沖縄の家畜由来細菌の薬剤耐性：II 豚および鶏糞便由来薬剤耐性大腸菌の R 因子について(畜産学科)

Drug Resistant Strains of Bacteria Isolated from Domestic Animals in Okinawa

II. Distribution of R factors in fecal *E. coli* strains isolated from pigs and chickens*

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I INTRODUCTION

The occurrence of drug resistant bacteria is a matter of great concern in both human and animal medicine. Furthermore, drug resistant strains carry transferable drug resistance factors.

It seems possible to conclude from many reports (1, 4, 6, 9) that the resistant strains have emerged mainly from the use of antimicrobial drugs in the treatment of clinical disease, from their uses as feed additives for the prevention of disease, or from their uses as feed additives for nutritional purposes.

Several Japanese investigators (4, 9, 15) have reported that the transferable drug resistence was prevalent among nonpathogenic *E. coli* strains in farm animals in Japan as a result of the use of the antimicrobial drugs. The antimicrobial drugs have also been used commonly for animals in Okinawa.

In a previous report (5), the author confirmed that the incidence of multiple drug resistant strains was widespread among pigs and chickens in Okinawa. However, the author could not demonstrate the distribution of R factors among these drug resistant *E. coli* strains in the report.

In the present study, therefore, the author tried to determine to what extent R factors-carrying *E. coli* strains are present in feces of healthy pigs and chickens in Okinawa.

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Ⅱ MATERIALS AND METHODS

1 Bacterial strains

The strains of *E. coli* used were isolated from fecal samples of apparently healthy pigs and chickens who were fed with commercial feeds, in all over the island of Okinawa.

The methods of isolation and identification of *E. coli* were described previously

A total of 373 strains, 152 from pigs and 221 from chickens, were tested. A part of them were same with those used in previous report (5).

2 Drugs and media

Aminobenzyl penicillin (APC), oxytetracycline hydrochloride (TC), streptomycin sulfate (SM), chloramphenicol (CP), kanamycin sulfate (KM), sulfadimethoxine (SA) and nalidixic acid (NA) were used.

BTB-lactose agar or McConkey agar (Eiken) was used for the isolation of *E. coli* organisms. Brain heart infusion broth and agar (BBL) were used for drug sensitivity tests.

3 Drug sensitivity test

The agar dilution method was used as described previously.

The resistance level was defined as the maximum concentration allowing bacterial growth. The strains tolerating 25 μg/ml of APC, TC, SM, CP, KM or 200 μg/ml of SA were considered resistant.

Muller Hinton Medium (BBL) was used for the determination of SA resistant.

4 Transfer of R factor

E. coli K−12 ML1410 strain, resistant to NA, was used as a recipient of the R factors. The strain was supplied by the courtesy of Dr. N. Terakado, the National Veterinary Assay Laboratory, Tokyo.

Each donor or recipient strain was grown in 3 ml of BHI broth at 37°C for 18 hours. Then, 0.2 ml portions of the donor and recipient cultures were mixed in a tube containing 3 ml of BHI broth. After the conjugation mixture had been incubated at 37°C for 18 hours, cultures were streaked on a selective BHI agar containing NA (50 μg /ml) and one of drugs with concentration of described above. Muller Hinton agar containing NA and SA was used as a selective medium when SA was selective marker.

These cultures were incubated overnight at 37°C. Then the resistance marker transferred was determined by observing bacterial growth on each selective medium.

Ⅲ RESULTS

1 Drug resistance patterns of fecal *E. coli* isolated from pigs and chickens

A total of 373 *E. coli* strains, 152 from pigs and 221 from chickens were tested for drug resistance to APC, TC, SM, CP, KM and SA.

Table 1 showed the drug resistance patterns and their isolation frequencies of the strains.

Table 1. Drug resistance patterns and their isolation frequencies of fecal *E. coli* strains isolated from pigs and chickens

	Resistance pattern*	Pig isolates (152) No. of strains		per cent**		Chicken isolates (221) No. of strains		per cent**	
6	APC TC SM CP KM SA	3		2.0		0		0	
5	APC TC SM CP SA	1	17	0.7	11.6	0	0	0	0
	APC TC SM KM SA	12		8.2		0		0	
	TC SM CP KM SA	4		2.3		0		0	
4	APC TC SM KM	30	59	20.4	40.1	7	12	3.3	5.7
	APC TC SM SA	0		0		0		0	
	APC TC CP KM	1		0.7		0		0	
	APC TC CP SA	1		0.7		0		0	
	TC SM CP SA	0		0		3		1.4	
	TC SM KM SA	27		18.4		2		1.0	
3	APC TC SM	1	31	0.7	21.1	2	111	1.0	53.1
	APC TC SA	4		2.7		7		3.3	
	TC SM KM	14		9.5		1		0.5	
	TC SM SA	11		7.5		100		47.8	
	TC KM SA	1		0.7		1		0.5	
2	APC TC	4	17	2.7	11.6	4	52	1.9	24.5
	APC SM	0		0		1		0.5	
	TC SM	9		6.1		19		9.1	
	TC KM	2		1.4		1		0.5	
	TC SA	2		1.4		27		12.9	
1	TC	20	20	13.6	13.6	25	34	12.0	16.3
	SM	0		0		1		0.5	
	SA	0		0		8		3.8	
Total of resistance		147		100(96.7)		209		100(94.6)	
0	Sensitive	5		(3.3)		12		(5.4)	
Total of tested		152		(100)		221		(100)	

* Abbreviations; APC, aminobenzyl penicillin; TC, oxytetracycline hydrochloride; SM, streptomycine sulfate; CP, chloramphenicol; KM, kanamycin sulfate; SA, sulfadimethoxine

** Percentages given in parentheses were calculated from the total number of strains tested.

Of 152 pig isolates, only 5 strains, or 3.3%, were sensitive to all six drugs, whereas 147, or 96.7%, were resistant to one or more drugs. Eighteen different drug resistance patterns were found among the 147 resistant strains.

Out of the 147 drug resistant strains, 20, or 13.6%, were resistant to a single drug and other 127, or 86.4%, were multiply resistant. Quadruple resistant strains were found in most frequently (40.1%), in which APC-TC-SM-KM and TC-SM-KM-SA resistances were the most frequently encountered patterns, occurring among 20.4% and 18.4% of the resistant strains, respectively. Triple resistant strains were also found at high frequency, 21.1%. In this case, resistance patterns of TC-SM-KM and TC-SM-SA were most common. Strains, singly resistant to TC only, were also isolated with a relatively high frequency, 13.6%. The occurrence of double and quintuple resistances was equally prevalent. Sextuple resistance was the least common, 2.0%.

On the other hand, of the 221 strains isolated from chickens, 209 strains, or 94.6%, were resistant strains and fell into 16 different resistance patterns. Among them, 34, or 16.3%, were singly resistant and other 175, or 83.7%, were multiply resistant. Multiple resistance included 111 (53.1%) triple, 52 (24.5%) double and 12 (5.7%) quadruple resistances, in descending order. Among chicken strains tested, there were no strains which showed resistance to 5 and 6 drugs used.

As was clear from the table, the triple resistance pattern of TC-SM-SA was found most frequently, 47.8%.

In table 2, above data were simply summarized. The strains resistant to 4 or more drugs were found in over half of total strains isolated from pigs. These results were contrary to those of chicken isolates in which only 5.4% was obtained.

Table 2. Multiple drug resistance of *E. coli* strains

Resistant to	Pig isolates			Chicken isolates		
	No. of strains resistant	Per cent of total isolates	Accumulated per centage	No. of strains resistant	Per cent of total isolates	Accumulated per centage
6 drugs	3	2.0	2.0	0	0	0
5 〃	17	11.2	13.2	0	0	0
4 〃	59	38.8	52.0	12	5.4	5.4
3 〃	31	20.4	72.4	111	50.2	55.6
2 〃	17	11.2	83.6	52	23.6	79.2
1 drug	20	13.2	96.8	34	15.4	94.6
0 (sensitive)	5	3.3	100.1	12	5.4	100
Total	152	100		221	100	

However, no difference was noted when the percentages of the strains resistant to more than 2 drugs were compared between pig and chicken isolates.

2 Drug resistance patterns of R factors in resistant *E. coli* strains

The distribution of R factors among the resistant *E. coli* strains was investigated. The isolation frequency of R factors with reference to resistance patterns was shown in tables 3 and 4.

Among the 147 drug resistant strains isolated from pigs, 65, or 44.2%, were found to carry R factors (table 3).

Table 3. Resistance patterns and R factors in resistant strains of *E. coli* isolated from pigs

Resistance pattern	No. of strains resistant	No. of R^+ strains*	Per cent	Resistance pattern of R factor	No. of strains
APC TC SM CP KM SA	3	0	0		
APC TC SM KM SA	12	10	83.3	APC TC SM KM SA	1
				APC TC SM KM	3
				APC SM KM SA	3
				APC SM KM	2
				TC	1
APC TC SM CP SA	1	0	0		
TC SM CP KM SA	4	3	75.0	TC CP	1
				KM	2
APC TC SM KM	30	28	93.3	APC TC SM KM	2
				APC SM KM	25
				TC SM	1
APC TC CP KM	1	0	0		
APC SM CP SA	1	0	0		
TC SM KM SA	27	15	55.6	TC SM KM SA	1
				TC SM KM	4
				TC KM SA	1
				TC KM	1
				SM KM	1
				SM SA	2
				TC	1
				SM	3
APC TC SM	1	0	0		
APC TC SA	4	0	0		
TC SM KM	14	1	7.1	TC SM	1
TC SM SA	11	3	27.3	SM CP	1
				TC	2
TC KM SA	1	0	0		
APC TC	4	2	50.0	TC	2
TC SM	9	2	22.2	TC	1
				SM	1
TC KM	2	0	0		
TC SA	2	0	0		
TC	20	2	10.0	TC	2
Total	147	65	44.2		65

* R^+ strain: R factor-carrying strain

A high percentage of transfer was noted for quadruple and quintuple resistant strains, namely, 93.3% of quadruple resistant strains with pattern APC-TC-SM-KM and 83.3% of quintuple strains with pattern APC-TC-SM-KM-SA transferred part or all of their resistance patterns to the recipient strains.

Table 4 Resistance patterns and R factors in resistant strains of *E. coli* isolated from chickens

Resistance pattern	No. of strains resistant	No. of R^+ strains	Per cent	Resistance pattern of R factor	No. of strains
APC TC SM SA	7	5	71.4	TC SM SA	1
				TC SM	2
				SM	1
				SA	1
TC SM CP SA	3	1	33.3	TC SM CP SA	1
TC SM KM SA	2	2	100.0	TC SA SM	1
				TC SM	1
APC TC SM	2	1	50.0	TC SM	1
APC TC SA	7	2	28.6	APC	1
				SA	1
TC SM KM	1	1	100.0	SM	1
TC SM SA	100	37	37.0	TC SM SA	6
				TC SM	9
				SM SA	4
				TC	8
				SM	10
TC KM SA	1	0	0		
APC TC	4	1	25.0	TC	1
APC SM	1	0	0		
TC SM	19	15	79.0	TC SM	4
				TC	3
				SM	8
TC KM	1	1	100.0	TC	1
TC SA	27	4	14.8	TC SA	1
				TC	2
				SA	1
TC	25	6	24.0	TC	6
SM	1	0	0		
SA	8	0	0		
Total	209	76	36.4		76

The resistance patterns of R factors of these 65 strains were classified into 16 different patterns (table 5).

Table 5 Resistance patterns of R factors identified in the R factors-carrying *E. coli* strains

Pig isolates (65)			Chicken isolates (76)		
Resistance patterns of R factors	Number of strains	Per cent	Resistance patterns of R factors	Number of strains	Per cent
APC SM KM	27	41.5	TC	21	27.6
TC	9	13.8	SM	20	26.3
APC TC SM KM	5	7.7	TC SM	17	22.4
TC KM SA	4	6.2	TC SM SA	8	10.5
SM	4	〃	SM SA	4	5.3
APC SM KM SA	3	4.6	SA	3	3.9
TC SM	2	3.0	TC SM CP SA	1	1.3
TC	2	〃	TC SA	1	〃
KM	2	〃	APC	1	〃
APC TC SM KM SA	1	1.5			
TC SM KM SA	1	〃			
TC KM SA	1	〃			
TC KM	1	〃			
TC CP	1	〃			
SM KM	1	〃			
SM CP	1	〃			
Total : 16 patterns	65	100	9 patterns	76	100

In regards to resistance patterns of R factors transferred, pattern APC-SM-KM was most frequently encountered, and pattern TC was the second. These 2 patterns of resistance comprised more than 50% of all R factors identified.

On the other hand, of the 209 drug resistant strains isolated from chickens, 76, or 36.4%, were found to transfer part or all of their resistance to the recipient strains (table 4).

In the case of chicken strains, quadruple resistant strains transferred their resistance at high percentages (66.7%, or 8 of 12 strains). Among the 76 strains, a total of 9 different patterns of R factors were identified (table 5).

The most commonly occurring resistance patterns of R factors were single patterns of TC and SM, and also combination thereof, pattern of TC-SM. These 3 patterns of resistance accounted for 76.3% of all R factors identified in chicken isolates (table 5).

3 Frequency of resistance and R factors to individual drugs in *E. coli* strains

Table 6 showed the isolation frequency of individual resistances to each of the 6 drugs and also showed frequency of R factors of single drug resistance appearing as part of multiply resistant patterns.

Of the 152 strains of pig origin, 147, or 96.7%, were resistant to at least one of the 6 drugs used. The highest frequency was obtained in TC, being 96.1%. Following in sequence of occurrence was SM (75.0%), KM (60.2%), SA (43.4%), APC (37.5%) and CP (5.9%).

On the other hand, of 221 strains isolated from chickens, 209, or 94.6%, were resistant strains to one of the drugs. A high percentage of resistance was noted for TC, KM and SM, being 90.1, 70.1 and 61.5%, respectively.

Regardless of their origins, the strains tested were similarly resistant to TC and sensitive to CP. However, results of KM-sensitivity were quite different between strains isolated from pigs and chickens. Namely, the isolation frequency of KM resistant strains was 60.2% in pig isolates whereas only 2.3% in chicken isolates.

Though the data were not tabulated, all strains tested were sensitive to NA at concentration of 50 $\mu g/ml$.

In regard to transferability of drug resistance, a high percentage of transfer was noted for APC (37 of 57, or 64.9%), KM (46 of 92, or 50%) and SM (51 of 114, or 44.7%) resistant strains in pig origin, and for SM (50 of 136, or 36.8%) resistant strains in chicken origin.

The percentages of transfer were somewhat different between pig and chicken isolates. For example, resistance to APC was able to transfer at high frequency, 64.7%, in pig isolates, whereas 0% in chicken isolates. Resistance to KM and its transferability was also different. In contrast, the TC-resistant strains which was the most frequently encountered among the both isolates, transferred equally their resistances with relatively low percentages.

IV DISCUSSION

In a previous report (5), the author showed that the multiple drug resistance was common among *E. coli* strains isolated from feces of healthy pigs and chickens in Okinawa. However, distribution of R factors among these resistant bacteria still remain unknown.

This study was, therefore, undertaken to assess the incidence of R factors among

Table 6. Frequency of resistance and R factors to individual drugs in *E coli* strains isolated from pigs and chickens

Drug to which resistant	Pig isolates (152 strains)				Chicken isolates (221 strains)			
	No. of resistant strains	Per cent*	No. of R^+ strains	Per cent**	No. of resistant strains	Per cent*	No. of R^+ strains	Per cent**
APC	57	37.5	37	64.9	21	9.5	0	0
TC	146	96.1	24	16.4	199	90.1	48	24.1
SM	114	75.0	51	44.7	136	61.5	50	36.8
CP	9	5.9	1	11.1	3	1.4	1	33.3
KM	92	60.2	46	50.0	5	2.3	0	0
SA	66	43.4	10	15.2	155	70.1	17	11.0
Total***	147	96.7	65	44.2	209	94.6	76	36.4

* Number of resistant strains per number of total strains tested

** Number of R factor-carrying strains per number of resistant strains

*** Resistant to at least one of the 6 drugs

the resistant *E. coli* strains. The strains and antimicrobial drugs used were increased in number those used in the previous test (5).

The conclusions obtained in the study was that drug resistant strains occur in significant numbers in both pig and chicken isolates, being 96.7 and 94.6%, respectively, and that infectious drug resistance is prevalent among these resistant strains, 44 and 36%.

There were several reports (4, 9, 15) about the distribution of drug resistant strains and R factors among *E. coli* strains in feces of apparently healthy farm animals. The first report respecting above in Japan was made by Mitsuhashi et al. and his co-workers (9, 12, 13). They surveyed for drug resistance and distribution of R factors among *E. coli* strains isolated from 151 pigs and 108 chickens. Their results obtained was that all of the pigs and 38% of the chickens excrete *E. coli* strains resistant to TC, CP, SM and SA. Among resistant strains from pigs and chickens, 87 and 76%, respectively, were multiply resistant and resistance pattern of TC-SM-SA was isolated most frequently in both isolates.

Of the resistant cultures isolated from pigs and chickens, 40 and 22%, respectively, carried R factors.

Tajima et al. (14) also surveyed for drug resistance to SM, TC and CP among *E. coli* strains isolated from healthy pigs and chickens. Thirty-one out of 33 strains from pigs and 303 out of 596 strains from chickens were resistant to one of these drugs. In both cases, resistance to TC was observed most frequently; resistance to CP was rare.

Kashiwazaki et al. (4) reported about distribution of R factors in fecal *E. coli* strains from SPF pigs. They isolated a total of 752 drug resistant *E. coli* strains from 66 fecal samples on McConkey agar containing each of SM, CP or TC. Strains of single (TC), double (SM-TC), and triple (SM-CP-TC) resistance were observed most frequently. Of 752 resistant strains, 366, or 49%, were found to have R factors.

Recently, Terakado et al. (1972) studied *E. coli* strains isolated from fecal samples of 91 healthy pigs in Hokkaido using 6 drugs, similar with those used in the present study. Of 385 strains isolated, 234 (60.8%) were resistant strains which included 18.5% of single; 25.5% of double; 12.0% of triple; 3.9% of quadruple; 0.3% of quintuple and 0.8% of sextuple resistances. Strains resistance to TC or SM were found in over half of them, whereas no strains resistance to CP were observed. Strains resistance to KM which were rare in isolates from human and cows, were isolated in 12.7%. They confirmed incidence of R factors in 39.7% (93 out of 234) of resistant strains.

From the data described above, it could be concluded that infectious drug resistance was prevalent among nonpathogenic *E. coli* strains from healthy pigs and chickens in Japan.

The fact that the strains resistance to TC, SM or SA were found most frequently appeared to be a reflection of both clinical and nonclinical antibiotic usage. TC, SM and SA were commonly used in animal feeds and also were widely used clinically in

Japan (2). In contrast, resistance to CP which was not as widely used, was found in only lower percentages. Concerning of this point, Mercer et al. (6) found a definite relationship between the use of antimicrobial drugs in feeds and the occurrence of resistant strains. But, unfortunately, a comparison of this type could not be made in the present study.

From the data of this and other studies (9, 11, 15), it could be said that the isolation frequency of drug resistant strains was high in isolates of pigs than those of chickens, that KM-resistant strains could be demonstrated in high frequency among pig isolates than those of chickens, and also that CP resistant strains were rare in number in both isolates.

Similar high incidence of infectious drug resistance among the fecal *E. coli* strains have been reported in foreign countries (1, 6, 11, 16).

Subsequently, as for the patterns of resistance and R factors, quadruple resistance patterns of APC-TC-SM-KM and TC-SM-KM-SA were found most frequently (40%) in pig isolates. R factors were also detected high frequencies in above 2 types, being 93.3% (28 out of 30 strains) and 55.6% (15 out of 27 strains), respectively. In contrast, the triple resistance pattern of TC-SM-SA was found in 47.8% (100 of 209) of the resistant strains isolated from chickens, but only 37% of them carried R factors.

Among resistant strains of chickens, R factors with pattern of TC-SM was found most frequently in 79% (15 of 19 strains).

As showh in table 5, the patterns of R factors which frequently observed were APC-SM-KM and TC in pig isolates, and TC, SM and TC-SM in chicken isolates. This seemed to be depended upon differences in sensitivity to APC and KM among these strains (table 6).

Mitsuhashi et al. (9) reported that resistant strains with pattern of TC-SM-SA were found most frequently in both pig and chicken isolates, being 47 and 33%, respectively.

Of these resistant strains, 40 and 22%, respectively, were found to have R factors.

According to the report of Kashiwazaki et al. (4), the patterns of TC, SM-TC and SM-TC-CP were most common among resistant strains isolated from pigs, and 49% of them carried R factors.

Terakado's investigation (15) of *E. coli* strains also collected from healthy pigs showed relatively lower percentage (60%) of resistance, in which patterns with TC-SM were found most common. Of these resistant strains, 39% carried R factors. The R factors with patterns of TC, SM and TC-SM were found in about 50%.

The data described above appeared to vary widely from one report to another. This variation could be artributed to variations in the selective techniques utilized by different investigators.

Similar high incidence of multiple resistance and R factors among *E. coli* strains, clinically isolated, have been reported by several authors (1, 3, 10).

In contrast, in the case of human strains, there was different incidence of resis-

tance and distribution of R factors between clinical and nonclinical isolates (3, 7, 8, 11).

This difference was believed to be a reflection of drugs used in feed additives in animals.

The conclusions to be drawn from the present study was that a significant level of R factors was prevalent among drug resistant *E. coli* strains isolated from pigs and chickens in Okinawa.

V SUMMARY

A total of 373 *E. coli* strains, 152 from pigs and 221 from chickens were surveyed for drug resistance and distribution of R factors.

Of 152 pig isolates, only 5 (3.3%) were sensitive to all 6 drugs (APC, TC, SM, CP, KM and SA) used, and other 147 (96.7%) were resistant to one or more drugs. Out of the 147 resistant strains, 65, or 44.2%, were found to transfer all or a part of their resistance patterns to the drug sensitive recipient strain.

The resistance patterns of R factors were classified into 16 different patterns, in which 2 patterns, APC-SM-KM and TC, comprised more than 50% of all R factors identified.

On the other hand, of 221 strains isolated from chickens, 209 or 94.6%, were drug resistant strains. Seventy-six of the 209 (36.4%) were found to carry R factors, and a total of 9 different patterns of R factors were identified.

The most commonly occurring resistance patterns of R factors were patterns of TC, SM, and TC-SM. These 3 patterns of resistance accounted for 76.3% of all R factors detected in chicken isolates.

In regard to data of drug resistance and distribution of R factors, there were some differences between strains isolated from pigs and chickens. However, it could be concluded that a high incidence of resistant strains did exist in these animals in Okinawa, and that R factors were widespread among these resistant strains.

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沖縄の家畜由来細菌の薬剤耐性

Ⅱ　豚および鶏糞便由来薬剤耐性大腸菌のR因子について

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要　　約

沖縄の各地で飼育されている健康な豚および鶏の糞便より大腸菌を分離し，ペニシリン（APC），テトラサイクリン（TC），ストレプトマイシン（SM），クロラムフエニコール（CP），カナマイシン（KM）およびサルフア剤（SA）など6種の薬剤に対する感受性試験を行なった。

さらに耐性菌についてはR因子の検出を試みた。得られた成績は次の通りである。

1. 豚由来152株中147株，96.7％が耐性菌で，しかも多剤耐性菌が全株の83.6％で高率であった。
2. 耐性菌147株中65株，44.2％に伝達可能なR因子が確認された。
   R因子の耐性型は16種にまたがったが，APC-SM-KMおよびTCの2型が全体の過半数を占めた。
3. 鶏由来221株中209株，94.6％が耐性菌であった。多剤耐性菌は79.2％で，うちTC-SM-SAの耐性型を有する3剤耐性菌が100株で、耐性菌の47.8％を示した。
4. 鶏由来の耐性菌209株中76株（36.4％）がR因子を有しており，その耐性型は9型に分類された。これら耐性型のうちTC，SM，TC-SMの3型が全体の76.3％を占めた。
5. 豚および鶏由来大腸菌株間に耐性菌の検出頻度およびR因子の分布の状況に差が認められたが，しかし上記の成績より，沖縄においてもR因子を有する耐性菌が広く分布していることが明らかにされた。

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