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Detection of *Mycoplasma pneumoniae* by Real-Time Nucleic Acid Sequence-Based Amplification

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Real-time isothermal nucleic acid sequence-based amplification (RT-NASBA) was applied to the detection of *Mycoplasma pneumoniae*. In vitro-generated *M. pneumoniae* RNA was used to assess the sensitivity of the assay. The 95% hit rate was 148 molecules of *M. pneumoniae* RNA in the amplification and 10^4 molecules of in vitro-generated RNA after nucleic acid extraction. The sensitivity of the RT-NASBA and the conventional NASBA assays corresponded to 5 color-changing units (CCU) of *M. pneumoniae*. In spiked throat swabs, nasopharyngeal aspirates, bronchoalveolar lavages, and sputum, the sensitivity of both NASBA assays corresponded to 5 to 50 CCU of *M. pneumoniae*. A total of 17 clinical specimens positive for *M. pneumoniae* by PCR were also positive by conventional NASBA, but one specimen was negative by RT-NASBA. These results indicate that the sensitivity of detection of *M. pneumoniae* by RT-NASBA in respiratory samples might be slightly reduced compared to that by conventional NASBA. However, the real-time assay is superior in speed and ease of handling.

Mycoplasma pneumoniae is a common etiologic agent of respiratory tract infections in humans and is responsible for 15 to 20% of all cases of pneumonia (4) and a wide range of mild to serious extrapulmonary complications (2, 8).

In the past, diagnosis of infection by this organism was usually based on serology, because growth in culture is slow and insensitive (6, 11). Therefore, nucleic acid amplification techniques have been introduced. PCR of fragments of the P1 gene or the 16S rRNA gene were shown to be considerably more sensitive than culture for the detection of *M. pneumoniae* (3, 7, 10, 17).

Nucleic acid sequence-based amplification (NASBA; bioMérieux, Boxtel, The Netherlands) is targeted at RNA. It makes use of the simultaneous enzymatic activities of avian myeloblastosis virus reverse transcriptase, RNase H, and T7 RNA polymerase under isothermal conditions.

Real-time NASBA uses molecular beacons, which are DNA hybridization probes that fluoresce only upon hybridization with their targets (12, 19). They have a stem-loop structure and contain a fluorophore and a quencher group. In its normal state, the stem keeps the fluorophore and the quencher together, preventing emission of fluorescence. In the presence of a sequence that is complementary to the loop sequence, the probe unfolds upon hybridization, the quencher no longer absorbs photons emitted by the fluorophore, and the probe starts to fluoresce. The whole process of amplification and detection takes place in a fluorescence reader. The produced amplicons can be used for conventional electrochemiluminescence (ECL) detection, allowing a comparison between both detection formats. Other real-time detection formats that are used with PCR, such as SYBR Green or the use of fluorescence-labeled oligonucleotide probes coupled to the endogenous 5′ exonuclease activity of *Taq* DNA polymerase as a means of cleaving a quenched fluorescent moiety from the probe (13), cannot be applied to NASBA reactions.

Ovyn et al. previously described the use of NASBA for the typing of *M. pneumoniae* strains and isolates (16) as well as for the detection of *M. pneumoniae* in spiked respiratory specimens (14). Here we applied real-time NASBA for the detection of *M. pneumoniae* RNA in clinical specimens and compared it with conventional NASBA on a number of clinical samples.

The bacterial strains used were described previously (14). Briefly, *M. pneumoniae* types 1 and 2, *M. fermentans*, *M. hominis*, *M. genitalium*, *M. orale*, *M. buccale*, *M. salivarium*, *M. pirum*, *M. arthritidis*, and *Ureaplasma urealyticum* were grown in cultures in spiroplasma (SP4) medium (18) without thallium acetate and supplemented with amphotericin B (0.5 mg/ml), polymyxin B (500 U/ml), glucose (0.5%), and arginine (0.25%) or urea (0.5%), depending on the nutritional needs of the species. *Legionella pneumophila*, *Haemophilus influenzae*, *Moraxella catarrhalis*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, viridans group streptococci, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli*, *Neisseria meningitidis*, and *Pseudomonas aeruginosa* were grown in cultures with supplements chosen depending on the nutritional needs of the species. *Chlamydia pneumoniae* was grown in cultures on HEp-2 cells.

M. pneumoniae strain PI 1428 was quantitated by incubation of 10-fold dilutions in SP4 medium at 37°C. The cultures were monitored for color change during 2 months. The titer was expressed in color-changing units (CCU) per milliliter. One CCU corresponds to 10 to 100 cells (1).

Throat swabs, bronchoalveolar lavages (BAL), nasopharyngeal aspirates (NA), sputum, and bronchus aspirates (BA) were obtained from the University Hospital Microbiology Laboratory and tested either as individual specimens or in pools of

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at least 10 specimens. Single and pooled samples were subjected to identical treatments. All had been previously tested and found to be negative for *M. pneumoniae* by PCR (7).

A total of 117 respiratory specimens from hospitalized patients with acute lower-respiratory-tract infections previously found to be *M. pneumoniae* negative ($n = 100$) or positive ($n = 17$) by PCR (7) were also analyzed by real-time and conventional NASBA.

Nucleic acids were extracted using a NucliSens basic kit extraction module (bioMérieux) (15) and stored at −80°C.

NASBA amplification was performed using a NucliSens basic kit amplification module with OT2157 (5′ AATTCTAATACGACTCACTATAGGGAGGTCCTTTCAACTTTGATTCA 3′) and OT2156NBK (5′ GATGCAAGGTCGCATATGAGGATCCTGGCTCAGGATTAA 3′); for ECL detection, amplicons were detected with the NBK ECL probe 5′ GATGCAAGGTCGCATATGAG 3′ in combination with the biotin capture probe pd1256 (5′ ATAATGGGGGATAACTAGTT 3′) (9). Detection with the NBK ECL probe and pd1256 was considered positive when the signal reached >0.02× that of the NBK reference solution (15). A molecular beacon, Mycobeacon01 (5′ FAM-CCATGGGTTGAAAGACTAGCTAATACCATGG-Dabsyl 3′), was constructed and hybridized only with *M. pneumoniae* amplification products.

The analytical sensitivity of the real-time *M. pneumoniae* 16S rRNA NASBA was compared to the sensitivity of the conventional NASBA in combination with ECL detection on 10-fold dilutions of suspensions of *M. pneumoniae* PI 1428 or dilutions of wild-type in vitro-generated RNA in water (14). To calculate the 95% hit rate with in vitro RNA, SAS (Cary, N.C.) version 6.12 software was used. Tenfold dilutions of *M. pneumoniae* PI 1428 added in quadruplicate to samples of the respiratory pools before protease treatment (14) were used to study the clinical sensitivity.

The intrarun and interrun variations in real-time and conventional NASBA were estimated by running a dilution series (50, 500, and 5,000 CCU/100 μl of sample) consisting of *M. pneumoniae* added in duplicate to BAL pools and analyzing five replicates of each nucleic acid extract.

M. pneumoniae 16S rRNA NASBA with primers OT2156NBK and OT2157 and the molecular beacon Mycobeacon01 for nucleic acid extracts from *M. pneumoniae* types 1 and 2 resulted in 100% specificity. Whereas conventional NASBA produced a positive result with *M. genitalium* RNA, this was not the case for the real-time NASBA. Molecular beacons are known to be more specific than their conventional counterparts (19), and obviously, the single mismatch towards the center of the detection region in *M. genitalium* 16S rRNA is sufficient to prevent the molecular beacon from interacting with amplicons generated from this target RNA. The biotin capture probe, on the other hand, matches 100% with both the *M. pneumoniae* and *M. genitalium* amplicons, yielding a positive result with both organisms.

When immediately added to the amplification reactions, the 95% hit rate for the analytical sensitivity of the *M. pneumoniae* 16S rRNA NASBA primers tested on dilutions of in vitro-generated WT RNA was 148 molecules. When extraction (i.e., the isolation of in vitro-generated RNA from lysis buffer) was done prior to the amplification, 5×10^4 molecules were needed for a 100% hit rate in the amplification reaction. However, it should be mentioned that only 10% of the extracted nucleic acid was used in the amplification reaction. When applied on dilutions of nucleic acids extracted from a culture of *M. pneumoniae*, the analytical sensitivity of the assay was 5 CCU; in both real-time and conventional NASBA on spiked clinical specimens, it ranged between 5 and 5×10^3 CCU (Table 1). No difference in sensitivity between the assays was seen.

TABLE 1. Sensitivity of real-time and conventional NASBA spiked in quadruplicate in pools of respiratory specimens

Sample	No. of samples with correct results/total no. of samples tested at a CCU value[a] of:				
	0	5	50	500	5,000
Lysis buffer[b]	0/4	3/4	4/4	4/4	ND[e]
Lysis buffer[c]	0/4	3/4	4/4	4/4	ND
Throat swab[b]	0/4	2/4	3/4[d]	4/4	ND
Throat swab[c]	0/4	2/4	4/4	4/4	ND
BAL[b]	0/4	2/4	4/4	4/4	ND
BAL[c]	0/4	2/4	4/4	4/4	ND
NA[b]	0/4	2/4	3/4	4/4	ND
NA[c]	0/4	3/4	3/4	4/4	ND
BA[b]	0/4	ND	1/4	2/4	4/4
BA[c]	0/4	ND	1/4	2/4	4/4
Sputum[b]	0/4	ND	3/6	4/6	5/6
Sputum[c]	0/4	ND	4/6	4/6	4/6

[a] Each CCU value is the number of CCU in nucleic acid extraction/100 μl of sample.
[b] Results of real-time NASBA.
[c] Results of conventional NASBA.
[d] Underlined numbers represent differences in number of samples with correct results between real-time and conventional NASBA.
[e] ND, not done.

The intrarun variability coefficients for the real-time detection of 50, 500, and 5,000 CCU were 26.4, 8.1, and 4.6, respectively; the interrun variation coefficients for the same inputs were 34.8, 10.7, and 13.9, respectively. The intrarun variation coefficients for the electrochemiluminescence detection of 50, 500, and 5,000 CCU were 61.8, 45.3, and 28.2, respectively; the interrun variation coefficients for the same inputs were 57.7, 78.2, and 42.6, respectively. The superior results obtained by the real-time technology may be ascribed to less sample handling and a different technology. The very good results of the intrarun variation coefficients also underline the robustness of the test.

A total of 16 out of 17 clinical specimens positive for *M. pneumoniae* by PCR and conventional NASBA were also positive by real-time NASBA. One specimen was repeatedly negative by real-time NASBA even after 1/5 and 1/10 dilution of the nucleic acid extract prior to amplification. However, the ECL counts of this sample were below 25,000, which is a weakly positive result indicative of a low concentration of the pathogen in the underlying specimen. Similar observations of somewhat lower sensitivity of the real-time NASBA compared to that of the conventional NASBA have been reported by Greijer et al. (5). None of 100 PCR-negative and conventional NASBA-negative clinical specimens gave real-time NASBA-positive results.

We conclude that real-time NASBA and conventional NASBA show high concordance in sensitivity and specificity, with a clear advantage for the real-time technology regarding handling, speed, and number of samples that can easily be

tested in a single run. Furthermore, the real-time NASBA assay, requiring fewer manipulations and producing results without postamplification processing, reduces the potential risk for product carryover. With the number of *M. pneumoniae*-positive samples we investigated, the real-time NASBA assay described for the detection of *M. pneumoniae* in respiratory specimens is promising. A large number of clinical specimens from patients with community-acquired pneumonia or other lower-respiratory-tract infections should be analyzed for further evaluation of the assay.

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