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Laboratory diagnosis of *Mycoplasma pneumoniae* infection. 4. Antigen capture and PCR-gene amplification for detection of the mycoplasma: problems of clinical correlation

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SUMMARY

Direct detection assays for *Mycoplasma pneumoniae* were established by PCR amplification of short sequences within the foot protein/adhesin (P1) gene and the 16S ribosomal RNA gene.

Specificity and sensitivity was excellent, no hybridization was observed with *M. genitalium* and other human *Mycoplasma* species. In nose and throat washings from subjects with respiratory infection a pattern of high counts (c.f.u./ml) of *M. pneumoniae* (deduced from the amount of amplified PCR product), and a positive antigen capture assay, was found in 83% of subjects with serological evidence of current infection with *M. pneumoniae*.

A small proportion of subjects with serological patterns suggesting infection in the more distant past had positive PCR assays. This was considered to represent either persistence of the organism from a previous infection or perhaps transient carriage during a reinfection, without substantial change in antibody response.

PCR-based assay of *M. pneumoniae* offers a powerful, rapid, and sensitive substitute for culture of the mycoplasma. Antigen capture, while less sensitive than PCR, offers the advantage that it is more often positive with samples from current infection and requires less stringent laboratory organization to contain false positive results. We conclude however that the laboratory diagnosis of a chosen clinical episode should not rest on the PCR or Ag-EIA assays alone, but must also include antibody assays to confirm whether infection is current or represents persistence from past exposure.

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INTRODUCTION

Previous papers in this series outlined the clinical importance of *M. pneumoniae* in respiratory infection and the shortcomings of conventional methods for its laboratory diagnosis [1, 2].

In attempts to improve the laboratory methods, direct and indirect antigen capture enzyme immunoassays (Ag-EIA) were devised [1] and compared with dot-blot hybridization using, as ^{32}P probes, single-stranded (M13; $\cong$ 500 nucleotides) and double-stranded ($\cong$ 1000 base pairs) sequences derived from the *M. pneumoniae* DNA cloned in *Escherichia coli*, for direct diagnosis (Harris and colleagues, unpublished). Ag-EIA was also compared with a ^{125}I probe against a specific rRNA sequence of the mycoplasma (*Mycoplasma pneumoniae* Rapid Diagnostic System: GenProbe, CA, USA). Dot-blot hybridization with cloned probes and Ag-EIA were of roughly equal sensitivity with simulated positive samples (nasopharyngeal exudates seeded with graded doses of a culture of *M. pneumoniae*) whereas GenProbe was much more sensitive. However, with specimens from naturally-infected subjects, Ag-EIA detected about 55% of serologically proven cases [1] but GenProbe only detected about a quarter of those identified by Ag-EIA [2].

The unexpected insensitivity of GenProbe (given its excellent performance with simulated samples) was attributed, speculatively, to the loss of the rRNA target in respiratory secretions during natural infection, once the membrane of the mycoplasma cell had been disrupted by immune response or chemotherapy. On the other hand, cell adherent mycoplasma proteins and glycolipid antigens might be cleared more slowly from the respiratory tract [1, 2].

As a response to this problem we thought that the use of the polymerase chain reaction (PCR) [3] to amplify short sequences of the genome of the mycoplasma might not only offer a much greater sensitivity than the methods already explored, but also might circumvent the problem of nucleic acid degradation by permitting the amplification of any residual DNA fragments remaining in damaged organisms.

The present report compares the specificity and sensitivity of PCR assays for DNA sequences specific for *M. pneumoniae* found within the P1 protein and the 16S ribosomal RNA genes of the mycoplasma, with the Ag-EIA for direct detection of the organism in respiratory exudates. Simulated positive and authentic respiratory tract specimens were used and particular attention is paid to the difficulties of laboratory diagnosis of a clinical episode with an organism that apparently persists in the respiratory tract for longer periods than previously appreciated. While this work was in progress, preliminary reports on PCR detection of *M. pneumoniae* [4, 5] and *M. genitalium* [6–10] have appeared.

MATERIALS AND METHODS

Culture and quantitation of laboratory strains of mycoplasma

Methods for the growth of the organisms and for determination of colony-forming units of *Mycoplasma* and *Acholeplasma* spp. were those described by Kok and colleagues [1]. Briefly, the standard strains were grown in SP4 broth culture

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medium [11]. Dilutions of these cultures were plated onto modified Hayflicks' mycoplasma agar [12] for up to 10 days. Colony-forming units were determined by counting colonies with a plate microscope (× 10).

Assay of DNA content of mycoplasmas

The DAPI (4′,6-diamidino-2-phenylindol) fluorescence methods were used as outlined by Harris and colleagues [2].

Serological assays

Antibody to *M. pneumoniae* was titrated by a modified haemagglutination assay for total (IHA(t)) and IgM antibody (IHA-IgM) [13]; also by a locally-developed EIA for IgM antibody and by complement fixation tests [12, 13].

Source of clinical samples

Nose and throat washings from patients with respiratory illness in a general survey of subjects for infection with respiratory viruses and other agents were collected and forwarded to us from the University of Newcastle by Professor G. Tannock.

Preparation of simulated positive and other samples for assay by PCR dot-blot hybridization (PCR-DBH)

Pooled sputa, shown to be negative for *M. pneumoniae* antigen by Ag-EIA, were solubilized with an equal volume of 'Sputolysin' (Behring Diagnostics) and dilutions of a broth culture of *M. pneumoniae* were added to give final counts ranging from 10^0–10^6 c.f.u./ml. 50 μl aliquots of the simulated positive specimens were centrifuged 10000 g for 30 min in a microfuge at RT. The pellet was resuspended in 20 μl of a mixture of 10 mM Tris-HCl (pH 7·5 at 37 °C), 0·01% NP40, containing 200 μg/ml proteinase K and incubated for 1 h at 37 °C. Samples were then boiled for 10 min to denature proteinase K which would otherwise subsequently degrade the Taq polymerase and interfere with the amplification reaction. The clinical specimens from the Newcastle survey were either throat or nasal washings and did not need Sputolysin treatment, otherwise they were processed in the same fashion.

PCR-dot-blot hybridization amplification assay for M. pneumoniae *P1 gene (P1-PCR-DBH)*

The P1-PCR-DBH assay was based on the PCR amplification of a 543 bp section of the P1 protein gene (external surface adhesin gene) of *M. pneumoniae*. The amplified product was examined by two methods. The first was gel electrophoresis, while the second utilized quantitative dot-blot hybridization (P1-PCR-DBH) using a synthetic ^{32}P 'hairpin' probe [14].

The primers were (*a*) PCR primer 1:

5′-CAAGCCAAACACGAGCTCCGGCC-3′

which is complementary to the P1 gene negative strand residues 3666–3688 (P1

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gene sequences as described by Inamine and colleagues [15], and (*b*) PCR primer 2:

5′-CCAGTGTCAGCTGTTTGTCCTTCCCC-3′

which is complementary to the positive strand residues 4208-4183. The ^{32}P hairpin hybridization probe was 69 nucleotides long,

5′-CCTCCTCCACCAACAACCTCGCGCC-T_{22}-AAA($[^{32}P]A)_{19}$-3′,

where $([^{32}P]A)_{19}$ refers to 19 [^{32}P]dAp residues. This consisted of a synthetic portion of 50 nucleotides in which nucleotides 1–25 were complementary to 4040–4065 of the negative strand of the P1 gene (i.e. within the PCR amplified P1 gene section), and nucleotides 26–50 act as template and primer in a hairpin configuration for fill-in by Klenow pol 1 fragment and [^{32}P]dATP (see below). This gave about 19 radiolabelled [^{32}P]dAP residues per probe and a specific activity of $\geqslant 10^9$ c.p.m./μg [14].

This choice of primers and detection probe is specific for *M. pneumoniae*. Although *M. genitalium* has a gene similar to the P1 gene of *M. pneumoniae* [16], the two PCR primers and ^{32}P hairpin probe do not bind to the *M. genitalium* gene because of the deliberate choice of differing sequence for primers and probe for the two organisms.

The PCR method followed that of Saiki and colleagues [3] and was that described in the protocol notes (1989) for AmpliTaq issued by Perkin Elmer-Cetus. The reaction mixture contained, in a final volume of 50 μl, 20 μl of proteinase K-treated sample (see above), 10 mM Tris-HCl pH 8·3 (at 25 °C), 50 mM KCl, 1·5 mM $MgCl_2$, and 0·02% (w/v) gelatin (Cat No. G2500, Sigma, St Louis, MO) added to offset the action of any residual, uninactivated proteinase K. The whole PCR buffer was autoclaved before use. The reaction mixture also contained 200 μM of each dNTP (PROMEGA), 0·6 μmol of each primer 1 and 2 and 2 units of cloned Taq polymerase. Each batch of tests included positive and negative controls consisting, respectively, of 100 pg *M. pneumoniae* DNA and 1 μg of *E. coli* DH1 DNA.

Thirty cycles of PCR were performed with each cycle consisting of 1 min at 94 °C for denaturation, 1 min at 55 °C for annealing and 2 min at 72 °C for primer extension. The final cycle incorporated an extension step of 10 min at 72 °C. The reaction mixture was then used for dot-blot hybridization (see below).

Treatment of PCR-amplified products for dot-blot hybridization

The PCR-amplified products from the test samples and from the control, *M. pneumoniae* and *E. coli* DNA, were denatured by incubation at RT with sodium hydroxide (final concentration = 0·5 M) for 15 min, followed by neutralization with ammonium acetate (final concentration = 1 M). Samples were loaded by gravity filtration onto Zeta-probe membranes using a Bio-Dot apparatus (Bio-Rad, Richmond, CA 94804). The filters were washed twice with 1 M ammonium acetate and then allowed to air-dry before baking in a vacuum oven for 2 h at 80 °C.

Hybridization and washing conditions

The hybridization and washing conditions for the 'hairpin' probe were those of Sriprakash and Hartas [14]. Membranes were autoradiographed and individual

spots were cut from the filters and the radioactivity quantitated by liquid scintillation counting. These c.p.m. values were used to calculate a sample ratio for the PCR-dot-blot hybridization assay as follows:

$$\text{Sample ratio} = \frac{\text{sample c.p.m.} - \text{background c.p.m.}}{\text{background c.p.m.}}.$$

The recommended 'cut-off' point for the above is a ratio of 2·0 and chosen on the basis that it was well above the value for all observed negatives. Positive and negative controls were included in every PCR test (see above).

PCR-dot-blot hybridization assay for the 16S rDNA gene of M. pneumoniae *and the P1-like gene of* M. genitalium

As a consequence of the initial testing of the clinical samples by Ag-EIA, and P1-PCR-DBH it became necessary to retest the samples for the presence of *M. pneumoniae* using primers to another gene and also for *M. genitalium* (see explanation under Results). The primers and probes chosen for this purpose, under the same general assay conditions, were:

(*a*) *M. pneumoniae*, specific sequences from 16S ribosomal RNA gene differing from those found in other *Mycoplasma* species [17]

Primer 1 (186–208):

5′-GAATCAAAGTTGAAAGGACCTGC-3′

Primer 2 (475–452):

5′-CTCTAGCCATTACCTGCTAAAGTC-3′

Hairpin probe (269–251):

5′-GGTAGGCCGTTACCCCACC-T_{30}-AAAA-($[^{32}P]A)_{26}$-3′

Product size: 290 bp.

The assay conditions were those described above except annealing was at 60 °C for 1 min

(*b*) *M. genitalium* P1 (foot protein) gene equivalent [16]

Primer 1 (2614–2637):

5′-GGTGGCTCCTCCAAACCAACCACC-3′

Primer 2 (2734–2712):

5′-GCAACAGTTGATTGCGCTGCGGG-3′

Hairpin probe (2650–2679):

5′-CCCAACAGTACTAGTCCCACCAGTGACTGG-T_{36}-AAAA-($[^{32}P]A)_{32}$-3′

Product size: 121 bp.

RESULTS

Elimination of proteinase K activity from processed samples before PCR amplification

In initial experiments, with simulated samples which had been proteinase K-treated and then boiled, low yields of amplification product were obtained. The

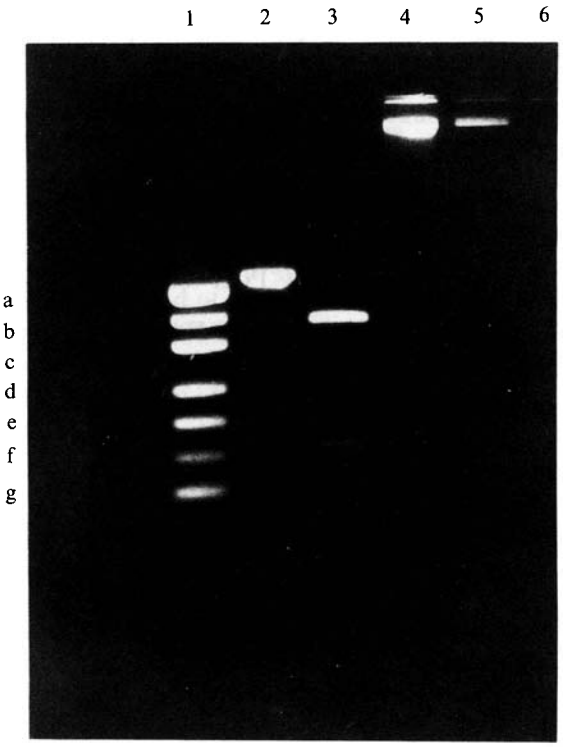

Fig. 1. Patterns obtained by gel electrophoresis of undigested and restriction enzyme-digested product from PCR amplification of a 543 bp fragment in the P1 gene of *M. pneumoniae* together with various controls. Lane 1, Mol. mass markers pUC19 digested with *Hpa* I (a = 501 bp, b = 404, c = 331, d = 242, e = 190, f = 147, g = 110; lane 2, PCR product from *M. pneumoniae*; lane 3, PCR product from *M. pneumoniae* digested with *Hind* III; lane 4, *E. coli* DNA as substrate (no product); lane 5, *M. genitalium* as substrate; lane 6, HeLa cell DNA as substrate.

decreased yield seemed likely to be due to the presence of residual proteinase K which degraded the Taq polymerase. Inclusion of 0·01 % v/v NP40 in the buffer used for proteinase K digestion promoted full inactivation of proteinase K during boiling. This step, plus the addition of gelatin to the reaction mixture, led to satisfactory product yields (Williamson and Harris 1991, unpublished).

Authenticity of the product of PCR amplification of a fragment of the M. pneumoniae *P1 gene*

Authenticity was established by

(i) Gel analysis of the product which visualized a DNA fragment of 540 bp (Fig. 1) corresponding to the expected size (543 bp) based on the sequence of the P1 gene [15, 18] and the spacing of the PCR primers (see Materials and Methods).

(ii) The 543 bp DNA fragment contains one *Hind* III restriction enzyme site and digestion of the product produced fragments of the predicted sizes (expected 387 and 156 bp; observed 396 and 157 bp) (Fig. 1).

(iii) The product hybridized to a ^{32}P hairpin probe directed against a region within the expected product (see Materials and Methods).

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Table 1. *Specificity of product produced by PCR amplification with primers to sequences within the P1 and 16S ribosomal genes and within the P1-like gene of* M. genitalium. *Analysis on gel electrophoresis and by dot blot hybridization assay when tested with a range of mycoplasma and other microbial species of human or animal origin as substrates for the PCR reaction*

	M. pneumoniae			*M. genitalium*
		PCR-DBH sample ratios		
Organism tested	Gel electrophoresis P1 gene	P1 gene	16S rDNA gene	P1-like gene
M. buccale	–	0·7	0·5	1·4
M. fermentans	–	1·5	0·6	1·0
M. genitalium	–	1·0	1·3	26·0
M. hominis	–	0·9	0·9	0·7
M. hyorhinis	–	1·4	0·8	0·7
M. pneumoniae	+	27·4	18·6	0·6
M. salivarium	–	0·9	1·0	1·2
A. laidlawii A	–	1·3	1·0	1·2
A. laidlawii B	–	1·2	0·6	1·6
Streptococcus MG*	–	0·6	1·1	0·5
E. coli DH 1 strain*	–	1·0	0·6	1·0
Pooled negative sputa	–	1·2	1·1	0·9
Cut-off values	Visible fragment†	2·0	2·0	2·0

Reaction mixtures included 0·05 ml of 10^6 c.f.u./ml of each mycoplasma. * Other organisms were tested using sufficient organisms to give 1 μg of DNA in the reaction mixture (see Materials and Methods).

† P1 gene PCR product (10 μl) analysed by agarose gel electrophoresis and ethidium bromide staining (see Fig. 1).

Amplification factor obtained with PCR of a fragment of the M. pneumoniae *P1 gene*

Comparison of peak areas obtained by scanning a Polaroid negative of an agarose gel of a range of DNA standards (plotted as peak area *v.* amount of DNA) and of the PCR-amplified P1 gene fragment, indicated an amplification factor of 6×10^6.

Specificity of the PCR assays

The specificity of the PCR reaction for *M. pneumoniae* was determined by testing a range of *Mycoplasma* species and bacteria. The mycoplasmas were tested at 10^6 c.f.u./ml, while *E. coli* and *Streptococcus* MG were tested using sufficient organisms to give 1 μg of DNA (DAPI method) per reaction. The specificity of the combined PCR, gel-electrophoresis assay was excellent; only *M. pneumoniae* gave a visible DNA fragment on gels stained with ethidium bromide (Table 1). The specificity of the product on dot-blot hybridization with the hairpin probe was also excellent; no cross-reacting organism was found (Table 1). Similar tests with the primers and probe to the 16S ribosomal RNA gene of *M. pneumoniae* also gave comparable specific results (Table 1). The specificity of the PCR amplification assay for *M. genitalium* was checked in the same way. No reciprocal cross-reacting organism was found (Table 1). Sensitivity was similar to that obtained for *M. pneumoniae*.

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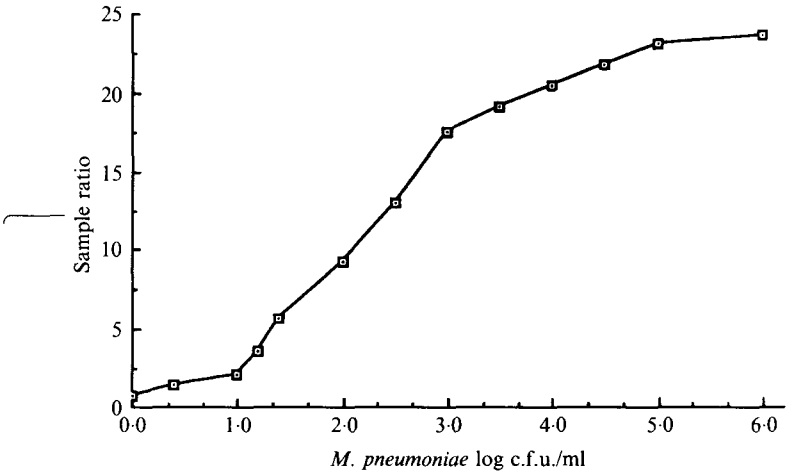

Fig. 2. Quantitation of *M. pneumoniae* by P1-PCR-DBH. A range of dilutions (10^0–10^{-6}) of a laboratory culture of *M. pneumoniae* (FH strain) was tested by P1-PCR-DBH and the sample ratios plotted against the c.f.u./ml of the mycoplasma.

Comparison of the sensitivity of M. pneumoniae *detection by P1-PCR-DBH and by Ag-EIA with stimulated clinical specimens*

This was determined as illustrated by Harris and colleagues [2]. A range of dilutions from 10^6 to 10^0 c.f.u./ml of *M. pneumoniae* was prepared in a pool of normal sputa (negative by Ag-EIA and P1-PCR-DBH). An aliquot of each dilution was assayed by P1-PCR-DBH followed by analysis of the product on gel electrophoresis (0·01 ml), and on dot-blot hybridization (0·05 ml). The volume of PCR reaction product analysed on gel was one fifth of the total reaction product. The residue, or later the total product, was analysed by dot-blot hybridization (unless otherwise stated). Ag-EIA (0·05 ml) was performed as described by Kok and colleagues [1]. Direct detection of the product by gel electrophoresis was found to be insensitive and difficult to quantitate (data submitted but not shown). The P1-PCR-DBH assay overcame both of these problems. Thus the most sensitive assay was PCR amplification followed by dot-blot hybridization which detected 50 c.f.u./ml of the mycoplasma, followed by PCR and gel electrophoresis which detected, 500 c.f.u./ml (note, however, that only 0·01 ml of the total product was analysed by gel electrophoresis). Lastly, the least sensitive was Ag-EIA with an end point around 10^3 c.f.u./ml. The absolute detection limit by PCR techniques was 2·5 c.f.u. (i.e. PCR detection limit = 50 c.f.u./ml × 0·05 ml tested = 2·5 c.f.u. detected), which is equivalent to approximately 400 genome copies (1 c.f.u. = approximately 160 genome copies [2]).

Quantitation of M. pneumoniae *by P1-PCR-DBH*

An important finding arising from application of the P1-PCR-DBH assay was that *M. pneumoniae* could be quantitated in specimens. This was determined by P1-PCR-DBH assay on aliquots of falling dilutions of a *M. pneumoniae* culture of known c.f.u./ml (Fig. 2). There was a usable semilinear relationship between sample ratio and c.f.u./ml over the range 10^1–10^5 and the content of organisms in a sample could be quantitated approximately from the sample ratio.

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Table 2. *Correlation of the results of detection of* M. pneumoniae *by Ag-EIA and P1-PCR-DBH with clinical category and stage of respiratory infection in individuals as assessed by serological criteria*

	Clinical category	Subjects (*n*)	Serological assessment of stage of infection	Results: Ag-EIA Pos	Ag-EIA Neg	P1-PCR-DBH Pos	P1-PCR-DBH Neg	Subtotal
(A) or (B)	URTI (43) or LRTI (10)	53	Current*	3	0	1	2	3§
			Recent†	7	13	5	15	20
			Past. distant past‡	4	26	3	27	30
(C)	No symptoms	9	Current*	3	1	4	0	4
			Recent†	0	0	0	0	0
			Past. distant past‡	1	4	1	4	5
	Total	62		18	44	14	48	62

* Current infection: ⩾ fourfold increase of titre by CFT, IHA(t) or IgM, or EIA-IgM between sera taken before, during or after the 'index episode'.

† Recent, but not current infection: unchanging titre IHA(t) > 320; IHA IgM positive between sera taken before, during or after the 'index episode'.

‡ Past infection: (unchanging titre IHA(t) < 320: IHA IgM neg.); distant past infection (unchanging IHA(t) $\leqslant 80$; IHA IgM neg.) with sera taken before, during or after the 'index episode'.

§ Includes one subject with possible reinfection.

Comparative sensitivity of detection of M. pneumoniae *by P1-PCR-DBH and by Ag-EIA with specimens from patients with or without serological evidence of recent or current* M. pneumoniae *infection*

A total of 62 subjects selected from the Newcastle survey of acute respiratory infection provided serum specimens, throat and nasal washings. Such samples had been collected at regular intervals during the survey which was undertaken primarily to ascertain the prevalence of respiratory viruses in the area. As the samples had been collected for virus isolation they contained antibiotics inhibitory for mycoplasmas so culture was not attempted. Preliminary serological tests in Newcastle revealed some rising CF antibody levels to *M. pneumoniae* antigen so it was decided to investigate in greater detail whether the mycoplasma was involved in episodes of upper or lower respiratory tract illness with the particular objective of determining whether assay of the nose and throat washing by Ag-EIA or PCR amplification of *M. pneumoniae* genes would give a decisive result identifying a particular episode of respiratory illness as caused by *M. pneumoniae*.

Table 2 summarizes the findings by Ag-EIA and P1-PCR-DBH in relation to clinical diagnosis, and by serological criteria for (*a*) current, (*b*) recent but not current infection and (*c*) infection in the past or distant past. The serological criteria are given at foot of Table 2; serum samples were available in the weeks before the illness, at the time of the clinical incident chosen for diagnosis ('index episode') or after the episode. Group C, with no recorded symptoms, had periodic serum sampling over the same period as those with URTI or LRTI.

Among the 53 subjects with symptomatic upper or lower respiratory tract

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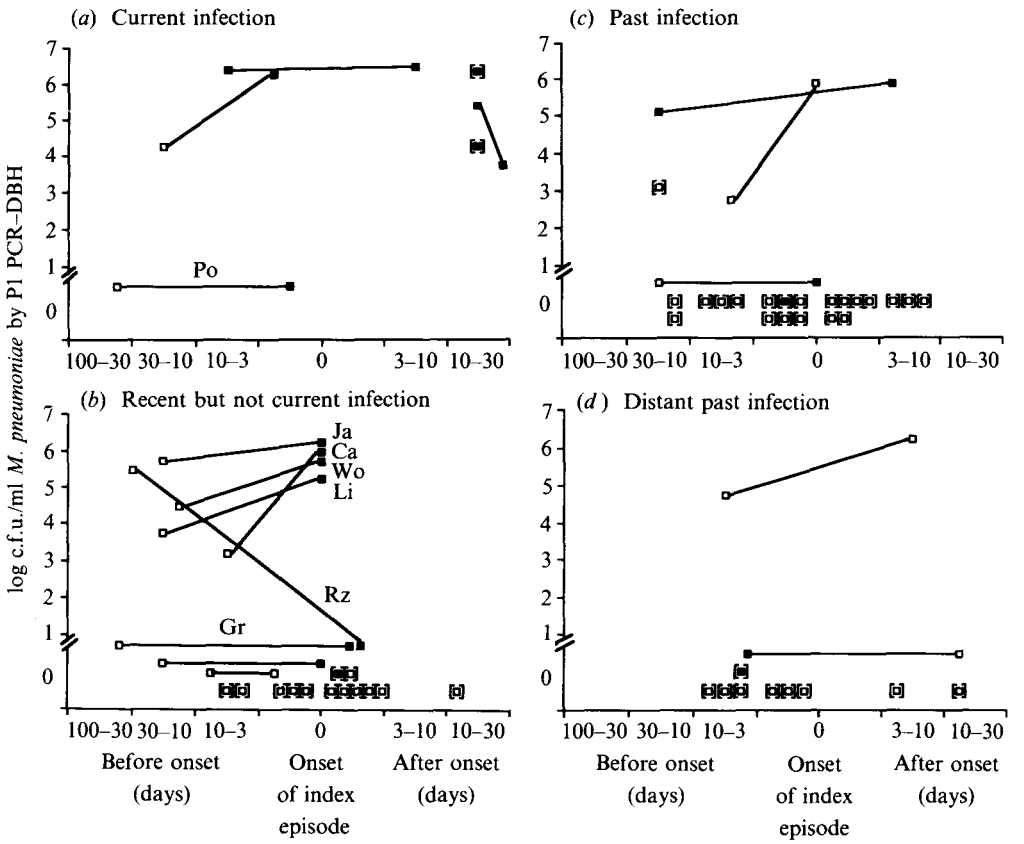

Fig. 3. Reactions by Ag-EIA (■, positive; □, negative) or numbers (c.f.u./ml) of *M. pneumoniae* detected by P1-PCR-DBH, in nose or throat washings of subjects in relation to the timing of a clinical or infection episode (index episode). Groups of subjects are arranged by the serological criteria given in Table 3.

infection (groups A and B combined) there were 3 (5·6%) with serological evidence of current infection; all were positive by Ag-EIA and one by P1-PCR-DBH. One of the three subjects-GR-with LRTI, had a rising HIA(t) antibody titre from 80 to 1280, without IgM class antibody by IHAIgM. Ag-EIA was positive on nasal washings but P1-PCR-DBH was negative (Fig. 3). This may have been an example of reinfection as described by Hu and colleagues [19]. The subject is shown as a current infection in Table 2 but has been reclassified as recent, not current infection in subsequent Tables and in Fig. 3, because of the absence of IgM antibody.

Twenty of the total group of 53 symptomatic subjects had serological evidence of recent but not current infection and of this subgroup, 7 (35%) were positive by Ag-EIA and 5 (25%) by P1-PCR-DBH. Again, most of these subjects complained of upper rather than lower tract symptoms. Smaller numbers of positives by Ag-EIA or P1-PCR-DBH were found in the subgroup with serological evidence of past, or distant past infection.

Lastly, there were 4 current infections (seroconversions) without symptoms in the remaining 9 subjects (group C); all had evidence of infection by Ag-EIA

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Table 3. *Summary of the number of subjects with URTI, LRTI, or with no symptoms, whose respiratory tract washings reacted in the Ag-EIA and/or P1-PCR-DBH assays*

	PCR-DBH		
Ag-EIA	Positive	Negative	Total
Positive	10	8	18
Negative	4	40	44
Total	14	48	62

χ_1^2 (association) 15·4, $P < 0{\cdot}01$: χ_1^2 McNemar = 0·75, $P > 0{\cdot}25$.

and/or P1-PCR-DBH and numbers of organisms above 10^4 c.f.u./ml by quantitative PCR.

In summary (Table 2), of those subjects (groups A, B and C combined) with either serological evidence of current, or of a recent but not current infection, a total of 13/27 (48%) and 10/27 (37%), were positive by Ag-EIA and P1-PCR-DBH respectively, as against 5/35 (14%) and 4/35 (11%) of those in the groups with serological evidence of infection in the past or distant past.

The general pattern therefore appeared to be that the respiratory infection episodes ('index episode') chosen for investigation were occurring in a population recently seeded by a widespread prevalence of *M. pneumoniae* with persistence of the organism as judged by the presence of antigen or DNA of the organism. Alternatively, the Ag-EIA or P1-PCR-DBH positives in persons with serological evidence of past or distant infection may represent reinfection (see Discussion). However, despite the small numbers (3 of 53) of authenticated *M. pneumoniae* infections in the symptomatic subjects (i.e. current infection determined serologically, plus positive Ag-EIA or P1-PCR-DBH), the involvement of identifiable respiratory viruses was also low and the etiology of many respiratory episodes remained unclear. Thus of the *M. pneumoniae*-negative symptomatic episodes under investigation, only 2 of the 43 patients with URTI had evidence of current infection with influenza B virus, while 2 of the 10 patients with LRTI had a current infection with parainfluenza virus and a third had influenza B.

Correlations and sensitivity of Ag-EIA and P1-PCR-DBH assays

Table 3 summarizes the overall relationship, in terms of subjects, between assay reactions with nose or throat washings in the Ag-EIA and the P1-PCR-DBH. It will be seen that although there is a general trend associating Ag-EIA + /P1-PCR-DBH + or Ag-EIA − /P1-PCR-DBH − χ_1^2(association) 15·4 ($P < 0{\cdot}01$), but there are discrepancies in both directions, most marked in the category Ag-EIA + /P1-PCR-DBH − . However there is no statistical support for the view that the values are from different universes of observations (χ_1^2 McNemar = 0·75; $P > 0{\cdot}25$). The discordant values between Ag-EIA and P1-PCR-DBH were explored further by considering the results from the examination of *all* specimens as distinct from *all* subjects. As before the clinical categories were separated by serological criteria. Overall, with all the samples, P1-PCR-DBH yielded more positives than Ag-EIA − 47 *v.* 27; 81% and 46% of the total of 58 positive samples respectively. The proportion of samples positive by one or other, or both assays was higher 18/22

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(82%) in the group with serological evidence of current infection as compared with the three other groups (37–27%).

Other analysis (data not shown) revealed that although P1-PCR-DBH gave the highest proportion (88%) of the total positives obtained with the specimens from the 'current infection' group, raised rates (80 and 84%) were also obtained with the 'recent, but not current' and 'past' infection groups, whereas the proportion with positive Ag-EIA, though lower (10/18, 55%) in the current infection group, fell away more sharply in the recent but not current infection group (9/20, 45%) to 8/20 (40%) in those infected in the past or distant past group. This difference was reflected in the increasing discordance between the two assays, which is least (10/18, 55% of total positives) with samples from 'current' infection and maximum (7/7, 100%) with those samples from subjects judged to have been infected with the distant past. However, analysis for discordance between the two assays by χ^2 McNemar did not however show a statistically significant superiority of P1-PCR-DBH over Ag-EIA except for the overall distribution for all specimens (χ^2 McNemar = 8·5, $P < 0{\cdot}005$).

This analysis takes no account of the relation between reactions by Ag-EIA or P1-PCR-DBH, the time of sampling in proximity to the 'index episode' (illness or serological conversion) under attempted diagnosis, and the number of organisms detected by P1-PCR-DBH. To remedy this, Fig. 3 sets out, (*a*) the mean number of c.f.u./ml of *M. pneumoniae*, determined by P1-PCR-DBH and (*b*) positive or negative reactions by Ag-EIA for samples taken at various time points before, during or after the 'index episode' in the 62 subjects. In Fig. 3, when only a single sample (NW or TW), was available at a particular time point, it is shown as a square inside a square bracket. When more than one sample was available the mean values for c.f.u./ml were derived and if antigen was present in any of the samples, a solid square is shown. Samples at different time points taken from the same individual are connected by a line. Data are grouped, as before, in the four serological categories.

Several points of interest emerge. Five (83%) of the six subjects with serological evidence of current infection had *M. pneumoniae* c.f.u./ml titres $\geqslant 10^4$ and all were Ag-EIA positive (Fig. 3*a*). The remaining patient (Po) in this group had an unchanging CF antibody titre of 8 between sera taken 35 days before the index episode (URTI) and a sample taken 14 days after the episode. However the HIA(t) titre rose from 160 to 2560 and IgM antibody developed. Although P1-PCR-DBH and 16S rDNA-PCR-DBH were negative, a sample (TW) taken during the index episode was Ag-EIA positive. One interpretation is that Po had URTI (unrelated) as an 'index episode' during the late convalescent phase of an *M. pneumoniae* infection, rather then an URTI due to a current infection with the mycoplasma (see Discussion).

In the group of recent but not current infections (Fig. 3*b*), 4 (19%) – subjects Ja, Li, Wo, Ca, – of the 21 subjects had high ($\geqslant 10^4$ c.f.u./ml) P1-PCR-DBH values and were also antigen positive, whereas all other subjects in this group are P1-PCR-DBH negative at the time of the index episode although four were Ag-EIA positive. Subjects Ja, Li, Wo and Ca all had raised HIA(t) titres 640–2560 and IgM antibody, although one (Li) showed falling HAI(t) levels and later reverted to IgM negative. The findings are interpreted as those in subjects sampled

Table 4. *Correlation of the numbers of* M. pneumoniae *organisms with the stage of infection as determined by serological pattern*

Serological category	Proportion with P1-PCR-DBH values $\geqslant 10^4$ c.f.u./ml *M. pneumoniae*			
	Subjects	(*n*)	Specimens	(*n*)
Current infection	5 (83%)	6	13 (59%)	22
Recent, not current	5 (24%)	21	11 (20%)	54
Past	3 (12%)	24	6 (12%)	48
Distant past	1 (9%)	11	3 (12%)	24
Total	14 (22%)	62	33 (22%)	148

in the later stages of an *M. pneumoniae* infection as described by Kok and colleagues [1]. A fifth subject (Rz) showed a steep decline in numbers of mycoplasmas between 30 days before the 'index episode' and its onset. This was accompanied by a decline in HIA(t) from 2500 to 80 and seroconversion to IgM negative. Rz is an example of a subject becoming P1-PCR-DBH negative but showing antigen in a later sample (Discussion). A sixth subject, Gr, mentioned in the analysis of Table 2, had a IHA(t) response without IgM and was Ag-EIA positive/P1-PCR-DBH negative.

In the two remaining groups with serological markers of past infection (Fig. 3*c*, *d*), most subjects were negative; 4 (11%) of the total of 35 subjects had raised P1-PCR-DBH values suggesting continuing carriage from a previous infection or perhaps reinfection. Table 4 summarises the same data for subjects or specimens; a level of $\geqslant 10^4$ c.f.u./ml discriminates well between the presumptive current infection group and the remainder.

The detection of more positives by P1-PCR-DBH than by Ag-EIA was not unexpected in view of the greater sensitivity of PCR. What was unexpected was the discordant group Ag-EIA+/P1-PCR-DBH negative. Although the χ^2_1 McNamar tests had indicated that the discordance between Ag-EIA and PCR assays is of borderline significance for most subgroups, and could be due to chance, it seemed necessary nevertheless to seek an explanation for the Ag-EIA positive/PCR negative subgroup brought out in Table 3 and illustrated in Fig. 3. Three can be envisaged.

First, the primers and probe used to detect the P1 gene might not detect strains of *M. pneumoniae* with a variant P1 gene whereas the mycoplasma antigens are invariant and would be detected by Ag-EIA. All samples were therefore retested under code with the primers and probe against sequences in the 16S ribosomal RNA gene which are specific for *M. pneumoniae* (see Materials and Methods). This revealed (Table 5) that, overall, all of the P1-PCR-DBH positive subjects reacted identically with the 16S rDNA PCR-DBH. However, when all samples are considered, there were four instances in which P1-PCR-DBH was negative but the 16S rDNA probe gave a positive result. One of the four samples was also Ag-EIA positive. Interpretations are offered in the Discussion.

Second, Ag-EIA might detect a genetically unrelated organism which fortuitously shares antigens with *M. pneumoniae*; this would not be detected with primers for the P1 protein or 16S rDNA genes used for PCR reaction. The question of

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Table 5. *Relation, in subjects and specimens, between Ag-EIA and PCR-DBH assays for P1 and 16S rDNA of* M. pneumoniae

		Reaction patterns in previous tests				
Group	Probe	AgEIA+ P1-PCR-DBH+	AgEIA− P1-PCR-DBH+	AgEIA+ P1-PCR-DBH−	AgEIA− P1-PCR-DBH−	Total
Subjects	16S rDNA+	10	4	0	0	14
($n = 62$)	16S rDNA−	0	0	8	40	48
	Subtotal	10	4	8	40	62
Specimens	16S rDNA+	16	31	1	3	52
($n = 148$)	16S rDNA−	0	0	10	87	96
	Subtotal	16	31	11	90	148

Table 6. *Prevalence and numbers of* M. genitalium *organisms by PCR assay, by subjects and by samples in the Newcastle survey of respiratory infection*

Category	Total subjects	Total samples	Subjects PCR positive for *M. genitalium*	Samples PCR positive for *M. genitalium*
Ag-EIA+, P1-PCR-DBH−	8	11	1	2*
Ag-EIA+, P1-PCR-DBH+	10	16	0	0
Ag-EIA−, P1-PCR-DBH+	4	31	0	0
Ag-EIA−, P1-PCR-DBH−	40	90	2	2†
Total	62	148	3	4

* One subject (two samples) positive for *M. genitalium* via quantitative PCR-DBH; samples gave 34 and 60 c.f.u./ml.

† Two subjects (one sample each) positive for *M. genitalium* as above; samples gave 80 and 2000 c.f.u./ml.

heterogenetic cross reactions has been investigated exhaustively [1, 2] and none were found in the Ag-EIA for *M. pneumoniae* with a wide range of common respiratory mycoplasmas and respiratory bacteria including, for example, pneumococci and *Streptococcus* MG which share carbohydrate or glycolipid haptens with the mycoplasma. There are however shared antigens between *M. pneumoniae* and *M. genitalium* which also has sequence homology (2–8%) with *M. pneumoniae* [20, 21]. As the P1 protein gene primers, and those for 16S rDNA were designed not to react with *M. genitalium*, it seemed possible that antigen positive, PCR negative reactions might arise from the presence of *M. genitalium*. Accordingly, all respiratory samples were tested under code with *M. genitalium* specific PCR primers and probe (see Materials and Methods). This revealed 3 *M. genitalium* positive individuals among the 62 subjects and 4 positive samples (Table 6). The positive samples, which had from 30–2000 c.f.u./ml of the mycoplasma, showed no consistent association with a positive reaction by Ag-EIA. It seems unlikely therefore that the presence of *M. genitalium*, which has been isolated from the respiratory tract, along with *M. pneumoniae* [22] explains the discrepancy between the Ag-EIA and P1-PCR-DBH assays.

Third, the possible effect of degradation of target DNA by respiratory nucleases, or of its masking by inhibitors was investigated by seeding 15 nose or throat washings from 9 subjects, which had given an Ag-EIA+/P1-PCR-DBH−

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reaction, with a small number (10^2 c.f.u./assay) of *M. pneumoniae* organisms, followed by quantitative examination in the P1-PCR-DBH. In each instance the organisms were recovered in closely comparable numbers to those seeded. The same samples were also treated by a phenol-based method to extract DNA and the extract examined for P1 gene sequences – all were negative. Consequently digestion of the nucleic acid target in *intact* mycoplasma cells, or the presence of an inhibitor of PCR amplification in the respiratory secretions, is unlikely as an explanation of the Ag-EIA+, PCR-DBH− discrepancy (see Discussion).

DISCUSSION

The reasons for investigating PCR amplification of a short sequence in a *M. pneumoniae* gene as a possible method for direct diagnosis of infection, are outlined in the Introduction. In the present work, two PCR-based assays for *M. pneumoniae* were established without difficulty; the first targeted a sequence in the P1, foot protein or adhesin gene, and the second an organism-specific sequence in the 16S ribosomal RNA gene. Specificity and sensitivity was excellent (Table 1, Fig. 1).

Cross-hybridization reactions with *M. genitalium*, which shares some nucleotide sequences with *M. pneumoniae*, were not a problem, although with the 16S ribosomal DNA primers and probe the temperature of annealing had to be increased from 55 to 60 °C. The sequence in the P1 gene was chosen partly because of the association of the gene product with pathogenicity and partly because multiple copies of the gene are present in the *M. pneumoniae* genome. The 16S rDNA-PCR-DBH assay was established to exclude antigen-positive; PCR – negative results as arising from a possible strain to strain variation in the P1 gene; it is presumed that the ribosomal DNA is highly conserved between strains of *M. pneumoniae*. The ever-present hazard of contamination of assays with DNA from PCR product (amplicon) or positive control samples, as a source of false positive results, was contained by microbiological asepsis and also excluded by repeated testing of samples under code which gave completely reproducible results.

Attention is drawn to the following technical features of the assays. First, the inclusion of 0·01 % v/v NP40 in the DNA extraction medium with the proteinase K leads to more effective destruction of the enzyme on boiling and prevents subsequent degradation of the Taq polymerase and consequent diminution of PCR product yield. Second, the properties of the synthetic ^{32}P hairpin probe are ideal for dot-blot hybridization with nucleic acids in clinical samples. The specific activity of the probe is on average 10 times greater than that of probes prepared by end labelling, or nick-translation, and there is no cross-hybridization with any DNA other than that from *M. pneumoniae*. This contrasts with cloned probes which may include sequences from the host or vector as impurities and may give false positives with DNA from *E. coli*, or plasmids related to the cloning vector if these happen to be present in bacteria in the clinical samples. Third, the dose-response curve for the P1-PCR-DBH assay showed a usable straight line or sigmoid curve relationship between the binding values and the numbers of organisms detected (Fig. 2) and proved useful in enumerating organisms present in respiratory exudates (Table 4, Fig. 3). At the cut-off point (i.e., sample ratio 2·0) approximately 2·5 c.f.u. of *M. pneumoniae* were detected. Fourth, the P1-PCR-

DBH was, as expected, considerably more sensitive that Ag-EIA, requiring 100–1000 fewer c.f.u./ml to give a positive result.

Despite the great sensitivity and specificity of the PCR amplification of *M. pneumoniae* genes as a method of detection of the organism it is not, in our opinion, a 'one-assay' substitute for earlier methods. Its effective use not only requires considerable space and technical commitment to contamination control but also requires quantitation of the number of c.f.u./ml of the mycoplasma in the sample and, in particular, integration of the findings with the measurement of the serological response in the patients. It appears to be a much more rapid and more sensitive substitute for culture on specimens from patients – as distinct from simulated positive samples made up with laboratory adapted strains. Thus, for example, of 96 cultured samples (NPA) kindly sent to us by Professor Lyn Gilbert and taken from children with clinically-suspect respiratory illness in the Royal Children's Hospital Melbourne, 4 were culture-positive in cell-free diphasic media, or on cell sheet culture. Of these, 4 were P1-PCR-DBH+ and 2 Ag-EIA+. However 5 of the remaining 91 culture-negative subjects were P1-PCR-DBH+ and 1 was Ag-EIA+.

Several conclusions may be drawn from the extensive analysis of the samples from the Newcastle survey. Detection of *M. pneumoniae* by PCR-DBH, with primers and probes within the P1 protein gene or specific sequences from the 16S ribosomal RNA gene was readily achieved with 10^6 or more c.f.u/ml being detected in some throat or nasal washings. Positive P1-PCR-DBH results correlated quite well with those of Ag-EIA when the samples came from subjects with serological evidence of a current infection with the mycoplasma. High c.f.u./ml values by PCR – $\geqslant 10^4$ – with a positive Ag-EIA was a characteristic pattern (83%) in subjects with a current infection as judged by serological criteria (Fig. 3). A similar pattern was seen in 5 (23%) of 21 subjects with serological responses suggesting a recent rather than current infection. These subjects were considered to have been sampled with late stages of infection. As the period from the presumptive moment of infection lengthened, again as judged by the pattern of residual antibody, the discordance between the two assays increased with, overall, the PCR-based method giving more positives with samples than Ag-EIA. This pattern is interpreted as persisting carriage of the mycoplasma, or carriage associated with reinfection in an immune subject.

Much of our view of the ecology and epidemiology of *M. pneumoniae* infection is conditioned by the results of culture of the organism and the complement fixation test for antibody responses; both are insensitive and PCR-based detection methods should clarify the question of long term persistance of the mycoplasma after infection.

This conclusion is reinforced from recent experiments (Marmion and colleagues 1992, unpublished) with guinea-pigs inoculated intranasally with *M. pneumoniae* which show that counts of c.f.u./ml of the mycoplasma in the lung estimated by P1-PCR-DBH rise to a peak between 12 and 30 days after inoculation but the organism is present in the lungs for at least 200 days at a lower titre. Diphasic or cell sheet culture, and Ag-EIA, were positive between 33 and 70 days but declined to negative levels by 200 days; culture was consistently three logs less sensitive than PCR assay. The insensitivity of the Ag-EIA may in fact be an advantage in

that it rapidly detects the larger number of organisms associated with the peak of the infection while not giving positive results with small numbers detectable by PCR and perhaps lingering from a previous infection.

The indications from our work that *M. pneumoniae* may persist in the respiratory tract, a behaviour familiar from other mycoplasmas such as *M. gallisepticum*, were foreshadowed in the human volunteer experiments of Couch and colleagues [23] in which *M. pneumoniae* was recovered at 27 days after inoculation, the longest period of sampling. In our earlier studies [1] we found that over 50% of cases tested in the 1983/4 and 1985/7 outbreaks in Adelaide were still culture- or antigen-positive 15–60 days after onset of illness.

Attention is drawn to the anomalous finding that the Ag-EIA may be positive when the P1-PCR-DBH is negative – a total 11 of the 148 samples from the 62 subjects showed this pattern (Table 5). We found that the discrepancy was not due to the presence of an inhibitor of the PCR reaction nor did it appear (Table 6) to be due to the consistent presence of *M. genitalium* which shares antigens with *M. pneumoniae*, but not the DNA sequences chosen for the P1-PCR-DBH.

This discrepancy was also checked by the use of the primers and probe for the 16S ribosomal RNA gene on the supposition that there might be strain-to-strain variation in the P1 gene leading to a failure of detection by the P1-primers and probe. This reappraisal showed that although there was an exact correspondence of positive subjects detected by either primer-probe system, in fact when the total collection of samples was considered, there were four 16S rDNA-PCR-DBH positive/P1-PCR-DBH negative samples. One of the four samples was also Ag-EIA positive. One subject, with serological evidence of current infection, had supplied three of the anomalous samples, including the Ag-EIA+/P1-PCR− DBH-ve/16S rDNA+sample. Another sample from the same subject at a different time point was however positive by P1-PCR-DBH. It is possible that there was simply a difference in sensitivity between the two PCR assays, or that there had indeed been a selective degeneration of the target containing P1 gene sequences, leaving those of the 16S rDNA gene. Seven subjects yielded 10 Ag-EIA positive/P1-PCR-DBH negative and 16S rDNA-PCR-DBH negative samples. It is noted that all but one of them came from individuals with serological evidence of recent or past rather than current infection (Fig. 3).

We are driven back to the explanation advanced earlier by Harris and colleagues [2] to explain a similar discrepancy between Ag-EIA and the GenProbe rRNA assay – namely that as a consequence of the developing immune response in the respiratory tract, or perhaps because of antibiotic therapy, the membrane of the mycoplasma is breached and the exposed nucleic acids are destroyed by nucleases in the respiratory secretions whereas debris of the organism and its antigens, in particular the glycolipids, are cleared more slowly from the respiratory tract and remain to be detected by Ag-EIA.

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