Molecular evidence for the existence of additional members of the order Chlamydiales

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Respiratory tract infections in man may be caused by several members of the genus Chlamydia and also by two Chlamydia-like strains, 'Simkania negevensis' (Z-agent) and 'Parachlamydia acanthamoebae' (Bn). To facilitate diagnostic procedures a PCR assay able to detect all known Chlamydiaceae sequences in one reaction was developed. For this purpose, primers were selected to amplify a fragment of the 16S rRNA gene. Characterization of the amplified fragments was done by hybridization with specific probes and by sequencing. PCR assays were carried out using DNA isolated from nose/throat specimens or from peripheral blood mononuclear cells of patients with respiratory tract infections, and from vessel wall specimens of abdominal aneurysms. Six of the 42 nose/throat swab specimens analysed yielded strong bands and one yielded a faint band. Three of these bands were identified as Chlamydia pneumoniae and one as Chlamydia trachomatis by sequencing. Analysis of the three other bands yielded two different new sequences. DNA isolated from peripheral blood mononuclear cells of one patient yielded a third new sequence. DNA isolated from peripheral blood mononuclear cells of four healthy controls was negative. One of the abdominal aneurysm specimens also yielded a strong band. Sequencing revealed a fourth new sequence. All negative controls included during specimen processing and PCR analysis remained negative. The typical secondary structure of microbial 16S genes was present in all four new sequences indicating the validity of the sequence data. All four new sequences were distinct from other bacteria and clustered together with known Chlamydiaceae sequences. Phylogenetic analysis suggested a new lineage, separating the four new sequences, 'S. negevensis' and 'P. acanthamoebae' from the genus Chlamydia with the four known chlamydial species. In conclusion, this study provides evidence for the existence of several new members of the order Chlamydiales. Since the source of the Chlamydia-like strains has not been identified and serological and/or molecular cross-reactivities may be expected, results of identification of infecting recognized organisms should be interpreted cautiously.

Keywords: Chlamydia, Parachlamydia, 16S rDNA, phylogeny

INTRODUCTION

Currently, four species are recognized within the genus Chlamydia in the family Chlamydiaceae in the order Chlamydiales. Two of these four species, Chlamydia trachomatis and Chlamydia pneumoniae, naturally infect humans. C. trachomatis is the leading cause of preventable blindness in developing countries (Schachter & Dawson, 1990), a major cause of sexually transmitted diseases throughout the world (Centers for Disease Control and Prevention, 1993; Taylor-Robinson, 1993; World Health Organization Working Group, 1989) and a cause of respiratory tract infections in infants. C. pneumoniae is a major cause of upper and lower respiratory tract infections and has been associated with cardiovascular diseases (Danesh et al., 1997; Gupta &
C. psittaci mainly infects animals, including birds, and is a cause of zoonotic respiratory tract infections. Chlamydia pecorum is not known to infect humans. Recently, two Chlamydia-like strains, ‘Simkania negevensis’, or Z-agent (Kahane et al., 1993), and ‘Parachlamydia acanthamoebae’, or Bn (Amann et al., 1997), have been described. Nucleic acid sequence analysis indicates that they probably belong to the family Chlamydiaceae, but not within one of the four currently recognized species. Both strains seem to be able to infect humans, causing respiratory tract infections (Birtles et al., 1997; Lieberman et al., 1997).

Thus, many different Chlamydia species may cause respiratory tract infections in humans. Since isolation in cell culture of the Chlamydia-like strains is probably difficult, we decided to use amplification techniques to study the epidemiology of human chlamydial respiratory tract infections. The use of broad range bacterial primers and nucleotide sequencing for identification is only useful when one species is expected (Goldenberger et al., 1997; Greisen et al., 1994). The presence of more than one species would necessitate lengthy cloning and sequencing procedures. Therefore, this approach cannot be applied to detect bacteria in swabs from mucosal sites. Application of specific probes might identify specific pathogens (Monstein et al., 1996), but interferences of other bacteria is likely to result in a low sensitivity for detection of some pathogens. Therefore, we developed a Chlamydiaceae-specific PCR assay able to detect all known Chlamydiaceae sequences in one reaction, including those of the recently described Chlamydia-like strains. Here we describe the design of the primers and probes used in this PCR assay and report on the analysis of the first results.

**METHODS**

**Reference strains.** C. pecorum strain E58 (ATCC, Manassas, VA, USA), C. psittaci (avian type, isolated from a human lung biopsy, typed by RFLP of the ompl gene) and C. trachomatis serovar L2 strain 434-B were propagated in monolayers of HeLa 229 (ATCC CCL2.1) cells. C. pneumoniae strain TW183 (Washington Research Foundation, Seattle, WA, USA) was propagated in monolayers of HeP2 cells (ATCC CCL23). Each monolayer was sonicated in 2 ml 0.4 M sucrose-phosphate buffer (45P) and stored aliquoted at −70 °C. Batches of C. pneumoniae and C. trachomatis were titrated by inoculating a tenfold serial dilution series onto 1-d-old monolayers in 96-well microtitre plates. After 2 or 3 d incubation the monolayers were fixed with methanol, stained with FITC-labelled anti-LPS monoclonal antibodies, and the number of inclusions was counted. Results were expressed as inclusion-forming units (f.i.u.) ml⁻¹.

**Clinical specimens.** Specimens were collected from a general practitioner’s surveillance of respiratory tract infections: 42 nose/throat swabs were collected in transport medium (5 ml) during the first 3 weeks of January 1997. Aliquots (1 ml) were centrifuged in a microcentrifuge, and the pellet was lysed in a buffer with proteinase K (Ossewaarde et al., 1992). After heating for 10 min at 100 °C, the specimens were used directly in the PCR assay. Peripheral blood mononuclear cells from one patient with acute bronchitis (from the acute and the convalescent phases) and from four healthy controls working in the same workplace were collected. Mononuclear cells purified from 2.5 ml blood using Histopaque (Sigma-Aldrich) were centrifuged in a microcentrifuge and the pellet was processed as described above. Vessel wall tissue was obtained from six patients during surgical treatment of their abdominal aneurysms. DNA was isolated from three specimens taken from different locations of the aneurysm of each patient using a PCR-inhibitor-free procedure as described previously (Meijer et al., 1998).

**Design of primers and probes.** The 16S rRNA gene was chosen as the target, since it was the only gene of which the sequence was available for all known members of the Chlamydiaceae. All sequences were downloaded from GenBank and aligned. Possible primer and probe locations compatible with all known Chlamydiaceae sequences were selected by comparing this alignment with a quantitative map of nucleotide substitution rates (Van de Peer et al., 1996). Next, the 3’ ends of the primers were analysed for their homology with human sequences using the K-tuple frequency method included in the computer program PC-Rare (Griffais et al., 1996). Thermodynamic properties of the primers and probes were analysed by the computer program Oligo 4.1 (Rychlik & Rhoads, 1989). Finally, the primers and probes were analysed for similarity with other DNA sequences using the output of the blast server (Altschul et al., 1990). Out of several sets, one set of primers and probes (Table 1) was selected according to the following criteria: compatibility with all known Chlamydiaceae sequences, lowest homology with human DNA at the 3’ ends of the primers, thermodynamic suitability, and no significant similarity with other DNA sequences. A general family-specific Chlamydiaceae probe was designed using all known Chlamydiaceae sequences and the sequences obtained in this study.

**PCR reaction and hybridization.** The PCR reaction mixture was as described previously including the dUTP-uracil-N-glycosylase contamination prevention system and anti-Taq antibodies (Meijer et al., 1998). The PCR assay was carried out in separate rooms using dedicated equipment (Ossewaarde et al., 1992). Negative controls were included after every three to five specimens during specimen processing and PCR analysis. The final volume was 25 μl including 5 μl of the specimen. Ten picomoles of each of the eight primers (two forward and six reverse primers) was used. After amplification using a ‘touch-down’ protocol, 10 μl reaction mixture was electrophoresed in a 2% agarose gel (Meijer et al., 1998). The gels were transferred to nylon membranes by vacuum blotting. The biotin-labelled probes were hybridized at 60 or 65 °C. Binding of the probes was detected by chemiluminescence using the ECL system (Amersham). Since this assay was originally developed to detect known respiratory tract pathogens, only the probes for ‘S. negevensis’, C. pneumoniae and C. psittaci were used for screening the clinical samples. In addition, the probe for ‘P. acanthamoebae’ and the general Chlamydiaceae probe were used to characterize the new sequences.

**Sequence analysis.** Strong bands in the agarose gels at or close to the expected length were sequenced directly using either the forward primer CHL16SF ORF2 or the general sequencing forward primer in the sequencing reaction with the Thermo Sequenase Dye Terminator Cycle Sequencing Pre-Mix kit (Amersham) or the Big Dye Terminator Cycle Sequencing Ready Reaction kit (ABI) on an automated sequencer. Reverse sequencing was carried out using either the resolved reverse primer or the general sequencing reverse primer. Specimens yielding faint bands were amplified from either the original
Positioning of the oligonucleotides was based on the alignment of complete 16S rDNA gene sequences.

**Table 1. Oligonucleotide sequences selected for primers and probes**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’ → 3’)</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Forward primers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHL16SFOR1</td>
<td>GTGGATGAGGCATGCAGTCGA</td>
<td>‘S. negevensis’</td>
</tr>
<tr>
<td>CHL16SFOR2</td>
<td>CGTGGATGAGGCATGCAAGTCGA</td>
<td>All others</td>
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<tr>
<td><strong>Reverse primers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHL16SREV1</td>
<td>CTCTCAGCCGCCCTAGAGCTTCTAG</td>
<td>‘S. negevensis’</td>
</tr>
<tr>
<td>CHL16SREV2</td>
<td>CAATCTCTCAATCCGCCCTAGAGCTTCTAG</td>
<td>‘P. acanthamoebae’</td>
</tr>
<tr>
<td>CHL16SREV3</td>
<td>ATCTCTCAATCCGCCCTAGAGCTTGAAGG</td>
<td>C. psittaci</td>
</tr>
<tr>
<td>CHL16SREV4</td>
<td>ATCTCTCAATCCGCCCTAGAGCTTGAAGGA</td>
<td>C. psittaci</td>
</tr>
<tr>
<td>CHL16SREV5</td>
<td>CAATCTCTCAATCCGCCCTAGAGCTTGAAGG</td>
<td>C. trachomatis</td>
</tr>
<tr>
<td>CHL16SREV6</td>
<td>CTCTCAGCCGCCCTAGAGCTTGAAGG</td>
<td>C. pecorum</td>
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<tr>
<td><strong>Probes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHL16SPROBE01</td>
<td>BIO*AGCTGGGGTACCTGGTTCTTT</td>
<td>‘S. negevensis’</td>
</tr>
<tr>
<td>CHL16SPROBE02</td>
<td>BIO*AAATGGTGGGTTGGTTAAATTTAC</td>
<td>‘P. acanthamoebae’</td>
</tr>
<tr>
<td>CHL16SPROBE03</td>
<td>BIO*CGAAGTGGTGTAGTTTAGCCATCCTAAGAC</td>
<td>C. psittaci</td>
</tr>
<tr>
<td>CHL16SPROBE07</td>
<td>B10*CGAAGTGGTGTAGTTTAGCCATCCTAAGAC</td>
<td>C. pneumoniae</td>
</tr>
<tr>
<td><strong>General 1</strong></td>
<td>B10*TATGCGGGGAGAGTGTAGATAATCAT</td>
<td>Chlamydiaceae</td>
</tr>
<tr>
<td><strong>General sequencing primers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>GTCGAGTGAGGCATGC(G/A)AGTCGA</td>
<td>Chlamydiaceae</td>
</tr>
<tr>
<td>Reverse</td>
<td>CTCTCAATCCGCCCTAGAGGTAG</td>
<td>Chlamydiaceae</td>
</tr>
</tbody>
</table>

specimen or the band recovered from the gel. The obtained sequences were compared with the sequences available in GenBank using the BLAST server (Altschul et al., 1990). The ‘Suggest Tree’ option of the Ribosomal Database Project (University of Illinois, Urbana-Champaign, USA) was used to add the new sequences to the existing tree of the Ribosomal Database Project. The ‘Check Chimera’ option was used to check for possible chimeric sequences created during amplification. All sequences were aligned manually taking into account the secondary structure obtained from the SSU database available at the Antwerp Ribosomal RNA Database (University of Antwerp, Antwerp, Belgium; Van de Peer et al., 1998). Phylogenetic analysis was carried out using the neighbour-joining method with a distance matrix calculated by the method of Jukes & Cantor (1969) using TREECON for Windows version 1.3b (Van de Peer & De Wachter, 1994). The corresponding sequence of the related bacterium *Rickettsia prowazekii* (GenBank M21789) was used to construct a root. The following sequences from GenBank were included: M83313, *C. trachomatis* (hamster); D85718, *C. trachomatis* (mouse); U73110, *C. trachomatis* (swine); D85721, *C. trachomatis* (man); D85717, *C. pecorum* (kola); D88317, *C. pecorum* (calf); D85708, *C. psittaci* (guinea pig); D85713, *C. psittaci* (bird); D85703, *C. psittaci* (cat); U73784, *C. pneumoniae* (horse); Z49873, *C. pneumoniae* (man); L27666, ‘S. negevensis’; Y07536, ‘P. acanthamoebae’; AF097184, Chlamydia Research Group 1 (CRG1); AF097185, CRG2; AF097186, CRG3; AF097187, CRG4.

**RESULTS**

First, the lower limit of the PCR assay was determined using a dilution series of DNA isolated from *C. pneumoniae* and *C. trachomatis* reference strains. The amount of DNA equivalent to 0.1 i.f.u. always yielded a clearly visible band of approximately 270 bp after agarose gel electrophoresis, and DNA equivalent to 0.01 i.f.u. yielded a clearly visible band in some experiments. DNA isolated from *C. psittaci* and *C. pecorum* reference strains equivalent to 0.1 i.f.u. also yielded a clearly visible band. In each run during this study, positive controls always confirmed this lower limit. Further optimization of the PCR assay was not attempted.

Forty-two patients with upper respiratory tract infections were studied. Six nose/throat swab specimens yielded a strong band in agarose electrophoresis after amplification of the DNA. One specimen yielded a faint band. The first series of hybridization experiments did not result in definite identification of all positive reactions. Using a hybridization temperature lower than optimal (60°C vs 65°C), five bands reacted weakly with the *C. pneumoniae*-specific probe, but none reacted with the *C. psittaci*-specific probe or with the ‘S. negevensis’-specific probe (data not shown). Therefore, all six strong amplification products were sequenced directly. The seventh specimen was re-amplified from the original specimen and subsequently sequenced. Two of these specimens were identified as *C. pneumoniae* and the third specimen as *C. trachomatis*. The fourth specimen yielded a mixture of sequences that could not be resolved. After re-isolation of the DNA from the original specimen and subsequent amplification in the PCR assay, the sequence obtained was identified as that of *C. pneumoniae*. The other three specimens yielded two
different new sequences not present in GenBank that clustered together with the Chlamydia-like strains. These sequences were designated CRG2 and CRG3.

The DNA isolated from the peripheral blood mononuclear cells from a patient with acute bronchitis yielded another bacterial sequence. Controls yielded a faint band slightly longer than the Chlamydia sequences, but distinct from other bacterial sequences. The Chlamydia-specific probe showed weak cross-reactions with C. pneumoniae and C. pecorum. In all hybridization experiments, strong reactions were always reproducible, but weak reactions were not always reproducible. When applied to blots of bands of non-Chlamydiaceae sequences such as the human sequences described above, the Chlamydiaceae-specific probe discriminated clearly between chlamydial sequences and sequences from other origins.

The validity of the sequence data was tested by constructing the secondary structure typical of all 16S rRNA helices 6 and 11 (Fig. 1). Nucleotide substitutions were observed throughout the sequence. The 'Suggest Tree' option of the Ribosomal Database Project suggested a phylogenetic tree in which all new sequences were joined in one branch together with 'S. negevensis' and close to other Chlamydia sequences, but distinct from other bacterial sequences.
New members in the order \textit{Chlamydiales}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{phylogenetic_tree}
\caption{Phylogenetic analysis of the amplified region (216–224 bp) of the four recognized species of the family \textit{Chlamydiaceae}, both \textit{Chlamydia}-like strains and the four new sequences obtained in this study. The sequence CRG1 was obtained from a vascular specimen; CRG2 and CRG3 from respiratory specimens; and CRG4 from a peripheral blood mononuclear cell specimen. Percentages represent the number of times the branch appeared in a tree during 1000 bootstrap samples. The genetic distance is represented as fixed mutations per site.}
\end{figure}

\begin{table}
\centering
\caption{Percentage nucleotide similarity of the four new sequences, the two \textit{Chlamydia}-like strains and example sequences of the four recognized \textit{Chlamydia} species}
\begin{tabular}{|c|c|c|c|c|c|c|c|c|c|c|c|c|c|}
\hline
 & \textit{C. pecorum} & \textit{C. psittaci} & \textit{C. trachomatis} & \textit{C. pneumoniae} & \textit{S. negevensis} & \textit{P. acanthamoebae} & CRG1 & CRG2 & CRG3 & CRG4 \\
\hline
\textit{C. pecorum}   & 100.0 & 93.5 & 88.6 & 89.0 & 71.0 & 73.9 & 70.2 & 76.7 & 66.9 & 73.9 \\
\textit{C. psittaci}  & 100.0 & 86.5 & 93.5 & 70.6 & 73.5 & 72.2 & 78.4 & 67.4 & 75.5 \\
\textit{C. trachomatis} & 100.0 & 84.1 & 71.0 & 71.8 & 71.4 & 75.3 & 75.9 & 79.6 & 69.0 & 76.3 \\
\textit{C. pneumoniae} & 100.0 & 71.4 & 73.5 & 75.5 & 78.8 & 75.1 & 72.7 & 72.7 & 74.3 \\
\textit{S. negevensis} & 100.0 & 75.5 & 72.7 & 72.7 & 72.7 & 74.3 & 74.3 & 74.3 & 74.3 & 74.3 \\
\textit{P. acanthamoebae} & 100.0 & 75.5 & 72.7 & 72.7 & 72.7 & 74.3 & 74.3 & 74.3 & 74.3 & 74.3 \\
\textit{CRG1} & 100.0 & 70.0 & 71.0 & 71.0 & 71.0 & 71.0 & 71.0 & 71.0 & 71.0 & 71.0 \\
\textit{CRG2} & 100.0 & 70.0 & 71.0 & 71.0 & 71.0 & 71.0 & 71.0 & 71.0 & 71.0 & 71.0 \\
\textit{CRG3} & 100.0 & 70.0 & 71.0 & 71.0 & 71.0 & 71.0 & 71.0 & 71.0 & 71.0 & 71.0 \\
\textit{CRG4} & 100.0 & 70.0 & 71.0 & 71.0 & 71.0 & 71.0 & 71.0 & 71.0 & 71.0 & 71.0 \\
\hline
\end{tabular}
\end{table}

sequences. The ‘Check Chimera’ option did not show evidence for chimeric sequences. All new sequences and example sequences from the four recognized \textit{Chlamydia} species, 'S. negevensis' and 'P. acanthamoebae' were manually aligned taking into account the secondary structure obtained from the SSU database available at the Antwerp Ribosomal RNA Database. Fig. 2 shows the phylogenetic analysis carried out using the neighbour-joining method with a distance matrix calculated by the method of Jukes & Cantor (1969) using \textsc{treecon} for Windows. The percentage similarity between sequences of the order \textit{Chlamydiales} ranged between approximately 65 and 95%. Percentage similarities of type strains are listed in Table 3.

\section*{DISCUSSION}

Using a new \textit{Chlamydiaceae}-specific PCR we have obtained molecular evidence for the existence of at least four new strains. Sequence analysis showed that these strains were distinct from all other known bacteria and clustered together with 'S. negevensis' and 'P. acanthamoebae' in a separate lineage in the order \textit{Chlamydiales}.

Currently, four species are recognized within a single genus \textit{Chlamydia} belonging to the only family, \textit{Chlamydiaceae}, within the order \textit{Chlamydiales}: \textit{C. pecorum}, \textit{C. pneumoniae}, \textit{C. psittaci} and \textit{C. trachomatis}. Molecular, microbiological and immunological analyses have improved this classification by reclassifying several strains; for example, a horse strain and a koala bear strain are now classified as \textit{C. pneumoniae} (Kaltenboeck et al., 1993; Storey et al., 1993). The species \textit{C. psittaci} is very heterogeneous. Microbiological studies have defined eight biotypes (Spears & Storz, 1979) and serological studies have defined nine immunotypes (Perez-Martinez & Storz, 1985). The type strains of both \textit{C. pneumoniae} and \textit{C. pecorum} were originally classified as \textit{C. psittaci}. Detailed molecular analysis of the nucleotide sequences of the 16S rRNA gene and of the ribosomal intergenic spacer has now revealed several sub-groups within \textit{C. psittaci} and \textit{C. trachomatis} (Everett & Andersen, 1997; Pudjiatmoko et al., 1997). Human \textit{C. trachomatis} is subdivided into three biovars: the mouse biovar with one serotype (the mouse pneumonitis agent); the trachoma biovar consisting of the serovars A–K; and the lymphogranuloma venereum biovar consisting of the serovars L1–L3.
Currently, 19 different human serovars of *C. trachomatis* are recognized (Grayston & Wang, 1975; Ossewaarde et al., 1994; Wang & Grayston, 1991). Recent evidence suggests that some strains isolated from swine and hamsters may also be classified as *C. trachomatis* (Everett & Andersen, 1997; Fox et al., 1993; Kaltenboeck & Storz, 1992; Pudimatoko et al., 1997). Two recently identified Chlamydia-like strains, *S. negevensis* and *P. acanthamoebae*, have not been definitely classified yet. Using phylogenetic analysis they appear to be in a sub-branch distinct from, but closely related to, all other Chlamydia strains. Genetic analysis suggested that *S. negevensis* might belong to a new genus within the family Chlamydiaceae (Kahane et al., 1995).

The new sequences cluster together with known Chlamydia sequences apart from other bacteria using data from large 16S sequence databases. The genetic distances between all Chlamydia sequences suggest a division in two lineages: the four recognized Chlamydia species in one lineage and the Chlamydia-like strains in a second lineage. The phylogenetic branch separating these lineages was highly significant by bootstrap analysis.

Although all our samples were from clinically ill patients, it is still too early to draw any conclusions on the pathogenicity of these new strains. One of the new sequences was derived from mononuclear cells of a patient with bronchitis. One and two weeks later comparable specimens from the same person were negative. In addition, four healthy controls, volunteers from the same workplace, were negative. These data suggest the involvement of this new Chlamydia strain in respiratory tract infections, but pathogenicity of these new strains has to be established in more extensive studies. On the other hand, both *S. negevensis* and *P. acanthamoebae* could be considered environmental contaminants: *S. negevensis* was originally found as a cell culture contaminant and *P. acanthamoebae* as an intracellular parasite of possibly free-living amoebae. Therefore, we cannot exclude the possibility that these new strains caused positive PCR assay results by contamination somewhere in the diagnostic chain, either in the patient or during specimen processing.

However, the existence of these new organisms might have significant consequences for serological and molecular laboratory diagnosis of chlamydial respiratory tract infections. So far, there are no data suggesting antigenic cross-reactions between the new strains and recognized species. If such cross-reactions exist, however, serological data should be more carefully interpreted. For example, serological cross-reactions between *C. pneumoniae* and *C. trachomatis* are well-documented (Kern et al., 1993; Moss et al., 1993). This study shows the existence of three regions of highly conserved sequences in the 16S rRNA gene within the family Chlamydiaceae. The existence of more regions can be expected in the remaining part of the 16S rRNA gene. If primers and probes have been chosen from these locations during the design of PCR assays, positive reactions might be incorrectly interpreted as a positive diagnosis of a *C. pneumoniae* infection. At least, the possible existence of serological or molecular biological cross-reactions should be taken into account when interpreting already published data.

In conclusion, we have identified four new previously unrecognized Chlamydiaceae sequences. Phylogenetic analysis strongly suggests that they belong to a separate lineage within the order Chlamydiales.

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REFERENCES


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