Journal of Food Protection Vol. 42, No. 12, Pages 950-953 (December, 1979)

Antibiotic-resistant Bacteria in Raw Milk and Ability of Some to Transfer Antibiotic Resistance to *Escherichia coli*

LESTER HANKIN[1]*, GEORGE H. LACY[1], GEORGE R. STEPHENS[1] and WALTER F. DILLMAN[2,3]

The Connecticut Agricultural Experiment Station, New Haven, Connecticut 06504, and Dairy Division, Department of Agriculture, Hartford, Connecticut 06115

(Received for publication February 27, 1979)

ABSTRACT

Raw milk samples were examined for number and percentage of bacteria resistant to seven antibiotics: penicillin, ampicillin, chloramphenicol, neomycin sulfate, polymyxin B sulfate, tetracycline and streptomycin sulfate. A significant negative correlation was found between the total aerobic count of the milk sample and the concentration (above 5 or 10% of the total count) of bacteria in each milk resistant to each of the antibiotics tested. Three of 42 gram-negative isolates were capable of transferring their antibiotic resistance to *Escherichia coli*. Substantial numbers of antibiotic-resistant bacteria in raw milk were found and some survived pasteurization. Inspection of farms failed to indicate a relationship between farm practices or use of antibiotics in feed or as pharmaceuticals and number of antibiotic-resistant bacteria in the raw milk.

Bacterial antibiotic resistance may arise by spontaneous mutation or extrachromosomal inheritance in man and other animals brought about by selective pressure of antibiotics used in therapy and prophylaxis or for growth promotion (*8*). Resistant bacteria are eliminated by animals and may contaminate the soil and other objects. Thus agricultural products may contain antibiotic-resistant bacteria, and if the products are consumed raw the resistant bacteria may invade man. An overview of the importance of antibiotic-resistant bacteria to food microbiology has been presented (*5*).

Antibiotic resistance can also be transferred from one bacterium to another as genetic elements on plasmids. Plasmids may mediate their own conjugal transfer or be cotransferred with another plasmid (*9*).

In this study we examined the number of bacteria, as well as the proportion of the total count found in raw milk, that are resistant to seven antibiotics. We also demonstrated that some gram-negative bacteria isolated from raw milk and resistant to a specific antibiotic may transfer this resistance to *Escherichia coli* via cell to cell contact, presumably by conjugation.

MATERIALS AND METHODS

Samples

Raw milk was collected at farms in 1-oz. sterile containers from refrigerated holding tanks by collectors licensed by the Connecticut Department of Agriculture and using prescribed procedures (*2*).

Media and counts

A total aerobic count was made by spreading 0.1 ml of appropriately diluted sample on previously poured and hardened Standard Methods agar (BBL, Cockeysville, MD). Plates were incubated at 30 C for 48 h. Counts of antibiotic-resistant bacteria were made in the same manner, using media containing antibiotics described below.

Antibiotic-containing media were prepared by adding sterile antibiotic solutions to melted and tempered (48-50 C) Standard Methods agar. The antibiotics used and the final concentration per ml in the test media were as follows: penicillin G (Benzylpenicillin, sodium salt, Sigma Chem. Co., St. Louis, MO), 500 and 10 units; ampicillin (Sigma), 500 μg; chloramphenicol (Chloromycetin, crystalline, Sigma), 100 μg; neomycin sulfate (Sigma), 50 μg; polymyxin B sulfate (Sigma), 500 and 50 units; tetracycline hydrochloride (crystalline, Sigma), 10 μg; streptomycin sulfate (B grade, Calbiochem, San Diego, CA), 100 μg. All antibiotic solutions were prepared in concentrated aqueous solutions and filter-sterilized so that when added to the autoclaved and tempered medium there was not more than a 1% change in medium concentration. Preparation of media containing antibiotics and their use has been described (*1,3,4*).

Transfer of antibiotic resistance

E. coli strain C600*nal* (a nalidixic acid-resistant strain obtained from Dr. R. B. Sparks, Jr., Genetics Department, this Station) and strain *E. coli* X-705 (a streptomycin-resistant strain obtained from Dr. R. Curtis III, University of Alabama) were used as the recipients for conjugal plasmids derived from the bacteria isolated from raw milk.

Cell to cell contact necessary for the conjugal transfer of plasmids was established on Plate Count agar (Difco, Detroit, MI) by a modification of a previously described technique (*6,7*). Plasmid recipient strain *E. coli* C600*nal* was streaked on the central portion of agar plates. Each antibiotic resistant, gram-negative bacterium obtained from the raw milk was streaked on the same agar plate at right angles to the recipient. After incubation for 18 h at 30 C, the cells on the agar surface were suspended in 2 to 3 ml of sterile water and 0.01, 0.1 and 1.0-ml portions of the cell suspension were placed in empty petri dishes and suspended in tempered Plate Count agar fortified with antibiotics. Putative plasmid transconjugants of *E. coli* C600*nal*R$^+$ were selected from the population of bacteria growing in the agar by their ability to grow in the presence of nalidixic acid (50 μg/ml of medium) as well as the antibiotic to which the gram-negative bacterium (donor) obtained from the raw milk was originally resistant.

As further evidence for infectious plasmid-like transfer, *E. coli* C600*nal*R$^+$ transconjugants were mated with *E. coli* X-705. Selection of *E. coli* X-705R$^+$ transconjugants was based on resistance to streptomycin sulfate (250 μg/ml in the medium) and the antibiotic whose resistance was conferred by the putative plasmid. Control plates were included to detect any spontaneous mutants among the recipient or donor bacteria to antibiotic resistance.

RESULTS AND DISCUSSION

Resistant bacteria in raw milk

A wide range in the total number of antibiotic-

[1]*Agricultural Experiment Station.*
[2]*Department of Agriculture.*
[3]*Deceased March 24, 1979.*

resistant bacteria as well as in the percentage resistant to any single antibiotic was observed in the 114 raw milk samples examined (Table 1). The average total bacterial count of the samples was 18,000 per ml. The average percentage of the total bacterial count resistant to any of the antibiotics was less than 12.5% except when polymyxin at the 50-unit level was used. However, the percentage of resistant bacteria ranged from 0 to 100% for individual samples. Fewer bacteria were resistant to chloramphenicol than to any other antibiotic. Decreasing the penicillin concentration in the medium from 500 to 10 units per ml doubled the average number of resistant bacteria. Decreasing the polymyxin level in the medium from 500 to 50 units per ml increased the average number of resistant bacteria more than 11-fold.

TABLE 1. *Average number of aerobic bacteria in raw milk resistant to seven antibiotics.*

Antibiotic in medium	Amount per ml medium	No. samples	Total count (log avg per ml)
None	—	114	17,500
Penicillin	10 units	38	597
	500 units	114	282
Ampicillin	500 μg	114	194
Chloramphenicol	100 μg	113	80
Neomycin	50 μg	114	285
Polymyxin	50 units	92	7,850
	500 units	61	696
Streptomycin	100 μg	114	504
Tetracycline	10 μg	114	407

Concentration of resistant bacteria in each milk sample

One measure of the relative concentration of bacteria able to resist an antibiotic is shown by the proportion of samples in which bacteria resistant to a given antibiotic equals or exceeds an arbitrary level of 5 or 10% of the total count (Table 2). For example, in only 4.4% of the samples were more than 10% of the total count resistant to chloramphenicol while 93.5% of the samples had more than 10% of the total count resistant to polymyxin (50 units/ml). The second and third highest percentages of samples containing a population at least 10% resistant were in the tests containing 500 or 10 units of penicillin. Most of the bacteria resistant to penicillin appeared to be fluorescent pseudomonads. When ampicillin, another form of penicillin, was used instead of penicillin, the proportion of resistant bacteria fell from 21.9 to 10.5%.

However, when the minimum percentage of resistant bacteria in any sample was set at 5%, the percentage of samples with resistant bacteria above this value increased about two- or three-fold over that with the 10% minimum for all antibiotics except penicillin and polymyxin (50 units/ml). For penicillin this phenomenon was not unexpected since one criterion for resistance to penicillin is production of β-lactamase. Therefore, resistance to penicillin is generally an all or none phenomenon. Resistance to polymyxin was overcome by increasing the concentration of this antibiotic in the medium (see polymyxin 500 level, Table 2).

Concentration of resistant bacteria and intercorrelations

If the proportion of bacteria resistant to a given antibiotic in a sample of raw milk was at least 5% (or 10%) of the total count, then that milk was considered to have a meaningful concentration of bacteria resistant to that antibiotic. Thus each sample of milk could have from none to seven meaningful concentrations or groups of antibiotic-resistant bacteria. Therefore, another method for assessing the resistance pattern of bacteria in raw milk is obtained.

Figure 1 shows the relation of total aerobic count of samples with at least 5 or 10% of the total count resistant to none or more antibiotics (groups of antibiotic-resistant bacteria). The regression of the logarithm of the total aerobic count of each milk sample on the number of groups (0 to 7) of antibiotic-resistant bacteria was calculated. The points are the mean aerobic counts at each level. Despite considerable variation, both regressions are highly significant ($p \leq .01$) and their slopes are negative. This indicates that bacteria from raw milk with a low total count were, in general, resistant to a wide range of antibiotics. That is, the lower the total count of the sample, the more antibiotics the bacteria resisted.

Similarly, when the proportion of bacteria in a sample resistant to an antibiotic was correlated with the total

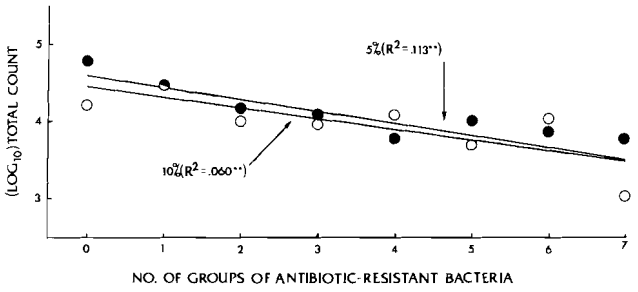

Figure 1. *Regression of total aerobic bacterial count on number of groups of antibiotic-resistant bacteria in raw milk. An antibiotic-resistant group comprises at least 5% or 10% of the total aerobic count for each sample. Each milk was assigned a numerical value of zero to seven corresponding to the number of groups of antibiotic-resistant bacteria. The points are the mean aerobic count values at each level. Solid circles = 5% or more of resistant bacteria, open circles = 10% or more of resistant bacteria, ** significant at $p \leq .01$. For 5% level, $F = 14.27^{**}(n = 114)$; for 10% level, $F = 7.09^{**}(n = 114)$.*

TABLE 2. *Percentage of raw milk samples with at least 5 or 10% of the total aerobic count resistant to the indicated antibiotic.*

Proportion of total count	Antibiotic								
	PEN[1]		AMP	CAM	NEO	PLM		STR	TET
	10U	500U	500 μg	100 μg	50 μg	50U	500U	100 μg	10 μg
	(% of samples)								
≥ 5%	44.7	27.2	18.4	10.6	18.4	96.7	41.0	39.5	29.8
≥ 10%	34.2	21.9	10.5	4.4	8.8	93.5	14.8	16.7	16.7

[1]Abbreviation: PEN, penicillin; AMP, ampicillin; CAM chloramphenicol; NEO, neomycin sulfate; PLM, polymyxin B sulfate; STR, streptomycin; TET, tetracycline. Concentrations are designated as units (U) or μg per ml of medium.

count of that sample all the correlations except that with neomycin were negative (Table 3, top row). Thus, as the total count increased there was a decrease in the proportion of bacteria in that total population resistant to a specific antibiotic. Although some of the correlations are statistically significant they account for only 5 to 26% of the observed variability.

Correlations were also calculated between the proportion of bacteria resistant to the individual antibiotics (Table 3). Several apparent anomalies in the correlations were noted. Although a significant correlation between the 10 and 500 unit levels of penicillin was observed, as expected, only the 500-unit level of penicillin and ampicillin were correlated. A correlation was found to exist between tetracycline and chloramphenicol ($r = .34$, $p \leq .01$, $n = 113$), antibiotics that inhibit protein synthesis. However, no correlation was found between neomycin and streptomycin ($r = .05$, $n = 114$) even though both antibiotics are chemically similar and inhibit protein synthesis. The lack of correlation between antibiotics with similar modes of action must assume either multiple resistance mechanisms in the same bacterium, or more likely, different populations of bacteria, each resistant to a different antibiotic.

Resampling of raw milk and examination of farms

Four months after the initial sampling, raw milk from 19 farms was resampled to determine if the antibiotic resistance patterns of the bacterial flora had changed. Although the proportion of the total count found to be resistant to any single antibiotic changed in some instances, a statistical analysis (t-test) revealed no significant difference in proportion of resistant bacteria between the two dates except for tetracycline. For this antibiotic the proportion of resistant organisms declined significantly ($t = 2.80$, $n = 38$) at the second sampling. Thus it appeared that the bacterial flora in raw milk from a specific farm remained essentially unchanged during 4 months, as determined by the proportion of the total count resistant to a specific antibiotic, except for tetracycline.

In an attempt to ascertain the reason for the variability among farms in the proportion of the total count resistant to an antibiotic, five farms from the same geographical area were selected for further study. On the first sampling, these five farms had raw milk containing high, medium and low proportions of the total bacterial count resistant to specific antibiotics. On-site inspection of these dairy farms was made to determine if antibiotics were present in either livestock feed or veterinary supplies. Use of these materials could lead to direct (intestinal selection) or indirect (manure contamination) enrichment for antibiotic-resistant bacteria. We found no antibiotic-containing feed. However, all the farms had variable but considerable quantities of penicillin-containing veterinary preparations used to treat mastitis. There was no obvious correlation for the proportion or number of antibiotic-resistant bacteria in raw milk with the presence of antibiotics used for therapy or prophylaxis.

Resistant bacteria in pasteurized products

We also tested seven commercially pasteurized dairy products for bacteria resistant to antibiotics. Although much variation was observed, bacteria resistant to penicillin, ampicillin and polymyxin were found. It was not possible from these few samples to ascertain whether the resistant bacteria survived pasteurization or were post-pasteurization contaminants.

To test this aspect, we laboratory-pasteurized 20 raw milk samples and examined them for bacteria resistant to antibiotics. Ten of the 20 laboratory-pasteurized samples contained no bacteria resistant to antibiotics. For the remaining 10 samples more bacteria were found

TABLE 3. *Correlation (r) of total aerobic bacterial count of raw milk [TCNT] with percentage of total count resistant to specific antibiotics and correlation (r) between percentage of total count resistant to each antibiotic.*

	Antibiotic								
	PEN 10[1]	PEN 500	AMP	CAM	NEO	PLM 50	PLM 500	STR	TET
TCNT	-.516**[2]	-.319**	-.175	-.259**	.053	-.236*	-.079	-.217*	-.068
	(38)[3]	(114)	(114)	(113)	(114)	(92)	(61)	(114)	(114)
PEN 10		.82**	.21	.44**	.55**	.30	—	.47**	.12
		(38)	(38)	(38)	(38)	(38)	(0)	(38)	(38)
PEN 500			.70**	.53**	.10	.06	.33*	.11	.16
			(114)	(113)	(114)	(92)	(61)	(114)	(114)
AMP				.60**	.05	-.01	.41**	.13	.23*
				(113)	(114)	(92)	(61)	(114)	(114)
CAM					.19*	.16	.05	.24**	.34**
					(113)	(91)	(61)	(113)	(113)
NEO						.24*	.10	.05	.03
						(92)	(61)	(114)	(114)
PLM 50							.25	-.04	.21*
							(39)	(92)	(92)
PLM 500								.09	-.02
								(61)	(61)
STR									.54**
									(114)

[1]Abbreviations: PEN, penicillin; AMP, ampicillin; CAM, chloramphenicol; NEO, neomycin sulfate; PLM, polymyxin B sulfate; STR, streptomycin, TET, tetracycline. See Table 1 for amounts per ml of medium.
[2]* significant at $p \leq .05$, ** significant at $p \leq .01$.
[3]Number in parentheses indicates number of samples.

resistant to streptomycin, tetracycline and polymyxin than to the other antibiotics. With streptomycin, the proportion of resistant bacteria averaged 26.5% of the total count. Before pasteurization of these same 10 samples, the average percentage of streptomycin resistant bacteria was only 4.9%. Thus it appears that bacteria resistant to streptomycin, tetracycline and polymyxin can survive laboratory pasteurization. Such information is important in light of the data presented below on R^+ plasmid transfer from bacteria in raw milk to *E. coli*.

Transfer of R^+ plasmids from antibiotic-resistant bacteria in raw milk to E. coli

From the bacteria in raw milk resistant to antibiotics, 42 gram-negative bacteria were chosen to study their ability to transfer their antibiotic resistance. Each appeared to be the predominant colony type on the medium from which it was isolated. Of these bacterial isolates 12 were resistant to penicillin, six to ampicillin, seven to streptomycin, five to neomycin, nine to tetracycline, and three to chloramphenicol. Only three isolates among the 42 selected were capable of transferring antibiotic resistance to *E. coli* C600*nal.* Two of the isolates were originally resistant to penicillin and one to tetracycline. All three *E. coli* C600*nal*R^+ transconjugants transferred resistance to *E. coli* X-705. No spontaneous mutants to antibiotic resistance were noted when *E. coli* C600*nal. E. coli* X-705 or isolates from raw milk were plated at similar concentrations in selective media.

Thus the data indicate that under our conditions, antibiotic resistance probably resident on conjugal plasmids can be transferred, but it was not the most prevalent type of resistance found among the gram-negative antibiotic-resistant bacteria isolated from raw milk. Further, none of the three putative antibiotic resistance plasmids carried resistance to a second or third antibiotic (among the seven antibiotics used in this study).

ACKNOWLEDGMENT

We thank Margaret Staba and Ruth Schlesinger for technical assistance.

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CONCLUSIONS AND RECOMMENDATIONS

Blood analysis may be used to estimate total DDT residues in beef fat before slaughter. It is considered that the proposed method can be used to infer whether a given sample will pass the limit or not. Analysis in triplicate is recommended for samples with residue levels close to the legal limit. It is considered that the proposed method can be used to infer whether a given sample will pass the limit or not.

Comparison of the present data with others published on the same subject indicates variations in the results, but the factors causing these variations are unknown.

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