Drug Resistant Strains of Bacteria Isolated from Domestic Animals in Okinawa

IV. Drug resistance and R factors of *E. coli* isolated from broiler chicken and pork*

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I INTRODUCTION

In previous report[6], it was demonstrated that drug resistant strains and R factors are widespread among *E. coli* strains isolated from feces of healthy pigs and chickens given foodstuffs containing antimicrobial drugs.

Smith[12,13] reported that R factors from nonpathogenic *E. coli* isolated from feces of healthy calves, pigs and fowls could be transferred to resident *E. coli* in humans. Furthermore, Kim and Stephens (1972) found that more than 10% of drug resistant *E. coli* isolated from ready-to-cook broilers could transfer resistance to a potential human pathogen, *S. typhimurium*. These may support to the idea that R factors-carring strains of *E. coli* associated with farm animals present a potential hazard to human.

On the other hand, Babcock et al. (1973) demonstrated a high incidence of transferable antibiotic resistance in *E. coli* strains isolated from dressed beef and also healthy humans. And they stated the following: If ingested, the R factor-bearing strains of *E. coli* associated with dressed beef could give rise to serious health problems. However, the *E. coli* strains already present in the humans sampled would seem to be even more potentially harmful because these strains tended to be more multiple resistant and were capable of transferring their resistances at higher frequencies than could the beef strains.

In any event, to confirm whether these *E. coli* strains from meat-producing animals serve as reservoirs of R factors for human enteric bacteria, the authors, firstly, surveyed for drug resistance and distribution of R factors among *E. coli* strains isolated from broiler chicken and pork samples.

Additionally, the data obtained here were compared with those of fecal samples of these animals.

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II MATERIALS AND METHODS

1 Isolation of *E. coli*

Ten packaged broiler chicken and 70 pork samples were purchased from retail markets.

Bacteriological sampling was mainly done following the methods used by Kim and Stephens[5]. After the overwrap of each packaged broiler was opened, an area of approximately 2 ～ 4 cm^2 of each site, except for fluid associated with each carcass, was swabbed. One swab was dipped into fluid collected between the container and carcass. The following 8 sites were examined: (1) the fluid, (2) the dorsal skin, (3) the thoracic skin, (4) the inside of the abdominal cavity, (5) the inner site of thigh muscle, (6) the inner surface of the gizzard, (7) the inner site of the heart muscle, and (8) the outer surface of the liver.

On the other hand, each pork sample was cutted sterilely and inner cutted site was swabbed with sterile moistened swab.

The swabs on which the samples were taken were plated directly onto McConkey agar plates. After the McConkey agar plates were incubated overnight at 37°C, 5 colonies having the typical appearance of *E. coli* from each plate were picked and confirmed as *E. coli* by biochemical reactions. Confirmed isolates were then subcultured and stored in cooked meat medium until used.

A total of 325 strains confirmed as *E. coli* in which 169 from chicken and 156 from pork were tested for drug resistance.

2 Drugs and media

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

Brain heart infusion was used as propagating medium for the transfer of R factors. Heart infusion (HI) agar was used for assay of drug resistance. For SA resistance assay, Muller-Hinton medium was used.

3 Tests for drug resistance and for transfer of drug resistance

Drug resistance was determined by the method described previously[6,7]. The strains tolerating 25 μ g/ml of APC, SM, TC, CP and KM or 200 μ g/ml of SA were considered resistant. *E. coli* K-12 ML1410, resistant to NA, was used as the recipient of the R factors. Equal volumes of overnight cultures of the recipient and of the test strains were mixed and incubated at 37°C. After 24 hours of incubation, the mixed culture was spotted on 6 different plates, i.e., HI agar plates containing 50 μ g/ml of NA and 25 μ g/ml of each APC, SM, TC, CP and KM and Muller-Hinton medium containing 50 μ g/ml of NA and 200 μ g/ml of SA. The resistance marker transferred was determined by observing bacterial growth on each selective plate.

III RESULTS

1 Isolation of *E. coli*

One-hundred and sixty-nine strains of *E. coli* were isolated from 54 of 80 chicken samples examined (Table 1).

E. coli isolates could always be obtained from the fluid, dorsal skin and thoracic skin but not from heart muscle.

On the other hand, 156 strains of *E. coli* were isolated from 70 pork samples examined.

Table 1. Isolation frequency of *E. coli* from broiler chickens

Sites examined	No. of samples	Isolation of *E. coli*	
		No. of samples	No. of strains
Fluid	10	10	26
Dorsal skin	10	10	32
Thoracic skin	10	10	27
Abdominal cavity	10	9	30
Thigh muscle	10	3	7
Gizzard	10	4	20
Heart muscle	10	0	0
Liver	10	8	27
Total	80	54 (67.5%)	169

2 Isolation frequency of drug resistant *E. coli*

A total of 325 strains of *E. coli* which were isolated from chicken and pork samples was surveyed for resistance to APC, SM, TC, CP, KM and SA.

As shown in table 2, of the 169 chicken isolates, 153, or 90.5%, were resistance to at least one of the 6 drugs used. TC resistant strains were most frequently (72.8%), followed by SA (59.2%), SM (47.9%) and KM (23.7%) resistant strains. The isolation frequency of APC or CP resistant strains was rather low being 10.7% each. Sixteen strains (9.5%) were sensitive to all 6 drugs used.

On the other hand, out of 156 isolates from pork, 75, or 48.1%, were resistance to one of the 6 drugs tested. This percentage was significantly different from that of chicken isolates. Resistance to TC and SM was most common among pork isolates (39.1% each). Few of the isolates were resistance to APC and CP.

Resistance to SM was most common among pork isolates as described, whereas this resistance was the third most common among chicken isolates. In contrast, resistance to SA, the second most common resistance for chicken isolates, was the fourth most common among pork isolates.

Table 2. Isolation frequencies of individual drug reistances among *E. coli* from meat samples

Drug to which resistant*1	Chicken isolates (169)		Pork isolates (156)	
	No. of strains resistant	Percent frequency	No. of strains resistant	Percent frequency
APC	18	10.7	5	3.2
SM	81	47.9	61	39.1
TC	123	72.8	61	39.1
CP	18	10.7	12	7.7
KM	40	23.7	46	29.5
SA	100	59.2	33	21.2
Total*2	153	90.5	75	48.1

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

*2 Resistance to at least one of the 6 drugs

To make comparison of these data with those of fecal *E. coli* from chickens and pigs, the data reported previously[6] were also tabulated in table 3.

Table 3. Isolation frequencies of individual drug resistances among *E. coli* from fecal samples

Drug to which resistant	Chicken isolates (221)		Pig isolates (152)	
	No. of strains resistant	Percent frequency	No. of strains resistant	Percent frequency
APC	21	9.5	57	37.5
SM	136	61.5	114	75.0
TC	199	90.1	146	96.1
CP	3	1.4	9	5.9
KM	5	2.3	92	60.2
SA	155	70.1	66	43.4
Total	209	94.6	147	96.7

Although order of frequency was similar among *E. coli* isolates obtained from meats and feces in both animals, the percentage of isolates having at least one resistance was significantly higher among *E. coli* isolates from pig feces (96.7%) than among isolates from pork (48.1%). Differences were also found in percentages of resistance to KM among chicken isolates and of resistance to APC among pig isolates.

3 Drug resistance patterns of *E. coli*

Twenty-four different drug resistance patterns were identified among the 153 resistant strains obtained from chicken, however, 74.5% of the isolates tested were grouped into 8 patterns (Table 4). Three of the most commonly found resistance patterns were resistance to SM-TC-SA, SM-TC-KM-SA, and TC. Nearly half of the resistant strains were classified into these 3 resistance patterns.

On the other hand, 7 most frequent of the 17 different resistance patterns exhibited by the 75 resistant pork isolates were also shown in the table 4. The 78.7% of the resistant isolates were fallen into these 7 resistance patterns. In this case, about one-third of the resistant strains had a resistance pattern SM-TC-KM. Multiple resistance to various combinations of SM, TC and SA was usually encountered among chicken and pork isolates.

Table 4. The most frequent resistance patterns exhibited by resistant *E. coli*

Chicken isolates (153)			Pork isolates (75)		
Resistance patterns	No. of strains	Percent frequency	Resistance patterns	No. of strains	Percent frequency
SM-TC-SA	31	20.3	SM-TC-KM	24	32.0
SM-TC-KM-SA	21	13.7	SM-TC-SA	9	12.0
TC	19	12.4	SM-TC-CP-KM-SA	7	9.3
TC-SA	15	9.8	SM-SA	6	8.0
SM-TC	9	5.9	TC	6	8.0
SA	8	5.2	SM-TC-KM-SA	4	5.3
APC	6	3.9	SM-KM-SA	3	4.0
APC-SM	5	3.3			
8 patterns	114	74.5	7 patterns	59	78.7

Table 5 illustrated the distribution patterns of singly and multiply resistant isolates. Among the 169 chicken isolates 45.5% were resistance to 3 or more drugs, and 68.6% were multiple resitance ones. The strains sensitive to all the 6 drugs used, were found in only 9.5%.

In contrast, among the 156 pork isolates, 43.0% were multiple resistance and more than half (51.9%) were sensitive ones. In both isolates, no strains resistant to all 6 drugs were encountered. Although the data were not tabulated the strains tested were all sensitive to NA.

4 Demonstration of R factors and their resistance patterns

All resistant isolates were tested for their ability to transfer their resistance to *E. coli* K-12 ML1410 recipients.

The isolation frequencies of R factors with reference to resistance patterns were presented in tables 6 and 7.

Table 5. Multiple drug resitance of *E. coli*

Resistance to	Chicken isolates (169)			Pork isolates (156)		
	No. of strains	Percent of total isolates	Accumulated percentage	No. of strains	Percent of total isolates	Accumulated percentage
6 drugs	0	0	0	0	0	0
5	2	1.2	1.2	8	5.1	5.1
4	30	17.7	18.9	9	5.8	10.9
3	45	26.6	45.5	38	24.4	35.3
2	39	23.1	68.6	12	7.7	43.0
1	37	21.9	90.5	8	5.1	48.1
0 (sensitive)	16	9.5	100	81	51.9	100
Total	169	100		156	100	

Table 6. Demonstration of R factors in drug resistant *E. coli* isolated from broiler chicken and their resistance patterns

Resistance patterns	No. of strains resistant	No. of R^+ strains*1	Percent*2	Resitance pattern of R factors	No. of strains
APC-SM-TC-SA	2	1	50.0	APC-SM-TC-SM	1
SM-TC-KM-SA	21	2	9.5	SM-SA	2
SM-TC-KM	2	2	100	SM	2
SM-TC-SA	31	9	29.0	SM-TC SM	6 3
TC-CP-SA	3	1	33.3	SA	1
APC-SM	5	5	100	APC-SM	5
SM-TC	9	5	55.6	SM-TC SM	3 2
TC-KM	1	1	100	TC	1
TC-SA	15	2	13.3	TC-SA TC	1 1
SM	3	3	100	SM	3
TC	19	7	36.8	TC	7
Other 13 patterns	42	0	0	–	0
Total	153	38	24.8		38

*1 R^+ strains: R factor-carring strains

*2 No. of R^+ strains per No. of resistant strains

Among the 153 resistant strains obtained from chicken, 38 strains (24.8%) were found to carry R factors. Although the 24 different resistance patterns were identified as described above, the 38 R factors-carrying strains were found among strains belonged to 11 groups of the resistance patterns. Quadruple (APC-SM-TC-SA) resistance pattern of R factor was found in one case, however, other transferable resistance patterns were of double or single. The resistance patterns of R factors of the 38 strains were classified into 8 different patterns. The pattern SM was most common (10 strains) and patterns TC and SM-TC were the second common (9 each). CP or KM resistance was not transferred from any of the resistant chicken isolates.

Table 7. Demonstration of R factors in drug resistant *E. coli* isolated from pork and their resistance patterns

Resistance patterns	No. of strains resistant	No. of R^+ strains*1	Percent*2	Resistance pattern of R factors	No. of strains
SM-TC-CP-KM-SA	7	1	14.3	SA	1
SM-TC-KM-SA	4	2	50.0	SM-KM	1
				TC-KM	1
SM-TC-KM	24	1	4.2	SM-TC	1
SM-TC-SA	9	4	44.4	SM	2
				SA	2
SM-KM-SA	3	1	33.3	SM-SA	1
APC-TC	1	1	100.	APC	1
SM-SA	6	5	83.3	SM-SA	4
				SM	1
TC	6	1	16.7	TC	1
Other 9 patterns	15	0	0	–	0
Total	75	16	21.3		16

See footnotes *1 and *2, table 6

In the case of pork isolates, 16 of the 75 (21.3%) resistant strains transferred a part or all of their resistance patterns to the recipients. As resistance patterns of R factors, the pattern SM-SA was most frequently, followed by SM and SA. CP resistance was not transferred from the 12 CP resistant pork isolates.

IV DISCUSSION

From the previous reports[6,7], the authors indicated a definite association between the drugs fed to the domestic animals and the isolation from their feces of *E. coli* resistant to these drugs and capable of transfering this resistance.

Because R factors could transfer their resistance markers not only from *E. coli* to *E. coli* but also from *E. coli* to other enteric bacteria with no relation to animal species from which these strains isolated, the potential public health significance of R factors is now of important. In fact, some invetigators[8,10,12] showed both direct animal to man transfer and indirect transfer by contaminated foods of animal origin could occur. Therefore, in several countries, including Japan, the practice of feeding some drugs to domestic animals was prohibited[9].

In the present report, the authors surveyed to what extent drug resistant *E. coli* and R factors-carrying *E. coli* were widespread among meat samples of broiler chicken and pork purchased at retail markets. The data obtained were also compared with those of fecal *E. coli* isolated from chickens and pigs. This was done as preliminary experiments to clear forementioned public health implications of R factors-carrying *E. coli* from farm animals given feeds containing antimicrobial drugs.

In the first place, as to broiler chicken samples, 90.5% (153 out of the 169 strains) of the isolated *E. coli* were resistant to at least one of the 6 drugs. This was almost similar with that obtained in chicken fecal *E. coli* (94.6%). The similarlity was also obtained in isolation frequency of multiple drug resistant strains (68.6% in meat and 79.2% in feces). In both cases, *E. coli* resistant to either one or various combinations of SM, TC and SA were frequently observed, particularly, triple (SM-TC-SA) resistant strains were the most common. In contrast, a difference was observed in percentage of KM resistant strains, i.e., 23.7% in meat isolates and 2.3% in fecal isolates. Of the resistant strains, R factors-carrying strains were detected in 24.8% of meat isolates while these were more frequently detected in fecal isolates (36.4%).

A few reports have been made concerning the incidence of drug resistance and of transferable drug resistance in *E. coli* isolated from poultry meat[2,5,14].

Kim and Stephens examined them using 226 *E. coli* strains isolated from ready-to-cook broilers. Among the 226 *E. coli* isolates tested, 96.5% were resistant to 3 or more drugs, and all isolates were resistant to at least one of 10 drugs used. A high percentage of the *E. coli* isolates were resistant to triple sulfa (100%), penicillin (69.9%), dihydrostreptomycin (61.9%) and chlortetracycline (48.2%). They also showed that more than 10% of resistant *E. coli* isolates transferred drug resistance to a susceptible, pathogenic *S. typhimurium*. Incidence of drug resistance and frequency of transfer of drug resistance depends upon the drugs used and methods used by the investigators, therefore, it is difficult to compare results obtained in the present report with those from other reports. However, it can be said that most of *E. coli* from chicken were drug resistance. The incidence of R factors-carrying strains was lower among meat isolates than those of fecal isolates.

As to pork samples, the authors demonstrated that 48.1% of *E. coli* isolates were resistant strains. This was about a half of 96.7% which obtained by ourselves from fecal *E. coli* of pigs. Similar difference was also observed in isolation frequency of multiple drug resistant strains, being

43.0% in meat and 83.6% in feces. The isolation frequency of strains resistant to TC, SM and KM, in descending order, was same in both isolates from meat and feces of pigs. The main difference observed was that the percentage of APC resistant strains was only 3.2% in meat isolates whereas it was 37.5% in fecal isolates. Regarding R factors, 21.3% (16 out of 75) of resistant strains detected from pork were found to carry them but this was less than a half of 44.2% which obtained from fecal *E. coli* isolates of pigs. Transferable KM resistance was demonstrated only in 2 out of 46 (4.3%) KM resistant strains of pork origin, whereas it was detected in 46 of the 92 (50%) KM resistant fecal strains of pigs.

Sato (1974) surveyed for drug resistance and for transferable drug resistance among *E. coli* strains isolated from pork using 6 drugs, the same used in the present study. All of 163 strains isolated were resistant to either one or various combinations of the 6 drugs, and 93% of the resistant strains were of multiple resistance. The highest incidence of drug resistance was encountered to TC (84.7%), SM (83.7%), SA (69.3%) and KM (44.2%). Few of the isolates were resistant to CP (5.5%) and APC (6.1%). The results were similar with those of our present study except the percentages of resistant strains to each drug. A higher percentage was obtained by Sato. Similarly, the incidence of transferable drug resistance among Sato's isolates (about 42%) was much higher than those of us (21.3%). Hence, such data reported by Sato were rather similar to those of our fecal isolates. If we compare the data obtained from broiler chicken and pork, we could find much differences than those found between meat and fecal samples of each animal. Particularly, the difference between meat samples of chicken and pork could be noticed in patterns of drug resistance and their R factors. The data obtained from meat samples of each animal seemed to be closser to those obtained from fecal samples of corresponding animal than those of any sample obtained from uncorresponding animals. Therefore, the fact suggested that the meat samples might be partially contaminated with fecal *E. coli* of same animal during the slaughter process.

As to contamination of carcass with fecal *E. coli* at slaughter, investigations were made by Howe et al.[3,4]. They demonstrated contamination of the abdominal cavity with fecal *E. coli* following slaughter and evisceration at the factory by comparing O-serotypes found in feces with those isolated from abdominal swabs[4]. They also found similar cross-contamination in calf carcass at slaughter[3]. One reason of lower incidence of drug resistance and transferable drug resistance in pork samples might be due to few chance of contaminations with fecal *E. coli* during slaughter and following process. However, the contamination of meat sold at retail markets was also possible from containers, from meat wrapping materials, from handling equipments, and even from the employee themselves.

Anyhow, the fact that meat products sold at retail markets were contaminated with R factors-carrying *E. coli*, is a potential public health problem. Therefore, we should make efforts to reduce R factors-carrying *E. coli* from animals and their environments.

From the data presented here, the authors could not conclude the effect of antibiotics added to animal feeds on occurrence of R factors-carrying *E. coli* and consequently on contamination of meat products with such R factors. Further experiments along this line will be necessary.

V SUMMARY

Three-hundred and twenty-five (325) *E. coli* examined were isolated from packaged broiler chickens and porks purchased from retail markets.

Of the 169 chicken isolates, 153 (90.5%) were resistant to at least one of 6 drugs (APC, SM, TC, CP, KM and SA) tested; TC resistant strains were most frequently (72.8%), followed by SA (59.2%), SM (47.9%) and KM (23.7%) resistant strains. Three of the most commonly found resistance patterns were SM-TC-SA, SM-TC-KM-SA and TC.

Out of the 153 resistant strains, 38 (24.8%) transferred a part or all of their resistance patterns to sensitive recipients. Most commonly encountered pattern of R factors was SM, followed by TC and SM-TC.

On the other hand, of the 156 pork isolates, 75 (48.1%) were resistant to one or more of the 6 drugs tested; 61 (39.1%) showed resistant to TC and SM, 46 (29.5%) to KM and 33 (21.2%) to SA. About one-third of the resistant strains had a triple (SM-TC-KM) resistance pattern.

R factors were demonstrated in 16 strains (21.3%). SM-SA was most frequently encountered pattern of R factors, followed by SM and SA.

Possibility of carcass contamination with drug resistant fecal *E. coli* of same animal was discussed.

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沖縄の家畜由来細菌の薬剤耐性

Ⅳ. 鶏肉および豚肉より分離された薬剤耐性大腸菌とそのR因子

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要　　約

畜産食品のR因子保有大腸菌による汚染状況を調べる目的で，市販の包装された鶏肉および豚肉を対象に大腸菌を分離し，APC，SM，TC，CP，KM，SAの6剤を用いて，薬剤耐性試験と，耐性菌についてはR因子の検索を行った。

1. 鶏肉由来169株中153株，90.5％が何れかの薬剤に耐性を示す耐性菌であった。

個々の薬剤別の耐性率は，TCで最も高く72.8％で，次いでSA（59.2％），SM（47.9％），KM（23.7％）の順であった。

耐性型ではSM-TC-SAの3剤型，SM-TC-KM-SAの4剤型およびTCの単剤型が多く，これら3型で全耐性菌の46.4％を占めた。

2. 耐性菌153株中38株，24.8％にR因子が確認され，伝達耐性型としてはSM，TC或いはSM-TC型が主で，SA型は比較的少なかった。

3. 豚肉由来156株中，耐性菌は75株，48.1％に認められた。薬剤別ではTC或いはSM耐性菌が多く，何れも39.1％で，次いでKM（29.5％），SA（21.2％）の順に検出された。

耐性型としては，SM-TC-KMの3剤耐性型が多く，この型だけで全耐性菌の1/3を占めている。

4. R因子は耐性菌中21.3％，16株に検出されたが，その耐性型はSM-SA，SA，SMが主であった。

5. 上記肉由来菌の成績を対応する動物の糞便由来大腸菌のそれと比較し，食肉の糞便由来菌による汚染，特に薬剤耐性を伝達するR因子保有大腸菌による汚染の可能性について考察した。

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