

## In Vitro Antimicrobial Activity against Reference Strains and Field Isolates of *Treponema hyodysenteriae*

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**The in vitro susceptibilities of eight isolates of *Treponema hyodysenteriae* from pigs naturally infected with swine dysentery between 1976 and 1983 were determined by an agar dilution technique. Carbadox, olaquinox, tiamulin, metronidazole, furazolidone, and monensin were the most active against these field isolates regardless of the year of recovery. The influence of inoculum size on the MICs against four reference strains of *T. hyodysenteriae* was studied. Various degrees of activities of ampicillin and lincomycin were found, depending on the inoculum size. The effect of successive in vitro subcultures on the susceptibility of a reference strain of *T. hyodysenteriae* was examined. The strain resistant to tylosin became susceptible to the drug.**

*Treponema hyodysenteriae* is considered the primary causative agent of swine dysentery, a disease characterized by severe bloody diarrhea (8, 24). Determination of the susceptibility of *T. hyodysenteriae* to antimicrobial agents is of great importance in the selective use of chemotherapeutics, the evaluation of new antimicrobial agents, and the development of drug resistance through continuous use of antimicrobial agents against field isolates (10, 15, 18, 26, 27). A commonly used method for in vitro susceptibility testing of the organism is an agar dilution technique (15, 27). However, the MICs are sometimes markedly influenced by the test conditions (4, 23). Inoculum size would be a key factor affecting the amount of antimicrobial agent required to inhibit the organism in vitro, because a large inoculum is required for the propagation of *T. hyodysenteriae* (13). Because the reference strain, as an indicator strain for MIC determination, should usually be maintained in vitro, successive subcultures in vitro may also affect its MICs since successive subcultures often cause decreased pathogenicity in vivo (12).

The purpose of the present study was to determine the in vitro activities of antimicrobial agents currently in medicinal and veterinary use and to investigate the influence of inoculum size and the effect of in vitro subcultures on the susceptibility of *T. hyodysenteriae*.

### MATERIALS AND METHODS

***T. hyodysenteriae* strains.** Four reference strains of *T. hyodysenteriae* (S73/2 [from D. J. Taylor, United Kingdom] [25], ATCC 31212 and ATCC 27164 [from D. L. Harris, United States] [14], and DJ70 [M. Kashiwazaki, Japan] [1]) were used to study the influence of inoculum size and the effect of successive subcultures on in vitro susceptibilities. Eight field isolates were examined for the activities of antimicrobial agents. These isolates were individually isolated from mucosal scrapings of colons or diarrheal feces with mucus and blood from pigs naturally infected with swine dysentery at different localities in Japan. Four isolates were recovered during sporadic outbreaks from 1976 to

1979, and four others were recovered from 1980 to 1983. They were maintained in vitro in our laboratory. Eight field isolates underwent less than 10 successive subcultures; S73/2, ATCC 31212, and ATCC 27164 underwent less than 50 successive subcultures; and DJ70 underwent both less than 50 and more than 150 successive subcultures. Prophylactic and therapeutic regimens for swine dysentery are unknown.

**Media.** An anaerobic diluent (11) or saline was used for diluting mucosal scrapings or feces. As a selective medium, Trypticase soy agar (TSA) (BBL Microbiology Systems, Cockeysville, Md.) containing 400 µg of spectinomycin hydrochloride (supplied by Upjohn Japan Co., Tokyo, Japan) or 200 U of polymyxin B sulfate (supplied by Taito Pfizer Co., Tokyo, Japan) or a combination of 200 µg of spectinomycin hydrochloride, 25 µg of vancomycin hydrochloride (purchased from Sigma Chemical Japan Co., Tokyo, Japan), and 25 µg of colistin sulfate (supplied by Kokin Chemical Co., Tokyo, Japan) per ml supplemented with 5% defibrinated sheep blood was used for the isolation of *T. hyodysenteriae* from infected materials. These were prepared by methods described previously (9, 11, 22). TSA medium without any antimicrobial agent was used for maintaining pure cultures of the treponemes and for antimicrobial susceptibility tests.

**Antimicrobial agents.** A total of 15 antimicrobial agents were examined in this study, and their sources were as follows: ampicillin, ceftizoxime, chloramphenicol, and thiopeptin, Fujisawa Pharmaceutical Co., Osaka, Japan; carbadox, Taito Pfizer Co., Tokyo, Japan; furazolidone, Kokin Chemical Co., Tokyo, Japan; gentamicin, lincomycin, and metronidazole, Sigma Chemical Japan Co., Tokyo, Japan; monensin sodium and oxytetracycline hydrochloride, Takeda Chemical Industries, Osaka, Japan; olaquinox, Toyo Jozo Co., Tokyo, Japan; tiamulin hydrogen fumarate, Sankyo Co., Tokyo, Japan; tylosin tartrate, National Veterinary Assay Laboratories (Japan), Tokyo, Japan; and virginiamycin, Nihon Zenyaku Co., Fukushima, Japan. Solutions containing 1,000 µg of active ingredient of each antimicrobial agent per ml were prepared fresh.

**Anaerobic systems.** For agar cultures, an anaerobic atmosphere of approximately 80% hydrogen and 20% carbon

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TABLE 1. MICs of 15 antimicrobial agents for eight field isolates of *T. hyodysenteriae* from 1976 to 1983

Antimicrobial agent	MIC ( $\mu\text{g/ml}$ ) for field isolates from:	
	1976-1979	1980-1983
Carbadox	$\leq 0.00625$	$\leq 0.00625$
Olaquinox	0.05-0.20	0.025-0.10
Tiamulin	0.025-0.05	0.025-0.05
Monensin	0.05-0.10	0.05-0.10
Metronidazole	0.10-0.39	0.05-0.20
Furazolidone	0.10	0.025
Ampicillin	0.78	0.39-0.78
Ceftizoxime	1.56	0.10-1.56
Chloramphenicol	1.56	1.56
Oxytetracycline	1.56-12.5	1.56-6.25
Gentamicin	3.13-6.25	1.56-6.25
Virginiamycin	0.78-3.13	0.39-1.56
Lincomycin	3.13-25	3.13-6.25
Thiopeptin	12.5-25	12.5-25
Tylosin	3.13->100	100->100

dioxide was generated by evacuation and refilling of vented GasPak jars (BBL) with cold palladium catalysts.

**Isolation and identification of treponemes.** To isolate the treponemes, infected materials were streaked onto the TSA selective medium. Plates were incubated anaerobically at 37 or 42°C for 3 to 6 days. Organisms were transferred from the selective medium by removing a few small plugs of beta-hemolytic areas with a loop (3 mm in diameter) and by streaking this material across TSA medium. The isolated organisms were identified as *T. hyodysenteriae* after phase-contrast microscopic observations for morphology and hemolytic tests, as described previously (15). Pure cultures of isolates were maintained routinely by transferring them to non-antibiotic-containing TSA medium.

**MIC determination.** The MICs of 15 antimicrobial agents were determined by agar dilution methods described previously (15).

The influence of three inoculum sizes was tested, with the

small inoculum ranging from  $10^4$  to  $10^5$  CFU/ml, the medium inoculum ranging from  $10^6$  to  $10^7$  CFU/ml, and the large inoculum ranging from  $10^8$  to  $10^9$  CFU/ml. Each MIC presented is the median value of five experiments.

## RESULTS

The ranges of MICs against eight field isolates of *T. hyodysenteriae* are shown in Table 1. Of 15 antimicrobial agents, carbadox was the most active against the organisms, inhibiting all eight isolates at  $\leq 0.00625$   $\mu\text{g/ml}$ . Olaquinox, tiamulin, monensin, metronidazole, and furazolidone were active, with MICs ranging from 0.025 to 0.20  $\mu\text{g/ml}$ .

The MICs for the four isolates obtained from 1976 to 1979 were almost the same as those for the four isolates obtained from 1980 to 1983.

Each MIC with large, medium, and small inocula is presented in Tables 2 and 3. Regardless of the inoculum size, carbadox, monensin, and gentamicin exhibited the same MICs. But the MICs of ampicillin and lincomycin varied depending on the inoculum size (small,  $10^4$  to  $10^5$  CFU/ml; large,  $10^8$  to  $10^9$  CFU/ml).

In comparing MICs for strains which underwent less than 50 successive subcultures and those for strains which underwent more than 150 successive subcultures, the activities of all antimicrobial agents against *T. hyodysenteriae* DJ70 were very similar except for tylosin. Strain DJ70, which was resistant to tylosin, changed its susceptibility after more than 150 successive subcultures in vitro.

## DISCUSSION

The results of earlier workers have shown that nitro compounds such as quinoxaline, nitroimidazole, and nitrofurans had strong in vitro activities against *T. hyodysenteriae* (10, 15, 18, 26, 27). It has also been shown that pleuromutilin (16) and polyether (17) are active against *T. hyodysenteriae*. These results are in agreement with the present findings. Our results indicated that the above-mentioned antimicrobial

TABLE 2. Influence of inoculum size and effect of in vitro subcultures on MICs for *T. hyodysenteriae*

Antimicrobial agent	MIC ( $\mu\text{g/ml}$ ) for S73/2 <sup>a</sup>			L/S <sup>b</sup>	MIC ( $\mu\text{g/ml}$ ) for DJ70 <sup>c</sup>				L/S <sup>d</sup>
	L	M	S		L	M <sub>1</sub>	M	S	
Carbadox	$\leq 0.00625$	$\leq 0.00625$	$\leq 0.00625$	1	$\leq 0.00625$	$\leq 0.10$	$\leq 0.00625$	$\leq 0.00156$	4
Olaquinox	0.20	0.05	0.025	8	0.05	$\leq 0.10$	0.05	0.025	2
Tiamulin	0.05	0.05	0.025	2	0.025	$\leq 0.10$	0.025	0.0125	2
Monensin	0.10	0.10	0.05	2	0.10	$\leq 0.10$	0.10	0.05	2
Metronidazole	0.78	0.39	0.10	4	0.78	$\leq 0.10$	0.39	0.20	4
Furazolidone	0.10	0.05	0.025	4	0.20	$\leq 0.10$	0.10	0.05	4
Ampicillin	3.13	1.56	0.20	16	1.56	0.78	0.78	0.20	8
Ceftizoxime	3.13	1.56	0.39	8	3.13	ND <sup>e</sup>	1.56	0.39	8
Chloramphenicol	1.56	1.56	0.78	2	1.56	1.56	1.56	0.78	2
Oxytetracycline	1.56	0.78	0.39	4	12.5	6.25	6.25	3.13	4
Gentamicin	6.25	6.25	6.25	1	6.25	6.25	6.25	3.13	2
Virginiamycin	3.13	1.56	1.56	2	1.56	0.78	0.78	0.78	2
Thiopeptin	25	25	12.5	2	25	12.5	25	12.5	2
Lincomycin	0.39	0.20	0.10	4	50	12.5	12.5	3.13	16
Tylosin	6.25	1.56	1.56	4	12.5	>100	3.13	1.56	8

<sup>a</sup> Successive in vitro subcultures with less than 50 serial passages. L, large inoculum; M, medium inoculum; S, small inoculum.

<sup>b</sup> Ratio of large-inoculum MIC to small-inoculum MIC for strain S73/2.

<sup>c</sup> Successive in vitro subcultures with more than 150 serial passages, except for M<sub>1</sub>. With this inoculum, the strain was successively subcultured in vitro with less than 50 serial passages.

<sup>d</sup> Ratio of large-inoculum MIC to small-inoculum MIC for strain DJ70.

<sup>e</sup> ND, Not done.

TABLE 3. Influence of inoculum size on MICs for *T. hyodysenteriae*<sup>a</sup>

Antimicrobial agent	MIC ( $\mu\text{g/ml}$ ) for ATCC 31212			L/S <sup>b</sup>	MIC ( $\mu\text{g/ml}$ ) for ATCC 27164			L/S <sup>c</sup>
	L	M	S		L	M	S	
Carbadox	$\leq 0.00625$	$\leq 0.00625$	$\leq 0.00625$	1	$\leq 0.00625$	$\leq 0.00625$	$\leq 0.00625$	1
Olaquinox	0.10	0.10	0.05	2	0.10	0.05	0.05	2
Tiamulin	0.05	0.025	0.0125	4	0.025	0.0125	0.0125	2
Monensin	0.10	0.10	0.10	1	0.10	0.10	0.10	1
Metronidazole	0.10	0.05	0.025	4	0.20	0.10	0.05	4
Furazolidone	0.10	0.025	0.0125	8	0.05	0.025	0.0125	4
Ampicillin	1.56	0.39	0.20	8	3.13	0.78	0.39	8
Ceftizoxime	3.13	1.56	0.39	8	3.13	1.56	0.78	4
Chloramphenicol	1.56	1.56	0.78	2	3.13	1.56	0.78	4
Oxytetracycline	6.25	3.13	3.13	2	1.56	0.78	0.39	4
Gentamicin	3.13	3.13	3.13	1	3.13	3.13	6.25	0.5
Virginiamycin	1.56	1.56	0.78	2	1.56	1.56	0.78	2
Thiopeptin	25	25	12.5	2	25	25	12.5	2
Lincomycin	50	12.5	1.56	32	0.39	0.20	0.10	4
Tylosin	>100	>100	100	>1	12.5	3.13	1.56	8

<sup>a</sup> For successive in vitro subcultures with less than 50 passages. L, large inoculum; M, medium inoculum; S, small inoculum.

<sup>b</sup> Ratio of large-inoculum MIC to small-inoculum MIC for strain ATCC 31212.

<sup>c</sup> Ratio of large-inoculum MIC to small-inoculum MIC for strain ATCC 27164.

agents were still active in vitro against field isolates regardless of the period from which they were recovered (1976 to 1979 or 1980 to 1983).

In susceptibility testing by agar dilution, the influence of inoculum size was examined. It was known that the influence of inoculum size varies with different antibiotics (4). This was seen in the results of experiments testing ampicillin against *Staphylococcus aureus* (21) and cephalosporins against members of the family *Enterobacteriaceae* (5). Susceptibility has been shown to depend on inoculum size and not on the  $\beta$ -lactamase activity of the organism against ampicillin (2). The present study showed that the activities of ampicillin and lincomycin varied, depending on inoculum size.

There are several reports of the effectiveness of lincomycin in vivo in the therapy of swine dysentery (6, 7, 20). The in vitro activity of lincomycin was, however, somewhat less than that of carbadox, which was by far the most effective of all antimicrobial agents. The present findings indicated that with a small inoculum, ampicillin and lincomycin were active against all four reference strains of *T. hyodysenteriae*. However, the activities of tylosin and lincomycin were poor with a large inoculum exposed for a short time to these antibiotics but better with a small inoculum exposed for a long period (18). These results suggest that a careful medication regimen should be started from the onset of dysentery.

*T. hyodysenteriae* DJ70, isolated from a pig naturally affected with swine dysentery, was originally resistant to tylosin, but after more than 150 serial passages in vitro, it became susceptible to the antibiotic. Reports indicate that with prolonged in vitro cultivation (over 150 subcultures), attenuation and loss of infectivity occur (12). The drug resistance of *T. hyodysenteriae* is reported to be mediated by R plasmids (19). In other bacteria, members of the family *Enterobacteriaceae*, for example, R-plasmid drug resistance is eliminated by serial passage in vitro (3). These findings emphasize the importance of assessing MICs with fresh isolates to determine the precise medication for swine dysentery.

The present findings indicated that, in selecting a thera-

peutic agent and determining MICs, the influence of inoculum size needs to be considered.

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