Assessment of Intercentre Reproducibility and Epidemiological Concordance of *Legionella pneumophila* Serogroup 1 Genotyping by Amplified Fragment Length Polymorphism Analysis

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**Abstract** The aims of this work were to assess (i) the intercentre reproducibility and epidemiological concordance of amplified fragment length polymorphism analysis for epidemiological typing of *Legionella pneumophila* serogroup 1, and (ii) the suitability of the method for standardisation and implementation by members of the European Working Group on Legionella Infections. Fifty coded isolates comprising two panels of well-characterised strains, a “reproducibility” panel (n = 20) and an “epidemiologically related” panel (n = 30), were sent to 13 centres in 12 European countries. Analysis was undertaken in each centre following a previously determined standard protocol. Results were analysed by the participants, using gel analysis software where available, and submitted to the coordinating centre. The coordinating centre reanalysed all results visually and selected data-sets with gel analysis software. Data analysis by participants yielded reproducibility (R) values of 0.20–1.00 and epidemiological concordance (E) values of 0.11–1.00, with 6 to 34 types. Following visual analysis by the coordinating centre, R = 0.78–1.00, and E = 0.67–1.00, with 10–20 types. Analysis of three data-sets by the coordinating centre using gel analysis software yielded R = 1.00 and E = 1.00, with 12, 13 or 14 types. This method can be used as a simple, rapid screening tool for epidemiological typing of isolates of *Legionella pneumophila* serogroup 1. Results demonstrate that the method can be highly reproducible (R = 1.00) and epidemiologically concordant (E = 1.00), with good discrimination. The electropherograms generated are amenable to computer-aided analysis, but strict adherence to a previously defined laboratory protocol is required. Following designation of representative type strains and patterns, this method will be adopted by the European Working Group on Legionella Infections as the first internationally standardised typing method for use in the investigation of travel-associated *Legionella* infections.
Introduction

*Legionella* infection (legionellosis) can vary from a mild respiratory illness to acute life-threatening pneumonia. Legionellosis is usually acquired by inhalation of an aerosol generated from a contaminated environmental source. The identification of the source of an outbreak can be aided by characterisation of the clinical and environmental strains. Although 20 of the 42 described *Legionella* species have been implicated in human disease [1], the vast majority of cases are caused by *Legionella pneumophila* strains, most of which belong to serogroup 1. Consequently, isolates of this common serogroup must be further differentiated if any convincing epidemiological link is to be established.

A strategy to develop a standardised approach to, and method for, genotyping *Legionella pneumophila* serogroup 1 in investigations of travel-associated legionellosis within Europe was proposed and agreed upon by members of the European Working Group on Legionella Infections (EWGLI) in 1996. A wide range of genotypic typing techniques was subsequently investigated [2]. One of these methods, amplified fragment length polymorphism (AFLP) analysis [3], appeared to offer a simple, rapid and reproducible means of differentiating epidemiologically related and unrelated strains [2]. Consequently, this second study was initiated to assess the reproducibility of the AFLP methodology when used in different European laboratories.

The aims of the present study were as follows: (i) to assess the reproducibility (R) and epidemiological concordance (E) [4] of the AFLP methodology, and (ii) to assess the suitability of the methodology for standardisation and implementation as a true epidemiological typing system within Europe by the EWGLI.

Materials and Methods

**Participants.** Participants from 13 centres, representing 12 European countries, took part in the study. Each of these centres acts as a local or national reference laboratory for *Legionella* infections. The study was coordinated at the Respiratory and Systemic Infection Laboratory, Public Health Laboratory Service Central Public Health Laboratory, London, UK.

**Bacterial Strains.** A collection of 50 *Legionella pneumophila* serogroup 1 isolates was obtained from nine of the participating countries. The characteristics of 20 of these isolates have been described previously [2]. The additional isolates have been extensively characterised from previous studies [5–8] or by the authors (see Table 1). This collection comprised one reproducibility panel of 20 strains and one epidemiologically related panel of 30 strains (Table 1). The reproducibility panel comprised ten duplicate isolates, and the epidemiologically related panel comprised nine epidemiologically related sets and one additional set comprising variants of the same strain.

As in the previous study [2], all isolates were given a unique European Union *Legionella* culture collection number (EUL no.), and their purity and identity were confirmed by standard methods. Isolates were archived by preservation in glycerol broth on glass beads at –80°C [9]. Replicates of each strain were prepared on BCYE agar (Oxoid, UK) slopes, randomly allocated a study code number by one of the authors (T.G.H.) and then distributed to each participant by courier.

**Study Design.** Each centre examined the coded isolates by the standard EWGLI AFLP protocol described below. Any deviation or variation was recorded by each centre and reported to the coordinating centre. Evaluation of the method was based on the Consensus Guidelines of the European Study Group on Epidemiological Markers [4] as described previously [2]. Briefly, the reproducibility (R) was considered to be the ability of the method to correctly identify epidemiologically related isolates of the same type. The epidemiological concordance (E) was expressed as the number of epidemiologically related strains found to be indistinguishable, divided by the total number of such sets in the study.

**Amplified Fragment Length Polymorphism Analysis.** AFLP analysis for *Legionella pneumophila* was originally described by Valsangiacomo et al. [3]. This method was used with modifications in a previous study [2]. The further modifications described here were designed to increase the reproducibility. The following is thus a description of the standard EWGLI AFLP protocol.

**Standard European Working Group on Legionella Infections Amplified Fragment Length Polymorphism Protocol.** *Legionella* strains were subcultured onto BCYE agar (Oxoid, UK) plates for 48–72 h. Bacterial growth was harvested from the plates and genomic DNA extracted using the Nucleon BACC2 DNA Extraction kit (Nucleon Biosciences, UK, or Amersham Pharmacia Biotech, UK). The concentration of genomic DNA was determined by measuring the absorbance at 260 nm. Restriction-ligation reactions were performed at 37°C for 3 h in a total volume of 20 ml. Each mix comprised approximately 1.5 μg of genomic DNA, 200 ng of each adapter-oligonucleotide (AFLP-LG1: 5′-CTCTGTAAGACTGCGTACATGCAGG-3′; and AFLP-LG2: 5′-TGATCACGGAGTCTAC-3′), 20 U of *Pst*I (Boehringer Mannheim, Germany), 1 U of T4 DNA ligase (Boehringer Mannheim) and 1 ligation buffer (10 ligation buffer is 660 mM Tris, pH 7.5, 50 mM MgCl2, 10 mM dithiothreitol, and 10 mM ATP; Boehringer Mannheim). Prior to the polymerase chain reaction (PCR), tagged DNA fragments were precipitated using a final concentration of 2.5 M ammonium acetate in 100 μl and an equal volume of absolute ethanol. After incubation for 5 min at room temperature, centrifugation was carried out at 12,000 × g for 10 min, and the pellet washed once with 70% ethanol. The precipitate was air-dried and resuspended in 100 μl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

Before the PCR reaction, a 1:100 dilution of the resuspended pellet was prepared, and 5 μl of this dilution was used as template DNA in the PCR reaction. PCR was performed in a reaction mixture of 25 μl using the Ready-To-Go PCR beads, 0.5 ml format (Amersham Pharmacia Biotech). Each reaction mix comprised template DNA and 75 ng of selective primer (AFLP-PsrI-G: 5′-GACTGCGTACATGCAGG-3′), with the magnesium concentration adjusted to 2.5 mM MgCl2. An overlay of mineral oil (Sigma, USA) was used to prevent evaporation. Amplification was performed using the following parameters: initial denaturation at 94°C for 4 min, followed by 33 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and elongation at 72°C for 2.5 min. Amplified products (20–40% of reaction mix, i.e. 5–10 μl) were separated by electrophoresis on 1.5% agarose (UltraPure agarose; Life Technologies, UK) gels in 1×TBE (0.089 M Tris-borate, 1 mM EDTA) for 4 h at 3.5 V/cm. The MBI Fermentas Ladder Mix (MBI Fermentas, USA) was used as molecular size markers (at 0.75–1.0 μg/lane). To aid normalisa-