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Screening for Antimicrobial Resistance in Fecal Samples by the Replica Plating Method

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Replica plating can be used for the detection of antibiotic resistance in normal flora. We have evaluated this application of the replica plating method by comparing it with a five-colony method. The replica plating method uses a single plate for each antibiotic, with a concentration just above that for borderline resistance. By the five-colony method, five colonies per sample were picked, chosen to represent all different colony morphologies present, and MICs were determined by a standard agar dilution method. The gram-negative, aerobic floras of 131 fecal samples were screened for resistance to ampicillin, cefuroxime, nalidixic acid, trimethoprim, sulfamethoxazole, and tetracycline by both methods. The rate of resistance detection by the two methods did not differ statistically for any of the antibiotics tested. The breakpoint concentrations used for the replica plates in the study gave results similar to those produced by the agar dilution method and the breakpoint values of the National Committee for Clinical Laboratory Standards and can thus be recommended. As the only currently used resistance detection method, replica plating facilitates an exact determination of the percentage of resistant colonies/total number of colonies (between 1 and 100%) in a sample. This revealed an uneven distribution, with only 23% of the samples having resistance frequencies in the range of 10 to 85%; usually, the resistant flora either was a small minority or was very dominant in samples with resistance. This phenomenon was present for all of the antibiotics.

The detection of resistant strains in the normal flora is a problem that has been approached in numerous ways. Much studied is the fecal gram-negative flora because of the multitude of potential pathogens and the sheer number of organisms presumably constituting a reservoir of resistance genes (16). Several methods have been used, combining in different ways the testing of isolated colonies and selective plating.

Plates selecting for antimicrobial resistance can be prepared in two ways: by using antibiotic-containing agar plates and plating several dilutions of the sample onto these (1, 2, 5, 7, 9, 15) or by plating the sample onto nutrient agar and adding antibiotic-containing disks and then picking colonies growing within the zone of inhibition for further testing (6, 15, 20, 25). Samples grown on agar not containing antibiotics must somehow be tested for resistance, and the most commonly used method is purifying a number of colonies and testing them. Different protocols for choosing these colonies include picking 1 colony (3) or 1 colony of each morphology type (10, 18, 19); picking 5 (13, 26), 10 (15, 21, 24), or 12 colonies (11); or picking 5 colonies of each colony type (4). Another approach, which was pioneered by Marshall and Levy and others (17, 21, 23) and which was based on the replica plating method of Lederberg and Lederberg (14), is to choose a not too densely grown master plate that allows the colonies to be counted and to replicate the colonies from that plate onto several different antibiotic-containing plates. This replica plating method and the method that uses selective antibiotic-containing plates are the only ones with which it is possible to determine the frequency of resistant/susceptible strains in each sample. The replica plating method is more exact in the detection of resistance between 1 and 100%, since by the selective plating method the frequency must be estimated by comparing dilutions on nonantibiotic-containing plates, while by the replica plating method exactly the same colonies are plated onto both antibiotic-containing and pure plates. The selective plating method is more sensitive, however, detecting as few as 100 resistant bacteria per g of feces (2).

Replica plating is a fast and practical method for testing large numbers of samples, but so far there have been no reports on how well it correlates with more conventional resistance testing methods. The inocula transferred by the velvet replication surface are dependent on the colony morphology, and can therefore be relatively uneven. In fact, replica plating cannot strictly be called a method for resistance testing, because it is impossible to standardize one of the most crucial parameters; the inoculum density. The cutoff concentrations used are also of great importance; if it is too low susceptible strains will grow through, and if it is too high resistant strains are missed. To test the reliability of the replica plating method, we compared the results produced by that method with those produced by a five-colony method with morphology-based selection by testing the colonies by an agar dilution method according to the guidelines given by the National Committee for Clinical Laboratory Standards (NCCLS) (22) and using the same material for both methods.

MATERIALS AND METHODS

Fecal samples. One hundred fifteen stool samples were collected during a study of the frequency of antibiotic and mercury resistance in the normal flora of healthy subjects. These persons were provided with sterile containers for stool collection and were instructed to bring a fresh sample, preferably collected in the morning of the same day that the sample was submitted. Sixteen additional samples collected by rectal swabs from hospitalized patients receiving long-term care were included to increase the number of samples with resistant isolates, giving a total of 131 stool samples. All samples were cultured before 4 p.m. on the day of collection. Four serial 10-fold dilutions of the stool samples were made in physiological saline and were plated onto MacConkey agar plates (Oxoid Ltd., Basingstoke, England), which is selective for aerobic gram-negative enteric bacteria, and the plates were incubated aerobically overnight at 35°C. Plates with 100 to 1,000 separate colonies (preferably, about 200; mean number of colonies, 322)

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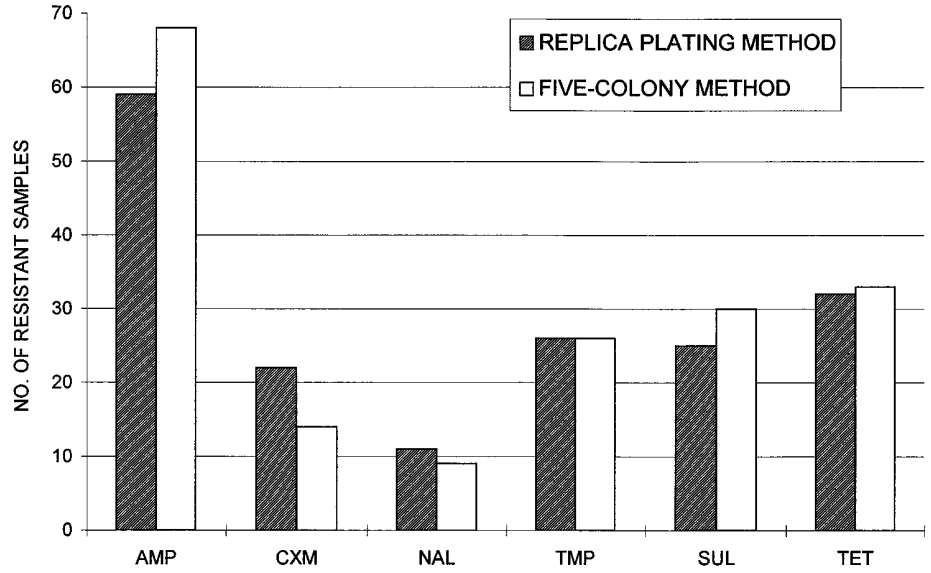

FIG. 1. Number of samples in which resistance was detected by either method (n = 131). None of the differences were statistically significant. AMP, ampicillin; CXM, cefuroxime; NAL, nalidixic acid; TMP, trimethoprim; SUL, sulfamethoxazole; TET, tetracycline.

were chosen for replica plating. For the five-colony method, five colonies from each sample were picked, carefully selecting colonies with different appearances when possible. These were stored at −20°C in tryptone-soy broth (Oxoid Ltd.) with 20% glycerol until they were tested for resistance by the agar dilution method.

Velvet replica plating method. The MacConkey agar master plates were replicated, using a stamp covered with a sterile piece of velvet and exerting only a light pressure, onto a series of Iso-Sensitest agar plates (Oxoid Ltd.) with fixed amounts of antimicrobial agents, as follows: ampicillin, 32 μg/ml; cefuroxime, 16 μg/ml; nalidixic acid, 32 μg/ml; trimethoprim, 8 μg/ml; sulfamethoxazole, 512 μg/ml; and tetracycline, 4 μg/ml. Control plates without antimicrobial agents were included at the beginning and end of each plating series. Each new batch of plates was checked with appropriate control strains. After overnight incubation at 35°C the number of colonies growing on the plates containing antibiotics was counted. When there was near to or apparent 100% growth, the antibiotic plate was compared with the control plate and the missing colonies were counted. If ≥1% of the colonies transferred from the master plate grew on an antibiotic-containing plate, the sample was considered to contain bacteria resistant to the agent.

Determination of MICs and selection of breakpoints. MICs were determined by an agar dilution method according to the guidelines set up by NCCLS (22). The breakpoints for resistance were those recommended by NCCLS (resistant and intermediate), as follows: ampicillin, ≥16 μg/ml; cefuroxime, ≥16 μg/ml; nalidixic acid, ≥32 μg/ml; trimethoprim, ≥16 μg/ml; tetracycline, ≥8 μg/ml; sulfamethoxazole, ≥512 μg/ml. All of the antimicrobial preparations used were from Sigma Chemical Co., St. Louis, Mo. When the MIC distributions of the isolates were plotted, these MIC breakpoints did indeed fall between the resistant and susceptible populations, despite the mix of gram-negative species (data not shown). The breakpoints of the replica plates were selected on the basis of preliminary experiments (data not shown). Although slightly different from those given in the NCCLS guidelines, they were also well within the area between susceptible and resistant; this area contained several dilutions for all antibiotics except ampicillin, and very few isolates had MICs within this range. This margin makes the choice of breakpoints easier, since a one-step dilution up or down does not affect the results much.

Statistical methods. The chi-square analysis-of-contingency table test was used to calculate the significance of the differences between groups. Correlation coefficients were calculated by the Spearman rank correlation test.

RESULTS

Detection of resistance. The number of resistant samples, defined as ≥1% resistant colonies by the replica plating method and at least one resistant colony out of five colonies tested by the five-colony method, did not differ statistically for any of the antibiotics tested (Fig. 1).

Correlation between the methods. For the replica plating method, the percentages of resistant strains were calculated for

TABLE 1. Correlation between replica plating and five-colony method[a]

Antimicrobial agent	% Resistant strains	No. of samples with the following no. of resistant isolates:					
		0	1	2	3	4	5
Ampicillin (r = 0.832; P < 0.0001)	<1	58	12	1	1	0	0
	1–10	4	3	11	1	0	0
	11–50	0	2	6	3	2	2
	51–99	0	1	0	4	1	2
	100	1	0	2	3	4	7
Cefuroxime (r = 0.545; P < 0.0001)	<1	105	3	1	0	0	0
	1–10	11	2	2	0	0	0
	11–50	1	2	1	1	0	0
	51–99	0	0	1	0	1	0
	100	0	0	0	0	0	0
Nalidixic acid (r = 0.862; P < 0.0001)	<1	119	1	0	0	0	0
	1–10	3	0	1	0	0	0
	11–50	0	0	1	0	0	1
	51–99	0	1	0	0	1	0
	100	0	0	0	1	0	2
Trimethoprim (r = 0.913; P < 0.0001)	<1	103	2	0	0	0	0
	1–10	1	0	2	0	0	0
	11–50	1	1	0	2	1	0
	51–99	0	1	0	4	2	2
	100	0	0	1	1	2	5
Sulfamethoxazole (r = 0.784; P < 0.0001)	<1	98	5	2	1	0	0
	1–10	2	0	2	0	0	0
	11–50	0	0	1	1	0	0
	51–99	0	1	1	1	2	2
	100	1	2	1	0	1	7
Tetracycline (r = 0.874; P < 0.0001)	<1	95	2	0	0	2	0
	1–10	3	1	1	2	1	0
	11–50	0	3	0	2	1	0
	51–99	0	0	3	2	1	0
	100	0	1	1	2	3	5

[a] Each sample from the replica plating method was grouped according to the frequency of resistance for that sample (vertical), and the results from the five-colony method were given as zero to five resistant isolates per sample (horizontal). Data for each sample were plotted according to the results of both methods. r is the Spearman rank correllation coefficient. P is the P value.

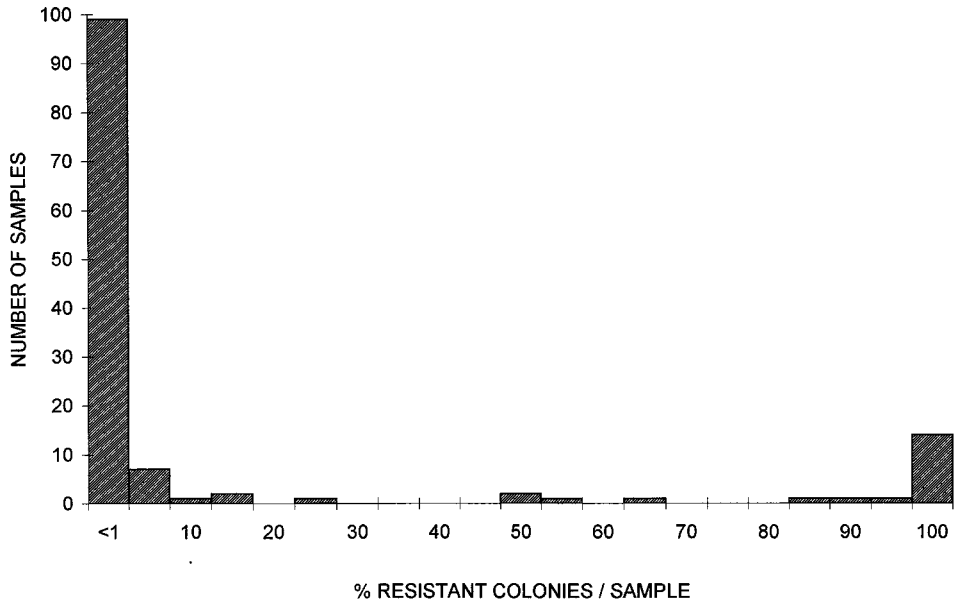

FIG. 2. Distribution of resistance frequencies in the replica plate samples (n = 131) for tetracycline. Most samples have either a very low or a very high percentage of resistant colonies. The bars represent groups of 5% (1 to 5%, 6 to 10%, etc.) except for the bar labeled <1, which indicates <1%.

each sample, and the results were grouped into the following five classes: <1%, 1 to 10%, 11 to 50%, 51 to 99%, and 100%. For the five-colony method the number of resistant isolates among all five colonies is given for each sample. The results produced by both methods for each sample were plotted against each other (Table 1). The correlation was very high for every antibiotic tested (mean correlation coefficient, 0.8; range, 0.5 to 0.9). The correlation coefficients, calculated by the Spearman rank correlation test, are given in Table 1.

Number of strains per sample. The number of isolates having different MIC profiles (differences greater than ±1 dilution) picked from each sample and tested by the five-colony method were as follows: 40% had only one strain, 37% had two different strains, 17% had three different strains, 6% had four different strains, and only 0.8% (one sample) had five different strains. The average number of different strains picked from each sample was 1.9.

Frequency distribution. The frequency distributions of the resistant strains in a sample, produced by the replica plating method, were uneven. Of the samples in which resistant isolates were detected, on average 32% had between 1 and 10% resistant colonies and 45% had between 85 and 100% resistant colonies on their plates, leaving only 23% of the samples with resistance frequencies of from 10 to 85%. This is illustrated in Fig. 2, in which the frequency distribution of tetracycline is presented. Tetracycline is chosen as an example, because the second-highest correlation between the methods was obtained with tetracycline and more samples contained isolates that were resistant to tetracycline than to trimethoprim, which had the highest correlation coefficient, but the trend was similar for all antibiotics.

DISCUSSION

The results obtained by the replica plating method correlated, perhaps surprisingly, well with those obtained by the five-colony method. Several investigators have found that when more colonies are studied, whatever trait is searched for will be found at a higher frequency (21, 27). Murray et al. (21) found more trimethoprim resistance by the replica plating method than by picking 10 random colonies from the same material (21). On the other hand, there has been evidence that five colonies might be an optimal number of colonies to be studied. Nearly 80% of the total number of different *Escherichia coli* O serogroups studied in stool samples by Vosti et al. (27) were found among the first five colonies examined. Apparently, if emphasis is put on picking as many different colonies as possible from a sample instead of picking them totally at random, it makes little difference if one examines 5 or 500 colonies. The two antibiotics producing the largest differences between the two methods were both β-lactams, although the differences were not statistically significant. This is not unexpected, since the inoculation effect is quite marked for these and also because the low-level production of extended-spectrum β-lactamases is not necessarily visible as a rise in the MIC (12). For cefuroxime the replica plating method may provide results that are closer to reality, because it also detects low-level β-lactamase producers. The cutoff for the ampicillin-containing replica plates (32 μg/ml) was higher than the MIC breakpoint (≥16 μg/ml) determined by population distributions, and it is hard to say which one is correct. The replica plating breakpoint was set with the inoculation effect in mind to avoid false-positive results; a lower concentration causes a marked overgrowth of susceptible strains (unpublished data). The very small difference for nalidixic acid is encouraging; heavy inocula could theoretically have been expected to produce false-positive results because of point mutations, and this should have been most visible for nalidixic acid, but apparently this does not happen frequently enough to affect the overall results.

The uneven frequency distribution found by the replica plating method, with most resistance in a sample being found among either less than 10% or more than 85% of the total number of colonies, may be caused by a number of mechanisms. There might be one very dominant strain or one plasmid that has spread to several strains, thus making the plasmid, not the strains, dominant, or there might be several strains or plasmids that happen to carry resistance to the same antibiotic. The work by Hartley and Richmond (8) showed that when

approximately 10 *E. coli* colonies were picked from a sample, they were often of the same strain or one strain was very dominant. All three mechanisms are worth considering. Exact data on resistance frequencies in a sample detected at this magnitude (between 1 and 100%) have not been presented before. As such, it is merely an intriguing fact, but it could be combined with data on species, and possibly plasmid, distributions to enable more interesting conclusions concerning the gram-negative populations inhabiting human intestines and their levels of resistance to be drawn.

In conclusion, replica plating with the cutoff concentrations tested by us has proven to provide essentially the same results as those obtained by a standard method and those obtained by using NCCLS breakpoints. For the screening of large materials, replica plating can be a faster, more practical method, and it also provides additional information on the population dynamics in a material which cannot be obtained by any other method.

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