

## *Mycoplasma pneumoniae* and *Mycoplasma genitalium* Mixture in Synovial Fluid Isolate

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**A mycoplasma cultured from synovial fluid specimens from a patient with pneumonia and subsequent polyarthritis was identified initially as *Mycoplasma pneumoniae*. In retrospective studies, the culture was shown also to contain *Mycoplasma genitalium*. In this paper, the laboratory techniques employed in the identification and separation of the two species are presented, and evidence to implicate postinfectious autoimmunity is provided. An increasing number of reports of *M. genitalium* in human tissue sites and difficulties in isolation and identification of the organism in the clinical laboratory suggest the need for more extensive application of rapid and specific detection systems for both *M. genitalium* and *M. pneumoniae* in the clinical laboratory.**

*Mycoplasma genitalium* was first isolated from the urogenital tracts of two patients with nongonococcal urethritis more than a decade ago (30). While subsequent experimental challenge studies with *M. genitalium* in primates suggested invasive and pathogenic qualities and an active role in urogenital tract disease (22, 32), repeated cultural studies failed to confirm this association in human genital infections. It was only recently, by using gene amplification techniques (PCR), that two independent research groups have provided more significant evidence of involvement of *M. genitalium* in human urethritis (9, 10).

Although earlier studies of *M. genitalium* clearly established it as a distinct species, it was apparent that the organism shared a number of important biological and serological properties with strains of *Mycoplasma pneumoniae* (30, 31). Investigations over the past decade have repeatedly confirmed the close structural, antigenic, and molecular relationships of these two organisms. Thus, the two mycoplasmas have similarly organized attachment structures (31), possess sequence homology between their adhesin genes (6), exhibit common epitopes among their adhesin and membrane proteins (16), and contain similar membrane glycolipids (13, 23). Since the respiratory tract was obviously established as the primary site of *M. pneumoniae* colonization, the interactions with *M. genitalium* were not originally thought to complicate the delineation of the role of *M. genitalium* in human genital tract infections.

However, the discovery in 1988 (3) of *M. genitalium*-*M. pneumoniae* mixtures in nasopharyngeal throat specimens from patients with acute respiratory disease not only contributed new concepts about the host distribution of *M. genitalium* but also prompted important questions about the potential pathogenicity of the organism and its relationship with *M. pneumoniae*. Frozen throat washings taken from U.S. Marine recruits during a vaccine field trial in 1974 and 1975 were cultured on the then newly developed SP-4 broth medium (29). At least 16 isolates, identified at the time by epi-immunofluorescence (8)

as *M. pneumoniae*, were grown in SP-4 broth and stored at  $-70^{\circ}\text{C}$ . About 14 years later, 4 of the 16 stored isolates were found to possess species-specific antigens to both the 170-kDa protein adhesin (P1) of *M. pneumoniae* and the 140-kDa protein adhesin (P140) of *M. genitalium* (3). The mixed species were subsequently separated by conventional mycoplasma cloning techniques (27), and purified strains of *M. genitalium* were deposited in the American Type Culture Collection.

The occurrence of mixed cultures of *M. pneumoniae* and *M. genitalium* prompted a reexamination of other *M. pneumoniae* isolates stored in the National Institute of Allergy and Infectious Diseases laboratory. In this report, we describe the isolation and identification of both mycoplasmas in a human synovial fluid isolate received in 1986 and discuss the possible basis for postinfectious autoimmunity.

### MATERIALS AND METHODS

**Patient history.** In April 1986, a patient with pneumonia and a history of arthritis was hospitalized at the University of Texas Medical Center in Galveston (7). A diagnosis of *M. pneumoniae* infection was established through serological techniques, using an indirect immunofluorescence test to detect elevations in both immunoglobulin M (IgM) and IgG *M. pneumoniae* antibody responses. Two weeks after admission, the patient developed new symptoms of pain and swelling in both knees and wrists. The serum gamma globulin level in the patient at the time was within the normal range. Synovial fluids from the left knee and right wrist were cultured in SP-4 broth medium, and mycoplasmas were isolated from both sites. Epi-immunofluorescence tests performed at the time in two separate laboratories, using agar colonies of the isolate (designated UTMB-10) stained with conjugated antiserum specific to *M. pneumoniae*, indicated the identification of the joint isolate as *M. pneumoniae* (7). The culture, which had undergone several passages in the Galveston laboratory and approximately five additional passages in the National Institute of Allergy and Infectious Diseases laboratory, was then stored at  $-70^{\circ}\text{C}$ .

**Laboratory identification of a mixed culture.** In late 1988, the UTMB-10 strain was revived from the frozen state by passage into 10 ml of SP-4 broth in a T-25 plastic tissue culture flask. After 5 days of incubation, the phenol red pH indicator in the broth exhibited a color change (yellow) toward the acid range, and adherent growth was apparent on the inner surface of the flask. The supernatant fluid was removed, and the attached mycoplasmas were detached from the plastic surface by addition of 4 ml of fresh SP-4 broth and a sterile rubber policeman. Five serial 10-fold dilutions of the concentrated culture were made in 2-ml volumes of SP-4 broth, and at least three SP-4 agar plates were then inoculated with 0.2-ml volumes of each serial dilution of UTMB-10. SP-4 agar plates were incubated at  $37^{\circ}\text{C}$  in an anaerobic atmosphere (GasPak jar; BBL Microbiology Systems, Cockeysville, Md.) for 5 days. Agar plates containing about 300 mycoplasma colonies of UTMB-10 were selected for direct epi-immunofluorescence

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TABLE 1. Epi-immunofluorescence tests for differentiation of agar colonies of *M. genitalium* from those of *M. pneumoniae*

Antigen (agar colonies)	Fluorescence of agar colonies treated with the indicated dilution of each specific conjugate					
	<i>M. genitalium</i>			<i>M. pneumoniae</i>		
	1:16	1:64	1:128	1:32	1:256	1:1,024
<i>M. genitalium</i>	4+	1+	±	3+	Negative	Negative
<i>M. pneumoniae</i>	3+	Negative	Negative	4+	2+	±
Strain UTMB-10	3+	±	Negative	4+	2+	±
Strain UTMB-10G	4+	1+	±	3+	Negative	Negative

testing, using appropriate predetermined dilutions of conjugated antiserum to either *M. pneumoniae* (FH strain) or *M. genitalium* (G37 strain). Each conjugate was prepared in the National Institute of Allergy and Infectious Diseases laboratory by direct labeling of the respective hyperimmune antiserum with fluorescein isothiocyanate (8). The selection of the specific conjugate dilution to employ in the test was based upon the ability of that dilution to stain only colonies of the homologous *Mycoplasma* species. Briefly, this procedure involved the removal of 16 small agar squares (12 by 12 mm) from agar plates containing mycoplasmal colonies and the transfer of each agar piece to a separate glass slide. Each individual agar piece was stained with a specific twofold dilution (in phosphate-buffered saline) of either conjugate, using a dilution range of 1:8 to 1:1,024. The *M. pneumoniae* conjugate had an immunofluorescence extinction titer against the homologous FH strain of 1:1,024, while the *M. genitalium* conjugate titer to the homologous strain G37 was 1:128 (Table 1). Several agar pieces containing the UTMB-10 culture were first stained with a 1:64 dilution of the *M. genitalium* conjugate. Few colonies (estimated to be about 1 to 2% of the population on the agar piece) stained with this dilution of conjugate. A clear majority of the agar colonies stained intensively with a 1:256 dilution of the *M. pneumoniae* conjugate. The rather low number of UTMB-10 colonies on the agar pieces that stained with the diluted *M. genitalium* conjugate was confirmed by staining a 60-mm-diameter agar plate containing mixed colonies with the same 1:64 conjugate.

**Purification of the mixed culture.** The UTMB-10 culture was eventually purified to distinct clones of *M. genitalium* and *M. pneumoniae*, using a modified growth inhibition technique. In this procedure, 0.2 ml of a logarithmic-phase culture (in SP-4 broth) of the mixed UTMB-10 mycoplasma culture was plated to a series of SP-4 agar plates, and the plates were allowed to dry. Three or four filter paper discs, saturated with hyperimmune rabbit antiserum to *M. pneumoniae* FH, were placed evenly on the surface of each agar plate, and the plates were again allowed to dry. Following appropriate incubation, pieces of agar containing colonies growing between the antiserum-saturated discs were passaged to 3-ml volumes of fresh SP-4 broth in 1-dram (ca. 4-ml) vials and incubated at 37°C. After several repeated trials with this technique, the mycoplasma population in the mixed UTMB-10 line had shifted to predominately *M. genitalium*. This partially purified culture, as well as the initial mixed culture, were then filter cloned to yield purified strains of *M. genitalium* and *M. pneumoniae*. The identities of the strains were confirmed by agar immunofluorescence and growth inhibition tests and by immunoblotting with monoclonal antibodies specific to the distinct epitopes of the adhesins of *M. pneumoniae* and *M. genitalium* (3).

**Serological analysis.** A single acute-phase serum sample from the patient was available for retrospective analysis, using both a metabolism inhibition test (21) and immunoblotting (3) with standard *M. genitalium* and *M. pneumoniae* prototype strains as antigens. To document the existence of cross-reactive antibodies to host tissue components in the patient's serum, immunoblots were performed with keratin, fibrinogen, and myosin antigens (Sigma Chemical, St. Louis, Mo.) (2, 4). Briefly, 15 µg of purified myosin (bovine muscle; Sigma M-6643), keratin (human epidermis; Sigma K0253), or fibrinogen (human type III; Sigma F-4129) per lane was loaded and separated by sodium dodecyl sulfate-7.5% polyacrylamide gel electrophoresis (SDS-7.5% PAGE). Gels were electrophoretically transferred to nitrocellulose, and immunoblots were performed as previously described (16) with the patient's serum diluted 1/200. Alkaline phosphatase-conjugated goat anti-human IgG (Zymed) at a 1:1,500 dilution in 1% BLOTTO was added to the membranes, and the filters were washed thoroughly prior to development with 5-bromo-4-chloro-3-indolyl phosphate-nitro blue tetrazolium (Sigma B5655). To determine whether antikeratin antibody in the patient's serum might recognize cross-reactive *M. genitalium* and *M. pneumoniae* epitopes, human keratin (300 µg) was separated by SDS-7.5% PAGE and transblotted onto nitrocellulose. Ponceau stain (Sigma P7170) defined the portion of nitrocellulose containing keratin antigen (see Fig. 3), and the remaining nitrocellulose was trimmed away. The keratin-containing strips were blocked with 3% BLOTTO and incubated with patient serum diluted 1:50 for 3 h at 37°C. Bound antibodies were eluted with 0.2 M NaCl-0.2 M glycine in 0.1% bovine serum albumin, pH 2.2 (14). Nitrocellulose strips with no or irrelevant antigens were incubated with the patient's serum and processed as described above. Each eluent was neutralized to pH 7.0 with 1 M Tris, pH 9. Nitrocellulose strips containing *M. pneumoniae* and *M. genitalium* total proteins were incubated with the keratin-eluted and control antibody fractions overnight at 37°C. Anti-human

IgG-IgA-IgM antibody conjugated with alkaline phosphatase (Zymed 68-8322) was used to probe for the presence of cross-reactive antibodies.

## RESULTS AND DISCUSSION

The immunofluorescence test on the stored synovial fluid isolate clearly identified both *M. pneumoniae* and *M. genitalium* in the culture. The low numbers of *M. genitalium* in the mixed culture might not reflect the population of this species in the original specimen, since the culture had been passaged in the laboratory more than five times. In other experiences with mixed cultures of these species (3), continued passage on artificial medium and the slower growth of *M. genitalium* favored the shift in population toward *M. pneumoniae*. Confirmation of the identity of each purified strain was established by epi-immunofluorescence and conventional growth inhibition tests and by immunoblotting with monoclonal antibodies specific for the distinct epitopes of the adhesins of *M. pneumoniae* and *M. genitalium* (3, 15, 16). Subcultures of each line were deposited in the American Type Culture Collection: *M. genitalium* UTMB-10G (ATCC 49899) and *M. pneumoniae* UTMB-10P (ATCC 49894).

An analysis of the patient's serological response to the two mycoplasmas was limited because only a single serum specimen was available. However, in both the metabolism inhibition test and immunoblotting, the patient showed moderate reactivities against proteins of both *Mycoplasma* species (Table 2 and Fig. 1), providing some limited support for the likelihood of a mixed infection with the two organisms.

As with the initial observation of a mixture of *M. pneumoniae* and *M. genitalium* in human throat specimens (3), the presence of the two organisms in synovial fluid of a patient with respiratory disease and arthritis raises further questions about the pathogenic roles that these mycoplasmas play individually or synergistically in disease development. For example, it is

TABLE 2. Metabolism inhibition tests with patient's convalescent-phase serum and mycoplasmal antigens

Serum	MI <sup>a</sup> titer with antigen:		
	<i>M. pneumoniae</i> FH	<i>M. genitalium</i>	
		G37	UTMB-10G
Patient	1:64	1:64	1:64
Control			
<i>M. pneumoniae</i> (FH)			
HRC 6-04-S	1:2,048	1:64	1:64
HRC 72-A-22	1:1,024	1:32	1:64
<i>M. genitalium</i> (G37)			
Rab. 680-681	1:256	1:2,048	1:2,048
Rab. 822-823	1:128	1:512	1:512

<sup>a</sup> MI, metabolism inhibition.

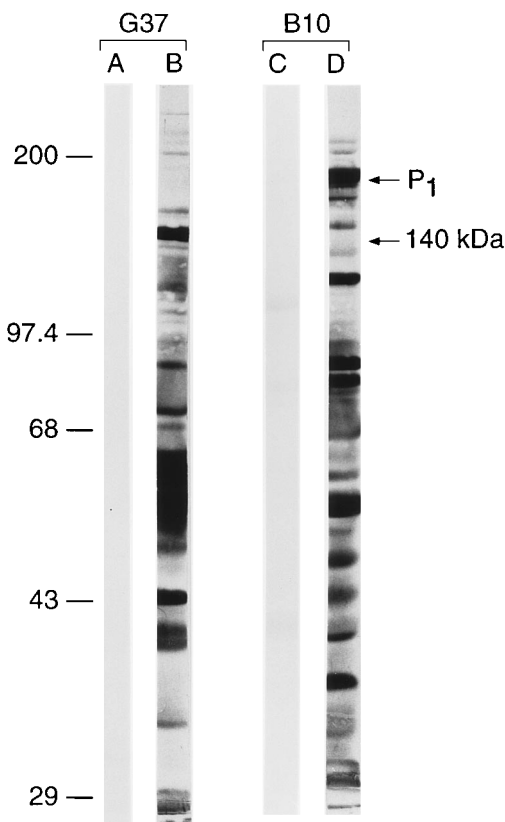


FIG. 1. Immunoblots with *M. genitalium* (G37) and *M. pneumoniae* (B10) total proteins and patient's serum. Lanes A and C, pooled normal human serum controls diluted 1/100. Lanes B and D, patient's serum diluted 1/100. Protein molecular weight markers (in thousands) are indicated at the left. The arrows designated P<sub>1</sub> and 140 kDa at the right indicate the locations of the P1 adhesin of *M. pneumoniae* and the 140-kDa adhesin of *M. genitalium*.

unclear what part *M. genitalium* may play in acute respiratory disease (3, 28) or whether *M. genitalium* and *M. pneumoniae* contribute to joint inflammation. However, *M. pneumoniae* has been reported as a primary cause of acute polyarthritis in humans (11, 25) and only in patients with hypogammaglobulinemia. For these reports, one cannot exclude the possibility that *M. genitalium* might have been present also, which is consistent with the fastidious nature of *M. genitalium* and difficulties in identifying the agent. Indeed, a report that describes the detection (by PCR assay) of *M. genitalium* in the synovial fluids of two patients with arthritis has just appeared (24). Clearly, natural infections with mycoplasmas in nonhuman mammals lead to acute and chronic arthritis (4). The detection of antimycoplasmal antibodies and mycoplasmal antigens in synovia of patients with arthritis disorders (4) implicates pathogenic mycoplasmas as etiologic agents in acute and chronic arthritides. Furthermore, it has been suggested for decades that mycoplasma-mediated infectious diseases are complicated by autoimmune disorders which are reflected in elevated titers of antibodies that are cross-reactive with mycoplasma and host antigens (4, 26). We have reported that a family of adhesin-related molecules exists among pathogenic mycoplasmas and that these molecules exhibit high levels of sequence homology with mammalian cytoskeletal components (2). An immune response directed against these mycoplasma antigens may provoke an antiself response, which would correlate with autoimmune-like mechanisms in mycoplasmal dis-

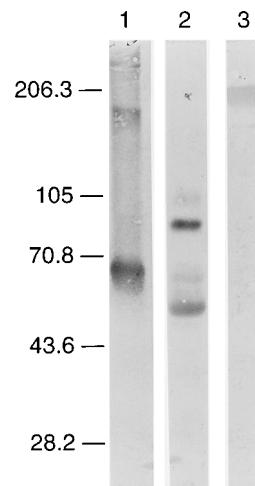


FIG. 2. Immunoblot with human keratin (lane 1), human fibrinogen (lane 2), and bovine myosin (lane 3) (all at 50 µg per lane and from Sigma) and patient's serum diluted 1/200. Ponceau staining of extracellular matrix proteins separated by SDS-PAGE and immunoblots with guinea pig antikeratin (Sigma), mouse antifibrinogen (Sigma), and rabbit antimyosin (Chemicon) revealed similar patterns. Numbers at the left indicate molecular weights in thousands.

ease (2, 4). Immunoblots with keratin, fibrinogen, and myosin antigens detected the presence of host cross-reactive antibodies in the patient's serum (Fig. 2). Elevated levels of antibodies to these human proteins (compared with levels in pooled normal human sera) were detected in the patient's serum (data not shown), with the highest antibody titers exhibited against keratin (Fig. 3). Immunoblotting of antikeratin antibodies from the patient's serum against *M. genitalium* and *M. pneumoniae* total proteins revealed strong immunoreactivities against the P140 adhesin and several other proteins of *M. genitalium* and weaker yet obvious immunoreactivities against *M. pneumoniae* proteins, including the P1 adhesin (data not shown). Consistent with these observations, we were able to demonstrate retrospectively a distinct seroconversion to keratin by using paired sera from two of four patients with documented mycoplasmal pneumonia infections (Table 3). In separate studies we have observed similar seroconversions to keratin and several other cytoskeletal proteins by using paired sera from patients diagnosed with *M. pneumoniae* infections in a recent outbreak of acute respiratory disease (unpublished data).

It remains uncertain whether mycoplasmas elicit immunopathological processes through molecular mimicry, through modulation of the immune response via immune cell activation and cytokine production, or both (5, 19, 20, 26). Nevertheless, the unique biological properties of pathogenic mycoplasmas, including the antigenic mimicry described above, are consistent with the inflammatory mechanisms and immunopathological changes observed in mycoplasma-associated diseases (4, 26).

The diagnosis of clinical *M. pneumoniae* infections is almost always established through serological analysis, frequently with the complement fixation test. This test is fairly insensitive and nonspecific (1, 17, 28), and most newly developed enzyme-linked immunosorbent assays (ELISAs) or passive agglutination tests reported over the past few years cannot differentiate antibody responses to *M. pneumoniae* from those to *M. genitalium* (1, 12, 18). However, immunoblotting of a patient's serum with the *M. pneumoniae* 170-kDa adhesin protein or the *M. genitalium* 140-kDa adhesin protein can show a specific antibody response in acute-phase sera (15). The eventual de-

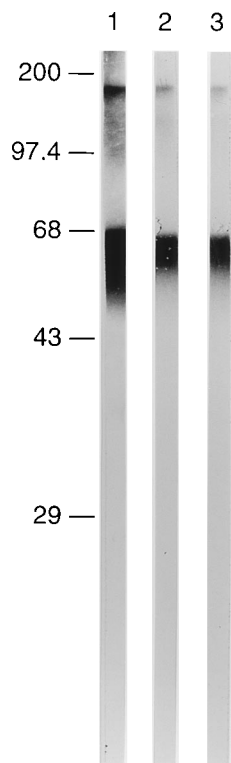


FIG. 3. Immunoblot with human keratin (50  $\mu$ g per lane; Sigma) and patient's serum diluted 1/200 (lane 1), 1/500 (lane 2), or 1/1,000 (lane 3). Ponceau staining of human keratin separated by SDS-PAGE and immunoblots with guinea pig antikeratin (Sigma) revealed similar protein patterns. Numbers at the left indicate molecular weights in thousands.

lineation of the role of *M. genitalium* in respiratory or extrapulmonary infections will depend upon further application of rapid specific detection methods such as PCR, laboratory isolation of the organism by conventional methodology, and serological analysis of host immune responses by differential techniques.

TABLE 3. Immunoreactivities of patient sera with extracellular matrix components<sup>a</sup>

Patient and phase	Immunoreactivity of serum with:		
	Keratin	Fibrinogen I	Fibrinogen III
1			
Acute	0.079 $\pm$ 0.008	0.066 $\pm$ 0.006	0.139 $\pm$ 0.040
Convalescent	0.514 $\pm$ 0.029	0.062 $\pm$ 0.013	0.153 $\pm$ 0.096
2			
Acute	0.073 $\pm$ 0.003	0.036 $\pm$ 0.008	0.155 $\pm$ 0.019
Convalescent	0.429 $\pm$ 0.098	0.085 $\pm$ 0.015	0.217 $\pm$ 0.026
3			
Acute	0.146 $\pm$ 0.009	0.047 $\pm$ 0.015	0.195 $\pm$ 0.016
Convalescent	0.205 $\pm$ 0.006	0.106 $\pm$ 0.035	0.177 $\pm$ 0.014
4			
Acute	0.162 $\pm$ 0.019	0.094 $\pm$ 0.021	0.132 $\pm$ 0.032
Convalescent	0.233 $\pm$ 0.021	0.129 $\pm$ 0.011	0.218 $\pm$ 0.012

<sup>a</sup> An ELISA format was used, which included 1/100 dilutions of acute- and convalescent-phase sera from patients with diagnosed mycoplasmal pneumonia (3, 4) and 2  $\mu$ g of human epidermis keratin (Sigma) and human fibrinogen types I and III (Sigma) per well. Immunoreactivities are expressed as the mean of absorbancy (490 nm)  $\pm$  the standard deviation of four determinations from two separate assays. A pool of normal human sera consistently provided baseline immunoreactivity values against keratin and fibrinogens I and III of less than 0.050.

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