APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1999, p. 3483–3486 Vol. 65, No. 8
0099-2240/99/$04.00+0
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Use of Antibiotic Resistance Analysis To Identify Nonpoint Sources of Fecal Pollution

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Received 22 March 1999/Accepted 27 May 1999

A study was conducted to determine the reliability and repeatability of antibiotic resistance analysis as a method of identifying the sources of fecal pollution in surface water and groundwater. Four large sets of isolates of fecal streptococci (from 2,635 to 5,990 isolates per set) were obtained from 236 samples of human sewage and septage, cattle and poultry feces, and pristine waters. The patterns of resistance of the isolates to each of four concentrations of up to nine antibiotics were analyzed by discriminant analysis. When isolates were classified individually, the average rate of correct classification (ARCC) into four possible types (human, cattle, poultry, and wild) ranged from 64 to 78%. When the resistance patterns of all isolates from each sample were averaged and the resulting sample-level resistance patterns were classified, the ARCCs were much higher (96 to 100%). These data confirm that there are measurable and consistent differences in the antibiotic resistance patterns of fecal streptococci isolated from various sources of fecal pollution and that antibiotic resistance analysis can be used to classify and identify these sources.

Contamination of surface water and groundwater with untreated manure and sewage continues to be a serious environmental problem (20). Exposure of the public to pathogens in drinking and recreational water is unacceptable, and high levels of nitrogen and phosphorus in the receiving waters can cause eutrophication. In many waters, contamination can occur from several possible sources, including animal sources in agriculture (e.g., cattle feedlots and poultry litter piles) as well as human sources (failing septic systems and leaking sewer lines), and thus the actual source(s) of the pollution is often unknown.

There have been many attempts to develop a method for source determination, including measurement of the ratio of fecal coliforms (FC) to fecal streptococci (FS) (1, 3, 16), detection of the presence of specific types of bacteriophages (4, 14, 17, 21) or FS (2, 18), molecular techniques such as pulsed-field gel electrophoresis (6, 19), and antibiotic resistance analysis (7, 9–12, 15). However, few have shown high levels of specificity and reliability. Among these, one promising approach is based on the analysis of differences in antibiotic resistance by using the multiple antibiotic resistance (MAR) index (7, 12, 15). In 1996, we reported a new type of antibiotic resistance analysis (ARA), which builds on the power of the MAR analysis (22) but with FS instead of FC. FS (a multispecies grouping of gram-positive, catalase-negative cocci that hydrolyze esculin and grow in 6.5% NaCl and at 45°C) were used instead of FC because FS survive well in the environment and are found in all potential pollution sources (e.g. composted poultry litter [23]), in contrast to FC. By increasing the number of concentrations for each drug tested from one to four and by using the power of discriminant analysis (a multivariate statistical technique [5]), we were able to correctly classify isolates of FS from known and unknown sources based on their antibiotic resistance profiles. For example, when isolates from cattle, human, poultry, and wild sources were analyzed, the average rate of correct classification (ARCC) was 84% (22).

Although our initial study showed good classification success, it was based on a relatively small number of isolates. In the present study, we used ARA to classify four new, larger data sets, collected over a 4-year period. By using more and/or different antibiotics, we obtained classification success rates that, although not as high as the initial rate, were still well above background (random) classification levels. Thus, these data support our hypothesis that ARA can be a useful tool in classifying and identifying potential sources of fecal pollution in natural waters.

MATERIALS AND METHODS

Sample collection. Four sets of samples were collected from June 1993 through April 1997 (Table 1). Samples were obtained from the following types of known sources: beef and dairy cattle feces (cattle), turkey and chicken feces (poultry), domestic sewage influent and septage (human), and pristine stream-water (wild). For each agricultural animal source, samples were collected from fresh feces from local farms located in Rockingham County, Va. Sewage samples were collected from the influent of local municipal sewage treatment plants (Fisherville, Stephens City, Middletown, and Parkers Mill, Va.). These facilities process only human waste, but the possibility of agricultural input from surface runoff cannot be excluded. Septage samples were obtained from septic trucks. Wild isolates were collected from pristine streams (Briery Branch, Upper Dry River) in the George Washington National Forest, Va. Because these pristine streams are not significantly impacted by humans (8), the stream samples were assumed to contain bacteria from the feces of wild animals only. After collection, all samples were placed on ice and processed within 6 h.

Isolation of FS. FS were isolated as described previously (22). Various amounts of fecal samples (0.1 to 1.0 g) were suspended in 50 ml of saline buffer (8.5 g of NaCl, 0.3 g of KH_2PO_4, and 0.6 g of Na_2HPO_4 per liter [pH 7.3]) and were filtered through 0.45-μm-pore-size filters (type GN-6; Gelman Sciences). The filters were then transferred to a 50-mm petri dish containing an absorbent pad soaked with 1.9 ml of Enterococcosel broth (BBL). The filters were incubated for 48 h at 37°C. After incubation, 48 (or, for some of the earlier samples, 96) colonies from each sample were randomly picked with sterile toothpicks, transferred to microwell plates containing 0.2 ml of Enterococcosel broth, and incubated for another 48 h at 37°C. Some isolates were randomly chosen for characterization, and 161 of the 190 isolates were classified as FS.

Antibiotics. Antibiotics were selected because of their widespread use in animals and/or humans: amoxicillin (AMX) (Sigma); ampicillin, sodium salt (AMP) (Sigma); chlortetracycline hydrochloride (CTC) (Sigma); erythromycin (ERY) (Sigma); gentamicin (GEN) (Sigma); oxytetracycline hydrochloride (OTC) (Sigma and ICN); salinomycin (SAL) (Agri-Bio and Sigma); streptomy-

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TABLE 1. Descriptions of the data sets used in the analyses

Data set	Collection dates (mo/yr)	No. of isolates (number of samples)					Antibiotics tested	Reference or source
		Cattle	Human	Poultry	Wild	Total		
1	1/93–3/93	568 (6)	181 (2)	552 (6)	134 (3)	1,435 (17)	CTC, OTC, SAL, STR	21
2	6/93–7/94	2,110 (27)	2,054 (23)	1,140 (13)	686 (9)	5,990 (72)	AMP, ERY, GEN, TET, VAN	This study
3	9/94–2/95	1,157 (13)	533 (6)	542 (7)	403 (5)	2,635 (31)	OTC, SAL, STR, TET, VAN	This study
4	6/95–8/95	1,174 (18)	837 (15)	737 (12)	96 (2)	2,844 (47)	AMX, ERY, OTC, STR, TET, VAN	This study
5	5/96–4/97	1,083 (25)	802 (7)	376 (11)	771 (18)	3,032 (69)	AMP, AMX, CTC, ERY, OTC, SAL, STR, TET, VAN	This study

cin sulfate (STR) (Sigma); tetracycline (TET) (Sigma); and vancomycin (VAN) (Sigma). All these drugs are used in both humans and animals except SAL (chickens only) and VAN (humans only) (13). Stock solutions of each drug were prepared in water (AMP, OTC, and STR), in 1:1 water-ethanol (CTC, ERY, SAL, TET, and VAN), or in 1:1 water-methanol (AMX), filter sterilized, and added to autoclaved Trypticase soy agar (BBL). The following final concentrations were used: 20, 40, 60, and 80 μg/ml for CTC, OTC, and STR; 5, 10, 15, and 20 μg/ml for AMX and GEN; 10, 15, 30, and 50 μg/ml for AMP, ERY, and TET; 5, 10, 15, and 30 μg/ml for VAN; and 1, 5, 10, and 15 μg/ml for SAL.

Antibiotic resistance was determined as described previously (22). The isolates were transferred with a 48-prong replica-plater (Sigma) from the Enterococcosel-containing microwells to a set of antibiotic-containing Trypticase soy agar plates. Each set consisted of one plate of each concentration of each antibiotic and one control plate containing no antibiotic. The plates were incubated for 24 h, and growth of each isolate on each concentration of each antibiotic was determined. An isolate was considered to be resistant to a given concentration of antibiotic if growth occurred on that plate. Esculin-negative isolates and isolates that did not grow on the control plates were not used in the analyses.

Discriminant analysis. Data for the ability of each of the known isolates to grow in the presence of each concentration of each antibiotic were analyzed by SAS software (VAX version 6.08; SAS Institute Inc.) using the procedure DISCRIM (prior probabilities, equal; covariance matrix, pooled). The ARCC for each combination of antibiotics was computed by averaging the percentages of correctly classified isolates (along the diagonal) (22).

Discriminant analysis was performed on five sets of isolates: one previously published (22) set (set 1) and four new sets (sets 2 to 5). The sets were collected during different times and differ in both the number and combination of antibiotics on which the isolates were tested (Table 1).

Discriminant analysis was performed in two ways: at the isolate level and at the sample level. For isolate-level analyses, each isolate was classified individually, based on its resistance to each concentration of each drug, into one of four types (cattle, human, poultry, and wild). For these analyses, the number of isolates per data set ranged from 1,435 to 5,990. For sample-level analyses, the resistance of all of the isolates from each sample was averaged for each concentration of each antibiotic. For example, if one-quarter of the isolates in a given sample were resistant to 40 μg of STR/ml, the average value of 0.25 would be used. Average values for each concentration of each drug were then used to classify the sample into one of the above four groups. The number of samples per data set ranged from 17 to 72.

RESULTS

Isolate-level analyses. Over a 4-year period, four sets of isolates were collected and analyzed (by using various numbers and combinations of antibiotics) to determine if the high rate of classification success that was obtained previously (22) could be obtained again. In the previously published set (set 1), the ARCC of those isolates was 84% when analyzed with four drugs: CTC, OTC, SAL, and STR (Table 6 in reference 22). Cattle and wild isolates were classified moderately successfully (79 and 75%, respectively), and human and poultry isolates were very well classified (93 and 88%, respectively).

To see if other drugs could provide better classification, a second set of isolates (set 2) was tested by using an entirely different set of drugs: AMP, ERY, GEN, TET, and VAN. These drugs were chosen because they are commonly used in humans. Unfortunately, when set 2 was analyzed, the ARCC was only 64% (Table 2). Cattle and poultry isolates were well classified, but human and wild isolates were very poorly classified. A third set (set 3) was tested by using a subset of the drugs from the two previous sets: OTC, SAL, STR, TET, and VAN. When set 3 was analyzed, the ARCC was 66% (Table 3). As with set 2, human isolates were poorly classified and poultry isolates were well classified. A fourth set (set 4) was tested by using six drugs: AMX, ERY, OTC, STR, TET, and VAN. AMX was added because of its high levels of use in humans. When set 4 was analyzed, the ARCC was 65% (Table 4). Human isolates were very poorly classified, but wild isolates were well classified.

To determine if data from more drugs would improve classification success, a fifth set (set 5) was tested by using AMP, AMX, CTC, ERY, OTC, SAL, STR, TET, and VAN. Additionally, this set included only septic sources for the human samples, while the previous sets contained samples of primary sewage influent, which could have been contaminated with other sources by overland flow. When set 5 was analyzed, the ARCC increased to 78% (Table 5). The classification success of human isolates increased to 82%. Poultry and wild isolates were well classified in this set, but cattle isolates were poorly classified.

Sample-level analyses. In addition to the isolate-level analyses, we analyzed all five data sets at the sample level. When the antibiotic resistance patterns of all the isolates in each sample were averaged, the classification of the samples was very high (Table 6). Three of the five data sets were perfectly classified at the sample level, and overall only 5 of 236 samples (2%) were incorrectly classified.

TABLE 2. Classification of 5,990 isolates of FS from known sources (set 2)[a]

Source	No. (%) of isolates classified as:			
	Cattle	Human	Poultry	Wild
Cattle (n = 2,110)	1,698 (80)	102 (5)	103 (5)	207 (10)
Human (n = 2,054)	451 (22)	994 (48)	342 (17)	267 (13)
Poultry (n = 1,140)	70 (6)	153 (13)	912 (80)	5 (1)
Wild (n = 686)	316 (46)	32 (5)	10 (1)	328 (48)

[a] The ARCC for this analysis (with AMP, ERY, GEN, TET, and VAN) was 64%.

TABLE 3. Classification of 2,635 isolates of FS from known sources (set 3)[a]

Source	No. (%) of isolates classified as:			
	Cattle	Human	Poultry	Wild
Cattle (n = 1,157)	740 (64)	158 (14)	77 (6)	182 (16)
Human (n = 533)	112 (21)	242 (45)	73 (14)	106 (20)
Poultry (n = 542)	16 (3)	25 (5)	483 (89)	18 (3)
Wild (n = 403)	92 (23)	27 (7)	17 (4)	267 (66)

[a] The ARCC for this analysis (with OTC, SAL, STR, TET, and VAN) was 66%.

TABLE 4. Classification of 2,844 isolates of FS from known sources (set 4)[a]

Source	No. (%) of isolates classified as:			
	Cattle	Human	Poultry	Wild
Cattle (n = 1,174)	732 (62)	135 (12)	162 (14)	145 (12)
Human (n = 837)	235 (28)	313 (37)	182 (22)	107 (13)
Poultry (n = 737)	125 (17)	73 (10)	529 (72)	10 (1)
Wild (n = 96)	4 (4)	4 (4)	3 (3)	85 (89)

[a] The ARCC for this analysis (with AMX, ERY, OTC, STR, TET, and VAN) was 65%.

TABLE 6. Sample-level classification of FS from known sources

Data set	No. of correctly classified samples/total no.				ARCC[a] (%)
	Cattle	Human	Poultry	Wild	
1	6/6	2/2	6/6	3/3	100
2	27/27	22/23	13/13	8/9	96
3	13/13	6/6	7/7	5/5	100
4	16/18	14/15	12/12	2/2	96
5	25/25	7/7	11/11	18/18	100

[a] The ARCC of the samples was determined by dividing the number of correctly classified samples for all source types by the total number of samples in that data set.

DISCUSSION

Isolate-level analyses. Although there were lower rates of classification success for the isolates in the larger datasets than was seen previously, many more isolates were correctly classified (greater than 60%) than would occur as a result of random classification into one of four groups (25%). Even though these subsequent sets of isolates were not classified as well as the initial set, the ARCCs were consistently more than 60% correct in samples that were collected from many different sources over a 4-year period.

Sets 2, 3, and 4 were similarly classified (similar ARCCs), but many of the individual sources showed variability in classification success. The reasons for this variability are unclear. The variation in classification success may be explained in part by the different number and combination of drugs used. In general, the number of drugs that were used in a classification was only weakly associated with classification success. No particular drug or drugs seem to be necessary for good classification in all datasets: removal of a given drug from the analysis may reduce the ARCC for one data set but may have no appreciable effect for another (analyses not shown). Thus, it seems likely that the more drugs that are used, the better the chances of getting a combination of drugs that is successful in discriminating among that particular set of samples.

Another hypothesis for the variation in classification success of individual sources from set to set could be changes in the populations from which the samples were taken. These samples were collected over a 4-year period from many different locations, and so this possibility cannot be excluded. If the resistance patterns do change over time or from location to location, the database that is used to classify unknown samples should be composed of recently collected, local samples.

The classification of human isolates improved markedly in set 5. This could have been a result of the use of more drugs, but it could also have been caused by the use of septage as the source of the human samples. Human samples in sets 1 to 4 were from influent to municipal sewage treatment plants. Although most of the sewage was human in origin, the influent in many of the plants could have included overland flow from agricultural land, which could have introduced contaminating bacteria from animal sources and thus reduced the classification success.

Poultry isolates were consistently very well classified. Because chickens and turkeys often receive regular exposure to antibiotics in their food or water, there is strong selection pressure on their fecal bacteria to become resistant to these drugs, and this is reflected in the high classification rates. Cattle isolates, however, were generally poorly classified, especially in the last set of isolates. Generally, cattle are only occasionally given antibiotics and thus would seem to be more similar to wild isolates, which receive no antibiotics. The high proportion of cattle isolates that were misclassified as wild in set 5 (24%) supports the similarity of cattle and wild isolates.

Sample-level analyses. The sample-level analyses strongly support our hypothesis that patterns of antibiotic resistance can be used to classify FS isolated from different sources. When the resistance data from all the isolates from each sample were averaged, the classification of all samples was excellent (98%). However, a drawback of sample-level analysis is the necessary assumption that all of the isolates in a given sample are from the same source (because they are all averaged together). This assumption is valid for known, homogeneous samples, such as those analyzed here but would not be valid for a sample which was contaminated by more than one source. If it is assumed that a sample has a single major source, the sample-level analysis would be preferred because of the high ARCCs. If this is not the case, the isolate-level analysis should be used, because it can assign each of the isolates to a specific source.

In conclusion, the large number of isolates tested so far confirms that sources of fecal pollution can be differentiated by using ARA. Although some isolates were misclassified, the majority of isolates were correctly classified in all sources from all data sets. The information contained in the resistance patterns thus seems strong enough to use for classification of unknown isolates from polluted waters, which may contain mixtures of different sources. However, further research is needed to determine if antibiotic resistance analysis can accurately identify the components of mixed samples.

TABLE 5. Classification of 3,032 isolates of FS from known sources (set 5)[a]

Source	No. (%) of isolates classified as:			
	Cattle	Human	Poultry	Wild
Cattle (n = 1,083)	581 (54)	141 (13)	95 (9)	266 (24)
Human (n = 802)	66 (8)	655 (82)	16 (2)	65 (8)
Poultry (n = 376)	21 (5)	10 (3)	341 (91)	4 (1)
Wild (n = 771)	83 (11)	1 (0)	11 (1)	676 (88)

[a] The ARCC for this analysis (with AMP, AMX, CTC, ERY, OTC, SAL, STR, TET, and VAN) was 78%.

ACKNOWLEDGMENTS

We thank J. Monroe and C. Hagedorn for critical reading of the manuscript and R. Domangue, R. Harris, I. Knight, and G. Wyngaard for discussion and helpful comments.

This work was supported by the James Madison University Department of Biology and by grants from the Virginia Water Resources Research Center and the Shenandoah Valley Soil and Water Conservation District of Virginia.

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