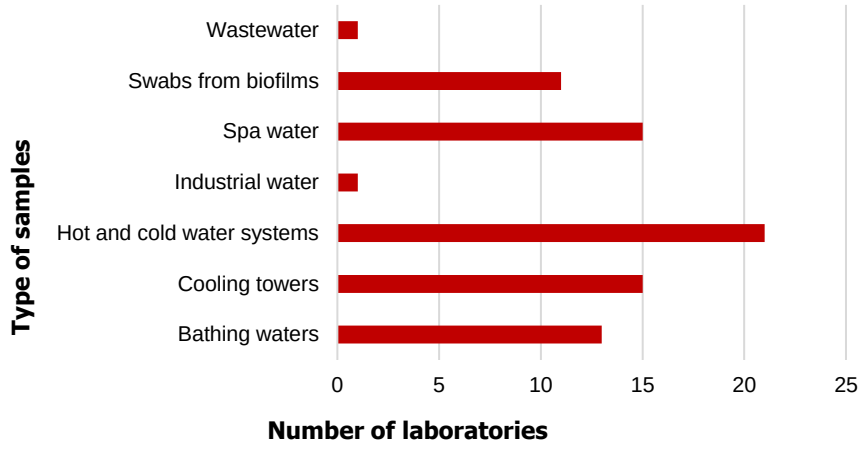
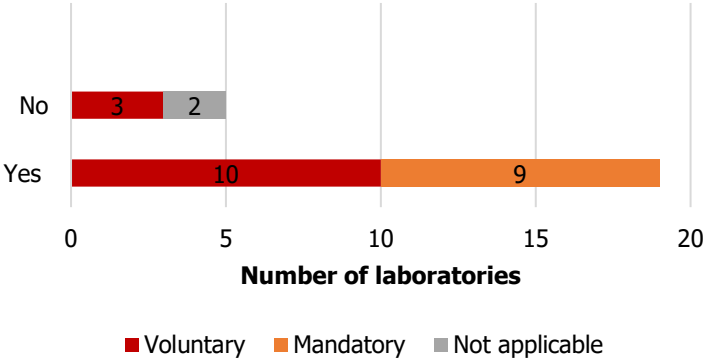


Figure A10 shows whether laboratories participated in their national EQA schemes programme and if these schemes are voluntary or mandatory (n=24).

Figure A10. Participation in national EQA schemes



Information on water examination

Figure A11 shows the published methods used to examine environmental and water samples (n=26)

Figure A11. Published methods used to examine swabs and water

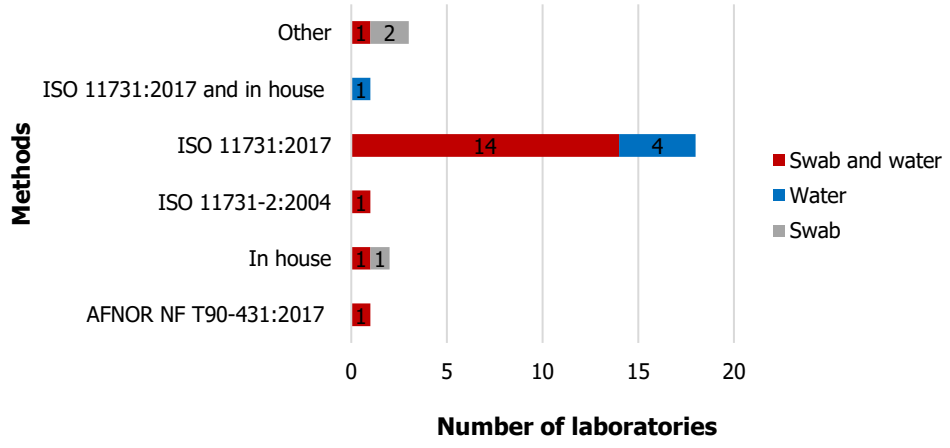


Figure A12 shows the method and volume used to examine the water samples in this exercise (n=24).

Figure A12. Method and volume used to examine water samples

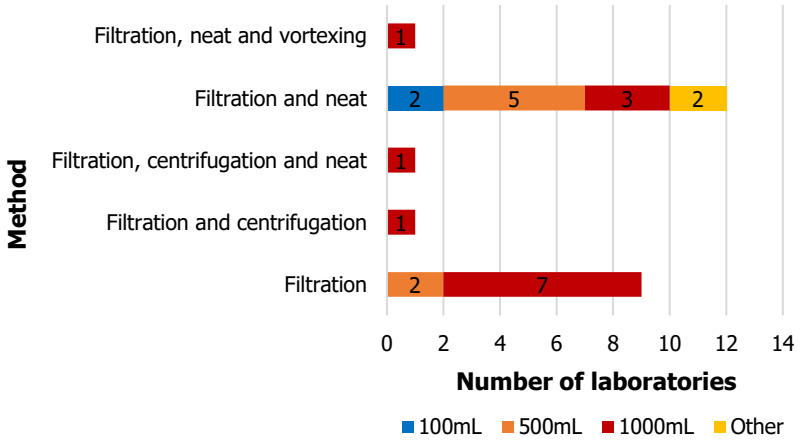
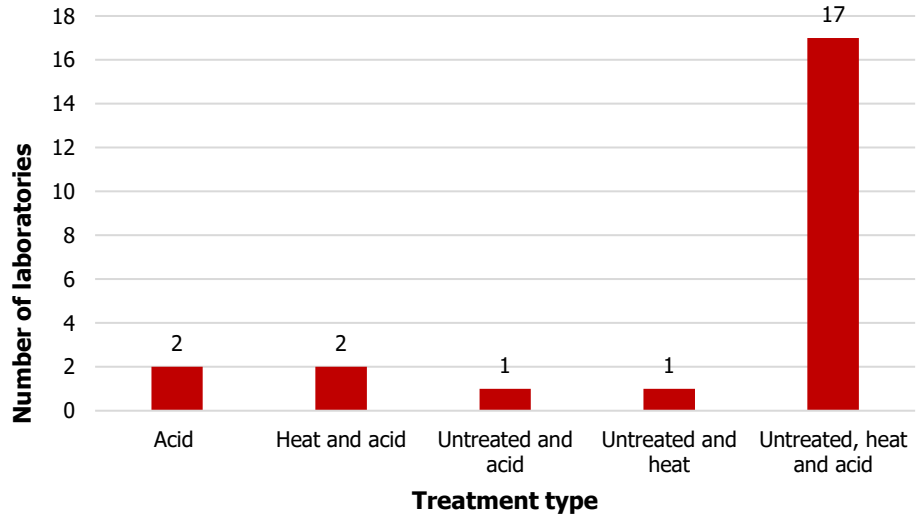


Figure A13 provides details of how the sample was processed (n=23).

Figure A13. Information on sample processing



For the laboratories that undertook acid treatment as part of the examination, the information in Figure A14 provides details on the stage of the process at which the acid was applied (n=21).

Figure A14. Stage at which acid was applied

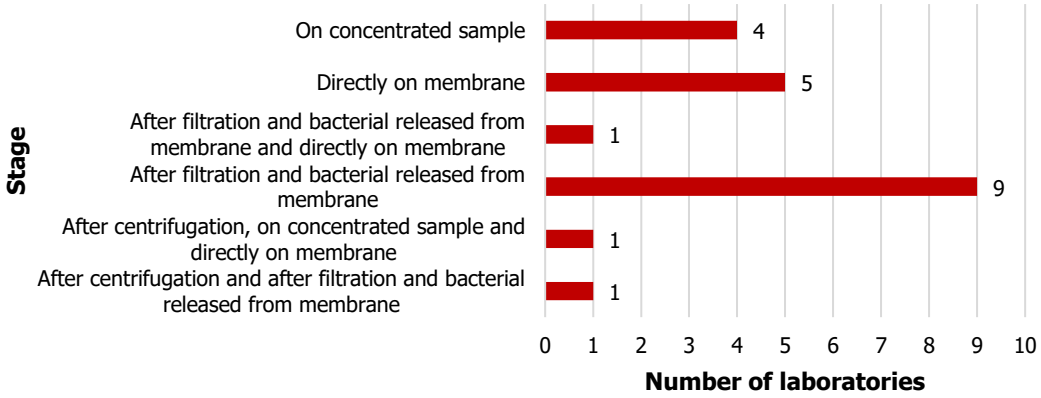
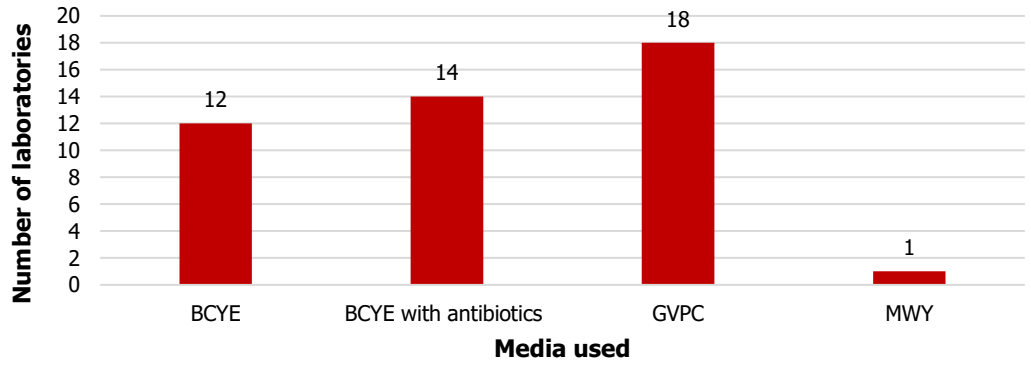


Figure A15 shows the different types of media used (n=24).

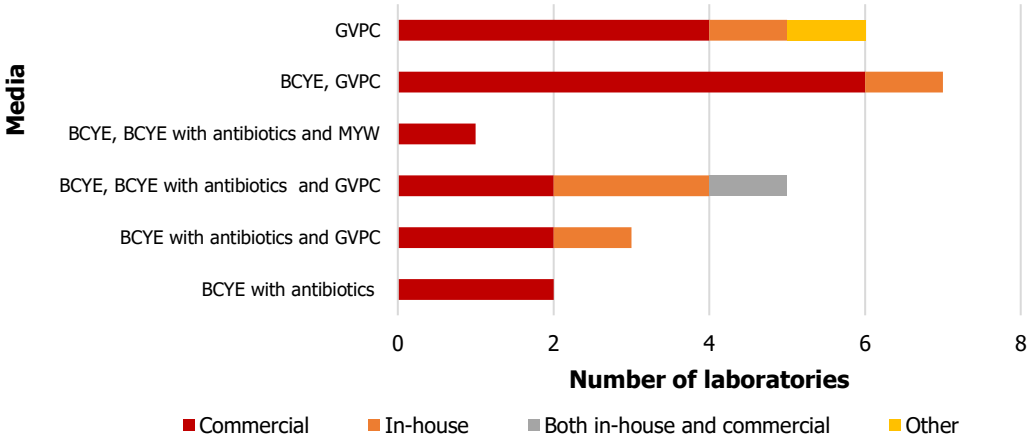
Figure A15. Different types of media used



Codes:
BCYE: Buffered charcoal yeast extract agar without L-cysteine
BCYE with antibiotics: Buffered charcoal yeast extract agar with selective supplements
GVPC: Glycine vancomycin polymyxin B cycloheximide
MWY: Modified Wadowsky Yee.

Figure A16 shows the combinations of media used and the source of this media (n=24).

Figure A16. All media used and source of media



Codes:

BCYE: Buffered charcoal yeast extract agar without L-cysteine
BCYE with antibiotics: Buffered charcoal yeast extract agar with selective supplements
GVPC: Glycine vancomycin polymyxin B cycloheximide
MWY: Modified Wadowsky Yee.

Table A20 provides information on the confirmation tests done on presumptive colonies of *Legionella* (n=24).

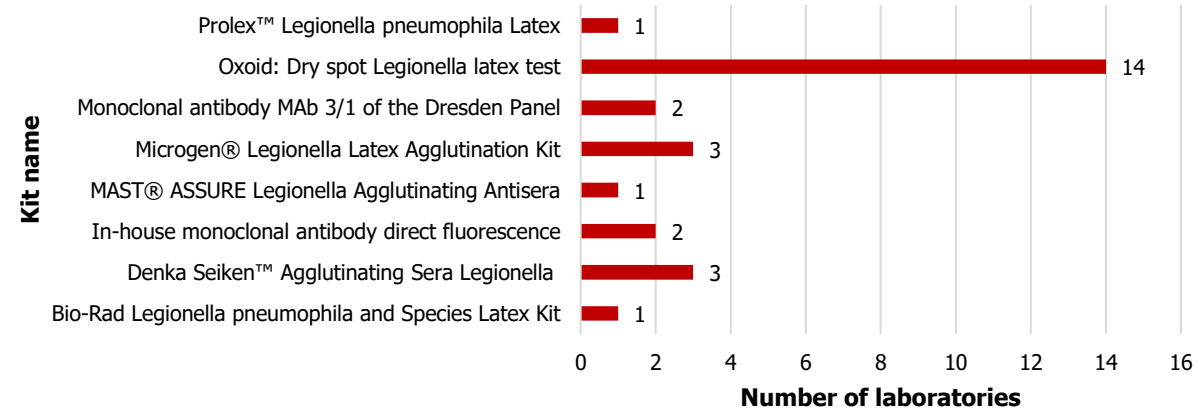
Table A20. Confirmation tests undertaken on presumptive colonies of *Legionella*

Confirmation tests	Number of laboratories
MALDI-TOF	1
Subculture in parallel on BCYE with and without antibiotics, and MALDI-TOF MS	1
Subculture on BCYE and BCYE with cysteine	6
Subculture on BCYE and PCR	1
Subculture on BCYE with cysteine	7
Subculture on BCYE with cysteine and MALDI-TOF	1
Subculture on BCYE with cysteine and PCR	1
Subculture on BCYE with cysteine, on TSASB and MALDI-TOF	1
Subculture on BCYE, BCYE with cysteine and PCR	2
Subculture on BCYE, BCYE without cysteine and R2A	1
Subculture onto GVPC and BCYE	1

Codes:
BCYE: Buffered charcoal yeast extract agar without L-cysteine
BCYE with antibiotics: Buffered charcoal yeast extract agar with selective supplements
GVPC: Glycine vancomycin polymyxin B cycloheximide
MWY: Modified Wadowsky Yee.
TSASB: Trypticase soy agar with blood

Figure A17 details the kits used to carry out the serogroup testing, with some laboratories using a combination of kits (n=27).

Figure A17. Kit used for serogroup testing



Information on molecular testing

Figure A18 shows the method used to determine the sequence base type (n=13).

Figure A18. Method used to determine the SBT

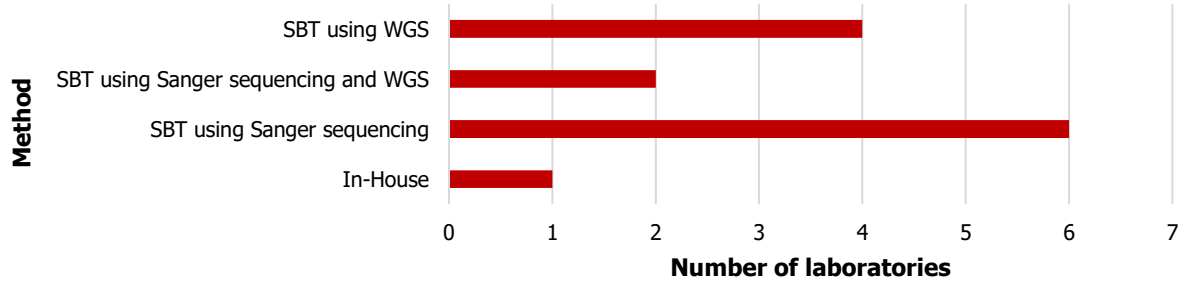


Table A21 below shows how samples are processed for molecular examination (n=6).

Table A21. Sample processing for molecular examination

Process	Number of laboratories
Filtration of 100 mL and filtration of 10 mL on 2 different membrane	1
Filtration of 1L of water sample	1
Filtration of 500 mL	2
Real time PCR was used on isolated Legionella strains from samples	2

The table below shows the DNA extraction kits used.

Table A22. List of DNA extraction kits used

DNA extraction kit	Number of laboratories
Bio-Rad Aquadien™ Bacterial DNA Extraction and Purification	2
Invitrogen™ i-prep	2
NucliSens easyMAG	1
Other	2
Roche Diagnostics MagNA Pure LC DNA Isolation Kit III	2
Sacace™ *Legionella pneumophila* Real™	1

The table below shows the volume of extracted DNA for use in assays.

Table A23. Volume of extracted DNA used in assays

Volume (mL)	Number of laboratories
2.5	1
5	5
10	2
Other	1

The table shows the commercial assay that was used.

Table A24. Commercial assays used

Commercial assay	Number of laboratories
AmpliSens® *Legionella pneumophila*-FRT	1
Bio-Rad iQ-Check® Quanti *Legionella* spp. and *L. pneumophila* Real-Time PCR Quantification Kit	1
Bio-Rad iQ-Check® Screen *Legionella* spp. and *L. pneumophila* Kit	1
In-house	1
Other	2
Sacace™ *Legionella pneumophila* Real™	1

The table below shows the amplification platforms used.

Table A25. Amplification platforms used

Amplification platform	Number of laboratories
Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System	1
Bio-Rad CFX96 Touch™ Deep Well RT-PCR Detection System	2
Other	1
Roche LightCycler ® 2.0	1
RotorGene	1

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