Epizootiological Studies on R Plasmid with Carbadox Resistance

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ABSTRACT. A total of 930 *Escherichia coli* strains, consisting of 307 strains isolated from cattle, 414 from swine, and 209 from chickens isolated during a period from 1976 to 1980, were examined for R plasmid with carbadox (Cdx) resistance. No Cdx-resistant strains were found among the strains of bovine and avian origin. All the bovine and avian strains were susceptible to 3.13 µg/ml of Cdx under anaerobic conditions. Of the 414 strains of porcine origin, however, 30 strains (7.2%) showed resistance to Cdx. All Cdx-resistant strains were originated from six farms where Cdx was used for preventing swine dysentery or promoting piglet growth. Of the 30 resistant strains, 21 (70%), the isolates on a farm in 1980, had conjugative R plasmid carrying the Cdx-resistance determinant. The character of Cdx-resistance was invariably transmissible by conjugation to *E. coli* K-12 jointly with the resistance to such other drugs as streptomycin, spectinomycin and ampicillin. Then, pNV 13 with Cdx-resistance, a representative R plasmid isolated from a porcine strain of *E. coli*, was examined for the host range. It was conjugally transmissible to *Klebsiella pneumoniae*, *Citrobacter freundii*, *Salmonella typhi*, *Salmonella typhimurium* and *Shigella flexneri* 3a, but not to *Treponema hyodysenteriae*. These transconjugants carrying pNV 13 showed resistance to Cdx; the minimum inhibitory concentrations of Cdx ranged from 6.25 to 12.5 µg/ml under anaerobic conditions.

Carbadox (Cdx) [4] is one of quinoxaline-di-N-oxide compounds, involving olaquinox [2] and quinoxin [6]. It is only Cdx that is approved for animal use for preventing and treating swine dysentery. The mechanism of action of these agents has not completely been clarified [1,13]. Recently, the appearance of R plasmid with Cdx resistance was demonstrated in *Escherichia coli* strains isolated from pigs [10]. Such an R plasmid, pNV 13, gives a character of Cdx-resistance to *E. coli* K-12 jointly with resistance to streptomycin, spectinomycin and ampicillin. The molecular weight of this plasmid is about 28×10^6 daltons. Little information is available, however, on the appearance of Cdx-resistance R plasmid among *E. coli* strains in domestic animals.

The present study was undertaken to

clarify the distribution of this R plasmid in *E. coli* strains isolated from domestic animals and to further investigate the stability of Cdx-resistance R plasmid, pNV 13, in the enterobacterial host cells and in *Treponema hyodysenteriae*.

MATERIALS AND METHODS

Bacterial strains: A total of 930 strains of *E. coli* were submitted for the assay for Cdx resistance. These strains were isolated on a total of 195 farms in Japan during a period from 1976 to 1980. They consisted of 307 strains (60 farms) from cattle, 414 (77) from pigs, and 209 (58) from chickens. All of them were originated from the feces of apparently healthy animals, and most of those from pigs were isolated on the farms where Cdx was commonly used. Substrains of *E. coli*

K-12, ML1410 and ML1410 Rfp^r were used as hosts and recipients of R plasmid [10]. The test for the host range of pNV 13, the representative R plasmid with Cdx resistance, was made using recipient strains of rifampicin (Rfp)-resistant mutants of various species *Citrobacter freundii* GN 587, *Klebsiella pneumoniae* GN 402, *Proteus mirabilis* GN 3167, *Proteus rettgeri* GN 3239, *Proteus vulgaris* GN 3213, *Pseudomonas aeruginosa* M 1292, *Salmonella typhi* TD, *Salmonella typhimurium* LT 2, *Shigella flexneri* 3a YSH 1 and *Yersinia enterocolitica* 7-3. *Treponema hyodysenteriae* ATCC 27167, S 73/2 and DJ 70, were also employed.

Media: Heart infusion broth (Difco), trypticase soy broth (BBL) and L broth [3] were used as liquid media. Deoxycholate-hydrogen sulfide-lactose (DHL) agar (Eiken), heart infusion agar (Difco), Mueller-Hinton medium (Difco), L broth agar and trypticase soy agar (BBL) were used as solid media.

Drugs: Oxytetracycline (Tc), chloramphenicol (Cm), dihydrostreptomycin sulfate (Sm), spectinomycin hydrochloride (Spc), kanamycin sulfate (Km), ampicillin (Apc), (Rfp), sodium sulfadimethoxine (Su), Cdx and nalidixic acid (Nal) were used.

Drug susceptibility: The agar dilution method was used for the determination of drug susceptibility. Bacterial susceptibility to Cdx was examined under anaerobic conditions with the gas pack system (BBL) [10]. The assay for drug susceptibility of *T. hyodysenteriae* strains was performed by the method of Kitai *et al.* [8]. In this method, the assay medium used was trypticase soy agar supplemented with 5% defibrinated sheep blood [7], and the final inoculum of *T. hyodysenteriae* was adjusted to a concentration of 10^6 to 10^7 colony-forming units per ml. The agar plates were incubated anaerobically

at 37°C for 3 days. The growth of *T. hyodysenteriae* was judged by the appearance of β -hemolysis on the plates.

Detection of R plasmids with Cdx resistance: Transmissibility of Cdx-resistant character was examined by mixed cultivation at 37°C for 1 hr [10]. *E. coli* ML 1410 was used as a recipient strain. Selection for the transconjugant was performed on DHL agar containing both Nal (25 μ g/ml) and any one of the following drugs: Tc, Cm, Spc, Km, and Apc (25 μ g/ml), Sm (12.5 μ g/ml), Cdx (1.56 μ g/ml) and Su (400 μ g/ml).

Host range of pNV 13: Isolation of pNV 13 [10] and drug resistance levels of its transconjugant *E. coli* strains are shown in Tables 2 and 4. The host range of pNV 13 was examined by mixed cultivation at 37°C for 2 hr and 24 hr. *E. coli* ML 1410 (pNV 13) was used as a donor strain. As for selectors of transconjugants used were Rfp (25 μ g/ml) and any one of Spc, Apc (25 μ g/ml) and any one of Spc, Apc (25 μ g/ml), Sm (12.5 μ g/ml), and Cdx (1.56 μ g/ml). When *T. hyodysenteriae* was used as a recipient, the transformation test was performed with plasmid DNA of pNV 13 by the method of Cohen *et al.* [3].

Stability of pNV 13 in host strains: The stability of pNV 13 in host strains was judged by curing the drug resistance. The host strains with pNV 13 were cultivated in drug-free trypticase soy broth to undergo ten passages. Each subculture was checked for Cdx resistance by examining about 200 colonies by the replica plating method [9].

RESULTS

Susceptibility to Cdx: Table 1 shows the antibacterial activities of Cdx against the 930 strains of *E. coli*. Under anaerobic conditions, the growth was inhibited with 3.13 μ g/ml of Cdx in all of the strains

from cattle and chickens. The mean minimum inhibitory concentrations (MIC) of Cdx ranged from 0.2 to 0.39 $\mu\text{g/ml}$. No Cdx-resistant strains were detected among them. On the other hand, the strains from pigs exhibited a lower sensitivity to Cdx than did those from the other animal species. Of 414 strains originating from pigs, 30 strains (7.2%) isolated on six farms showed MIC values of Cdx higher than 12.5 $\mu\text{g/ml}$. Those showing MIC values of Cdx exceeding 25 $\mu\text{g/ml}$ were isolated only in 1980 and on a farm where Cdx was used for preventing swine dysentery.

Conjugal transmission of Cdx resistance: Table 2 shows the results of conjugal transfer experiments between the Cdx-resistant strains and ML 1410. Of 30 Cdx-resistant strains showing MIC values of Cdx higher than 12.5 $\mu\text{g/ml}$, 21 (70%) isolated on a farm in 1980 carried conjugative R plasmid with Cdx resistance. From such strains that showed MICs of Cdx higher than 25 $\mu\text{g/ml}$, Cdx-resistance R plasmid was isolated at a frequency of 87.0% (20/23). The Cdx-resistance was almost always transmitted jointly with Sm Spc Apc-resistance.

Host range of pNV 13: The transmissibility of pNV 13 to several enteric bacteria is shown in Table 3. The conjugal transfer of pNV 13 was achieved at a frequency of 10^{-4} to 10^{-7} to *K. pneumoniae*, *C. freundii*, *S. typhi*, *S. typhimurium*, and *S. flexneri* 3a in 24 hr of conjugation. *K. pneumoniae*, when used as a recipient, exhibited a frequency of transfer of 10^{-4} . In serial transfer experiments, the phenotype of resistance to Cdx Sm Spc Apc was transmitted to another ML 1410 strain from the pNV 13-carrying transconjugants of different enteric bacteria as one unit by conjugation.

On the other hand, no transconjugant was obtained when *Proteus*, *Pseudomonas* and *Yersinia* were used as recipients. Similarly, when *T. hyodysenteriae* was used as a recipient, no transconjugant or transformant with pNV 13 was obtained.

Table 4 shows the levels of drug resistance of enteric bacteria possessing pNV 13. For all the transconjugant strains except *S. flexneri* 3a, MICs of Cdx ranged from 6.25 to 12.5 $\mu\text{g/ml}$ under anaerobic conditions. The host strain, *S. flexneri* 3a, presented a lower Cdx resistance level than did any other bacterial strain.

Table 1. Antibacterial activity^a of carbadox against *Escherichia coli* isolated from domestic animals

Animal species	Year of isolation	Number of farms	Number of isolates with MIC ($\mu\text{g/ml}$) of:											Total
			0.1	0.2	0.39	0.78	1.56	3.13	6.25	12.5	25	50	100	
Cattle	1976	20	27	51	40	9	4	2	0	0	0	0	0	133
	1977	20	18	83	38	7	2	0	0	0	0	0	0	148
	1978	20	2	6	5	2	8	3	0	0	0	0	0	26
Pig	1976	20	3	32	37	19	19	12	13	1	0	0	0	136
	1977	20	1	26	45	29	11	8	9	1	0	0	0	130
	1978	20	0	15	17	11	3	2	6	2	0	0	0	56
	1979	16	0	0	4	10	2	2	0	2	0	0	0	20
	1980	1	0	0	6	12	12	6	12	1	7	15	1	72
Chicken	1976	20	15	37	27	2	3	0	0	0	0	0	0	84
	1978	20	8	24	18	14	5	0	0	0	0	0	0	69
	1980	18	7	13	12	8	6	0	0	0	0	0	0	56

a: Under anaerobic conditions.

Table 2. Conjugal transfer of carbadox resistance to *E. coli* K-12 ML 1410^a

MIC of carbadox ($\mu\text{g/ml}$)	Donor strains tested		Transconjugant strains ^b	
	Resistance pattern	Number of strains	Resistance pattern	Number of strains
12.5	Cdx Tc Cm Sm Spc Su Km Apc	1	Cdx Sm Spc Apc Km	1
	Cdx Tc Cm Sm Spc Apc	1	None	
	Cdx Tc Sm Km	2	None	
	Cdx Tc Km	3	None	
25	Cdx Tc Sm Spc Su Km Apc	5	Cdx Sm Spc Apc	4
	Cdx Tc Sm Spc Su Apc	2	Cdx Sm Spc Apc	2
50	Cdx Tc Sm Spc Su Km Apc	12	Cdx Sm Spc Apc	10 ^c
	Cdx Tc Sm Spc Su Apc	3	Cdx Sm Spc Apc	3
100	Cdx Tc Sm Spc Su Km Apc	1	Cdx Sm Spc Apc	1
Total		30		21

a: MIC of carbadox for *E. coli* ML 1410 was 6.25 $\mu\text{g/ml}$ in aerobic incubation and 0.2 $\mu\text{g/ml}$ in anaerobic incubation.

b: Selection was made with Tc-nalidixic acid (Nal), Cm-Nal, Sm-Nal, Spc-Nal, Km-Nal and Apc-Nal.

c: Including pNV 13.

Table 3. Host range of pNV 13 in enteric bacteria tested by conjugation

Donor	Recipient	Transfer frequency ^a	
		2 hr	24 hr
<i>E. coli</i> K-12 ML 1410	<i>K. pneumoniae</i> GN 402	10^{-6}	10^{-4}
	<i>C. freundii</i> GN 587	10^{-7}	10^{-6}
	<i>S. typhi</i> TD	10^{-7}	10^{-6}
	<i>S. typhimurium</i> LT 2	* ^b	10^{-7}
	<i>S. flexneri</i> 3a YSH 1	*	10^{-7}
	<i>P. mirabilis</i> GN 3167	*	*
	<i>P. rettgeri</i> GN 3239	*	*
	<i>P. vulgaris</i> GN 3213	*	*
	<i>P. aeruginosa</i> M 1292	*	*
	<i>Y. enterocolitica</i> 7-3	*	*

a: Transfer frequency was calculated as the number of exconjugant recipients having acquired drug resistance, divided by the number of donor cells after mixed culture at 37°C for 1 hr.

b: Transfer frequency was lower than 10^{-8} .

Stability of pNV 13 in the host strain: When *S. typhi* and *S. typhimurium* were used as host strains, pNV 13 was eliminated at a frequency of 1 to 3% of about 200 colonies examined at the 10th passage. The character of Cdx Sm Spc Apc-resistance was jointly cured of. For *C. freundii*, the curing rate of pNV 13 was

about 10%. For *K. pneumoniae*, however, pNV 13 was eliminated by about 60% at the 6th passage, and by about 90% at the 10th passage. On the other hand, when *S. flexneri* 3a was used as host strain, all pNV 13 was eliminated at the 3rd passage, indicating that it was very unstable in this host strain.

Table 4. Levels of drug resistance ^ain enteric bacteria possessing pNV 13

Strain		MIC ($\mu\text{g/ml}$)			
		Sm	Spc	Apc	Cdx
<i>E. coli</i> ML 1410	pNV 13	>100	>100	>100	12.5
	—	3.13	12.5	3.13	0.2
<i>K. pneumoniae</i> GN 402	pNV 13	>100	>100	>100	12.5
	—	1.56	12.5	100	0.78
<i>C. freundii</i> GN 587	pNV 13	>100	>100	>100	12.5
	—	>100	50	6.25	0.39
<i>S. typhi</i> TD	pNV 13	>100	>100	>100	6.25
	—	25	6.25	0.78	0.2
<i>S. typhimurium</i> LT 2	pNV 13	>100	>100	>100	12.5
	—	12.5	25	1.56	0.39
<i>S. flexneri</i> 3a YSH 1	pNV 13	>100	>100	>100	1.56
	—	12.5	25	1.56	0.39

a: Under anaerobic conditions.

DISCUSSION

Cdx has been used as an animal drug for prevention or treatment of swine dysentery during the first half of the 1970s. It was authorized in 1976 to use Cdx as a feed additive for promotion of growth of piglets less than 2 months old [14]. Since then it has commonly been used in pigs in Japan.

It is known that the use of an antimicrobial agent induces drug-resistant strains [11, 12]. The present investigation revealed a relation between the appearance of Cdx-resistant strains and the use of this agent. The strains originating from cattle and chickens with no history of Cdx treatment were susceptible to the agent, but those from pigs were not. It is not difficult to see that the use of Cdx brought about Cdx resistant *E. coli*.

R plasmid with Cdx resistance was obtained at a high frequency from such strains that showed MICs higher than 25 $\mu\text{g/ml}$. Such highly resistant strains were isolated exclusively from pigs in 1980. Therefore, it is assumed that R plasmid may have appeared very recently.

Besides, pNV 13 with Cdx resistance is transferable to some species of enteric bacteria. It was kept stable when *Salmonella* was a host strain.

On the other hand, the antibacterial activity of Cdx against *T. hyodysenteriae*, a primary causative agent of swine dysentery [5], was reported by Kitai *et al.* [8], who failed to obtain Cdx-resistant *T. hyodysenteriae*. In the present experiment, Cdx-resistant *T. hyodysenteriae* was not obtained by either conjugation or transformation with pNV 13. From these results, it seems hardly possible that *T. hyodysenteriae* becomes resistant to Cdx with R plasmid.

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要 約

Carbadox 耐性形質を有する R plasmid の疫学的検討： 大前憲一・米沢昭一・寺門誠致¹⁾ (農林水産省動物医薬品検査所, ¹⁾農林水産省家畜衛生試験場) — 1976年から1980年にかけて分離された大腸菌 930株 (牛由来株 307株, 豚由来株 414株および鶏由来株 209株) を用いて Carbadox (Cdx) 耐性形質を有する R plasmid の分布状況について検討した。Cdx 耐性株は, 牛由来株および鶏由来株では検出されなかった。これらの菌株は, 嫌気性下で Cdx 3.13 µg/ml に対して感受性を示した。一方豚由来株では, 414株中 30株 (7.2%) に Cdx 耐性株が認められた。これらはすべて Cdx が豚赤痢の予防又は成長促進に使用されていた農場から分離されたものであった。またこれら耐性株中 1980年に1農場に由来した 21株 (70%) から, Cdx 耐性形質を有する R plasmid が検出された。これら Cdx 耐性形質は, 混合培養により *E. Coli* K-12 株へ常に Streptomycin, Spectinomycin および Ampicillin 耐性を伴って伝達可能であった。つぎにすでに報告した豚由来大腸菌から検出された Cdx 耐性形質を有する pNV 13 を用いてその宿主域について検討したところ pNV 13 は, *Klebsiella pneumoniae*, *Citrobacter freundii*, *Salmonella typhi*, *Salmonella typhimurium* および *Shigella flexneri* 3a に対して接合伝達が可能であったが, *Treponema hyodysenteriae* では認められなかった。これら pNV 13 を保有する transconjugant の嫌気性下の Cdx 耐性値は, 最小発育阻止濃度で 6.25~12.5 µg/ml であった。