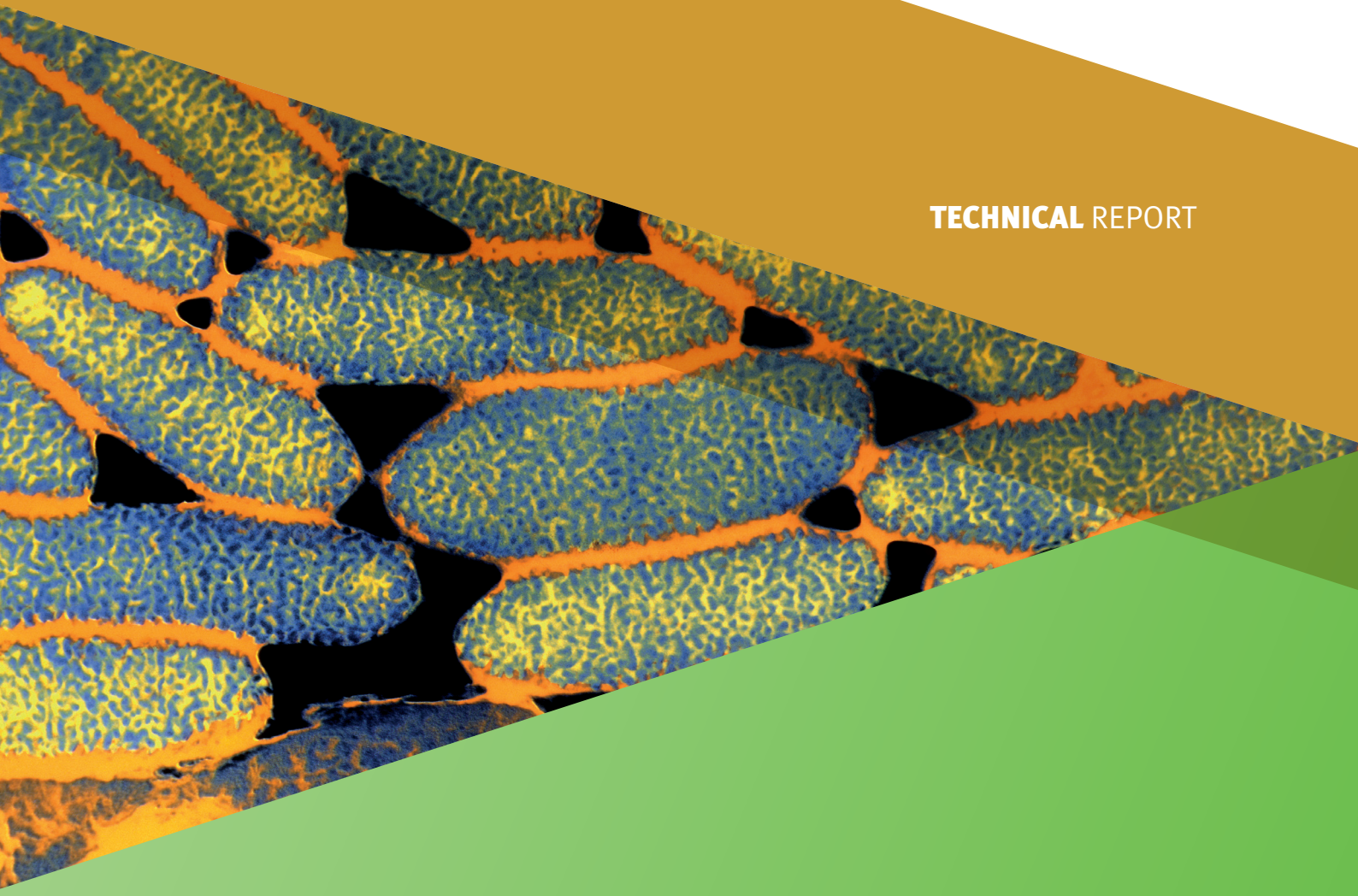

TECHNICAL REPORT

External quality assessment schemes to support European surveillance of Legionnaires' disease 2020-2021

EU/EEA countries

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ECDC TECHNICAL REPORT

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Abbreviations

BCYE	Buffered charcoal yeast extract
CFU	Colony forming unit
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
LUA	*Legionella pneumophila* urinary antigen
MALDI-TOF	Matrix Assisted Laser Desorption Ionisation Time-of-Flight
MLST	Multilocus Sequence Typing
MVLA	Multiple-Locus Variable-Number Tandem-Repeat Analysis
NFP	National Focal Point
PCR	Polymerase chain reaction
PFGE	Pulsed Field Gel Electrophoresis
PHE	Public Health England
RT-PCR	Real-time polymerase chain reaction
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
UAT	Urinary antigen
UK NEQAS	United Kingdom National External Quality Assessment Service
WGS	Whole genome sequencing

Executive summary

In 2020, the European Centre for Disease Prevention and Control (ECDC) requested the start of the second year of an External Quality Assessment (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).

These EQA schemes provide 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 two 2020–2021 EQA exercises was to continue monitoring the accuracy of *Legionella* testing and results reported by individual laboratories, to enable comparison of results between laboratories and within countries across Europe. This report presents an analysis of participants' results for the 2020-2021 EQA exercises for the European Union/European Economic area (EU/EEA) countries.

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

Two rounds were completed during 2020–2021, with EQA distributions sent on 9 November 2020 and 1 March 2021. This was comprised of distributions 4895 (clinical) and 4896 (environmental) in November 2020 and distributions 5113 (clinical) and 5114 (environmental) in March 2021. A total of 30 EU/EEA countries were invited to take part in these EQA distributions via their national focal points (NFP) for Legionnaires' disease, with a maximum of two laboratories per country to be proposed based on their involvement in the management of public health incidents associated with *Legionella* in their country.

Each distribution comprised a total of 20 simulated samples: 10 representing clinical material and 10 representing environmental samples. Strains of *Legionella* were provided by the Respiratory and Vaccine Preventable Bacteria Reference Unit (RVPBRU) and these strains were fully characterised using conventional and molecular methods.

Laboratories only needed to examine samples/specimens they would routinely test or process and report whether the sample/specimen contained a *Legionella* spp., and then provide identification, enumeration, serogroup (Sg) and sequence type (ST) where relevant.

November's distribution represented an outbreak associated with a fountain. The outbreak strain of *Legionella pneumophila* Sg 1, ST109 used was isolated from patients and domestic water system. It has been isolated from the environment in several countries across Europe, Canada, and the United States.

March's distribution represented an outbreak associated with a hospital water system. The outbreak strain of *Legionella pneumophila* Sg 1, ST62 was isolated from clinical specimens and environmental samples. It has been associated with community-acquired, nosocomial and travel-related cases. The majority of *L. pneumophila* ST62 strains identified to date belong to Sg 1.

This annual report is split into two sections, each covering the two distributions sent. It also includes results from a survey undertaken with the participating laboratories on methods/kit information and frequency of testing performed for each method/kit by the laboratories.

For the clinical element in the November 2020 distribution, 23 laboratories examined the sputum specimens and 22 examined the urine specimens. For the March 2021 distribution, 20 laboratories examined the sputum specimens and 18 examined the urine specimens.

For the environmental element, 17 of the laboratories examined both the water and swab samples in November's distribution and 16 laboratories for both sample types in March's distribution.

Where results reported were not in accordance with the intended exercise, laboratories were advised by the organisers to investigate to determine the root cause.

The November 2020 distribution was associated with an outbreak in a water fountain. The outbreak strain was *Legionella pneumophila* Sg1 ST109, which was present in patients one, two, and three. Patient four had clinical details commonly seen with Legionnaires disease but did not contain a *Legionella* spp. Patient five contained a *Legionella pneumophila* not associated with the outbreak (ST047) and patient six contained a *Legionella longbeachae*. This non-*Legionella pneumophila* was added to determine laboratories' capacity to correctly identify a non-pneumophila Legionella and the reporting procedures i.e. would laboratories report to species level or as a non-*Legionella pneumophila*?

The March 2021 distribution was associated with an outbreak related to a hospital setting. As per the November distribution, patients one, two, and three contained the outbreak strain, *Legionella pneumophila* Sg1 ST62. Patient two comprised a negative sputum specimen and a positive urine sample. Lower concordance was attained with this paired specimen. Patient four contained a non-serogroup 1 strain of *Legionella pneumophila.* As urinary antigen kits can only identify Sg1, this was therefore reported as negative by all participants. As per the November distribution,

one specimen (patient six) contained a non-*Legionella pneumophila.* Similar findings were obtained in both distributions, and a lower concordance was achieved for the non-*Legionella pneumophila*.

Overall concordance was excellent (100%) across both distributions for identification of *L. pneumophila.* However, concordance with intended results was reduced for negative samples, those not containing *Legionella* spp.*,* and those containing non-*Legionella pneumophila.* Concordance was excellent (100%) for both distributions for reporting the Sg, with the large majority of laboratories performing serogrouping, with only one participant not reporting a result. In both distributions only a few laboratories reported ST results. For the November distribution (4895), a total of 15/23 laboratories reported ST for patient 1, followed by 14/23 laboratories for the remainder of the clinical specimens. For the March distribution (5113) a total of 10/20 laboratories reported a result against the ST. From the participants reporting a result, concordance was lower for a non-*L. pneumophila* Sg1 included in distribution 4895.

In total, 20 environmental laboratories were sent this EQA, 17 for distribution in November and all laboratories returned results. For March's distribution 20 laboratories were sent the EQA, of which 16 returned a result.

The environmental laboratories represented a total of 23 EU/EEA countries across the two distributions.

For the environmental laboratories, for November's distribution 4896, 17 laboratories reported a result for isolation and identification, up to 17 reported a Sg, up to 16 reported an enumeration count and up to ten a ST. For molecular methods, up to seven laboratories analysed the samples for *L. pneumophila* and up to six for *Legionella* spp. The overall isolation performance for culture was very good with 98.7% over the 10 samples, with 17 reporting a result. Performance with reporting a correct identification, when a *Legionella* spp. was in the sample, was also high, with 98.1%.

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.

For March's distribution 5114, 16 laboratories reported a result for isolation and identification, up to 15 reported a Sg, up to 15 reported an enumeration count and up to eight a ST. For molecular methods, up to six laboratories analysed the samples for *L. pneumophila* and for *Legionella* spp. The overall isolation performance for culture was very good with 94.8% over the 10 samples, with 16 reporting a result. Performance with reporting a correct identification, when a *Legionella* spp. was in the sample, was also high with 99.1%.

Only one laboratory reported an incorrect isolation three times, once in distribution 4896 and twice in distribution 5114.

The overall performance for molecular methods was not calculated due to low numbers of laboratories analysing the sample by this method. However, for distribution 4896 one laboratory reported an incorrect result for five of the samples for *Legionella* spp. for samples that contained a *L. pneumophila*. This laboratory did not participate in distribution 5114 to determine if improvements were made through these EQA exercises. For distribution 5114, no laboratory reported an incorrect result twice.

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.

In conclusion for the 2020-2021 scheme, 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. These data provide 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.

Overall, the performance of laboratories participating in the 2020-2021 EQA was very good. There were no significant issues arising for species identification, serogroup, enumeration, or sequence type.

The performance of laboratories from a total of 23 EU/EEA countries in these exercises was very good for culture-based/detection methods used by both the clinical (96.9%) and environmental laboratories (96.8%).

From the results data collated in this EQA, it was ascertained that for clinical specimens the majority of laboratories identified the pathogen and Sg, but a significant number did not report the ST (Tables 6 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.

In replies to an ECDC evaluation questionnaire, laboratories indicated that this EQA exercise was considered very useful, and overall there was continued very positive feedback on the 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 human 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. 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 the European level since 1987, firstly through a dedicated surveillance network funded by the European Commission and then, since April 2010, through the European Legionnaires' Disease Surveillance Network (ELDSNet), 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 are 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; and
- 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 from 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 2020–2021

The purpose of these EQA exercises 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 2020-2021 EQA exercises 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 2020–2021 EQAs were:

- to continue understanding the baseline level of testing undertaken in laboratories in response to routine outbreak scenarios, for both clinical and environmental samples;
- to continue assessing if there were any general performance issues over the two EQAs sent to determine specific issues relating to the different species, levels and background organisms included; and
- to provide individual technical support to laboratories as a follow up to the exercises, if requested by the countries.

2. Study design and methods

Organisation of the EQAs

The two EQAs were 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 (NFP) 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 dispatched as part of this contract.

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 username and passwords generated for each one. This allowed the laboratory to return results and view individualised reports through a secure web portal, the results were anonymised for ECDC.

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

- The EQA distributions were sent on 9 November 2020 and 1 March 2021 to a maximum of two laboratories per country.
- Prior to each exercise, ECDC invited ECDC National Focal Points for Legionnaires' disease to propose up to two laboratories per country to take part in the EQA exercise; 30 EU/EEA countries were contacted. One laboratory that undertakes clinical examination of specimens and one that examines environmental samples was required. One laboratory could also be nominated to participate in both clinical and environmental, if they usually processed both types of samples. 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.
- Each 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/specimens 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 (sero-grouping) (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.
- November's distribution simulated an outbreak associated with fountain water. The outbreak *Legionella pneumophila* strain chosen was Sg 1 and ST109.
- March's distribution simulated an outbreak associated with a hospital water system. The outbreak *Legionella pneumophila* strain chosen was Sg 1 and ST62.

A total of 17 environmental laboratories from 17 EU/EEA countries and 23 clinical laboratories from 23 EU/EEA countries took part in November's EQA (Table 1).

In the March EQA, a total of 16 environmental laboratories from 16 EU/EEA countries and 23 clinical laboratories from 23 EU/EEA countries took part (Table 1).

Each laboratory was provided with a unique laboratory identification. Of those taking part, 17/23 participating countries tested both the clinical specimens and environmental samples in November, and 15/23 in March. Hungary only took part in November's distribution, and Romania only took part in March's distribution.

13/20 laboratories from a total of 20 countries took part in both environmental distributions.

15/23 laboratories from a total of 23 countries took part in both distributions for the clinical distributions.

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

	November 2020 distribution			March 2021 distribution		
Country	Clinical EQA samples – 4895	Environmental EQA samples – 4896	Number of participating laboratories per country	Clinical EQA samples – 5113	Environmental EQA samples – 5114	Number of participating laboratories per country
Austria	Yes	-	1	Yes	-	1
Belgium	Yes	-	1	Yes	Yes	2
Bulgaria	Yes	Yes	2	Yes	Yes	2
Cyprus	Yes	-	1	Yes	-	1
Czechia	Yes	Yes	2	Yes	-	1
Denmark	Yes	Yes	2	Yes	Yes	2
Estonia	Yes	Yes	2	Yes	Yes	2
Finland	Yes	Yes	2	Yes	Yes	2
France	Yes	Yes	2	Yes	Yes	2
Germany	Yes	Yes	2	Yes	Yes	2
Greece	Yes	Yes	2	Yes	-	1
Hungary	Yes	Yes	2	-	-	0
Ireland	Yes	Yes	2	-	Yes	1
Italy	Yes	Yes	2	Yes	-	1
Latvia	Yes	Yes	2	Yes	Yes	2
Lithuania	Yes	-	1	Yes	-	1
Netherlands	Yes	-	1	Yes	Yes	2
Norway	Yes	Yes	2	Yes	Yes	2
Portugal	Yes	Yes	2	Yes	Yes	2
Romania	-	-	0	Yes	Yes	2
Slovak Republic	Yes	Yes	2	Yes	Yes	2
Slovenia	Yes	Yes	2	Yes	Yes	2
Spain	Yes	-	1	Yes	-	1
Sweden	Yes	Yes	2	Yes	Yes	2

The panel of EQA samples/specimens were dispatched within approved United Nations containers, including an EQA protocol. This protocol contained information on the sample/specimen details, instructions on how to process samples/specimens' safety data information, and instructions on how to enter the results online. The information was also available electronically to all participants and NFPs for Legionnaires' disease in ELDSNet via the UK NEQAS web portal.

A dedicated page was available on the UK NEQAS website for laboratories to enter and submit their results. Laboratories 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 identification 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, a copy of the web reply form was available for laboratories to download to facilitate the manual recording of test results prior to submission online. Laboratories were allowed six weeks (42 days) from the date of dispatch of both clinical specimens 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 SBT.

Six weeks after dispatch dates, the web platform was closed for results submission and the intended results were published on the UK NEQAS secure website. Laboratories were notified by email that the intended results were available for viewing. Individual reports were made available in March 2021 for November 2020 distribution and in June 2021 for the March 2021 distribution.

For November's distribution, laboratories were asked about basic method information for processing the samples/specimens. This was captured at the same time the laboratories reported their results and the findings are shown in Annex 1.

After the close of March's distribution, an additional method questionnaire was sent electronically to the laboratories that took part in both distributions. A summary of the findings is found in Annex 2.

Between 28 June and 31 July 2021, 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 of this report.

Certificates of participation were sent electronically to the laboratories on 31 March 2021 for November's distribution and 15 July 2021 for March's distribution. A hard copy of the certificate was available on request.

EQA exercise scenario and sample design

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

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

The individual laboratory reports detailed a laboratory's reported results for each examination requested and the microbiological contents for each sample/specimen. This included the identification of the *Legionella* species, Sg, ST and enumeration results, where applicable. The report also provided an overall performance for each examination based on all the laboratories reported results.

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.

9 November 2020 distribution

Five environmental samples were supplied to represent an outbreak associated with a fountain. Samples provided were water from a fountain, and the inlet of the fountain, water from a nearby cooling tower, swabs from the hose of the fountain and the filter pump. In addition, five routine monitoring samples were supplied: water and swabs from hot and cold water systems, water from a spa pool, and river water from a sewage effluent.

Simulated clinical samples were taken from six patients with suspected symptoms of Legionnaires' disease (sputum and or urine specimens).

The outbreak strain of *Legionella pneumophila* serogroup 1, ST109 used in this EQA exercise has been isolated from patients and domestic water systems. This strain has been isolated from the environment in several countries across Europe, Canada and the United States and is the first strain on the *Legionella* sequence-based typing database listed.

Simulated specimens included *Legionella pneumophila* serogroup 6, ST68. This sequence type of *Legionella pneumophila* has been isolated from patients as well as domestic and hospital water systems. Whole sequence analysis suggest it is persistent in a water system for a considerable period of time.

Another strain distributed in this panel included *a Legionella pneumophila* serogroup 1, ST47. ST47 is the leading cause of legionellosis in north-western Europe, this ST is rarely isolated from the environment.

Legionella jordanis, a species that is rarely encountered, was included in the environmental distribution. This species was originally isolated in 1978 from the Jordan River in Indiana following an outbreak of Legionnaires' disease in that region. The same researchers isolated a second strain two years later from a sewage plant in Georgia (1982). The clinical significance is unknown but occasional clinical case reports have been documented including in immunocompromised patients.

Legionella longbeachae was included for the clinical distribution, the most common *Legionella* species after *Legionella pneumophila* to be isolated from humans. *L. longbeachae* was first isolated in 1980 from a patient with pneumonia in Long Beach, California, United States. There are two serogroups of *Legionella longbeachae*. This species has been associated with horticultural growth medium and is found in patients that undertake gardening and has been detected in tree bark [5].

Samples/specimens were prepared and quality-controlled by the EQA organisers and the panels were dispatched as distributions 4895 (clinical) and 4896 (environmental).

1 March 2021 distribution

Five environmental samples were supplied to represent an outbreak associated with a hospital water system. Samples provided were waters from hot and cold-water system and shower water from an orthopaedic ward, water from hot and cold system from estates department, water from a cooling tower situated on the hospital grounds, and a swab of a biofilm from the hot and cold-water system. In addition, five routine monitoring samples were supplied: water and swabs from hot and cold-water systems, water from a spa pool, potable water, and water from a cooling tower.

Clinical specimens were taken from six patients with suspected symptoms indicating Legionnaires' disease (sputum and or urine samples).

The outbreak strain of *Legionella pneumophila* Sg 1, ST62 used for this EQA exercise has been isolated from clinical and environmental samples. It has been associated with community-acquired, nosocomial, and travel-related cases. Majority of *L. pneumophila* ST62 strains identified to date belong to Sg 1.

Legionella pneumophila serogroup 6, ST2923 was also included. This sequence type of *Legionella pneumophila* is unique and has only been identified once from an environmental sample in the United Kingdom (UK).

Legionella anisa was included in the environmental distribution. This species was first isolated from water during a nosocomial outbreak in the United States between March 1980 and June 1981. *Legionella anisa* is one of the most frequent species of *Legionella* other than *Legionella pneumophila* in the environment and may be hospital-acquired in rare cases. A distinguishing characteristic is the ability of colonies to exhibit blue-white autofluorescence when viewed under ultraviolet light. As a result, *L. anisa*, along with several other species of *Legionella*, is sometimes referred to as 'blue-white' *Legionella*.

Legionella bozemanii was included in the clinical distribution. This species was first isolated from lung tissue in 1959. The isolate came from a healthy scuba diver in Florida with fatal bronchopneumonia [6]. The second isolate, was recovered in 1979 from lung tissue from a patient with lymphatic leukemia in whom a fulminant pneumonia developed after submersion in brackish swamp water [7]. *L. bozemanii* is within the top five most common *Legionella* species isolated from water distribution systems [8].

Samples/specimens were prepared and quality-controlled by the EQA organisers and the panels were dispatched as distributions 5113 (clinical) and 5114 (environmental).

Clinical

Ten clinical specimens were prepared in each set (five simulated sputum and five liquid urine specimens). An overview of specimens is provided in Tables 2 and 4.

Participants were requested to provide an organism identification, Sg and ST (simulated sputum samples) and *Legionella pneumophila* urinary antigen (LUA result) (urine specimens). Simulated sputum specimens 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 to resemble 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 Sg 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.

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.

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; and
- 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

An overview of samples provided is shown in Tables 3 and 5.

For each distribution, 10 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; and
- the sample contents matched those obtained from RVPBRU for identification, Sg and ST.

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 were displayed graphically. Detected/not detected, the Sg and ST 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 these EQA exercises and findings can be found in Annex 1 and 2. Key results are integrated into the separate sections below.

Intended results for the 2020–2021 exercise

Sample contents for the specimens included in the clinical and environmental distributions are described in Tables 2-5, including the serogroup and sequence base type when *Legionella pneumophila* was present.

Table 2. Clinical specimens 6364-6373 provided in distribution 4895 (9 November 2020)

Specimen number	Patient	Specimen	Specimen type	Specimen contents	Sg	ST	Details
6364	1	1	Sputum	*Legionella pneumophila, Streptococcus oralis, Streptococcus mitis*	1	109	Fever, headache and muscles pains. Visited water fountain.
6365		2	Urine	*Legionella pneumophila*	1	109	
6366	2	1	Sputum	*Legionella pneumophila, Moraxella catarrhalis, Streptococcus oralis,*	1	109	Confusion and headache. Recent contact with water fountain.
6367		2	Urine	*Legionella pneumophila*	1	109	
6368	3	1	Sputum	*Legionella pneumophila, Streptococcus mitis*	1	109	Diarrhoea and mental confusion. Drank from water fountain four days previously.
6369		2	Urine	*Legionella pneumophila*	1	109	
6370	4	1	Sputum	*Streptococcus oralis, Streptococcus salivarius*			Dizziness and muscle pain. Lives close to water fountain.
6371		2	Urine	Negative			
6372	5	5	Urine	*Legionella pneumophila*	1	047	Trouble sleeping and thinking clearly. Visited water fountain.
6373	6	6	Sputum	*Legionella longbeachae, Streptococcus mitis, S. salivarius*			Shortness of breath and loss of appetite in an immunocompromised patient.

Table 3. Environmental samples 6374-6383 provided distribution 4896 (9 November 2020). Levels of *Legionella* spp. in the sample is shown as approximate colony forming units (cfu) per litre

Specimen number	Sample type	Sample contents	Sg	ST	Comments
6374	Fountain water	*L. pneumophila* ($1.5x10^5$) *Brevundimonas vesicularis* *Pseudomonas fluorescens*	1	109	Samples taken as part of one outbreak investigation.
6375	Water from a cooling tower near the location of the fountain	*Enterococcus faecalis* *Roseomonas aestuarii* *Pseudomonas aeruginosa*			
6376	Swab from the hose of the fountain	*L. pneumophila* ($1.8x10^4$) *Roseomonas aestuarii* *Citrobacter braakii*	1	109	
6377	Inlet water sample to the fountain	*Pseudomonas fluorescens* *Pseudomonas putida* *Staphylococcus epidermidis*			
6378	Swab from the filter pump of the fountain	*L. pneumophila* ($1.8x10^2$) *Staphylococcus epidermidis* *Escherichia coli*	1	109	
6379	Hot and cold water system	*L. pneumophila* ($2.6x10^3$) *Staphylococcus saprophyticus* *Escherichia coli*	1	47	Samples taken as part of routine quality monitoring of water.
6380	Biofilm swab from hot and cold water system	*Klebsiella pneumoniae* *Staphylococcus haemolyticus* *Enterococcus faecium*			
6381	Spa water	*L. pneumophila* (45) *Brevundimonas vesicularis* *Enterococcus faecalis*	6	68	
6382	River water near a sewage effluent	*Legionella jordanis* ($5.8x10^4$) *Staphylococcus epidermidis*			
6383	Biofilm swab from hot and cold water system	*L. pneumophila* ($2.6x10^3$) *Staphylococcus saprophyticus* *Escherichia coli*	1	47	

Table 4. Clinical specimens 7034-7043 provided in distribution 5113 (1 March 2021)

Specimen number	Patient	Specimen	Specimen type	Specimen contents	Sg	ST	Details
7034	1	1	Sputum	*Legionella pneumophila* *Streptococcus oralis*	1	62	Muscle aches and headache following recent stay on orthopaedic ward.
7035		2	Urine	*Legionella pneumophila*	1	62	
7036	2	1	Sputum	*Moraxella catarrhalis* *Streptococcus oralis*			Previous hospitalisation following bone fracture repair. Patient now presenting with shortness of breath.
7037		2	Urine	*Legionella pneumophila*	1	62	
7038	3	1	Sputum	*Legionella pneumophila* *Streptococcus mitis* *Streptococcus oralis*	1	62	Persistent cough, fever and headache following hospital stay 10 days previously.
7039		2	Urine	*Legionella pneumophila*	1	62	
7040	4	1	Sputum	*Legionella pneumophila* *Streptococcus mitis* *Streptococcus oralis*	6	2923	Persistent cough, fever and headache following hospital stay 10 days previously.
7041		2	Urine	Negative *(Legionella pneumophila* Sg6*)*	6	2923	
7042	5	5	Urine	Negative			Fever of 104F (40°C) and confusion. Query secondary infection following discharge from hospital.
7043	6	6	Sputum	*Legionella bozemanii*			Cough, bringing up mucus and muscle pains for five days.

Table 5. Environmental samples 7044-7053 provided distribution 5114 (1 March 2021). Levels of *Legionella* spp. in the sample is shown as approximate cfu per litre

Specimen number	Sample type	Sample contents	Sg	ST	Comments
7044	Water from hot and cold water system from orthopaedic ward	*L. pneumophila* ($2.9x10^3$) *Brevundimonas vesicularis* *Aerococcus viridans*	1	62	Samples taken as part of one outbreak investigation.
7045	Shower water from orthopaedic ward	*L. pneumophila* ($5.7x10^4$) *Pseudomonas putida* *Staphylococcus epidermidis*	1	62	
7046	Water from hot and cold water system from estates department	*Acinetobacter junii* *Pseudomonas fluorescens*			
7047	Biofilm swab from hot and cold water system	*Legionella anisa* ($9.1x10^2$) *Citrobacter braakii*			
7048	Water from a cooling tower located in the hospital ground	*L. pneumophila* ($6.8x10^4$) *Roseomonas aestuarii* *Pseudomonas aeruginosa*	1	62	
7049	Potable water	*L. pneumophila* ($8.1x10^3$) *Microbacterium luteolum*	1	1	Samples taken as part of routine quality monitoring of water.
7050	Water from cooling tower	*Staphylococcus saprophyticus*			
7051	Pool Water from a spa pool	*L. pneumophila* ($1.4x10^4$) *Klebsiella pneumoniae* *Staphylococcus haemolyticus* *Enterococcus faecium*	1	1	
7052	Water from hot and cold system	*Enterococcus faecalis*			
7053	Biofilm swab from hot and cold water system	*L. pneumophila* ($2.8x10^2$) *Klebsiella pneumoniae* *Staphylococcus haemolyticus* *Enterococcus faecium*	6	2923	

Scoring applied to the examinations

All distributions were scored for the main examinations, either with a score of zero if not correct or 2 if correct. The allocation of scores is a means of drawing attention to differences between a participant's result and what has been designated as the intended result or the 'assigned value'. Scores help laboratories to identify whether there is a problem with their testing.

Clinical scoring applied: A score of 2 was given for the following:

- For reporting a correct identification of the *Legionella* spp.
- For reporting a correct *Legionella* Sg.
- For reporting a correct urinary antigen result.

Environmental scoring applied: A score of 2 was given for the following:

- For reporting a correct isolation result.
- For reporting a correct identification of the *Legionella* spp.
- For reporting a correct Sg.
- For reporting an enumeration value within the expected range. This was calculated by using the participants' median was used as the assigned value and then the expected range calculated using 5 and 95 percentiles – however, there were not enough data sets to score this examination.

9 November 2020

Clinical distribution 4895

A total of 23 of participating laboratories from 23 countries reported results for the simulated sputum samples compared with 22 laboratories who reported results for the urine samples.

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

Table 6. Examinations performed and concordance achieved for distribution 4895

Sample number	Contents	Identification		Serogroup		Sequence type		Urinary antigen		Overall % performance by sample
		N	%	N	%	N	%	N	%	
6364	*Legionella pneumophila* Sg 1 ST109, *Streptococcus oralis, Streptococcus mitis*	23/23	100	23/23	100	15/15	100	-	-	100
6365	*Legionella pneumophila*	-	-	-	-	-	-	22/22	100	100
6366	*Legionella pneumophila* Sg 1 ST109, *Moraxella catarrhalis, Streptococcus oralis,*	23/23	100	23/23	100	13/14	92.9	-	-	97.6
6367	*Legionella pneumophila*	-	-	-	-	-	-	22/22	100	100
6368	*Legionella pneumophila* Sg 1 ST109, *Streptococcus mitis*	23/23	100	23/23	100	13/14	92.9	-	-	97.6
6369	*Legionella pneumophila*	-	-	-	-	-	-	21/22	95.5	95.5
6370	*Streptococcus oralis, Streptococcus salivarius*	20/23	87	-	-	-	-	-	-	87
6371	No organisms	-	-	-	-	-	-	22/22	100	100
6372	*Legionella pneumophila*	-	-	-	-	-	-	22/22	100	100
6373	*Legionella longbeachae, Streptococcus mitis, S. salivarius*	21/23	91.3	- Not reported	-	-	-	-	-	91.3
Overall performance by examination			95.7		100		95.3		99.1	

Patient 1:

Specimen 6364: Excellent concordance, with intended results with 100% (23/23), 100% (23/23), and 100% (15/15) of participants reporting a correct identification for this specimen, Sg and ST respectively.

Specimen 6365: An excellent performance, with 100% (22/22) concordance with intended results from participants returning a result. One laboratory stated not examined for all UAT.

Patient 2:
Specimen 6366: An excellent performance, with 100% (23/23) of participants reporting the correct result for identification, a 100% (23/23) with correct Sg and 92.9% (13/14) with correct ST. One laboratory reported 'other' and listed ST109, ST277 or new ST.

Specimen 6367: The specimen was positive for *L. pneumophila* urinary antigen. An excellent performance, with 100% (22/22) of participants reporting the correct result.

Patient 3:
Specimen 6368: An excellent performance, with 100% (23/23) of participants reporting the correct identification, a 100% (23/23) reporting the correct Sg and 92.9% (13/14) reporting the correct ST. One laboratory, the same as for specimen 6366, reported 'other' and listed ST109, ST106, ST591 or new ST.

Specimen 6369: A total of 95.5% (21/22) of participants returning a result for this specimen reported the correct result, with one laboratory reporting the incorrect result of negative.

Patient 4:
Specimen 6370: A good performance was noted, with 87% (20/23) of participants returning a result of no pathogens isolated. Of these three laboratories, two reported the presence of *L. pneumophila* and one reported *Legionella spp.* not *L. pneumophila.* Of those laboratories reporting the presence of *L. pneumophila* (n=2), one laboratory stated culture negative, polymerase chain reaction (PCR) positive and no further tests were reported. The other laboratory reported a *L. pneumophila* Sg 1.

Specimen 6371: An excellent performance, with 100% (22/22) of participants reporting the correct result.

Patient 5:
Specimen 6372: An excellent performance, with 100% (22/22) of participants reporting the correct result.

Patient 6:
Specimen 6373: A good performance with 91.3% (21/23) of participants returning a result for this specimen reported the correct result (not *L. pneumophila* isolated was considered correct).

Environmental distribution 4896

Ten simulated environmental samples were sent to 17 laboratories in 17 EU/EEA countries. All 17 laboratories returned a result.

Sample numbers: 6374-6378 were samples taken as part of one outbreak investigation.

Sample numbers: 6379-6383 were samples were taken as part of routine monitoring.

Sample numbers: 6376, 6378, 6380 and 6383 were swab samples.

Performance of the laboratories on these samples were split into culture-based methods (Table 7) and molecular methods (Table 8). Culture-based method analysis included results reported for isolation, identification, enumeration, Sg and ST results. An overall performance assessment column as a percentage has been captured for culture-based method results by sample number and by each examination. Overall performance by sample was calculated using the mean value across a maximum of the five examinations. For molecular methods, the overall performance has not been calculated, as the number of data sets returned for analysis is too low to provide robust performance data.

Table 9 shows in more detail the enumeration results reported by the laboratories. The participants' median is used as the assigned value and the expected range is calculated using 5 and 95 percentiles.

Table 7. Examinations done on cultured samples

Sample number	Contents	Isolation		Identification		Enumeration		Serogroup		Sequence type		Overall % performance by sample
		N	%	N	%	N	%	N	%	N	%	
6374	*L. pneumophila* sg 1, ST109	17/17	100	17/17	100	14/16	87.5	17/17	100	9/9	100	97.5
6375	No *Legionella*	16/16	100	-	-	-	-	-	-	-	-	100
6376	*L. pneumophila* sg 1, ST109	16/17	94.1	16/16	100	-	-	16/16	100	9/9	100	98.5
6377	No *Legionella*	17/17	100	-	-	-	-	-	-	-	-	100
6378	*L. pneumophila* sg 1, ST109	17/17	100	17/17	100	-	-	17/17	100	10/10	100	100
6379	*L. pneumophila* sg 1, ST47	17/17	100	17/17	100	14/16	87.5	17/17	100	7/7	100	97.5
6380	No *Legionella*	17/17	100	-	-	-	-	-	-	-	-	100
6381	*L. pneumophila* sg 6, ST68	17/17*	100	11/11	100	9/11	81.8	10/11	90.9	5/5	100	94.5
6382	*L. jordanis*	14/15	93.3	13/14	92.8	12/14	85.7	-	-	-	-	90.6
6383	*L. pneumophila* sg 1, ST47	17/17	100	16/17	94.1	-	-	15/15	100	6/6	100	98.5
Overall performance by examination			98.7		98.1		85.6		98.5		100	

** Sample 6381: The sample Legionella pneumophila serogroup 6 sequence type ST68. The reason for this low performance is due to the low level of Legionella pneumophila in the sample which was approximately 45 colony forming units per litre which is below the lower detection limit as reported in this distribution by some participants for the methods used by laboratories. Therefore, the reporting of a not detected result is considered correct.*

Table 8. Molecular methods

Sample number	Identification	Intended results for *Legionella pneumophila*	Molecular results *Legionella pneumophila*	Intended results for *Legionella* spp.	Molecular results *Legionella* spp.
6374	*L. pneumophila*	Detected	7/7	Detected	4/4
6375	No *Legionella*	Not detected	5/5	Not detected	5/5
6376	*L. pneumophila*	Detected	5/5	Detected	3/4
6377	No *Legionella*	Not detected	4/5	Not detected	4/5
6378	*L. pneumophila*	Detected	6/6	Detected	3/4
6379	*L. pneumophila*	Detected	7/7	Detected	4/5
6380	No *Legionella*	Not detected	2/3	Not detected	2/3
6381	*L. pneumophila*	Detected	6/7	Detected	3/5
6382	*L. jordanis*	Not detected	6/6	Detected	6/6
6383	*L. pneumophila*	Detected	6/6	Detected	3/4

Table 9. Data on enumeration results where reported

Sample number	Identification	Number of results	Participants median (cfu/L)	Intended range (cfu/L)	Number of outlying counts
6374	*L. pneumophila*	16	$8.3x10^4$	$5.4x10^3 – 2.2x10^5$	2 (1 low and 1 high)
6379	*L. pneumophila*	16	$6.6x10^2$	$33 – 5.2x10^3$	2 (1 low and 1 high)
6381	*L. pneumophila*	11	$1.5x10^2$	$24 – 1.4x10^3$	2 (1 low and 1 high)
6382	*L. jordanis*	14	$5.1x10^4$	$1.0x10^4 – 9.1x10^4$	2 (1 low and 1 high)

Sample 6374: Performance was excellent, with 17/17 (100%) of participants reporting the correct isolation result, 17/17 (100%) for identification, 14/16 (87.5%) of the laboratories reporting a count within the intended range, 17/17 (100%) reporting the correct Sg and 9/9 (100%) of the laboratories reporting the correct ST. The overall performance for examinations by culture was 97.5%.

Seven laboratories examined the sample using a molecular kit that only detects *L. pneumophila* and all 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 6375: Performance was excellent, with 16/16 (100%) of the laboratories reporting the correct isolation result.

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

Sample 6376: Performance was very good, with 16/17 (94.1%) of participants reporting the correct isolation result, 16/16 (100%) for identification, 16/16 (100%) reporting the correct Sg, and 9/9 (100%) of the laboratories reporting the correct ST. The overall performance for examinations by culture was 98.5%.

Five laboratories examined the sample using a molecular kit that only detects *L. pneumophila* and all reported the correct result. In addition, all five of these laboratories also examined the sample using a molecular kit that detects *Legionella* spp., and 3/4 reported a correct result.

Sample 6377: Performance was excellent, with 17/17 (100%) of the laboratories reporting the correct isolation result.

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

Sample 6378: Performance was excellent, with 17/17 (100%) of participants reporting the correct isolation result, 17/17 (100%) for identification, 17/17 (100%) reporting the correct Sg and 10/10 (100%) of the laboratories reporting the correct ST. The overall performance for examinations by culture was 100%.

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

Sample 6379: Performance was excellent, with 17/17 (100%) of participants reporting the correct isolation result, 17/17 (100%) for identification, 14/16 (87.5%) of the laboratories reporting a count within the intended range, 17/17 (100%) reporting the correct Sg and 7/7 (100%) of the laboratories reporting the correct ST. The overall performance for examinations by culture was 97.5%.

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

Sample 6380: Performance was excellent, with 17/17 (100%) of the laboratories reporting the correct isolation result.

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

Sample 6381: Performance was excellent, with 17/17 (100%) of participants reporting the correct isolation result, 11/11 (100%) for identification, 9/11 (81.8%) of the laboratories reporting a count within the intended range, 10/11 (90.9%) reporting the correct Sg and 5/5 (100%) of the laboratories reporting the correct ST. The overall performance for examinations by culture was 94.5%.

Seven laboratories examined the sample using a molecular kit that only detects *L. pneumophila* and 6/7 reported the correct result. In addition, five of these laboratories also examined the sample using a molecular kit that detects *Legionella* spp., and 3/5 reported a correct result.

Sample 6382: Performance was very good, with 14/15 (93.3%) of participants reporting the correct isolation result, 13/14 (92.8%) for identification and 12/14 (85.7%) of the laboratories reporting a count within the intended range. The overall performance for examinations by culture was 90.6%.

Six laboratories examined the sample using a molecular kit that only detects *L. pneumophila* and all reported the correct result. In addition, all six of these laboratories also examined the sample using a molecular kit that detects *Legionella* spp., and all reported a correct result.

Sample 6383: Performance was excellent, with 17/17 (100%) of participants reporting the correct isolation result, 16/17 (94.1%) for identification, 15/15 (100%) reporting the correct Sg and 6/6 (100%) of the laboratories reporting the correct ST. The overall performance for examinations by culture was 98.5%.

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

1 March 2021

Clinical distribution 5113

A total of 20 of participating laboratories from 20 countries reported results for the simulated sputum specimens compared with 18 laboratories who reported results for the urine specimens.

Table 10. Examinations performed and concordance achieved for distribution 5113

Sample number	Contents	Identification		Serogroup		Sequence type		Urinary antigen		Overall % performance by sample
		N	%	N	%	N	%	N	%	
7034	*Legionella pneumophila* Sg 1 ST62, *Streptococcus oralis*	20/20	100	19/19	100	10/10	100	-	-	100
7035	*Legionella pneumophila*	-	-	-	-	-	-	18/18	100	100
7036	*Moraxella catarrhalis* *Streptococcus oralis*	18/20	90	-	-	-	-	-	-	90
7037	*Legionella pneumophila*	-	-	-	-	-	-	13/18	72	72
7038	*Legionella pneumophila* Sg 1 ST62, *Streptococcus mitis* *Streptococcus oralis*	20/20	100	19/19	100	10/10	100	-	-	100
7039	*Legionella pneumophila*	-	-	-	-	-	-	18/18	100	100
7040	*Legionella pneumophila* Sg 6 ST2923 *Streptococcus mitis* *Streptococcus oralis*	20/20	100	19/19	100	6/7	85.7	-	-	95.2
7041	Negative	-	-	-	-	-	-	18/18	100	100
7042	Negative	-	-	-	-	-	-	18/18	100	100
7043	*Legionella bozemanii*	19/20	95	-	-	-	-	-	-	95
Overall performance by examination			98		100		92.5		94.4	

Patient 1:
Specimen 7034: The specimen contained *Legionella pneumophila* serogroup 1: ST62. Excellent concordance with intended results with 100% (20/20), 100% (19/19) and 100% (10/10) of participants reporting a correct identification, correct Sg and correct ST respectively for this specimen.

Specimen 7035: The specimen was positive for *L. pneumophila* urinary antigen. An excellent performance with 100% (18/18) concordance with intended results from participants returning a result.

Patient 2:
Specimen 7036: The specimen was negative for *Legionella pneumophila.* A good performance with 90% (18/20) of participants reporting the correct result. Two participants incorrectly reported the presence of a *Legionella bozemanii.*

Specimen 7037: The specimen was positive for *L. pneumophila* urinary antigen. Only 72% (13/18) of participants reported the correct result.

Patient 3:
Specimen 7038: The specimen contained *Legionella pneumophila* serogroup 1: ST62. An excellent performance with 100% (20/20) 100% (19/19) and 100% (10/10) of participants reporting the correct identification, Sg and ST respectively.

Specimen 7039: The specimen was positive for *L. pneumophila* urinary antigen. An excellent performance with 100% (18/18) of participants returning a result for this specimen reported the correct result.

Patient 4:
Specimen 7040: The specimen contained *Legionella pneumophila* serogroup 6: ST2923. An excellent performance was noted with 100% (20/20) of participants returning a correct result, 100% (19/19) reporting the correct Sg and 85.7% (6/7) reporting the correct ST. One laboratory incorrectly reported ST1734.

Specimen 7041: The specimen was positive for *L. pneumophila* urinary antigen. An excellent performance with 100% (18/18) of participants reporting the correct result.

Patient 5:
Specimen 7042: The specimen was positive for *L. pneumophila* urinary antigen. An excellent performance with 100% (18/18) of participants reporting the correct result.

Patient 6:
Specimen 7043: The specimen contained *Legionella bozemanii* with 95% (19/20) of participants returned a correct identification for this specimen. Reporting a result of 'Not *L. pneumophila'* isolated was considered correct. One participant incorrectly reported the presence of *L. anisa.*

Environmental distribution 5114

Ten simulated environmental samples were sent to 20 laboratories in 20 EU/EEA countries. 16 laboratories returned a result. Two laboratories informed the organisers that they would not be able to examine the samples and two laboratories did not return a result.

Sample numbers: 7044-7048 were samples taken as part of one outbreak investigation.

Sample numbers: 7049-7053 were samples were taken as part of routine monitoring.

Sample numbers: 7047 and 7053 were swab samples.

Performance of the laboratories on these samples were split into culture-based methods (Table 11) and molecular methods (Table 12). 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. Overall performance by sample was calculated using the mean value across a maximum of the five examinations. For molecular methods the overall performance has not been calculated as the number of data sets returned for analysis is too low to provide robust performance data.

Table 13 shows in more detail the enumeration results reported by the laboratories. The participants' median is used as the assigned value and the expected range is calculated using 5 and 95 percentiles.

Table 11. Examinations done on cultured samples

Sample number	Contents	Isolation		Identification		Enumeration		Serogroup		Sequence type		Overall % performance by sample
		N	%	N	%	N	%	N	%	N	%	
7044	*L. pneumophila* sg 1, ST62	13/16	81.2	13/13	100	11/13	84.6	11/11	100	5/5	100	93.2
7045	*L. pneumophila* sg 1, ST62	16/16	100	16/16	100	14/15	93.3	14/14	100	8/8	100	98.6
7046	No *Legionella*	15/16	93.7	-	-	-	-	-	-	-	-	93.7
7047	*L. anisa*	16/16	100	15/16*	93.7	-	-	-	-	-	-	96.8
7048	*L. pneumophila* sg 1, ST62	15/15	100	15/15	100	12/13	92.3	14/14	100	7/7	100	98.5
7049	*L. pneumophila* sg 1, ST1	16/16	100	16/16	100	15/15	100	15/15	100	7/7	100	100
7050	No *Legionella*	12/14	85.7	-	-	-	-	-	-	-	-	85.7
7051	*L. pneumophila* sg 1, ST1	16/16	100	16/16	100	13/15	86.6	15/15	100	6/7	85.7	94.5
7052	No *Legionella*	16/16	100	-	-	-	-	-	-	-	-	100
7053	*L. pneumophila* sg 6, ST2923	14/16	87.5	13/13	100	-	-	12/13	92.3	5/7	71.4	87.8
Overall performance by examination			94.8		99.1		91.4		98.7		92.8	

**identification results reported as* L. anisa*,* Legionella *spp. or* Legionella *species not* pneumophila *were accepted as being fully correct. 12/16 laboratories reported the full identification as* L. anisa.

Table 12. Molecular methods

Sample number	Identification	Intended results for *Legionella pneumophila*	Molecular results *Legionella pneumophila*	Intended results for *Legionella* spp.	Molecular results *Legionella* spp.
7044	*L. pneumophila*	Detected	5/5	Detected	5/5
7045	*L. pneumophila*	Detected	6/6	Detected	6/6
7046	No *Legionella*	Not detected	4/4	Not detected	3/4
7047	*L. anisa*	Not detected	4/4	Detected	4/4
7048	*L. pneumophila*	Detected	6/6	Detected	6/6
7049	*L. pneumophila*	Detected	6/6	Detected	6/6
7050	No *Legionella*	Not detected	3/3	Not detected	2/3
7051	*L. pneumophila*	Detected	6/6	Detected	6/6
7052	No *Legionella*	Not detected	4/4	Not detected	4/4
7053	*L. pneumophila*	Detected	4/4	Detected	4/4

Table 13. Data on enumeration results where reported

Sample number	Identification	Participants median (cfu/L)	Intended range (cfu/L)	Number of results	Number of outlying counts
7044	*L. pneumophila*	$1.1x10^3$	$1.1x10^2 – 3.9x10^3$	13	2 (1 low, 1 high)
7045	*L. pneumophila*	$2.5x10^4$	$1.2x10^3 – 1.1x10^5$	15	1 (1 high)
7048	*L. pneumophila*	$1.6x10^4$	$2.3x10^2 – 6.4x10^5$	13	1 (1 low)
7049	*L. pneumophila*	$3.8x10^3$	$6.1x10^2 – 1.9x10^4$	15	-
7051	*L. pneumophila*	$5.0x10^3$	$8.7x10^2 – 7.9x10^4$	15	2 (1 low, 1 high)

Sample 7044: Performance was very good, with 13/16 (81.2%) of participants reporting the correct isolation result, 13/13 (100%) for identification, 11/13 (84.6%) of the laboratories reporting a count within the intended range, 11/11 (100%) reporting the correct Sg and 5/5 (100%) of the laboratories reporting the correct ST. The overall performance for examinations by culture was 93.5%.

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

Sample 7045: Performance was excellent, with 16/16 (100%) of participants reporting the correct isolation result, 16/16 (100%) for identification, 14/15 (93.3%) of the laboratories reporting a count within the intended range, 14/14 (100%) reporting the correct Sg and 8/8 (100%) of the laboratories reporting the correct ST. The overall performance for examinations by culture was 98.6%.

Six laboratories examined the sample using a molecular kit that only detects *L. pneumophila* and all reported the correct result. In addition, all six of these laboratories also examined the sample using a molecular kit that detects *Legionella* spp., and all reported a correct result.

Sample 7046: Performance was very good, with 15/16 (93.7%) of the laboratories reporting the correct isolation result.

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

Sample 7047: Performance was excellent, with 16/16 (100%) of the laboratories reporting the correct isolation result and 15/16 (93.7%) for identification. The overall performance for examinations by culture was 96.8%.

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

Sample 7048: Performance was excellent, with 15/15 (100%) of participants reporting the correct isolation result, 15/15 (100%) for identification, 12/13 (92.3%) of the laboratories reporting a count within the intended range, 14/14 (100%) reporting the correct Sg and 7/7 (100%) of the laboratories reporting the correct ST. The overall performance for examinations by culture was 98.5%.

Six laboratories examined the sample using a molecular kit that only detects *L. pneumophila* and all reported the correct result. In addition, all six of these laboratories also examined the sample using a molecular kit that detects *Legionella* spp., and all reported a correct result.

Sample 7049: Performance was excellent, with 16/16 (100%) of participants reporting the correct isolation result, 16/16 (100%) for identification, 15/15 (100%) of the laboratories reporting a count within the intended range, 15/15 (100%) reporting the correct Sg and 7/7 (100%) of the laboratories reporting the correct ST. The overall performance for examinations by culture was 100%.

Six laboratories examined the sample using a molecular kit that only detects *L. pneumophila* and all reported the correct result. In addition, all six of these laboratories also examined the sample using a molecular kit that detects *Legionella* spp., and all reported a correct result.

Sample 7050: Performance was very good, with 12/14 (85.7%) of the laboratories reporting the correct isolation result.

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

Sample 7051: Performance was excellent, with 16/16 (100%) of participants reporting the correct isolation result, 16/16 (100%) for identification, 13/15 (86.6%) of the laboratories reporting a count within the intended range, 15/15 (100%) reporting the correct Sg and 6/7 (85.7%) of the laboratories reporting the correct ST. The overall performance for examinations by culture was 94.5%.

Six laboratories examined the sample using a molecular kit that only detects *L. pneumophila* and all reported the correct result. In addition, all six of these laboratories also examined the sample using a molecular kit that detects *Legionella* spp., and all reported a correct result.

Sample 7052: Performance was excellent, with 16/16 (100%) of the laboratories reporting the correct isolation result.

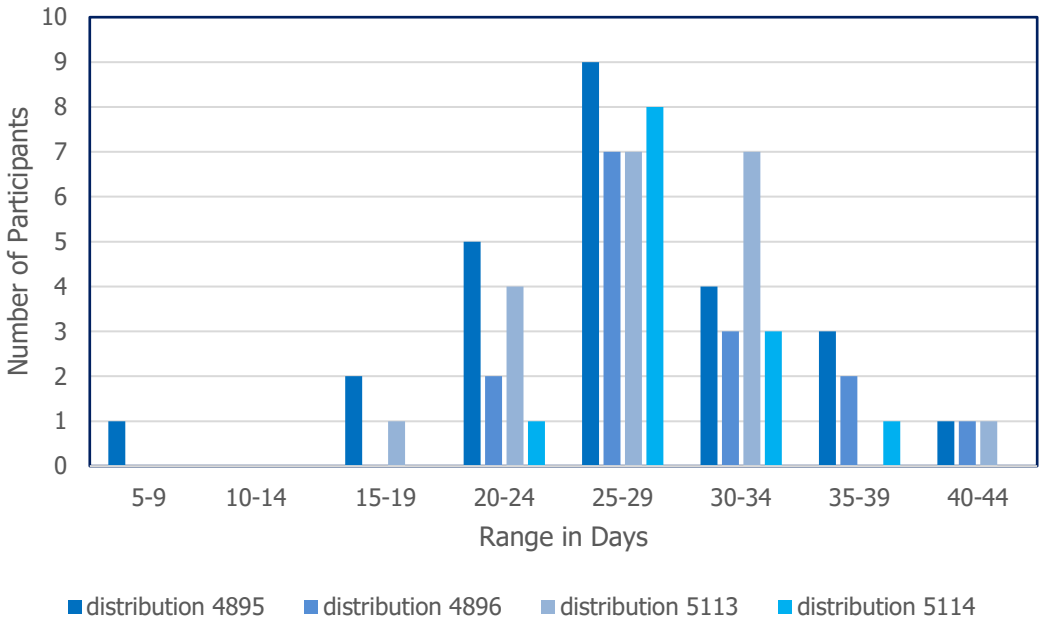
Four laboratories examined the sample using a molecular kit that only detects *L. pneumophila* and all reported the correct result. In addition, all four of these laboratories also examined the sample using a molecular kit that detects *Legionella* spp., and all reported a correct result.

Sample 7053: Performance was very good, with 14/16 (87.5%) of participants reporting the correct isolation result, 13/13 (100%) for identification, 12/13 (92.3%) reporting the correct Sg and 5/7 (71.4%) of the laboratories reporting the correct ST. The overall performance for examinations by culture was 87.8%.

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

The turnaround time of reporting results for all the exercises was analysed and is shown in Figure 1. Most results were reported between 20–34 days.

Figure 1. Turnaround times to reporting results via the online secure UK NEQAS system for clinical distributions (4895, 5113) and environmental distributions (4896, 5114)



Distribution 4895: November 2020 clinical, distribution 4896: November 2020 environmental, distribution 5113: March 2021 clinical and distribution 5114: March 2021 environmental

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/ specimens 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 and reliable. Participation also ensures high quality of the surveillance data reported.

Overall, the performance of laboratories participating in the 2020-2021 EQA was very good. There were no significant issues arising for species identification, serogroup, enumeration, or sequence type. There was, however, one urine specimen positive for *L. pneumophila* urinary antigen, for which only 72% of participants reported the correct result. This was unexpected, as the concordance with other positive urinary antigen specimens was excellent.

November's distribution represented an outbreak associated with a fountain. The outbreak strain of *Legionella pneumophila* Sg 1, ST109 used was isolated from patients and a domestic water system. It has been isolated from the environment in several countries across Europe, Canada and the United States and the first strain on the *Legionella* sequence-based typing database listed.

March's distribution represented an outbreak associated with a hospital water system. The outbreak strain of *Legionella pneumophila* Sg 1, ST62 was isolated from clinical specimens and environmental samples. It has been associated with community-acquired, nosocomial and travel-related cases. The majority of *L. pneumophila* ST62 strains identified to date belong to Sg 1.

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

From the results data collated in this EQA, it was ascertained that the majority of laboratories for clinical specimens identified the pathogen and Sg, but a significant number did not report the ST (Tables 6 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.

Clinical discussion

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

Based on published guidance by PHE in the UK, the three most commonly described specimen 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 2) was sent out simultaneously with distribution 5113, and this confirmed the most common specimen types examined routinely by participating laboratories to be sputum 92.9% (13/14), urine 85.7% (12/14) and BAL 100% 14/14.

From the methods questionnaire, it was determined that a total of 9/15 participants had indicated they were clinical diagnostics laboratories, as well as reference laboratories. Five participants were clinical diagnostics laboratories only, with three stating they had access to a reference laboratory, while two did not.

A total of 5/15 laboratories participated in a national EQA scheme. However, this was only a mandatory exercise for one of them. This is a very low number, especially as nine laboratories are noted to be reference laboratories and EQA is a requirement for accreditation. The most commonly tested specimen types reported by the 15 laboratories for routine testing were sputa, broncho-alveolar lavage, tracheal fluid and urine. BCYE with cysteine (n=10) and BCYE without cysteine (n=8) were the most frequently used media for the isolation of *Legionella* spp. (See results in Annex 2).

Distribution 4895: Three paired (sputum/urine) simulated specimens (6364,6365; 6366, 6377; 6388, 6399) with relevant accompanying clinical details were sent for evaluation. Distribution 5113: Two paired (sputum/urine) simulated specimens (7034,7035; 7038, 7039). These paired specimens were designed to mimic an outbreak.

Identification:
- Overall concordance was excellent (100%), across both distributions for identification of L. pneumophila.
- Concordance with intended results was reduced for negative samples, those not containing Legionella spp., and those containing non-Legionella pneumophila.

Serogroup:
- Overall concordance was excellent (100%), for both distributions for reporting the Sg.
- The large majority of laboratories performed serogrouping, with only one participant not reporting a result in distribution 5113.

Sequence type:
- A reduced number of laboratories reported against ST for both distributions. Distribution 4895: A total of 15/23 laboratories reported ST for patient 1, followed by 14/23 laboratories for the remainder of the specimens. For distribution 5113 a total of 10/20 laboratories reported a result.
- From the participants reporting a result, concordance was lower for a non-L. pneumophila Sg1 included in distribution 4895.
- One laboratory listed several possible STs for two out of three outbreak associated specimens.

Urinary antigen testing:
- Specimen 7037 obtained a low concordance with only 72% of laboratories reporting the correct positive intended result.
- Similar findings with specimen 6369, where two laboratories incorrectly reported UAT not detected.

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 (RT 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 the majority of participants (21/23) reported culturing the simulated sputum specimens, the remaining two performed PCR direct from the specimen. Confirmatory tests included: conventional single target PCR (n=1), RT PCR single target (n=1), RT PCR multiplex (n=1), MALDI ToF (n=1), serology (n=4) and mip sequencing (n=1).

Environmental discussion

The environmental aspect of these EQA's 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 with accompanying Sg and ST for water samples.

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. 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.

A methods survey questionnaire (Annex 2) was sent out in April 2021 to the 16 laboratories that took part in distribution 5114; only 10 responses were received.

The overall performance of the laboratories in the EU/EEA countries was very high. The 10 laboratories providing method data, nine stated they examined the water samples for *Legionella* bacteria using ISO 11731:2017 (Water

quality - Enumeration of *Legionella*). Most of the laboratories that returned information about their method responded that they filtered the water sample and would carry out culturing on untreated, acid and heat-treated samples.

13/21 laboratories in total took part in both distributions. These laboratories will be able to assess their performance over time, which EQA is a valuable tool to provide this.

For distribution 4896 the maximum results reported was 17 and for distribution 5114 this was 16. Not all laboratories examined all the samples or undertook all the examinations when the sample contained a *L. pneumophila.*

Isolation:

- The overall performance for isolation of *Legionella* was very good with performance of 98.7% for distribution 4896 and 94.8% for distribution 5114. Only one laboratory reported an incorrect isolation three times, once in distribution 4896 and twice in distribution 5114.
- The performance for sample 6381 in distribution 4896 was (65%). The reason for this low performance is due to the low level of *L. pneumophila* in the sample which was approximately 45 colony forming units per litre which is below the lower detection limit for methods used by laboratories. Therefore, the reporting of a not detected result was considered correct.
- Two samples (6382 and 7047) from the two distributions contained a non-*pneumophila* species of *Legionella*. 6382 contained *L. jordanis* and 7047 contained *L. anisa*. Isolation performance was very good, 93.3% and 100% respectively.
- Over the two distributions the overall performance for six water samples in distribution 4896 was 98.9 and 95.1% for distribution 5114 containing eight water samples.
- Over the two distributions the overall performance for four swab samples in distribution 4896 was 98.5% and 93.8% % for distribution 5114 containing two swab samples.
- Over the two distributions the overall performance for 14 samples containing a *Legionella* spp. was 96.9%.
- Over the two distributions the overall performance for six samples not containing a *Legionella* spp. was 96.6%.
- Over the two distributions the overall performance for the 10 outbreak investigation samples was 96.9%.
- Over the two distributions the overall performance for the 10 routine monitoring samples was 96.7%.
- 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.

Identification:

- Over the two distributions the performance for the correct identification of *Legionella* spp. for 14 samples containing this organism was 98.6%.
- 12 samples contained a *L. pneumophila* and performance was 99.5%. Two samples contained non-*pneumophila* strains, the performance for correct identification for these strains was 93.3%.

Enumeration:

- The number of data sets returned over the two distributions varied between 11-16 for the samples. Therefore, this is below the number required to produce robust performance data.
- When statistical calculation is based on 10-19 results, they should be interpreted with caution as they may be overly influenced by outlying results. When there are fewer than 10 reported results, the statistics are not considered robust enough.
- Enumeration results can only be provided for positive water samples that contain a *Legionella* spp. This equated to a total of nine samples over the two distributions, performance was consistent by sample which ranged from 100% - 81.8% of results reported in the expected range. The performance by distribution was 85.6 for distribution 4896 and 91.4 for distribution 5114, which is very good.
- Further analysis showed that one laboratory reported a low outlying count once in distribution 4896 and once in distribution 5114. Another laboratory reported a high outlying count three times over the two distribution, once in distribution 4896 and twice in distribution 5114.
- The number of outlying results by samples over the two distribution was not influenced by the level of the *Legionella* spp. in the sample.

Serogroup:

- Over the two distributions, 10 samples contained a *L. pneumophila* serogroup 1, performance excellent with 100%. Two samples contained non-serogroup 1 and performance was 91.6%.

Sequence type:

- The overall performance over the two distributions for 12 samples in reporting a correct ST was 96.4%.
- The overall performance over the two distributions for six samples as part of the outbreak investigation was 100%.
- The overall performance over the two distributions for six samples as part of the routine monitoring was 92.3%.
- The overall ST performance was 100% for distribution 4896, where three samples contained ST109, one contained ST47, one contained ST47 and one contained ST68.
- The overall ST performance was 85.7% for distribution 5114, where three samples contained ST62, two contained ST1 and one contained ST2923. The three laboratories reporting an incorrect sequence type were all different.
- Method used for ST analysis was Sanger sequencing and/or WGS.

Molecular methods:

- The number of laboratories examining the samples by molecular methods is low. For distribution 4896, between 3-7 laboratories examining the samples used a kit that detects *L. pneumophila* and between 3-6 for *Legionella* spp. For distribution 5114, between 3-6 laboratories used a kit that detects *L. pneumophila* and between 3-6 for *Legionella* spp. Therefore, performance as a percentage for results reported using molecular methods has not been calculated.
- The overall performance with distribution 5114 was much higher compared to distribution 4896. For distribution 4896, an incorrect result was reported for three of the samples compared to none in distribution 5114 for *L. pneumophila* kits used. For distribution 4896, an incorrect result was reported for seven of the samples compared to two in distribution 5114 for *Legionella* spp. kits used.
- For distribution 4896, one laboratory reported an incorrect result for five of the samples for *Legionella* spp. for samples that contained a *L. pneumophila*. This laboratory did not participate in distribution 5114 to determine if improvements were made through these EQA exercises. For distribution 5114, no laboratory reported an incorrect result twice.
- An analysis of the kits from the method questionnaire did not indicate that one specific molecular test was commonly being used.

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, so 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 was allocated to allow sufficient time for the panel to arrive at the laboratories via air freight to the various countries. 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). The mean value for returning results was determined to be 28 days for both clinical specimens and environmental samples. The mean values illustrate a significant improvement from the first EQA in 2019, in which the mode was 35 days for clinical and 40 days for environmental. Nevertheless, the turnaround times to report results indicate 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). One limitation to the system used to report the EQA results is there is no facility to capture the reporting of preliminary results, as some laboratories do.

The clinical specimens 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 the detection of urinary antigens were authentic clinical liquid urine, spiked with species antigen, and provided in plastic tubes to resemble a true specimen.

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, but is not representative of how a swab sample would be received in a laboratory for analysis.

For environmental samples, the enumeration results could not be evaluated due to the low number of data sets 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; and
- 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 to acknowledge compliance with defined objectives and quality standards such as those detailed in ISO 17025:2017 or ISO 15189:2012.

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

Participant feedback on this EQA to ECDC

A short feedback evaluation survey was sent directly by ECDC to all participating laboratories of the November 2020 and/or March 2021 distributions, with the online survey open between 28 June and 31 July 2021.

A total of six 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 the March 2021 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 find the outbreak scenario with different sample types (either clinical or environmental schemes) of additional benefit to your laboratory than a method-based EQA scheme?

Question 6. Do you consider that this EQA survey provides insight into your laboratory capacity for processing samples under a suspected Legionella outbreak investigation?

The following results were summarised and provided by ECDC experts.

According to ECDC, feedback was provided by 16 of the 24 EU/EEA laboratories participating in the November 2020 and/or March 2021 distributions, 12 of which participated in both and four only in November 2020. This represents a response rate of 66% (16/24).

Among the respondents, 10/16 had participated in both clinical and environmental distributions, 4/16 in the clinical distribution, and two only in the environmental distribution.

Four of the EU/EEA respondents indicated that corrective actions were taken based on the EQA distribution results (10 did not need to due to conforming results and two were unable to process samples due to shipment delay at borders). The types of corrective action included concentrating negative samples from bigger volumes to detect low numbers of *Legionella* and changing the UAT test type used.

In all, 7/12 laboratories participating in the March 2021 distribution indicated they would use the results of the EQA exercise as evidence for accreditation and/or licencing purposes for the methods used. Among the five not using it, two indicated that this was because they were unable to consequent to custom delays on samples and one laboratory intended to start using it for accreditation in next rounds.

All 16 laboratories reported that they considered the exercise important for their laboratory to assure its diagnostic capability and only one laboratory was not completely satisfied with the report format for their results as they would have liked allele profile as well as sequence type to be indicated. The outbreak scenario was considered of additional benefit than a method-based approach EQA by 14 laboratories, with two reporting no opinion on this question. Eight laboratories also considered that the EQA survey provided insight into the laboratory capacity for processing samples under an outbreak scenario and in particular:

- Reviewing process and practical coordination with environmental or other laboratories under an outbreak scenario; and
- Quality control and testing of WGS or other molecular-based methods.

Although the comments and feedback represent only half of the laboratories that participated in this distribution, overall the feedback of this second EQA year continue to indicate that this EQA design is considered to be of value by the ELDSNet network in support to surveillance and outbreak investigations.

5. Conclusions

The performance of laboratories from a total of 24 EU/EEA countries participating in this year's exercises was very good for culture-based/detection methods used by both the clinical (96.9%) and environmental laboratories (96.8%).

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* and other *Legionella* spp. More EQA data are required to determine the actual on-going performance of the laboratories with the varying design of EQA sample/specimen sent. 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, 23/23 laboratories examined the sputum samples in the November distribution and 20/20 laboratories in the March distribution. A total of 22/23 laboratories examined the urine specimens in the November distribution, compared with 18/20 laboratories in the March distribution. This indicates that fewer participating laboratories processed urine samples than sputum. For environmental samples, 17 of the laboratories examined both water and swab samples in November's distribution and 16 laboratories examined both sample types in March's distribution. 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, in the November distribution 4895, all 23 reported an identification, 23 for Sg and between 15-15 for ST. The large majority of laboratories reported isolation and identification using culture-based methods, with two performing PCR direct from the specimen. 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.

For the environmental laboratories, in November's distribution 4896, 17 laboratories reported a result for isolation and identification, up to 17 reported a Sg, up to 16 reported an enumeration count and up to 10 a ST. For molecular methods, up to seven laboratories analysed the samples for *L. pneumophila* and up to six for *Legionella* spp. The overall isolation performance for culture was 98.7%, with 17 reporting a result. For March's distribution 5114, 16 laboratories reported a result for isolation and identification, up to 15 reported a Sg, up to 15 reported an enumeration count and up to eight a ST. For molecular methods, up to six laboratories analysed the samples for *L. pneumophila* and for *Legionella* spp.. The overall isolation performance for culture was 94.8%, with 16 reporting a result. Only one laboratory reported an incorrect isolation three times, once in distribution 4896 and twice in distribution 5114.

The overall performance for molecular methods was not calculated due to low numbers of laboratories analysing the sample by this method. However, for distribution 4896 one laboratory reported an incorrect result for five of the samples for *Legionella* spp. for samples that contained a *L. pneumophila*. This laboratory did not participate in distribution 5114 to determine if improvements were made through these EQA exercises. For distribution, 5114 no laboratory reported an incorrect result twice.

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 second year of the ECDC EQA *Legionella* scheme was very positive.

6. Recommendations

These exercises will continue to provide a baseline understanding of the level of testing undertaken in the laboratories, determine performance issues and, where possible, provide support to laboratories/countries that 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 these EQA exercises and include different *L. pneumophila* Sg, 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 continue through further EQA exercises if there are issues when less commonly encountered species, Sg or ST are included.
- For environmental samples to include levels of *Legionella* spp. that are at the lower end of the detection limit for culture.
- 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 continue to gather 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 carry out routinely but did not report a result for or instances where the organisers did not allow for these examination results to be reported.

Future objectives

- To continue improving the awareness of the different *Legionella* spp. that may be isolated from clinical specimens and environmental samples.
- To continue raising awareness of the confirmatory tests done and their limitations when confirming *Legionella* spp. isolates in samples.
- To continue raising 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 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 continue exploring participants' feedback from the evaluation survey to develop the exercises (e.g. improving information in the individual EQA reports).
- To continue communicating the results of these EQAs at ELDSNET and *Legionella* conferences to increase the awareness of the importance of EQAs for the quality of *Legionella* detection in laboratories.

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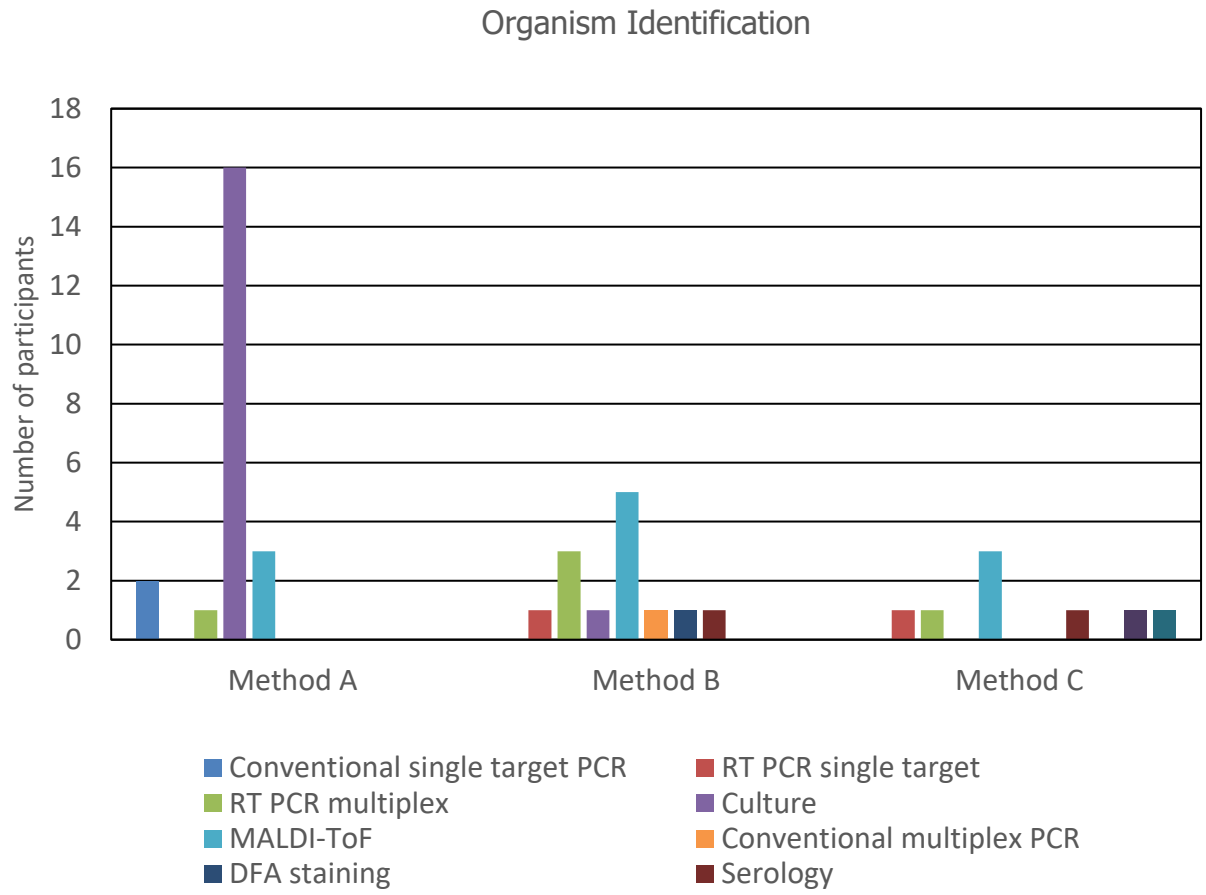
Annex 1. Findings from method capture

As part of the EQA exercise, a questionnaire was sent out on the methods used to analyse the samples/specimens. This information was gathered when the laboratory returned their results online. The data presented below are for all EU/EEA countries.

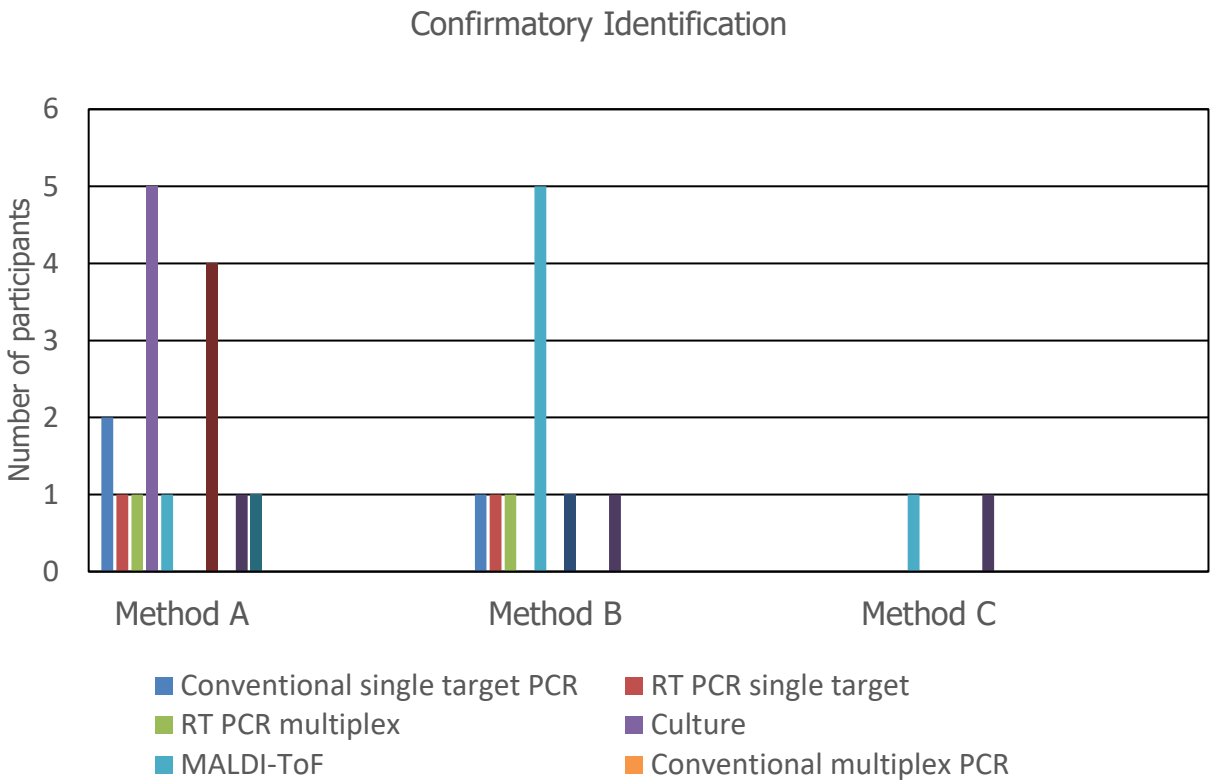
Methods capture for clinical distribution 4895

1. Which methods were used to identify the organism?



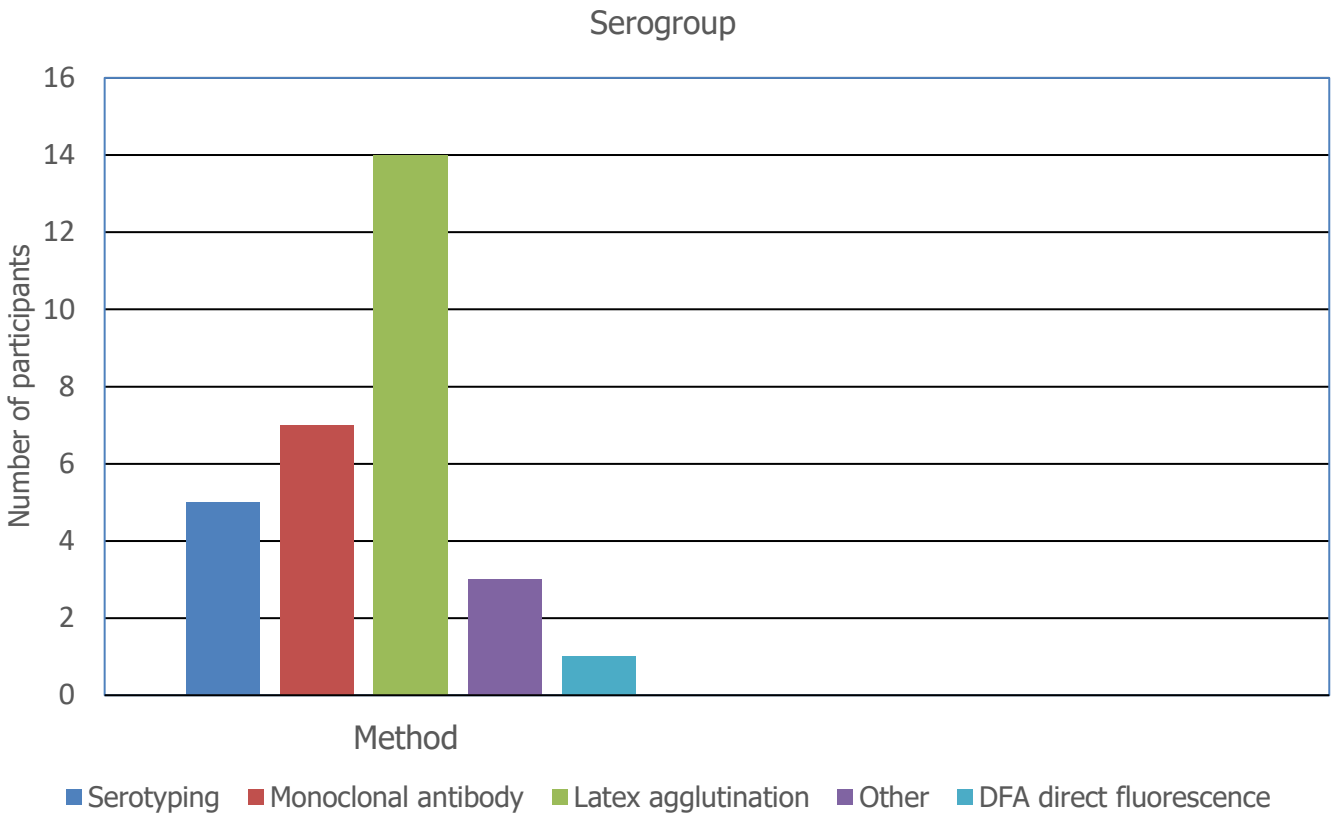
Method A, B and C relate to the order in which participants stated they performed testing.

2. Which methods were used to confirm the identification?

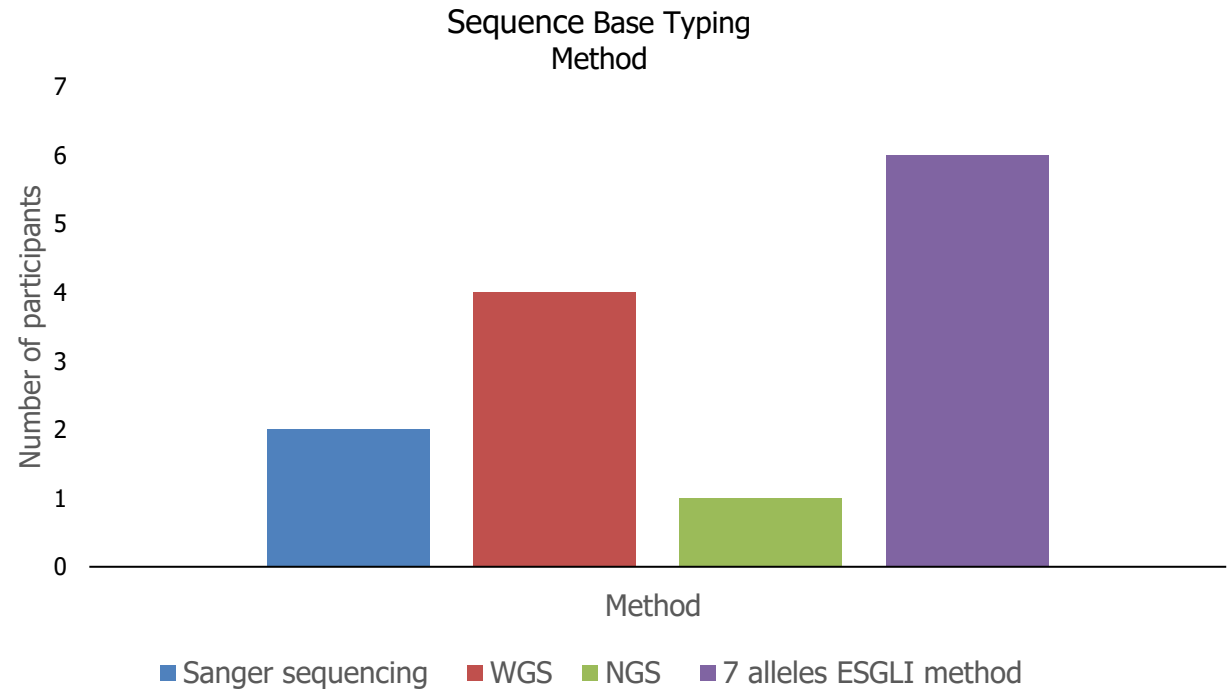


Method A, B and C relate to the order in which participants stated they performed testing.

3. Which methods were used to perform serogrouping?

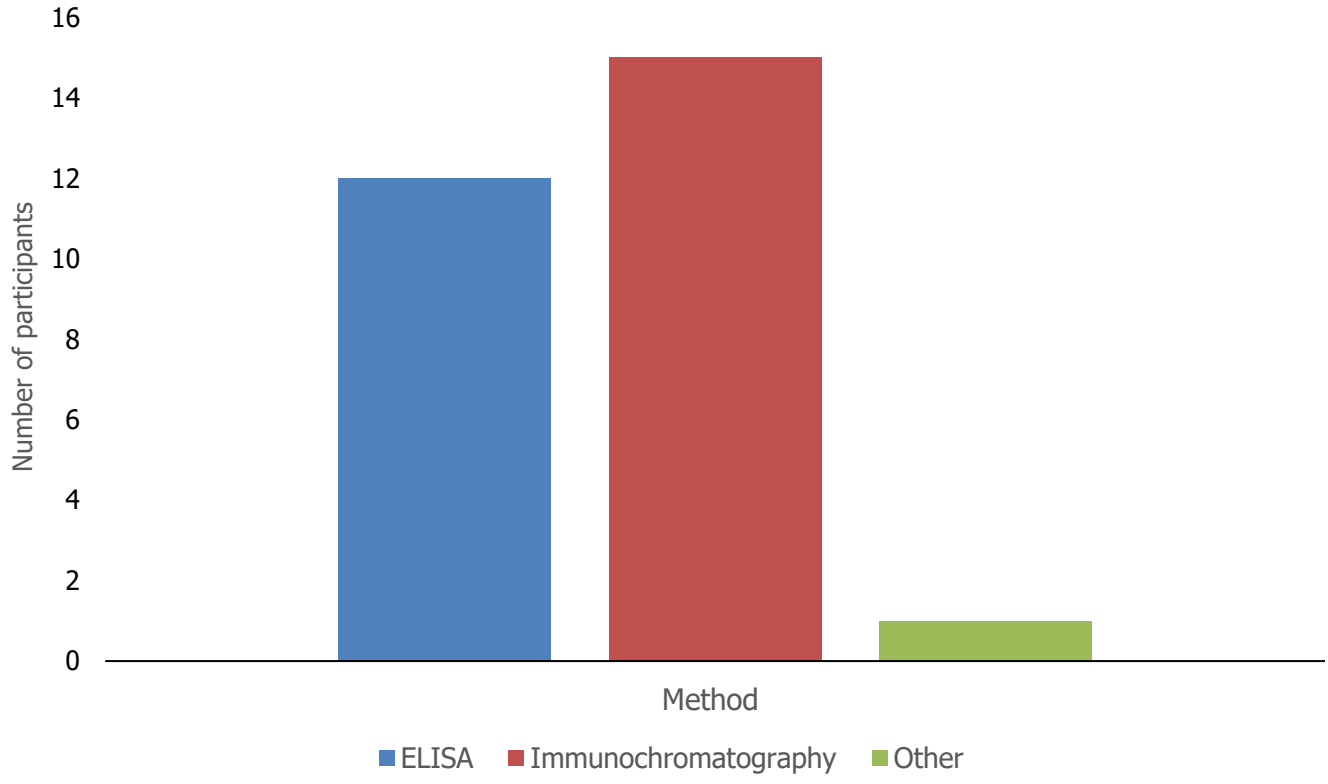


4. Which methods were used to confirm the sequence base typing?



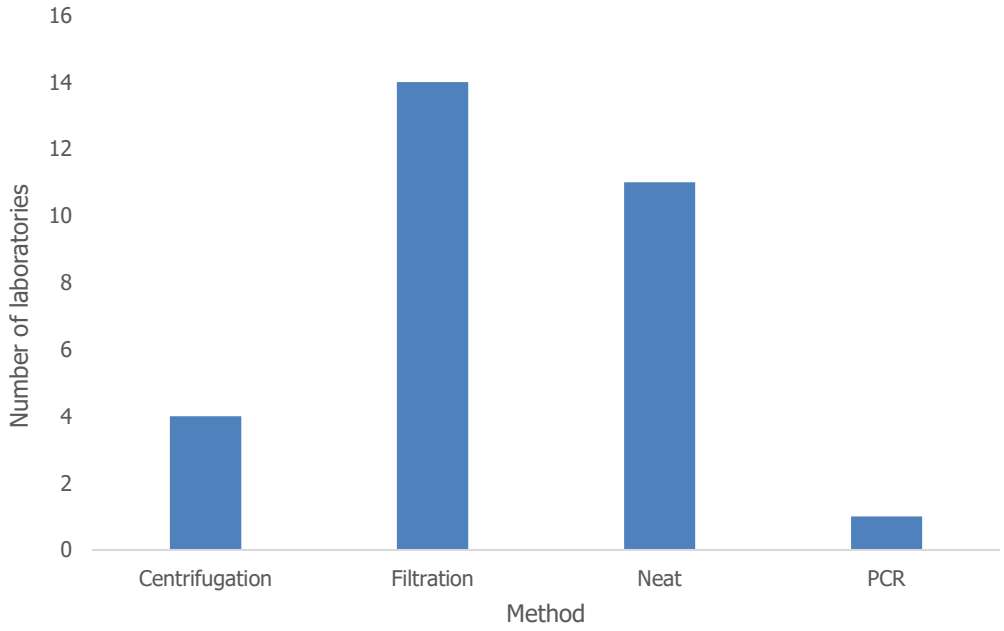
NGS- Next generation sequencing, WGS- Whole genome sequencing

5. Which methods were used to test for urinary antigens?

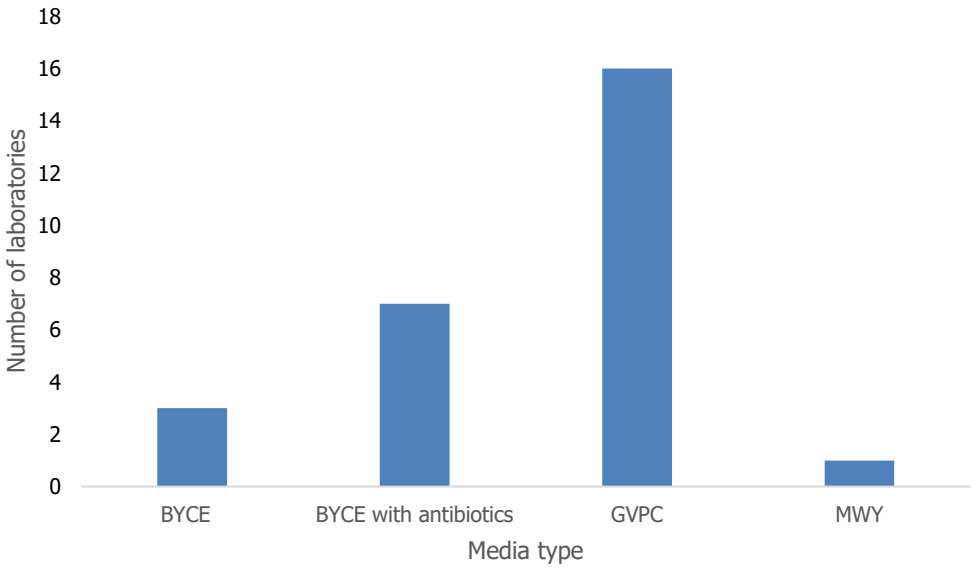


Methods capture for environmental distribution 4896

1. Which methods were used to process the samples?

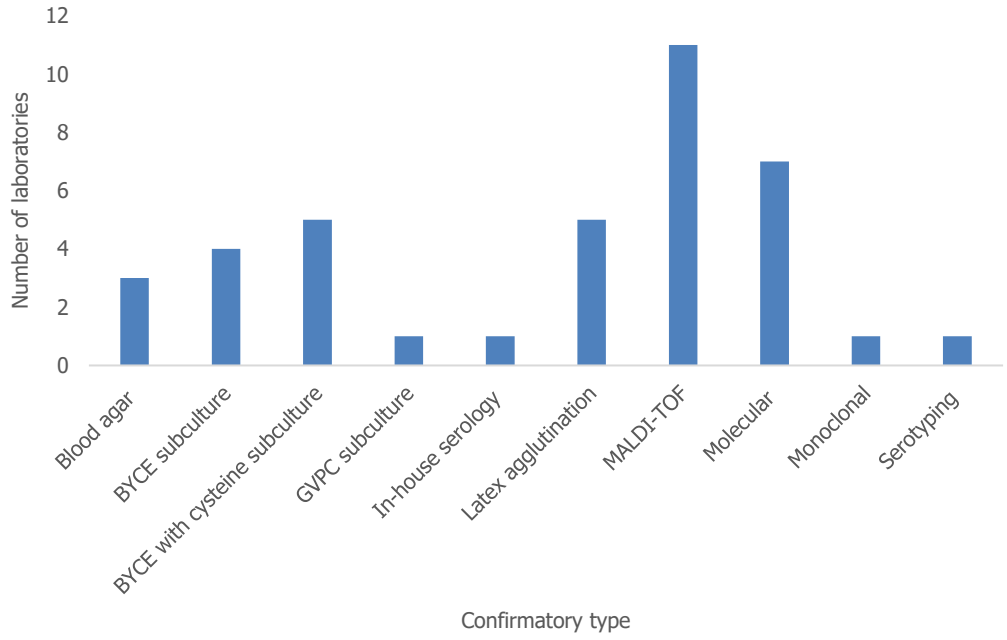


2. Which media were used to process the samples?

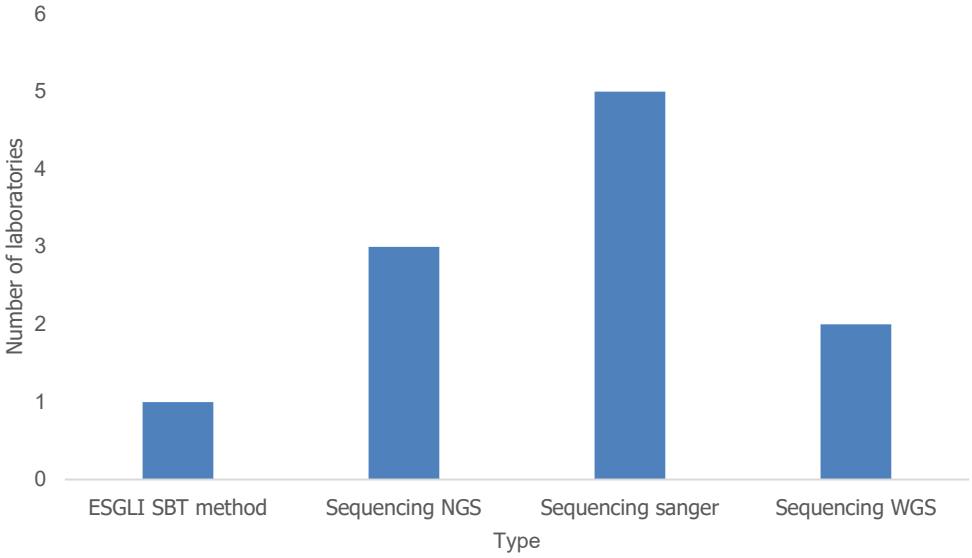


BCYE: Buffered charcoal yeast extract agar without L-cysteine; BCYE with antibiotics: Buffered charcoal yeast extract agar with selective supplements; GVPC: Glycine vancomycin polymyxin B cycloheximide; MWY: Modified Wadowsky Yee.

3. What confirmatory tests were done on the presumptive *Legionella* spp. colonies?



4. What sequencing method was used?



Annex 2. Findings from method questionnaire

A. Methods survey findings for clinical specimens

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

A questionnaire sent out and was completed by 14 of the participating laboratories in the 23 EU/EEA countries. Laboratories completing the questionnaire included; Belgium, Bulgaria, Cyprus, Estonia, Finland, France, Greece, Ireland, Lithuania, Norway, Portugal, Slovenia, Spain, and Sweden.

The method data shown are 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.

General information on questions asked

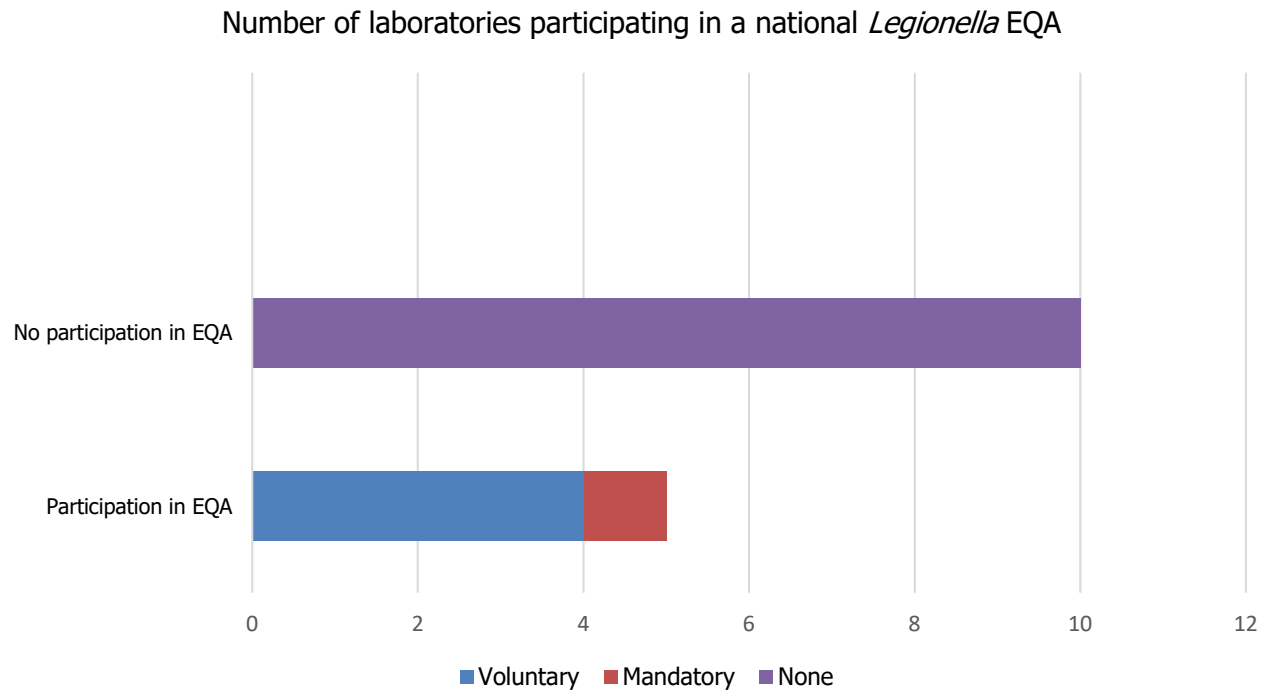
1. Are you a clinical diagnostic laboratory?
2. Are you a National Reference Laboratory?

A total of 14/15 reported they were clinical diagnostic laboratories. Of these 14 laboratories, nine were also reference laboratories. One laboratory was a reference laboratory only. From the remaining five laboratories who were not reference laboratories, three stated they did have access to a reference lab, while two did not.

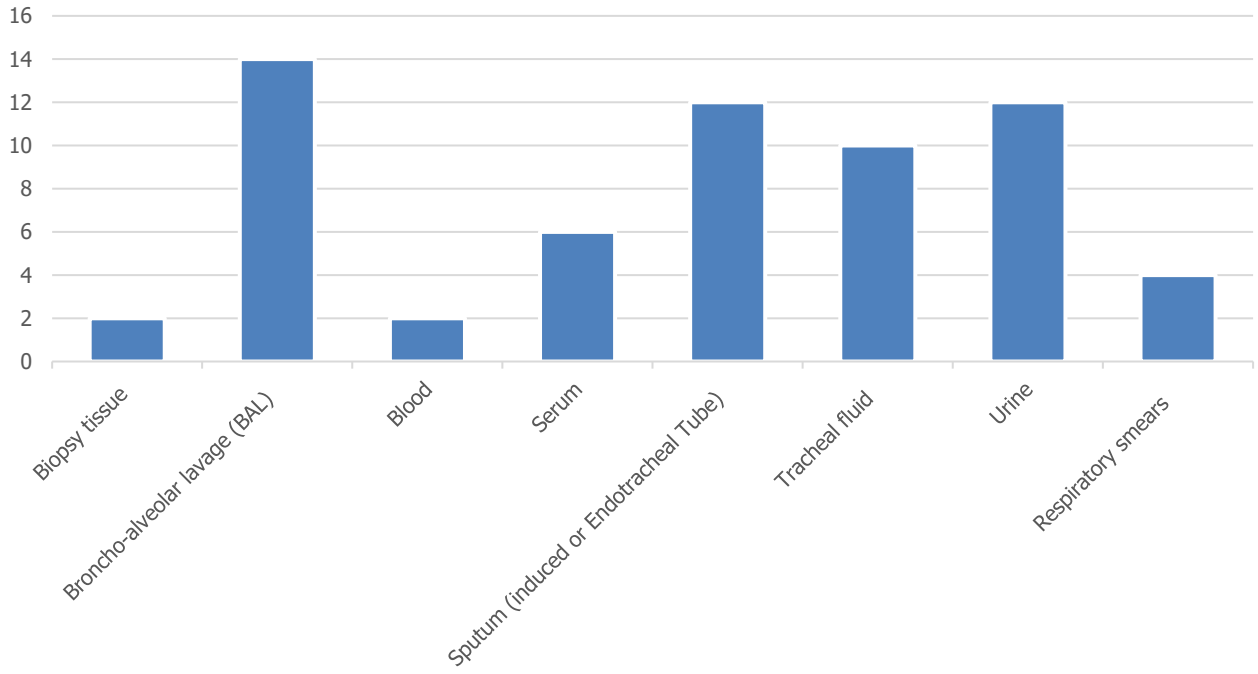
3. Approximately what percentage of all Legionnaires' disease cases in your country in 2020 involved a surveillance notification from your lab?

The percentage of Legionnaires disease in 2020 which involved surveillance notification by those replying to the methods survey ranged from none to 100%, with a large variation in reporting.

4. Does your laboratory participate in a National EQA scheme for the detection of *Legionella* species?
4a. Is this a voluntary participation?
4b. Or a mandatory requirement?

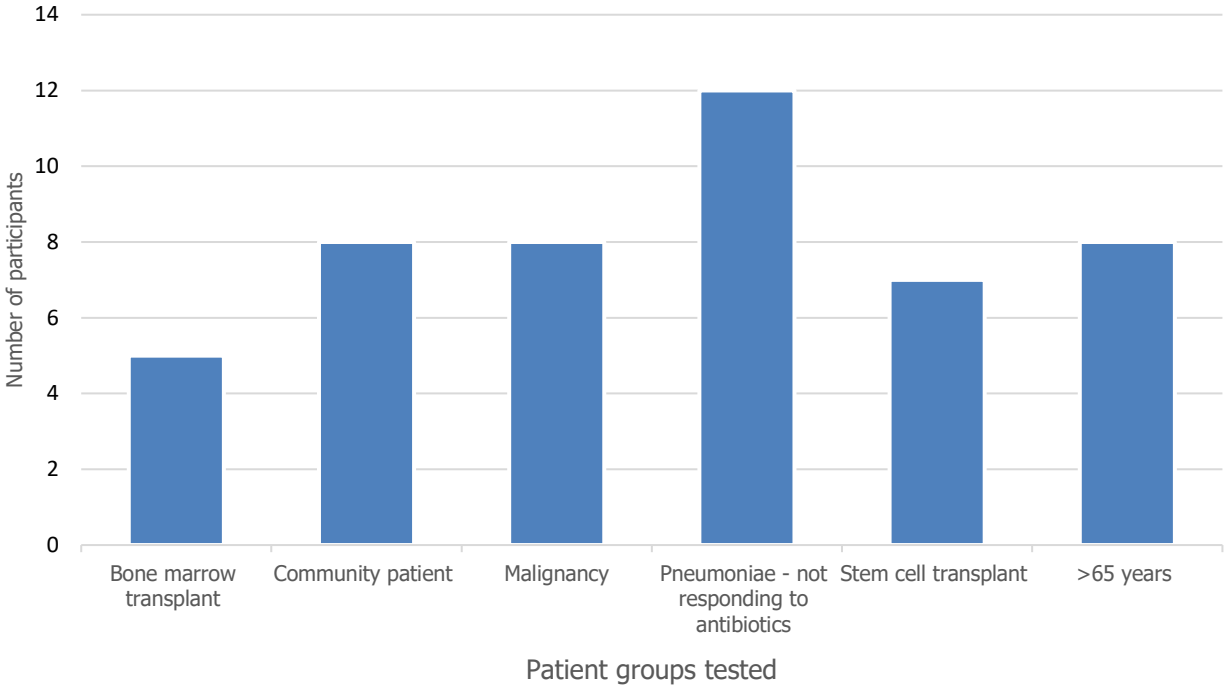


5. Which type of clinical specimen does your laboratory receive for Legionella infection investigations?



Three laboratories noted additional specimen types including: bronchial aspirates, pericardic and pleural fluids, post-mortem specimens, sputum – non-induced and synovial fluid.

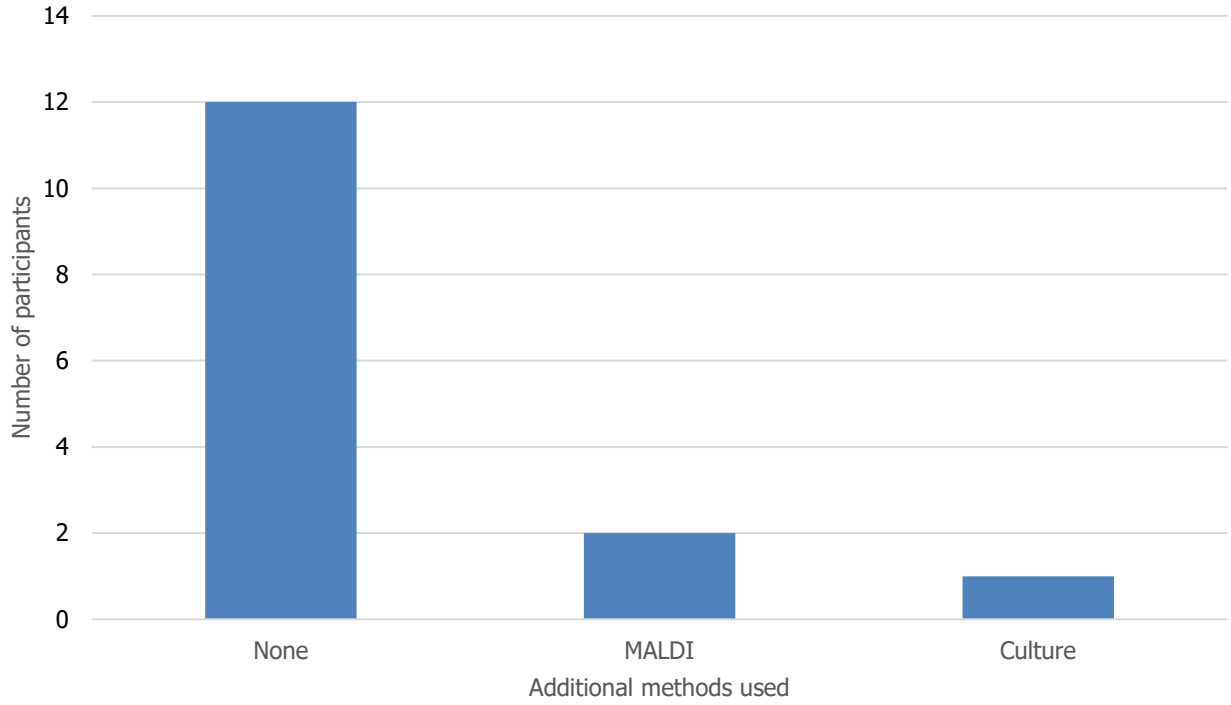
6. Which of the following patient groups do you receive samples routinely for *Legionella pneumophila* testing in your laboratory?



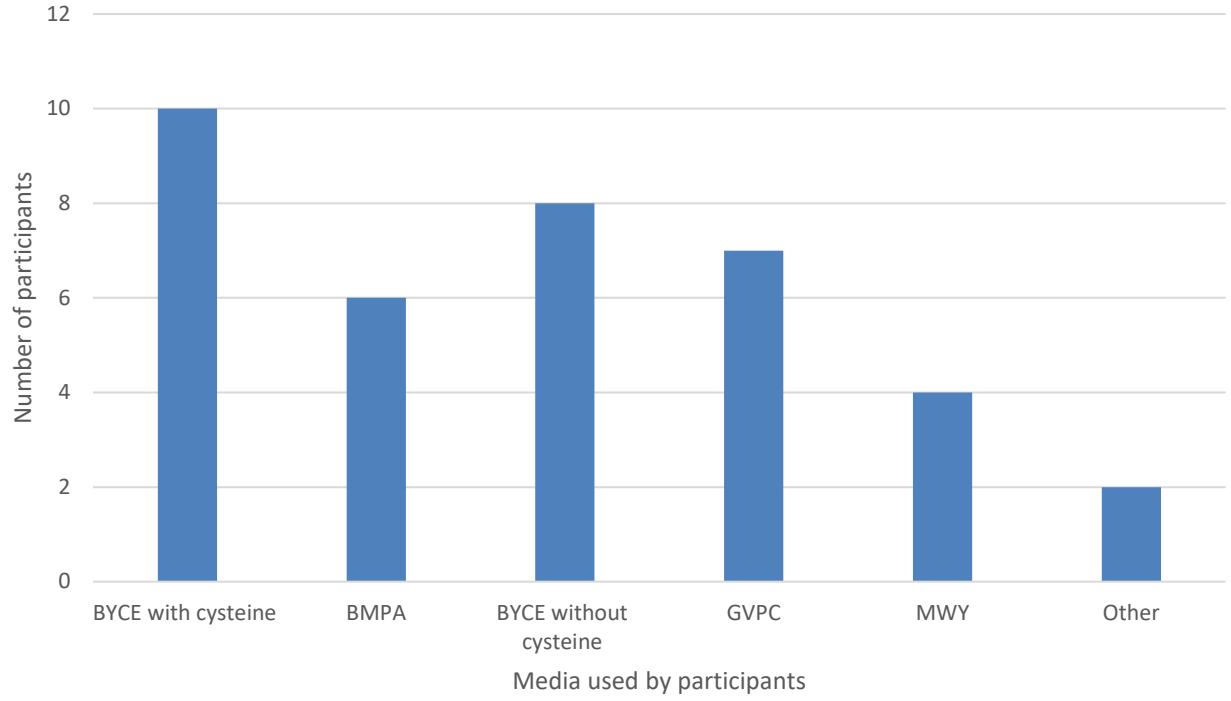
7. Did you outsource any tests used in this distribution to another laboratory?

The large majority (n= 11) did not outsource any tests to other laboratories. Of the three that did, tests outsourced included SBT, WGS and serotyping. One laboratory noted that there is a legal requirement to send all isolates to the same national reference lab.

8. What, if any, other methods/elements other than your routine protocol did you apply to this EQA?



9. What media do you use in your laboratory?



Other: Legionella agar Base and vials of legionella BCYE Supplement without L-Cysteine and 5 % Blood agar.
The media used was reported to be in-house n= 6 and commercial n= 8.
BCYE: Buffered charcoal yeast extract agar; GVPC: Glycine vancomycin polymyxin B cycloheximide; MWY: Modified Wadowsky Yee; BMPA: Legionella BMP selective media with activated charcoal, yeast extract and ACES buffer.
All laboratories provided a response.

10. How long do you incubate your culture plates for?

Incubation ranged from 3-10 days (n=1), 7-9 days (n=4), 11-14 days (n= 1), 10 days (n=7) and >14 days (n=1).

11. Do you use a moist chamber when culturing samples for Legionella?

During incubation, the large majority (n=11) laboratories stated they incubated in a moist chamber, one participant did not provide a response and two stated they didn't use a moist chamber.

12. If your laboratory performs urinary antigen testing, please state the kit and manufacturer used.

Urinary antigen testing kit	Number of laboratories
BinaxNOW Abbott	5
nal von minden	1
ImmuViewÂ® S. pneumoniae and L. pneumophila Urinary Antigen	1
Diagnostic automation	1
Legionella pneumophila rapid test	1
Sofia Legionella FIA, quidel corporation	1
Microgen Legionella Latex agglutination kit	1

13. If you use multiplex PCR, which other respiratory pathogens does the assay include?

Additional respiratory targets in multiplex PCR	Number of participants
Legionella species	2
Chlamydophila pneumoniae, Chlamydia pneumoniae	3
Legionella pneumophila	3
Bordetella pertussis/parapertussis	3
Streptococcus pneumoniae	2
Haemophilus influenzae	2
L. pneumophila serogroup 1	2
Chlamydophila psittaci	1
Mycoplasma pneumoniae	3

14. If you use PCR methods, please state the kit and manufacturer used.

PCR methods kit	Number of participants
ARGENE Legio pneumo/Cc R-GENEÂ® (BioMerieux)	1
'in-house' method adapted from the literature	4
Allplex RP4, Seegene	2
L.spp / L. pneumophila : R-DiaLeg kit (Diagenode)	1

15. Which Legionella pneumophila serogroups do you have serology tests for?

Serogroups	Number of participants
1-6	2
1-14	8
1-15	1

16. Approximately how many *Legionella* spp clinical tests for culture isolation and identification, serology and PCR are undertaken in your laboratory each year?

Number of tests performed	None	<10	10-99	100-499	500-1 000
Culture	1	2	6	2	2
PCR tests	2	1	2	6	1
7 alleles ESGLI method	6	2	2	1	0
Direct Fluorescent Antibody (DFA) staining	9	2	0	0	0
Mip sequencing	5	6	0	0	0
Serotyping	4	1	4	2	0
Monoclonal antibody	6	2	2	0	0
Latex agglutination	3	2	4	2	0
Sequencing (sanger & WGS)	5	2	2	2	0

Methods survey findings for environmental samples

A questionnaire was sent to all participants who participated in distribution 5114. The objective was to gather information on the method/processes used for this EQA exercise. The laboratories were only asked to provide information on the methods they use in their laboratory.

In all, 10/26 (62.5%) of the participating laboratories provided information on their methods/processes. Responses were received from Belgium, Denmark, Estonia, France, Ireland, Latvia, Norway, Portugal, Sweden and Slovakia Republic.

The total numbers will not always correspond to 10 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.

General information on questions asked

1. Are you a National Reference Laboratory for environmental and water samples?

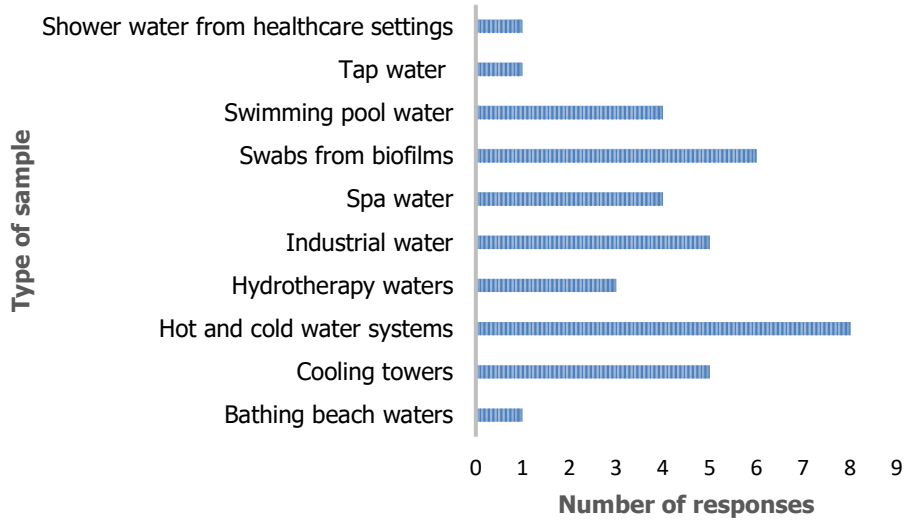
From 10 responses, seven stated they were a reference laboratory, the three that were not indicated that if required they do have access to one.

2. Does your laboratory participate in a National EQA scheme for the detection of Legionella species?

Of the 10 responses, six stated they did not take part in national EQA programmes. Of the four that did take part, two responded that this was on a voluntary basis and one stated it was mandatory.

3. Which type of specimen does your laboratory receive for *Legionella* investigations?

Nine responses were received, as shown in the graph below:



4. Approximately how many *Legionella* spp. tests for culture isolation and identification, serology and PCR are undertaken in your laboratory in the previous calendar year?

The table below provides the responses received.

	None	<10	10-99	100-499	500-1000	>1 000	Total number of responses
Culture isolation and identification	0	0	1	3	3	2	9
Serotyping	2	0	2	3	0	1	8
Molecular	1	0	5	0	1	0	7
Sequencing (sanger & WGS)	3	1	2	1	0	0	7

5. Did you outsource any tests used in this distribution 5114 to another laboratory?

Of the 10 responses, nine stated they did not outsource any of the tests in this EQA exercise, one laboratory outsourced the test for sequenced based typing of *L. pneumophila* and WGS of *L. pneumophila.*

6. Did you have any difference in protocol for processing routine samples or outbreak samples in this EQA distribution?

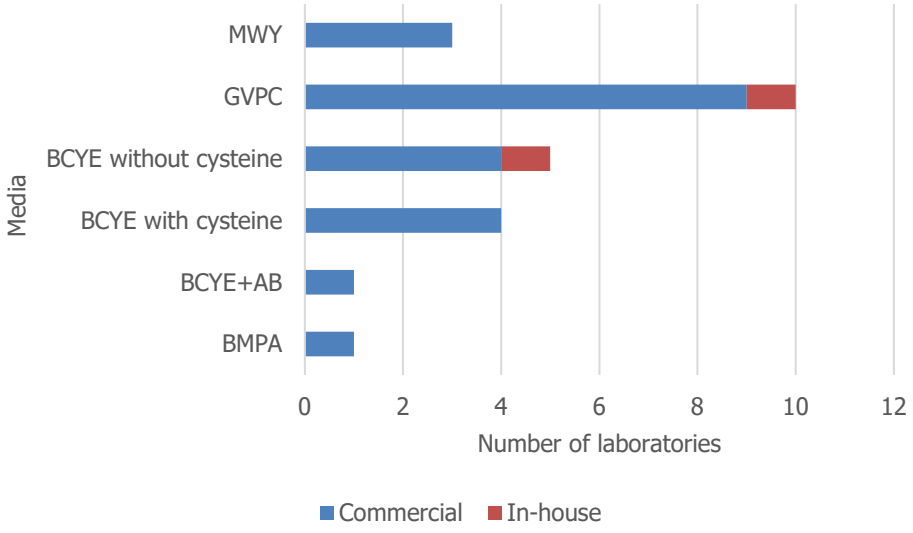
Of the 10 responses received, none stated they did not process the routine and outbreak samples differently. One laboratory stated the SBT and WGS was sent to another laboratory.

7. Which published method does your laboratory follow for analysing water/swab samples?

Of the 10 responses received nine used ISO 11731: 2017 for both water and swab samples, one laboratory used NF T90-431 v.2017 for water and an in-house method for swab samples.

8. What media do you use in your laboratory?

Of the 10 responses seen the below graph shows the media used. Most laboratories used more than one media.



BMPA: Legionella BMPA Selective Agar; 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.

9. How long do you incubate your culture plates for?

Of the 10 responses received, one laboratory incubated agar plates for 3-5 days, three for 7-10 days and six for 10 days.

10. Do you use a moist chamber when culturing samples for *Legionella*?

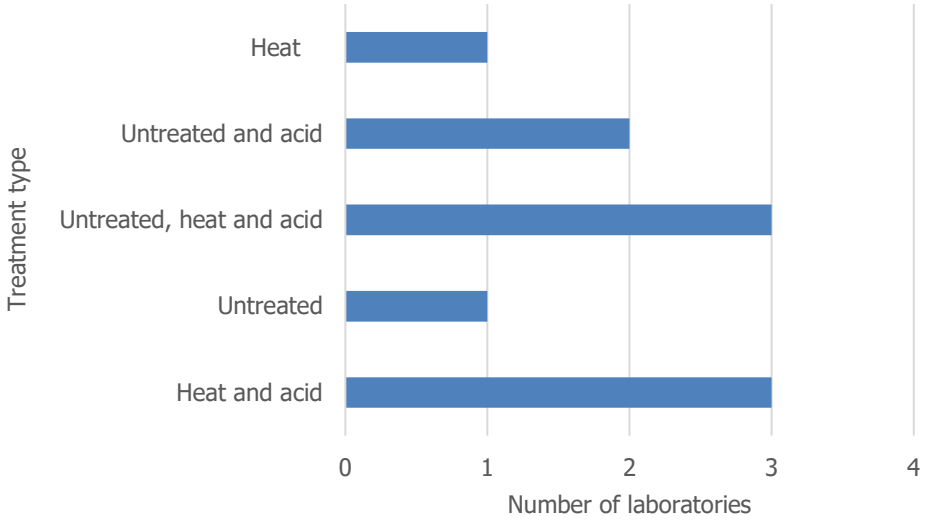
Of the 10 responses received, three laboratories did not use a moist chamber and seven did.

11. What volume was used to examine the water samples for filtration method?

Of the 10 responses received, one laboratory would filter between 10–100mL, two laboratories 100mL, one laboratory 500mL, and six laboratories would process 1000mL of sample.

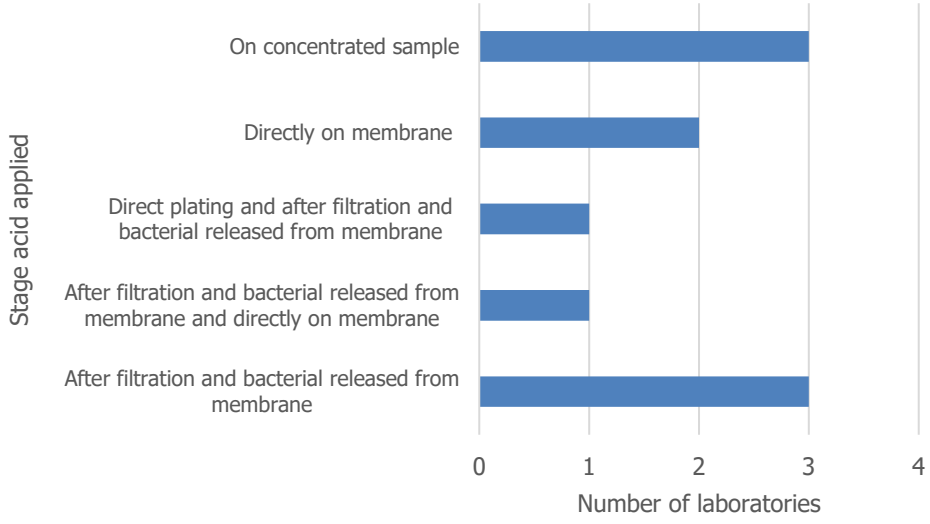
12. Do you perform direct/ heat/acid/ untreated testing of the sample?

The graph below shows the responses received from 10 laboratories.



13. At what stage of the processing is acid applied?

The graph below shows the different described categories.



14. Do you perform molecular work direct from the sample?

Of the 10 responses received, four laboratories do not undertake any molecular examinations on samples. Of the six that did, only two undertook molecular testing directly from the sample.

The two laboratories undertaking molecular examination do include an internal control as part of their process.

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