ESGLI ESCMID STUDY GROUP FOR LEGIONELLA INFECTIONS

European Society of Clinical Microbiology and Infectious Diseases

Evaluation of 18 assays for the qualitative detection of *Legionella pneumophila* antigen in urine samples from patients with pneumonia

Background

Legionnaires' disease (LD) is a severe pneumonia that is transmitted through inhalation of aerosols of contaminated water. *Legionella* has been clearly identified as one of the most common causes of severe community-acquired pneumonia (CAP): LD accounts for 2%–8% of CAP cases (1). LD notification is mandatory in all 30 European Union and European Economic Area (EU/EEA) countries, which reported 5,500 to 6,500 LD cases annually between 2005 and 2010 (2). Since 2011, notified LD cases are increasing every year in Europe with a fatality rate of about 10%. Among 60 species of *Legionella*, the species *L. pneumophila* is responsible for more than 90% of cases of LD diagnosed worldwide. This species can be subdivided into 16 serogroups and most human disease are caused by *L. pneumophila* serogroup 1 (Lp1) (2).

It has been well established that the clinical syndrome of *Legionella* pneumonia is nonspecific and that accurate diagnosis requires laboratory testing that is specific for *Legionella* species. Two methods are of major interest for early diagnosis: the detection of urinary antigen and the use of molecular techniques. Today, urinary antigen tests account for 70% to 80% of cases that are diagnosed in Europe and United States corresponding to the first-line diagnostic tests for LD (2). The development and spread of rapid urinary antigen detection kits such as lateral flow immunochromatographic assays or fluorescent immunoassays has revolutionised the diagnosis of legionellosis and allows early adaptation of the antibiotic therapy. These methods can be implemented in a point-of-care format with low-complexity tests. In this context, many commercialised tests are regularly placed on the market and are proposed in all European labs. These tests are widely used and the positive ratio is about 1-2%.

The antigen detected is the bacteria lipopolysaccharide (LPS) (3), which diversity in structure and antigenicity is the basis for Lp serogroups identification. The commercialized tests detect mainly *L. pneumophila* serogroup 1 LPS. The good performances of these tests are crucial: the presence of false negative results has an impact on the treatment of the patient and the outcome of the disease; on the other hand, false positive results may lead to misidentification of cases and have a significant impact on epidemiological surveillance. False positive results with enzyme immunoassays on urine samples were firstly described in the early 80s. Since the *Legionella* antigen detected in urine is heat stable, heating of specimens was used to verify positive results. Boiling (100°C) liberates bacterial polysaccharides from antibody complexes and eliminates the nonspecific interferences in enzyme-linked immunosorbent assays (4). The evaluation of commercial urinary antigen tests (UATs) are regularly published, however these studies compare one test to another and no study comparing several tests on the same urine samples is available. Studies showed a specificity of nearly 99% for the better tests but showed also important differences in term of specificity among UAT. The major drawback is a sensitivity estimated at 80% to 90% for diagnosis of LD

caused by Lp1, and from 14% to 69% for other Lp strains (5-7). The sensitivity of tests seems also to be Lp1 strains dependent (8); LD caused by some Lp1 strains, called the Lp1 mAb 3/1-negative strains (non-Pontiac group), are significantly less frequently diagnosed by commercially available assays. The diversity of distribution of particular strains or clone in Europe may influence the performance of diagnosis. There is no internationally validated assay standard or agreed unit.

These data reinforce the need for proposing a large multicentric European study including a large diversity of urine samples and strains associated to infection. Due to a low availability of samples from patients with LD, the evaluation of UAT on different types of Lp strains remains difficult. Recently, a new method was evaluated to compare the limit of detection (LOD) of 3 commercial UATs by using extracted LPS of several *L. pneumophila* strains (8). The amount of LPS can be indirectly determined by the amount of 2-keto-3-deoxyoctonate (KDO) (9). Thanks to KDO standardization, this method based on extracted LPS can represent a standardized way for comparing limit of detection of numerous UAT.

Objectives

The main objective of this project is **to compare the performance of 18 UAT** for the qualitative detection of *Legionella pneumophila* antigen in urine samples, including 13 immunochromatographic tests, 2 fluorescent immunoassays and 2 EIA tests, **in 9 European National Reference Centers for *Legionella*.**

This comparative study will be done by using (1) urine samples from patients with pneumonia collected in France, Denmark, Germany, Switzerland, UK, The Netherlands, Italy, Belgium and Slovenia; (2) and a pool of negative urine samples surcharged by fixed amount of extracted LPS of *L. pneumophila*.

In particular the specific objectives are:

- **Objective#1**. To evaluate the sensitivity of the tests for *L. pneumophila* sg1 and *L. pneumophila* non-sg1 LD
- **Objective#2**. To evaluate the specificity of the tests and the rate of false positive results
- **Objective#3**. To evaluate the usefulness of sample boiling for the elimination of false positive results
- **Objective#4**. To evaluate the contribution of technology coupled with an automatic reader on the performance of tests
- **Objective#5**. To evaluate inter-laboratory agreement
- **Objective#6**. To evaluate the contribution of the method based on extracted LPS for comparing sensitivity of UATs
- **Objective#7**. To obtain limit of detection data and assay range detection to inform construction of future international standard and agreed unit of measurement
- **Objective#8**. To establish an ESGLI recommendation for *Legionella* urinary antigen testing

The purpose of this project is to realize for the first time a European multicentric study independent from urinary antigen test producers in order to establish an ESGLI recommendation for the diagnosis of LD using *Legionella* urinary antigen testing.

RESEARCH PLAN

1- Design

Evaluation of 18 urinary *Legionella* antigen tests by a European study in 9 National Reference Centers using

(1) prospective samples from patient with pneumonia and submitted for *Legionella* urinary antigen;

(2) repository urine samples collected from patients with known LD;
(3) urine samples charged with *L. pneumophila* LPS extract from several serogroups of *L. pneumophila* strains.

2- Material & methods

2.1- Urinary antigen tests

The tests available on the market were checked for the presence of the CE mark (Directive 98/79/EC) of the European Parliament on *in vitro* diagnostic medical devices. In total, 18 kits were selected:

Immunochromatographic tests

- BinaxNOW® *Legionella* Urinary Antigen Card (Abbott ARD)
- Biosynex L. pneumophila (Biosynex)
- CerTest (Biotec)
- Immunocatch™ Legionella (EIKEN CHEMICAL CO., LTD)
- Immuview *S. pneumoniae* and *L. pneumophila* Urinary Antigen Test (SSI Diagnostica)
- *Legionella K-Se*T (Coris Bioconcept)
- Legionella Monlab Test (Monlab)
- Legionella Rapid Test Cassette (JustChek Acro™ Biotec)
- Legionella Vitassay, (single or with *S. pneumococcus*)
- Nadal® Legionella Test 552022
- SD Bioline Legionella Urinary Ag test (Standard Diagnostics)
- Sofia *Legionella* FIA (Quidel / Eurobio Scientific)
- Standard F Legionella Ag FIA (SD Biosensor, Orgentec)
- TRU *Legionella* (Meridian Bioscience)
- Urisign Legionella color (Servibio)
- Simple/Stick Legio pneumo (Operon)

EIA tests

- Binax™ Legionella Urinary Antigen EIA (Alere)
- RIDASCREEN Legionella (R-Biopharm)

Terms of inclusion in the study: The kits needed for this study will be provided free of charge by the producers. The protocol is nonnegotiable and all data will be published independently from the outcome. All the kits provided should belong to the same batch (with the exception for the one lab who will test urines on different batches).

2.2- Participating Laboratories

Nine European National Reference Centers (NRC) for *Legionella* will participate to this study:

- French NRC will function as investigator center (writing of project in coordination with ESGLI members, interpretation and analysis of data, sensitivity using LPS from Lp isolates).
- Denmark NRC will function as ESGLI coordination center (interpretation and analysis of data, inter-laboratory agreement tests).
- Each center (9 NRC) will analyze a proficiency panel of 10 urines chosen sent from the ESGLI coordination center with all 18 tests.
- Each center (9 NRC) will analyze 55 fresh urine samples from their own routine
- Each center will analyze 5 UTI urine samples
- Each center will provide 5 urine samples selected from the most recent culture-proven LD cases to the coordination lab

2.3- Clinical specimens

Sensitivity panel: A total of 45 urine samples from *L. pneumophila* sg1 culture proven LD cases will be collected by the coordination center from the nine participating laboratories. For this purpose, each laboratory should send to the coordination center 5 urine samples of at least 5 mL selected among the most recent culture proven LD cases. Urines should be collected during the days between clinical suspicion of pneumonia and final confirmation of legionellosis by culture. These urine samples should also be positive when tested by own routine UAG method. These urine samples can be frozen samples or not.

Sensitivity using *L. pneumophila* LPS: A total of 25 Lipopolysaccharide (LPS) extracts from *Legionella pneumophila* strains belonging to different serogroups and monoclonal antibody subtypes will be purified and analyzed at the NRC in Lyon (France) in order to assess the sensitivity of the tests (8). A pool of sterile urine samples will be constituted and negativity using the 18 UATs confirmed. A fixed amount of LPS will be added to this urine pool and each LPS preparation will be simultaneously tested in one experiment, using the 18 UAT.

Proficiency Panel: 10 urines (negative and positive samples) will be chosen and distributed from the ESGLI coordinator lab to each of the 9 participating centers. This panel will be used to determine inter-laboratory agreement. These samples will be tested with all the kits only once after boiling treatment (see paragraph - confirmation of positive results).

Routine Panel: A total of 495 fresh urine samples will be tested. Each participating Centre will analyze 55 consecutive urine samples from their own routine. Only fresh urines from patients for whom the *Legionella* urinary antigen test was prescribed by a clinician for the diagnosis of Legionnaires' disease will be included in the study. While waiting for analysis, the urine samples with or without preservative (at least 5 mL) may be stored at room temperature for max. 24 hours or at 4°C for max. 7 days. Frozen samples or samples with smaller volumes will not be included in the study. After testing, all urines will be stored at -20°C until the end of the study. For a given urine sample, when a positive result will be obtained by at least one of evaluated tests, the pneumonia should be a x-thorax confirmed pneumonia or at least confirm by physician.

UTI Panel: Each participating laboratory will also test at least 5 urine samples from patients without symptoms of pneumonia but with a confirmed urinary tract infection.

Respiratory samples
For antigen positive patients (especially for discordant ones) it is recommended when possible to perform PCR and culture from a pulmonary sample in order to confirm the diagnosis of legionellosis. The PCR-negative and culture-negative from respiratory samples result cannot exclude a diagnosis of legionellosis.

In total, 675 urine samples from patients will be tested using 18 *Legionella* urinary antigen tests.

2.4- Laboratory testing

The tests will be performed according to the manufacturers' instructions. All urines samples will be anonymized before testing.

Urinary antigen detection
The same urine sample will be analyzed in parallel, the same day for the lateral flow tests and the next day for the ELISA tests, with all 18 UAT according to manufacturer's instructions.

- Results (only qualitative) will be interpreted in accordance to the instructions of the producers.
- No concentration of urines will be performed additionally.
- A positive and a negative control will be tested for each lot or in accordance to the manufacturer's instructions.
- All the rapid ICT tests (with the exception of tests equipped with an automated reader) will be read by two independent readers and will also be checked a second time after 60 minutes in order to evaluate the stability of the assay. Should there be a discrepancy between the 2 readings, a third reader will read a third time.
- For tests equipped with an automated reader, a visual reading will be also performed (if possible).
- For invalid tests (absence of control strip), the samples will be tested again in the same conditions. The number of invalid tests will be indicated for all tests.

Confirmation of the positive results

All positive urine samples will be retested with the kits giving a positive result before heat treatment. This will be performed by boiling a 3 mL portion of the sample during 5 minutes at 100°C in a dry bath followed by a centrifugation of 5 min at 1000 x g. The supernatant will be used for the confirmation tests. See also ANNEX 1. The presence of false positive reactions will be considered if the result turns out to be negative after heat treatment of the sample.
The final result considered for comparative analysis will be only the result after heat treatment. Results before and after heat treatment will be indicated in the results tables.

Discordant results

When discordant results among different assays will be observed, *Legionella* detection will be performed by culture or PCR in respiratory samples if those samples are available. Legionellosis will be considered if the patient presents signs of pneumonia and results on respiratory samples are positive. The negative results (PCR and culture) from respiratory samples result cannot exclude a diagnosis of legionellosis.

3- Data analysis

Anonymized data from each lab will be collected and analyzed by the principal investigator and the coordination Lab. A common table (excel file) will be given to each participant for filling the results and information needed.

Data from readers should be available for Labs and can be given if needed to the principal investigator and to the coordination Lab.

Exclusion criteria:
The proportion of valid tests will be calculated for all methods and reported for information. Only valid samples from valid tests will be taken into account in the analysis. The sample with invalid result with a test, will be excluded only for this test.

Test agreement and inter-laboratory agreement will be calculated. Sensitivity, specificity, and the rate of false positive and false negative tests (PPV, NPV) will be calculated for each test.
For the interpretation we will consider:

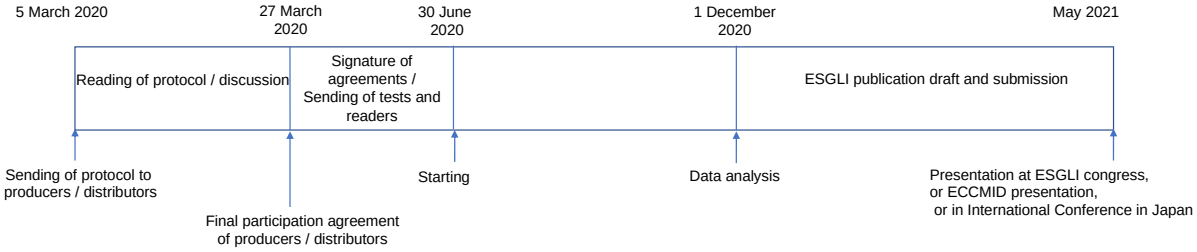
- a false positive result if the test becomes negative after heating the sample
- a false negative result if other diagnostic tests such as culture, PCR, serology, etc. are positive for Lp1
- a presumptive false positive result if the majority of the UAG tests are negative
- a presumptive false negative result if the majority of the UAG tests are positive

For all patients yielding a positive result with at least one of the tests, a PCR and culture on respiratory samples will be performed if those samples were prescribed by a clinician for the diagnosis of the pneumonia.

4- Report

All the results obtained with all the assays by the participating laboratories and the coordination center will be published in a peer reviewed journal. An “ESGLI recommendation” for *Legionella* UAG testing will be included in the publication. The results will be also present at national and international level including the annually ESGLI conference and will be submitted for the ECCMID 2021.

5- Study schedule



6- Industrial producers

- Site training and monitoring:

Labs are all expert for diagnosis of Legionella. If industrial producers would like to perform training of operator(s), it will take place on all sites before the start of the study. This training is not mandatory.

- Distribution of tests:

In total, producers will distribute 1125 urinary tests including

- 100 urinary tests for each lab (except coordination lab and investigator lab)
- 200 urinary tests for coordination lab (45 additional positive US)
- 225 urinary tests for investigator lab (100 additional tests for testing 25 LPS, 5 dilutions of LPS).

- Distribution of readers:

For tests equipped with an automatic reader, producers will provide a reader for each lab. Data from readers have to be available for labs.

REFERENCE

1- Bartlett JG. Diagnostic tests for agents of community-acquired pneumonia. Clin Infect Dis. 2011 May;52 Suppl 4:S296-304
2- Beauté J; The European Legionnaires' Disease Surveillance Network. Legionnaires’ disease in Europe 2011-2015. Euro Surveill. 2017 Jul 6;22(27).
3- Williams A, Lever MS. Characterisation of *Legionella pneumophila* antigen in urine of guinea pigs and humans with Legionnaires’ disease. J Infect 1995. 30:13–16.

4- Birtles RJ, Harrison TG, Samuel D., Taylor AG. Evaluation of urinary antigen ELISA for diagnosing Legionella pneumophila serogroup 1 infection. J Clin Pathol. 1990 43(8):685-690.
5- Helbig JH, Uldum SA, Lu¨ck PC, et al. Detection of Legionella pneumophila antigen in urine samples by the BinaxNOW immunochromatographic assay and comparison with both Binax Legionella Urinary Enzyme Immunoassay (EIA) and Biotest Legionella Urin Antigen EIA. J Med Microbiol 2001;50:509–16. ;
6- Helbig JH, Uldum SA, Bernander S, et al. Clinical utility of urinary antigen detection for diagnosis of community-acquired, travel-associated, and nosocomial legionnaire's disease. J Clin Microbiol 2003;41:838–40 ;
7- Benson RF, Tang PW, Fields BS. Evaluation of the Binax and Biotest urinary antigen kits for detection of Legionnaire's disease due to multiple serogroups and species of Legionella. J Clin Microbiol 2000;38:2763–5.
8- Ranc AG, Carpentier M, Beraud L, Descours G, Ginevra C, Maisonneuve E, Verdon J, Berjeaud JM, Lina G, Jarraud S. Legionella pneumophila LPS to evaluate urinary antigen tests. Diagn Microbiol Infect Dis. 2017 Oct;89(2):89-91.
9- Karkhanis YD, Zeltner JY, Jackson JJ, Carlo DJ. 1978. A new and improved microassay to determine 2-keto-3-deoxyoctonate in lipopolysaccharide of Gram-negative bacteria. Anal Biochem 85:595–601.

PERSONNEL AND CO-INVESTIGATORS

Participating laboratories - Contacts

This protocol has been discussed and approved by all participants.

Principal investigator:
Sophie Jarraud : sophie.jarraud@univ-lyon1.fr

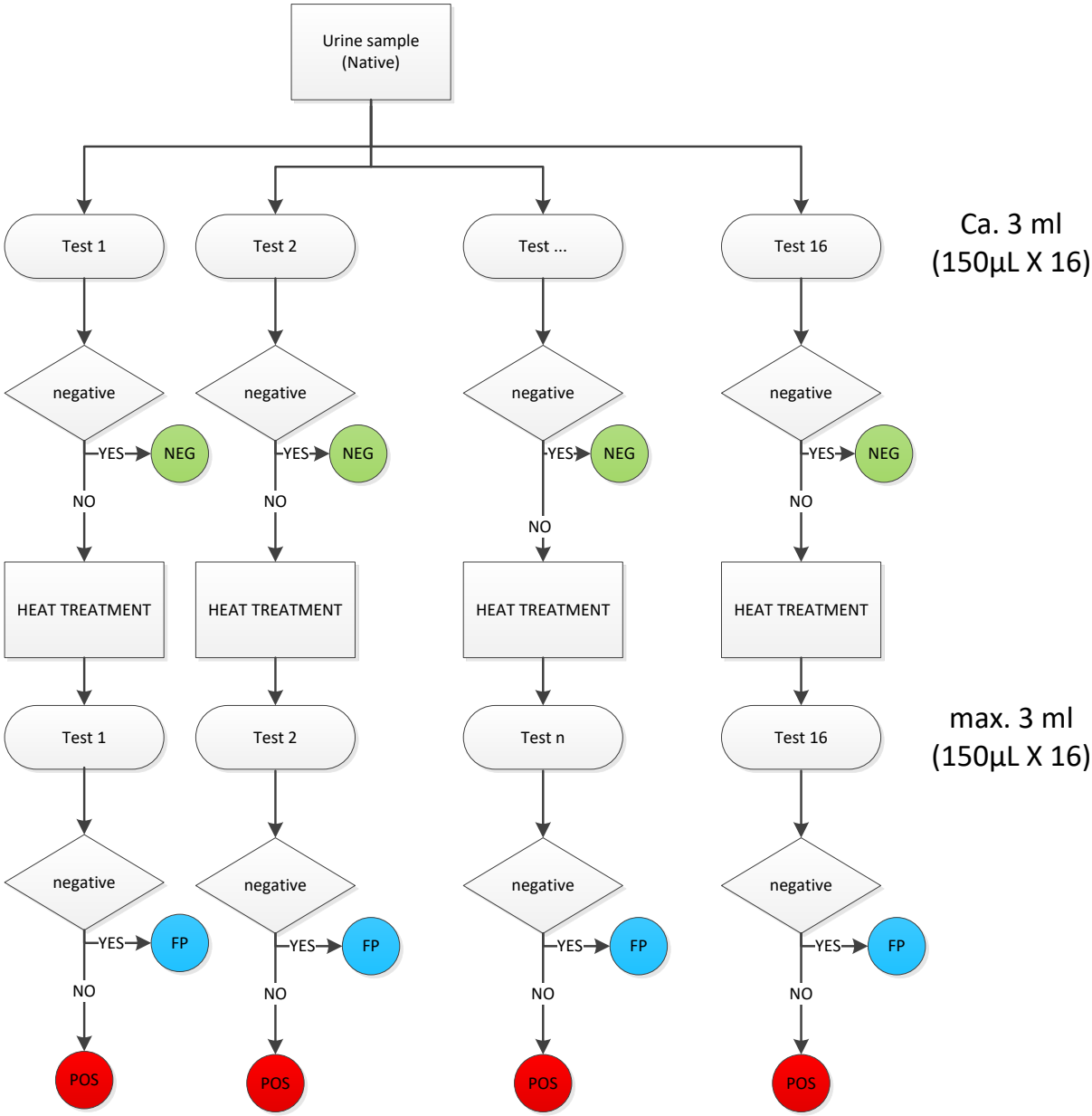
Coordination Lab:

- Soren Uldum Ph.d., M.Sc., senior scientist. Head of the Aptypical Pneumonia Unit and head of the Danish national reference laboratory for *Legionella* at Statens Serum Institut, Copenhagen, Denmark
- Charlotte Sværke Jørgensen Ph.d., M.Sc. Head of the Serology Section, Microbiological Diagnostics & Virology, Statens Serum Institut, Copenhagen Denmark.

Name of investigators	**Country (contact)**
Pr Sophie Jarraud (Principal Investigator) Dr Ghislaine Descours, Dr Laetitia Beraud and Dr Camille Allam	*Legionella* National Reference Center Lyon - France sophie.jarraud@univ-lyon1.fr
Dr Søren Anker Uldum and Charlotte Sværke (Coordination Lab)	Statens Serum Institut - *Legionella* National Reference Center Copenhagen - Denmark SU@ssi.dk csv@ssi.dk
Dr Christian Lück	Reference Laboratory Legionella Technische Universität Dresden - Germany Christian.Lueck@tu-dresden.de
Dr Valeria Gaia	Legionella Reference Center Servizio di microbiologia EOLAB

	Bellinzona - Switzerland Valeria.Gaia@eoc.ch
Dr Vicki Chalker	Respiratory & Vaccine Preventable Bacteria Reference Unit - Legionella Reference Center PHE Microbiology Reference Services London - UK Vicki.Chalker@phe.gov.uk
Dr Sjoerd Euser	Streeklaboratorium - Regional Public Health Laboratory Kennemerland - Legionella Reference Center Haarlem – The Netherlands S.Euser@streeklabhaarlem.nl
Dr Maria Luisa Ricci	Istituto Superiore di Sanità - Legionella Reference Center Rome - Italy marialuisa.ricci@iss.it
Dr Fedoua Echahidi	UZ Brussel Hospital - Legionella Reference Center Brussel - Belgium fedoua.echahidi@uzbrussel.be Alaeddine.Meghraoui@erasme.ulb.ac.be
Dr Darja Kese	University of Ljubljana | Faculty of Medicine Institute of microbiology and immunology Ljubljana - Slovenia darja.kese@mf.uni-lj.si

ANNEX 1: Analysis of the samples (example for 16 tests)



Ca. 3 ml
(150µL X 16)

max. 3 ml
(150µL X 16)