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Quantitative real-time PCR tests for diagnostic and prognostic purposes in cases of legionellosis

M. Maurin[1,2], L. Hammer[3], B. Gestin[1], J. F. Timsit[3,4], O. Rogeaux[5], F. Delavena[6], J. Tous[6], O. Epaulard[7], J. P. Brion[7] and J. Croizé[1]

1) *Laboratoire de Bactériologie, Centre Hospitalier Universitaire de Grenoble, Université Joseph Fourier, Grenoble,* 2) *Laboratoire Adaptation et Pathogénie des Micro-organismes, CNRS UMR 5163, Université Joseph Fourier, Grenoble cedex 9,* 3) *Réanimation Médicale, Centre Hospitalier Universitaire de Grenoble, Université Joseph Fourier, Grenoble,* 4) *INSERM U823—Team 11: Epidemiology of cancer and critical illnesses, Albert Bonniot institute, La Tronche,* 5) *Service des Maladies Infectieuses and* 6) *Laboratoire de Biologie, Centre Hospitalier Régional de Chambéry, Place du Dr François Chiron, Chambéry and* 7) *Service des Maladies Infectieuses, Centre Hospitalier Universitaire de Grenoble, Université Joseph Fourier, Grenoble, France*

Abstract

The usefulness of two quantitative real-time PCR assays (qrt-PCRmip targeting *Legionella pneumophila*, and qrt-PCR16S targeting all *Legionella* species) performed on lower respiratory tract (LRT) samples for diagnostic and prognostic purposes in 311 patients hospitalized for community-acquired pneumonia (CAP) in Rhône-Alpes (France) was evaluated. The Now *Legionella* urinary antigen test (UAT) from Binax (Portland, ME, USA) was used as a reference test. Samples were divided into two groups. Group A included 255 CAP patients admitted to Chambery hospital in 2005 and 2006. The Now *Legionella* UAT was positive in 14 patients. Sensitivities, specificities, positive predictive and negative predictive values for both qrt-PCR tests were 63.6, 98.7, 77.7 and 97.4%, respectively. Group B included 56 consecutive legionellosis patients diagnosed during a 4-year period (2003–2006) at the Grenoble University Hospital. The qrt-PCR16S and qrt-PCRmip displayed a sensitivity of 82.14 and 80.4%, respectively. Among the 70 legionellosis cases, *L. pneumophila* serogroup 1 was isolated in 15; qrt-PCRmip was positive in another 36, suggesting *L. pneumophila* infection, whereas the *Legionella* species involved could not be determined in the remaining 19 cases. The *Legionella* burden in LRT samples at the time of admission was determined in 46 patients using qrt-PCR16S tests, 44 for qrt-PCR mip groups A and B patients. It varied from 1.9 to 8.35 $\log_{10}$ DNA copies/mL of LRT sample for qrt-PCR16S and from 1.9 to 8.11 $\log_{10}$ DNA copies/mL of sample for qrt-PCRmip. High bacterial loads in LRT samples at hospital admission were significantly associated with higher Fine classes, the need for hospitalization in an intensive care unit and for prolonged hospitalization.

Keywords: Diagnosis, *Legionella*, legionellosis, prognosis, quantitative real-time PCR
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Corresponding author and reprint requests: M. Maurin, Laboratoire de Bactériologie, CHU de Grenoble, Université Joseph Fourier, BP 217, 38043 Grenoble, Cedex 9, France
E-mail: mmaurin@chu-grenoble.fr

Introduction

Legionellosis is a pneumonia caused by inhalation of *Legionella* species-contaminated aerosols [1]. It is responsible for 2–15% of all community-acquired pneumonias (CAP) requiring hospital admission [2]. Of the 50 species and 72 serogroups belonging to the genus *Legionella*, *L. pneumophila* serogroup one (sg1) is responsible for at least 80% of human infections [1–3]. Mortality rates of 10–15% are usually reported [1,2] but may be higher in immunocompromised patients.

The severity of legionellosis depends primarily on the immune status of the patient and the speed of diagnosis and administration of an appropriate antibiotic therapy [1,4]. Culture-based diagnosis of legionellosis remains fastidious and its sensitivity is poor [1]. Direct fluorescence antibody staining of *Legionella* spp. in respiratory samples is considered unreliable [1,2,5,6]. Serological diagnosis lacks both sensitivity and specificity [5,7], and only provides a retrospective diagnosis. In recent years, *L. pneumophila* urinary antigen tests (UATs) have become reference tests allowing rapid legionellosis diagnosis [5,8–14]. These tests are considered highly sensitive, although they may be negative at the early stage of legionellosis, and highly specific, although urinary antigens

may persist for months in some patients [12,15]. However, these tests detect primarily *L. pneumophila* sg1 antigens and are much less sensitive in detecting other *Legionella* serogroups or species [5,8,12]. PCR-based techniques have been developed as rapid diagnostic tools, potentially allowing amplification of DNA from all *Legionella* species and serogroups [5,16–27], but these tests are poorly standardized.

In the present study, taking the Now *Legionella* UAT as a reference test, we evaluated the relative sensitivities and specificities of two qrt-PCR assays for legionellosis diagnosis in CAP patients requiring hospitalization. Additionally, in a subset of legionellosis patients, we quantified *L. pneumophila* DNA burden in lower respiratory tract (LRT) samples at hospital admission and tentatively correlated bacterial loads with disease severity.

Materials and Methods

Patients

CAP patient inclusion criteria were as follows: patients > 18 years old, admitted to hospital with clinical and radiological findings suggestive of pneumonia (fever, cough, expectoration, dyspnoea, thoracic pain, new or progressive infiltrate on chest X-ray), in whom the infection occurred outside the hospital setting. The Fine class, a pneumonia severity index [28], was determined at the time of admission.

Patients were considered legionellosis cases when clinical signs compatible with CAP were present, and a *Legionella* strain was grown in culture and/or a Now *Legionella* UAT was positive.

Patients were divided into two groups. Group A included consecutive patients hospitalized for CAP from November 2004 to March 2006 at the General Hospital of Chambery, France. Group B included consecutive legionellosis cases, hospitalized at the University Hospital of Grenoble during a 3-year period (2003–2006).

Microbiological investigations

Usual microbiological investigations were carried out at hospital admission: two sets of blood cultures (Bactec 9240; Becton Dickinson, Grenoble, France); culture of LRT samples [i.e. bronchial aspirations and/or bronchoalveolar lavage (BAL) specimens and/or sputum samples], including *Legionella* culture on buffered charcoal yeast extract medium supplemented with α-ketoglutarate (Oxoid, Wesel, Germany); an indirect immunofluorescence assay (IFA) for detection of serum antibodies directed against *L. pneumophila* sgs1–6 (Meridian Diagnostics Inc., Cincinnati, OH, USA); the Now *Legionella* UAT (Binax, Portland, ME, USA) performed on urine samples 25-fold concentrated by selective ultrafiltration (Minicon B15; Millipore Corp., Bedford, MA, USA). Additionally, two *Legionella* qrt-PCR assays were performed on LRT samples, either tested immediately after collection or frozen at −80°C for several days to 1 month before testing.

qrt-PCR assays

DNA was extracted from the patients' LRT samples using QIAamp DNA Mini kit (QIAGEN, Hilden, Germany). Two qrt-PCR assays previously described in the literature [29], which we had used to quantify *Legionella* spp. in water samples [30], were used. Analytical sensitivities, specificities, and quantification limits of these techniques have been previously described [29,30]. The qrt-PCR16S amplifies a 386-bp portion of 16S ribosomal RNA-encoding genes of all *Legionella species* [29,30]. The qrt-PCRmip amplifies a 186-bp portion of the *mip* (macrophage internalization potentiator) gene of all serogroups of *L. pneumophila* [29,30]. Primers, probes and amplification protocols were as previously described [30], and results obtained with the Light Cycler 2.0 instrument (Roche, Meylan, France). A 374-bp internal inhibitor control (3000 copies per reaction) was used in all experiments [30].

Mip and 16SrRNA gene amplification and sequencing

In patients with positive qrt-PCR tests but negative cultures, we attempted to amplify and sequence nearly complete *mip* and 16S rRNA genes directly from LRT samples, in order to identify the *Legionella* species involved. PCR amplification was performed using the *mip* gene primers and the procedure previously reported by Ratcliff *et al.* [31] and Stolhaugh *et al.* [32] and using the following primers for the 16SrRNA gene: Lg16SFw (5′-TTAACACATGCAAGTCGAACGG-3′) and Lg16SRv (5′-ACCGGAAATTCCACTACCCT-3′).

Statistical analysis

Using qrt-PCR assays, we evaluated the bacterial burden in LRT samples at the time of admission, and tentatively correlated this biological marker with age (<60 or >60 years), the Fine class at hospital admission, the need for hospitalization in an intensive care unit (ICU), and duration of hospitalization (1–14 days vs. >14 days), using the two-tailed Student's *t*-test at the 95% confidence limit.

Results

Group A included 255 CAP patients, with a Fine class of 2 for 31 patients, 3 for 39 patients, 4 for 61 patients, 5 for 30 patients, and an undetermined Fine class for 94 patients.