DIAGNOSTIC MICROBIOLOGY

Widely used, commercially available *Chlamydia pneumoniae* antigen contaminated with mycoplasma

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Mycoplasma contamination was detected in a widely used commercially available *Chlamydia pneumoniae* antigen preparation. Contamination was studied with a mycoplasma group-specific 16S rRNA polymerase chain reaction (PCR) and sequence analysis. Several lots of the purified *C. pneumoniae* antigen from the Washington Research Foundation appeared to be contaminated with the same *Mycoplasma* species, which appeared to be closely related to *M. arginini*. Antigen slides prepared for the detection of chlamydia antibodies by MRL Diagnostics were contaminated with the same *Mycoplasma* sp. Chlamydia antigen slides from Labsystems OY and two chlamydia complement fixation reagents (Virion International Distribution Ltd and Behring Werke) were not contaminated. It is concluded that commercially available *C. pneumoniae* antigens may contain mycoplasma antigens as well. Although the impact of such mycoplasma contamination on the results of chlamydia serology may not be significant, routine screening of all antigen preparations obtained by tissue culture before their distribution and use is recommended.

Introduction

Serodiagnosis of *Chlamydia pneumoniae*, *C. trachomatis* and *C. psittaci* infection is usually based on complement fixation tests (CF), micro-immunofluorescence tests (MIF) or enzyme-immunoassay (EIA) [1–3]. The antigens used in these tests are purified from chlamydia-infected cell cultures. Worldwide, many cells used for cell culture are contaminated with mycoplasma [4]. As interpretation difficulties arose with the MIF for the detection of *C. pneumoniae*-specific antibodies due to non-specific background fluorescence, the mycoplasma contamination of these commercially available chlamydia antigens was investigated with a mycoplasma group-specific 16S rRNA PCR. Sequence analysis was done to identify the species found by PCR.

Materials and methods

Chlamydia antigens

Purified *C. pneumoniae* elementary bodies were obtained from the Washington Research Foundation

(WRF; Seattle, WA, USA; lot numbers 6, 7 and 17). Slides coated with chlamydia antigen were obtained from MRL Diagnostics, Cypress, CA, USA, and from Labsystems OY, Helsinki, Finland (lot numbers R047994 and 83 PB 1, respectively). These slides are marketed for the specific purpose of serological diagnosis of chlamydial infection. *C. psittaci* complement fixation (CF) reagents were obtained from Virion International Distribution Ltd, Chan, Switzerland and from Behring Werke, Marburg, Germany (lot numbers 293616.08 and 413588B, respectively).

Mycoplasma PCR

Chlamydia antigen slides from MRL Diagnostics and from Labsystems OY were washed as described by Gilroy *et al.* [5]. Briefly, three antigen slides were used for each isolation. Sterile distilled water (50 µl/slide) was added to the slide with an aerosol-resistant tip. The surface was washed and scraped with the tip to dislodge the material and suspend it in the water. Purified *C. pneumoniae* antigen (*c.* 10⁹ organisms/ml) and *C. psittaci* CF antigen were diluted 1 in 100 and 1 in 10 with sterile distilled water, respectively. Diluted antigen suspension (100 µl) or chlamydial antigen recovered from slides (100 µl) were pretreated as described by Boom *et al.* [6].

Mycoplasma DNA was amplified by PCR and analysis of the amplified samples was performed as described by van Kuppeveld *et al.* [7]. A mycoplasma group-specific primer set was used, which amplifies a 280-bp fragment with the following primers: forward primer GPO-3 (5'-GGGAGCAAACAGGATTAGATACCCT-3') and reverse primer MGSO (5'-TGCACCATCTGTCACTCTGTAAACCTC-3'). PCR products (40 µl) were analysed by electrophoresis on an agarose 2% gel stained with ethidium bromide. Southern blot hybridisation was performed with [γ -³²P]ATP-end-labelled internal oligonucleotide GPO-2 (5'-CTTAAAGGAATTGACGGGAAC CCG-3'). The sensitivity of the genus-specific mycoplasma PCR with *Mycoplasma pneumoniae* was *c.* one cfu [8]. All chlamydia antigen preparations were also amplified with a chlamydia-specific PCR to monitor the DNA isolation. *C. pneumoniae*-containing antigens were amplified by a *C. pneumoniae*-specific PCR and analysis of the amplified samples as performed as described by Campbell *et al.* [9]. *C. psittaci* CF antigens were amplified with a PCR that amplifies both *C. pneumoniae* and *C. psittaci*, as described by Tong *et al.* [10]. PCR products (40 µl) were analysed by electrophoresis on an agarose 2% gel stained with ethidium bromide; 20% of the PCR reaction tubes were used for negative controls. All pipetting was done with aerosol-resistant tips and the recommendations of Kwok *et al.* were used to prevent carryover contamination [11].

Mycoplasma hybridisation assay

The GenProbe Mycoplasma tissue culture hybridisation assay (GenProbe Inc., San Diego, CA, USA) was used according to the manufacturer's instructions.

DNA sequencing and phylogenetic tree construction

The 373 DNA Sequencing System (Perkin Elmer, Foster City, USA) was used to sequence the amplified fragments according to the manufacturer's protocol. Sequence homology searches were done by use of the European Molecular Biology Laboratory nucleotide sequence database (EMBL; Heidelberg, Germany) and the GenBank nucleic acid database (National Center for Biotechnology Information, Bethesda, MD, USA). The GenBank nucleic acid database was used for the sequence retrieval of other frequent contaminants of tissue cultures and other bacteria. The CLUSTAL W program (EMBL), release 1.5, was used to calculate the homology of the sequences and to construct multiple alignments of the sequences [12]. The PHYLIP program (Phylogeny Inference Package; University of Washington, Seattle, USA), version 3.5c, was used to construct a phylogenetic tree [13]. The maximum likelihood method was used to estimate the phylogenetic distances between the sequences [14].

Serology

A MIF assay with purified *C. pneumoniae* antigen from the WRF as described before was used to measure *C. pneumoniae*-specific IgG, IgM and IgA antibodies [3]. In addition, a commercially available MIF assay was used to compare *C. pneumoniae*-specific IgG, IgM and IgA antibody titres (Labsystems OY). Sera were obtained from patients with clinical and serological evidence of recent respiratory tract infection due to *M. pneumoniae* (n = 32) or *C. pneumoniae* (n = 156).

Results

Four batches of *C. pneumoniae* antigen from the WRF were analysed. Lot numbers 7 and two batches of 17, which were provided in 1992, 1994 and 1995, respectively, were all mycoplasma PCR positive. Lot number 6, which was provided in 1991, was mycoplasma PCR negative. The microscopy slides from MRL for the detection of chlamydial antibodies were also positive for mycoplasma, as determined by PCR, while the slides from Labsystems for the detection of chlamydial antibodies and the *C. psittaci* CF antigen products were mycoplasma PCR negative. The specificity of the amplified products was confirmed by hybridisation with the internal probe GPO-2. Detectable chlamydial DNA was present in all samples, as determined by chlamydia-specific PCR, while results with all negative controls remained negative. All mycoplasma PCR positive chlamydia antigen samples were confirmed by the GenProbe Mycoplasma hybridisation assay. However, a total of five MRL slides were needed to obtain a weak positive signal in this hybridisation assay.

A DNA fragment of 280 bp was PCR amplified with the primers GPO-3 and MGSO. After sequencing the product in both directions, a consensus sequence was constructed. The length of the remaining sequence was 200 nucleotides. This fragment contained the two variable regions V3 and V5 of the 16S rRNA gene. Comparison of the nucleic acid sequences of all mycoplasma-positive *C. pneumoniae* antigen lot numbers of the WRF, including the mycoplasma-positive *C. pneumoniae* antigen slides from MRL Diagnostics, showed a high degree of similarity (Table 1).

The nucleic acid sequences of the *Mycoplasma* sp. found in the WRF and MRL products were further aligned with those of a group of nine known *Mycoplasma* spp. – *M. hyosynoviae*, *M. hyopneumoniae*, *M. hominis*, *M. orale*, *M. arginini*, *M. fermentans*, *M. pneumoniae*, *M. genitalium* and *Acholeplasma laidlawii*. Six other 16S rRNA sequences were also aligned – *C. pneumoniae*, *C. psittaci*, *C. trachomatis*, *Staphylococcus aureus*, *S. epidermidis* and *Escherichia coli*. A phylogenetic tree was constructed to investigate the relationships among the group *Mycoplasma* spp. found in the WRF antigen, the mycoplasma found

Table 1. Homology values (%) between 16S rRNA sequences of bacterial contaminants found in three different *C. pneumoniae* antigen batches of the WRF and the MRL chlamydia antigen slides and of some other bacterial species

	WRF17a	WRF17b	MRL	<i>M. orale</i>	<i>M. fermentans</i>	<i>M. arginini</i>	<i>A. laidlawii</i>	<i>M. pneumoniae</i>	<i>C. pneumoniae</i>	<i>C. psittaci</i>	<i>C. trachomatis</i>
WRF7	99	98	98	86	85	93	74	74	65	66	65
WRF17a	—	97	96	87	86	92	74	73	65	65	65
WRF17b	—	—	97	87	86	92	74	73	65	66	65
MRL	—	—	—	88	88	93	76	75	67	68	67
<i>M. orale</i>	—	—	—	—	83	87	70	69	63	63	62
<i>M. fermentans</i>	—	—	—	—	—	88	76	76	66	66	65
<i>M. arginini</i>	—	—	—	—	—	—	76	75	68	68	67
<i>A. laidlawii</i>	—	—	—	—	—	—	—	77	69	68	67
<i>M. pneumoniae</i>	—	—	—	—	—	—	—	—	68	67	67
<i>C. pneumoniae</i>	—	—	—	—	—	—	—	—	—	93	93
<i>C. psittaci</i>	—	—	—	—	—	—	—	—	—	—	97

Homology values were determined with the CLUSTAL W program.

in the MRL antigen slides and other bacteria. Analysis of the phylogenetic tree revealed that the mycoplasma sequences from the WRF and MRL antigen products were very similar to those published for *M. arginini* (Fig. 1).

Serum samples (188) were used to determine the impact of mycoplasma contamination of these preparations on their use in serology. No fluorescence pattern was found compatible with cross-reactivity in the *C. pneumoniae* MIF assay when 32 serum samples from patients with recent *M. pneumoniae* infection were used. Also, no significant differences were observed in the *C. pneumoniae*-specific IgG, IgM and IgA titres when the contaminated and clean *C. pneumoniae* antigens were used and their results were compared (data not shown).

Another confounding factor studied was the possibility that such mycoplasma contamination could alter the expression of antigens in the *C. pneumoniae* membrane. MIF results from patients with recent *C. pneumoniae* infection were compared with the contaminated and clean *C. pneumoniae* antigens; 156 serum samples were compared. Again, no significant difference was found in *C. pneumoniae*-specific IgG, IgM and IgA titres (data not shown).

Discussion

The class Mollicutes consists of a very large group of prokaryotes distinguished phenotypically from other bacteria by their lack of cell walls. The largest group is formed by the genus *Mycoplasma*, of which > 90 species have been described. Because of their small size (300–800 nm) and flexibility, mycoplasma can pass easily through 220- and 450-nm filters used in cell culture laboratories [15]. Cell cultures are widely used in both diagnostic tests and in biomedical research settings, as well as in the production of antigens and vaccines by industry. Approximately 15% of all continuous cell lines are estimated to be contaminated with mycoplasmas worldwide [4]. At present, five species account for > 95% of these infections. The most prevalent contaminants are two human strains, *M. orale* and *M. fermentans*, two bovine Mollicutes, *M. arginini* and *A. laidlawii* and a porcine Mollicute, *M. hyorhinis* [16]. Mycoplasmal infection may induce various degrees of cytopathic effects (CPE) in cells. However, infection often remains inapparent. These inapparent infections may result in unreliable experiments as seen in the study by Tan *et al.* [17]. The operon described in their study has been found to be of mycoplasmal origin and not of chlamydial origin as initially reported. Also, mycoplasma infection can be

Fig. 1. Phylogenetic tree of the WRF and MRL mycoplasma contaminant and other bacteria based on 16S rRNA sequences. The phylogenetic tree was calculated by the maximum likelihood procedure of the PHYLIP program.

responsible for the distribution of unsafe biological products, including vaccines and antigen preparations for serological diagnosis [18, 19].

Serological diagnosis of chlamydia infections is often based on CF or MIF results [1, 3]. The antigens used in these tests are commonly purified from chlamydia-infected cell lines. Messmer *et al.* reported that mycoplasma contamination of *C. pneumoniae* isolated from clinical specimens often occurs [4]. Their findings suggest that the *C. pneumoniae* isolates were contaminated by isolation of respiratory specimens, or by propagation in contaminated cell lines.

In the present study, mycoplasma contamination was observed in commercially available antigens marketed for the detection of chlamydia-specific antibodies. Several lot numbers of the purified *C. pneumoniae* antigen from the WRF were contaminated with identical mycoplasmas, as determined by PCR and sequence analysis. Also, the MRL antigen slides for the detection of chlamydial antibodies were PCR positive for the same mycoplasma. One of the explanations for this result can be found in the sharing of cell lines or the use of the same organism for the production of the antigen, or both. Both companies use the AR-39 organism and HeLa 229 cell line for the production of the *C. pneumoniae* antigen. Of interest, the organism used by MRL was provided by the WRF.

Analysis of the phylogenetic tree revealed that the mycoplasma sequences from the WRF and MRL antigen products were closely related, but not identical, to *M. arginini*. This indicates the possibility of a new *Mycoplasma* species, which has not previously been submitted to the GenBank and EMBL databases.

Several methods were used by MRL Diagnostics, Labsystems and the WRF to detect mycoplasmal contamination before culture of the chlamydial antigens. Mycoplasma detection at MRL diagnostics was done by the GenProbe mycoplasma screening assay (Dr W. Hogrefe, personal communication). Failure to detect mycoplasmal contamination by hybridisation assay was probably due to the small number of organisms present in the samples, as seen in the present study. For every new chlamydia antigen lot the cell culture system from Labsystems was tested with fluorochromic dye (DAPI). Also, the cells were tested twice a year by the mycoplasma PCR ELISA from Boehringer Mannheim (Dr M. Ristola, personal communication). The WRF tested only the stock HeLa 229 cells by DAPI staining twice a year (Dr C. C. Kuo, personal communication). Despite these prevention strategies, contamination with mycoplasma has apparently occurred frequently. These results demonstrate that the PCR may currently be the preferred tool for the detection of mycoplasma

contamination in cell cultures and chlamydial strains. Although this particular mycoplasma contamination had no significant impact on serodiagnosis of *C. pneumoniae* infection by MIF, caution should be exercised. Inapparent cell line contamination with human *Mycoplasma* spp. is always possible, which can have a major impact on the serodiagnosis of many bacterial and viral infections, and may confound research efforts.

In conclusion, mycoplasma contamination of commercially available antigens for the serodiagnosis of chlamydia infections has occurred, and therefore routine screening of all antigen preparations obtained by tissue culture by sensitive techniques is recommended before their distribution and use.

This work was presented in part at the 36th Interscience Conference on Antimicrobial Agents and Chemotherapy, New Orleans, USA, 15–18 Sept. 1996, and the 3rd meeting of the European Society for Chlamydia Research, Vienna, Austria, 11–14 Sept. 1996.

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