IN VITRO SENSITIVITY OF MYCOPLASMAS ISOLATED FROM VARIOUS ANIMALS AND SEWAGE TO ANTIBIOTICS AND NITROFURANS

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(Received for publication March 8, 1971)

A total of 15 strains of *Mycoplasma* were examined for *in vitro* sensitivity to 22 commonly used antibiotics and 9 nitrofurans. They were strains of *Mycoplasma mycoides* var. *mycoides*, *M. mycoides* var. *capri*, *M. hyorhinis*, *M. suipneumoniae* (*hyopneumoniae*), *M. granularum*, *M. canis*, *M. pulmonis*, *M. arthritidis*, *M. neurolyticum*, *M. gallisepticum*, and *M. laidlawii*, all of which were isolated from various animals, except for one strain of *M. laidlawii* which was isolated from sewage. The sensitivity was determined by observing inhibition of growth in the agar and broth dilution systems. Among all the mycoplasmas examined, there were no marked differences in susceptibility to these drugs, with the exception of erythromycin and oleandomycin. Antitumor antibiotics, *i. e.*, actinomycin D and mitomycin C, were the most active of all the agents. Tylosin, bottromycin, spiramycin and tetracycline followed them in activity. Kasugamycin, polymyxin B and colistin were noninhibitory. *M. suipneumoniae*, which is known as the etiological agent of swine enzootic pneumonia (SEP), and other species of respiratory mycoplasmas of swine were compared with regard to minimum inhibitory concentrations (MIC) upon these drugs. The sensitivity of *M. suipneumoniae* to the drugs used was similar to that of other mycoplasma. Among the new nitrofurans tested, drugs with high activity against the mycoplasma were discovered.

Many studies have been made previously on the sensitivity of various mycoplasmas to antimicrobial drugs. Because of the high host specificity of mycoplasmas, strains derived from the same individual host species were chiefly used in such studies. Reports published to date deal with studies on strains isolated from human beings,[1~9] chickens,[10~16] cattle[17~19] and tissue culture contaminants[20~24]. Tests of mycoplasmas were usually made *in ovo* and *in vitro*, by using tissue culture, dilution methods and sensitivity discs. Recently, a microtechnique with "microtiter" plates has been developed to facilitate the simultaneous testing of a large number of specimens[7]. The procedures of these tests and the composition of culture medium vary with the authors. In some studies, no clear MIC has been indicated. NEWNHAM *et al.*[14] reported the comparative *in vitro* effects of various drugs on the avian, mammalian and saprophytic strains of mycoplasmas and the L-form of bacteria. Since then, very few papers except that of TAYLOR-ROBINSON[7] have been published on such effects upon other animal strains.

The purposes of the present experiments were to determine the concentration of the drug required for the complete growth inhibition and to compare the sensitivity of different animal origin mycoplasma species to antimicrobial drugs. This paper deals with the *in vitro* activity of various antibiotics and nitrofurans on respiratory mycoplasmas, including those believed to be the etiological agents of some respiratory diseases and those inhabiting the respiratory tract. These results are thought to be of significance for the treatment and prevention of micoplasmosis, as well as for the explanation of the mechanism of action of certain drugs on mycoplasmas.

Materials and Methods

1. Strains used

Table 1 shows the 15 strains (of 11 species) of mycoplasma used in the present experiments and their hosts. These strains consisted of 9 strains supplied in 1963 by Dr. D.G.ff. EDWARD of the Wellcome Research Laboratories, England, 1 strain supplied by Dr. N. ISHIDA of the Tohoku University, Japan, and maintained in a modified EDWARD's medium[25] for passage at this laboratory, 2 strains supplied in 1968 by Dr. W.P. SWITZER of the Iowa State University, U.S.A., 1 strain by Dr. R.F.W. GOODWIN of the School of Veterinary Medicine, University of Cambrige, England, and 1 strains by Dr. T. ESTOLA of the State Veterinary Medical Institute, Finland. All of the strains which originated from swine had been maintained in a modified GOODWIN's liquid medium.[26]

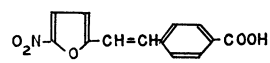
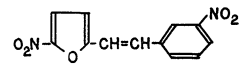
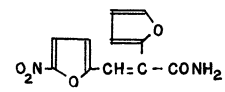
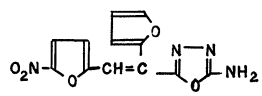
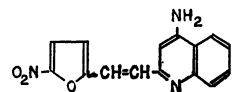
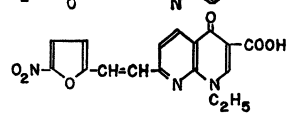
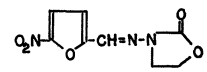
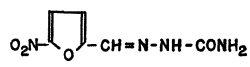
2. Antibiotics and nitrofurans used

A total of 22 antibiotics and 9 nitrofurans were tested. The antibiotics were divided into 5 groups; *viz.*, a tetracycline group (tetracycline, chlortetracycline and methacycline),

Table 1. Mycoplasma strains used in sensitivity tests

Host	Mycoplasma species	Strain	Origin
Cattle	*M. mycoides* var. *mycoides*	PG-1	EDWARD
	M. laidlawii B	PG-10	〃
Goat	*M. mycoides* var. *capri*	PG-3	〃
Swine	*M. hyorhinis*	P. 49-TP3 P-1 strain 7	SWITZER
		Haapalehto/1	ESTOLA
	M. suipneumoniae (*M. hyopneumoniae*)	M244-67-1	GOODWIN
		SEP 46-4	Isolated at this laboratory
	M. granularum	#39 P-16	SWITZER
Dog	*M. canis*	PG-14	EDWARD
Rodent	*M. pulmonis*	PG-22	〃
		T	ISHIDA
	M. arthritidis	PG-6	EDWARD
	M. neurolyticum	PG-28	〃
Chicken	*M. gallisepticum*	PG-31	〃
Sewage	*M. laidlawii* A	PG-8	〃

Table 2. Structure of nitrofuran derivatives

NF-137	
NF-463	
AF-2	
NF-161 (Furamizol)	
2Q-4N	
NF-620	
Furazolidone	
Nitrofurazone	

a macrolide group (tylosin, erythromycin, oleandomycin, leucomycin and spiramycin), a peptide group (actinomycin D, mikamycin complex, bottromycin, colistin and polymyxin B), a glycoside group (streptomycin, kanamycin, neomycin and kasugamycin) and a miscellaneous group (puromycin, chloramphenicol, lincomycin, mitomycin C and morimycin-similar to hygromycin A). The nitrofurans tested were NF-137, NF-463, AF-2, 2Q-4N, 2Q-4N (lactate), NF-161, NF-620, furazolidone and nitrofurazone. The chemical structural formulae of these nitrofurans are given in Table 2.

3. Media used

The solid medium was the same as the agar plate described by CHANOCK *et al.*[27], except that the antimicrobial drugs were eliminated from the plate. It consisted of 70 ml of Difco PPLO agar, 20 ml of horse serum, and 10 ml of fresh yeast extract (25 %). Modified GOODWIN's liquid medium was used for *M. suipneumoniae* (*hyopneumoniae*) derived from swine, since this organism is unable to grow in CHANOCK's medium. It consisted of 40 ml of HANKS' solution, 30 ml of HARTLEY's broth, 10 ml of 5 % lactalbumin hydrolysate (NBC), 20 ml of swine serum, and 5 ml of fresh yeast extract (25 %).

4. Assay methods for drugs. Determination of minimum inhibitory concentration (MIC)

Agar dilution method: Each antibiotic was dissolved in sterile distilled water to give a concentration of 2,000 mcg/ml. For water-insoluble antibiotics, a small amount of methanol was added to the water. The nitrofurans were first dissolved in dimethylformamide. Serial dilutions of each drug were added to PPLO broth without crystal violet (Difco). Agar plates were prepared aseptically so that they might contain the final drug concentrations of 50, 25, 10, 5, 2.5, 1, 0.25, 0.13, 0.05, 0.03, 0.01 and 0.005 mcg/ml of agar respectively. The size of inoculum was determined in the following manner. At first, 0.01 ml of $10^{5\sim6}$ CFU*/ml of 3-day-old culture in liquid medium was dropped on each plate with its surface thoroughly dried. All plates were placed in a polyethylene bag with moisture and incubated at 37°C for 5 days. The colonies grown on the plates were observed under a dissecting microscope (Nikon type SMZ). The lowest concentration of a drug that had inhibited the growth of colonies completely was regarded as the MIC of the drug.

Broth dilution method: Each 2 ml of the modified GOODWIN's liquid medium containing 10^5 CCU**/ml of fresh mycoplasma liquid culture was placed in small test tubes. To this was added 0.5 ml of the drug solution at a concentration of 250 mcg/ml prepared in the same way as described in the agar method. Then serial-five fold dilution was added so that the final drug concentration in the medium was reduced from 100 mcg/ml to 0.0064 mcg/ml. The medium was incubated at 37°C for 7 days. During the period of incubation, it was examined daily for change in pH by observing, for comparison, uninoculated and inoculated media containing no drugs. The lowest concentration of a drug inhibiting the color change completely was regarded as the MIC of the drug.

Results

The sensitivities of various mycoplasmas to some antibiotics and nitrofurans, as determined by the agar dilution method, are given in Tables 3 and 4. The sensitivities of the swine strains by the broth dilution method, are listed in Table 5.

Many of the macrolides were found to be effective. The most prominent inhibitory effect was shown on all the strains by tylosin, bottromycin, tetracycline, spiramycin and lincomycin. The anti-tumor antibiotics (actinomycin D and mitomycin C) demonstrated the strongest growth inhibition of all strains at a concentration below 0.25 mcg/ml.

* Colony-forming units ** Color-changing units

Table 3. Sensitivity of various mycoplasmas to 22 antibiotics (Agar dilution method)

	Drug	Minimum inhibitory concentration (mcg/ml)													
		0.005	0.01	0.03	0.05	0.13	0.25	0.5	1	2.5	5	10	25	50	>50
Tetracyclines	Tetracycline					1, 3		9	2		4, 5, 6, 7, 8, 10, 12		11		
	Chlortetracycline										1, 9, 10	3	2, 4, 6, 7	5, 8, 11, 12	
	Methacycline						1, 9		3, 4, 10		2, 5, 6, 7, 8, 11, 12				
Macrolides	Tylosinn		1		10	2, 6, 7	4, 9, 12	11	5		3, 8				
	Erythromycin			1, 4, 10, 12	2	11									3, 5, 6, 7, 8, 9
	Oleandomycin						1,2,10,12	4	11						3, 5, 6, 7, 8, 9
	Spiramycin					10	1	3, 8,9, 12	2, 4	5, 11	6, 7				
	Leucomycin					10	1		2, 4, 8, 9	3	11, 12	5, 6, 7			
Peptides	Actinomycin D			1, 3, 4, 5, 6,7,9,10	8, 11, 12	2									
	Mikamycin complex							1, 3, 10	9		2, 4, 6, 7, 11, 12	5, 8			
	Bottromycin	1, 10		9	2, 5			3, 6, 7	8		4, 11, 12				
	Colistin														1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12
	Polymyxin B														1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12
Glycosides	Streptomycin									12	1, 4		10	2, 5, 11	3, 6, 7, 8, 9
	Kanamycin								3, 5		7, 8, 9	6	1, 4	2, 11, 12	10
	Neomycin											3, 5, 7, 8	4,6,9, 11, 12	1	2, 10
	Kasugamycin													3, 9	1, 2, 4, 5, 6, 7, 8, 10, 11, 12
Others	Puromycin								1, 2, 10	3,4,5,6, 7, 8, 9, 12	11				
	Chloramphenicol								1		2, 3, 4, 5, 6, 7, 8, 9, 10, 12		11		
	Lincomycin						3, 12	1,4, 9, 11	2, 8	5, 6, 7	10				
	Mitomycin C			1, 4, 8, 10, 12	2, 7, 9	3, 6, 11	5								
	Morimycin								5,7,10,11,12	3, 4, 6, 9	1	2, 8			

1. *M. mycoides* var. *mycoides*
2. *M. mycoides* var. *capri*
3. *M, hyorhinis*
4. *M. granularum*
5. *M. canis*
6. *M. pulmonis* (PG-22)
7. *M. pulmonis* (T)
8. *M. arthritidis*
9. *M. neurolyticum*
10. *M. gallissepticum*
11. *M. laidlawii* A
12. *M. laidlawii* B

Table 4. Sensitivity of various mycoplasmas to 9 nitrofurans
(Agar dilution method)

	Minimum innibitory concentration mcg/ml												
	0.005	0.01	0.02	0.05	0.1	0.25	0.5	1	2	5	10	20	>20
NF-137		2					1	4, 6, 7	3, 10	9		8	5, 11, 12
NF-463				2			1, 7	4, 6	10	3		9	5, 8, 11, 12
AF-2		2			1		3, 4, 6, 7, 9, 10, 12		8	5, 11			
2Q-4N		3, 6, 10, 12	2, 7	1, 4, 5, 9, 11			8						
2Q-4N Lactate		3, 6	1,2, 4, 7, 10, 12	5, 9, 11			8						
Furamizol NF-161		1	4, 6, 7	2, 3, 10	9	12	5, 8, 11						
NF-620		3, 6, 7	10	1, 4, 12	9	2, 11		5	8				
Furazolidone									1	3	2, 6, 7, 9, 10		4, 5, 8, 11, 12
Nitrofurazone								2			1, 7	3,6, 9, 10	4, 5, 8, 11, 12

Table 5. Sensitivity of swine mycoplasmas to antibiotics and nitrofurans
(Broth dilution method)

Drug		Minimum inhibitory concentration (mcg/ml)						
		0.0064	0.032	0.16	0.8	4	20	>100
Tetracyclines	Tetracycline			A, C	B, D	E		
	Chlortetracycline					A, B, C	D, E	
	Methacycline			A	B, C, D	E		
Macrolides	Tylosin			A, C, D, E	B			
	Erythromycin			E			A	B, C, D
	Leucomycin				A, B, C, D	E		
	Spiramycin			A	B, C, D, E			
Peptides	Actinomycin D	C, D	E	A, B				
	Mikamycin complex			C, D	A, B	E		
	Bottromycin					A, B, C, D	E	
Glycosides	Streptomycin						E	A, B, C, D
	Kanamycin				A, B		C, D, E	
	Neomycin					B	A, C, D, E	
Others	Puromycin				A, B, C, D	E		
	Chloramphenicol					A, B, C, D	E	
	Mitomycin C	B, C, D	A	E				
	Morimycin				A, B	C, D, E		
Nitrofurans	Furamizol (NF-161)		A, B, C, D		E			
	NF-620	A, B, C, D			E			

A: *M. hyorhinis* (W.P. SWITZER). B: *M. hyorhinis* (T. ESTOLA). C: *M. suipneumoniae* (*hyopneumoniae*) (R.F.W. GOODWIN). D: *M. suipneumoniae* (*hyopneumoniae*) SEP 46-4. E: *M. granularum*

Table 6. Comparison of broth dilution method and agar dilution method

Drug		*M. hyorhinis*		*M. granularum*	
		Broth dilution	Agar dilution	Broth dilution	Agar dilution
Tetracyclines	Tetracycline	0.16*	0.2	4	5
	Chlortetracycline	4	10	20	25
	Methacycline	0.16	1	4	1
Macrolides	Tylosin	0.16	2	0.16	0.2
	Erythromycin	20	50	0.16	0.02
	Leucomycin	0.8	2	4	1
	Spiramycin	0.16	0.5	0.8	1
Peptides	Actinomycin D	0.16	0.02	0.032	0.02
	Mikamycin complex	0.8	0.5	4	5
	Bottromycin	4	0.5	20	5
Glycosides	Streptomycin	100	50	20	5
	Kanamycin	0.8	1	20	25
	Neomycin	20	10	20	25
Others	Puromycin	0.8	2	4	2
	Chloramphenicol	4	5	20	5
	Lincomycin	0.8	0.2	0.8	0.5
	Mitomycin C	0.032	0.1	0.16	0.02
	Morimycin	0.8	2	4	2

* Minimum inhibitory concentration (mcg/ml) after 7 days

Sensitivity to erythromycin varied widely among the mycoplasma species studied. There was over 1,000-fold variation between the lowest and highest concentrations which permitted the organisms to grow. A similar tendency was noted with oleandomycin. Sensitivities of the mycoplasmas to the other drugs did not vary as much.

Of the nitrofurans tested, 2Q-4N, NF-161 and NF-620 inhibited growth at a concentration ranging from 0.01 to 0.5 mcg/ml—far lower than those of commonly used nitrofurans.

For *M. hyorhinis* and *M. granularum*, sensitivity values determined by the agar dilution method were compared with those by the broth dilution method. The results obtained are presented in Table 6. Despite the difference in composition of the medium and procedure between the two methods, the MIC largely agreed, within five-fold variation in approximately 90% of the specimens estimated by the two methods.

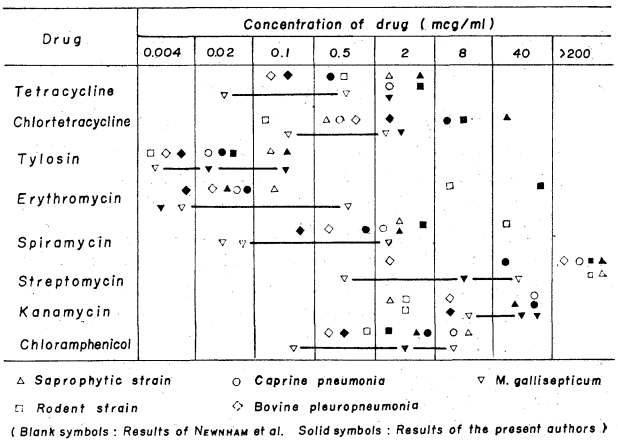
Discussion

To evaluate the *in vitro* activity of a drug against avian mycoplasmas, Zolli *et al.*[28] grew the organisms in broth culture and color change produced by acid formation was taken as indicator of end-point of growth inhibition. Methods in which glucose is used instead of maltose are widely used, but they cannot be applied to mycoplasmas not fermenting glucose. Godzeski and his co-workers[29] devised a rapid sensitivity test in which resazurin was used and determination was made in 18~24 hours. Their method seems to be in the experimental stage and it is not yet commonly used. The present authors adopted the agar dilution method to sensitivity test of a large number of species

of mycoplasmas under almost similar conditions.

The results obtained in these studies were compared with those of NEWNHAM *et al.*[14] As seen in Table 7, in spite of the differences in the strains, composition of the culture media and procedures, there was no large difference in results between the present and the previous studies. Chlortetracycline in this study, however, appeared to be the least active in contrast to the findings of NEWNHAM *et al.* The MIC value of streptomycin was 2 mcg/ml against *M. mycoides* var. *mycoides*, while it was 1,000 mcg/ml against NEWNHAM's strains. The former value was closer to those of OMURA *et al.* (10 mcg/ml)[19] and TURNER (7.8 mcg/ml)[17].

Table 7. *In vitro* sensitivity of mammalian, avian and saprophytic mycoplasmas to drugs (comparative data of NEWNHAM *et al.* and the present authors)



The great variabilities in sensitivities to erythromycin among the species of mycoplasma have long been noted in human strains and tissue culture contaminant strains.[7,14,21]. Of the species tested in the present experiments, all the species of murine origin (*M. pulmonis*, *M. arthritidis*, and *M. neurolyticum*), two of swine origin (*M. suipneumoniae* and *M. hyorhinis*) and one of canine origin (*M. canis*) were nonsensitive. Oleandomycin gave similar results to erythromycin. Such differences in sensitivity among the species of mycoplasma may be due to the differences in the mechanism of transport of drugs into the microbial cells or in the site of action in the inhibition of protein synthesis. Clarification of the inhibition mechanism must await further studies.

The anti-tumor antibiotics (actinomycin D and mitomycin C) were highly active against all the mycoplama species tested. Inhibition of growth of mycoplasmas by anti-tumor antibiotics was reported previously by KATAGIRI *et al.*[30] and ARAI *et al.*[5] This is of interesting from the viewpoint of relationship between mycoplasmas and mammalian cells.

M. granularum was found to grow on a medium devoid of serum fractions containing cholesterol and to produce carotenoid pigments in essentially the same manner as *M. laidlawii*[31]. Recently, it has been proposed that a second genus and a new family be re-established within the order Mycoplasmatales for those species of sterol-nonrequiring mycoplasmas[32~33]. In the present work, two strains of *M. laidlawii* and one strain of *M. granularum* were susceptible to each drug within a narrow range of concentration of the drug. There were no differences between sensitivities of the sterol-nonrequiring mycoplasmas and the sterol-requiring mycoplasmas to the drugs tested.

Comparatively numerous reports[14,15,34] have been published on the sensitivities of the avian strains of mycoplasma to nitrofurans, but few studies deal with mammalian strains. While those reports have given the values of such sensitivity ranging from 0.5 to 50 mcg/ml, the present observations indicate that 2Q-4N, NF-161 and NF-620 have an inhibitory effect at concentrations below 0.5 mcg/ml. It is expected that an *in vivo* activity tests will be of considerable significance.

Acknowledgements

The authors wish to express their gratitude to Dr. N. TANAKA of the Institute of Applied Microbiology, University of Tokyo, for many antibiotics used in the present experiments, and to Dr. S. WATANABE of the Laboratory of the Ueno Pharmaceutical Co., Ltd., for the nitrofurans.

This report was presented in a summarized form to the 67 th Meeting of the Japanese Society of Veterinary Science in 1969.

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