T-STRAIN MYCOPLASMA* SELECTIVE INHIBITION BY ERYTHROMYCIN *IN VITRO*

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The precise role of the Mycoplasma in the aetiology of human non-gonococcal urethritis has been a controversial subject for nearly 25 years. Mycoplasma were first isolated from a human lesion by Dienes and Edsall (1937). The organisms they recovered in primary culture produced colonies which were highly characteristic in morphology and large in size. We refer to organisms of the pleuropneumonia group which produce these large, characteristic colonies on agar as the *classic* Mycoplasma. With the exception of the reports by Shepard (1956, 1959, 1960; Shepard, Alexander, Lunceford, and Campbell, 1964), and subsequently by Ford (Ford, Rasmussen, and Minkin, 1962; Ford and MacDonald, 1963; Ford and Du Vernet, 1963), all studies reported in the literature on the role of Mycoplasma in the aetiology of human non-gonococcal urethritis were concerned exclusively with human *classic*-type Mycoplasma. Similarly, studies on the *in vitro* effect of various antimicrobial agents against the Mycoplasma relate to the *classic* Mycoplasma only.

A previously undescribed member of the Mycoplasma group was isolated from the urethrae of patients with non-gonococcal urethritis by Shepard (1954) and described in greater detail in subsequent reports (Shepard, 1956, 1959, 1960; Shepard and others, 1964). The minute colony size and morphology of this new Mycoplasma was radically different from that of previously described *classic* human genital organisms. It was designated a *T-strain* (Shepard, 1954, 1956) pleuropneumonia-like organism (Mycoplasma) to distinguish it from the *classic* human genital group. The *T-strain* organism was associated in approximately 70 per cent. of over 1,000 non-gonococcal urethritis patients studied

(Shepard, 1959, 1960; Shepard and others, 1964). Although the existence of *T-strain* Mycoplasma was rejected (Klieneberger-Nobel, 1959), and their status as members of the Mycoplasma group questioned (King, 1964), the findings reported by Shepard (1954, 1956, 1959, 1960; Shepard and others 1964) were confirmed by Ford (Ford and others 1962; Ford and MacDonald, 1963), who reported that *T-strain* Mycoplasma were associated in 79 of 100 cases of non-gonococcal urethritis in Vancouver (Ford and DuVernet, 1963). In addition, convincing confirmatory evidence was presented which firmly established the *T-strain* organisms as true Mycoplasma (Ford and MacDonald, 1963).

All experimental evidence to date indicates that the human genital *classic* Mycoplasma are insensitive to erythromycin *in vitro*. The earliest report of the *in vitro* action of erythromycin on Mycoplasma is that of Keller and Morton (1953), who found that three human genital *classic* Mycoplasma strains were highly resistant to the drug and grew in the presence of 200 µg./ml. of erythromycin in fluid culture. Rubin, Somerson, Smith, and Morton, (1954) confirmed this observation by further showing that erythromycin had no discernible effect in the elimination of human genital *classic* Mycoplasma in the discharges of female patients with gonorrhoea treated with the drug. The dosage used was 300 mg. four times a day for 3 days (total = 3.6 g.). The earliest reported trial of erythromycin in the treatment of human non-gonococcal urethritis was that of Haight and Finland (1952) who found the drug without effect. Three acute cases were treated (Mycoplasma status unknown); one received 5 g. erythromycin over 3 days, and two received 2.4 g. over 2 days.

Quite opposite clinical findings were reported by Willcox (1954), who treated 25 previously untreated cases of non-gonococcal urethritis with a deliberately

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low dose of 2.4 g. erythromycin (100 mg. four times daily over 6 days). Of 21 cases followed, fourteen (66.6 per cent.) were cured and seven required retreatment. In a subsequent study, Willcox (1955) treated 53 previously untreated cases of non-gonococcal urethritis with 6 g. erythromycin (250 mg. four times a day) for 6 days. Of fifty cases followed, 39 (78 per cent.) were cured—a 17 per cent. increase in cure rate with a 2.5-fold increase in total dosage. Thus, the clinical effectiveness of erythromycin (Willcox, 1954, 1955), coupled with the demonstrated resistance *in vitro* and *in vivo* of human genital classic Mycoplasma to the drug (Keller and Morton, 1953; Rubin and others, 1954), provided strong evidence *against* these organisms as causative agents of human non-gonococcal urethritis. The above findings also supported the conclusions reached by Nicol and Edward (1953), who believed that the (classic) Mycoplasma were commensal organisms of the human genito-urinary tract and unrelated to non-gonococcal urethritis.

Material and Methods

Sixteen human Mycoplasma strains were employed as test organisms. These included five T-strains, five untyped human genital classic strains, five recognized human Mycoplasma species, and a tissue culture contaminant of human origin.

T-strains 960, K12, K71, and F354 were originally isolated from patients with non-gonococcal urethritis and were subsequently propagated in an all-agar system. T-strain F354 was isolated by Dr D. K. Ford in Vancouver, B. C. T-strain 19 was isolated from a urethritis-free carrier.

The five untyped human genital classic strains were isolated from patients with urethritis.

The remaining organisms included *M. salivarium* (PG-20); *M. hominis*, Type 1 (PG-21); *M. hominis*, Type 2 (PG-27); and *M. fermentans* (PG-18). Also included was the human upper respiratory tract pathogen, *M. pneumoniae* (Chanock, 1963), and *M. pharyngis* (Clyde, 1964), a human classic upper respiratory tract Mycoplasma and tissue culture contaminant ("Duke" strain).

Culture plates of TDA-16 agar medium (tryptic digest-plasma agar) were prepared containing dilutions of standard erythromycin to provide final concentrations ranging from 100 to 0.047 µg./ml. erythromycin. Freshly-prepared stock solutions were made by first dissolving erythromycin standard powder* in 70 per cent. methyl alcohol to a concentration of 10,000 µg./ml. erythromycin, and storing this solution at 4° C. for 24 hrs. to ensure complete solution. Further dilutions were made in M/15 phosphate buffer pH 7.0, to a concentration of 1,000 µg./ml. erythromycin. Final dilutions of the drug were made in melted complete agar medium at 45° to 50° C. to

yield desired concentrations. For use in a broth dilution system, final dilutions of the drug were made in tryptic digest-horse serum (20 per cent.) broth. All tests were conducted at pH 6.0 and at pH 7.5.

For evaluating the *in vitro* action of erythromycin in primary cultures inoculated with clinical material, plates of TDA-16 agar medium (pH 7.5) were prepared containing 6.25 µg./ml. erythromycin.

Female urethral specimens were collected on small, sterile calcium alginate wool swabs (Calgiswabs®)† and placed in tubes containing 0.25 ml. sterile broth. Specimens were held in the refrigerator at 4° C. until paired culture plates were inoculated—usually within a period of 3 hrs. A few specimens which were collected late in the day were stored in the frozen state overnight.

Results

All of the ten classic Mycoplasma strains examined were found to be highly resistant (>100 µg./ml.) *in vitro* to erythromycin (Table I). This high degree of resistance was unaffected by the pH of the culture system (6.0 or 7.5). Our observations thus confirm the original findings of Keller and Morton (1953).

TABLE I
INHIBITION OF HUMAN MYCOPLASMA BY
ERYTHROMYCIN *IN VITRO*

Mycoplasma	No. of Strains	M.I.C. (µg./ml.)‡	
		pH 7.5	pH 6.0
T-strain	5	0.78	3.12
Classic, genital*	5	> 100**	> 100**
<i>M. salivarium</i>	1	> 100	> 100
<i>M. hominis</i> -1	1	> 100	> 100
<i>M. hominis</i> -2	1	> 100	> 100
<i>M. fermentans</i>	1	> 100	> 100
<i>M. pharyngis</i>	1	> 100	> 100
<i>M. pneumoniae</i>	1	0.025	NT‡‡

* Untyped human genital classic Mycoplasma.

† Minimum inhibitory concentration resulting in complete inhibition of Mycoplasma colony development.

** Highest concentration tested (no inhibition).

‡‡ NT=Not Tested. *M. pneumoniae* does not initiate growth at pH 6.0.

In striking contrast to the confirmed insensitivity of classic human genital Mycoplasma to erythromycin, T-strains were found to be selectively inhibited by the drug *in vitro*, employing the same test system (Table I). Five T-strains, isolated from different patients with non-gonococcal urethritis, were completely inhibited by 0.78 µg./ml. erythromycin at pH 7.5, and by 3.12 µg./ml. erythromycin at pH 6.0. A similar degree of selective inhibition of T-strain Mycoplasma by erythromycin was obtained in a fluid culture system (Figure, opposite). Two factors probably explain the 4-fold increase in drug concentration that was required to achieve complete inhibition of T-strain growth at pH 6.0, versus that

* Erythromycin base standard powder was made available through the courtesy of Dr R. S. Griffith, Lilly Laboratory for Clinical Research, Indianapolis, Ind.

† Colab Laboratories, Inc., Chicago Heights, Illinois, USA.

FIGURE.—Inhibition of T-strain Mycoplasma by erythromycin *in vitro* when cultivated in a fluid medium of pH 6.0 and pH 7.5.

at pH 7.5. First, it is well known that erythromycin is least active in slightly acid solutions. Second, the optimum (H)⁺-ion concentration for maximal growth of T-strain Mycoplasma was recently (Shepard and Luncford, 1965) demonstrated to be pH 5.5 to 6.0. Thus, a reaction of pH 6.0 is the most favourable for growth of T-strains and the least favourable pH for inhibitory action of the drug.

T-strain Mycoplasma in mixed primary cultures from the urethral tract of 124 females were also selectively inhibited by erythromycin incorporated in the agar medium (Table II). Paired culture plates (one of which contained 6.25 μg./ml. erythromycin) were uniformly inoculated from a broth suspension prepared from a urethral swab specimen. The control medium *without* inhibitor yielded 69 isolations of T-strain Mycoplasma, eleven of "intermediates", and twenty of classic human genital Mycoplasma. The companion medium containing erythromycin

was completely inhibitory for T-strain and "intermediate" Mycoplasma. Classic human genital Mycoplasma were unaffected by the drug. In fact, three isolations of classic Mycoplasma were obtained on the erythromycin-containing medium that were missed on the control medium without inhibitor. Of the classic Mycoplasma isolated from twenty females on the control medium, seventeen were mixed with T-strain Mycoplasma. All isolations of classic Mycoplasma on the control medium were duplicated on the medium containing erythromycin, with the exception of the three cultures referred to above. Colonies that were intermediate in size between T-strain and classic Mycoplasma in primary cultures we have designated "intermediates". They were generally few in number and relatively large in size (up to 40 μ), but bore strong resemblance to T-strains in morphology, colony structure and staining reaction. Their sensitivity to erythromycin (Table II) suggests that "intermediate" Mycoplasma are T-strains which grow to relatively large size under certain conditions, or when few in number. Intermediates are frequently encountered in primary cultures from the female genito-urinary tract, but are rarely encountered in primary cultures from male non-gonococcal urethritis patients.

It was of interest to note that *Mycoplasma pneumoniae* (Chanock, 1963), a recently demonstrated pathogenic human Mycoplasma from the upper respiratory tract, was also found to be sensitive to erythromycin *in vitro* when tested under the same conditions as the other fifteen human

TABLE II

SELECTIVE INHIBITION BY ERYTHROMYCIN *IN VITRO* OF T-STRAIN MYCOPLASMA IN MIXED PRIMARY CULTURE FROM THE FEMALE URETHRA

Genital Mycoplasma Isolated	No. of Females Cultured	Recovery of Mycoplasma	
		Inhibitory Agent	
		None	Erythromycin*
T-strain	124	69	0
Intermediates		11	0
Classic		20	23

* Erythromycin, 6.25 μg./ml. incorporated in TDA-16 agar medium of pH 7.5.

Mycoplasma strains. *Mycoplasma pneumoniae* (strain NMFRL) was completely inhibited by 0.025 µg./ml. erythromycin in an agar dilution system of pH 7.5 (Table I). The organism was not tested at pH 6.0 because of its inability to grow at this pH (Shepard, 1965). The value of 0.025 µg./ml. is in agreement with that found by Griffith (1963) for the Eaton and Mac strains of *M. pneumoniae* (0.025 and 0.05 µg./ml. respectively).

Summary

The selective inhibition of T-strain Mycoplasma by erythromycin *in vitro* provides a biochemical method of distinguishing T-strains from human genital classic Mycoplasma in the form of an erythromycin inhibition test. T-strain Mycoplasma, associated with 70 to 80 per cent. of cases of non-gonococcal urethritis in human males, have now been shown to be susceptible to an antibiotic which is effective in treatment of the disease.

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Le mycoplasme de souche T. L'inhibition sélective par l'érythromycine *in vitro*

RÉSUMÉ

L'inhibition sélective du mycoplasme de souche T par l'érythromycine *in vitro* donne une méthode biochimique permettant de différencier le mycoplasme de souche T du mycoplasme classique génital humain en faisant le test d'inhibition par l'érythromycine. Le mycoplasme de souche T, associé à 70 à 80 pour-cent des cas d'urétrite non-gonococcique chez les hommes, a maintenant été prouvé sensible à un antibiotique, qui est aussi efficace contre la maladie.