HYGROMYCIN A, AN ANTITREPONEMAL SUBSTANCE

I. SCREENING METHOD AND THERAPEUTIC EFFECT FOR *TREPONEMA HYODYSENTERIAE*-CAUSED INFECTION IN CF-1 MICE

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In vitro and *in vivo* screening methods were established for the discovery of new active substances against *Treponema hyodysenteriae*. During the screening methods, hygromycin A produced by *Streptomyces hygroscopicus* KA-355 was found to be active against *T. hyodysenteriae*. Hygromycin A did not show high antitreponemal activity in *in vitro* test using the paper disc method on the agar plate inoculated with *T. hyodysenteriae*. However, the antibiotic exhibited highly therapeutic effect in CF-1 mice, compared with of lincomycin, tiamulin, lankacidin C or olaquindox drinking water. The effective dose (ED_{50}) of hygromycin A was 1.1 μg/ml.

Swine dysentery (SD) is a severe muco-hemorrhagic diarrheal disease that primarily affects pigs during the growing-finishing period. An anaerbic spirochete, *Treponema hyodysenteriae* is considered to the primary etiologic agent of SD.[1~4] Some antitreponemal drugs, carbadox,[5,6] lincomycin,[5] tiamulin,[7] olaquindox and lankacidin C (sedecamycin) and others have been used clinically to data. Recently, serious problems such as the appearance of drug-resistant strain of *T. hyodysenteriae* in the field and the side effect of the drugs have arisen.[8,9] Several attempts have been made to produce a model infection with *T. hyodysenteriae* in laboratory animals.[10~12] SUENAGA and YAMAZAKI[13] have reported that CF-1 mouse shows the highest susceptibility to *T. hyodysenteriae*. Further, JONES *et al.*[10] reported that cecal lesions similar to SD developed in CF-1 mice inoculated orally with *T. hyodysenteriae*. In order to find and evaluate a more effective drug of microbial origin, we established the primary *in vitro* and *in vivo* screening programs. As a result, we found that hygromycin A[14] produced by a *Streptomyces hygroscopicus* showed high therapeutic effect against *T. hyodysenteriae-in vivo* test using CF-1 mice. In this paper, we wish to describe *in vitro* screening method, the susceptibility of *T. hyodysenteriae* to some antibiotics, the susceptibility of several mouse strains to *T. hyodysenteriae* and the therapeutic effect of hygromycin A and other drugs such as lincomycin, lankacidin C, carbadox and olaquindox for swine dysentery using CF-1 mice.

Materials and Methods

In Vitro Screening

T. hyodysenteriae Strain and the Culture Medium

T. hyodysenteriae B-78 (ATCC 27164) and DJ70 were provided by Dr. M. KASHIWAZAKI (National Institute of Animal Health) and were used as the test organism. Brain heart infusion broth (Difco) supplemented with 2% fetal calf serum (BHI) was used for cultivation the organisms, and Trypticase soy agar (BBL) supplemented with 5% defibrinated sheep blood (TSA) was used for assay of antitreponemal activity. The organisms were cultivated anaerobically in BHI and TSA.

Assay and Screening Method of Antitreponemal Activity

The *in vitro* antimicrobial activity against *T. hyodysenteriae* B-78 was assayed by the paper disc method. An agar plate was prepared as follows: The cell of a stock culture of *T. hyodysenteriae* were transferred into 7 ml of BHI and incubated statically at 37°C for 24 hours under anaerobic conditions. TSA was seeded using the culture broth adjusted to 1×10^7 cells/ml of TSA for 24 hours under anaerobic condition after putting the paper discs on the plate.

An agar-diffusion disc assay was used to screen for antitreponemal substances in fermentation broths. A paper disc (diameter; 8 mm) dipped in the broth was applied to an agar plate for the assay. After incubation for 24 hours under anaerobic condition, the resulting hemolytic inhibition zone was measured.

In Vivo Screening

Test Organism

Three strains of *T. hyodysenteriae* DJ70, DJ70 P-3, and B-78 (ATCC 27164) were used for *in vivo* test. Strain DJ70 P-3 was obtained by the passage of a strain of DJ70 at three times through mice.

Mice

Seven kinds of female mice were used for this experiment. Mouse strain was as follows: 5 to 7-week-old CF-1 strain (Charles River Laboratories, Inc., Atsugi, Japan), 5-week-old Jcl: ICR strain (CLEA Japan, Inc., Tokyo, Japan), 5-week-old Slc: BALB/C strain, Slc: C57/6 strain, Slc: C3H/He strain, Slc: DBA/2 strain, SLC-CDF1 strain (Shizuoka Laboratory Animal Center, Hamamatsu, Japan). Mice were housed in cages (five per cage). The bedding (wood shavings) was not changed after inoculation of *T. hyodysenteriae*. Mice were fed a pellet ration *ad libitum*, except during the fasting period.

T. hyodysenteriae Culture Preparation

For the susceptibility test, *T. hyodysenteriae* DJ70 was cultured anaerobically on Trypticase soy agar (BBL) plate supplemented with 10% defibrinated sheep blood (TSA) at 37°C for 3 days. The cell suspension of *T. hyodysenteriae* DJ70 was collected by pouring Trypticase soy broth (BBL) on the plate. *T. hyodysenteriae* B-78 was cultured anaerobically in brain heart infusion broth (Difco) supplemented with 2% calf serum at 37°C for 24 hours. For therapeutic testing, *T. hyodysenteriae* DJ70 P-3 was cultured in brain heart infusion broth (Difco) supplemented with 2% calf serum at 37°C for 24 hours.

Experimental Procedure

1) Mice Susceptibility Test: Mice were inoculated at one time (and/or two times) orally with 1 ml of the prepared culture of *T. hyodysenteriae* DJ70 or B-78 after 24 or 48 hours fasting period. At 10 days post-inoculation, post mortem examination was carried out.

2) Therapeutic Effect by Oral Administration: Method 1; mice were inoculated orally with 1 ml of the prepared culture of *T. hyodysenteriae* DJ70 P-3 after 24 hours fasting period. The culture contained approximately 1×10^8 cfu/ml. The test drugs were orally administered to mice at 2 hours post-inoculation. A feed was given to mice again. Drug administration was once a day for 4 days. At 7 days post-inoculation, post mortem examination was carried out.

Method 2; mice were inoculated orally with 1 ml of the prepared culture of *T. hyodysenteriae* DJ70 P-3 after 24 and 48 hours fasting period. The culture contained approximately 1×10^8 cfu/ml. The test drugs were orally administered to mice at 2 hours post-inoculation. A feed was given to mice again. Drug administration was once a day for 6 days. At 7 days post-inoculation, post mortem examination was carried out.

3) Therapeutic Test in Drinking Water Adminstration: At the same time as fasting, medicated water was given to mice. After 24 and 48 hours fasting period, mice were inoculated orally with 1 ml of the prepared culture *T. hyodysenteriae* DJ70 P-3. The culture contained approximately 1×10^8 cfu/ml. At 2 hours second inoculation, feed was given to mice again. At 5 days post-inoculation, post mortem examination was carried out.

Post Mortem Examination

The ceca were examined for signs of inflammation and scored as either positive and negative. Cecal contents were cultured anaerobically at 37°C on a selective media for *T. hyodysenteriae* (on TSA plate supplemented with 10% sheep blood, 400 μg/ml spectinomycin, 50 μg/ml vancomycin, 50 μg/ml colistin) to detect *T. hyodysenteriae*.

Results

Identification of KA-355

The antitreponemal substance produced by a strain KA-355 was identified with hygromycin A[14] from the physico-chemical properties. The producing strain was also identified as a *Streptomyces hygroscopicus*.

The Susceptibility of *T. hyodysenteriae* B-78 to Some Antibiotics

The susceptibility of *T. hyodysenteriae* to several antibiotics was tested using TSA and a paper disc. As shown in Table 1, benzylpenicillin, chloramphenicol, kitasamycin, tylosin, lincomycin, clindamycin, tiamulin (dynamutilin) and lankacidin C showed high activity at 5 μg against *T. hyodysenteriae* B-78. However, nucleosides, polyenes, peptides and aminoglycosides except for gentamicin did not exhibit acivity at this concentration. KITAI *et al.*[15] and KINYON and HARRIES[8] have reported the antitreponemal activity of antibiotics against isolates of *T. hyodysenteriae* by the agar dilution technique. Although small differences in susceptibility patterns of agents against *T. hyodysenteriae* were observed, both assay systems yield similar results.

Table 1. Susceptibility of *Treponema hyodysenteriae* to antimicrobial agents.

Antimicrobial agents[a]	Inhibition zone[b]	Antimicrobial agents[a]	Inhibition zone[b]
Benzylpenicillin	+++	Blasticidin S	−
Sulbenicillin	+	Ezomycin	−
Cefazolin	−	Bacitracin	−
Chloramphenicol	+++	Cycloserine	−
Tetracycline	+	Gramicidin S	−
Chlortetracycline	−	Amphotericin B	−
Gentamicin	++	Nystatin	−
Kanamycin	−	Lincomycin	+++
Streptomycin	−	Clindamycin	+++
Erythromycin	+	Dynamutilin	+++
Kitasamycin	++	Lankacidin A	+
Oleandomycin	−	Lankacidin C	++
Spiramycin	−	Novobiocin	−
Tylosin	++	Vancomycin	−

[a] The amount of the antibiotic on the disc is 5 μg.

[b] Inhibition zone: +++; 30~40 mm, ++; 20~30 mm, +; 20~10 mm.

Antitreponemal Activity of Hygromycin A

Hygromycin A exhibited weakly antimicrobial activity against Gram-positive and Gram-negative bacteria. Hygromycin A shows a characteristic antimicrobial activity against anaerobic bacteria and mycoplasma. The antitreponemal activity (MIC) against *T. hyodysenteriae* B-78 and DJ70 was 6.24 and 3.12 μg/ml, respectively.

The Susceptibility of Mice to *T. hyodysenteriae*

As shown in Table 2, CF-1 mice showed high susceptibility to *T. hyodysenteriae*, but the other strains of mice were not susceptible. CF-1 mice were optimally infected using the inoculum size of 10^8+10^8 (or 10^8) cfu of *T. hyodysenteriae* DJ70 P-3. However, the inoculation at 10^8+10^8 cfu of *T. hyodysenteriae* DJ70 and B-78 showed infection ranging from 2/5 to 3/5. From the result in the inoculation with 10^8+10^8 cfu of each strain (separate challenges), a strain DJ70 seems to be more pathogenic than B-78. CF-1 mice inoculated with 10^8+10^8 (or 10^8) cfu of the strain DJ70 P-3 was infected perfectly at 5 or 7 days post-inoculation. Cecal lesions consisted of a shrinkage, edema, hyperemia and large quantities of mucous exudate. Gas was usually present.

Table 2. Comparison of the susceptibility of various mouse strains of *Treponema hyodysenteriae* infection on various mice strains.

Strain of mice	Strain of *T. hyodysenteriae*	No. of inoculated *T. hyodysenteriae* (cfu/mouse) × 2 times	Positive cecal lesion	Isolation of *T. hyodysenteriae*
CF-1	I[a]	10^8	4/4	4/4
		10^8 [d]	2/5	4/5
		10^7 [d]	3/5	5/5
	II[b]	10^8	1/2	2/2
	III[c]	10^8	3/5	3/5
		10^8 [d]	2/5	5/5
		10^7 [d]	3/5	4/5
ICR	I	10^8	0/5	0/5
		10^8 [d]	0/5	0/5
		10^7 [d]	0/5	0/5
	III	10^8	0/5	0/5
C3H/He	I	10^7	0/5	0/5
	II	10^8	0/2	0/5
	III	10^8	0/5	0/5
BALB/C	I	10^7	0/2	0/5
	II	10^8	0/4	0/5
	III	10^8	0/3	0/5
SLC-CDF1	I	10^7	0/4	0/5
	II	10^8	0/1	0/5
	III	10^8	0/3	0/5
DBA/2	I	10^7	0/5	0/5
	II	10^8	0/4	0/5
	III	10^8	0/4	0/5
C57BL/6	I	10^7	0/5	0/5
	II	10^8	0/5	0/5
	III	10^8	0/5	0/5

[a] *T. hyodysenteriae* DJ70.
[b] *T. hyodysenteriae* DJ70 P-3.
[c] *T. hyodysenteriae* B-78.
[d] The mice were inoculated once.

Therepeutic Test by Oral Administration

Method 1

Therapeutic effects of hygromycin A and some drugs by oral administration are shown in Table 3. Cecal lesions were not observed in the mice administered with hygromycin A at 5 mg/kg×4 times, however *T. hyodysenteriae* was detected in cecal contents at the first day. Cecal lesions were observed in the mice treated with hygromycin A at 1 mg/kg×4 times, indicating no therapeutic effect at this dose. On the other hand, lankacidin C, carbadox and olaquindox showed a little therapeutic effect at the dose of 4 mg/kg×4 times.

Method 2

The comparative therapeutic effect of hygromycin A with carbadox by oral administration is shown in Table 4. Although cecal lesions were not observed in the mice administered with hygromycin A at 5 (or 1) mg/kg×6 times, *T. hyodysenteriae* was detected in the cecal contents at third day. The rate of cure by hygromycin A was 40% at each dose. Carbadox was effective at 5 mg/kg×6 times, but not effective at 1 mg/kg×6 times. At the dose of 5 mg/kg×6 times, carbadox was more effective than hygromycin A. As the results, the therapeutic effects of hygromycin A by oral administration seem to be slightly lower than those of carbadox and other drugs.

Table 3. Therapeutic effects of hygromycin A and some antitreponemal compounds by oral administration of experimentally infected mice with *Treponema hyodysenteriae* DJ70 P-3.

Compounds	Dose of treatment (mg/kg)	No. of mice	Cecal[a] lesion	Isolation of *T. hyodysenteriae*[b]		Rate of cure (%)
				1 day[c]	3 days[c]	
Hygromycin A	5	5	0	2	5	0
	1	5	2	4	5	0
Lincomycin	5	5	0	0	1	80
Lankacidin C	5	5	3	4	4	20
Carbadox	5	5	0	0	2	60
Olaquindox	5	5	0	0	3	40
Control	None	5	5	5	5	0

[a] Number of mice showing cecal lesion.
[b] Isolation of *T. hyodysenteriae* by direct plating of cecal material on TSA.
[c] Incubation period.

Table 4. Therapeutic effects of hygromycin A and carbadox by oral administration on experimentally infected mice with *Treponema hyodysenteriae* DJ70 P-3.

Compounds	Dose of treatment (mg/kg)	No. of mice	Cecal[a] lesion	Isolation of *T. hyodysenteriae*[b]		Rate of cure (%)
				1 day[c]	3 days[c]	
Hygromycin A	5	5	0	0	3	40
	1	5	0	0	3	40
Carbadox	5	4	0	0	0	100
	1	5	4	4	5	0
Control	None	4	4	4	4	0

[a] Number of mice showing cecal lesion.
[b] Isolation of *T. hyodysenteriae* by direct plating of cecal material on TSA.
[c] Incubation period.

Table 5. Therapeutic effects of hygromycin A and some antitreponemal compounds in drinking water on experimentally infected mice with *Treponema hyodysenteriae* DJ70 P-3.

Compounds	Dose of treatment (μg/ml)	No. of mice	Cecal[a] lesion	Isolation of *T. hyodysenteriae*[b]		Rate of cure (%)	ED_{50} (μg/ml)
				1 day[c]	3 days[c]		
Hygromycin A	15	5	0	0	0	100	1.1
	3.75	5	0	0	0	100	
	1.86	5	0	0	0	100	
	0.94	5	0	0	3	40	
Lincomycin	60	5	0	0	0	100	13
	30	5	0	0	0	100	
	15	5	0	0	0	60	
	7.5	5	0	0	4	20	
Tiamulin	60	5	0	0	0	100	40
	30	5	0	0	0	40	
	15	5	0	0	4	20	
	7.5	5	3	5	5	0	
Lankacidin C	60	5	0	0	1	80	40.5
	30	5	0	0	2	60	
	15	5	0	0	5	0	
	7.5	5	3	3	5	0	
Olaquindox	60	5	3	3	5	0	>60
	30	5	5	5	5	0	
	15	5	5	5	5	0	
	7.5	5	5	5	5	0	
Control	None	5	5	5	5	0	

[a] Number of mice showing cecal lesion.
[b] Isolation of *T. hyodysenteriae* by direct plating of cecal material on TSA.
[c] Incubation period.

Therapeutic Test in Drinking Water

Therapeutic effects of hygromycin A and other drugs such as lincomycin, tiamulin (dynamutilin), lankacidin C and olaquindox are shown in Table 5. When administered in the drinking water, hygromycin A demonstrated the highest therapeutic effect (ED_{50} value: 1.1 μg/ml) compared with those of lincomycin (ED_{50}: 13 μg/ml), tiamulin (ED_{50}: 40 μg/ml), lankacidin C (ED_{50}: 40.5 μg/ml) and olaquindox (ED_{50}: >60 μg/ml). Comparison of the effect of hygromycin A with carbadox could not be examined because of the insolubility of carbadox in water. During the experimental period, medicated water consumption was about 200 ml per each group and avarage dose of drug administration at each ED value was as follows: Hygromycin A; 0.31 mg/kg/day, lincomycin; 3.71 mg/kg/day, tiamulin; 11.4 mg/kg/day, lankacidin C; 11.5 mg/kg/day, olaquindox; 17.1 mg/kg/day.

Discussion

The *in vitro* activity of hygromycin A against *T. hyodysenteriae* B-78 and DJ70 was less when compared with those of the other drugs such as lincomycin, tiamulin and lankacidin C. It has been reported that hygromycin A is a specific inhibitor of the peptide bond formation step of protein synthesis. GUERRERO and MODOLELL[16] have pointed out that the action of hygromycin A on peptidyl transfer is similar to that of chloramphenicol which shares some common structural features with hygromycin A. Recently, YOSHIDA *et al.*,[17] have isolated hygromycin A and methoxyhygromycin during the screening

for inhibitors of K88 antigen synthesis that suppress hemagglutination by *Escherichia coli*. This suggests that hygromycin A is one of the possible candidates for a chemotherapeutic agent which inhibits bacterial adhesion to host tissues.

In the mice susceptibility test, all mouse strains used in this experiment except for CF-1 mice, did not show the susceptibility for *T. hyodysenteriae*. This suggests that the infection of *T. hyodysenteriae* might be influenced by intestinal flora.[18] In the present data, differences in the composition of intestinal flora among mouse strains might play a role in the susceptibility of the mice to the infection.

Although hygromycin A did not show a therapeutic effect by oral (gavage) administration, it showed high therapeutic effect when administered in the drinking water. Comparing total dose and rate of cure (%) of hygromycin A by two methods of administration, the rate of cure was 100% at the total dose of 3.71 mg/kg in drinking water. On the other hand, the rate of cures were 40 and 9% at the total dose of 30 and 6 mg/kg by oral (gavage) administration, respectively. This indicates that the therapeutic effect in drinking water is superior to that by oral (gavage) administration for hygromycin A. The times of administration rather than the amount of total dose may explain the high therapeutic effect as indicated in drinking water administration. This means that the residence time of hygromycin A in the intestine may be short and the metabolic rate may be fast in the case of compulsory oral administration. These data suggest that hygromycin A in feed or in drinking water seems to be an excellent therapeutic drug for swine dysentery. The therapeutic effects of hygromycin A in swine test will be described in our second paper.[19]

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