沖縄の家畜由来細菌の薬剤耐性：III
牛,山羊および馬糞便由来薬剤耐性大腸菌とその R 因子について(畜産学科)

Drug Resistant Strains of Bacteria Isolated from Domestic Animals in Okinawa

Ⅲ. Drug resistance and distribution of R factors among facal *E. coli* strains isolated from cattle, goats and horses*

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I INTRODUCTION

In previous reports (4, 5), the authors demonstrated high incidence of drug resistance and transferable drug resistance among fecal *E. coli* strains isolated from healthy pigs and chickens in Okinawa. These animals were usually fed with commercial feeds which contained antimicrobial drugs for prevention of diseases and for nutritional purposes.

Several reports (2, 3, 7～10) have suggested that the emergence of the drug resistant fecal *E. coli* strains may occur as a result of the continuous feeding of the antimicrobial drugs to such farm animals.

To make clear the above relation, fecal *E. coli* strains isolated from cattle, goats and horses were used for investigations of drug resistance.

These farm animals in Okinawa were not fed with commercial feeds containing antimicrobial additives.

The present report deals with a survey of drug resistance and transferable drug resistance of fecal *E. coli* strains isolated from cattle, goats and horses. The data obtained were also compared with those of pigs and chickens.

II MATERIALS AND METHODS

1 Bacterial strains

The strains of *E. coli* used were isolated from fecal samples of apparently healthy cattle, goats and horses. These farm animals were usually not fed with commercial feeds supplemented with antimicrobial drugs but fed with grass and bran.

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A total of 986 strains, 405 from cattle, 371 from goats and 210 from horses, were used.

E. coli K-12, ML 1410 strain, resistant to NA, was used as a recipient of R factor.

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. McConkey agar (Eiken) was used for the isolation of *E. coli* strains. The strains isolated were purified and stored in cooked meat medium (Eiken). Brain heart infusin broth (BBL) or tryptosoy broth (Eiken) was used for liquid culture. Brain heart infusion agar was used for the determination of drug resistance. For the SA-resistance assay, Muller-Hinton medium (BBL) was used.

3 Tests for drug resistance and transfer of drug resistance

The methods used were described in previous reports (4, 5). Drug resistance of each strain was determined by the agar dilution method. The resistance level was expressed as the maximum concentration of the drugs which allowed bacterial growth. The strains tolerating 25 $\mu g/ml$ of APC, SM, TC, CP, and KM or 200 $\mu g/ml$ of SA were considered resistant.

Brain heart infusion agar containing 50 $\mu g/ml$ of NA and indicated concentration of each of selective drugs was used as a selective medium for the transfer of R factor to recipient strain, ML 1410.

The resistance marker transferred was determined by observing bacterial growth on each selective medium.

III RESULTS

1 Isolation frequency of drug resistant strains and their resistance patterns

A total of 986 *E. coli* strains, 405 from cattle, 371 from goats and 210 from horses were surveyed for resistance to APC, SM, TC, CP, KM and SA. Table 1 showed the drug resistance patterns and their isolation frequencies of the strains.

Of 405 cattle isolates, 312 strains (77.0%) were sensitive to all 6 drugs used, and 93 strains (23.0%) were resistant to one or more drugs.

Twenty-two different resistance patterns were found among the 93 resistant strains. Single resistant strains were isolated most frequently (49.5%), followed by double (26.9%), triple (10.8%) and quadruple (10.8%) resistant strains. Quintuple resistant strains were isolated 2.2% but no sextuple resistant strain was isolated. The most frequently isolated resistance patterns were TC (29%), followed by SA (12.9%), TC-SA (7.5%), APC-SA (6.5%), SM-TC (6.5%) and APC-SM-TC-SA (5.4%). The isolation frequencies of other patterns were below 5%.

Of 371 goat isolates, 298 strains (77.6%) were sensitive and other 83 strains (22.4%) were resistant. Twenty-two different resistance patterns were observed.

Table 1. Drug resistance patterns and their isolation frequencies of fecal E. coli strains isolated from cattle, goats and horses

	Resistance pattern*	Cattle isolates (405)			Goat isolates (371)			Horse isolates (210)		
		No. of strains	Per cent**		No. of strains	Per cent**		No. of strains	Per cent**	
6	APC SM TC CP KM SA	0	0		1	1.2		0	0	
5	APC SM TC CP SA	0	0		0	0		1	1.6	
	APC SM TC KM SA	2	2.2	2.2	0	0	3.6	0	0	1.6
	SM TC CP KM SA	0	0		3	3.6		0	0	
4	APC SM TC SA	5	5.4		0	0		0	0	
	APC TC CP SA	0	0		2	2.4		0	0	
	APC TC KM SA	0	0		1	1.2		0	0	
	SM TC CP SA	2	2.2	10.8	1	1.2	4.8	1	1.6	3.2
	SM TC KM SA	3	3.2		0	0		0	0	
	SM CP KM SA	0	0		0	0		1	1.6	
3	APC SM TC	2	2.2		0	0		0	0	
	APC SM CP	1	1.1		1	1.2		0	0	
	APC SM SA	1	1.1		1	1.2		0	0	
	APC CP SA	0	0		3	3.6		0	0	
	SM TC CP	1	1.1	10.8	0	0	14.5	0	0	14.7
	SM TC KM	2	2.2		4	4.8		1	1.6	
	SM TC SA	2	2.2		3	3.6		7	11.5	
	SM CP SA	0	0		0	0		1	1.6	
	TC KM SA	1	1.1		0	0		0	0	
2	APC SA	0	0		4	4.8		1	1.6	
	APC TC	0	0		1	1.2		0	0	
	APC CP	1	1.1		0	0		0	0	
	APC KM	1	1.1		0	0		1	1.6	
	APC SA	6	6.5		4	4.8		8	13.1	
	SM TC	6	6.5	26.9	6	7.2	28.9	2	3.3	27.8
	SM CP	0	0		1	1.2		0	0	
	SM SA	4	4.3		3	3.6		3	4.9	
	TC SA	7	7.5		1	1.2		2	3.3	
	CP SA	0	0		4	4.8		0	0	
1	APC	2	2.2		9	10.8		8	13.1	
	SA	2	2.2		0	0		2	3.3	
	TC	27	29.0		12	14.5		3	4.9	
	CP	0	0	49.5	2	2.4	47.0	0	0	52.4
	KM	3	3.2		0	0		0	0	
	SA	12	12.9		16	19.3		19	31.1	
Total of resistance		93	100 (23.0)		83	100 (22.4)		61	100 (29.0)	
Sensitive		312	(77.0)		288	(77.6)		149	(71.0)	
Total of tested		405	(100)		371	(100)		210	(100)	

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

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

Among resistant strains, single resistant strains were most frequently encountered (47.0%), followed by double (28.9%), triple (14.5%), quadruple (4.8%) and quintuple (3.6%). The strain resistant to all six drugs used was also isolated.

The most frequently isolated resistance patterns were SA (19.3%), TC (14.5%), APC (10.8%), and SM-TC (7.2%). Other resistance patterns were isolated less than 5%.

On the other hand, out of 210 strains isolated from horses, 149 strains, or 70.9%, were sensitive ones and other 61 strains, or 29.1%, were resistant to one or more drugs. These 61 resistant strains were fallen into 16 different resistance patterns. Of them, SA resistance pattern was most frequently isolated (31.1%), followed by APC (13.1%), APC-SA (13.1%), and TC-SM-SA (11.5%).

Similarly, single resistant strains were most frequently encountered (52.4%) and following descending orders: double (27.8%), triple (14.7%), quadruple (3.2%) and quintuple (1.6%) resistant strains.

As was clear from the table, the strains with single resistance pattern, especially resistant to SA or TC, were similarly found most frequently among resistant strains isolated from 3 animal species.

In table 2, above date were simply summarized. The multiply resistant strains were relatively fewer in number among strains of any animal origins. In contrast, the sensitive strains were found more than 70%.

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

Resistance pattern	Cattle isolates			Goat isolates			Horse isolates		
	No. of strains resistant	Per-cent	Accumulated percentage	No. of strains resistant	Per-cent	Accumulated percentage	No. of strains resistant	Per cent	Accumulated percentage
Sextuple	0	0	0	1	0.3	0.3	0	0	0
Quintuple	2	0.5	0.5	3	0.8	1.1	1	0.5	0.5
Quadruple	10	2.5	3	4	1.1	2.2	2	1.0	1.5
Triple	10	2.5	5.5	12	3.2	5.4	9	4.3	5.8
Double	25	6.2	11.7	24	6.5	11.9	17	8.1	13.9
Single	46	11.4	23.1	39	10.5	22.4	32	15.2	29.1
Sensitive	312	77.0	100	288	77.6	100	149	71.0	100
Total	405	100		371	100		210	100	

2 Demonstration of R factors and their resistance patterns

The demonstration of R factors among the drug resistant, *E. coli* strains was examined.

The isolation frequencies of R factors with reference to resistance patterns were shown in tables 3, 4 and 5.

Among 93 resistant strains isolated from cattle, 12 strains (12.9%) were found to carry R factors (table 3). These 12 strains were fallen into 6 resistance patterns and transferred part or all of their resistance patterns to the recipient strain.

Five quadruple resistant strains with pattern APC-SM-TC-SA transferred all of their resistance patterns and also 3 of 6 double resistant strains with pattern SM-TC transferred all of their SM-TC resistance markers.

The resistance patterns of R factors of these 12 strains were classified into 4 different patterns. The pattern APC-SM-TC-SA was most frequently encountered, and pattern SM-TC was the second.

Table 3. Demonstration of R factors in drug resistant *E. coli* strains isolated from cattle and their resistance patterns

Resistance patterns	No. of strains resistant	No. of R^+ strains*1	Per-cent*2	Resistance pattern of R factor	No. of strains	Per-cent*3
APC SM TC SA	5	5	100	APC SM TC SA	5	41.8
APC SM TC	2	1	50	SM TC	1	
SM TC KM	2	1	50	SM KM	1	8.3
SM TC SA	2	1	50	SA	1	33.3
SM TC	6	3	50	SM TC	3	
SA	12	1	8.3	SA	1	16.6
Other 16 patterns	64	0	0	-		
Total	93	12	12.9*4		12	100

*1 R^+ strain: R factor-carrying strain

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

*3 No. of strains with indicated resistance markers per total number of R^+ strains, 12

*4 Total No. of R^+ strains per total No. of resistant strains

Nextly, of the 83 drug resistant strains isolated from goats, 9, or 10.8% were found to transfer part or all of their resistance to the recipient strain (table 4).

As shown in the table, all 4 resistant strains with APC-SM resistance markers transferred all of their markers. The quadruple resistant strain transferred only TC resistance marker.

The resistance patterns of R factors of the 9 strains were classified into 5 different patterns in which APC-SM was superior in numbers.

In the case of horse isolates, 6 (9.8%) out of the 61 resistant strains transferred their resistance patterns to the recipient, ML 1410 strain (table 5).

As shown in the table, these 6 R factor-carrying strains were a part of 13 resistant strains which were fallen into 4 indicated different resistance patterns and their resistance patterns of R factors were classified into 4 different ones.

Table 4. Demonstration of R factors in drug resistant *E. coli* strains isolated from goats and their resistance patterns

Resistance patterns	No. of strains resistant	No. of R^+ strains*1	Per-cent*2	Resistance pattern of R factor	No. of strains	Per-cent*3
APC TC KM SA	1	1	100	TC	1	
APC SM	4	4	100	APC SM	4	44.4
APC SA	4	1	25	APC	1	11.1
SM TC	6	2	33.3	SM TC	1	11.1
				SM	1	11.1
TC	12	1	8.3	TC	1	22.2
Other 17 patterns	56	0	0			
Tatal	83	9	10.8*4		9	100

See footnotes *1, *2, *3 and *4, table 3.

Table 5. Demonstration of R factors in drug resistant *E. coli* strains isolated from horses and their resistance patterns

Resistance patterns	No. of strains resistant	No. of R^+ strains*1	Per-cent*2	Resistance pattern of R factor	No. of strains	Per-cent*3
SM TC KM	1	1	100	SM TC	1	16.7
SM TC SA	7	3	42.9	SM SA	1	16.7
				SA	2	33.3
SM TC	2	1	50	SM	1	33.3
SM SA	3	1	33.3	SM	1	
Other 12 patterns	48	0	0			
Total	61	6	9.8*4		6	100

See footnotes *1, *2, *3, and *4, table 3.

3 Frequency of resistance and R factor mediated resistance to individual drugs

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 405 strains of cattle-isolates, 93 (23%) were resistant to either one of the 6 drugs used. Of which, 61 strains were TC-resistant and 45 strains were SA-resistant. These 2 types of resistance patterns were superior in number.

In the case of goat-isolates, 83 (22.4%) of 371 strains were drug resistant. A high percentage of resistance was noted for SA, TC, APC and SM, being 11.9, 8.6, 7.3 and 7.3%, respectively.

On the other hand, of 210 strains isolated from horses, 61, or 29.1%, were strains resistant to, at least, one of the 6 drugs. The highest isolation frequency was obtained in SA, being 20.5%. Following sequence of occurrence was SM (9.5%), APC (9.1%) and TC (8.1%).

Table 6. Frequency of resistance and R factor-mediated resistance to individual drugs

Drug to which resistant	Cattle isolates (405)				Goat isolates (371)				Horse isolates (210)			
	No. of resistant strains	Percent*1	No. of R^+ strains	Percent*2	No. of resistant strains	Percent*1	No. of R^+ strains	Percent*2	No. of resistant strains	Percent*1	No. of R^+ strains	Percent*2
APC	20	4.9	5	25.0	27	7.3	5	18.5	19	9.1	0	0
TC	61	15.1	4	6.6	32	8.6	3	9.4	17	8.1	1	5.9
SM	32	7.9	10	31.3	27	7.3	6	22.2	20	9.5	4	20.0
CP	4	1.0	0	0	18	4.9	0	0	4	1.9	0	0
KM	12	3.0	1	8.3	9	2.4	0	0	3	1.4	0	0
SA	45	11.1	7	15.6	44	11.9	0	0	43	20.5	1	2.3
Either one*3	93	23.0	12	12.9	83	22.4	9	10.8	61	29.1	6	9.8

* 1 Number of resistant strains per number of total strains tested

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

* 3 Resistant to either one of the 6 drugs used

Regardless of their origins, SA-resistant *E. coli* strains were isolated most frequently, followed by TC- or SM-resistant strains.

In contrast, KM- and CP-resistant strains were fewer in numbers. Though the data were not tabulated, all strains tested were sensitive to NA at concentration of 50 $\mu g/ml$.

As for the R factors, SM- and APC-resistant strains of cattle- and goat-origins transferred their resistance markers, SM or APC, with higher percentages. In the case of horse-origin, SM-resistant ones also transferred highly, but no transfer was noted in APC-resistant strain.

The SA-resistant strains were equally isolated most frequently from any animal species but their transferbilities were somewhat different, namely, 15.6% in cattle-origin and 2.3% in horse-origin, whereas 0% in goat-origin.

IV DISCUSSION

The *E. coli* strains isolated from feces of healthy cattle, goats and horses were examined for drug resistance and transferable drug resistance. These animals were usually fed with grass and bran in Okinawa.

As expected, the results obtained in the present study were different with those of previous reports (4, 5) in which fecal *E. coli* strains isolated from pigs and chickens which were usually fed with commercial feeds containing antimicrobial drugs were tested. Namely, the percentages of drug resistant strains; 23.0 in cattle-, 22.4 in goat- and 29.1% in horse-isolates, were less than one-third in comparison with those of pigs (96.7%) and chickens (94.6%). Furthermore, even among resistant strains isolated from the former animals, single resistant strains were most frequently encountered (about 50%) and followed by double-, triple- and quadruple-resistant strains. In contrast, the majority of drug resistant strains of the latter animals were multiple drug resistance.

Isolation frequency of resistance to TC and SA was high in cattle- and goat-isolates, and to SA and SM in horse-isolates; whereas, isolation frequency of resistance to CP and KM was low in any animal-isolates.

Among these resistant strains, R factor-carrying strains were found 12.9 in cattle-, 10.8 in goat- and 9.8% in horse-isolates, respectively. These data were also lower than 44.2 in pig- and 36.4% in chicken-isolates.

High incidence of R factors was obtained in following cases: all 5 quadruple-(APC-SM-TC-SA) resistant strains isolated from cattle transferred all of their resistance patterns to the recipient strain, all 4 double- (APC-SM) resistant strains of goats transferred their APC-SM resistance patterns and 3 of 7 triple-(SM-TC-SA) resistant strains of horses transferred all or part of their resistance patterns. It was an interest to know that the strains of each 3 above mentioned cases were isolated from animals of same farm.

In regard to the individual drugs used, SM-resistant strains of any animal origin, were found to carry R factors with a high percentage. The APC-resistant

strains isolated from cattle and goats also carried R factors in high proportion but no APC-resistant strains isolated from horses had R factors. The CP-resistant strains were isolated rarely and they had no R factors. Among the KM-resistant strains, only 1 strain isolated from cattle carried R factor.

Though isolation frequency of resistance to SA was relatively high, distribution of R factors among these strains was low.

The data obtained in the present study were nextly compared with those of other investigators. But few reports concerning to drug resistance fecal *E. coli* strains isolated from cattle, goats and horses, were seemed to be published.

Suzuki et al. (10) reported that of the 62 cattle examined, 21 (34%) harbored drug resistant *E. coli* strains neither from 4 sheep nor from 14 goats. Of 21 cattle carrying resistant *E. coli* strains, 9 animals (43%) excreted *E. coli* strains harboring R factors.

Terakado et al. (12) surveyed for drug resistance and R factors among *E. coli* strains isolated from pigs, cattle and human being. According to their results, of the 136 strains from cattle, 124 (91%) were sensitive to 13 drugs used, and only 12 (9%) were drug resistant strains. Majority of the 12 resistant strains had TC-resistance in their resistance patterns. R factor-carrying strains were found in 7 (58.3%) out of the 12 resistant strains. Percentages of drug resistant strains were low in cattle-isolates than those of pig and human-isolates, whereas percentages of R factor-carrying strains were high in cattle-isolates.

Tajima et al. (11) reported that among 69 fecal *E. coli* strains isolated from horses, 10 were drug resistance, mostly of resistant to TC. But they did not examined for R factors.

From the data of the present authors and others (10～12) mentioned above, it could be said that the drug-resistant *E. coli* strains were not so widespread among cattle, goats and horses which had not usually been fed on drug-containing feeds. Judging from another point of view, these surveys indicated a definite association between the drugs applied to the animals as feed additives and tne isolation from their feces of *E. coli* strains resistant to these drugs. 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 usages. These drugs were commonly used in Japan(1).

The effect of the use of antimicrobial drugs, particularly as feed additives, on the emergence of drug-resistant *E. coli* strains in animals has been reviewed by Smith (7).

Smith and Crabb (8) studied the effect of the continuous feeding of diets containing TC on the *E. coli* population of the alimentary tract of pigs and fowls. All the fecal *E. coli* from the animals in the herds in which diets containing TC were fed were TC-resistant. By contrast, TC-resistant strains either were absent or formed only small proportion of the total *E. coli* in the animals in herd in which TC feeding had not been practiced. The similar results have been confirmed by other workers for calves as well as for pigs and fowls (2, 3, 11).

However, the authors, in the present study, demonstrated 22.4% of drug-resistant strains isolated from goats which were never fed on drug-containing feeds and also never treated with drugs. Therefore, other indirect factors should participate in appearrance of drug resistant strains. In any event, however, there was little doubt that the continuous feeding of animals on drug-containing feeds had contributed significantly to the high incidence of drug resistant bacteria, so that the use of antimicrobial drug in feeds should be controlled by regulations.

V SUMMARY

A total of 986 fecal *E. coli* strains isolated from healthy cattle, goats and horses were examined for drug resistance and distribution of R factors. These animals were usually never fed with antimicrobial drug-containing feeds in Okinawa.

Of 405 cattle-isolates, 93, or 23.0%, were resistant to one or more drugs used. Thirteen percent (12 strains) of the reistant strains transferred all or a part of their resistance patterns to the sensitive recipient strain. Of the 93 resistant strains, 49.5% were singly resistant and 50.5% were multiply resistant strains. The most common resitant patterns were TC (27 of 93), SA (22 of 93) and TC-SA (7 of 93).

Of 371 strains isolated from goats, 83 (22.4%) were found to be resistant. Among the 83 resistant strains, 39, 24, 12, 3 and 1 strains were single-, double-, tripl-, quadruple- and quintuple-resistant, respectively. The SA-resistant strains were isolated most frequently followed by TC- and APC-resistant ones in decreasing order. The R factors were demonstrated at a frequency of 10.8% (9 strains) among 83 resistant strains.

Of 210 horse-isolates, 61, or 29.1%, were resistant strains. Among them, 19, 8 and 8 strains were SA-, APC-SA- and APC-resistant ones, respectively. Six (9.8%) of the 61 resistant strains were found to carry R factors.

In any cases, R factors were demonstrated mostly among multiple resistant strains, particularly, among those possessing SM-reistance pattern. None of CP-resistant strains was found to carry R factors.

These results indicated that drug resistant strains and R factors were not so widespread among the animals which were usually not fed with antimicrobial drug-containing feeds.

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沖縄の家畜由来細菌の薬剤耐性

Ⅲ 牛、山羊および馬糞便由来薬剤耐性大腸菌とそのR因子について

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要約

一般に抗菌剤を含まない飼料で飼育されている健康な牛，山羊および馬の糞便より分離した大腸菌計986株を対象に，APC，SM，TC，CP，KM，SAの6剤を用いて，薬剤耐性試験とR因子の検索を行なった。

1. 牛由来405株中93株，23.0％が何れかの薬剤に耐性を示す耐性菌であった。耐性菌中，約半数の46株が単剤耐性菌で，多剤耐性菌の占める率は比較的低い。耐性パターンではTC（27株），SA（12），TC-SA（7）が主で，これら3型で耐性菌の半数を占めた。

2. 耐性菌93株中12株，12.9％にR因子が確認され，SMあるいはAPCを耐性型に持つ多剤耐性菌に比較的高率に検出された。

3. 山羊由来371株中83株，22.4％が耐性菌で，うち39株は単剤，24株は2剤耐性菌であった。主たる耐性パターンはSA，TCおよびAPCの単剤型であったが，使用した6剤すべてに耐性の株も1株分離された。耐性菌83株中R因子を確認できたのは9株で，10.8％に相当した。

4. 馬由来株では，210株中61株，29.1％が耐性菌で，耐性パターンとしてはSA単剤型が最も多く，次いでAPC-SA，APC型が多く検出された。耐性菌の52.4％が単剤耐性菌である。

R因子は6株，9.8％に検出され，これらは何れも多剤耐性菌に属した。

5. 上記3家畜由来株に共通した点は，耐性菌が30％以下，またR因子の検出率も13％以下で共に比較的低率であり，さらに耐性菌の中でも単剤耐性菌が約50％，2剤耐性菌が約25％を占め，多剤耐性菌が少ないことであった。

薬剤別にみると，SAに対する耐性菌が最も多く，次いでTC，SM，APCの順で，逆に，CPおよびKMに対する耐性菌は比較的少数であった。

6. 以上の成績を，抗菌剤添加飼料で飼育されている豚および鶏より分離した大腸菌についての，前報の成績と比較し，考察を試みた。

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