APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 1983, p. 165–170
0099-2240/83/070165-06$02.00/0

Vol. 46, No. 1

Multiple Antibiotic Resistance Indexing of *Escherichia coli* to Identify High-Risk Sources of Fecal Contamination of Foods†

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Received 27 September 1982/Accepted 19 April 1983

Escherichia coli isolates taken from environments considered to have low and high enteric disease potential for humans were screened against 12 antibiotics to determine the prevalence of multiple antibiotic resistance among the isolates of these environments. It was determined that multiple-antibiotic-resistant *E. coli* organisms exist in large numbers within the major reservoirs of enteric diseases for humans while existing in comparatively low numbers elsewhere. These differences provide a method for distinguishing high-risk contamination of foods by indexing the frequency with which multiple-antibiotic-resistant *E. coli* organisms occur among isolates taken from a sample.

The native habitat of *Escherichia coli* is the enteric tract of humans and other warm-blooded animals. Its presence in food or water is generally considered to indicate direct or indirect fecal contamination and the possible presence of enteric pathogens. Since Escherich identified *E. coli* (*Bacillus coli*) as an indicator of fecal pollution, attempts have been made to define the sanitary significance of this microorganism (6). Much has been published about the merits of *E. coli* as a fecal indicator (1–4, 6, 9, 10, 15), but several problems persist. Few have challenged the validity of *E. coli* as an indicator for fecal contamination of water, but the application of water standards to foods has raised many questions as well as criticisms (13, 16). *E. coli* is widely disseminated in the environment through the feces of humans and other animals. This, coupled with the ability of the bacterium to survive for months external to the colon (5, 14, 19), makes the bacterium almost ubiquitous and the significance of its presence in food equivocal. The inability to differentiate significant contamination continues to make it difficult for regulatory agencies to discriminate between cases in which the assumption of probable contamination is justified and cases in which it is not (20).

The greatest risk of fecal contamination to humans originates from humans, poultry, or swine. Human feces potentially carry all enteric diseases to which humans are susceptible. Poultry is the primary and permanent reservoir for *Salmonella* spp., whereas swine harbor *Salmonella* spp., *Shigella* spp., enteropathogenic *E. coli*, and other disease-causing microorganisms. A procedure which would distinguish between *E. coli* originating from these high-risk environments and *E. coli* originating from other sources would provide a quantum of definition not possible with current laboratory procedures. Indexing *E. coli* isolates obtained from food according to the frequency with which multiple antibiotic resistances (MAR) occur may provide a relatively easy method for making this distinction.

There is a large body of literature recently reviewed by Novick (18) demonstrating that the subtherapeutic use of antibiotics in the mass production of poultry, eggs, and pork has promoted the emergence of and maintains the prevalence of multiple-antibiotic-resistant (MAR) *E. coli* in the fecal environment of these animals. The wide use and abuse of antibiotics in human therapy has produced MAR *E. coli* in the feces of humans as well (5, 11, 18). These practices have resulted in the coexistence of MAR *E. coli* within these major reservoirs of enteric disease for humans. The consequence of these practices may provide a fortuitous opportunity to identify *E. coli* contamination of food originating from these high-risk environments by MAR indexing of *E. coli* isolates obtained from food. Data presented in this paper indicate that this possibility exists, at least within the geographic area of the study.

MATERIALS AND METHODS

Samples. Human. *E. coli* was obtained from a large cross section of the human population of the Willamette Valley of Oregon by sampling raw sewage water from seven municipal wastewater disposal systems serving populations ranging from 1,200 to over 300,000

† Technical paper no. 6544 from the Oregon Agriculture Experiment Station.

people. Isolates of *E. coli* were also taken by rectal swab from 102 human subjects.

Food animals. Animal feces were collected from food animal production facilities with high population densities and placed in 32-oz. (ca. 0.946-liter) Whirl-Pac disposal bags. Included were three poultry brooder houses and two laying houses randomly selected and located in the north, central, and southern sections of the Willamette Valley; 10 cattle feedlots, which included all of the major lots located in eastern Oregon, eastern Washington, and western Idaho supplying a major portion of beef to the Oregon market; and one major piggery in eastern Oregon and two smaller piggeries in the Willamette Valley.

Farm animals. Fecal samples were randomly collected from grazing sheep, horses, and cattle throughout the Willamette Valley. Samples included pastured animals on small family-owned farms, as well as animals winter grazing large grass seed farms and orchards of the Willamette Valley. Feces of cattle on the open rangeland of central Oregon were also included.

Wild animals. Feces of common wild animals and birds of the Willamette Valley were collected, which included deer, fox, opossum, rabbit, raccoon, rat, mouse, and skunk. Fecal samples were taken from two game refuges, which were essentially natural undisturbed habitats, and from farms and ranches of the Willamette Valley. Deer feces were collected from forested areas surrounding the Willamette Valley and rangelands of central Oregon. Fecal samples from deer were also collected in and near urban areas of the Willamette Valley.

Isolation of cultures. Solid samples were prepared by adding all or a portion of the sample to 50 ml of sterile 1% peptone water contained in a 0.5-pint (ca. 0.236-liter) Osterizer jar and blended for 1 min. A 1-ml amount of blended material was pipetted into each of 10 test tubes containing 10 ml of sterile EC medium (Difco Laboratories). Liquid samples were added directly to EC medium without blending or dilution. The soiled end of the human rectal swabs was placed in the mouth of tubes containing EC broth, and the applicator stick was cut immediately above the swab with sharp sterile scissors. The applicator stick was used to submerge the freed swab in the broth.

Inoculated tubes were incubated at 45.5°C in a water bath until the first indication of gas (usually less than 8 h). A loopful of broth culture from each positive tube was streaked onto plates of Levine EMB agar (Difco). The plates were incubated for 48 h at 37°C. Ten typical *E. coli* colonies were randomly selected by lifting them from the plate with sterile wooden toothpicks and transferring them to a master plate of tryptone-peptone-extract agar. The master plates had been previously scored with a hot 30-probe nichrome wire stab replicator to provide a pattern for inoculation and subsequent replication. All isolates were replicated on Simmons citrate agar (Difco) and evaluated after 96 h of incubation at 37°C. Citrate-positive isolates were not included in this study. Approximately 10% of the citrate-negative isolates were randomly selected throughout the study for further diagnostic evaluation by the indole–methyl red–Voges-Proskauer–citrate reactions to confirm the consistent selection of *E. coli* isolates by the procedure.

Antibiotic resistance testing. Antibiotics were selected to include those commonly used in human therapy, as well as those allowed and commonly used in food animal feeds. The human use of antibiotics is ostensibly for the single purpose of combating disease. They may be administered orally or by injection; the single exception, among those used for screening MAR *E. coli*, is nitrofurazone, which is limited to topical applications in humans. Antibiotics have multiple uses in the production of food animals and are, for the most part, administered orally in feeds. All antibiotics used for screening MAR *E. coli* are or have been used for human therapy; seven have been used almost exclusively, whereas five are currently used in the United States for both human therapy and food animal production. Table 1 summarizes the use and route of application.

Antibiotic resistance testing was a modified procedure of Kelch and Lee (12). Master plate colonies incubated for 48 h were replicated onto tryptone-peptone-extract agar plates, each containing a separate antibiotic at the concentration indicated in Table 2. The final plate replicated was one of plain tryptone-peptone-extract agar. The results from this plate confirmed the successful replication of colonies on the preceding replicated plates and provided a fresh master plate of cultures. The concentrations of antibiotics used in the medium were based upon active antibiotic exclusive of associated anions or carrier materials or both. The antibiotics were stirred into the melted agar at 45°C and immediately poured into petri dishes to minimize exposure to elevated temperatures. Unused agar plates were stored at 1°C and discarded if not used within 7 days. Stocks of antibiotics were held at 1°C for no longer than 6 months. Monthly tests over a 6-month period with known sensitive isolates of *E. coli* indicated adequate storage stability for all antibiotics stored under these conditions. Powders were weighed to 0.1-mg accuracy; liquids were quantified by micropipette.

Data processing and indexing. *E. coli* isolates were considered resistant to an antibiotic only if their

TABLE 1. Antibiotics and their application to humans and food animals

Antibiotic generic name	Spectrum of use[a] in:	
	Humans	Food animals
Chloramphenicol	O, I, 1	R
Streptomycin	O, I, 1	R
Ampicillin	O, I, 1	R
Tetracycline	O 1	R
Chlortetracycline	O 1	P 1, 2, 3, 4; C 1, 2, 3; S 1, 2, 3
Oxytetracycline	O 1	P 1, 2, 3, 4; C 1, 2, 3, 5; S 1, 2, 3
Neomycin	O, I, 1	P 1; C, R; S 1
Nitrofurazone	TR 1	P 1; C, R; S 1
Nalidixic acid	O 1	R
Kanamycin	O, I, 1	R
Penicillin G	O, I, 1	P 1, 2, 3; C, R; S 2, 3
Sulfathiazole	O 1	R

[a] C, Cattle; I, injection; O, oral; P, poultry; R, restricted, not allowed, or not applicable; S, swine; T, topical; 1, disease control; 2, feed efficiency; 3, growth promotion; 4, egg production; 5, milk production.

TABLE 2. Antibiotics and concentrations used for determining MAR in *E. coli* isolates

Antibiotic generic name	Trade name	Vendor	Use concn (μg/ml)
Chloramphenicol	Chloromycetin	Parke-Davis	10.0
Streptomycin	Streptomycin	Lilly	12.5
Ampicillin	Amcill S	Parke-Davis	10.0
Tetracycline	Tetracycline	Parke-Davis	25.0
Chlortetracycline	Auredmycin	Lederle	25.0
Oxytetracycline	Terramycin	Pfizer	25.0
Neomycin	Mycifradin	Upjohn	50.0
Nitrofurazone	Furacin	Eaton	28.0
Nalidixic acid	Neggram	Winthrop	25.0
Sulfathiazole	Sulfathiazole	Merck	500.0
Kanamycin	Kantrex	Bristol	25.0
Penicillin G	Crysticillin	Squibb	75.0[a]

[a] Penicillin G is given in international units.

growth in the presence of the antibiotic was as well developed as their growth on the control plate. Any sign of inhibition or sensitivity was considered to be indicative of nonresistance. This strict definition of resistance was necessary to make the interpretation of results easier and consistent.

Antibiotic resistance was scored on optical scan sheets (utility layout sheet, National Computer Systems), and the data were evaluated by computer. The program provided MAR indices and MAR profiles, as well as other data not presented in this paper. The data could be grouped to provide profiles or indices for a single isolate, sample, environment, food process, or any other designation or grouping of data where an MAR index or profile would provide useful information for the evaluation of a health risk. The MAR index, when applied to a single isolate, is defined as a/b, where a represents the number of antibiotics to which the isolate was resistant, and b represents the number of antibiotics to which the isolate was exposed. For example, if the isolate were exposed to 12 antibiotics and were resistant to 6, the index for the isolate would be 6/12, or 0.50. If indexing is applied to a sample from which several isolates were taken, the index of the sample would be $a/(b \cdot c)$, where a is the aggregate antibiotic resistance score of all isolates from the sample, b is the number of antibiotics, and c is the number of isolates from the sample. For example, if the aggregate antibiotic resistance score of 30 isolates taken from a sample were 240, the MAR index of the sample would be $240/(12 \times 30)$, or 0.66.

RESULTS AND DISCUSSION

The procedure used for the isolation of *E. coli* was found to be both simple and reliable. Less than 2% of the total isolates were citrate positive, whereas all randomly selected citrate-negative isolates screened by the indole–methyl red–Voges-Proskauer–citrate test were confirmed as *E. coli*. It was found to be important to remove isolates from the EC medium at the first indication of gas production to avoid the overgrowth of *E. coli* by *Klebsiella* spp., *Proteus* spp., or *Enterobacter* spp. Early removal was also imperative to minimize plasmid exchange, which may rapidly occur where these species coexist under conditions of vigorous growth (17). It is of interest that the MAR index for isolates from human raw sewage was greater than the index found among isolates recovered by direct anal swabbing. Although there may be several factors contributing to this observed difference, it is likely that plasmid exchange, occurring in the sewerage system, is the most significant cause. This assumption is in harmony with observa-

TABLE 3. Sources of *E. coli* isolates with MAR indices of 0.199 or less

Source	No. of sites sampled	No. of samples	No. of isolates	% of isolates resistant to three or more antibiotics	MAR index
Domestic animals					
Sheep (grazing)	10	50	500	0	0.070
Cattle (grazing)	15	75	750	0	0.130
Cattle (feedlot)	10	130	3,370	7.8	0.120
Wild animals					
Deer	7	10	100	0	0.120
Raccoons	8	15	150	9	0.111
Opossums	5	7	70	1.5	0.115
Coyotes	4	10	100	0	0.142
Birds	30	150	1,500	0	0.080
Rural vector animals					
Rats	5	9	45	0	0.090
Mice	3	3	15	0	0.070
Insects	7	7	0	0	0.000
Orchard soil	23	115	563	0	0.080

tions made by Graybow et al. (7, 8), who demonstrated that plasmid exchange readily occurs between *E. coli* and other coliform bacteria in the stagnant areas of wastewater systems.

All sampling was highly randomized and believed to be representative of the animals and environments sampled. With the exception of cattle feedlot samples, all samples were taken within the state of Oregon, most of which were from the agricultural areas of the Willamette Valley. Raw sewage samples and the mouse and rat feces from metropolitan areas were mostly collected from the sewerage systems of the major population centers of Oregon (Portland, 366,383; Eugene, 105,624; Salem, 89,233; Springfield, 41,621; and Corvallis, 40,960). Three other communities with populations of less than 2,500 were also included. Sewage samples were taken on four separate occasions at 3-month intervals with no significant difference in the MAR index among samplings or areas samples.

Very few MAR *E. coli* isolates were found among animals in which antibiotics are seldom or never used. Over 9,000 *E. coli* isolates were evaluated for the prevalence of MAR *E. coli*. Data presented in Table 3 are derived from isolates taken from sources having MAR indices of 0.199 or less, whereas Table 4 lists sources from which isolates had MAR indices of 0.200 or greater. Also listed are percentages of isolates resistant to three or more antibiotics, which gives some indication of the distribution of MAR *E. coli* within the sample. Isolates of *E. coli* from humans, commercial poultry farms, swine, dairy cattle, and vector animals associated with these environments could easily be identified and distinguished from *E. coli* isolates originating from other sources. These primary reservoirs for high-MAR *E. coli* are also the major reservoirs for enteric diseases which are transmitted to humans through food and water. The data strongly suggest that, within the geographic limits of this study, fecal contamination from these high-risk sources can be distinguished. The ability to make this distinction has obvious usefulness to the food industry, federal and state regulatory authorities, and public health agencies.

The choice of an MAR index of 0.200 to differentiate between low- and high-risk contamination is arbitrary. Indices of between 0.200 and 0.250 are in a range of ambiguity, and samples in this range require careful scrutiny. For example, Table 5 contains six hypothetical examples, each representing conditions giving an MAR index of 0.200 but each likely representing different degrees of risk. Example 1 is a situation in which 6 (20%) of 30 isolates from a sample are resistant to all 12 antibiotics, and the remaining 24 isolates are resistant to none. This sample would likely represent contamination from two or more sources, one of which is highly significant. Example 6, on the other hand, is a case in which 24 (80%) of 30 isolates are resistant to 3 of 12 antibiotics, and the remaining 6 isolates are resistant to none. This sample likely represents a single source of *E. coli* and a contamination of passing concern.

The following are two examples of how MAR indexing was used to identify high-risk contamination potentially hazardous to humans. It is common practice in the Willamette Valley to pasture sheep in orchards during winter months. Although this practice is of concern and discouraged, sheep are not considered to be high-risk

TABLE 4. Sources of *E. coli* isolates with MAR indices of 0.200 or more

Source	No. of sites sampled	No. of samples	No. of isolates	% of isolates resistant to three or more antibiotics	MAR index
Humans					
Raw sewage	8	32	332	84.6	0.630
Anal swabs	102	100	351	47.5	0.370
Poultry					
Brooder houses	4	40	400	88.0	0.537
Laying houses	2	20	210	100.0	0.457
Swine, piggeries	2	32	330	88.0	0.595
Metropolitan vector animals					
Rats	7	17	34	51.6	0.430
Mice	4	4	123	100.0	0.473
Insects	4	5	126	53.0	0.312
Dairy cows	18	18	182	64.0	0.410

TABLE 5. Six hypothetical examples of *E. coli* isolates having various degrees of antibiotic resistance among 30 isolates of each example but where the MAR index for each group is 0.200

Example	Total no. of isolates from sample	No. of MAR isolates	No. of antibiotics to which MAR isolate is resistant	No. of isolates having no resistance
1	30	6	12	24
2	30	8	9	22
3	30	9	8	21
4	30	12	6	18
5	30	18	4	12
6	30	24	3	6

animals, and there has been no known outbreak of enteric disease from this practice; yet a potential risk exists. Sheep are not usually exposed to antibiotics. They were found to have an MAR index of 0.075. One herd, however, was found to have an index of 0.275, considerably higher than expected. Further sampling showed that their drinking water was contaminated by *E. coli* having an MAR index of 0.375 and originating from a failed septic tank system of a nearby residence. Fecal contamination of an orchard crop from this source would carry a higher-than-normal risk which, in this case, was identified by MAR indexing.

The second example relates to poultry. Several poultry production facilities located in the Willamette Valley are surrounded by orchards and other farm crops. Poultry, one of the major reservoirs of *Salmonella* spp., is considered to be a high-risk source of fecal contamination. It was found that feces from birds and animals located in the vicinity of poultry brooder and laying houses have MAR indices similar to poultry. Wild birds often have access to poultry feeds containing antibiotics, or they consume poultry droppings. Rodents have the same access, whereas predatory animals such as coyotes and raccoons acquire MAR *E. coli* presumably from consuming rodents, escaped poultry, or discarded poultry carcasses. It was also found that nitrofurazone-resistant *E. coli* organisms were frequently isolated from the poultry environment but seldom elsewhere. As mentioned earlier, nitrofurazone has very limited use but is allowed in animal feeds for the control of coccidiosis in poultry and bacterial enteritis (scours) in swine. Nitrofurazone may prove to be a useful marker, signaling fecal contamination from this source. Although vector animals, particularly wild birds, are of concern, the primary threat comes from the use of raw poultry manure as fertilizer. This practice has been difficult to detect in the past because of other innocuous sources of *E. coli* contamination of these crops. The use of MAR indexing can easily detect the use of poultry manure not only by the increase in the index but also by the presence of isolates resistant to nitrofurazone.

Both of these incidences are illustrative of how MAR indexing might apply. The MAR index of the isolates in both cases was higher than normally expected; both cases signaled the exposure of the source animals to high-risk contamination with the increased possibility of shedding microorganisms harmful to humans. These investigations suggest that an unexpected increase in the MAR index of *E. coli* isolates from food should prompt an immediate investigation even though the number of *E. coli* organisms present is below the established guideline or standard.

It is not intended that MAR indexing replace current methodology or standards for *E. coli* in foods or water but, rather, that it supplement them by providing additional information about the origin of contamination, information which has not been available in the past.

MAR indexing is likely to provide a useful tool for better risk assessment by identifying contamination from high-risk environments. The presence or absence of MAR *E. coli* would give more significance to current arbitrary numerical standards.

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