

RNA Amplification by Nucleic Acid Sequence-Based Amplification with an Internal Standard Enables Reliable Detection of *Chlamydia trachomatis* in Cervical Scrapings and Urine Samples

SERVAAS A. MORRÉ,¹ PETER SILLEKENS,² MARCEL V. JACOBS,¹ PIERRE VAN AARLE,²
SJOERD DE BLOK,³ BOB VAN GEMEN,² JAN M. M. WALBOOMERS,¹ CHRIS J. L. M. MEIJER,¹
AND ADRIAAN J. C. VAN DEN BRULE^{1*}

Department of Pathology, Section of Molecular Pathology, University Hospital Vrije Universiteit, 1081 HV Amsterdam,¹
NASBA Development Unit, Organon Teknika B.V., 5281 RM Boxtel,² and Department of Obstetrics and Gynaecology,
OLVG Hospital, 1091 HA Amsterdam,³ The Netherlands

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In the present study, the suitability of RNA amplification by nucleic acid sequence-based amplification (NASBA) for the detection of *Chlamydia trachomatis* infection was investigated. When comparing different primer sets for their sensitivities in NASBA, use of both the plasmid and *omp1* targets resulted in a detection limit of 1 inclusion-forming unit (IFU), while the 16S rRNA appeared to be the most sensitive RNA target for amplification (10^{-3} IFU). In contrast, for DNA amplification by PCR, the plasmid target was optimal (10^{-2} IFU), which is 10 times less sensitive than rRNA NASBA. To exclude false negativity in NASBA detection because of inhibition of amplification and/or inefficient sample preparation, an internal standard was developed. The internal control was added prior to sample preparation. This 16S rRNA NASBA with an internal control was compared with a plasmid DNA PCR by using a group of *C. trachomatis*-negative ($n = 41$) and -positive ($n = 37$) cervical scrapings, as determined by enzyme immunoassay (EIA). In addition, urine samples from the EIA-positive women were tested ($n = 17$). Both NASBA and PCR assays were able to detect *C. trachomatis* in all EIA-positive cervical scrapings, the corresponding urine samples, and two samples from the EIA-negative group. The internal NASBA standard was found clearly in all EIA-negative samples. In conclusion, these results indicate that detection of *C. trachomatis* by RNA amplification by NASBA with an internal standard is a suitable and highly sensitive detection method, with potential use in the diagnosis of urogenital *C. trachomatis* infections with cervical scrapings as well as urine specimens.

Chlamydia trachomatis infection is a leading cause of sexually transmitted disease in the United States and Europe, where 3×10^6 to 4×10^6 and 3×10^6 cases, respectively, are reported annually (6, 9). *C. trachomatis* infection may cause urethritis, cervicitis, and pelvic inflammatory disease in females. Many of these *C. trachomatis* infections run an asymptomatic course. Because these infections remain undetected and subsequently untreated, they may result in severe sequelae like ectopic pregnancy and tubal infertility (17).

Until now, *C. trachomatis* infections have been diagnosed by growth of the microorganism in cell culture or by non-culture-based techniques (e.g., direct immunofluorescent staining with monoclonal antibodies, enzyme immunoassays [EIAs], and direct DNA hybridization [8, 22, 34]). While for cell culture viable *Chlamydia* particles are necessary, the other conventional microbiological assays (direct immunofluorescence and EIA) have limitations in sensitivity and specificity (23, 29).

Recently, nucleic acid amplification techniques have been successfully introduced for the routine diagnosis of *C. trachomatis* infections. They have the advantage of higher sensitivities and specificities compared with those of conventional tech-

niques for the detection of *C. trachomatis* (23, 29). In addition to homemade PCR assays (21, 26), commercially available systems can also be used, i.e., Amplicor (PCR; Hoffmann-La Roche) and the ligase chain reaction (Abbott Diagnostic Division). Both the PCR (13, 18) and the ligase chain reaction (33) assays that have been described have the advantage that they use simple pretreatment of clinical samples like cervical scrapings and urethral swabs, which facilitates routine diagnosis. The reliable detection of *C. trachomatis* in urine specimens from both men and women by amplification methods (3, 7, 39) represents a significant development. This noninvasive sampling will greatly facilitate large screening programs for asymptomatic *C. trachomatis* infection and will also facilitate epidemiological studies.

Although PCR and ligase chain reaction show improved sensitivities and specificities compared with those of EIA and direct immunofluorescence, these DNA amplification techniques also detect nonviable *C. trachomatis*, for example, in patients with recently cured infections or self-limiting infections. On the other hand, the detection of microorganisms by an RNA-based amplification technique will probably be more sensitive because of the presence of multiple RNA copies and might strongly imply biological activity. In addition, the amplification methods used to date often lack controls to exclude false-negative results. These could result from improper sample preparation or inhibition of amplification. In some cases, human β -globin-specific PCRs are used to check for the suitability of a processed sample for amplification purposes (17,

* Corresponding author. Mailing address: Department of Pathology, Section of Molecular Pathology, University Hospital Vrije Universiteit, De Boelelaan 1117, 1081 HV, Amsterdam, The Netherlands. Phone: 31-20-4440503 and 31-20-4444023. Fax: 31-20-4442964. Electronic mail address: vandenbrule@azvu.nl.

31). However, the need for a proper marker is evident and must be evaluated.

Recently, the RNA amplification techniques nucleic acid sequence-based amplification (NASBA; Organon Teknika [14]) and 3SR (self-sustained sequence replication [11]) have appeared. NASBA is a powerful RNA amplification method which makes use of the simultaneous enzymatic activities of avian myeloblastosis virus reverse transcriptase, RNase H, and T7 RNA polymerase under isothermal conditions, resulting in a 10^9 -fold amplification of the target RNA. The NASBA technique has already been successfully applied for the detection of human immunodeficiency virus type 1 (HIV-1) RNA (5, 13, 40–42), citrus tristeza virus (16), and *Campylobacter jejuni* in foods (36) and for the detection and identification of mycobacteria (37, 38).

In this study, the suitability of the NASBA technique for the detection of *C. trachomatis* RNA was investigated. Therefore, *C. trachomatis*-specific primer sets were compared for their sensitivities in NASBA by using RNA from three different target genes: the gene coding for the major outer membrane protein (MOMP), the 16S rRNA gene, and the endogenous plasmid of *C. trachomatis*. Furthermore, NASBA was compared with PCR by using the most sensitive targets. To exclude either inhibition of amplification or inefficient sample preparation, an internal standard, which is not amplified by NASBA in the presence of wild-type *C. trachomatis*, was developed. The performance of NASBA was evaluated with cervical scrapings and urine specimens.

MATERIALS AND METHODS

***C. trachomatis* strain and specimen collection.** The serovar L2 strain of *C. trachomatis* was used in this study (30). Cervical scrapings and first-void urine specimens were obtained from patients attending the Department of Gynaecology, OLVG Hospital, Amsterdam. Those patients presenting with a variety of gynecological complaints were routinely tested for *C. trachomatis* by EIA (Chlamydiazyme; Abbott Diagnostic Division). Samples were collected with cervical brushes (Rover BV) placed in 3 ml of lysis buffer (50 mM Tris HCl [pH 6.4], 20 mM EDTA, 1.3% [wt/vol] Triton X-100, 5.25 M guanidinium thiocyanate, and after vortexing, the samples were transported to the laboratory at room temperature on the day of collection. The scrapings were further diluted with 7 ml of lysis buffer and were stored at -80°C . First-void urine samples were collected in sterile containers, transported at room temperature, and processed immediately by centrifuging 1.5 ml at 14,000 rpm (Merck Eppendorf centrifuge 5415C) for 10 min. The supernatants were discarded, and the pellets were stored at -80°C .

***C. trachomatis* L2 dilution series.** *C. trachomatis* serovar L2 was cultured as described previously (30). The titer of the *Chlamydia* stock was estimated as 600 inclusion forming units (IFU) per μl . Nucleic acid from the *C. trachomatis* stock was extracted as described by Boom et al. (4). Briefly, 100 μl (60,000 IFU) of the *Chlamydia* stock was lysed in 1 ml of lysis buffer. The nucleic acid released in the lysate was bound to activated silica (70 μl of silica [Fluka] as a 1-g/ml suspension in 0.1 N HCl), which was added to the lysate prior to 10 min of incubation at room temperature. After washing and drying of the silica, nucleic acid was eluted in 100 μl of distilled water and a dilution series ranging from 10^1 to 10^{-4} IFU was made. Nucleic acid was stored at -80°C .

Pretreatment of clinical specimens for NASBA and PCR. To 1 ml of the lysed cervical cell suspension, 70 μl of activated silica was added, and the mixture was incubated for 10 min at room temperature, with vortexing every minute. The scrapings were subsequently processed as described above, except that the DNA or RNA was eluted in 75 μl of distilled water. The silica-based method was used for DNA or RNA purification from the 1.5-ml urine pellets, as described for the cervical scrapings, after adding 1 ml of lysis buffer to the urine pellets.

Internal NASBA standard. To generate internal standard RNA, a transcription vector was constructed. The vector contained the wild-type *C. trachomatis* target of interest (plasmid, the *omp1* gene, or the 16S rRNA gene) in which an additional DNA sequence was cloned. The wild-type *C. trachomatis* target of interest was cloned into pG30 (a pGEM3 derivative [Promega] lacking the *Pst*I and *Sph*I sites in the multiple cloning sequence and into which a new *Bam*HI site has been introduced after removing the *Bam*HI site in the multiple cloning sequence). The additional DNA sequence is a 134-bp fragment with 5' *Pst*I and 3' *Sph*I ends from the HIV-1 pv22 sequence (24) comprising nucleotides 1015 to 1146 from the 5' noncoding region. The cloning of this fragment into the wild-type *C. trachomatis* target of interest is described in detail in the Results section.

Both the wild-type and the internal standard plasmids were linearized with *Bam*HI. In vitro RNA was generated from these two constructs by using T7 RNA

polymerase as described previously (32). The reaction mixtures were treated with DNase I to remove plasmid DNA. After phenol extraction and ethanol precipitation, the recovered in vitro-generated RNA was quantitated spectrophotometrically. All RNA stocks were stored at -80°C . From the wild-type stock a 10-fold dilution series was made from 2×10^5 to 2×10^{-1} molecules per μl , from which 5 μl was used for NASBA, generating positive controls from 10^4 to 10^0 target molecules in the final NASBA reaction mixtures.

From the internal standard or wild-type RNA, dilution series were made from 10^5 , 10^4 , 10^3 , and 10^2 molecules, and each of these was added to 1 ml of an EIA-negative cervical scraping. After nucleic acid extraction, 5 μl was used for NASBA to determine the optimal number of molecules of the internal standard which need to be spiked before sample preparation.

Detection by NASBA. Primers (see Table 1) specific for either *C. trachomatis* endogenous plasmid (primers OT1241-OT1240 and OT1583-OT1584), the *omp1* gene (primers OT1958-OT1960 and OT1828-OT1829), or 16S rRNA (primers OT1257-OT1259 and OT1257-OT1252) were synthesized and purified as described previously (14).

NASBA amplifications were carried out as described by Kievits et al. (14), with minor modifications. Reactions were performed in a 20- μl reaction mixture containing 40 mM Tris (pH 8.5; Sigma), 12 mM MgCl_2 (Sigma), 70 mM KCl (Baker), 5 mM dithiothreitol (Sigma), 15% (vol/vol) dimethyl sulfoxide (DMSO; Sigma), 1 mM (each) deoxynucleoside triphosphate (dNTP; Pharmacia), 2 mM rATP, rUTP, and rCTP and 1.5 mM rGTP (Pharmacia), 0.5 mM ITP (Boehringer), 2.1 μg of bovine serum albumin (Boehringer), 0.08 U of RNaseH (Pharmacia), 6.4 U of avian myeloblastosis reverse transcriptase (Seikagaku), 32 U of T7 RNA polymerase (Pharmacia), 0.2 μM (each) primer, and 5 μl of isolated nucleic acid. Reactions were performed at 41°C for 90 min and were stopped by placing the reaction mixtures on ice. For NASBA, precautions similar to those for PCR were taken to prevent contamination. Detection of the amplified product was done either by Northern blot analysis or enzyme-linked gel assay (ELGA) (40). For Northern blot analysis, 1.5 μl of NASBA RNA was separated through a 1% agarose gel and transferred to a nylon membrane (Qiabran; Qiagen). Hybridizations were performed with specific ^{32}P -end-labelled oligonucleotide probes (see Table 1). Autoradiography was performed at -80°C by using Kodak Royal X-Omat films and intensifying screens. For detection by ELGA, 3 μl of RNA from NASBA, 1 μl of $6 \times \text{SSC}$ ($1 \times \text{SSC}$ is 0.15 M NaCl plus 0.015 M sodium citrate), 1 μl of the layer mixture, and 1 μl of target-specific horseradish peroxidase (HRP) probe (see Table 1) at 3.3×10^{-8} M were incubated for 15 min at 45°C . Three microliters was used for electrophoresis on a 7% polyacrylamide-bisacrylamide gel containing 0.04% dextran sulfate. The gel was stained for 10 min on a rotation shaker until the blue bands became clearly visible by using a TMB substrate solution (0.5 ml of TMB-DMSO [5 mg/ml], 24.5 ml of TMB buffer [10 mM citrate, 10 mM EDTA; pH 4.8], and 2.5 μl of H_2O_2).

Detection by PCR. The plasmid-specific primers (purchased from Perkin-Elmer) used for PCR amplification are described in Table 1: CtP1-CtP2 (10) (identical to NASBA primers OT1541-OT1540 but without the T7 tail) and PL6.1-PL6.2 (identical to NASBA primers OT1583-OT1584 but without the T7 tail). PCRs for *C. trachomatis* were performed as described previously (18, 30) in a total volume of 50 μl containing 50 mM KCl, 1.5 mM MgCl_2 , 0.2 mM (each) dNTP, 25 μM of each primer, 1 U of *Taq* polymerase (Amplitaq; Cetus, Emeryville, Calif.), and 5 μl of isolated nucleic acid. Amplification by PCR consisted of denaturation for 4 min at 94°C . Forty cycles of amplification followed the denaturation step: each cycle consisted of 1 min at 95°C , 1 min at 55°C , and 1.5 min at 72°C . The final elongation step was extended for another 4 min. The amplified DNA was analyzed by 1.5% agarose gel electrophoresis and ethidium bromide staining. Additional Southern blot analysis was performed with pCtP5 and OT1570 as specific ^{32}P -end-labelled oligonucleotide probes (Table 1). Autoradiography was performed at -80°C with Kodak Royal X-Omat films and intensifying screens.

RESULTS

Comparison of different primer sets in NASBA and PCR. By using 10-fold dilutions (corresponding to 10^1 down to 10^{-4} IFU per 5 μl) of nucleic acid isolated from the *C. trachomatis* L2 stock, the sensitivities of different primer sets in NASBA were determined. From the three different targets used in NASBA, i.e., plasmid, *omp1*, and 16S rRNA, the 16S rRNA target appeared to be the most sensitive, as summarized in Table 2. Both 16S rRNA primer sets tested revealed a detection limit of 10^{-3} IFU, which is 1,000 times more sensitive than those of the *omp1* and plasmid targets. The three targets had identical sensitivities in both ELGA and Northern blot analyses (Table 2). In Fig. 1, the products generated by the 16S rRNA primer set by NASBA, resulting in an amplicon of 151 nucleotides, are shown. In addition to the wild-type and free HRP probe, a larger amplicon of approximately 300 nucleotides was generated (indicated by an arrow in Fig. 1). Although

TABLE 1. Sequences of primers and probes used in this study for NASBA and PCR amplification and detection

| Method and oligonucleotide | Sequence | Function | Generated length | Target |
|----------------------------|--|------------------------|---------------------|---------------------------|
| PCR | | | | |
| CtP1 | 5'-TAGTAACTGCCACTTCATCA-3' | Probe | 201 bp | Ct ^a plasmid |
| CtP2 | 5'-TTCCCTTGTAAATTCGTTGC-3' | | 201 bp | Ct plasmid |
| pCtP5 | 5'-ATCTCATTACCATGCATTAGCAGCTATCCA-3' | | Ct plasmid | |
| PL6.1 | 5'-AGAGTACATCGGTCAACGA-3' | Probe | 130 bp | Ct plasmid |
| PL6.2 | 5'-TCACAGCGGTTGCTCGAAGCA-3' | | 130 bp | Ct plasmid |
| OT1570 | 5'-CGTGCGGGTTATCTTAAAAGGGAT-3' | | Ct plasmid | |
| NASBA | | | | |
| OT1257 | 5'-AATTCTAATACGACTCACTATAGGGCTCGGATGCCAAATATCGCCACA-3' | T7 primer | 182 nt ^b | Ct 16S rRNA |
| OT1259 | 5'-GATCTTGGTTCAGATTGAACGCTG-3' | | 182 nt | Ct 16S rRNA |
| OT1252 | 5'-GATGAGGCATGCAAGTCGAA-3' | | 151 nt | Ct 16S rRNA |
| OT1253 | 5'-AGCAAATTGTTTCGGCAATTGTTT-3' | Probe | | Ct 16S rRNA |
| OT1390 | 5'-NH ₂ AGCAATTGTTTCGGCAATTGTTT-3' | HRP probe ^c | | Ct 16S rRNA |
| OT1541 | 5'-AATTCTAATACGACTCACTATAGGGTTCCTTGTAAATTCGTTGC-3' | T7 primer | 226 nt | Ct plasmid |
| OT1540 | 5'-TAGTAACTGCCACTTCATCA-3' | | 226 nt | Ct plasmid |
| OT1542 | 5'-ATCTCATTACCATGCATTAGCAGCTATCCA-3' | Probe | | Ct plasmid |
| OT1734 | 5'-NH ₂ ATCTCATTACCATGCATTAGCAGCTATCCA-3' | HRP probe | | Ct plasmid |
| OT1583 | 5'-AATTCTAATACGACTCACTATAGGGCAAGAGTACATCGGTCAACGA-3' | T7 primer | 155 nt | Ct plasmid |
| OT1584 | 5'-TCACAGCGGTTGCTCGAAGCA-3' | | 155 nt | Ct plasmid |
| OT1570 | 5'-CGTGCGGGTTATCTTAAAAGGGAT-3' | Probe | | Ct plasmid |
| OT1733 | 5'-NH ₂ CGTGCGGGTTATCTTAAAAGGGAT-3' | HRP probe | | Ct plasmid |
| | 5'-AATTCTAATACGACTCACTATAGGGAGAGAATACATCAAACGATCCC-3' | T7 primer | 267 nt | Ct MOMP VD-1 ^d |
| OT1958 | | | | |
| OT1960 | 5'-TGCATCCTTGCACCACTTGG-3' | | 267 nt | Ct MOMP VD-1 |
| OT1959 | 5'-GACCGTGTTTT (C/A) AACAGAT-3' | Probe | | Ct MOMP VD-1 |
| OT1996 | 5'-NH ₂ GACCGTGTTTT (C/A) AACAGAT-3' | HRP probe | | Ct MOMP VD-1 |
| OT1828 | 5'-AATTCTAATACGACTCACTATAGGGAGACTACTGCAATACCGCAAGAT-3' | T7 primer | 226 nt | Ct MOMP VD-4 |
| OT1829 | 5'-TACATTGGAGTTAAATGGTCT-3' | | 226 nt | Ct MOMP VD-4 |
| OT1827 | 5'-ATGCAAATCGTTTCCTTGCA-3' | Probe | | Ct MOMP VD-4 |
| OT1995 | 5'-NH ₂ ATGCAAATCGTTTCCTTGCA-3' | HRP probe | | Ct MOMP VD-4 |

^a Ct, *C. trachomatis*.^b nt, nucleotides.^c HRP probes were used for detection by ELGA.^d VD, variable domain.

the origin of this amplicon is unknown, it is only observed in *C. trachomatis*-positive specimens. The 16S rRNA target was chosen as the target in further experiments in this study with the primer set which generates the smallest fragment (151 nucleotides; Table 1). A dilution series from wild-type in vitro-generated 16S rRNA in water showed an analytical sensitivity

of 10 to 100 molecules in NASBA. For PCR, only two plasmid primer sets were tested because the plasmid target has been shown to be the most sensitive in other studies (30). Table 2 shows that the sensitivities of both plasmid primer pairs in PCR were equal. An equivalent of 10⁻¹ IFU could be detected upon gel analysis. Compared with gel analysis, the sensitivity of

TABLE 2. Sensitivities of the primer sets for different targets of *C. trachomatis*

| Target | Primer set for NASBA or PCR | Sensitivity (IFU) | | |
|------------------|--|-------------------|-------------------|---|
| | | NASBA | | PCR ^a with Southern blotting |
| | | ELGA | Northern blotting | |
| 16S rRNA | OT1257-OT1259 OT1257-OT1252 | 0.001 0.001 | 0.001 0.001 | |
| <i>omp1</i> VD-1 | OT1958-OT1960 | 1 | 1 | |
| <i>omp1</i> VD-4 | OT1828-OT1829 | 1 | 1 | |
| Plasmid | OT1541-OT1540 or CtP1-CtP2 OT1583-OT1584 or PL6.1-PL6.2 | 1 1 | 1 1 | 0.01 0.01 |

^a Only the plasmid target was tested, since this target was previously found to be the most sensitive for PCR (30).

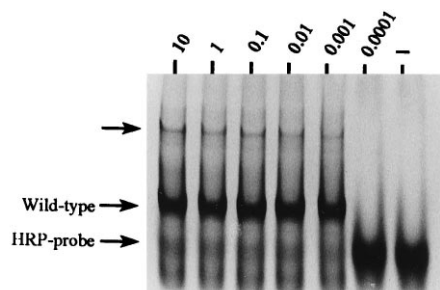


FIG. 1. NASBA on a 10-fold dilution series in water (10^1 to 10^{-4} IFU, plus a negative control) of *C. trachomatis* L2 nucleic acid with the OT1257-OT1252 primer set. The NASBA products were visualized after electrophoresis on a polyacrylamide gel (7%) by ELGA detection. The wild-type 16S rRNA (151 nucleotides) and the free HRP probe are indicated. At the top of the ELGA gel, the unknown amplifier is indicated with an unlabeled arrow.

PCR increased 10-fold after hybridization. The Ctp plasmid primer set was chosen for further experiments in this study.

Internal standard for NASBA. The most sensitive target for the *C. trachomatis*-specific NASBA is the 16S rRNA. Therefore, part of the cDNA comprising nucleotides 1 to 312 (serovar L2; GenBank/EMBL accession number M59178) was cloned into *EcoRI*-*Csp45I* sites in plasmid pG30. This pG30 16S cDNA (wild type) was used to construct an internal standard. The 134-bp non-*Chlamydia* fragment (see Materials and Methods) was introduced into the 16S cDNA by replacing the wild-type sequence between positions 96 and 118. To establish this, two PCR fragments were generated, i.e., fragment 1 with primers P1 (containing a 5' *EcoRI* site) and P2 (containing a 5' *PstI* site) and fragment 2 with primers P3 (containing a 5' *SphI* site) and P4 (containing a 5' *EcoRI* site). These two PCR fragments and the 134-bp fragment were digested with *PstI* and *SphI*, and subsequently, a triple ligation was made. The ligation product was subjected to PCR with the P1 and P2 primers. The generated PCR product of 416 bp and pG30 were digested with *EcoRI* and *Csp45I* and were subsequently ligated and transformed to *Escherichia coli* DH5 α (Fig. 2 outlines the cloning strategy). Nucleotide sequence analysis did not reveal any mutations in the primer or probe annealing sites. This plasmid was used for the large-scale generation of runoff transcripts of 1.2 kb after linearization with *Bam*HI.

Determination of the optimal concentration of the internal standard. To determine the optimal concentration of the internal standard which will not interfere with *C. trachomatis* wild-type 16S rRNA detection by NASBA, the following was done. First, a 10-fold dilution series of in vitro-generated internal standard RNA or wild-type RNA was made in a background of *C. trachomatis*-negative cervical scrapings (as determined by EIA and plasmid PCR). By NASBA, upon detection by ELGA, a minimum of 10^4 molecules of the internal standard could be detected (Fig. 3A). When only wild-type RNA was present, a detection limit of 10^4 molecules could be reached after ELGA (Fig. 3B). This optimal number of internal standard molecules was mixed with a wild-type dilution series to investigate the competitive character of the wild type compared with that of the internal standard. When adding 10^4 molecules of the internal standard RNAs to a dilution series of the wild-type rRNA, the detection limit of 10^4 molecules could still be reached, which indicates that the addition of internal standard did not interfere with the detection efficiency of wild-type 16S rRNA. Indeed, in cases in which no wild-type 16S rRNA was detected (Fig. 3C, lanes 3 and 4), the internal standard was clearly amplified. In those lanes in which wild-

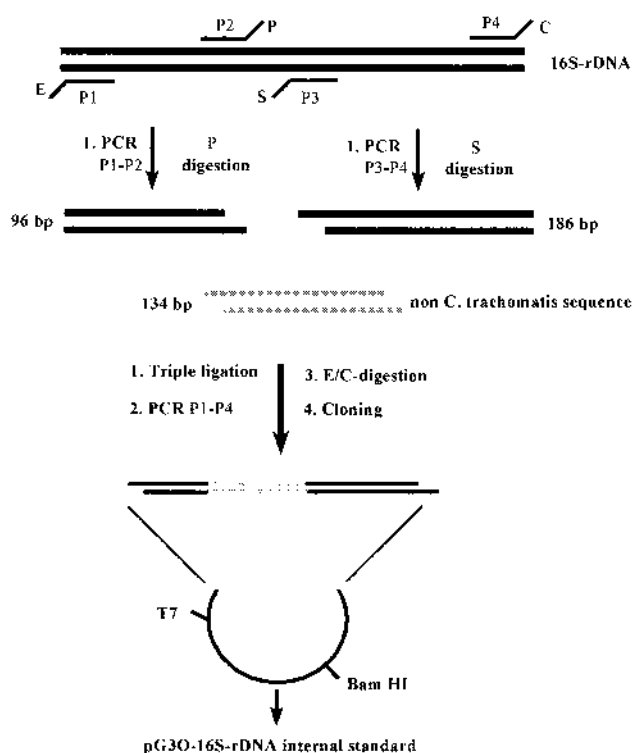


FIG. 2. Outline of the cloning of the NASBA 16S rRNA internal standard. E, *EcoRI*; P, *PstI*; S, *SphI*; C, *Csp45I*; P1 to P4, primers introducing the E, P, S, or E restriction sites.

type rRNA was amplified, the larger amplicon was generated at a low intensity, as mentioned above in the description of Fig. 1.

The minimum number of internal standard molecules which must be added before sample preparation to guarantee successful rRNA detection is 10^4 molecules. Although this seems to be rather high, the final number of molecules used in the NASBA reaction appears to be 200 molecules (per 5- μ l reaction mixture) when taking into account the fact that approximately 55% is lost during the silica-based extraction method

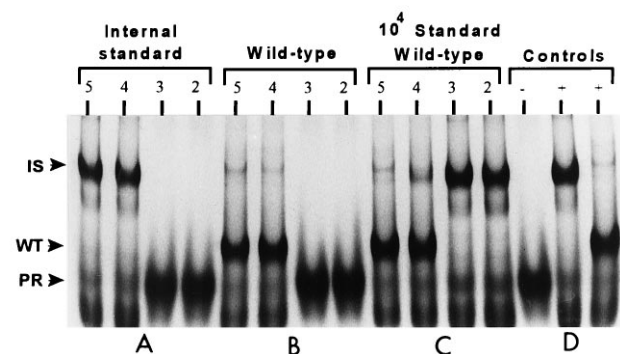


FIG. 3. *C. trachomatis* NASBA products detected by ELGA are shown for a dilution series (10^5 to 10^2 molecules) of in vitro-generated 16S rRNA added to cervical scrapings before nucleic acid isolation: (A) Internal standard: a 16S rRNA dilution of the internal standard. (B) Wild type: a wild-type 16S rRNA dilution. (C) The minimal amount of internal standard molecules (10^4 ; see panel A) with a dilution of the wild type. (D) Controls: NASBA negative, internal standard positive, and wild type positive. IS, internal standard (285 nucleotides); WT, *C. trachomatis* wild-type 16S rRNA (151 nucleotides); PR, probe.

TABLE 3. Comparison of *C. trachomatis* positivity by NASBA and PCR with clinical specimens

| Clinical specimen | No. positive/no. tested ^a | | |
|---------------------------------|--------------------------------------|---------------------|---------------------|
| | NASBA 16S rRNA | | PCR plasmid, CT |
| | CT | IS | |
| EIA-positive cervical scrapings | 36/37 | 1/37 | 36/37 |
| EIA-negative cervical scrapings | 2/41 | 39 ^b /41 | 2/41 |
| Urine samples | 16 ^c /17 | 1/17 | 16 ^c /17 |

^a CT, *C. trachomatis*; IS, 16S rRNA internal standard.

^b Including one sample showing inhibition or for which there was inefficient sample preparation. A new nucleic acid isolation resulted in a visible internal standard after NASBA detection.

^c Including one sample showing inhibition or for which there was inefficient sample preparation. A new nucleic acid isolation resulted in *C. trachomatis* positivity by NASBA and PCR.

(4). This was the concentration used in this study for analysis of the cervical scrapings. In a similar way, the minimum number of internal standard molecules was determined for urine specimens and also appeared to be 10^4 molecules (data not shown). Besides the 134-bp non-*C. trachomatis* fragment that was introduced, internal standards with a 63- and a 189-bp insert were also tested. However, the best results were obtained with the 134-bp fragment (data not shown), which was therefore used in the remainder of this study for the evaluation of NASBA with clinical samples.

Detection of *C. trachomatis* in cervical scrapings. Comparison of NASBA with PCR for the detection of *C. trachomatis* was performed with cervical scrapings from either *C. trachomatis* EIA-positive ($n = 37$) or -negative ($n = 41$) patients. The results are summarized in Table 3. Among the 37 EIA-positive cervical scrapings, 36 were positive for *C. trachomatis* by both NASBA and PCR. Indeed, for all *C. trachomatis*-positive samples, no amplification of the internal standard was found by NASBA. However, the remaining sample, which was negative for *C. trachomatis* by NASBA and PCR, showed no inhibition since the internal standard was successfully amplified. These results are presented in Fig. 4A. Of the five EIA-positive cervical scrapings, four showed a *Chlamydia*-specific fragment of

151 nucleotides (Fig. 4A, lanes 1 to 4), while one sample only generated the internal standard (285 nucleotides) (lane 5).

Among the 41 EIA-negative cervical scrapings, 2 samples were positive by both NASBA and PCR. The results are summarized in Table 3. The internal standard was successfully amplified from 38 samples which were also negative for *C. trachomatis* by PCR. However, the remaining sample, which was negative for *C. trachomatis* by NASBA and PCR, showed no amplification of the internal standard, indicating inhibition or inefficient sample preparation. These results are presented in Fig. 4C. Of the seven EIA-negative cervical scrapings, five showed the expected *Chlamydia* internal standard amplicon of 285 nucleotides (Fig. 4C, lanes 1 to 5), while in the two remaining samples, the *Chlamydia* amplicon of 151 nucleotides was amplified (lane 6) or no amplification occurred at all (lane 7).

Detection of *C. trachomatis* in urine specimens. Comparison of NASBA with PCR for the detection of *C. trachomatis* was also performed with first-void urine specimens from 17 patients of the group with *C. trachomatis* EIA-positive cervical scrapings. The results are summarized in Table 3. Among the 17 urine samples, 15 were positive by both NASBA and PCR. These *C. trachomatis*-positive urine samples showed no amplification of the internal standard by NASBA. However, one sample, negative for *C. trachomatis* by both NASBA and PCR, showed no inhibition since the internal standard was successfully amplified. The remaining sample showed no amplification at all, indicating inhibition or inefficient sample preparation. These results are presented in Fig. 4B, which contains urine samples from the five patients whose cervical samples were positive by EIA (Fig. 4A). Of the five urine samples, four showed a *Chlamydia*-specific amplicon of 151 nucleotides (Fig. 4B, lanes 1 to 4), while one sample only generated the internal standard (285 nucleotides) (lane 5). These results are identical to the results obtained for the corresponding cervical scrapings (Fig. 4A).

DISCUSSION

This study shows that detection of *C. trachomatis* RNA in clinical specimens by NASBA is as successful as the widely used DNA detection by PCR. From the three targets analyzed, i.e., RNA derived from the endogenous plasmid, the *omp1* gene, and the 16S rRNA gene, the rRNA gene proved to be the most sensitive target in NASBA, resulting in a detection limit of 10^{-3} IFU of *C. trachomatis* serovar L2. A sensitivity of less than 1 IFU can be reached since detection by nucleic acid amplification methodologies is not dependent on infectious *Chlamydia* particles, as previously shown by PCR (30). The improved sensitivity of the 16S rRNA target over those of the plasmid and *omp1* RNA targets is most likely due to the high expression and enhanced stability of the 16S rRNA. In contrast, in PCR it was previously shown that use of the plasmid target was more sensitive than use of either the rRNA gene or the *omp1* target (30), but in our hands, it was still 10 times less sensitive than the 16S rRNA NASBA.

For NASBA, an internal control was developed, since DNA and RNA amplification methods with clinical specimens might give false-negative results because of improper sample preparation, a possible loss of RNA during sample transport, or inhibition of amplification. Indeed, the internal standard was visible by ELGA detection for all *C. trachomatis*-negative clinical samples except for those samples for which inhibition or inefficient sample preparation was found (Table 3). For *C. trachomatis*-positive samples, only the wild-type sequence was amplified and no internal standard was detected (Fig. 4). Wild-

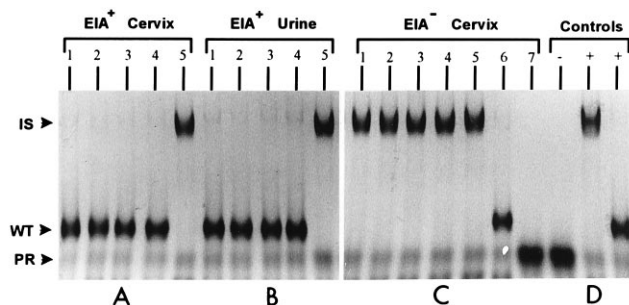


FIG. 4. *C. trachomatis* amplification by 16S rRNA NASBA with clinical samples after detection by ELGA. The results of NASBA are presented. (A) EIA⁺ Cervix, cervical scrapings from patients 1 to 5 positive for *C. trachomatis* by EIA. (B) EIA⁺ Urine, urine samples from patients 1 to 5 whose cervical scrapings were analyzed in panel A. (C) EIA⁻ Cervix, cervical scrapings from seven patients negative for *C. trachomatis* by EIA. (D) Controls: NASBA negative, internal standard positive, and wild-type positive. IS, internal standard (151 nucleotides); WT, *C. trachomatis* wild-type 16S rRNA (285 nucleotides); PR, probe.

type RNA is preferentially amplified because of its shorter length and the addition of a minimal amount of internal standard to the samples. In the future the NASBA internal standard will be added to the sample collection buffer for the additional monitoring of possible nucleic acid degradation during transport. As shown in other studies, an internal standard is indispensable for the reliable detection of microorganisms in clinical samples by DNA PCR (e.g., mycoplasmas [15]) or RNA PCR (*Mycobacterium* detection [35] and HIV-1 detection [42]). Indeed, depending on the source of the clinical specimens tested, high percentages of false-negative results (up to 15%) have been reported (15).

In the evaluation of the *C. trachomatis* detection in cervical scrapings, all results were identical for NASBA and PCR for the EIA-positive as well as for EIA-negative cervical scrapings. An EIA-positive sample (Table 3) negative by both NASBA and PCR had a clearly visible internal standard by ELGA detection (Fig. 4A, lane 5), suggesting that this sample was falsely positive by EIA. Also, the corresponding urine sample from this asymptomatic patient was negative for *C. trachomatis* by both PCR and NASBA, supporting false positivity by EIA. Among the 41 EIA-negative cervical scrapings, 2 samples were found to be positive by both NASBA and PCR. This indicates cervical scrapings false negative for *C. trachomatis* by EIA and supports previous reports showing the superiority of amplification methods in detecting *C. trachomatis* in clinical specimens (21, 23, 26, 29).

Of 39 cervical scrapings, 38 were negative for *C. trachomatis* by NASBA and PCR, and for these negative cervical scrapings, the internal standard was clearly visible by ELGA; no inhibition of NASBA amplification was shown for these samples. From the remaining EIA-negative sample which showed no internal standard (Fig. 4C, lane 7), nucleic acid was isolated again and tested. This time the internal standard was amplified, indicating inhibition or inefficient sample preparation the first time.

Also in urine samples derived from EIA-positive patients, *C. trachomatis* was successfully detected by both NASBA and PCR. However, aberrant results were found for two patients (Table 3). In one sample, which was *Chlamydia* negative by both PCR and NASBA, the internal standard was visible (Fig. 4B, lane 5). Interestingly, this is the urine sample from the patient with the false-positive cervical scraping as determined by EIA (Fig. 4A, lane 5). For another urine specimen no internal standard was visible by NASBA. However, a new extraction of nucleic acid from the sample resulted in successful amplification of both the wild-type 16S rRNA target by NASBA and the plasmid target by PCR, indicating inefficient extraction the first time.

The results obtained for the urine samples are identical to those obtained for the corresponding cervical scrapings, indicating the value of this noninvasive sampling method. The high sensitivity of the *Chlamydia* 16S rRNA NASBA with urine could be explained by the fact that the *C. trachomatis* 16S rRNA present is derived from lysed cells containing metabolically active reticulate bodies and/or the fact that the metabolically inactive *C. trachomatis* elementary bodies still contain approximately 10^3 16S rRNA molecules (2). The usefulness of urine for the detection of RNA is supported by others, although this was done by reverse transcription PCR (including reverse transcription PCR for enterovirus and rhinovirus RNAs [12, 25], human cytomegalovirus [28], hepatitis C virus RNA [20], and HIV-1 RNA [19]).

All results obtained by NASBA with cervical scrapings and urine specimens are identical to those obtained by plasmid PCR, which has proven to be a valid method for *C. trachomatis*

detection in several laboratories (21, 26, 30). In addition, the internal NASBA standard will prevent false-negative results. This indicates that NASBA is a sensitive and reliable amplification system. Moreover, all EIA-positive samples, which correspond to samples with high *C. trachomatis* loads, were confirmed to be positive by both PCR and NASBA. However, in this study only a small group of the EIA-negative cervical scrapings was evaluated. These scrapings were derived from asymptomatic women and could be weakly positive for *C. trachomatis*. The low frequency of *C. trachomatis* positivity (2 of 41 specimens; Table 3) by NASBA and PCR is in agreement with those obtained in other studies investigating asymptomatic populations. Although the PCR-positive specimens were also positive by NASBA, further study is needed to evaluate NASBA with a larger group of cervical scrapings and urine specimens, including samples either negative or only weakly positive for *C. trachomatis*. Preliminary data indicated that in another asymptomatic female population, identical *C. trachomatis* prevalences (5%) were found in urine specimens by either PCR or NASBA detection.

Use of the 16S rRNA target in NASBA will ensure detection of all *C. trachomatis*-positive patients. This is of importance since the endogenous plasmid is used as a target in most amplification systems for the routine diagnosis of *C. trachomatis*, and the existence of plasmid-free *C. trachomatis* variants has been reported (1, 2, 27). The use of these plasmid-based tests for *C. trachomatis* could result in *C. trachomatis*-positive patients being missed. Use of the 16S rRNA target will give reliable detection because of the presence of rRNA in all *Chlamydia* particles. A valuable application of RNA-based detection by NASBA compared with DNA-based detection methods might be the measurement of biological activity, i.e., the detection of transcriptionally active genes and the monitoring of microbial viability. To investigate the latter, a follow-up study of *C. trachomatis*-positive patients after antibiotic treatment is in progress, in that study DNA- and RNA-based amplification methods are being compared. A disadvantage of RNA-based amplification methods is the need to extract nucleic acids from cells, which is time-consuming, whereas PCR can be efficiently performed with crude cell suspensions (18). Automation of silica-based DNA and RNA extraction methods will greatly facilitate the use of NASBA for routine diagnosis.

In conclusion, in this study it was shown that NASBA is a powerful amplification technique with a high sensitivity for the detection of *C. trachomatis* 16S rRNA. The use of an internal standard for NASBA will exclude false-negative results. This assay enables the reliable and sensitive diagnosis of *C. trachomatis* infection not only in cervical scrapings but in urine specimens as well. If the sample processing could be automated, NASBA might be a powerful tool for the routine diagnosis of *C. trachomatis* infection and might be useful for large-scale epidemiological studies and screening programs.

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