Pediatr Infect Dis, 5:S223–S231, 1986
0277-9730/86/$02.00/0

Vol. 5, No. 6
Printed in U.S.A.

Ureaplasma urealyticum: history and progress

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My first introduction to mycoplasmas occurred at the time of a venereal disease symposium in Roanoke, VA, over 35 years ago. I was a young bacteriologist and commissioned officer in the United States Public Health Service, Venereal Disease Division. I had already established a small research laboratory at the United States Public Health Service (USPHS) Eastern Medical Center, Durham, NC, to undertake studies of granuloma inguinale and other minor venereal diseases. At the time of the Roanoke symposium, I had the opportunity to hear Dr. Justina Hill of The Johns Hopkins University discuss a little known and even less well understood group of microorganisms known as pleuropneumonia-like organisms (PPLO). Dr. Hill had been invited by the USPHS to discuss PPLO in relation to a sexually transmitted disease known as nonspecific urethritis, the incidence of which was showing an alarming increase. No one knew the cause of this infection or how to treat it. I gleefully accepted the challenge to commence research studies on the possible role of PPLO in this perplexing disease, nonspecific urethritis. Little did I know then how long the road would be or what surprises lay in store for me.

In April, 1950, I spent a week in the laboratory of Dr. Louis Dienes, the first American mycoplasmologist, in Boston, MA. I came to learn all I could about the then known mycoplasmas. At the end of the week and with much encouragement from Sarabelle Madoff, Dr. Dienes' able research assistant, I returned to my laboratory in Durham, armed with great hope and a reference culture of strain CAMPO, a classic, large colony mycoplasma, and commenced to investigate the possible role of mycoplasmas in nongonococcal urethritis (NGU). Since my laboratory was located in a medical center for the treatment of sexually transmitted diseases, I was not wanting for a continuous supply of fresh clinical specimens.

The history of *Ureaplasma urealyticum* began 5 months following my visit to Dr. Dienes' laboratory. Back in my laboratory in Durham, the first task was to make a difficult to prepare agar medium known as "boiled blood ascitic agar," originally described by Klieneberger[1] and modified by Dienes.[2] To the agar was added 2% packed horse red blood cells, and the mixture was boiled briefly, cooled to 50°C and aseptically filtered through a cotton gauze filter while hot into a sterile dispensing vessel. This operation required the patience of Job and skill of a magician, and often even that was not sufficient for success, but to make the basal tryptic infusion agar into which the horse red cells were introduced was something else. The basal broth was made from something called "tryptic digest concentrate," after a formula used at Harvard Medical School during World War I, by V. J. Fields. One started with about 10 pounds of fresh bovine hearts obtained from a nearby slaughterhouse. We also filter-sterilized our ascitic fluids, often collected in 1- to 2-gallon volumes from some unfortunate patient. The laboratory filtration was performed using large, sterile 10- by 2-inch Mandler diatomaceous earth candles. More great patience and frustration. The final boiled blood ascitic agar was supplemented to 30% with sterile human ascitic fluid (often of very questionable performance). The reaction of the agar was adjusted to a high pH of 7.8 to 8.0 in accordance with standard *Mycoplasma* practice, which I followed to the letter. Aside from the agony of preparing the boiled blood agar medium, it was a difficult agar to use due to its cloudiness and content of red blood cell debris.

During the first year of my investigation into the possible role of mycoplasmas in NGU, careful measurements of colony size were made, in addition to recording variations in colony morphology. Examinations were made by viewing the colonies through the agar, under low power (× 100) with the closed plate inverted on the microscope stage. On September 22, 1950, 5 months after my visit to Dr. Dienes' laboratory in Boston, I recorded in my research notebook the first observation and recognition of *Ureaplasma* colonies. "Patient No. U-50: *Minute forms*, 0/10, 0/10, 0/12 μ. Patient No. U-52: Many *minute* forms, 0/8, 0/6, 0/8 μ." The numerator in the measurements indicated no surface growth zone, so characteristic of the large colony mycoplasmas (e.g. 105/50 μm). It was soon realized that some very tiny, minute *Mycoplasma*-like colonies or artifacts were appearing in the urethral and urine cultures from NGU patients, often alone or mixed with large colony, classic mycoplasmas. These

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tiny colonies initially were poorly stained and difficult to recognize due to the cloudy nature of the agar. Their size range was generally from about 7 to 20 μm in diameter. As shown in Figure 1, the extremely poor growth and frail appearance of these early *Ureaplasma* colonies is evident. Figure 2 shows three of these tiny colonies at higher magnification. These were the first photomicrographs of ureaplasma colonies taken in my

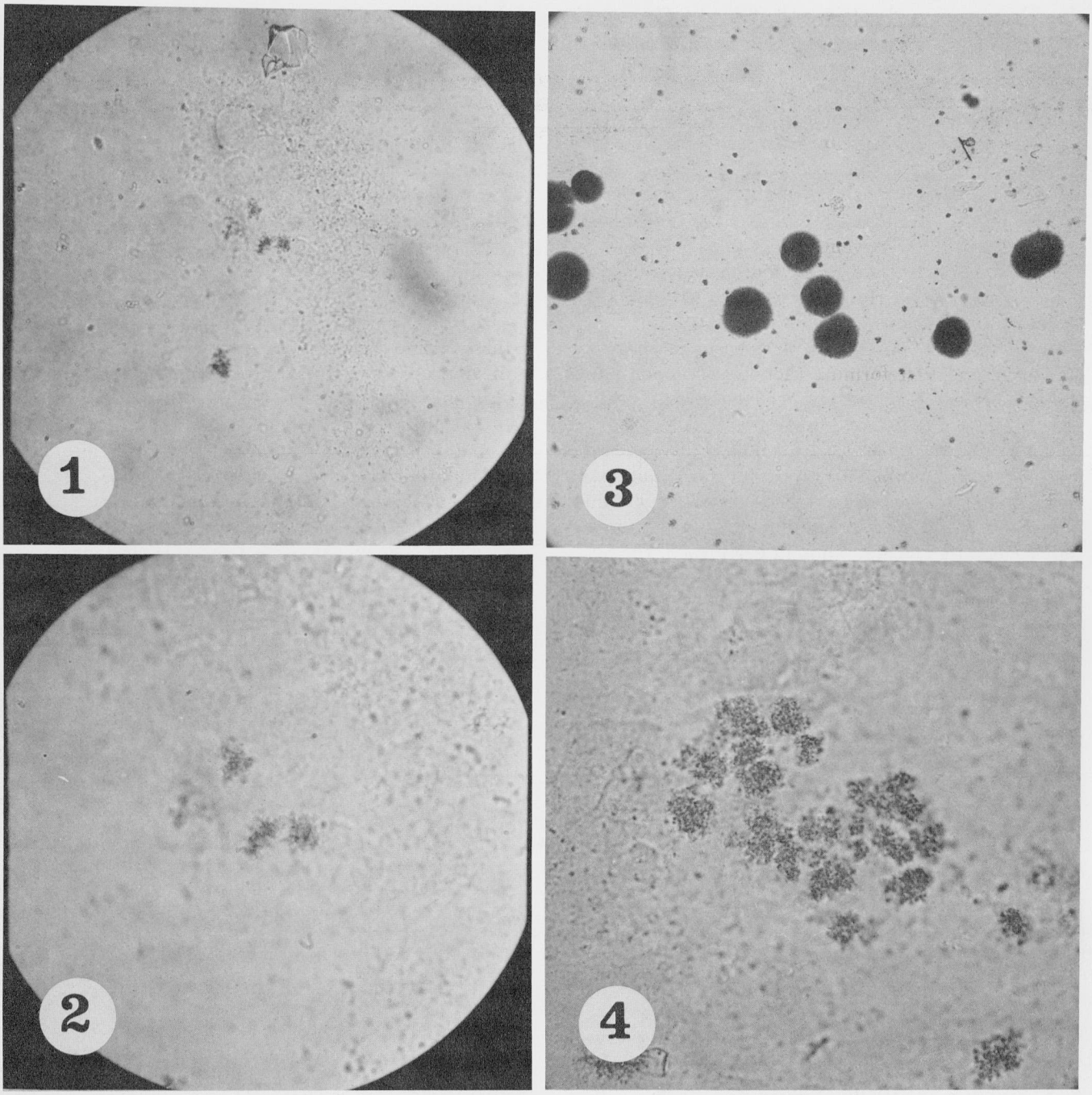

FIG. 1. The first photomicrograph of the tiny colonies of *U. urealyticum*, isolated April 26, 1951, on the boiled blood-ascitic agar of Klieneberger, as modified by Dienes. From urethral exudate of a male patient with nongonococcal urethritis. × 440. From Shepard,[3] with permission.

FIG. 2. The same microscopic field as in Figure 1, but at a higher magnification to emphasize the characteristic features of three of the four *Ureaplasma* colonies. × 950. From Shepard,[3] with permission.

FIG. 3. Minute *Ureaplasma* colonies intermixed with classic, large colony mycoplasmas (*M. hominis*, deeply stained in this wet Dienes-stained agar block preparation). The more than 50 *Ureaplasma* colonies can easily be overlooked. × 85.

FIG. 4. Multiple colonies of *U. urealyticum* growing into the agar medium beneath two *Ureaplasma*-infected urethral epithelial cells. Urethral exudate from a patient with nongonococcal urethritis, a treatment failure on 500 mg of oxytetracycline four times a day for 9 days. Wet Dienes-stained agar block preparation. × 750. From Shepard,[4] with permission.

laboratory at Eastern Medical Center, on April 26, 1951, and first published in 1954.[3] The earliest description of *Ureaplasma* colonies, recorded in my notebook in the fall of 1950, further read, "Surface growth absent; growing down into the agar; not compact, but loosely-bound; moderately well-stained (often poorly stained); and *very tiny*—about 7–12 microns, and irregular in outline." My thoughts were, "What are they?" They had no resemblance to the then known PPLO or *Mycoplasma* colonies, except that they exhibited one common feature; the tiny colonies grew down into the agar, and this was significant. It is of historical interest that one of the first ureaplasmas (early T-strain) isolated was from a patient with Reiter's syndrome, on November 1, 1950.

In June, 1952, I was transferred to the USPHS Medical Center at Hot Springs National Park, AR, where I continued my studies on the role of *Mycoplasma* in NGU. While there, subsequent improvements in the agar formula (notably the omission of the boiled blood component) yielded a clear medium which allowed easier detection and recognition of the tiny colonies in primary cultures of urethral exudates and urine sediments. It was soon evident that T colonies were often the only colonies growing out of such clinical specimens, frequently in large numbers and in apparent pure culture. Thus in early 1953, the accumulated evidence, plus the findings on a particular NGU patient cultured at the Hot Springs Medical Center, was sufficient to recognize these tiny colonies as those of a self-replicating agent (not artifact) and a previously undescribed member of the *Mycoplasma* group. The first written description of *U. urealyticum* was included in a handout paper, September 16, 1954, at the Symposium on Non-Gonococcal Urethritis, under the auspices of the United Nations World Health Organization, in the principality of Monaco. I had come to Camp Lejeune, NC, in July, 1953, to commence research studies on NGU for the Navy Medical Department. But it was a Navy officer who made the trip, not I, who distributed my handout paper entitled, "Some preliminary observations: Pleuropneumonia-like organisms in men with non-gonococcal urethritis at Camp Lejeune, NC." The first published description of *Ureaplasma* colonies was in the legend accompanying the first photomicrographs published of "tiny-form PPLO colonies," in 1954.[3]

Figure 3, a wet Dienes-stained agar block preparation, further illustrates the colonial morphology of the early ureaplasmas. At first glance all one sees are the large colony, classic *Mycoplasma* colonies which are deeply stained. However, among and surrounding them are more than 50 minute, stained T colonies (and you can readily see from this photomicrograph why I called them "T" or tiny colonies). One of the characteristic features of ureaplasmas in primary cultures of clinical specimens is their close association with urethral epithelial cells in urethral exudates from NGU patients. This is well-illustrated in Figure 4 which shows 18 multiple *Ureaplasma* colonies growing into the agar beneath a pair of infected urethral epithelial cells.

The organism itself is generally round to ovoid and approximately 350 μm in diameter. It is enclosed by a single trilaminar membrane about 10 nm thick which in turn appears to be covered by an extramembranous layer of polyanions about 15 to 30 nm thick, as demonstrated by a ruthenium red staining procedure, examined in thin sections and viewed by electron microscopy.[5] Organisms in agar colonies appear to be embedded in a gelatinous matrix, and I have observed elongated flocks of *Ureaplasma* organisms attached to glass surfaces in fluid cultures, waving like sea weeds when the flask was gently rocked. The organisms in fluid culture occur singly, in pairs, in triads of three, in short chains of three to five organisms and in small clumps. The morphology of *U. urealyticum* grown in fluid culture is illustrated in Figure 5. All of the elements just described are visible in this photomicrograph. This photomicrograph was made from an 80-fold concentrate from a 17-hour broth culture, which was resuspended in Tyrode's solution containing 1.0% formalin. The smear made from this suspension was stained in the hematoxylin-Giemsa stain of Shepard.[6] Electron micrographs of *U. urealyticum* from young broth cultures often exhibit micro-growth flocks which develop in polydirectional, branched growth ramifi-

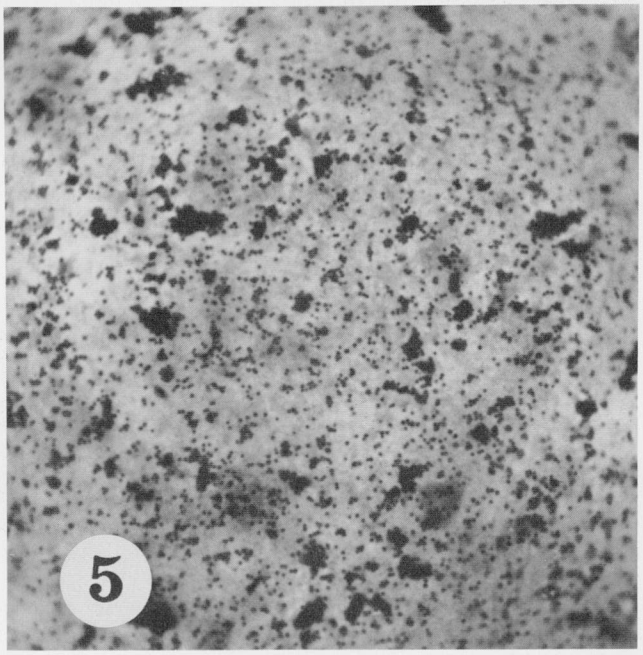

FIG. 5. *U. urealyticum* from a 17-hour, U-17B broth culture concentrated 80-fold. Washed and resuspended in Tyrode's buffer, pH 7.0, containing 0.8% formaldehyde. Hematoxylin-Giemsa stain of Shepard. × 1000. From Shepard,[6] with permission.

cations and enclose the organisms in a gelatinous matrix.

In clinical exudates *Ureaplasma* organisms can be demonstrated directly in smears of urethral exudate collected by epithelial scraping from male NGU patients and stained by Giemsa. In such preparations the organisms typically stain pale lavender and are intimately associated with infected urethral epithelial cells, occurring singly or in small clusters of organisms. It is these small, multiple foci of infection that give rise to multiple colony infection of epithelial cells seen in primary agar cultures of urethral exudates, as mentioned earlier. This feature is also seen in cultures of NGU patients who are treatment failures, often to a marked degree.

The first individual to confirm the existence of ureaplasmas in a different geographic area (Canada) was Denys Ford, in early 1962.[7] Our very early, dismal failures to subculture ureaplasmas serially in agar cultures were probably due to a nutritionally poor medium containing 30% human ascitic fluid enrichment of questionable performance, coupled with high medium alkalinity (pH 7.8 to 8.0). Successful serial passages in agar cultures, by the agar push-block method, were finally achieved after we abandoned the ascitic fluid enrichment in favor of horse serum supplementation, first suggested by Ford.[8] Unknown to me at the time was the important value of horse serum in providing a required substrate for ureaplasmas, which was subsequently shown to be urea.

Serial cultivation of ureaplasmas in broth cultures had its own special frustrations. Our early practice of transferring broth cultures every 48 hours (accepted *Mycoplasma* technique), resulted routinely in moribund cultures. Again it was Ford[8] who helped point the way by observing that ureaplasmas grew rapidly in broth cultures, attaining maximal titers after only 16 hours of incubation. The titers rarely exceeded 10^6 colony-forming units/ml. Even today titers rarely exceed 10^7 to 10^8 colony-forming units/ml.

Another important discovery was reported in 1965 by Shepard and Lunceford,[9] but it was sparked by a puzzling observation. A single *Ureaplasma* colony, adjacent to the outer border of a urethral *Streptococcus* colony, was 100% larger than any of the more distant *Ureaplasma* colonies. Efforts to demonstrate a stimulatory streptococcal accessory growth factor failed. However, since the agar medium contained dextrose, the possibility of a pH effect was considered and this proved to be correct. The acid produced by the *Streptococcus* colony by fermentation of the dextrose lowered the reaction of the medium to a favorable pH for this single, adjacent *Ureaplasma* colony. It was subsequently shown that the optimal reaction for cultivation of ureaplasmas was in the acid range of pH 5.5 to 6.5, and we recommended a reaction of pH 6.0 for most purposes.

In May, 1963, our laboratory discovered that ureaplasmas could hydrolyze urea with the production of ammonia. This was a significant milestone in our understanding of the biochemistry and nutritional requirements of ureaplasmas. Our findings were first published in 1966[10] (and independently by Purcell et al.[11]) and presented in detail in 1967.[12] These findings were confirmed the same year by Ford and MacDonald,[13] who described the influence of urea on the growth of ureaplasmas. Urea is an essential, growth-limiting substrate for ureaplasmas, and this was confirmed by Kenny and Cartwright in 1977[14] and by Sayed and Kenny in 1979.[15] On the basis of this special property a new genus, *Ureaplasma*, in the family *Mycoplasmataceae* was proposed in 1974 by Shepard et al.[16]; it contained a single species, *U. urealyticum*. We can now understand why the early agar colonies of the 1950s and 1960s were so tiny that they were called "T" colonies. In addition to possibly unknown factors we failed to supply two fundamental requirements of the organism, a low pH and an adequate supply of a required substrate, urea.

A new animal species, *Ureaplasma diversum*, was proposed in 1982 by Howard and Gourlay.[17] Like the human species (*U. urealyticum*) *U. diversum* is serologically heterogeneous, consisting of approximately 11 serovars which share no common antigens with human *U. urealyticum* serovars, of which there are presently 14.

Before leaving 1966 mention should be made of our finding that *U. urealyticum* was selectively inhibited and susceptible to erythromycin, an antibiotic which is also effective in the treatment of NGU.[18] A year later we reported that ureaplasmas were selectively inhibited by the anti-herpes virus compound, 5-iodo-2′-deoxyuridine, and also by hydroxyurea.[19] Both compounds are thymidine-blocking agents, and complete inhibition of *Ureaplasma* growth by either agent could be reversed by the addition of thymidine.

Since *U. urealyticum* was the only member of the mycoplasmas known to hydrolyze urea, ureaplasmas could now be detected and identified in cultures by demonstration of urease activity. Our first step in this direction was the development of a sensitive urease color test broth for detection of the organisms in clinical exudates and urine specimens. Our first color test broth was Medium U9, reported in 1970.[20] It was supplemented with only 5% horse serum and used phenol red as the indicator to detect urea hydrolysis and release of ammonia with the resultant rise in pH to pH 8 or 9, indicated by change from yellow to red. It was also a useful medium for quantifying ureaplasmas in exudates, urines or broth cultures. The U9 broth was especially useful in detecting ureaplasmas

in clinical specimens, but recovery of the organisms from incubated U9 broth was difficult, since transfers to fresh broth or agar had to be made early, before full red color change was reached. We always said, "Red is dead." U9 broth was subsequently improved by the incorporation of 0.01% L-cysteine HCl and was then designated U9B broth in 1976.[21] The viability problem with U9 and U9B was overcome by the development of our 10B urease color test broth in 1978,[22] which was more highly buffered and preserved the viability of ureaplasmas (primary isolates or stock strains) for much longer periods of time. For example stock strains remained viable in the refrigerator for 20 to 30 days, and seven of the first nine serovars were viable in 10B broth at 4°C for 92 days in one holding experiment. It is also excellent for storage of *Ureaplasma* strains in the frozen state at −60°C to −70°C for long periods.

There was an equally pressing need for detecting urease activity in *Ureaplasma* colonies in primary cultures of clinical specimens for detection and positive identification. What we needed was a simple, reliable spot test for urease which could be applied directly to suspected *Ureaplasma* colonies, much in the manner of the oxidase test for the identification of *Neisseria* colonies. The rationale of such a spot test seemed simple enough. We would apply a drop of enzyme substrate (urea) to the suspected *Ureaplasma* colony, wait a few minutes for the release of ammonia from the colony (by hydrolysis of urea by ureaplasmal urease) and apply a second drop of a reagent to detect the ammonia. We tested compounds like phenol red, Nessler's reagent and Berthelot's reagent but found them too weak and too generalized to be useful. They did not show where the ammonia was coming from; i.e. the colonies remained unchanged and showed no color development.

A test for gaseous ammonia developed by Feigl,[23] with the use of filter paper strips moistened with Feigl's reagent, looked promising. Feigl's reagent consisted of a mixture of silver nitrate and manganous nitrate. In the presence of ammonia a blackening of the filter paper occurred. After many trials and errors we subsequently developed a satisfactory direct spot test for urease in colonies of *Ureaplasma*.[24] The original spot test reagent consisted of two separate solutions: a 10% solution of urea; and an 0.8% solution of manganous chloride. It was later found that the strength of the urea solution could be reduced to 1.0%, and 0.8% manganous chloride was combined with the urea solution to yield a single spot test reagent solution.[25] The test was usually performed by placing a 1-cm square of agar (cut from an agar primary culture plate growing out suspected *Ureaplasma* colonies on standard agar) on a glass slide and applying the spot test reagent. The preparation was then placed on the microscope stage and examined under low power (×100). Colonies of *Mycoplasma hominis* are frequently also isolated from male urethritis patients. Since *M. hominis* is urease-negative, this species is unreactive in the spot test and *M. hominis* colonies remain unchanged, whereas the *Ureaplasma* colonies are seen as deep brown manganese accretion colonies. The contrast between colonies of *M. hominis* (and other large colony mycoplasmas) and those of *U. urealyticum* is dramatically increased by counterstaining the preparation on which the spot test has been performed. This is accomplished by placing a Dienes-stained coverslip on the agar block. Colonies of all large colony mycoplasmas stain bright blue in contrast to the deep brown of the *Ureaplasma* colonies. The spot test for urease is simple to perform, rapid, reliable and specific for identification of all *Ureaplasma* species.

In 1969 we set out to develop a differential agar medium which would provide the same picture as that obtained by the urease spot test, by incorporating the biochemical principle of the spot test into our A5H standard agar formula. The resultant medium was designated differential agar medium A7 and its formula was published in 1976.[21] Three years later an improved version was developed which was supplemented with a polyamine, putrescine HCl, and designated A7B.[26] The divalent cation indicator in the A7 and A7B differential agars was 0.88 mM manganese sulfate in combination with 0.1% added ultrapure grade urea. The ureaplasma colonies are identified by their deep brown reaction product, whereas *M. hominis* colonies are unreactive on this differential agar. If the preparation is counterstained with a Dienes-stained coverslip, the ureaplasma colonies remain unaltered and the colonies of *M. hominis* take up the blue dye of the counterstain. Thus the picture is essentially identical to that obtained by application of the spot test for urease activity on standard agars. Since the manganese reaction product is a metallic oxide complex coating the outer surface of the *Ureaplasma* colony, its appearance is a function of the manner in which it is illuminated. If colonies reacted to the spot test (or growing on differential agar) are examined in oblique, indirect light, the *Ureaplasma* colonies appear white. If the same *Ureaplasma* colonies are examined instead by the usual direct transmitted illumination through the substage condenser of the microscope, the colonies are deep brown. This feature of dual appearance is useful, because inoculated, incubated culture plates of differential agar can be examined in a preliminary manner by holding the culture plate up to a gooseneck lamp. Any *Ureaplasma* colonies, either single or in masses, will appear white in the beam.

Robertson and Chen[27] reported that certain serotypes of *U. urealyticum* were sensitive to 1.0 mM

manganese. Two broad biotypes of strains were recognized. The first biotype included serovars 1, 3, 6 and 14, and inhibition of growth in broth was temporary. The second biotype included serovars 2, 4, 5, 7, 8, 9, 10, 11 and 12, and inhibition of growth in broth was permanent. Robertson's two broad biotype groups of serovars was in agreement with the findings of Mouches et al.,[28] who found that serovars within the same biotype groups also contained similar proteins, and were identified as Groups A and B. We have used manganese sulfate in our A7 and A7B differential agars at 0.88 mM without apparent inhibition or problems. However, we thought it prudent to test a different divalent cation possibly to circumvent the inhibition by manganese of certain serovars. In 1983 we published the formula for A8 differential agar,[29] in which 1.0 mM calcium chloride is used as the divalent cation indicator of ammonia from urea. The microscopic picture is identical with that seen with A7 and A7B differential agars. Recent evidence suggests that even higher levels of $CaCl_2$ may be beneficial (2.0 or 3.0 mM), without attendant problems of growth inhibition of certain serovars.

By 1964 we summarized the clinical, laboratory and epidemiologic findings in NGU and first suggested that *Ureaplasma*-associated NGU was a sixth venereal disease.[30] In 1967 our knowledge concerning the cultivation and properties of the ureaplasmas was updated and summarized,[19] following a New York Academy of Sciences Conference on the Biology of the Mycoplasmas, held in New York in May, 1966. If my memory serves me correctly, it was during this conference that David Taylor-Robinson became interested in beginning studies of my "T strains." In 1970 our accumulated evidence supporting the role of ureaplasmas in NGU was published in *Journal of the American Medical Association*.[31] Our closing statement read, "The close association of "T" mycoplasmas with nongonococcal urethritis, their intimate association with the clinical course of the disease, their response to treatment of NGU, and their reappearance following reinfection (from consort or wife) suggests that "T" mycoplasmas play an important role in the etiology of the disease. Evidence accumulated to date strongly supports their role as one of the major causative agents of venereal nongonococcal urethritis in the human male." In addition the biology of the ureaplasmas was also presented. The isolation rate of ureaplasmas from male patients with NGU varied between 60 and 93% (number of patients not stated, but well over 2000 at that time). However, the isolation rate of ureaplasmas from a control group of 576 healthy, urethritis-free United States Marine Corps recruits at Camp LeJeune, NC, matched for age, occupation and sexual activity was only 25.8%.[31]

It soon became apparent that wide differences between isolation rates of ureaplasmas from NGU patients and from healthy controls in the world literature were not contributing to a resolution of the role of ureaplasmas in NGU. In an attempt to clarify their etiologic role in NGU, we conducted the first quantitative studies of the association of ureaplasmas with NGU.[32, 33] If ureaplasmas were closely associated with NGU and etiologically involved, as we believed, it should be possible to demonstrate quantitatively a close relationship of the organism to the clinical events of the disease. The most significant information would be that obtained in the condition of relapse where quantitative changes could be measured. We selected a reduced treatment regimen for *Ureaplasma*-positive NGU patients in the study which would assure that some of the patients would relapse following treatment. The drugs used were oxytetracycline, 250 mg four times daily over 4 days (total, 4.0 g), or tetracycline, 250 mg daily over only 3 days (total, 2.4 g). Assessment of the *Ureaplasma* status of the patient utilized freshly voided first morning urine specimens collected daily, before, during and following treatment of the NGU patients with the reduced regimens of tetracyclines. Each fresh urine specimen collected was sampled for determination of *Ureaplasma* titer in U9 urease color test broth, using 10-fold serial dilutions through eight tubes.

Thirty-two *Ureaplasma*-positive patients were treated with reduced regimens of tetracyclines. Of these 23 were successfully followed. Twelve were cured, two were treatment failures and nine patients relapsed after an eclipse period of 8 to 45 days (mean, 22 days), during which all clinical and laboratory findings were normal. The typical findings in one of our patients who was treated with a reduced regimen of tetracycline and who subsequently relapsed will serve as an example of our findings in this relapse group. Before treatment the NGU patient (Case A1481) presented with a history of sexual exposure approximately 3 weeks earlier and clinical signs of moderate mucopurulent urethral discharge, dysuria and frequency of urination. Pretreatment agar cultures were positive for *U. urealyticum* in large numbers. His urine titer for *U. urealyticum* was 1×10^5 color-changing units (CCU)/ml. The patient was then treated with 250 mg tetracycline three times a day over 3 days (total tetracycline, 2.4 g), during which time symptoms subsided and agar cultures became *Ureaplasma*-negative. After the first day of treatment the urine titer fell from 1×10^5 CCU/ml to negative. It looked as though we might have a cure. All signs, symptoms and cultures remained negative through Day 7 (4 days posttreatment). However, on Day 8 the agar culture became positive (small numbers of *Ureaplasma* colonies) and the urine titer rose to 1×10^1 CCU/ml. The patient remained asymptomatic. Over

the next 7 days the agar cultures remained positive with increasing numbers of colonies, and the urine titer climbed to the original pretreatment titer of 1×10^5 CCU/ml. On Day 13 (10 days posttreatment) the patient complained of dysuria; 1 day later urethral discharge appeared and NGU became reestablished. The patient was retreated with a full course of tetracycline (500 mg four times a day for 10 days) and cured. The convincing evidence in this study,[33] showing a close, quantitative relationship of *U. urealyticum* to the clinical course of NGU in relapse, strongly supported a pathogenic role for ureaplasmas in the etiology of NGU.

In 1978 we reported the results of a serologic survey of strains of *U. urealyticum* isolated at Camp Lejeune.[22] Cloned isolates from males with primary and recurrent NGU, patients with genitourinary tract infections and other disorders and asymptomatic carriers were serotyped by an agar growth inhibition test, with the use of antisera to serovars 1 through 8.

Ureaplasma isolates from 122 male NGU patients at Camp Lejeune were serotyped. Serovar 4 was associated most frequently (52%), and serovar 2 was next (17%); all the remaining serovars (except serovars 5 and 7, which were not isolated) were associated with NGU less than 10% in frequency of isolation. Among 91 symptomatic (but nonurethritis) patients with other conditions, including chronic prostatitis, renal stones, unexplained pyuria, etc., serovar 4 was again the most frequently isolated serovar. Among 125 asymptomatic carriers (controls), *U. urealyticum* serovars 4 and 8 were most frequently isolated.

In retrospect we are not sure what all this means. Serovars 4 and 8, for example, may be predominant among the prostitutes in the Camp Lejeune area. However, many of the Marine Corps enlisted men whom we saw with NGU were very recent returnees from overseas stations and training cruises. Port cities for shore leave such as Athens, Barcelona and many others, particularly around the Mediterranean area, are hot spots for acquiring *Ureaplasma*-associated NGU. The more recent serotyping studies of Lin and Kass[34] and of Robertson and Stemke[35] have extended the number of *U. urealyticum* serovars to 14.

One of the characteristic features of NGU, and a very useful clinical sign in the diagnosis of the disease, is the presence of mucous threads in the first-glass urine specimen. Following completion of therapy the persistence or absence of mucous threads in the urine is an important clinical sign in assessing response to treatment.[36, 37] Following our development of the differential agars, which specifically detect and identify colonies of ureaplasmas by virtue of a deep brown manganese reaction product, an additional, significant capability was observed for these agars. It was noted that urine sediments, planted on A7 differential agar, frequently contained mucous threads, intact or more frequently broken up. However, such mucous thread fragments often exhibited strong urease activity, thus signifying the presence of ureaplasma organisms. In view of the importance of urinary mucous threads in NGU, a study of intact, unaggregated mucous threads was undertaken to determine whether there was a significant association of *U. urealyticum* with such infected mucous threads.

Intact mucous threads were collected from 20 to 30 ml of freshly voided morning urine specimens from 170 men with NGU. They were planted without delay on the surface of A7 differential agar, incubated for 48 hours and examined under the microscope for evidence of *Ureaplasma*-infected mucous threads. Of the 170 men in the study 56 men (33%) were negative for ureaplasmas and had no threads. Fourteen men (8.0%) were *Ureaplasma*-positive but showed no threads. One hundred of the men (59%) were *Ureaplasma*-positive and all displayed *Ureaplasma*-infected mucous threads.

Ureaplasma-infected mucous threads were easily recognized by virtue of the deep brown manganese reaction product associated with such threads, and generally large numbers of *Ureaplasma* microcolonies were situated within such infected threads. *Ureaplasma*-infected threads often showed intense, extensive development of deep brown reaction product, together with a strong leukocyte response, indicating very heavy infection, often throughout their entire length. As with individual *Ureaplasma* colonies on A7 agar, *Ureaplasma*-infected mucous threads are white when viewed by indirect, oblique illumination. By way of contrast, healthy asymptomatic carriers of genital tract ureaplasmas show no threads, and the occurrence and distribution of *Ureaplasma* colonies in normal urinary mucus is random.

The history of the ureaplasmas is far from complete without acknowledging the early and more recent work with bovine ureaplasmas, ureaplasmas of sheep and those of other lower animals. Very significant and important findings have been reported in this area.

Ureaplasmas are becoming increasingly important in human disease. There is now convincing evidence that ureaplasmas are a significant cause of NGU in the human male. Circulating antibody to ureaplasmas was reported from NGU patients by metabolic inhibition test[11] and more recently by enzyme-linked immunosorbent assay.[38] In females with spontaneous pregnancy loss, serologic evidence of *Ureaplasma* infection was also recently reported.[39] Ureaplasmas are also a cause of persistent NGU that is associated with tetracycline- and minocycline-resistant strains of ureaplasmas in *Chlamydia*-negative men.[40, 41] Good evidence has been presented that ureaplasmas play a role in sexually transmitted Reiter's syndrome.[42]

Ureaplasmas are associated with male infertility[43] and were shown to be of etiologic importance in chronic prostatitis.[44] A causal role for ureaplasmas in reproductive failure in the female and in neonatal infections is now strongly supported by recent studies which will be reported to you during this symposium.

I should like to pay tribute to four individuals among the many who have contributed significantly to our knowledge of the ureaplasmas and to their role in human infectious diseases. First to Denys Ford, who was the first investigator to confirm, in a different geographic region (Canada), the existence of "T-strains" (*U. urealyticum*).[7] To Ruth Kundsin,[45] who in 1967 published a paper entitled "Strain of mycoplasma associated with human reproductive failure" (I named it the "Boston T-strain"). She pointed the way which has climaxed in the advent of this symposium. To David Taylor-Robinson[46] who fulfilled Koch's Postulates for *U. urealyticum* in NGU in a bold and difficult, well-controlled human volunteer experiment, and whose continuing studies have further supported a causal role for ureaplasmas in this disease. And to Patricia Quinn for her important contributions, including her serologic studies, supporting a causal role for ureaplasmas in pregnancy loss and neonatal infection.

REFERENCES

1. Klieneberger E: Further studies on *Streptobacillus moniliformis* and its symbiont. *J Pathol Bacteriol* 42:587–598, 1936
2. Dienes L: "L" organism of Klieneberger and *Streptobacillus moniliformis*. *J Infect Dis* 65:24–42, 1939
3. Shepard MC: The recovery of pleuropneumonia-like organisms from Negro men with and without nongonococcal urethritis. *Am J Syph Gonor Vener Dis* 38:113–124, 1954
4. Shepard MC: *The Mycoplasmatales and the L-Phase of Bacteria.* Edited by L Hayflick. New York, Appleton-Century-Crofts, 1969
5. Robertson J, Smook E: Cytochemical evidence of extramembranous carbohydrates on *Ureaplasma urealyticum* (T-strain mycoplasma). *J Bacteriol* 128:658–660, 1976
6. Shepard MC: Biology and taxonomy of *Ureaplasma urealyticum. Nongonococcal Urethritis and Related Infections.* Edited by D Hobson, KK Holmes. Washington, DC, American Society for Microbiology, 1977, pp 345–357
7. Ford DK, Rasmussen G, Minkin J: T-strain pleuropneumonia-like organisms as one cause of non-gonococcal urethritis. *Br J Vener Dis* 38:22–25, 1962
8. Ford DK: Culture of human genital "T-strain" pleuropneumonia-like organisms. *J Bacteriol* 84:1028–1034, 1962
9. Shepard MC, Lunceford CD: Effect of pH on human *Mycoplasma* strains. *J Bacteriol* 89:265–270, 1965
10. Shepard MC: Human *Mycoplasma* infections. *Health Lab Sci* 3:163–169, 1966
11. Purcell RH, Taylor-Robinson D, Wong D, et al: Color test for the measurement of antibody to T-strain mycoplasmas. *J Bacteriol* 92:6–12, 1966
12. Shepard MC, Lunceford CD: Occurrence of urease in T-strains of *Mycoplasma. J Bacteriol* 93:1513–1520, 1967
13. Ford DK, MacDonald J: Influence of urea on the growth of T-strain mycoplasmas. *J Bacteriol* 93:1509–1512, 1967
14. Kenny GE, Cartwright FD: Effect of urea concentration on growth of *Ureaplasma urealyticum* (T-strain *Mycoplasma*). J Bacteriol 132:144–150, 1977
15. Sayed IA, Kenny GE: Effects of reducing agents, catalase, and reuse of medium on toxicity of media for growth of *Ureaplasma urealyticum. J Infect Dis* 139:720–724, 1979
16. Shepard MC, Lunceford CD, Ford DK et al: *Ureaplasma urealyticum* gen nov., sp. nov.: Proposed nomenclature for the human T (T-strain) mycoplasmas. *Int J Syst Bacteriol* 24:160–171, 1974
17. Howard CJ, Gourlay RN: Proposal for a second species within the genus *Ureaplasma, ureaplasma diversum* sp. nov. *Int J Syst Bacteriol* 32:446–452, 1982
18. Shepard MC, Lunceford CD, Baker RL: T-strain mycoplasma: Selective inhibition by erythromycin *in vitro. Br J Vener Dis* 42:21–24, 1966
19. Shepard MC: Cultivation and properties of T-strains of *Mycoplasma* associated with non-gonococcal urethritis. *Ann NY Acad Sci* 143 (Art 1):505–514, 1967
20. Shepard MC, Lunceford CD: Urease color test medium U-9 for the detection and identification of "T" mycoplasmas in clinical material. *Appl Microbiol* 20:539–543, 1970
21. Shepard MC, Lunceford CD: A differential agar medium (A7) for identification of *Ureaplasma urealyticum* (human T mycoplasmas) in primary cultures of clinical material. *J Clin Microbiol* 3:613–625, 1976
22. Shepard MC, Lunceford CD: Serological typing of *Ureaplasma urealyticum* isolates from urethritis patients by an agar growth inhibition method. *J Clin Microbiol* 8:566–574, 1978.
23. Feigl F: *Spot Tests in Organic Analysis.* Fifth Edition. Amsterdam, Elsevier Publishing Company, 1956, pp 91–92
24. Shepard MC, Howard DR: Identification of "T" mycoplasmas in primary agar cultures by means of a direct test for urease. *Ann NY Acad Sci* 174:809–819, 1970
25. Shepard MC: Differential methods of identification of T-mycoplasmas based on demonstration of urease. *J Infect Dis* 127 (suppl):S22–S25, 1973
26. Shepard MC, Combs RS: Enhancement of *Ureaplasma urealyticum* growth on a differential agar medium (A7B) by a polyamine, putrescine. *J Clin Microbiol* 10:931–933, 1979
27. Robertson JA, Chen MH: Effects of manganese on the growth and morphology of *Ureaplasma urealyticum. J Clin Microbiol* 19:857–864, 1984
28. Mouches C, Taylor-Robinson D, Stipkovits L, et al: Comparison of human and animal ureaplasmas by one- and two-dimensional protein analysis on polyacrylamide slab gel. *Ann Microbiol* 132B:171–196, 1981.
29. Shepard MC: Culture media for ureaplasmas. *Methods in mycoplasmology.* Vol 1. Edited by S Razin, JG Tully. New York, Academic Press, 1983
30. Shepard MC, Alexander CE, Lunceford CD, et al: Possible role of T-strain mycoplasma in nongonococcal urethritis: A sixth venereal disease? *JAMA* 188:729–735, 1964
31. Shepard MC: Nongonococcal urethritis associated with human strains of "T" *Mycoplasma. JAMA* 211:1335–1340, 1970
32. Shepard MC: T-strain mycoplasmas as a cause of urethritis. *Epidemic Venereal Disease: The Proceedings of the Second International Venereal Disease Symposium.* St. Louis, MO. N Miami, FL, Symposia Specialists, 1973, pp 83–87
33. Shepard MC: Quantitative relationship of *Ureaplasma urealyticum* to the clinical course of nongonococcal urethritis in the human male. Les Colloques de l'Institut National de la Santé et de la Recherche Médicale. Les Mycoplasmes/Mycoplasmas. *INSERM* 33:375–380, 1974
34. Lin JSL, Kass EH: Fourteen serotypes of *Ureaplasma urealyticum* (T-mycoplasmas) demonstrated by the complement-dependent mycoplasmacidal test. *Infection* 8:152–155, 1980
35. Robertson JA, Stemke GW: Expanded serotyping scheme for *Ureaplasma urealyticum* strains isolated from humans. *J Clin Microbiol* 15:873–878, 1982
36. Harkness AH: *Non-gonococcal Urethritis.* Edinburgh, E and S Livingston, 1950
37. Messent JJ: Association of threads in the urine with non-gonococcal urethritis and prostatitis. *Br J Vener Dis* 46:469, 1970
38. Brown MB, Cassell GH, Taylor-Robinson D, et al: Measurement of antibody to *Ureaplasma urealyticum* by an enzyme-

linked immunosorbent assay and detection of antibody responses in patients with nongonococcal urethritis. *J Clin Microbiol* 17:288–295, 1983

39. Quinn PA, Shewchuk AB, Shuber J, et al: Serologic evidence of *Ureaplasma urealyticum* infection in women with spontaneous pregnancy loss. *Am J Obstet Gynecol* 145:245–250, 1983
40. Stimson JB, Hale J, Bowie WR, et al: Tetracycline-resistant *Ureaplasma urealyticum*: A cause of persistent nongonococcal urethritis. *Ann Intern Med* 94:192–194, 1981
41. Magalhães M: Persistent nongonococcal urethritis associated with a minocycline-resistant strain of *Ureaplasma urealyticum*: A case report. Sex Transm Dis 10:151–152, 1983
42. Ford DK, da Roza DM, Shah P, et al: Cell-mediated immune responses of synovial mononuclear cells in Reiter's syndrome against ureaplasmal and chlamydial antigens. *J Rheumatol* 7:751–755, 1980
43. Swenson CE, Toth A, O'Leary WM: *Ureaplasma urealyticum* and human infertility: The effect of antibiotic therapy on semen quality. *Fertil Steril* 31:660–665, 1979
44. Weidner W, Krause W, Schiefer HG, et al: Ureaplasmal infections of the male urogenital tract, in particular prostatitis, and semen quality. *Urol Int* 49:5–9, 1985
45. Kundsin RB, Driscoll SG, Ming PML: Strain of *Mycoplasma* associated with human reproductive failure. *Science* 157:1573–1574, 1967
46. Taylor-Robinson D, Csonka GW, Prentice MJ: Human intraurethral inoculation of ureaplasmas. *Q J Med* (New Ser) 46:309–326, 1977