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Measurement of Antimicrobial-Resistant *Escherichia coli* in Pig Feces with a Hydrophobic Grid Membrane Filter Interpreter System

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Hydrophobic grid membrane filter technology was used to measure resistance among *Escherichia coli* in pig fecal samples to ampicillin, sulfisoxazole, and tetracycline. The method accurately measured resistance, with sensitivities ranging from 96.5 to 99.5% and specificities ranging from 87.0 to 98.3%, and it identified *E. coli* with 96% confidence.

Escherichia coli is an important commensal and pathogen that inhabits the gastrointestinal tracts of humans and animals (9), and it is regarded as an important source of antimicrobial resistance determinants for other human and animal pathogens (19). There is considerable uncertainty regarding the risk of transferring bacterial antimicrobial resistance from food animals to humans, and methods that reliably and accurately measure the abundance of antimicrobial-resistant (AR) bacteria in both populations will help to resolve this uncertainty. The abundance of AR *E. coli* has been expressed as a concentration (14) or as a proportion (13, 14, 16, 27, 29).

The traditional approach for quantifying AR *E. coli* is based on comparing the number of colonies that grow on selective media with and without antimicrobial substances (14, 20, 27). However, few methods have applied new technologies, such as hydrophobic grid membrane filter (HGMF) technology, that permit reliable replica plating (6) and electronic enumeration (26). Because of these advantages, and the availability of approved HGMF-based methods for enumerating *E. coli* in food and water (2), we developed an accurate method (hereafter referred to as HGMF-RES) for measuring the abundance of AR *E. coli* in pig feces with the HGMF system.

Two groups of fecal samples were collected. The first group consisted of 20 fecal samples collected from each of 34 grower-finisher pig (75 to 110 kg live weight) populations. The second group consisted of 35 fecal samples collected from grower-finisher pigs from a single farm. For group 1, the 20 samples from each farm were composited by combining 5 ml of each fecal sample and then diluting them with 0.9% saline solution (1:2, vol/vol) and stomaching for 2 min (25). A 25-ml aliquot of each stomached sample was collected by pipette through a sterile, disposable prefilter with 200-μm-diameter pores (Filtaflex Ltd., Almonte, Canada) and then vortexed with 25 ml of tryptic soy broth (Difco Laboratories) and glycerol (1:1, vol/vol) (Fisher Scientific). One-milliliter subsamples were stored at −70°C. Group 2 samples were processed individually; otherwise, the same methods were used as described for group 1 samples.

Preliminary coliform counts were estimated for each processed fecal sample by the Spiral Plate Model D system (Spiral Systems, Inc., Cincinnati, Ohio) to inoculate predried MacConkey agar plates (Difco Laboratories). The plates were incubated at 35°C for 18 h. Based on the preliminary coliform count, another subsample was serially diluted to about 50 CFU per ml. Then about 150 CFU were inoculated onto a sterile 45-μm-pore-size ISO-GRID HGMF (Gelman Sciences, Laurent, Canada) by using a spread filter (Richard Brancker Research Ltd., Ottawa, Canada).

Inoculated HGMFs were transferred onto predried tryptic soy agar (Difco Laboratories) with 1.5% (wt/vol) magnesium sulfate and incubated at 35°C for 4 h (10) to resuscitate injured cells. Subsequently, HGMFs were transferred onto violet red bile agar (VRBA; Difco Laboratories) and incubated at 37°C for 18 h. These inoculated HGMFs were called "master HGMFs."

Replica plating from master HGMFs to copy HGMFs was performed with the RP-100 replicator (Richard Brancker Research Ltd.) (24, 26). A maximum of six copies of each master HGMF were made with each load of the replicator (Fig. 1). The first copy was placed onto tryptone bile agar (TBA; Oxoid), the last was placed onto Mueller-Hinton (MH) agar, and the middle copies were placed onto antimicrobial-containing MH agar plates. The MI-100 interpreter system (Richard Brancker Research Ltd.) was used to electronically count and record the growths on each copy HGMF in ASCII data files (26).

Bacterial growths on TBA after incubation at 44.5 ± 0.2°C for 18 to 24 h in a water-jacketed incubator (National Appliance Co., Portland, Oreg.) which stained pink with indole reagent were classified as presumptive *E. coli* (10).

Fecal *E. coli* were tested for resistance to the following antimicrobials and breakpoint concentrations: 16 μg of ampicillin (AMP)/ml, 256 μg of sulfisoxazole (SUL)/ml, and 8 μg of tetracycline (TET)/ml. Breakpoint concentrations were chosen according to the National Committee for Clinical Laboratory Standards (NCCLS) guidelines for routine testing of *Enterobacteriaceae* (18). The antimicrobials were added to commercially prepared media, using NCCLS guidelines (18), by a

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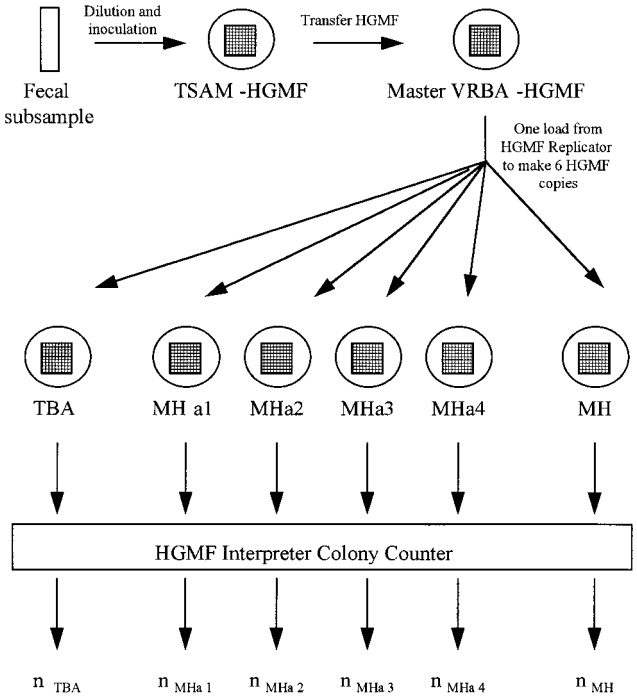

FIG. 1. Preparation of master HGMF from a fecal subsample and its replication, and placement of HGMF copies on media for determination of prevalence of antimicrobial resistance. An example calculation of the proportion (p) of *E. coli* organisms resistant to antimicrobial $a1$ is as follows: $p(a1) = (n_{MHa1} \cap n_{TBA} \cap n_{MH})/(n_{TBA} \cap n_{MH})$, where n is the number of colonies that grew on the respective agar plate. TSAM, tryptic soy agar with 1.5% (wt/vol) magnesium sulfate incubated at 35°C for 4 h; VRBA, violet red bile agar incubated at 37°C for 18 h; TBA, tryptone bile agar incubated at 44.5°C for 18 to 24 h; MH, Mueller-Hinton agar incubated at 35°C for 18 to 20 h (a1, etc., supplemented with antimicrobial 1, etc.).

NCCLS-accredited laboratory (Cathra International, Inc., St. Paul, Minn.).

HGMFs on antimicrobial-containing MH agar plates were incubated at 35°C for 18 to 20 h. Bacterial growths that were convex or dome shaped (33) and occupied at least one-half of an HGMF grid cell on a particular antimicrobial-containing agar were classified as resistant to that antimicrobial. Growths were recorded by the MI-100 interpreter system and manually verified on all HGMFs with a dissecting microscope.

Quality control of the HGMF-RES consisted of using reference strains of *Enterobacteriaceae* (ATCC 35555, ATCC 19606, ATCC 29993, ATCC 35028, CATHRA 23, and ATCC 25922) that were sensitive or resistant to the antimicrobials at the chosen breakpoint concentrations. Reference strains were placed directly onto the agar beside the HGMF with Cathra point inoculators at inocula densities of 0.5 McFarland standard (3). In addition, growths had to have been successfully replica plated across to TBA and MH agar plates before their resistance status was measured.

The accuracy of HGMF-RES in identifying and characterizing *E. coli* isolates was assessed by measuring sensitivity, specificity, and observed agreement between HGMF-RES and NCCLS-accepted tests conducted in a NCCLS-accredited collaborating laboratory. Sensitivity and specificity are used here in the epidemiological sense and describe the proportions of isolates correctly classified by HGMF-RES as resistant and nonresistant, respectively, relative to NCCLS-accepted, designated "gold standard" tests (15). Observed agreement is a parameter that is often used to measure agreements among antimicrobial susceptibility tests (3, 5, 22, 28, 32). Degener et al. (5) categorized observed agreement as follows: satisfactory, >95%; moderate, 85 to 95%; insufficient, <85%.

A total of 304 presumptive *E. coli* colonies were randomly selected from HGMFs on MH agar incubated at 35°C to determine the accuracy of HGMF-RES *E. coli* identification. The genus and species of the 105 group 2 colonies were identified with the GNI system (Vitek Systems, Hazelwood, Mo.) (22), while the 196 group 1 colonies were identified with the Repliscan system (Cathra International, Inc.) (3). The antimicrobial resistance characteristics of the group 1 colonies were independently determined by a NCCLS-accredited laboratory (N. Smart, Veterinary Laboratory Services, Ontario Ministry of Agriculture, Food and Rural Affairs) with the Repliscan system. Both the Repliscan and the HGMF-RES methods used the same commercial media (Cathra), although from different batches.

One technician was able to measure the resistance of over 1,000 *E. coli* colonies per day on an ongoing basis with HGMF-RES. Although coliforms were the predominant bacteria that grew on HGMFs placed on VRBA, in a few samples *Enterococcus* spp. accounted for about 50% of the bacterial colonies. Except for one sample that contained 242 colonies, the number of colonies that grew on HGMF copies placed on TBA ranged between 52 and 200, with a median of 120.

The colonies that grew on VRBA, TBA, MH agar, TET, and SUL plates were easily read by the HGMF interpreter, as the colonies that grew on these plates occupied at least three-quarters of the occupied HGMF grid cell. The interpreter often had difficulty distinguishing large indole-negative colonies from faint indole-positive colonies on the TBA plate; therefore, some colonies were manually marked with a disposable dark pen to make the electronic counting easier. A few colonies that grew on AMP also had to be marked manually to ensure that the appropriate grid cells were recorded by the interpreter. Appropriate growths (or lack of growth) of resistant and sensitive quality control strains were observed on all antimicrobial-containing plates.

Ninety-seven percent (102 of 105) of the isolates from individual fecal samples tested by the Vitek GNI system were identified as *E. coli* with greater than 96% confidence. Two isolates were identified as *Escherichia hermanii* and were from pigs in different rooms. One isolate was *Yersinia enterocolitica* biotype 4 serotype O:3. Ninety-five percent (187 of 196) of the isolates from composite samples tested by Repliscan were identified as *E. coli* with greater than 90% confidence (3). Nine isolates produced indole and were probably *E. coli*; however, the Repliscan system could not rule out *Citrobacter* sp., *Enterobacter* sp., *Yersinia* sp., *Shigella* sp., or *Aeromonas* sp. Indole was produced by 97% (190 of 196) of the group 1 isolates. Ninety-six percent (182 of 190) of these indole-positive isolates were also classified as indole positive on the HGMF indole test, while only 50% (3 of 6) of the indole-negative colonies on Repliscan were indole negative on the HGMF indole test. Further, all indole-negative colonies were identified as *E. coli* by Repliscan. Based on these findings, all bacterial growths on the TBA-HGMF incubated at 44.5°C were considered presumptive *E. coli*, irrespective of indole production.

The sensitivity, specificity, and observed agreement of the HGMF-RES method compared with those of the Cathra Repliscan method that was used by a NCCLS-accredited laboratory in measurement of *E. coli* resistance to AMP, SUL, and TET are presented in Table 1. There was excellent agreement between the two methods.

The HGMF-RES method accurately identified *E. coli* and its

TABLE 1. Comparison of results of HGMF-RES and Cathra Repliscan system methods[a]

Antimicrobial (breakpoint concn [μg/ml])	Proportion of AR *E. coli* by Repliscan	Sensitivity[b] (%)	Specificity[c] (%)	Observed agreement (%)
AMP (16)	0.30	98.3	87.0	90.3
SUL (256)	0.43	97.6	92.9	94.9
TET (8)	0.87	96.5	92.3	95.9

[a] Antimicrobial resistance characteristics of 196 isolates of *E. coli* randomly selected from composite fecal samples were measured.
[b] Percentage of the resistant isolates detected by HGMF-RES.
[c] Percentage of the susceptible isolates detected by HGMF-RES.

resistance to AMP (16 μg/ml), SUL (256 μg/ml), and TET (8 μg/ml) in grower-finisher pig feces. In addition, other work in our laboratory (6) has shown that HGMF-RES estimates *E. coli* concentrations in grower-finisher feces within the precision specifications for the HGMF enumeration technique for *E. coli* (7). Furthermore, HGMF-RES offers a number of advantages over other screening methods, including the ability to measure multiple resistance attributes of colonies, the ability to use an approved method for identifying *E. coli*, and electronic counting and recording of colonies. Thus, this method offers a useful new approach for identifying and quantifying, either as a concentration or as a proportion, AR *E. coli* colonies within pig fecal samples.

The ability of the HGMF-RES method to identify *E. coli* in individual and composite grower-finisher fecal samples is similar to those of approved methods used to identify *E. coli* in foods (1). Previous studies have shown that over 90% of lactose-fermenting coliforms from pig feces selected from MacConkey agar incubated at 37°C are *E. coli* (17, 34), and while it appears that elevating the incubation temperature does not greatly improve the probability of growths being classified as *E. coli*, we nevertheless recommend that bacterial growth at 44.5°C be used as a criterion for identifying presumptive *E. coli*, in keeping with the accepted procedures.

Other workers have measured the proportion of AR *E. coli* by selecting bacterial isolates from MacConkey agar that was incubated at 44°C (2, 30); however, there is evidence of plasmid loss among *E. coli* at incubation temperatures of 44.5°C (11, 12). As resistant genes are often located on plasmids, primary isolation of *E. coli* from CFU incubated on media at 44.5°C may bias the resistant proportion downwards. Therefore, we recommend that master HGMF plates be incubated at 37°C.

Theoretically, when less than 200 colonies grow on an HGMF, it is estimated that over 93% of these colonies originate from single CFU (26). As the HGMF-RES method was designed to have approximately 150 colonies grow on the master HGMFs, it was concluded that for practical purposes these colonies can be considered pure cultures. On this basis, the HGMF-RES can justifiably be used to measure the antimicrobial resistance characteristics of colonies in situ on grids, using media and quality control strains according to accepted standards (18).

The overall observed agreements between HGMF-RES and Repliscan for AMP, SUL, and TET not only surpassed the criteria of Rand et al. (23) of 0.8 for a resistance test to be considered useful but were comparable to those reported between Food and Drug Administration-approved methods (5, 32). A major limitation of observed agreement as a measure of accuracy is that it does not correct for the agreement that occurs due to chance nor does it indicate a test's ability to discriminate between susceptible and resistant isolates, i.e., the test's relative specificity and sensitivity, respectively (15). Thus, we recommend that relative specificity and sensitivity of resistance tests should be presented along with observed agreement when describing a test's accuracy.

One limitation of HGMF-RES is the difficulty in controlling the amount of inoculum transferred to copy HGMFs. Studies have shown that the inoculum dose of *Enterobacteriaeae* effects in vitro resistance to β-lactams, cephalosporins, and sulfonamides (8, 31). However, based on our results with AMP, inoculum effects did not appear to be of practical significance. This may not be the case for other antimicrobials or for other breakpoint concentrations. We advise that additional repeatability and accuracy studies be conducted before using other antimicrobials and breakpoint concentrations (6).

In conclusion, the HGMF-RES method is accurate, precise (6), and capable of handling large numbers of isolates with reasonable laboratory resource demands. In addition, there is the potential to screen colonies on HGMFs for the presence of particular genes (21). The method should therefore help to improve our understanding of the ecology and medical importance of AR bacteria. Previous studies investigating the ecology of AR *E. coli* have generally been constrained by the number of isolates that can be examined (4, 30), but the HGMF-RES method can determine the antimicrobial resistance characteristics of up to 200 *E. coli* colonies without the need to select, subculture, and identify isolates. The ability to measure attributes of large numbers of isolates is an asset to population studies, as it permits the development of efficient sampling plans which are necessary for obtaining estimates of population parameters sufficiently precise to draw reasonable inferences and to test hypotheses.

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