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(54) Title: ASSAY

(57) Abstract: A detectably-labelled molecule for the detection of Chlamydia DNA, comprises the nucleotide sequence identified as SEQ ID NO. 1 or 2.
ASSAY

Field of the Invention

This invention relates to the detection of Chlamydia microorganisms, using specific oligonucleotide probes.

Background to the Invention

Chlamydia pneumoniae is an obligate intracellular bacterium acting mainly as a respiratory pathogen. However, it has recently been linked to a number of chronic diseases, including coronary arteropathies, multiple sclerosis, and Alzheimer's disease.

Laboratory methods for detecting individuals infected with C. pneumoniae have relied mainly on serological analyses, since culturing the organism from clinical samples is difficult and associated with low yield. While the serological "gold standard" remains the microimmunofluorescence (MIF) technique, C. pneumoniae detection by methods based on the polymerase chain reaction (PCR) have been shown to be sensitive and specific but are not yet standardized or widely available; see Boman et al, J.Clin. Microbiol. 37: 3791-3799 (1999).

The most widely used C. pneumoniae gene targets in current PCR methods are the 437 bp species-specific PstI fragment (Campbell et al, J.Clin. Microbiol. 30: 434-439 (1992), and also 16S rRNA and omp1 gene (major outer membrane protein; MOMP). However, C. pneumoniae DNA detection in atheromatous lesions by PCR assays remains problematic because the reported positivity rate ranges from 0 and 100%, and no correlation has been found between C. pneumoniae DNA positivity and severity of the atheromatous lesions.

In the last decade, the real-time PCR-based fluorescence assay was introduced as a new tool in PCR methods. It is an automated technology which offers advantages such as high sensitivity and specificity, less chance of contamination and quantitative analyses. In addition, its results can be visualized faster than gel-based PCR assays. This technology has recently been used for detecting C. pneumoniae in human samples, but not in atheromatous plaques.

There is a lack of a gold standard for PCR detection of C. pneumoniae in clinical samples. Guidelines for standardizing C. pneumoniae assays have been published in a report (Dowell et al, Clin. Infect. Dis. 33: 492-503 (2001)) which summarizes many in-house PCR methods, and DNA target regions used for detection of C. pneumoniae in
various samples. However, sensitivity and specificity of these tests remain unknown. Specific recommendations regarding DNA extraction procedures from clinical samples are presented, the quality and the purity of the DNA template being among the most important factors for an efficient and powerful PCR assay. In particular, difficulty in extraction of DNA from atheromatous plaques can be due to the calcification of the sample. Consequently, the loss or the lack of DNA might result in false-negative results, independent of the performance of PCR-assays. As expected, there are ample variations in the number of *C. pneumoniae* positive atherosclerotic plaques reported in the literature, which may reflect either the differences in lesion severity and anatomical site or the histochemical or PCR techniques adopted or both.

There is therefore a need for improved methods for the detection of *Chlamydia* microorganisms, particularly in atherosclerotic plaques.

**Summary of the Invention**

The present invention is based on the finding that a real-time polymerase reaction can be used to detect accurately and specifically the presence of *Chlamydia* DNA in a sample from an atheromatous plaque.

According to a first aspect of the invention, an assay for the detection of *Chlamydia* DNA in a DNA sample obtained from an atheromatous plaque, comprises performing a real-time polymerase reaction on the sample using primers specific for *Chlamydia* DNA, and one or more labelled probes that hybridise under stringent conditions to *Chlamydia* DNA, and detecting the presence of a probe hybridised to a *Chlamydia* DNA molecule.

According to a second aspect of the invention, a labelled molecule for the detection of *Chlamydia pneumoniae* DNA, comprises the nucleotide sequence identified in SEQ ID NOS. 1 or 2, or a sub-sequence thereof that permits the specific hybridisation to *Chlamydia pneumoniae* DNA, under stringent hybridisation conditions.

**Description of the Invention**

The present invention uses real-time PCR to detect the presence of *Chlamydia* DNA in a sample.

Real-time PCR is a technique known in the art and the apparatus and reagents required to carry out this procedure are available from commercial sources. Labelled probes are required for detecting and quantifying the presence of the target DNA from
In the preferred embodiment, the probes are oligonucleotides derived from the PstI fragment of *Chlamydia pneumoniae*. Probes suitable for use in the present invention will comprise either or both of the sequences identified herein as SEQ ID NOS. 1 or 2, or a sub-sequence thereof that permits specific hybridisation to *Chlamydia pneumoniae* DNA, under stringent conditions. A suitable sub-sequence will usually be smaller than the sequences shown in SEQ ID NOS. 1 and 2, by only 1 or 2 nucleotides (removed from the 3' or 5' terminus). The sub-sequence will not usually differ in the remaining sequence. In a more preferred embodiment, two probes are used, and these consist of either of the sequences shown as SEQ ID NOS. 1 and 2. The probes are labelled with a fluorescent moiety. In the preferred embodiment, one probe is labelled with fluorescein and the other with LightCycler Red 640.

The sample under study may be from any source. In the preferred embodiment, the sample is from an artherosclerotic plaque.

The invention is further described in the following Example.

**Example**

Fifteen carotid plaques were obtained by endoarterectomy from 15 patients (mean age 68; range 61-72 years) with atherosclerotic arteriopathy. All patients had high titers (IgG> 256) of anti-*C. pneumoniae* antibodies, as tested by a commercial MIF assay (Labsystems Gy, Helsinki, Finland). DNA from each plaque, weighing 25 mg and previously homogenized by razor blade, was extracted by Nucleospin Tissue kit (Macherey-Nagel GmbH, Diiren, Germany) using the standard protocol for human tissue, according to the manufacturer’s instructions.

The assay was assessed for specificity and quantification by construction of a standard curve with purified genomic *C. pneumoniae* DNA. *C. pneumoniae* Parola strain was grown as previously described (Ciervo), and microorganisms were purified by a gradient centrifugation (Schachter). Prokaryotic DNA was obtained by using the aforementioned commercial kit adapted for DNA extraction from bacteria. Concentration and purity of *C. pneumoniae* genomic DNA were determined by measuring the optical density at 260 and 280 nm and serial dilutions (10³ to 1 copy numbers) were used to generate a standard curve for DNA quantification.

Two labelled probes were designed based on the nucleotide sequences of the *C. pneumoniae* PstI fragment, one of the best targets for a species-specific PCR
(Campbell et al, Supra). Primers and probes are shown in Table 1 ("Maass" is Angiology 48: 699-705 (2000)). A BLAST search was performed to check the specificity of the product target sequence (128 bp) of the primer and probe sets.

5 **TABLE 1.** Primers and probes used throughout this study

<table>
<thead>
<tr>
<th>Primers or probes</th>
<th>Sequence&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Reference</th>
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<tr>
<td>Primers</td>
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<tr>
<td>In-1</td>
<td>5'-AGTTGAGCATATTCGAGG-3'</td>
<td>Maass</td>
</tr>
<tr>
<td>In-2</td>
<td>5'-TTTATTTTCGTTCTGACAG-3'</td>
<td>Maass</td>
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<tr>
<td>Probes</td>
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<td>5'-CTCTTGGAGGCAACGTAAC-X-3'</td>
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<tr>
<td>CP1-LC</td>
<td>5'-LC640-TAACTTGGCGAATGACACCA-p-3'</td>
<td>SEQ ID NO. 2 (acc. no. AJ488062)</td>
</tr>
</tbody>
</table>

<sup>a</sup> X, fluorescein; LC640, LightCycler Red 640; p, phosphate.

The PCR reaction was performed in 10 µl (final volume) into glass capillary tubes. The PCR mixture contains 1 µl of DNA template (100 ng of human DNA from atherosclerotic plaques, or C. pneumoniae DNA ranging from 10<sup>5</sup> to 1 copies), 1 µl of commercial ready-to-use mixture LightCycler-DNA Fast Start master hybridization probes (Roche Diagnostics), 5 mM MgCl<sub>2</sub> (final concentration), primers and probes at final concentrations of 5 and 1 µM, respectively. Real-time PCR was carried out on a LightCycler instrument (Roche Diagnostics GmbH, Germany) (Meuer). The conditions for thermal cycling were as follows: initial denaturation for 10 min, followed by 55 amplification cycles at 95°C for 8 s, 55°C for 8 s, and 72°C for 6 s. Fluorescence was measured at 640 nm (F2 channel) to the end of each annealing phase. The amplification was followed by a melting program, which started at 45°C for 15 s and then increased to 95°C at 0.1°C/s, with the fluorescence signal continuously monitored on-line.

In order to verify the species-specificity of the assay, *Chlamydia trachomatis*, *Chlamydia psittaci* and human genomic DNAs were tested. No PCR products or hybridization were detected.
A quantitative analysis was performed by standard curve generated from the crossing point parameter (CP; Fit Point algorithm) of the standard \textit{C. pneumoniae} genomic DNA dilution series (10^5 to 1 copy) by the LightCycler quantification software (Fig. 1A-B). As shown in Fig 1C, the melting curve profile of hybridization probes gave the theoretically expected, specific melting temperature of 60.8°C. To confirm the specific amplification size, PCR products were resolved on 2% agarose gel by electrophoresis and visualized under UV light with ethidium bromide (Fig.1C, inset).

To assess the CP reproducibility, each dilution of the standard was measured twice in 10 independent runs. Replicate results from different dilution series and different runs showed a coefficient of variation between 1.3% and 2.1%, and consequently, the reproducibility of the assay was considered to be fairly acceptable. For the lowest concentration, 1 copy was detected in 7 out of 10 run tests. Failure of detecting 1 copy is not attributable to the PCR technology but rather dependent in pipetting inaccuracy or Poisson error statistics (Mygind 2001).

Excellent correlation was found between log-copy numbers and CPs with a regression coefficient of 0.98 (Fig. 1B).

Fifteen atherosclerotic plaques were examined, and 100 ng of purified total DNAs from plaques were tested in a parallel study with real-time PCR and nested PCR (Maass). DNA samples were analysed in three independent aliquots of each plaque by both PCR methods, and negative samples were checked for the presence of PCR inhibitors with \textit{C. pneumoniae} DNA-spiked samples (100 copies). All spiked specimens were positive in nested and real-time PCR. The latter method showed CP values similar to those obtained with the same copy number in the standard curve (data not shown). All DNA samples were negative in nested PCR while 3 samples were positive in real-time PCR experiment. The theoretical corresponding DNA copy number was calculated by plotting CP of each positive sample versus the standard curve, resulting into 50 ± 3, 37 ± 2, 24 ± 2 copies (the mean ± standard deviation of three determinations) (Fig. 1B).

The PCR amplification assay using the LightCycler lasted typically 40-60 min, but for samples with high target copy numbers it is possible to detect on-line the positivity in few minutes. In contrast, amplification by nested PCR and agarose gel detection of amplicons typically lasted 5.5 to 6 h for completion and accurate reading.
By the application of the probes, an internal region of the PstI fragment is targeted, and this permits genomic quantification by LightCycler real-time PCR. Purified DNAs from atheromatous plaques were analysed in parallel by real-time PCR and nested PCR. This study demonstrates clearly that real-time PCR is specific and more sensitive than nested PCR, because the former method resulted in detection of 3 positive samples against none by the latter method.

LightCycler real-time PCR combined with FRET hybridization probes, has several advantages as diagnostic method. Its high degree of automation, the availability of commercial kit in experimental setting up for gene amplification and hybridization, the resulting quantitative measurement, coupled with the sensitivity, specificity and the reproducibility, hold promise for a useful diagnostic tool and a potential standardisable method for detection of C. pneumoniae DNA in histological lesions.
CLAIMS

1. A detectably-labelled molecule for the detection of Chlamydia DNA, comprising
   the nucleotide sequence identified as SEQ ID NO. 1 or 2.
2. A molecule according to claim 1, consisting of less than 30 nucleotides.
3. A molecule according to claim 1 or claim 2, consisting of less than 25 nucleotides.
4. A molecule according to any preceding claim, consisting of the nucleotides
   identified as either SEQ ID NO. 1 or 2.
5. A molecule according to any preceding claim, wherein the label is fluorescent.
6. A molecule according to claim 5, wherein the molecule is fluorescein or
   LightCycler Red 640.
7. An assay for the detection of Chlamydia DNA in a DNA sample obtained from an
   atheromatous plaque, comprising
   performing a real-time polymerase reaction on the sample using primers specific
   for Chlamydia DNA, and one or more labelled probes that hybridise under stringent
   conditions to Chlamydia DNA, and
   detecting the presence of a probe hybridised to a Chlamydia DNA molecule.
8. An assay according to claim 7, wherein the probe has a nucleotide sequence that
   corresponds to a sequence in the PsI fragment of Chlamydia pneumoniae.
9. An assay according to claim 7 or claim 8, wherein the probe is one or more of
   those defined in claims 4 to 6.
10. An assay according to any of claims 7 to 9, wherein two probes are used, the
    probes being those defined in claim 4.
Figure 1

A

Fluorescence (F2)

Cycle Number

B

Crossing point (Cycles)

Log copy number

R = 0.98

y = -2,1839x + 39,509

C

Tm 60.8°C

Fluorescence -d[F2]/dT

Temperature (°C)
SEQNCE LISTING

<110> Cassone Dr, Antonio

<120> Assay

<130> JWJ01071WO

<140> Not yet known
<141> 2003-09-29

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<170> PatentIn Ver. 2.1

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