Special Lecture

ANTIBIOTICS IN THE CONTROL OF MYCOPLASMA DISEASES OF ANIMALS AND PLANTS

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While most of the mycoplasma studies were restricted to the avian mycoplasma, bovine pleuropneumonia and *Mycoplasma pneumoniae*, the work of a group of Japanese workers in 1967 indicated that yellows and little-leaf diseases of plants may be due to mycoplasma-like organisms rather than viruses, and that they were tetracycline susceptible. Despite their differences in specific characters, source and distribution, the animal and plant mycoplasmas have remarkable homogenity in their basic ultrastructure, colony morphology, nucleic acid composition and cultural requirements.

The highly contagious nature of chronic respiratory disease of chicks, bovine pleuropneumonia, agalactia of sheep and goats called for control measures either as vaccines or more recently as antibiotics and chemotherapeutic agents. Plant mycoplasma were almost invariably discovered and distinguished from true virtues by their susceptibility and reversion to healthy state by use of tetracycline group of antibiotics.

The mode of action of the antibiotics also were studied, that including the development of resistant strains. In rarein stances, as in *M. laidlawi* which if grown in cholesterol medium is inhibited by polyene antifungal antibiotics. With the epidemics of chronic respiratory disease of chicks becoming serious, decontamination of eggs with tylosin, erythromycin is essential. The new antibiotic DOL was found by Singh to be very effective in controlling *M. gallisepticum*. Enzootic pneumonia of pigs due to *M. hypopneumoniae* has been treated with I.M. injections of tylosin and lincomycin.

About 55 or more plant diseases with yellows and proliferation of axillary buds with little leaf are known to be incited by *Mycoplasma*-like bodies. Discovery of these bodies in electron microscope, and remission of the disease symptoms by treatment with tetracycline group of antibiotics have been the basis for separating them from viruses. Few of the phytopathogenic mycoplasmas have been cultured on cell free media. Using tetracyclines and few other antibiotics, control of several of the plant diseases like aster-yellows, spike disease of sandal ,little leaf of brinjal, citrus greening disease, stubborn disease of citrus, etc. have been reported, but the remission of the disease is only temporary, since the organism multiplies again. The 'static' nature rather than 'cidal' condition of tetracyclines may explain this. Recently, a new chemotherapeutic agent BP-101, has been used very successfully against the citurs greening disease, and also the grassy shoot of sugarcane. The remission of the disease is permanent, and there appears to be good scope of control of mycoplasma diseases using this chemotherapeutic agent. Cases of other mycoplasma disease treated with antibiotics include rice yellow dwarf, bunchy top disease of papaya, and others. The future holds great promise of detection and cure of these mycoplasma diseases of animals and of plants, and more attention will be paid for finding out new chemotherapeutic substances rather than making basic studies.

INTRODUCTION

The outstanding work of Nocard and Roux at the Pasteur Institute in 1898, that the bovine pleuropneumonia was caused by a filterable organism, which they cultured on a cell-free medium, has remained to this day, unique and unparalleled. While describing it as ultramicroscopic, they were able to establish the etiological agent, which now goes under the modern nomenclature *Mycoplasma mycoides* var *mycoides*. Borrel *et al.* (1910) proposed the binomial *Asterococcus mycoides* for the culturable agent of bovine pleuropneumonia, and this term remained in use even up to 1940. By this time the terms PPLO (pleuropneumonia-like organisms), L-organisms (L = Lister Institute) came into general use, and these included the L-forms of true bacteria. This confusion of L-phase of bacteria and the organisms causing pleuropneumonia of bovines was resolved by Freundt (1955) and Edward (1955) who accepted the genus *Mycoplasma* proposed by Nowak in 1929.

As it always happens with classical work, not much credence was given to the discoveries of Nocard and Roux, and since the organism was filterable, the causal agent of pleuropneumonia was considered as a virus. Even so, the yellows and little leaf diseases of some plants even six years back were all considered to be viruses, due to their symptom picture, transmission by insects and grafting. It was only since the investigations by Doi, Teranaka and Asuyama (1967) and Ishiie *et al.* (1967) that many of these yellows and little leaf diseases of plants are accepted to be incited by mycoplasma-like organisms. Numerous studies on their morphology, particularly ultrastructure, based on culture on cell-free media has enabled us to characterise them. They have the following characteristics (*i*) the smallest reproducing cells of 125 mμ and below, (*ii*) highly pleomorphic since they lack rigid cell wall, (*iii*) resistant to penicillin, but sensitive to tetracyclines, (*iv*) can be grown in cell-free media and are filterable, (*v*) growth is inhibited by specific antibody and (*vi*) mycoplasmas do not revert to or form bacteria-like parental forms. In other words, they are different from the L-phase of certain bacterial species.

Their great importance lies in the fact that they incite very important diseases in plants, animals, and human beings. Recent work on the cell morphology and biochemical make up of the animal mycoplasmas, and those of plants both in the plant host and insect vector have shown that they are very closely related, and perhaps in the near future, some of the mycoplasmas may be found which may infect both animals and plants.

Among the human mycoplasma, *M. pneumoniae* is known to cause from subclinical to severe cases of pneumonia and bronchitis. *M. hominis* and *M. pharyngitis* are the other two mycoplasmas of the respiratory tract. T-strains of mycoplasma are associated with nongonococcal urethritis.

The avian mycoplasmas have been studied in great length and have been successfully controlled also. *Mycoplasma gallisepticum* in poultry, *M. meleagridis* in turkey, and *M. hominis* are well known. *M. hyorhinus* causes lesions in pigs. The contagious agalactia in goats is caused by *M. agalactiae*. *M. mycoides* var *capri* also causes contagious caprine pleuropneumonia of goats.

Testing Methods for *in vitro* Sensitivity of *Mycoplasma* Species to Antibiotics

The fact that animal mycoplasma have been cultured in cell-free media provided the opportunity to screen large number of antibiotics and chemicals for inhibitory activity and thereafter use them for *in vivo* therapy. Many of the *Mycoplasma* species and their strains are inhibited by specific groups of antibiotics, but almost all of them are resistant to penicillins, bacitracin, polymyxins and the sulfonamides. There have been good number of reviews on this topic by Adler (1965), Mirande and Sussell (1967), Taylor-Robinson (1967), Gale *et al.* (1967) and Smith (1971).

Schutze (1968) tested various antibiotics against different *Mycoplasma* species and found that tylosin, erythromycin, lincomycin, dihydrostreptomycin, tetracycline, leucomycin and chloramphenicol were effective. He found that rifamycin SV, novobiocin, polymyxin-B were less effective, while penicillin, cephalothine, nystatin and bacitracin had no effect at all. Prokofeva *et al.* (1966) found some of the avian mycoplasma were inhibited by high concentration of streptomycin (5000 μg/ml) and neomycin (100 μg/ml). Nicolet and Meuron (1970) found that many pathogenic bovine mycoplasma were sensitive to spiramycin. Newnham (1965) studied the inhibitory action of 19 antibacterial, antifungal and antiprotozoal antibiotics on avian mycoplasmas and found that tylosin and demethyl-chlortetracycline showed the highest inhibitory action followed by erythromycin, spiramycin, tetracycline and oxytetracycline. Miller *et al.* (1968) showed that the antibiotic bottromycin was highly active against five species of mycoplasma. Hoshino *et al.* (1970) tested numerous antibiotics against a variety of mycoplasma and found that leucomycin, spiramycin, tylosin of the macrolide group were effective, while erythromycin and oleandomycin also macrolides had very little or no activity. Hamdy and Blanchard (1970) showed that spectinomycin and lincomycin were both active more individually than in combination. Fallon (1970) showed that the cephalosporin group of antibiotics were active only at very high concentrations of 1000 μg/ml and above. Singh and Singh (1967) working in India on PPLO of avian origin, studied the effects of antibiotics penicillin G, streptomycin and dihydrostreptomycin sulphate, erythromycin thiocyanate, tylosin tatrate and DOL, an antimycoplasma agent from Hindustan Antibiotics. They reported that the antibiotics were found in the following order of activity. DOL and tylosin were active at levels of 0.01 μg to 0.1 μg/ml, erythromycin 0.1 to 1 μg/ml, streptomycin 1000 μg/ml, dihydrostreptomycin 5000 to 10,000 μg/ml and penicillins with negligible or no activity at all.

Arai *et al.* (1966) and (1967) tested number of macrolides, nucleosides and tetracyclines against *M. pneumoniae*. They showed that all macrolides, nucleosides and tetracyclines, as well as the antitumor antibiotics so far tested, were inhibitory on the growth of mycoplasma and at the same time had antiprotozoal activity. All the penicillins, aminoglycosidic antibiotics and chloramphenicol had low or no antimycoplasmal activity or antiprotozoal activity. They concluded that there was some co-relation between antimycoplasmal and antiprotozoal activities.

As regards methods of handling the screening programme for *in vitro* activity, the following few methods may be described. An agar diffusion method for screening antimycoplasmal candidates was outlined by Perlman and Schwartz (1968),

Perlman *et al.* (1972). *Mycoplasma laidlawii* was used, and the media consisted of soypeptone broth supplemented with yeast extract, NaCl, and human serum as outlined by Kenny and Pollack. This solution is solidified with Noble agar. Ten ml of this poured over 150 mm Petri plates, allowed to harden, and 5 ml of the same agar with *Mycoplasma* cells 10^8 was poured as seed layer. Paper discs containing the antibiotics is placed and allowed to remain overnight, during which period the killing action of the mycoplasma cells by the antibiotic, if any, will take place. The plate is then flooded with 0.05 per cent of 2-6 dichlorophenolindophenol for 5 min and incubated for 60 to 90 min. The living cells will reduce the colour of the dye while the dead cells get deeply stained. The diameter of the coloured zone will indicate the amount of activity. A dose response curve can be drawn based on the diameter of the zone, and can be used in assay procedure. Ogata *et al.* (1971) outlined a method of testing *in vitro* testing. Fifteen mycoplasma species, and 22 antibiotics were evaluated. The organisms were *M. mycoides* var *mycoides*, *M. mycoides* var *capri*, *M. hyorhinus*, *M. suipneumoniae*, *M. granularum*, *M. canis*, *M. pulmonis*, *M. arthritidis*, *M. neurolyticum*, *M. gallisepticum*, and *M. laidlawii*. The last one was isolated from sewage. Agar dilution and broth dilution methods were used for the assay. Antibiotics chosen were actinomycin-D, mitomycin-C, tylosin, bottromycin, spiramycin, tetracycline, kasugamycin and polymyxin. For the plate assay 70 ml Difco PPLO agar, 20 ml of horse serum, 10 ml fresh yeast extract (25 per cent) were used. M.I.C. was determined by growth on the plates after 7 days at 37°C. For broth dilution assay, the colour change due to *p*H could be taken as a criterion. The antitumor antibiotics were most effective inhibitors. Tylosin, spiramycin and bottromycin were very effective in preventing growth at low concentrations. Kasugamycin, and polymyxin were ineffective. Erythromycin and oleandomycin were insensitive on some of the strains, *M. pulmonis*, *M. arthritidis*, *M. neurolyticum*, *M. hyorhinus* and *M. suipneumoniae*. The antitumor antibiotics being active against mycoplasma suggests the close relationship of mycoplasma with mammalian cells. *M. laidlawii* and *M. granularum* which do not require sterol, may have to be placed in a new genus.

Hoshino *et al.* (1970) in their screening system used Hoekken mycoplasma agar, which they considered as superior to others. It consisted of 0.7 per cent Hoekken heart extract, 1 per cent purified peptone, 0.1 per cent yeast extract and 0.5 per cent sodium chloride with 0.8 per cent agar, autoclaved at 121°C for 15 min. The mycoplasma colonies appear by about 96 hr at 37°C. They studied the *Mycoplasma* strains contaminating the tissue cultures, and after the incorporation of the antibiotics at various concentrations, their presence was tested by growth on the Hoekken mycoplasma agar. The M.I.C. was thus noted.

Antibiotic Testing *in vivo*

This is of utmost practical interest in the control of the diseases. Several animal models have been used in this system which gave valuable data. The chronic respiratory disease in chicks which is transmitted through eggs offers a good means of testing antimycoplasmal substances. The eggs are dipped in antibiotic solution at the incubator temperature for 30 minutes. The antibiotics used were 1000 and 2000 ppm

of tylosin, erythromycin and DOL which is also water soluble. Isolations are made from the eggs for the mycoplasma organisms and chicks after hatching, both cultturally and serologically. Some of the results have shown that DOL and tylosine tartrate are most effective in making the flocks free of the CRD inciting organisms.

Gale *et al.* (1967) used *M. gallisepticum* in six-day-old sex-linked pullets by inoculating them via the right thoracic air-sac. The inoculum was developed in embroynated eggs, and 0.5 ml was used for each chick. While there was no mortality, loss in weight gain, increase in specific agglutins, internal lesions, etc., were some of the common features. The gain in weight alone was sufficient for evaluation. The antibiotics were used along with drinking water at levels of 2000 mg per gallon of water. *E. coli* infection which is usually associated with *M. gallisepticum* can also be evaluated in this screening.

Hoshino *et al.* (1970) used *M. hominis* type-2 called the Campo strain by injecting through I. P. route in mice. After the 3rd day, the mice are sacrificed, and the internal organs, particularly the trachaea and lungs were plated on Hoekken agar for the number of colonies. Antibiotics are used for evaluating the type and dosage. The reduction in number of colonies is evaluated. Tylosin, leucomycin, and less of carbomycin were found effective, while the effect of erythromycin was erratic.

Practical Control of Mycoplasma Diseases by Antibiotics

Mycoplasma gallisepticum incites high morbidity and loss of weight gain in chicks due to poor feeding efficiency. The decontamination of eggs and making the flock free of the disease has already been pointed out by several workers including Singh and Singh (1967) in India. Tylosin tartrate was recommended at levels of 1600 ppm. Tylosin has no effect on Salmonellosis and *E. coli* which usually accompanies *M. gallisepticum,* and in this respect DOL which is highly active against Gram-negative organisms, and orally non-toxic up to level of 2 g/kg is a very useful chemotherapeutant. Olesuik *et al.* (1965) were able to check *M. gallisepticum* infection in chicken by adding 5 g of tylosin in a gallon of drinking water. Yamano *et al.* (1967) used chlortetracycline to eradicate respiratory mycoplasmosis in chicken. Layton and Kemp (1968), Minamimoto (1968) and others reported the effective control of *M. gallisepticum* infection by tylosin while Kumar *et al.* (1966) reduced the infection in turkeys by dipping in 1000 ppm tylosin preheated to 45°C. Tanaka *et al.* (1968) found bottromycin as eradicant of *M. gallisepticum.* Hamdy *et al.* (1969) used lincomycin and spectinomycin successfully controlling airsacculitis in turkey due to *M. meleagridis.* Bigland (1970) used 1200 ppm of chloramphenicol for decontaminating eggs in turkey infected with *M. meleagridis.* As a prophylaxis, chlortetracycline was found very effective when used as feed additive by Ookubo *et al.* (1966).

Enzootic pneumonia in swine can be as high as 40 to 50 per cent in some cases and Guljarani and Beveridge (1951) in U.K. first showed that the bacteria-free filterable inoculum produced the disease, and hence assumed to be a virus. Betts and Campbell (1956) showed that tetracyclines and not streptomycin cured the disease. Whittlestone (1967) cultured and identified the organism as *Mycoplasma suipneumoniae,* which was pleomorphic. *M. hypopneumoniae* also occurs in pigs, but the organism is resistant to tylosin and erythromycin, but susceptible to chlortetracycline. Wilson (1970) showed that lincomycin is very effective against this infection.

The mycoplasma strains producing mastitis in cattle in England, *M. bovigenitalium* was identified, and *M. laidlawii* was found in the nasal and genital tracts. The bovine pleuropneumonia has been well controlled by antibiotics, and Hudson and Etheridge (1969) showed that with seven 12 hourly I.M. injections of tylosin tartrate at level of 7.5 mg/kg controlled the disease.

Atypical pneumonia in human beings which causes severe lung infections with cough and fever, has been investigated in detail. Smith *et al.* (1967) in experimentally inoculated human volunteers (45 persons in all) showed that demethylchlortetracycline hydrochloride and tetracycline hydrochloride were superior in treatment to that of vaccine, which, while producing antibodies was not potent enough to afford protection from the severe symptoms. The volunteers were inoculated with the organism *M. pneumoniae* grown on PPLO-broth with 1000 units of penicillin. Nasal and throat sprays gave 100 per cent infection of the volunteers who came down with cough and fever.

Mycoplasma Diseases of Plants

Numerous reports of mycoplasmal diseases of plants have appeared since Doi *et al.* (1970) and Ishiie *et al.* (1967) suggested that Mycoplasma may be causal agents of plant diseases. Their evidence was based on the presence of Mycoplasma-like bodies in the phloem cells of infected plants and the disappearance of those bodies and the disease symptoms when treated with tetracycline antibiotics. In about 60 plant diseases, yellowing, little leaf, proliferation of axillary buds, general stunting have been the main symptoms. Some of the species have been isolated and cultivated *in vitro* in cell-free media, but this has not been as extensive as in animal mycoplasma.

The most interesting feature of plant mycoplasma has been that their mode of transmission, etc. perfectly coincided with that of viruses, so that in their being filterable through candles holding back bacteria, transmission through insect vectors and other means specific for viruses, made every one believe virus as being the causative agent. Even now, mycoplasmal etiology is first indicated by the response of the plant to tetracycline treatment, by their recovery from disease symptoms, and then consequent finding of the mycoplasmal bodies in ultrathin sections and electron microscopy. Tetracyclines have served as diagnostic agents for detecting mycoplasma diseases of plants. Both the plant host and the insect vector have the mycoplasma bodies, and they disappear after treatment with tetracyclines. Recently some needless confusion was thrown in by Atanasoff (Aug. 1972 *Phytopath. Zeit.*) by speculative hypothesis that the mycoplasma may be innocent symbionts in plants and animals and in some cases serve as vectors of viruses just as insects, nematodes, fungi, etc. The disappearance of disease symptoms as well as the mycoplasma after antibiotic treatment puts this hypothesis as truly speculative and without much value. If the etiological agent was a virus transmitted by *Mycoplasma,* the removal of the *Mycoplasma* by tetracycline would not take away the disease symptoms, since the virus itself is not inactivated by tetracyclines.

The Mycoplasma diseases of plants are of much significance since they are all very important economically. Shikata *et al.* (1969) showed the mycoplasma-like bodies in white-leaf of sugarcane and their control by tetracyclines. Lin *et al.* (1970)

made *in vitro* culture of this mycoplasma and also produced artificial inoculation. The first use of tetracycline in controlling mycoplasma was by Ishiie *et al.* in 1967 against yellow dwarf of mulberry. Hundred ppm of the antibiotic was used as dip treatment and spray. Davis *et al.* (1968, 1969, 1970) in series of experiments showed that aster-yellows was controlled by treatment with chlortetracycline and oxytetracycline, and the disease always reappeared.

In this place we may note that Freundt's suggestion that a filamentous phase of growth forms an essential part of life-cycle of Mycoplasma was not accepted by many workers who affirmed that it is true only of few species like *M. mycoides* var *mycoides*. This aspect is now being accepted, and even in plant mycoplasma same features are seen, as in the figures published in stubborn disease of citrus by Calavan (1971).

Excellent reviews on the mycoplasma diseases of plants have been published by Hull (1971), Ghosh and Raychaudhuri (1972), Hampton (1972) and others. Since this field of plant mycoplasma is new, the numerous references give only discoveries of the mycoplasmal body and their control temporarily by spraying with one of the tetracyclines, CTC, OT, TC or DEMCTC. The descriptions of the disease symptoms are given.

Freitag and Smith (1969) reported aster-yellows affected plantago, and celery, were seen recovering after treatment with chlortetracycline. The leaf-hopper *Macrosteles facifrons* also failed to acquire the disease from the treated plants. Anjaneyulu and Ramakrishnan (1969) and Raychaudhuri *et al.* (1970) reported remission of the little leaf of egg plant by spraying with tetracyclines. To make the antibiotic get into the plant, wick type of injection inside was found more feasible. Galvez and Shikata (1969) showed that the yellow dwarf of rice had the mycoplasma bodies and reported their remission by tetracycline treatment. Cousin and Staron (1969) tested tetracyclines, erythromycin and tylosin against the *Vinca rosea* mycoplasma and showed from point of phytotoxicity, and remission of the disease symptoms, only tetracyclines were effective. The bunchy top disease of papaya in Dominican Republic was shown by Story and Halliwell (1969) to be mycoplasma, controlled by tetracyclines. Varma *et al.* (1969), Hull *et al.* (1969) and Raychaudhuri *et al.* (1972), showed that the spike disease of sandal was due to mycoplasma and tetracyclines give temporary and partial remission. Among the various other mycoplasma diseases treated with tetracyclines have been, corn stunt (Grandos 1969), cassava witches' broom, opuntia tuna witches' broom, stubborn disease of citrus, purple top wilt and marginal flavescens of potato etc.

Citrus greening and die-back is a major disease in several countries, particularly India, Far Eastern countries, Africa and other places. It was long thought to be a virus disease, but recently the mycoplasma etiology has been established. The disease is transmitted by the psyllid, *Diphorina citri*. Martinez *et al.* (1970) reported remission of disease symptoms by spraying tetracycline. In India Nariani *et al.* (1971) have shown similar results, and Ghosh *et al.* (1971) have also cultured the mycoplasma on cell-free media. Capoor and Thirumalachar (1973) reported the disease in all details and showed that tetracycline only temporarily brings back healthy symptoms, and once the antibiotic is discontinued, the disease reappears. The die-back symptom is a secondary manifestation of the primary etiological agent, viz. the mycoplasma. It is similar in model to that of opportunistic fungi known in case of human infections

following broad-spectrum antibiotic therapy. The weak pathogenic fungi can invade the weakened body.

B.P. 101 is one of the new chemotherapeutic agent very active and effective against plant mycoplasma, developed by late Prof. M. J. Narasimhan in collaboration with the present author. It was very effectively tested against the grassy shoot of sugarcane. When the sets were dipped in a 100 ppm solution overnight and planted the healthy shoots came up, without the disease symptoms, and they remained healthy upto the time of maturity. Following this, Capoor and Thirumalachar (1973) tested it on citrus greening and die-back, using a 100 ppm solution which was made to diffuse into the stem through a funnel or some receptacle. The treatment was done with enough material as much as half a gram per tree. After three months the treated plants grew very healthy with abundant shoots and fruits. The control and cure of the citrus die-back and greening disease is now a possibility in all affected areas.

Development of resistant strains among mycoplasma to various antibiotics is always a possibility. Several workers reported this already. Chelton (1966) isolated resistant strains of *M. mycoides* var. *capri* to tetracyclines. Similarly in *M. gallisepticum* resistant strains to tylosin were reported, and with the withdrawal of the antibiotic, resistance also disappeared (Zanella 1966). Schwartz and Perlman (1971) showed that in *M. laidlawii* which can grow in medium without sterols, there was complete resistance to antifungal antibiotics amphotericin-B and fillipin. When sterols are given in the medium, the organism was completely inhibited by amphotericin-B. This showed that the mode of action of the polyene antibiotics which act by binding the sterols of the cell membrane. Schwartz and Perlman (1971) also reported the mode of resistance in *M. laidlawii, M. salivarium, M. hominis, M. pharyngis,* and *M. fermentans.* The antibiotics used were tetracyclines, kanamycin, gentamycin, streptomycin, chloramphenicol, and spectinomycin. Using resistant strains, studies were made for possible degradation of the antibiotic. The type of degradation have been known, acetylation by R-factor in case of chloramphenicol (Shaw) ,phosphorylation and acetylation in case of kanamycin, and adenylation in case of streptomycin. As suggested by Corcoraan in case of erythromycin resistant organisms, the resistance was due to low uptake and accumulation of the antibiotic. Even so it is assumed that in the present case the resistance may be due to lower uptake of the antibiotic substance.

The future of Mycoplasmatology, a group already well demarcated would be very exciting. Animal Mycoplasmatales is already well in advance of plant Mycoplasmatales. More *in vitro* cultural work characterisation and study of life-cycle of the different pathogens would lead to devising of new control measures. Plant Mycoplasmatology would get the same importance as plant virology, if it has not been there already.

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