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Real-Time PCR for *Chlamydia pneumoniae* Utilizing the Roche Lightcycler and a 16S rRNA Gene Target

Justin Hardick,* Nancy Maldeis,* Mellisa Theodore,* Billie Jo Wood,* Samuel Yang,† Shin Lin,† Thomas Quinn,*‡ and Charlotte Gaydos*

From the Johns Hopkins University, School of Medicine, Baltimore, Maryland; National Institute of Allergic and Infectious Diseases,† National Institutes of Health, Bethesda, Maryland; and the Department of Emergency Medicine,† Johns Hopkins University, School of Medicine, Baltimore, Maryland*

Chlamydia pneumoniae* (CPN) causes pneumonia in humans, and has emerged as an important respiratory pathogen. There are also established links between CPN infection and coronary artery disease. Traditional culture methods for CPN detection can be time consuming and difficult. There are a variety of molecular-based amplification methods for CPN detection. These methods are more sensitive than culture, but have the disadvantage of being inconsistent and non-comparable across studies. In this paper, we describe the adaptation of the existing primer set CPN 90/CPN91 for use in a real-time PCR assay using the Roche Lightcycler and a Taqman probe. This assay had an analytical sensitivity of between 4 and 0.4 infection-forming units (IFUs)/PCR reaction. A total of 355 samples were tested for validation of the assay. Tested samples included two standardized panels of blinded samples from culture (N = 70), archived specimens consisting of a CPN dilution series, CPN-spiked porcine aortal tissue and endarterectomy specimens (N = 87). The third group consisted of prospectively collected PBMCs from clinical samples (N = 198). Results were compared to nested PCR, which targets the *ompA* gene of CPN; TETR PCR, which targets the 16S rRNA gene of CPN; or the known result for the sample. Overall, the assay had a sensitivity of 88.5% (69 of 78) and a specificity of 99.3% (275 of 277). This method should prove useful for accurate, high throughput detection of CPN. *(J Mol Diagn 2004, 6:132–136)

Chlamydia pneumoniae (CPN) causes pneumonia in humans, and has associated links with coronary artery disease.[1–4] Culture and serology are the traditional methods of isolation and diagnosis, and each method has its own limitations.[5,6] Using culture for isolation and identification of CPN can be time consuming, costly, and potentially inaccurate because the organism is required to be viable for proper detection, and a dead organism cannot be grown in culture. Serology is also limited, as it provides only a retrospective diagnosis; cannot differentiate between previous, acute, or chronic infection; and can be subjective in nature.[5]

There are a variety of molecular methods available for CPN detection. These methods, including nested PCR and touchdown enzyme time release (TETR) PCR, have improved sensitivity over traditional methods of CPN detection.[5,7–9] However, these methods also have their limitations. Traditional PCRs typically rely on post-PCR processing for the determination of a result. In addition, recent reports suggest that there are inherent problems in using traditional PCRs for detection of CPN due to lack of standardization between laboratories that can result in increased variation.[5,10–12]

Real-time PCR offers the combination of amplification and detection in one step, eliminating the need for post-PCR processing. Real-time PCR also offers improved sensitivity over traditional PCR as well as the ability to quantify the amount of amplicon generated from a reaction.[13] Quantification may prove useful in differentiating previous, acute, and chronic CPN infections.

In this study, a previously published primer set CPN90 and CPN91 targeting a 197-bp region of the 16S rRNA gene of CPN was used with a Taqman probe (CPNTM) and the Roche Lightcylcer platform to develop a method for real-time detection of CPN from peripheral blood mononuclear cells (PBMCs) and respiratory specimens. This method has several advantages over traditional PCRs including decreased labor by eliminating the need for post-PCR processing of the sample and comparable, if not increased, sensitivity and specificity.

Materials and Methods

Preparation of Standards and Positive Controls

Standards and positive controls for use with the CPNTM PCR assay were derived from CPN strains A03, AR39, and AR388. A 1×10^5 infection-forming units (IFU)/ml aliquot was taken from each strain, and serial dilutions were performed to yield standards ranging from 400

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Address reprint requests to Dr. Charlotte Gaydos, John Hopkins University Department of Infectious Diseases, Ross Building, Room 1147, 720 Rutland Avenue, Baltimore, MD, 21205. E-mail: cgaydos@jhmi.edu.

IFU/PCR reaction to 0.4 IFU/PCR reaction. DNA extractions of each standard (400, 40, 4, and 0.4 IFU/PCR reaction) were performed using the Roche MagNA Pure LC robotic instrument (Roche Molecular Diagnostics, Indianapolis, IN). Although capable of extracting, DNA, RNA, or total nucleic acid only reagents from the MagNA Pure LC DNA Isolation Kit I (Roche) were used, and DNA extraction was carried out according to the instructions supplied for the MagNA Pure LC program "DNA I Blood Cells High Performance Serum" protocol.

Specimen Preparation

Three different sets of specimens were used for assay validation. The first set consisted of two blinded panels of pre-extracted CPN DNA from culture ($N = 70$). The second set consisted of archived specimens consisting of a CPN dilution series, CPN-spiked porcine aortal tissue and endarterectomy specimens ($N = 87$),[12] while the final set consisted of prospectively collected PBMCs from a heart disease study ($N = 198$). The first set of samples from culture, which were previously DNA extracted, was thawed and subjected to the CPNTM PCR assay. The second and third set were extracted using the Roche MagNA Pure LC robotic instrument in an identical manner to the standards, and subjected to the CPNTM PCR assay. Due to the variation in sample type and age, comparison of results between the sample sets was inappropriate, therefore, individual sensitivities and specificities for each set were calculated.

Primer and Probe Design

A previously published set of primers CPN90 (5′ggtctcaaccccatccgtgtcgg 3′) and CPN91 (5′ tgcggaaagctgtatttctacagtt 3′) was used for the CPNTM PCR assay.[7] A probe (CPNTM, 5′6FAM-atgccgcctgaggagtacactcgcaatamra 3′), using Taqman technology, was developed for detection of a 197-bp region of the 16S rRNA gene amplicon (TIB Molbiol, Adelphia, NJ).

PCR Conditions

PCR reactions contained 18 μl of master mix and 2 μl of template DNA. The final reaction mixture contained: 4 mmol/L $MgCl_2$, 20 pM of the primers CPN90 and CPN91, 20 pM of the CPNTM probe, and 1X LC FastStart DNA Master Hybridization Probes buffer based on the manufacturer's recommendations (Roche). Cycling conditions included a pre-incubation step of 4 minutes at 95°C followed by 45 cycles of 95°C for 0 seconds, 65°C for 10 seconds, and 72°C for 5 seconds. The amplification protocol was followed by a cooling period of 40°C for 30 seconds. Data collections were performed during extension and were monitored through the F1 channel of the instrument. Data analyses were performed using the Fit Points method through the Lightcycler software to minimize noise.

Nested PCR and TETR PCR were performed as previously described, and gel electrophoresis was used to determine the positivity of the samples.[7,8] TETR PCR targeted the 16S rRNA gene that is the same target as the CPNTM PCR assay, while nested PCR targeted the *ompA* gene, thus providing an alternative target for assay comparison.

Reproducibility Study

Standards (strain A03) of 400 IFU/PCR reaction, 40 IFU/PCR reaction, 4 IFU/PCR reaction and 0.4 IFU/PCR reaction were used to determine the reproducibility of the CPNTM PCR assay. Standards were serially diluted, extracted as described above, and subjected to the CPNTM PCR assay by two different technicians on two separate days. Standards were tested with eight replicates per standard. Variability is shown as the SD (SD). Statistical and regression analyses were carried out with STATA Version 7.0 (Stata Corp., College Station, TX) and Sigma Plot Software (SPSS Inc., Chicago, IL).

Specificity Testing

Both *Chlamydia psittaci* (VR125) and *Chlamydia trachomatis* (Serovar E) were tested to determine any potential cross-reactivity between the primers and probes with these organisms. Both *Chlamydia psittaci* and *Chlamydia trachomatis* samples had concentrations of 40 IFU/PCR reaction.

Sample Testing

A processing negative control, positive controls, consisting of a group of CPN standards (strains A03 or AR39 or AR388) of 400 IFU/PCR reaction, 40 IFU/PCR reaction, 4 IFU/PCR reaction, and 0.4 IFU/PCR reaction were included in all runs of tested samples. True positives were defined as positive either by a known result provided by an investigator based on culture results in the case of the standardized panels, or positive by two of the three PCRs used, ie, CPNTM PCR, nested PCR, or TETR PCR. Discrepant analysis was not performed in most cases due to limited sample volume.

Results

The CPNTM PCR assay had an analytical sensitivity between 4.0 and 0.4 IFU/PCR reaction (2000 and 200 IFU/ml, respectively), where 400 IFU/PCR reaction (200,000 IFU/ml), 40IFU/PCR reaction (20,000 IFU/ml) and 4.0 IFU/PCR (2000 IFU/ml) were positive 100% (32 of 32) of the time and 0.4 IFU/PCR reaction (200 IFU/ml) was positive 71.8% (23 of 32) of the time. These endpoints were comparable to those obtained by nested PCR or TETR PCR when performed on the same day.

Reproducibility Studies

On two separate days, two different technicians tested a panel of four standards with concentrations of 400 IFU

Table 1. Reproducibility of the CPNTM PCR Assay

Standard (known concentration)	Technician 1 Calculated concentration ± SD concentration*	Crossing point (C_t) ± SD crossing point (C_t)†
1 (400)	436.8 ± 157.6	30.7 ± 0.761
2 (40)	48.8 ± 8.72	34.6 ± 0.317
3 (4)	2.79 ± 1.15	39.9 ± 0.736
4 (0.4)	0.58 ± 0.27	42.9 ± 0.917
Standard (known concentration)	Technician 2 Calculated concentration ± SD concentration*	Crossing point (C_t) ± SD crossing point (C_t)†
1 (400)	462.5 ± 102.9	29.5 ± 0.427
2 (40)	42.7 ± 16.5	34.1 ± 0.772
3 (4)	3.10 ± 1.60	39.2 ± 0.874
4 (0.4)	1.10 ± 0.01	41.0 ± 0.102
Standard (known concentration)	Pooled means Calculated concentration ± SD concentration*	Crossing point (C_t) ± SD crossing point (C_t)†
1 (400)	449.7 ± 129.3	30.1 ± 0.860
2 (40)	45.7 ± 12.1	34.3 ± 0.617
3 (4)	2.9 ± 1.3	39.5 ± 0.861
4 (0.4)	0.68 ± 0.32	42.4 ± 1.12

Reproducibility data for Technician 1 and Technician 2. All calculated concentrations, standard deviations and crossing points are mean values based on 8 replicates analyzed for each technician, and 16 replicates analyzed for the pooled means.

*Calculated concentrations are given in CPN IFU per PCR reaction followed by standard deviations of the calculated concentrations. Calculated concentrations were generated by the Lightcycler utilizing C_t and the known concentration of the standards.

†Crossing point (C_t) followed by the standard deviation of the crossing point (C_t) was generated based on the cycle number where the Lightcycler software interpreted a logarithmic increase in fluorescence of the given sample.

per PCR reaction, 40 IFU/PCR reaction, 4 IFU/PCR reaction, and 0.4 IFU/PCR reaction. Results, including calculated concentrations and cycling times/crossing points (C_t) are indicated in Table 1, as well as in Figure 1. To assess the reproducibility, multiple linear regression was performed to create standard curves corresponding to the two runs. C_t was regressed on the log concentrations generated by Technician 1 and Technician 2 with a model allowing the intercepts and slopes of the standard curves corresponding to the two separate runs to differ. Analysis showed the fitted lines to be statistically different ($F(2.53) = 5.25$, $P = 0.0091$), although the overall variation in end point detection was not different for practical purposes (Figure 1).

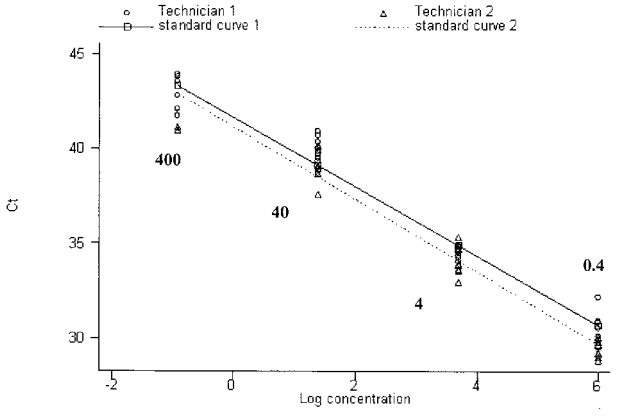

Figure 1. Standard curves generated by data from Technician 1 and Technician 2, respectively, from the reproducibility study. The concentration for each data point is given in CPN IFU/PCR reaction. Cycle number indicates the cycle that the given standard experienced a logarithmic change in fluorescence.

Specificity Studies

No signal was detected from either *Chlamydia psittaci* (VR125) or *Chlamydia trachomatis* (VR1477) when the CPNTM PCR assay analyzed these organisms at concentrations of 40 IFU/PCR reaction, as was previously demonstrated in earlier studies.[7] Additionally, the specificity of primer set CPN90 and CPN91 has been previously demonstrated with a large panel of organisms.[7]

Sample Testing

A total of 355 samples were tested over 23 runs in which positive control standards of 400 IFU/PCR reaction, 40 IFU/PCR reaction, 4 IFU/PCR reaction and 0.4 IFU/PCR reaction (strains A03, AR39, or AR388) and negative controls were used in every run to determine the C_t for positive samples, and account for background fluorescence.

The sensitivity and specificity of the CPNTM PCR assay were determined by comparing results to nested PCR, TETR PCR, or the known result for the sample from previous study.[12] Overall, the assay had a sensitivity of 88.5% (69 of 78) and a specificity of 99.3% (275 of 277). Tested samples included two panels of blinded samples from culture ($N = 70$), archived specimens consisting of a CPN dilution series, CPN spiked porcine aortal tissue and endarterectomy specimens ($N = 87$), and PBMCs from clinical patients ($N = 198$). Sensitivities and specificities were also calculated separately for each group due to variations in sample type, age of sample, and extraction method. It should also be noted that because there was variation in the treatment of the different sam-

Table 2. Results for "Gold Standards" Utilized in the CPNTM PCR Assay Development

Sample set	Positive	Negative	Gold standard	Source
1 (N = 70*)	54	16	Known panel result†	DNA extracts§
2 (N = 87*)	24	63	Nested (8‡) or TETR PCR (7‡)	PBMCs
3 (N = 198*)	0	198	Nested PCR (7‡)	PBMCs
Total (N = 355*)	78	277		

*Indicates the number of samples in the sample set.
†Indicates that the sample result was provided by an investigator based on culture results.
‡Indicates the appropriate reference.
§Indicates that the samples were DNA extracted prior to evaluation by an outside technician.

ple sets, the results are not necessarily comparable across the sets. The sensitivity and specificity for the standardized panels were 94.4% (51 of 54) and 100% (16 of 16), respectively, for the archived specimens 75% (18 of 24) and 100% (63 of 63) respectively, and for the PBMCs zero positives were detected, and the specificity was 98.9% (196 of 198) (Tables 2 and 3).

Using a gold standard of 2 of 3 positive PCR assays to define true positives resolves the sensitivity and specificity of the TETR and nested PCR for sample set 2 (N = 87) to be 100% (24 of 24) and 100% (63 of 63) respectively. Sensitivity and specificity cannot be calculated for sample set 1, as the results for these samples were derived from diluted cultures, and neither TETR nor nested PCR were performed on this sample set. For sample set 3 (N = 198), the sensitivity and specificity for the TETR PCR cannot be calculated because this assay was not performed on these samples. For the nested PCR, the sensitivity and specificity compared to the CPNTM PCR assay was 0% (0 of 2) and 100% (196 of 196), respectively.

Discussion

Although there are many diagnostic assays available for the detection of CPN, there is still a need for a simple, reproducible, and standardized assay for CPN detection. Due to the possibility of contamination in PCR-based testing, an assay that provides minimal sample manipulation is also desirable. The primer set CPN90 and CPN91 are sensitive, specific, and have proven useful in CPN detection. Robotic nucleic acid extraction from clinical samples is largely hands-off. Real-time PCR technology provides a platform for rapid amplification and detection of CPN while eliminating the need for post-PCR handling and processing, such as gel electrophoresis.

The CPNTM PCR assay combines the sensitivity and specificity of the CPN90 and CPN91 primer set with the speed, accuracy, and ease of real-time PCR technology. This assay is rapid, taking less than 3 hours from DNA extraction to completion of the PCR and sensitive, with a detection limit of 4 to $<$ 1 IFU/PCR reaction.

The overall sensitivity and specificity of the CPNTM PCR assay is comparable to other real-time methods available, including *ompA*-based PCRs.[5] Additionally, this method is comparable to nested PCR and TETR PCR, the gold standards used in development of this assay.[7,8] Based on other inter-method comparisons, the CPNTM PCR assay is comparable to recently available commercial research kits for CPN detection (PCR research assay, Abbott Laboratories).[5]

The sensitivity of the CPNTM PCR assay varied depending on the sample set. Because the samples differed in sample type, age, and extraction method, it was reasonable to calculate separate sensitivities and specificities, as well as the overall. The variation in treatment between the different sample sets makes comparison of the differing results difficult. However, the CPNTM PCR assay was still comparable to other available methods for CPN detection in terms of the overall sensitivity and specificity. For sample set 1 that consisted of two blinded panels of samples from culture, the sensitivity (94.4%) and specificity (100%) of the CPNTM PCR assay was comparable to other real-time methods available.[5] However, for sample set 2 that consisted of archived specimens consisting of a CPN dilution series, CPN-spiked porcine aortal tissue and endarterectomy specimens, the sensitivity of the assay was much lower (75%) than the overall sensitivity (88.5%), and much lower than other real-time methods for CPN detection.[5] The lower sensitivity is most likely due to DNA degradation through mul-

Table 3. Sensitivity and Specificity of the CPNTM PCR Assay for Three Sample Sets and Totals

Sample set	+/+	+/−	−/−	−/+	Sensitivity*	Specificity*
1 (N = 70)	51	0	16	3	94.4% (51/54)	100% (16/16)
2 (N = 87)	18	0	63	6	75% (18/24)	100% (63/63)
3 (N = 198)	0	2	196	0	NA	98.9% (196/198)
Total (N = 355)	69	2	275	9	88.5% (69/78)	99.3% (275/277)

*Sensitivity and specificity of the CPNTM PCR assay as determined by utilizing the nested PCR result, the TETR PCR result or the known result from culture as the gold standard. Set 1, two panels of blinded samples from culture that were DNA extracted by an outside technician. Set 2, archived specimens consisting of a CPN dilution series, CPN spike porcine aortal tissue and endarterectomy specimens. Set 3, PBMCs from prospectively collected clinical samples. +/+, Sample result was positive by both CPNTM PCR and nested PCR or TETR PCR. +/−, Sample result was positive by CPNTM PCR and negative by nested PCR or TETR PCR. −/−, Sample result was negative by all methods. −/+, Sample result was negative by CPNTM PCR and positive by nested PCR or TETR PCR.

tiple freeze-thaw processes and time. For sample set 3 that consisted of prospectively collected PBMCs, the sensitivity of the CPNTM PCR assay was non-calculable because there were no positives detected by the gold standard assays. The two positives that were identified by the CPNTM PCR assay were not confirmed by nested PCR or by TETR PCR. Although these two positives were not confirmed, it is possible that the CPNTM PCR is more sensitive, and that the two unconfirmed positives were true positives. Alternatively, the two unconfirmed positives could represent false positives, or potentially contaminated samples.

Cost is a limitation of the assay, as the equipment used in the assay can cost up to $50,000, and PCR reactions can cost approximately $8.00 per reaction without accounting for technician time. In addition, the cost of automated nucleic acid extraction can be considerable, as the instrument alone costs upwards of $80,000. However, despite this limitation, this real-time method appears to be a practical method of accurate, high throughput detection of CPN, and should serve to improve the reliability of diagnostic testing for CPN while minimizing manual processing, sample handling, and post-amplification manipulation. Future studies with this method should include prospective analysis of a large sample group, treated and extracted the in the same manner with comparison to a truly accurate gold standard.

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