

## Development of conventional and real-time NASBA<sup>®</sup> for the detection of *Legionella* species in respiratory specimens

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### Abstract

Isothermal nucleic acid sequence-based amplification (NASBA) was applied to detect *Legionella* 16S rRNA. The assay was originally developed as a *Legionella pneumophila* conventional NASBA assay with electrochemiluminescence (ECL) detection and was subsequently adapted to a *L. pneumophila* real-time NASBA format and a *Legionella* spp. real-time NASBA using molecular beacons. *L. pneumophila* RNA prepared from a plasmid construct was used to assess the analytical sensitivity of the assay. The sensitivity of the NASBA assay was 10 molecules of in vitro wild type *L. pneumophila* RNA and 0.1–1 colony-forming units (CFU) of *L. pneumophila*. In spiked respiratory specimens, the sensitivity of the NASBA assays was 1–10 000 CFU of *L. pneumophila* serotype 1 depending on the background. After dilution of the nucleic acid extract prior to amplification, 1–10 CFU of *L. pneumophila* serotype 1 could be detected with both detection methods. Finally, 27 respiratory specimens, well characterized by culture and PCR, collected during a *L. pneumophila* outbreak, were tested by conventional and real-time NASBAs. All 11 PCR positive samples were positive by conventional NASBA, 9/11 and 10/11 were positive by *L. pneumophila* real-time NASBA and *Legionella* spp. real-time NASBA, respectively.

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### 1. Introduction

Pneumonia is the most frequent manifestation of *Legionella* infection in man and may be nosocomial or community-acquired. The real incidence of legionellosis among cases of community-acquired pneumonia is unknown, but in the US it is considered to be responsible for 1% to 5% of pneumonias in adults (Marston et al., 1994, 1997; Fang et al., 1990). The

incidence of nosocomial pneumonia caused by *Legionella pneumophila* varies considerably and is believed to represent between 5% and 15% of all nosocomial pneumonias (Garbe et al., 1985).

Although the number of species within the genus *Legionella* continues to increase, the vast majority of infections are caused by *L. pneumophila*, particularly serotypes 1 and 6.

For diagnosis of a *Legionella* infection culture on selective media is considered the gold standard with a high specificity but low sensitivity (Yu, 1988). Serology doesn't provide an adequate solution due to sensitivity and specificity problems (Edelstein et al., 1980; Winn, 1995; Casal et al., 1992). An alternative tool is the

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detection of soluble *Legionella* antigens in urine. A disadvantage is the narrow scope of *Legionella* strains recognised by the antibody: most urinary antigen tests only detect *L. pneumophila* serotype 1 (Dominguez et al., 1999).

To improve the diagnosis of legionellosis, nucleic acid amplification techniques have been introduced. PCR of fragments of the macrophage infectivity potentiator (*mip*) (Schlenk et al., 1993; Bernander et al., 1997; Jaulhac et al., 1992), the 16S rRNA gene (Jonas et al., 1995), the 5S rRNA gene (Mahbubani et al., 1990) or amplification of a DNA fragment with unknown function (Starnbach et al., 1989) were shown to be considerably more sensitive than culture for the detection of *L. pneumophila*.

Real-time nucleic acid sequence-based amplification (NASBA, bioMérieux, Boxtel, The Netherlands) is targeted at RNA. The detection of microorganisms by a ribosomal RNA-based amplification technique such as NASBA might be more sensitive than PCR because of the presence of multiple RNA copies and detection of RNA also implies biological activity that avoids detection of dead organisms.

Afterwards, the same amplicons can be used for conventional electrochemiluminescence detection. The real-time NASBA technique has already been successfully applied for the detection of *Mycoplasma pneumoniae* (Loens et al., 2003) and *Chlamydomphila pneumoniae* (Loens et al., 2006).

The aim of the study was to develop a real-time NASBA assay for the detection of *Legionella* spp. in respiratory specimens based on NASBA amplification of a 16S rRNA target sequence using the NucliSens Basic Kit and compare the results with conventional NASBA results.

## 2. Materials and methods

### 2.1. Bacterial strains

The *Legionella* strain collection used to test the specificity of the NASBA primers included 34 *L. pneumophila* strains as well as 21 non-*L. pneumophila* strains and is listed in Table 1. In addition, specificity tests were performed with 21 bacterial species that may be present in respiratory specimens. *Legionella* strains were grown on buffered charcoal-yeast (BCYE; Oxoid Ltd., Belgium) agar plates at 37 °C for 48–72 h and titrated in fourfold to determine the amount of CFU/ml. All other bacteria were cultured and identified as described before (Loens et al., 2006). Reference strains: *M. pneumoniae* PI 1428 (ATCC 29085), *M. pneumoniae* MAC (ATCC

15492), *M. fermentans* (NCTC NC 10117), *M. hominis* (NCTC NC10111), *M. genitalium* G-37 (ATCC 33530), *M. orale* (NCTC NC10112), *M. buccale* (NCTC NC10136), *M. salivarium* (NCTC NC10113), *M. pirum* (NCTC NC11702), *B. pertussis* (LMG15587), *C. pneumoniae* (ATCC VR-1355), *S. pneumoniae* (ATCC49619), *S. aureus* (ATCC29213), *P. aeruginosa* (ATCC27853), *N. meningitidis* (93/1); and the following clinical isolates: *M. catarrhalis*, *H. influenzae*, *S. pyogenes*, *S. viridans*, *K. pneumoniae*, and *E. coli*.

### 2.2. Respiratory specimens

Pools of throat swabs, bronchoalveolar lavages (BAL), nasopharyngeal aspirates (NPA), sputum and bronchus aspirates (BA) obtained from the Microbiology Laboratory of the University Hospital of Antwerp that tested individually negative for *L. pneumophila* by culture were used as suspension medium. Furthermore, 100 respiratory specimens to be tested individually [20 throat swabs, 20 bronchoalveolar lavages (BAL), 20 nasopharyngeal aspirates (NPA), 20 sputum and 20 bronchus aspirates (BA)] were collected from the Microbiology Laboratory of the University Hospital of Antwerp and tested negative for *L. pneumophila* by culture. Specimens collected during a *Legionella* outbreak in Kapellen, Belgium (de Schrijver et al., 2003) previously tested for the presence of *L. pneumophila* by PCR (Van der Zee et al., 2002) and/or culture were used to evaluate the NASBA assay: 16 sputum specimens, 5 lung biopsy specimens, 1 pleural fluid, 2 BALs, 2 NPAs, 1 BA, and 1 bile fluid. All but one PCR positive specimen contained *L. pneumophila* serotype 1 (de Schrijver et al., 2003).

### 2.3. Nucleic acid extraction

Nucleic acids from 100 µl of protease treated specimens (individual specimens and aliquots of the respiratory specimen pools) (Loens et al., 2002) or aliquots of a bacterial culture were extracted as described previously, (Loens et al., 2006) using the NucliSens Basic Kit extraction module (bioMérieux, The Netherlands).

### 2.4. Primer design, biotin capture probe and molecular beacons

Oligonucleotide primers, ECL-probe and molecular beacons were derived from the *L. pneumophila* 16S rRNA sequence: P1 primer: 5'AATTCTAATACGACTCACTATAGGGAGGCTAATCTTAAAGCGCCA3'

Table 1  
*Legionella* species and strains tested by conventional and real-time NASBA

Species	Serotype	No. of strains	Strains <sup>a</sup>	Detection limit <sup>b</sup>		
				Conventional NASBA	Real-time NASBA L. pneu	Real-time NASBA L. spp.
<i>L. pneumophila</i>	1	15	CDC BC 1636, 14 clinical isolates UZA	0.1	1	0.1
<i>L. pneumophila</i>	2	1	CDC BC 1637	0.1	0.1–1	1
<i>L. pneumophila</i>	3	1	SEH	ND	ND	0.1
<i>L. pneumophila</i>	4	6	Strain Los Angeles, 5 clinical isolates UZA	100–1000	Neg	0.1
<i>L. pneumophila</i>	5	1	Strain Dallas	100–1000	Neg	0.1–1
<i>L. pneumophila</i>	6	4	Clinical isolates UZA	10	10–1000	0.1–1
<i>L. pneumophila</i>	7	1	SEH	ND	ND	0.1–1
<i>L. pneumophila</i>	8	1	Strain Concor	10	10	0.1–1
<i>L. pneumophila</i>	9	1	SEH	ND	ND	0.1–1
<i>L. pneumophila</i>	10	1	SEH	ND	ND	1
<i>L. pneumophila</i>	11	1	SEH	ND	ND	0.1
<i>L. pneumophila</i>	14	1	SEH	ND	ND	1
<i>L. longbeachae</i>	1	1	SEH	ND	ND	1–10
<i>L. longbeachae</i>	2	1	SEH	ND	ND	0.1
<i>L. longbeachae</i>	4a	1	Clinical isolate UZA	10	10	1–10
<i>L. longbeachae</i>	4b	1	Clinical isolate UZA	10	1000	10
<i>L. anisa</i>	1	1	SEH	ND	ND	0.1
<i>L. bozemanii</i>	1	1	SEH	ND	ND	0.1
<i>L. bozemanii</i>	2	1	SEH	ND	ND	0.1
<i>L. dumoffii</i>	1	1	SEH	ND	ND	0.1
<i>L. feelei</i>	1	1	SEH	ND	ND	10
<i>L. feelei</i>	2	1	SEH	ND	ND	10–100
<i>L. gormanii</i>	1	1	SEH	ND	ND	0.1
<i>L. hackeliae</i>	1	1	SEH	ND	ND	0.1
<i>L. maceachernii</i>	1	1	SEH	ND	ND	1–10
<i>L. micdadei</i>	1	1	SEH	ND	ND	10
<i>L. moravica</i>	1	1	SEH	ND	ND	0.1–1
<i>L. oakridgiensis</i>	1	1	SEH	ND	ND	10
<i>L. parisiensis</i>	1	1	SEH	ND	ND	0.1
<i>L. rubrilucens</i>	1	1	SEH	ND	ND	0.1
<i>L. sainthelensis</i>	1	1	SEH	ND	ND	0.1
<i>L. tucsonensis</i>	1	1	SEH	ND	ND	0.1
<i>L. wadsworthii</i>	1	1	SEH	ND	ND	0.1

L. pneu.: *L. pneumophila* real-time NASBA. L. spp.: *Legionella* species real-time NASBA. Neg: negative. ND: not done.

<sup>a</sup> CDC, Center for Disease Control, USA; SEH, Saint Elisabeth Hospital, Tilburg, The Netherlands; UZA: Clinical isolate, Universitair Ziekenhuis Antwerpen, Edegem, Belgium.

<sup>b</sup> Detection limit. Input in CFU in the extraction procedure.

and P2 primer 5'GATGCAAGGTCGCATATGAGCATTGTCTAGCTTGCTAGACA3', ECL capture probe for conventional *L. pneumophila* NASBA: 5'biotin-TAATGTCTGAGGACGAAAGCTGGG3', *L. pneumophila* molecular beacon 5'FAM-CCATGCGAATGTCTGAGGACGAAAGCTGGGCGCATGG-dabsyl3'. The generic ECL detection probe was used as provided in the NucliSens Basic Kit. For the *Legionella* spp. real-time NASBA the following primers and beacon were used: P1 primer: 5'AATTCTAATACGACTCACTATAGGGAGGCTAATCTTAAAGCGCCA3' and P2 primer 5'GATGCAAGGTCGCATATGAGATCCTGGCTCAGATTGAACGCT3' and *Legionella* spp. molecular beacon 5' FAM-CCGAGCTGAGTAACGCGTAGGAATATGCTCGG-dabsyl 3'.

## 2.5. Wild-type RNA

For the generation of wild type RNA, cDNA from part of the 16S rRNA from *L. pneumophila* serotype 1 CDC BC 1636, obtained by RT-PCR using adapted versions of the NASBA primers, containing an *EcoRI* site and a *Csp* 45I site, was inserted into a modified pGEM vector resulting in plasmid pG30 Lp 16S rDNA and processed as described previously (Loens et al., 2006).

## 2.6. NASBA amplification and detection

NASBA reactions were performed using the NucliSens Basic Kit amplification module (bioMérieux). In

negative control reactions, target nucleic acid was replaced by RNase-/DNase-free water. The whole real-time amplification process was run in a fluorescent reader, the NucliSens EasyQ Analyzer (bioMérieux). Results were calculated with the Ascent software (bioMérieux). Thereafter, the same amplification products were diluted 1:20 and also identified by electrochemiluminescence detection (ECL) using the NucliSens Basic Kit detection module and the NucliSens Reader (bioMérieux). Measured ECL counts were processed and validated by the Basic Kit user software. U1A (Nelissen et al., 1991) NASBA – to verify the presence of nucleic acid after nucleic acid extraction of specimens – was performed using the NucliSens Basic Kit® amplification module (bioMérieux, Boxtel, The Netherlands) according to the instructions of the manufacturer in a separate reaction tube.

### 2.7. Sensitivity study

The sensitivity of the NASBA based *Legionella* 16S-amplification assays was studied using 10-fold dilutions of wild type in vitro generated 16S rRNA in water, 10-fold dilutions of suspensions of different *L. pneumophila* serotypes or *Legionella* species in PBS and using 10-fold dilutions of *L. pneumophila* serotype 1 added in four-fold to protease-treated samples of the respiratory specimen pools (Loens et al., 2002).

### 2.8. Reproducibility of conventional NASBA and real-time NASBA

The intrarun and interrune variations in conventional and real-time NASBAs were estimated by running a 10-fold dilution series of *L. pneumophila* serotype 1 added in 5-fold to 2 BAL pools.

### 2.9. RNA degradation

RNA degradation was monitored by adding a dilution series of *L. pneumophila* serotype 1 to BAL pools. The resulting specimens were stored at 4 °C or at room temperature for different periods of time after which lysis buffer was added. The specimens were stored at –80 °C prior to extraction.

## 3. Results

### 3.1. Specificity of *Legionella* 16S rRNA NASBA primers

Using the primers and the biotin capture probe for ECL detection for conventional *L. pneumophila* NASBA,

positive results were obtained with nucleic acid extracts from different *Legionella* species and serotypes (Table 1), but with none of the other organisms.

### 3.2. Sensitivity of *Legionella* 16S rRNA primers

The sensitivity of *Legionella* conventional and real-time NASBAs measured by testing 10-fold dilutions of in vitro generated RNA was 10 molecules.

Applied on 10-fold dilutions of nucleic acids extracted from suspensions of cultures of *Legionella* in PBS, the sensitivity depended on the serotype and the species tested: with the conventional NASBA, as little as 0.1 CFU of *L. pneumophila* serotypes 1 and 2 and 10 to 1000 CFU of the other *L. pneumophila* serotypes and *Legionella* species could be detected. With the *L. pneumophila* real-time, NASBA using similar results were obtained, except that serotypes 4 and 5 were not detected at all. The detection level of *L. pneumophila* serotype 6 strains varied from strain to strain. Since preliminary results obtained with the *L. pneumophila* specific real-time assay, indicated that a higher sensitivity could be obtained with the *Legionella* spp. real-time assay only a limited number of *L. pneumophila* serotypes were investigated by this assay. A broader range of 10-fold dilutions of *Legionella* serotypes and species in PBS were tested with the *Legionella* spp. assay (Table 1). In contrast, the *Legionella* spp. assay detected between 0.1 and 1 CFU of most *L. pneumophila* serotypes and *Legionella* species (Table 1).

When conventional and real-time NASBAs were applied on protease-treated samples of the respiratory specimen pools spiked in quadruplicate with 10-fold dilutions of *L. pneumophila* serotype 1, the sensitivity was 1 CFU/100 µl in 3/4 throat swabs, 1/4 BAL and NPA samples. An input of 10 CFU/100 µl in the extraction was always detected in these samples. When BA and sputum pools were spiked in quadruplicate with *L. pneumophila* serotype 1, the obtained sensitivity dropped to more than 10<sup>3</sup> CFU.

However, the sensitivity of the assay, especially in sputum and BA increased to 10–100 CFU by diluting the nucleic acid extract five-fold prior to nucleic acid amplification. For the real-time NASBAs, dilution increased the fluorescence markedly making interpretation of results more clear-cut.

### 3.3. Reproducibility of conventional NASBA and real-time NASBA

The intrarun variability coefficients for the detection of 1, 10, 100 and 1000 CFU varied from 103.6, to 39.6

and from 58.5, to 9.5 by conventional and real-time NASBAs, respectively.

The interrun variation coefficients for the same inputs varied from 118.0 to 54.4 and from 76.6 to 8.9 by conventional and real-time NASBAs, respectively.

### 3.4. RNA degradation

The results of *L. pneumophila* RNA degradation at 4 °C and at room temperature are presented in Fig. 1. Results show that most degradation of *L. pneumophila* RNA seems to occur within 48 h and when specimens are kept at room temperature.

### 3.5. Detection of *L. pneumophila* in clinical and control specimens by conventional and real-time NASBA

All 100 routine samples were negative by both conventional and real-time NASBAs except one specimen that was *Legionella* positive by all assays.

### 3.6. Detection of *L. pneumophila* in clinical specimens by conventional and real-time NASBA. Specimens from a *L. pneumophila* outbreak

Twenty-eight clinical specimens collected during a *Legionella* epidemic in Kapellen, Belgium were investigated by both conventional and real-time NASBAs (Table 2). All lung biopsy specimens and most sputum specimens contained inhibitory factors as was shown by the absence or a low positive result in the U1A NASBA; positive results were only obtained after 1:5 or even 1:10 dilutions of the nucleic acid extract prior to amplification. Twelve respiratory specimens were *Legionella* PCR positive. Amplification of the undiluted nucleic acid extract yielded 6, 1, and 7 *Legionella* positive results by conventional NASBA, *L. pneumophila* real-time NASBA and *Legionella* spp. real-time NASBA, respectively. A 1:5 and a 1:10 dilution of the nucleic acid extract prior to amplification increased the number of positive results to 8 and 11 for conventional NASBA, to 5 and 8 for the *L. pneumophila* real-time

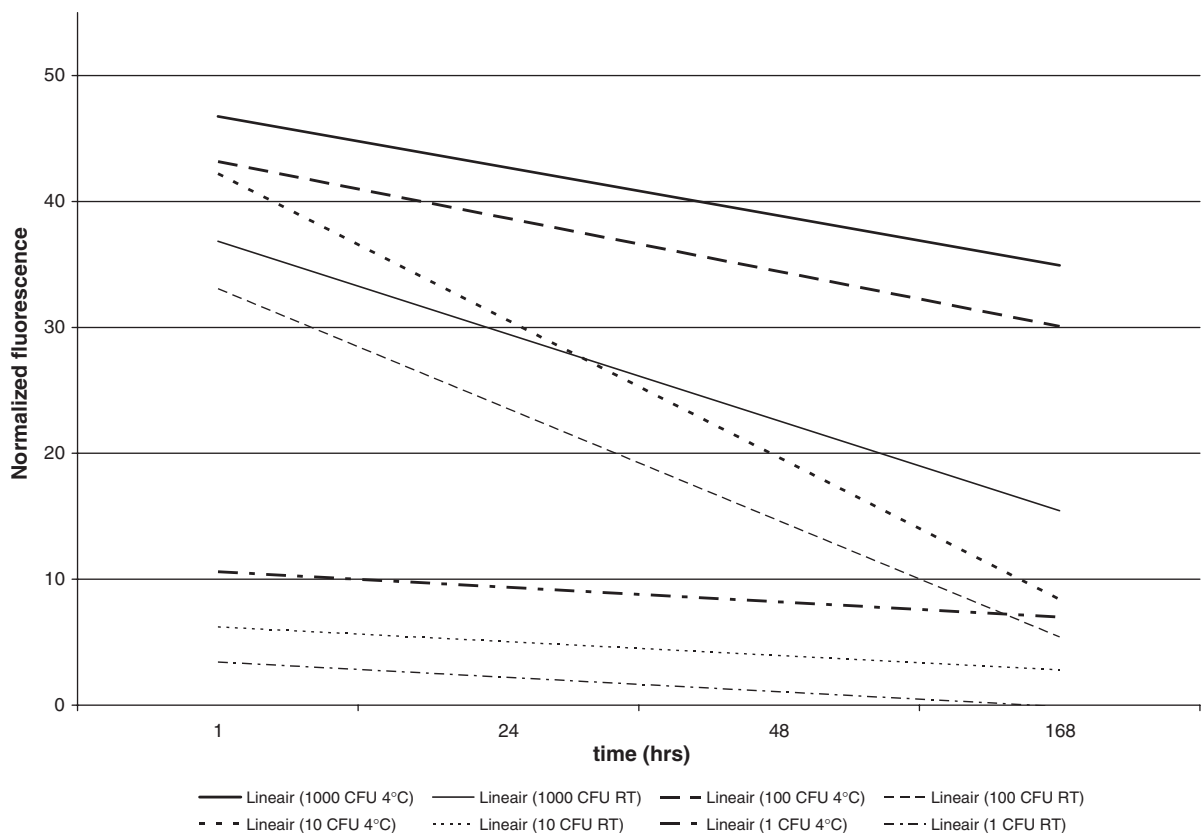


Fig. 1. *L. pneumophila* RNA degradation at 4 °C and at room temperature. Input in extraction (CFU). RT: room temperature.

Table 2  
Specimen characteristics from the Kapellen epidemic

No.	Specimen	PCR	Culture	NASBA ECL	Real-time NASBA L. pneu	Real-time NASBA L. spp.
1	Sputum	ND	–	–	–	–
2	Sputum	ND	–	–	–	–
3	Sputum	ND	–	–	–	–
4	Sputum	ND	–	–	–	–
5	Sputum	ND	–	–	–	–
6	Sputum	ND	–	–	–	–
7	Lung	+	+	+	+	+
8	Lung	+	+	+	+	+
9	Lung	+	–	+	+	+
10	Sputum	+	–	+	–	–
11	Sputum	NI	–	–	–	–
12	Sputum	+	+	+	+	+
13	Sputum	+ <sup>a</sup>	–	–	–	–
14	Sputum	ND	–	–	–	–
15	Bile fluid	–	–	–	–	–
16	Pl. fluid	–	–	–	–	–
17	Sputum	+	–	+	+	+
18	Lung	+	+	+	+	+
19	Lung	+	+	+	+	+
20	BAL	ND	–	+	–	–
21	BAL	+	–	+	+	+
22	Sputum	–	–	+	–	–
23	Sputum	+	–	+	+	+
24	Sputum	+	+	+	–	+
25	BA	–	–	–	–	–
26	Sputum	–	–	–	–	–
27	NA	–	–	–	–	–
28	NA	ND	–	–	–	–

BAL: bronchoalveolar lavage, BA: bronchus aspirate, L. pneu: *L. pneumophila*, L. spp.: *Legionella* spp., NA: nasopharyngeal aspirate, ND: not done, NI: not interpretable, Pl. fluid: pleural fluid.

<sup>a</sup> PCR positive for *Legionella* but not *L. pneumophila*. It was sampled on November 15, 2000 and it arrived in our laboratory on November 29, 2000.

NASBA and to 9 and 9 for the *Legionella* spp. real-time NASBA, respectively. NASBA failed to detect *Legionella* spp in sputum from patient 13 (Table 2). One PCR negative respiratory specimen and 1 specimen not examined by PCR were found positive by conventional NASBA (Table 2, patient nos. 20 and 22).

#### 4. Discussion

The aim of this study was to develop a conventional and a real-time NASBA assay to detect *L. pneumophila* based on 16S rRNA in clinical specimens. For real-time NASBA, two different assays were evaluated. The *L. pneumophila* real-time NASBA had a very low sensitivity for some *L. pneumophila* serotypes and *Legionella* species. Therefore, the *Legionella* spp. real-time NASBA was designed. With this new assay the clinically

most relevant *Legionella* serotypes and species, *L. pneumophila* serotypes 1 and 6, *Legionella micdadei*, *Legionella bozemanii* and *Legionella dumoffii* were tested and indeed detected.

A comparable detection level was obtained with 15 *L. pneumophila* serotype 1 strains whereas the detection level of *L. pneumophila* serotype 6 strains varied from strain to strain. This might be explained by the findings of McKinney et al. (1989) who reported that the mean genetic diversity for serotype 6, as determined from the degree of variability at 20 loci (McKinney et al., 1989), was found to be essentially the same as that for *L. pneumophila* subsp. *pneumophila* as a whole.

The sensitivity in spiked clinical specimens was determined with a *L. pneumophila* serotype 1 strain. In our hands the detection limit for *L. pneumophila* serotype 1 in clinical specimens depended on the respiratory specimens tested and varied between 10 CFU/ml and >100,000 CFU/ml for the more difficult specimens for both conventional and real-time NASBAs. Rantakokko-Jalava and Jajava found detection levels of 2000–200,000 CFU/ml in spiked BAL and sputum specimens with conventional PCR and of 20–200,000 CFU/ml in the same specimens with real-time PCR (Rantakokko-Jalava and Jajava, 2001). In their hands the use of the High Pure PCR Template Preparation Kit increased the analytical sensitivity to 2–200,000 CFU/ml and to 200–2000 CFU/ml after application of their conventional and real-time PCR respectively. We could increase the sensitivity of our assays to 10–1000 CFU/ml by diluting the nucleic acid extract 5-fold, suggesting the presence of inhibitors in the undiluted samples when tested on spiked clinical specimens and on documented specimens from a *L. pneumophila* serotype 1 epidemic in Kapellen, Belgium (de Schrijver et al., 2003). All *L. pneumophila* serotype 1 culture and/or PCR positive specimens were positive by conventional NASBA. Moreover, two additional specimens were found to be *Legionella* positive. One of these specimens was from a patient who was clinically suspected of having a *Legionella* pneumonia, although it was never confirmed with culture, PCR or a urinary antigen test. However, paired serum samples showed IgG seroconversion for *L. pneumophila*. The other patient also visited the fair in Kapellen, presented with respiratory tract symptoms at the hospital but did not develop pneumonia. There was insufficient material available from these patients to confirm our findings. A third discordant result was obtained with a sputum specimen from the outbreak: *Legionella* PCR was positive but probe hybridization revealed that it was positive for a *Legionella* species

other than *L. pneumophila*. This sample was negative with both conventional and real-time NASBAs. This might result from the delay in transmission of the specimen that arrived in our laboratory 14 days after sampling. Since RNA stability is critical for RNA based amplification techniques such as NASBA and weakly positive *L. pneumophila* specimens become NASBA negative after storage for a week at 4 °C (Fig. 1), RNA degradation seems to be the factor to blame.

Most degradation of *L. pneumophila* RNA seems to occur within 48 h (Fig. 1) especially when high numbers of organisms are present. Our findings correlate with those of Bruisten et al. (1997) studying the stability of HIV-1 RNA in whole blood, plasma and serum, before and after addition of lysis buffer. The authors concluded that the specimens could be kept at 4 °C provided that transportation time was as short as possible since most RNA degradation is likely to occur during transportation of the specimens. Specimens should preferably be processed on the day of sampling or stored at –70 °C thus stabilizing the RNA for at least 6 months as found previously for NASBA for *M. pneumoniae* (Loens et al., 2002), for *C. pneumoniae* (Loens et al., 2006) and for human rhinoviruses (Loens et al., in press).

One of the negative clinical samples was positive by conventional and real-time NASBAs although this could not be confirmed by a *L. pneumophila* specific PCR.

NASBA inhibitors may be present in clinical specimens and give rise to false negative or invalid results. In the present study false negative results were observed by the absence of a positive U1A NASBA result with undiluted nucleic acid extracts from bronchus aspirate, sputum, and lung biopsy samples indicating that an internal control is indispensable and needs to be implemented for the reliable detection of *L. pneumophila* in respiratory specimens.

The application of the *Legionella* spp. real-time assay increased the sensitivity of our real-time NASBA suggesting that the differences between both real-time assays might be due to the characteristics from the two beacons and the primers used.

Conventional NASBA seems to be more sensitive than the *L. pneumophila* real-time assay, especially when weakly positive samples are analyzed (ECL counts below 20000). This is in line with results of Hardegger et al. (2000) reporting that their real-time PCR slightly reduced the sensitivity as compared to conventional PCR for the detection of *M. pneumoniae*. The reasons for this are unclear but may be related to the different assay formats. However, we conclude that conventional and real-time NASBA, show high con-

cordance in test results with clear advantage of the real-time technology regarding handling, speed and number of samples that can easily be analyzed in a single run.

In summary, we showed that both conventional and real-time NASBA provide a sensitive and specific method for the detection of *Legionella* and that there is less intra- and interrun variation with the real-time NASBA compared to conventional NASBA with ECL detection. With the number of *L. pneumophila* positive samples that we could investigate, the *Legionella* spp. real-time NASBA assay described is promising for the detection of *Legionella* spp. in respiratory specimens although a larger number of clinical specimens need to be analyzed.

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