Drug Resistance and Transferable R Plasmids in *Edwardsiella tarda* from Fish Culture Ponds

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One hundred sixty eight strains of *Edwardsiella tarda* collected from cultured eel (*Anguilla japonica*), tilapia (*Tilapia nilotica*) and channel catfish (*Ictalurus punctatus*), and from water and plankton of culture ponds were studied for their sensitivities to 10 chemotherapeutic agents: chloramphenicol (CM), tetracycline (TC), streptomycin (SM), kanamycin (KM), aminobenzyl penicillin (ABP), cefazolin (CEZ), nalidixic acid (NA), furazolidone (NF), sulfamonomethoxine (SA) and trimethoprim (TMP). All strains were highly sensitive to CEZ and TMP. Only 32 of 168 strains were sensitive to all the drugs tested. The remaining 136 strains were resistance to various combinations of the 8 drugs (CM, TC, SM, KM, ABP, NA, NF and/or SA). The most common types of drugs resistance were to combinations of CM, TC, NF and/or SA.

Transferable R plasmids were detected in 38 out of 136 resistant strains. The most common type of resistance markers of R plasmid was CM, TC and SA. Other R plasmids had markers for resistance to TC, SA, TC SA and CM TC SM KM SA.

Cryptic plasmids were also detected in all strains *E. tarda* tested by agarose gel electrophoresis.

Eel culture is the most popular of all freshwater fish culture in Japan. The annual production reached about 32,000 tons in 1978. In recently years, eel culture has been carried out in closed systems in warm water ponds enclosed in green houses. Consequently, eel culturists are now able to expect short term culture and high production. They usually keep as many fish as possible in a limited pond space in order to increase productivity per unit space. This naturally causes unfavourable conditions for cultured fish, and incidences of bacterial infections, such as edwardsiellosis have been frequent (WAKABAYASHI and EGUSA, 1973). Under warm water conditions, edwardsiellosis can occur in both adult and young eel throughout the year (MIYAZAKI and EGUSA, 1976). Accordingly, antibiotics and synthetic chemotherapeutics have been used for the treatment of edwardsiellosis. The application of chemotherapeutics to eel ponds have caused the increase of drug resistant strains of *Edwardsiella tarda* and transferable R plasmids have been detected (AOKI *et al.*, 1977). As a result, chemotherapy of edwardsiellosis has become very difficult, and high levels of various chemotherapeutics have been introduced in eel culture.

Edwardsiellosis has been seen not only in cultured eel but also for other cultured freshwater fish; channel catfish (*Ictalurus punctatus*) (MEYER and BULLOCK, 1973), large mouth bass (*Micropterus salmoides*) (WHITE *et al.*, 1973) and tilapia (*Tilapia nilotica*) (unpublished data), as well as for marine fish; mullets (*Mungil cephalus*) (KUSUDA *et al.*, 1976) and sea bream (*Evynnis japonicus*) (KUSUDA *et al.*).

In this study, strains of *E. tarda* collected from fish culture ponds were tested for their susceptibilities to selected drugs. Drug resistant strains detected were further studied for the presence of transferable R plasmids.

CROSA *et al.* (1977) reported that a high virulent phenotype of the bacterial fish pathogen, *Vibrio anguillarum* is correlated with their specific plasmid. We also tried to detect cryptic other than R plasmids in our strains of *E. tarda* by assaying their plasmid DNAs by agarose electrophoresis.

Materials and Methods

One hundred sixty eight strains of *Edwardsiella tarda* were collected from cultured eel, tilapia and channel catfish and from culture pond water and

Table 1. Sources of strains of *Edwardsiella tarda*

Area	Year							Total
	1972	1973	1975	1976	1977	1978	1979	
Shizuoka	29	1		58				88
Aichi							3	3
Tokushima				3	9	21		33
Nagasaki					4		2	6
Ooita						2		2
Kagoshima						4	3	7
Miyazaki			1		2	9	2	14
Okinawa							2	2
Taiwan				2	10			12
USA		1						1
Total	29	2	1	63	25	36	12	168

plankton (Table 1). The strains were tested for their sensitivities to various chemotherapeutic agents. The serial, two-fold dilution method in agar medium recommended by the Japan Society of Chemotherapy (1975) for determination of the minimal inhibitory concentrations (MICs) of various drugs, was followed. The incubation temperature used was 30°C.

The chemotherapeutics used were chloramphenicol (Sankyo) (CM), tetracycline hydrochloride (Lederle) (TC), streptomycin sulphate (Meiji) (SM), kanamycin sulphate (Meiji) (KM), aminobenzyl penicillin (Fujisawa) (ABP), cefazolin (Fujisawa) (CEZ), nalidixic acid (Daiichi) (NA), furazolidone (Takeda) (NF), sulfamonomethoxine (Daiichi) (SA) and trimethoprim (Takeda) (TMP).

Penassay broth (Difco) was used as the medium for pre-culture and Heart infusion agar (Nissui) was used as the medium for sensitivity tests to antibiotics, NA and NF. Sensitivity disc medium (modified Mueller-Hinton agar) (Nissui) was used to test sensitivity to SA and TMP.

Each drug resistant strain of *E. tarda* was incubated in penassay broth with aeration at 30°C to about 5×10^8 cells/ml. *Escherichia coli* RC85 *nal* (nalidixic acid resistant) was incubated in penassay broth with aeration at 37°C to an equal density. One ml of *E. tarda* culture was mixed with 1 ml of *E. coli* RC85 in a test tube, incubated overnight at 37°C, and then plated on BTB-lactose nutrient agar (WATANABE *et al.*, 1971) containing NA and each one of the drugs to which the *E. tarda* strain was resistant. However, NA

resistant strains were mixed with *E. coli* W3102 *str* (streptomycin resistant) instead of RC85. Each of the clones of RC85 which received a drug resistance plasmid was reisolated for purification on a similar selective medium. The purified clones were then tested for their resistance to unselected drugs by their MICs.

Eleven strains were selected at random from the collection of naturally occurring *E. tarda* strains. Each strain was incubated at 30°C in penassay broth to about 5×10^8 cells/ml. Thirty ml of each culture was washed by centrifugation and re-suspended in 0.2 ml of a Tris-sucrose-EDTA buffer. The cell suspension was lysed by adding 20 μ l of a 10% sodium dodecyl sulfate solution, and a clear supernatant was obtained after centrifugation for 20 min at 18,000 rpm at 5°C. Aliquots of 10–40 μ l of supernatant (cleared lysate) of each preparation and standard DNA of Col K (KUNIGATA and MATSUHASHI, 1970) were electrophoresed in 0.8% agarose gels.

Results

The distribution of MICs of 10 chemotherapeutic agents against 168 strains of *E. tarda* are shown in Fig. 1. The strains were classified into apparently discrete groups which were sensitive or resistant to CM as determined by their MICs of about 0.8 μ g/ml and 50 μ g/ml, respectively. The pattern of MICs of TC resistance also gave two groups, 96 sensitive and 72 resistant strains. As for SM, KM, and ABP, the strains were also

Fig. 1. Number of naturally occurring *Edwardsiella tarda* strains with MICs ($\mu\text{g/ml}$).

Abbreviations: CM, chloramphenicol; TC, tetracycline; SM, streptomycin; KM, kanamycin; ABP, aminobenzyl penicillin; CEZ, cefazolin; NA, nalidixic acid; NF, furazolidone; SMM, sulfamonomethoxine; TMP, trimethoprim.

classified into two apparent groups and showed the presence of some resistant strains for these drugs. Interestingly enough, all strains tested were highly sensitive to CEZ.

Twentyfive strains were found to be resistant to NA. Only 43 of 168 strains were sensitive to NF. As for SA, the strains also fell into two groups, 82 strains were resistant to SA. The MIC of TMP against the strains was from 0.025 to 1.6 $\mu\text{g/ml}$. All strains were highly sensitive to TMP.

Thirtytwo of 168 strains were found to be sensitive to all drugs tested. While the remaining 136 strains were resistance to various combinations of the 8 drugs (Table 2). Of the 136 strains, 45 were

Table 2. Resistance markers and transferable R plasmids of *Edwardsiella tarda* isolated from freshwater fish culture ponds

Resistance marker of original strain	R ⁺ strains/Strains studied	Resistance marker of R plasmid
Sensitive strains	32	
NA ^a	0/1	
NF	0/40	
SA	0/2	
TC NF	0/3	
TC SA	0/2	
KM NF	0/2	
ABP NF	0/2	
NA NF	0/3	
NF SA	3/9	SA
CM TC SA	0/2	
TC NA NF	1/1	TC
TC NA SA	3/5	TC SA
TC NF SA	4/9	TC SA
KM NF SA	0/1	
CM TC SM NF	0/1	
CM TC NA SA	0/1	
CM TC NF SA	13/14	TC SA (2), CM TC SA (11)
CM NA NF SA	0/1	
TC SM NA NF	0/1	
TC SM NF SA	0/4	
TC ABP NF SA	0/1	
TC NA NF SA	3/3	TC SA
SM ABP NF SA	0/1	
KM NA NF SA	0/2	
CM TC SM NF SA	0/3	
CM TC KM NF SA	0/3	
CM TC ABP NF SA	5/7	CM TC SA
CM TC NA NF SA	5/6	CM TC SA
TC SM ABP NF SA	0/1	
TC SM NA NF SA	0/1	
CM TC SM KM NA SA	1/1	CM TC SM KM SA
CM TC ABP NA NF SA	0/2	
CM TC SM KM ABP NF SA	0/1	
Total	38/168	

^a Abbreviations: CM, chloramphenicol; TC, tetracycline; SM, streptomycin; KM, kanamycin; ABP, aminobenzyl penicillin; NA, nalidixic acid; NF, furazolidone; SA, sulfamonomethoxine.

resistant to one, 21 resistant to two, 19 resistant to three, 28 resistant to four, 21 resistant to five, 3 resistant to six and the remaining one was resistant

Fig. 2. Agarose gel electrophoresis of plasmid DNAs from 11 strains of *Edwardsiella tarda* and standard DNA Col K. Cleared lysate was electrophoresed on 0.8% agarose gels and visualized after ethidium bromide staining. No. 1, TYO46; No. 2, TYO88; No. 3, TYO112; No. 4, T1; No. 5, T33; No. 6, M1; No. 7, M8; No. 8, O1; No. 9, O3; No. 10, K1; No. 11, USA1; No. 12 and No. 13, Col K. Transferable R plasmid was detected in only two strains of No. 1 and No. 3.

to seven drugs. The most common type of the drug resistant strains had the markers of resistance to the combination of CM, TC, NF and/or SA.

Transferable R plasmids were detected in 38 of 136 resistant strains. The most common type of R plasmids determined resistances to CM, TC and SA. Other R plasmids had markers for resistance to TC, SA, TC SA and CM TC SM KM SA. Resistance to NA and NF was not transferred to *E. coli* from any strains of *E. tarda*.

Electrophoretic patterns in agarose gels of plasmid DNAs of 11 strains of *E. tarda* are shown in Fig. 2. Transferable R plasmids were detected in two strains, No. 1 and No. 3, and not detected in other strains. All strains were found to have more than one plasmid and up to eight different plasmid molecules (DNA from No. 2) except transferable R plasmid.

Discussion

Incidences of resistant strains of *E. tarda* in eel culture ponds have been increasing. This increase seems to follow the increase use of chemo-

therapeutics in eel culture. Because the resistance markers of *E. tarda* obtained in this study were CM, TC, NF and/or SA, attention should be paid to the fact that chloramphenicol, tetracycline, nitrofurantoin derivatives and sulfonamides have been frequently used for the treatment of edwardsielliosis of cultured eel. These drugs are not used so widely in tilapia culture and multiple drug resistant strains of *E. tarda* were not isolated from tilapia culture ponds. These facts clearly indicate that drug resistant strains of *E. tarda* have increased in parallel with the increased use of these chemotherapeutics. If these chemotherapeutics are widely used for all fish culture as they were for eel culture pond, it will soon be impossible to treat all infectious bacterial diseases of cultured fish. Fish culturists should not use chemotherapeutics indiscriminately. They should be concerned about a good environment of culture ponds, health of fish and prevention of pathogenic bacteria entering fish farm ponds.

Thirtyeight of 136 drug resistant strains of *E. tarda* isolated from fish culture ponds in various areas were found to carry transferable R plasmids. Drug resistance markers of the 38 R plasmids detected in *E. tarda* strains isolated from eel culture ponds were as follows: one, TC, three, SA; twelve, TC SA; twentyone, CM TC SA; and one, CM TC SM KM SA.

In other words, the resistance markers of R plasmids in *E. tarda* were followed by the chemotherapeutics used for treatment of edwardsielliosis of cultured eel. Incompatibility grouping of part of these plasmids were carried out and all of those R plasmids were found to be long to group A as described in a previous paper (AOKI *et al.*, 1977). The origin of the R plasmids detected in relation to fish culture is not yet known. But since group A R plasmids in *E. tarda* might not come from enteric bacteria of human and domestic animals, the formation of these R plasmids could be generated in *E. tarda* presumably as the results of "transposon" (CALOS and MILLER, 1980; STARLING, 1980).

E. tarda belongs to Enterobacteriaceae and this organism is well known as one of the intestinal flora of snake (SAKAZAKI, 1965) and as a pathogen of man (JORDAN and HADLEY, 1969). There have been no reports of conjugative R plasmids of *E. tarda* from snakes and human sources. This fact may also be explained by the abundant use of

chemotherapeutics for eel culture. Since *E. tarda* strains isolated from cultured fish directly infect humans and also the R plasmids can be transferred to human pathogenic bacteria, this could possibly cause a potential hazard to public health.

Cryptic plasmids were detected in all tested strains of *E. tarda*. The molecular sizes of these plasmid DNAs was about 10^6 daltons or less. The function of cryptic plasmids detected from *E. tarda* is not yet known and calls for future investigation.

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