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Citation	Japanese Journal of Veterinary Research, 22(3), 72-79
Issue Date	1974-07
DOI	10.14943/jjvr.22.3.72
Doc URL	http://hdl.handle.net/2115/2044
Type	bulletin (article)
File Information	KJ00002371159.pdf



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APPEARANCE OF R-FACTOR-MEDIATED DRUG
RESISTANCE IN *SALMONELLA TYPHIMURIUM*
EXCRETED BY CARRIER CALVES ON A FEEDLOT*

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(Received for publication, February 14, 1974)

A group of 36 calves, most of which were excreting *Salmonella typhimurium* sensitive to tetracycline (TC), were brought to a feedlot on which a high dose of chlortetracycline was fed. Four of the calves excreted the organism resistant to TC once or repeatedly during a period from 8 to 20 days after introduction. R factors were detected from the TC-resistant *S. typhimurium* strains. Of the strains, 6 from 2 calves had the R factor conferring TC-resistance, which was detected only by mixed cultivation at room temperature (about 25°C).

INTRODUCTION

It is generally accepted that the administration of antibacterial drugs to human or animals results in the selection or survival of resistant bacteria and increases the incidence of multiple drug resistant bacteria carrying an R factor. As reviewed by SMITH⁹⁾, the remarkably sharp increase in the incidence of drug resistant strains of *Salmonella typhimurium* in calves in Britain in 1960's coincided with the rapid development of the intensive system of calf rearing. The system is not only conducive to the spread of Salmonella among the calf population, but is one in which many different antibacterial drugs are used on a large scale. Thus it has been thought that an intensive calf rearing unit or feedlot plays an important role in the emergence and spread of drug resistant bacteria. Moreover, increases in the incidence of drug resistant *Escherichia coli*, especially the organism with R factors, in calves given different drugs in calf rearing units were reported^{3,4)}. However, little information has been given on the change in the incidence of drug resistant *S. typhimurium* carrying R factor in calves fed antibacterial drugs during their stay on the unit. LOKEN et al. could not indicate the in vivo acquisition of the R factor by *S. typhimurium* var. *copenhagen* in carrier calves brought into a commercial veal-rearing unit, where the prevalence of antibiotic resistance in cultures of *E. coli* isolated from

* Presented at the 76th Meeting of the Japanese Society of Veterinary Science, Oct. 15~16, 1973, Kagoshima-shi

the calves fed neomycin was relatively high and a large number of the cultures had R factors. With the exception of streptomycin, *S. typhimurium* var. *copenhagen* strains were quite sensitive to the drugs tested.

This paper deals with the appearance of R-factor-mediated drug resistance in *S. typhimurium* excreted by carrier calves 8 days after having been introduced into a feedlot. The calves excreted tetracycline (TC)-sensitive *S. typhimurium* at admission and were fed chlortetracycline (CTC) after admission. In addition, the paper gives an interesting finding that a certain type of R factor in *S. typhimurium* was not detected by mixed cultivation made at 37°C, but at room temperature (about 25°C).

MATERIALS AND METHODS

Salmonella isolation from rectal swabs of calves

Rectal swabs were sampled from calves of male Holstein-Friesian on arrival at a feedlot in Hokkaido and thereafter a number of the calves were sampled occasionally. However, the calves from Shizuoka and Shiraoui shown in table 1 were sampled repeatedly. The swabs in 15 ml of selenite brilliant green broth (Nissan) were incubated at 43°C overnight. The broth cultures were subcultured onto brilliant green agar plates (Eiken). Salmonella-like colonies on the plates were examined biochemically and serologically. Biotyping was made by the procedure of BRANDIS. The isolates were kept mainly on Dorset's egg medium (Nissan).

Drug sensitivity test

First, the sensitivity of Salmonella strains to antibacterial drugs was determined by the single disc method. The cultures were screened for drug resistance with the following discs (Showa), using sensitivity test agar (Eiken); oxytetracycline (OTC) 200 µg, streptomycin (SM) 50 µg, chloramphenicol (CP) 100 µg, kanamycin (KM) 50 µg, aminobenzyl penicillin 30 µg, furazolidone 20 µg, and sulfadimethoxine (SA) 400 µg. Then the minimal inhibitory concentration (MIC) of all Salmonella strains were examined by the agar dilution method using tetracycline hydrochloride (TC) (Banyu), streptomycin sulfate (Pfizer), sulfadimethoxine (Daiichi) and nalidixic acid (NA) (Daiichi). Heart infusion agar (Eiken) was used for the test, but Mueller Hinton agar (Eiken) was used for the test with SA. A strain was recorded as resistant when growth was not inhibited by the following drugs of specified concentration on the plates; SM (25 µg/ml), TC (25 µg/ml), SA (200 µg/ml) and NA (50 µg/ml).

Detection of the R factor

The detection of the R factor was made according to TERAKADO et al.¹⁰ *E.*

coli ML 1410 (NA-resistant, methionin-requiring F⁻ derivate of K-12) was used as a recipient. *E. coli* W 3630 (maltose-nonfermenting) was used as the secondary recipient from the converted recipient (MLR⁺). Each of Salmonella strains isolated (donor) was cultivated in brain heart infusion (BHI) broth (Eiken) at 37°C for 18 hr. The ML 1410 strain was cultured in a similar way. Two ml of the broth in a test tube was inoculated with 0.2 ml of each donor broth culture and an equal amount of the recipient culture. The mixture was incubated at 37°C for 18 hr. A loopful of each mixed culture was subcultured on a selective agar plate containing NA (50 µg/ml) and one of the following 3 drugs, TC (25 µg/ml), SM (25 µg/ml) and SA (200 µg/ml). In this experiment, a heart infusion agar was used for TC, Mueller Hinton agar for SA, and DHL agar (Eiken) for SM. The heart infusion or Mueller Hinton agar was added 4 ml of 0.2 % BTB solution and 1.5 g of lactose per 100 ml.

The selective media subcultured were incubated at 37°C for 1 or 2 days. The resistance marker transferred was determined by observing colonies grown on each selective medium. To confirm converted recipients and determine their resistance pattern, 3 colonies of the MLR⁺ on each selective medium were purified and examined for resistance to the drugs applied. When the *E. coli* W 3630 was used as the second recipient from MLR⁺, AL agar²⁾ added each drug in the absence of NA was used as selective agar. The inoculated plates were incubated at 37°C for 2 days. The resistance pattern transferred was checked similarly.

RESULTS

Salmonella status of the feedlot

Outbreaks of clinical *S. typhimurium* infection in calves and occasional isolations of the organism from carrier calves were reported on the feedlot in 1970 and 1971. In 1972, about 1,000 male Holstein-Friesian calves were kept permanently on the feedlot, but no Salmonella was isolated from the rectal swabs of 1,048 calves newly introduced and 59 calves diseased or culled. Samples from calf milk replacer and the environment (pen floor, drain, troughs and soil in paddocks) did not give Salmonella. On this feedlot, newly introduced calves (about 1 week old) were housed for about one month by groups of about 5 calves in each pen with a concrete floor covered with new sawdust. Each of the calves introduced was daily given about 250 mg of CTC during the first month. The antibiotic was used as a feed additive and a drug for prophylaxis.

In February and March of 1973, 2 groups of 36 and 3 calves respectively were introduced to the feedlot and a total of 36 calves of both groups (34 and 2 calves each) gave *S. typhimurium* at or after admission, as indicated in table 1.

TABLE 1 Isolation of *S. typhimurium* from calves and calf milk replacer and its drug resistance

SOURCE OF CALVES	SHIZUOKA (34)* ¹							SHIRAOI (2)		MILK REPLACER	TOTAL
Days after admission of calves, on which Salmonella isolation was made	0	8	12	14	16	18	20	0	4	.	
No. of strains tested	33	4	1	2	2	1	1	1	1	1	47
MIC of tetracycline* ²											
1.56	33* ³	0	0	0	0	0	0	0	0	0	33
3.13	0	0	0	1	0	0	0	1	1	0	3
6.25	0	0	0	0	0	0	0	0	0	1	1
:											
400	0	4	1	1	2	1	1	0	0	0	10
Drug resistance pattern* ⁴											
TC-SM-SA	0	4	1	1	2	1	1	0	0	0	10
SM-SA	30	0	0	1	0	0	0	1	1	1	34
SA	2	0	0	0	0	0	0	0	0	0	2
not resistant	1	0	0	0	0	0	0	0	0	0	1
No. of strains carrying R factor	0	4	1	1	2	1	1	0	0	0	10

*¹ No. of excretors*² Minimal inhibitory concentration ($\mu\text{g/ml}$) (Data of other drugs are excluded.)*³ No. of strains*⁴ Results obtained by the agar dilution methodRecorded as resistant when the growth was not inhibited by the following drugs of specified concentration on the plates; TC (25 $\mu\text{g/ml}$), SM (25 $\mu\text{g/ml}$), SA (200 $\mu\text{g/ml}$) and NA (50 $\mu\text{g/ml}$)

Also, *S. typhimurium* was isolated from a milk replacer sample. However, epidemiological examination did not indicate further transmission of the Salmonella on the feedlot.

Drug sensitivity and other characteristics of Salmonella strains

All Salmonella strains shown in table 1 had 0-5 antigen and belonged to biotype 1. They utilized d-tartrate.

The drug sensitivity test with a disc indicated that most of the strains were resistant to SM and SA, and some of them to OTC, too. Next, the MIC of the Salmonella strains were examined for TC, SM, SA and NA. In table 1 only details of MIC data of TC are given. As shown in the table, only 10 of the 11 strains isolated from 4 calves from Shizuoka 8~20 days after having been brought to the feedlot were resistant to TC (MIC=400 $\mu\text{g/ml}$). Also, 44 of the

47 *Salmonella* strains shown in the table were resistant to SM; MIC=800 $\mu\text{g/ml}$ (19 strains) and >1,600 $\mu\text{g/ml}$ (25 strains). Forty-six of the 47 strains were resistant to SA; 800 $\mu\text{g/ml}$ (1 strain) and >1,600 $\mu\text{g/ml}$ (45 strains). All of the strains tested were judged to be susceptible to NA (MIC=1.56~6.25 $\mu\text{g/ml}$).

Detection of R factors from *S. typhimurium* strains

As can be seen in table 1, R factors were detected from 10 strains of the resistance pattern of TC-SM-SA, which were isolated from calves 8 days or later after introduction. The resistance pattern of the R factor of 4 of the 10 strains was TC-SM-SA. MLR⁺ detected from the 4 strains on TC-selective medium was resistant only to TC, and those selected on SM- or SA-medium were resistant to TC, SM and SA, or SM and SA.

On the other hand, the remaining 6 strains of the same resistance pattern gave no MLR⁺ when the mixed broth culture incubated at 37°C overnight was subcultured on selective media. To ascertain the result, the next day, the mixed cultures which had been left at room temperature (about 25°C in the summer) were reinoculated onto the selective media. The second subculture indicated the transfer of only TC resistance to the recipient. Repeated tests gave the same results.

The converted recipient (MLR⁺) transferred its R factor to the second recipient (*E. coli* W 3630). However, in the case of the TC-resistance-carrying R factor, which was detected only by the mating culture made at room temperature, the secondary transfer of the resistance was clearly recognized when the mixed cultivation was made at room temperature.

TABLE 2 *Fecal excretion of S. typhimurium by calves and the drug resistance pattern of R factors in the organisms*

NO. OF CALF	PEN	DAYS AFTER ADMISSION OF CALVES							
		0	4	8	12	14	16	18	20
1	a	.*1	?*2	(TC)*4	?	?	?	?	?
2	b	(-)*3	?	(TC-SM-SA)
3	b	(-)	?	(TC-SM-SA)	.	(TC-SM-SA)	(TC-SM-SA)	.	.
4	b	(-)	?	(TC)	(TC)	(-)	(TC)	(TC)	(TC)
5*5	c	?	(-)	?	?	?	?	?	?

*1 Tested but not isolated

*2 Not tested

*3 R factor was not detected.

*4 Resistance pattern of R factor

R (TC) was detected only by mixed cultivation at room temperature.

*5 Introduced from Shiraoi

Relation of resistance pattern of R factors in *S. typhimurium* to excreting calves or housing pens

Table 2 indicates that 4 calves already harbored *S. typhimurium* carrying the R factors 8 days after they had been introduced to the feedlot. Moreover, each of Nos. 3 and 4 calves persistently excreted Salmonella carrying specific types of R factor in spite of being housed on the same floor. It was interesting that Nos. 1 and 4 housed separately gave the R factor of TC resistance which could be detected only by room-temperature incubation. This finding seems to show that this type of R factor was spread already among the calves of the same group at the time when they reached the feedlot after a 2-day trucking.

DISCUSSION

ROYAL et al. dosed approximately 10^6 organisms of CTC-sensitive *S. typhimurium* to a 2-day-old calf from which CTC-resistant *E. coli* had been isolated. The calf excreted *S. typhimurium* carrying R-factor-mediated CTC resistance from the day following dosing for 3 weeks during which CTC of 100 mg/day was fed. It has been believed that this was an example of in vivo transfer of the R factor. SMITH⁸⁾ demonstrated R factor transfer from *E. coli* to a recipient *S. typhimurium* in the alimentary tract of 3 of 8 calves used in the absence of drugs. A small portion of the inoculated *S. typhimurium* cells isolated at most of the daily examination of the feces of the 3 calves possessed the resistance pattern. He stated that, usually, antibiotic administration increased the rate of in vivo transfer of the R factor in animal experiments.

As indicated in the results, 4 calves, 3 of which had been excreting TC-sensitive *S. typhimurium* at admission, already excreted *S. typhimurium* carrying R-factor-mediated TC-resistance 8 days after admission to the feedlot. It was necessary to know how the TC-resistant *S. typhimurium* appeared in the carrier calves on the feedlot. First, the possibility of the in vivo transfer of the TC-resistance to *S. typhimurium* was considered. Previous studies^{11,12)} indicated that *E. coli* strains isolated from calves in 1970 and 1971 on the feedlot as well as *S. typhimurium* were resistant to one or more drugs and a large number of the organisms had R factors. A few months after the isolation of *S. typhimurium* from calves in 1973, fecal samples were collected from newly introduced calves and from the pens of rearing houses, and swabs from feeding buckets. All of 13 fecal samples gave *E. coli* resistant to one or more of the drugs tested (TC, SM, CP & SA) and one of 5 bucket-swabs did similarly. Twenty-six (66.7%) of the 39 *E. coli* strains isolated had R factors and 22 of the 26 strains transferred TC-resistance to the recipient. In addition, the epizootiological data showed that

R-factor-carrying *S. typhimurium* did not occur on the feedlot at the time when the carrier calves were introduced from Shizuoka. From these findings, it is evident that the Salmonella-excreting calves may have been introduced into the environment contaminated heavily with *E. coli* carrying R factors. Moreover, in an experiment, in vitro receptiveness of TC-sensitive *S. typhimurium* strains obtained from the carrier calves at admission to an R factor from *E. coli* strain was proved, indicating a potential for in vivo acquisition of R factor. However, in this study, no effort was made to isolate enteric bacteria other than Salmonella from the carrier calves. Therefore, it could not be directly proved what donor transmitted in vivo the TC-resistance-carrying R factors to *S. typhimurium*, and when the R factor transfer occurred. *S. typhimurium* having the unusual R factor showing in vitro transfer only at room-temperature incubation was isolated from 2 calves housed separately. This appears to indicate that cross infection of bacteria to be donor of the R factor may have occurred among the calves before they were housed in different pens.

On the other hand, it can also be considered that a very small number of *S. typhimurium* cells carrying R factors may have been overlooked in the carrier calves at admission. Anyhow, both in this case and in in vivo transfer of TC resistance, there is no doubt that high-dose-CTC medication would provide the selection pressure resulting in the dominant population of *S. typhimurium* having R-factor-mediated TC resistance.

Another important finding in this study was the detection of the R factor transferred only by a mixed cultivation at room temperature. *S. typhimurium* strains having the R factor were isolated collectively. An additional investigation⁷⁾ indicated that the in vitro transfer of the R factor occurred predominantly at 25°C, but that it was inhibited at 37°C. The transfer frequency of the R (TC) at 25°C was about $10^3 \sim 10^6$ times higher than at 37°C in all combinations of the donor and recipient strains used. The isolation of this type of R factor has not been described, excepting the R factor of KM resistance from *Proteus vulgaris*¹³⁾. The elimination frequency of the R (TC) factor in the cells of original *S. typhimurium* strain was 81.5% at 42°C incubation, 18.2% at 37°C and about 7.4% at 25°C⁷⁾. As described above, the R factor detected is very labile in the original host cell. As shown in table 2, calf No. 4 excreted TC-sensitive Salmonella 14 days after introduction. This fact appears to have been due to the labile characteristics of the R factor. However, it remains unsolved whether or not the transfer of the peculiar R factor which is inhibited in vitro at 37°C can occur in the alimentary tract of calves. Most recently, YOSHIDA and TERAWAKI isolated a temperature-sensitive R factor from a *S. typhimurium* strain derived from a patient with enteritis in 1969 in Tokyo. The R factor had

a resistance marker of TC-SM-SA, but only the transfer of TC resistance was temperature-sensitive.

ACKNOWLEDGMENT

The authors thank Dr. N. TERAKADO, The National Veterinary Assay Laboratory, Kokubunji, Tokyo, for his help during this study.

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Note added in proof

Phage type (ANDERSON, 1964) of *S. typhimurium* strains isolated from the calves of Shizuoka was 2, and that of strains from milk replacer and the calves of Shiraoui was 2c.