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Probability of Recovering Pathogenic *Escherichia coli* from Foods

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The probability of recovering pathogenic *Escherichia coli* from food by the *Bacteriological Analytical Manual* method was determined by the effects of several factors: the number of strains per food, the ability of pathogenic strains to survive enrichment, and the frequency of plasmid loss during enrichment. Biochemical patterns indicated the presence of about six *E. coli* strains per food sample. About half of the strains isolated from humans did not survive enrichment. Among those which grew, plasmid loss, as determined by gel electrophoresis and DNA colony hybridization, ranged from 20 to 95%. The combined effects of failure to survive enrichment and plasmid loss decreased the relative numbers of these strains and reduced the chance of detecting pathogens. To counteract this tendency and obtain a 90 to 95% probability of recovering a given pathogenic strain, 40 to 50 colonies per food sample should be picked during the routine testing of foods.

Selective enrichment is often required to isolate a particular indigenous or contaminant species of microorganism from a food. Because the *Escherichia coli* cells which contaminate foods may be present in low numbers or may be overgrown on culture media by other species of the indigenous microflora, food microbiologists have developed a physiologically demanding two-step enrichment process (2). Although this procedure is well suited for enumerating *E. coli* by a most-probable-number determination, the enrichment may be inefficient for the recovery of pathogenic strains. We attempted to determine the probability of recovering and identifying pathogenic strains by the procedure described in the U.S. Food and Drug Administration *Bacteriological Analytical Manual* (BAM; 22). Factors affecting the recovery of strains included the number of strains recovered from a food, the ability of a particular strain (isolated from humans) to survive the enrichment procedure, and the frequency of plasmid loss during selective enrichment.

MATERIALS AND METHODS

Bacterial strains. The *E. coli* strains used in this study (references in parentheses) were K334C2 (29), TD427C2 (26), E2534 (18), and 1184/68 (28).

Bacterial isolation procedure. Retail food samples were analyzed for *E. coli* as detailed in the BAM (22), except that no most-probable-number determinations were made and brilliant green-lactose-bile broth was not used. Isolates obtained from the food samples were stored in 10% glycerol at −70°C until tested; organisms not classified as *E. coli* (22) were omitted from further study. All media were prepared as directed by the manufacturer, and the rate of plasmid loss and the number of strains present in a food were determined by recovering up to 12 isolates from some foods.

Plasmid analysis. Plasmid DNA was isolated from frozen cultures grown overnight in 5 ml of brain heart infusion broth by the method of Birnboim and Doly (5) and analyzed by agarose gel electrophoresis (25). Plasmid molecular weights were determined by comparison of their migration rates with plasmids in *E. coli* strains IEC-78 (31) and V517 (20).

Biotyping. Carbohydrate utilization was tested by using Andrade indicator in carbohydrate-peptone broth (19, 34). Antibiotic susceptibilities were determined by the disk diffusion method (3). The abilities of isolates to degrade arginine, lysine, and ornithine were recorded (19).

DNA colony hybridization. Culture samples (0.1 ml) were spread onto nitrocellulose filters (BA85; Schleicher & Schuell, Inc., Keene, N.H.) and incubated overnight at 37°C on brain heart infusion agar or tryptic soy agar. Radioactively labeled DNA fragments carrying the genetic information for the heat-labile enterotoxin (LT) of *E. coli* were incubated with lysed colonies (12).

Probability calculations. The number of colonies that must be picked to ensure a given probability of recovering a particular strain is shown by the formula $N = \ln(1 - P)/\ln(1 - f)$, where N is the number of colonies to be picked, P is the confidence level for recovering a particular strain, and f is the fraction of the entire population that is represented by the strain that is being sought. N was rounded to the next whole number.

Data analysis. All data, including biochemical profiles, antibiograms, plasmid characterizations, and food product information, were entered into and analyzed by the Microbial Information System (Micro-IS; 4, 15, 16, 21, 35; V. M. Jones, F. Tenover, W. E. Hill, F. A. Benedict, and M. I. Krichevsky, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, X5, p. 312). Species were assigned by numerical taxonomy by the gram-negative, facultative aerobe probabilistic identification matrix (MAT 2) of the Micro-IS. Cluster analyses were performed, and similarity triangles and dendrograms were constructed by using the Jaccard coefficient (SJ) and unweighted average linkage clustering (36–38).

RESULTS

Foodborne *E. coli* isolation and characterization. A total of 153 isolates of *E. coli* were recovered from 36 retail foods. To avoid analyzing multiple isolates of genetically identical microorganisms, we considered a strain unique (i.e., different from other isolates in the same food) if the bacteria exhibited either a different biotype or a different plasmid profile that could not be explained by simple plasmid loss. A total of 84 strains were considered to be unique (Table 1).

The ability of the BAM procedure to select for *E. coli* and discriminate against other species was tested by comparing

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TABLE 1. Information on *E. coli* recovered from foods

Food type	No. of samples	Total isolates	Unique isolates[a]	Average no. of unique isolates
Ground beef	6	61	39	6.5
Pork sausage	7	63	31	4.4
Other[b]	5	29	14	2.8
Total	18	153	84	4.7

[a] Isolates that were not identical to other strains recovered from the same food samples.
[b] Cottage cheese, chicken, frozen turkey and beef pies, and alfalfa sprouts.

TABLE 3. Antibiotic resistances of *E. coli* isolates from foods

Food	No. of isolates	No. of resistant isolates (%)[a]	Average no. of resistances per resistant isolate
Ground beef	39	21 (54)	1.8
Pork sausage	31	22 (70)	3.0
Other[b]	14	5 (36)	3.0
Total	84	48 (57)	2.5

[a] Isolates resistant to at least one antibiotic. See Table 4 for antibiotics tested.
[b] See Table 1, footnote *b*.

the biochemical profiles of the food isolates with the bacterial profiles stored in the Micro-IS probabilistic identification matrix for facultative, gram-negative bacteria. Among the 84 strains considered to be unique isolates, 49 biochemical patterns were observed, and 59 (70%) isolates were considered to be *E. coli* at the 95% confidence level or above. In 16 additional cases, *E. coli* was the most likely identification. In 9 cases (11%), the most probable species were *Klebsiella rhinoscleromatis* (four cases), *Klebsiella ozaenae* (three cases), *Citrobacter amalonaticus* (three cases), *Yersinia enterocolitica* (one case), and *Erwinia stewartii* (one case). In some foods, as many as seven unique isolates were recovered. The biochemical reactions of the isolates are presented in Table 2, a summary of antibiotic resistances is shown in Table 3, and the percentages of resistant isolates are listed in Table 4.

Characteristics were examined in pairs to determine whether certain biochemical features tended to occur together more frequently than would be expected if they were randomly assorted. Associations that were significantly more frequent than expected were found among antibiotic resistances to pairs of the antibiotics ampicillin, streptomycin, tetracycline, and Triple Sulfa (BBL Microbiology Systems, Cockeysville, Md.). Simultaneous resistance to two of these antibiotics occurred about twice as often as it would if antibiotic resistance was randomly distributed and not genetically linked.

Dendrograms constructed by use of biochemical profiles revealed no tendency for strains isolated from a particular food type to be more similar to each other than to strains originating from other foods.

Recovery of *E. coli* of human origin from enrichment medium. Strains of *E. coli* that had been isolated from humans were tested to determine how many could survive the BAM procedure. Approximately 10^6 cells were added to 10 ml of lauryl sulfate tryptose broth to represent a level of contamination of about 10^8 cells per g of food. Of 22 strains analyzed, 15 (68%) produced gas 48 h after transfer to EC broth at 45.5°C.

Loss of plasmids during enrichment. The distribution of plasmid profiles for 147 isolates is shown in Table 4. The rate of plasmid loss was estimated in two ways. First, the plasmid complement of each isolate was determined (5) and compared with the plasmid profiles of biochemically identical isolates from the same food. Of the 24 instances in which such comparisons could be made, at least one plasmid was lost in 13 (54%) of the cases. Second, pure cultures of *E. coli* that produced LT were subjected to the two-step BAM enrichment procedure. Samples were spread plated onto nitrocellulose filters on an agar medium before and after enrichment. After overnight incubation at 37°C, the colonies were counted, lysed, and hybridized with radioactively labeled gene fragments that coded for LT (12). Colonies retaining the LT gene were represented by black spots on autoradiograms. To calculate the fraction of cells still harboring the LT gene, the number of spots was compared with the number of colonies (Table 5). Between 20 and 96% of the cells containing plasmids lost the LT gene and presumably the plasmids that carried it.

TABLE 2. Incidence (%) of positive biochemical reactions of *E. coli* strains isolated from foods

Test substance	% Positive reactions of isolates from:			Total (%) (n = 84)	Positive (%)[a]
	Ground beef (n = 39)	Pork sausage (n = 31)	Other[b] (n = 14)		
Dulcitol	80	84	100	85	67
Glycerol	95	94	93	94	97
Raffinose	74	71	93	76	52
Rhamnose	92	94	100	94	87
Salicin	59	84	93	74	48
Sorbitol	100	100	100	100	81
Sucrose	82	74	93	81	59
Xylose	100	100	100	100	89
Arginine	0	0	0	0	17
Lysine	85	84	64	81	88
Ornithine	72	19	79	54	63
Esculin	6	13	8	9	31

[a] Biochemical characteristics of *E. coli* isolates from a general survey (8).
[b] See Table 1, footnote *b*.

TABLE 4. Incidence (%) of antibiotic-resistant isolates of *E. coli* isolated from foods

Antibiotic	Resistant isolates (%) from:			Average % (n = 84)
	Beef (n = 39)	Pork (n = 31)	Other (n = 14)[a]	
Ampicillin	10	29	14	18
Chloramphenicol	0	3	7	2
Streptomycin	21	42	29	30
Tetracycline	49	65	21	50
Cephalothin	0	0	7	1
Gentamicin	0	0	0	0
Kanamycin	3	13	0	6
Neomycin	0	13	0	5
Penicillin G	0	0	0	0
Polymyxin B	0	0	0	0
Triple Sulfa	13	52	14	28
Avg	9	20	8	13

[a] See Table 1, footnote *b*.

TABLE 5. Incidence of loss of the LT gene from *E. coli* during enrichment[a]

Strain	% Positive		% Positive after enrichment/ % positive before enrichment
	Before enrichment	After enrichment	
K334C2	80	59	0.74
TD427C2	66	53	0.80
E2534	80	9.2	0.12
1184/68	24	1.0	0.04

[a] Fraction of cells harboring the LT gene was determined before and after enrichment by DNA colony hybridization (12).

TABLE 6. Distribution of the number of plasmids among *E. coli* isolates from foods

Food type	No. of isolates with the following no. of plasmids:							Average no. of plasmids per isolate
	0	1	2	3	4	5	6	
Ground beef	22	22	13	2	0	1	1	1.1
Pork sausage	19	23	6	9	1	0	2	1.3
Other[a]	7	15	3	2	1	0	0	1.1
Total	48	60	22	13	2	1	3	1.1
Observed % of total	33	41	15	9	1	1	1	
Expected % of total[b]	33	37	20	7	2	0.4	0.01	

[a] See Table 1, footnote *a*.

[b] Expected percent based on Poisson distribution, with average of 1.1 plasmids per isolate. Expected distribution was not significantly different from observed distribution at the 95% confidence level.

DISCUSSION

The isolation of *E. coli* from foods by using lauryl sulfate tryptose broth at 37°C followed by EC broth at 45.5°C (the BAM procedure) is time-consuming; however, it does discriminate against other species. In 89% of the isolates reported here, *E. coli* was the most probable species. This percentage rate of identification was relatively low because we omitted the biochemical results for identifying *E. coli* that were the same for all isolates (for example, indole–methyl red–Voges-Proskauer–citrate patterns, green sheen on eosin-methylene blue agar). Because the data that were included on biochemical characteristics vary in this species, the net effect was to eliminate those characteristics that are most typical of *E. coli* and could provide the highest confidence for identification by probabilistic comparison. As a result, those characteristics that are most variable were overemphasized in the analyses. In another performance test, Micro-IS identified 99% of the *E. coli* strains (15).

Tables 1 to 6 summarize the characteristics of the 84 unique strains of *E. coli* recovered from foods. Table 1 shows that ground beef harbored more strains than did the other foods, but additional comparisons were not significantly different at the 95% confidence level. The percentage of isolates able to carry out selected biochemical reactions (Table 2) was generally the same as those reported by Ewing and Martin (8); however, no statistical comparisons were made. Isolates from ground meats were more likely to be resistant to antibiotics than were the other isolates (Table 3) and tended to be resistant to more than one antibiotic. *E. coli* strains from pork sausage exhibited higher percentages of resistance to ampicillin, streptomycin, and Triple Sulfa than did those from other sources (Table 4). When considered as a group, antibiotic-resistant strains of *E. coli* were isolated from pork sausage about twice as often as from the other sources. Plasmids were found in 67% of the isolates, with no significant differences with respect to the source of the isolates (Table 5).

Although the enrichment method serves well for most-probable-number determinations, it is less reliable for the recovery of strains that may be pathogenic for humans. About 30% of the human isolates did not survive enrichment even when added in large numbers and without competition from other species (data not shown). Mehlman and Romero (23) found that only 31% of pathogenic *E. coli* strains could be quantitatively recovered by the Association of Official Analytical Chemists method (2) and that 30% of the strains were inhibited by MacConkey agar, 43% were inhibited by eosin-methylene blue agar, and 17% were inhibited by tryptone-bile salts agar.

Because some pathogenic determinant genes in *E. coli* are carried on plasmids, a procedure in which plasmids are lost would decrease the efficiency of recovering virulent strains. In the foodborne isolates examined, plasmid loss was observed about half of the time. Previous reports also revealed that the extent of plasmid loss during enrichment reached 99.95% when antibiotic resistance was used as a plasmid marker (10, 11). Four strains were tested by DNA colony hybridization to determine the fraction of cells losing the plasmidborne LT gene. Strains differed in their ability to retain plasmids, with losses ranging from 20 to 95% (Table 6). Even though the isolates had been stored at −70°C, 76% of strain 1184/68 cells had lost the LT gene before being subjected to enrichment. This strain also had the greatest degree of plasmid loss (96%), suggesting that the combination of this strain and its LT plasmid may be particularly unstable. It is not known whether the bacterial host cell or the plasmid plays the more important role in determining plasmid retention under enrichment conditions.

Because *E. coli* strains often differ in their ability to metabolize the substrates chosen for biotyping, we had a greater chance to observe differences between isolates than we would have if we had examined only the characteristics that are more uniform throughout the species. In addition to estimating the number of strains present in a particular food sample, this biotyping permitted us to determine whether the obtaining of two different plasmid patterns resulted from plasmid loss or from the presence of two different isolated strains.

Finding more than one strain (biovar) of *E. coli* in foods (Table 1) has serious implications for the recovery of one particular strain (Table 7). Because an average of five strains per food was observed (Table 1), 11 to 14 colonies would have to be picked to achieve a 90 to 95% probability of

TABLE 7. Effect of multiple *E. coli* strains in a food on the number of colonies that must be picked to recover a particular strain

No. of strains per food	No. of colonies that must be picked to recover a particular strain at a probability level of:		
	50%	90%	95%
1	1	1	1
2	1	4	5
5	4	11	14
10	7	22	29

TABLE 8. Combined effects of number of *E. coli* strains present, chance of survival in enrichment, and loss of plasmids on the relative frequency of a particular strain in three cases

Factor	Case		
	Best	Average	Worst
No. of strains	1	0.22	0.14
Survival in enrichment	0.70	0.50	0.10
Plasmid loss	0.80	0.55	0.04
Combined effects[a]	0.56	0.056	0.00057

[a] Product of the independent factors.

TABLE 9. Combined effects of factors that reduced recovery of a particular *E. coli* strain in best, average, and worst cases: number of colonies versus probability of recovery

Case[a]	No. of colonies that must be picked to recover a particular strain at a probability level of:		
	50%	90%	95%
Best	1	3	4
Average	13	41	53
Worst	1,216	4,039	5,225

[a] The effect of each case on relative strain frequency is shown in Table 8.

recovering a particular strain (assuming that all strains are present in equal numbers) (Table 7). With only two to five colonies selected in the standard BAM methodology, the chance that a particular strain will be recovered is only about 50%.

Other factors that may reduce the probability of recovering plasmid-harboring pathogenic strains are loss of plasmids during the enrichment procedure, failure to recover all pathogenic strains from the enrichment medium, and a further reduction in the relative frequency of plasmid-bearing strains, which tend to have slower growth rates than plasmid-free strains (40). We attempted to estimate the combined effect of these factors on the relative frequency of recovering a particular strain (Table 8). As this frequency decreases, more colonies must be picked to improve recovery of the strain (Table 7). To assess the effects of the factors listed in Table 8, we estimated the probability of strain recovery in the best, typical, and worst situations. To calculate the combined effect of these three factors, we assumed that the effects are independent of each other. (There may, in fact, be an interaction between survival rate and plasmid complement, as there is between growth rate and plasmid size [40], but for purposes of our estimates this was disregarded.) If we assume independence, we can determine the combined effect by multiplication of the individual effects (Table 8). For example, if one pathogenic strain coexists with about four other strains in a food, the relative frequency will be reduced by a factor of 0.22 (i.e., number of strains per food, 4.7) times 0.5 (about 50% of the strains survive enrichment) times 0.5 (about 50% of the strains lose a plasmid) = 0.056 (i.e., the strain of concern has a relative frequency of about 1 in 20). Table 9 shows how decreases in relative frequencies (Table 8) can increase the number of colonies that must be picked. In a typical situation, it might be necessary to analyze 40 to 50 colonies to achieve a 90 to 95% probability of recovering a particular pathogenic strain. Thus, it is difficult to recover a particular strain unless mass screening methods, which do not require pure cultures, are used.

Genetic probes have been used successfully for screening large numbers of clinical isolates (27, 33). Because these procedures do not require the isolation of pure bacterial cultures, the time required can often be reduced considerably. Such techniques have also been used to examine foods for the presence of particular species (9) or pathogenic strains (12, 14). DNA hybridization methods for *Salmonella* spp. compare favorably with culture enrichment and immunological tests (9a). Correct detection of enterotoxigenic *E. coli* was greater than 95% in a recent interlaboratory study (13). The advent of nonradioactive labels for probe DNAs (17, 32) and the development of synthetic DNA probes (24, 39; W. E. Hill, W. L. Payne, G. Zon, and S. L. Moseley, submitted for publication) coupled with end labeling (6) should lead to even further widespread implementation of tests based on these methods.

In summary, the BAM (22) enrichment method for isolating *E. coli* from foods is quite selective; in 75 of 84 (89%) of the isolations, *E. coli* was the most likely species. However, approximately 50% of the *E. coli* strains from human sources would not grow in the enrichment medium, even though large inocula of pure cultures were used. About half of the plasmid-containing strains lost a plasmid during enrichment, with plasmid loss as high as 96% in pure cultures. In addition, the presence of up to seven strains in a particular food sample indicated that at least 10 to 15 colonies should be analyzed.

Hence, the BAM enrichment procedure is not well suited for the efficient and reliable recovery of pathogenic strains of *E. coli* from foods. New enrichment methods are being developed (1, 7, 30; I. J. Mehlman and B. A. Wentz, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, P3, p. 200). However, unless these methods are designed to select for pathogens, the presence of multiple strains in a food will necessitate further testing of an impractically large number of isolates.

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