Multicenter Comparison of Molecular Methods for Detection of 
Legionella spp. in Sputum Samples

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Legionellosis can be diagnosed by PCR using sputum samples. In this report, the methods of nine laboratories for 12 sputum samples with Legionella pneumophila and Legionella longbeachae are compared. We conclude that (i) liquefaction prevents PCR inhibition, (ii) the employed mip gene PCRs detected L. pneumophila only, and (iii) the 16S rRNA gene PCR detected both Legionella species and is preferred for the diagnosis of legionellosis.

Legionellosis is routinely diagnosed by detection of Legionella DNA in sputum samples. A range of molecular methods is described in the literature for the detection of Legionella spp. (1, 3–8, 10–14). In this report we compared the methods that are performed by nine laboratories in The Netherlands. The aim of the study was to compare these methods and to identify critical steps in the procedures, thus aiding in the improvement and standardization of Legionella detection.

A series of 12 sputum samples were prepared for this survey by the Regional Laboratory of Public Health in Haarlem, The Netherlands. By using sputum as a matrix, the samples of the survey closely resemble samples from patients, with its characteristic abundant microbial flora and mucoproteins.

Each sample consisted of 200 μl of sputum that was prepared from discarded patient material after it was screened for Legionella bacteria by culture and PCR. Samples 1 to 12 were spiked with Legionella bacteria according to the scheme shown in Table 1. The Legionella strains were a Legionella pneumophila serogroup 1 strain and a Legionella longbeachae strain. Legionella longbeachae is included in the panel because this species is the second most common causative agent of legionellosis and should be detected by the laboratories (9).

Sample sets were transported on ice together with a questionnaire on sample preparation, DNA isolation, and PCR conditions. We invited 10 laboratories that routinely perform Legionella PCR on sputum to participate. Nine laboratories participated in the trial and returned the data and questionnaire. One laboratory failed to return the data and questionnaire. The participating laboratories were situated in academic hospitals, regional hospitals, and public health laboratories. The laboratories returned their data within 2 months of delivery of the samples. During this 2-month period, the stability of the frozen sputum samples was checked by the Regional Laboratory of Public Health in Haarlem, using a real-time PCR with the 16S rRNA primers described by Reischl et al. and SYBRgreen detection with melting-curve analysis (8). No differences were found between three sample sets that were analyzed at 4-week intervals (data not shown).

The performances of the laboratories are indicated in Table 2. For each correctly analyzed sample, i.e., the presence of a species and the correctly identified Legionella species, 2 points were obtained by the laboratory. If only the presence of an organism of the genus Legionella was reported, without the correct species name, 1 point was obtained. No points were obtained for a wrong diagnosis of a sample. Using Fisher’s exact test, the P value between data was calculated. A P value of <0.05 was considered statistically significant.

The results of the survey and scores are listed in Table 2. All laboratories except laboratory H used a real-time PCR method to detect Legionella DNA. Laboratory H used an endpoint PCR and detection by agarose gel electrophoresis. The PCR targets were either the 16S rRNA gene or the mip gene or both (Table 2). Laboratory B also used the 5S rRNA gene as a target. The laboratories used a variety of methods to prepare
the samples for PCR. All laboratories used a commercial system to extract DNA. The MagNA Pure (Roche) system was used by five laboratories. Prior to the DNA isolation, seven laboratories pretreated the sputum to liquefy the sample. The liquefaction involved enzymatic (proteinase K), chemical (lysis buffer; the sputolysin was dithiothreitol), and mechanical (MagNA Lyser, Roche) processes. Laboratories A and G did not liquefy the samples prior to DNA isolation. Laboratory A reported inhibited PCRs for five samples, while laboratory G reported inhibited PCRs for seven samples. Laboratory G correctly analyzed the samples after dilution of the DNA preparation, but dilution will inevitably reduce the analytical sensitivity of the test. Therefore, we conclude that liquefaction of the sputum samples prior to the DNA isolation is preferred to prevent the inhibition of the PCR. Previously reported PCR inhibition by mucolytic agents was not encountered in this study (2), possibly due to the introduction of improved DNA purification methods.

The nine participating laboratories used five different protocols for DNA isolation. These methods encompass automated methods using magnetic affinity beads (the MagNA Pure and MagNA Pure Compact systems and the NucliSENS easyMAG system) and manual methods using affinity matrices (the QIAamp DNA mini kit and Puregene DNA purification kit) (Table 2). The five laboratories that used the MagNA Pure system had the lowest scores for samples 1 to 8, which were spiked with *L. pneumophila* only, due either to PCR inhibition or to a false-negative result for *L. pneumophila*. However, the correlation between a low score and the use of the MagNA Pure system might be coincidental, since in a controlled study, Wilson et al. reported that automated DNA extraction systems, including the MagNA Pure system, performed significantly better than manual extraction methods (13).

A blank sample (sample 9) that was added to the sputum sets was found negative by all participating laboratories (except laboratory A, which reported inhibition), indicating that *Legionella* contamination of laboratory reagents did not play an important role in this survey.

The PCR methods that were used by the laboratories differed considerably. The applied target genes were 16S rRNA, *mip*, and 5S rRNA genes (Table 2). Real-time PCR was done on LightCycler (Roche), iCycler (Bio-Rad), or ABI PRISM (Applied Biosystems) instruments, and the detection was done by TaqMan or fluorescence resonance energy transfer probes. Endpoint PCR products were detected by agarose gel electrophoresis or enzyme-linked immunosassay. Despite all these differences, neither the analytical sensitivity nor the proneness to inhibition of the PCRs is correlated to any of these parameters (Table 2).

As expected, a clear difference was found between the 16S rRNA and *mip* gene PCRs for their ability to detect *L. longbeachae*. The *mip* PCRs were designed to specifically detect *L. pneumophila*, while the 16S rRNA gene PCRs were designed to detect other *Legionella* species as well (3, 8–11, 14). This is clearly reflected in the scores for sample 10, which was spiked with *L. longbeachae*. None of the laboratories that used only a *mip* PCR (labs E, F, and G) detected *Legionella* in this sample, while the laboratories that used a 16S rRNA PCR (except lab C) correctly detected the *Legionella* species in this sample. The scores for samples 11 and 12, which contained both *L. pneumophila* and *L. longbeachae*, were comparable to samples 3 to 6, which were spiked with the same amounts of *L. pneumophila*.

The detection of *Legionella* species other than *L. pneumophila* is relevant for the diagnosis of legionellosis, since this disease can be caused by species other than *L. pneumophila*, such as *L. longbeachae* in immunocompetent patients and *Legionella bozemanii* in immunocompromised patients. The cor-
rect identification of the species is also relevant for epidemiological studies and for the identification of sources of infection. Patients infected with any of the *Legionella* species are treated the same. Therefore, the high specificity of the *Legionella pneumophila* *mip* gene-targeted PCRs is not advantageous for the diagnosis of legionellosis.

We conclude that the pretreatment of the sputum is important to prevent inhibition of the PCR. The DNA isolation method is of less importance; the low scores with the MagNA Pure and the MagNA Pure Compact systems might be coincidental. The two target genes, the 16S rRNA gene and the *mip* gene, perform equally well in detecting the lowest level of *L. pneumophila* in the samples. However, the 16S rRNA gene PCR is able to detect *L. longbeachae*, while the *mip* gene PCR does not detect this clinically relevant species. Therefore, in our view, the 16S rRNA gene PCR is preferred for the identification of patients with Legionnaires’ disease caused by either *L. pneumophila* or *L. longbeachae*.

REFERENCES


