J Vet Diagn Invest 18:172–181 (2006)

Development of a polymerase chain reaction assay for the detection of antibiotic resistance genes in community DNA

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Abstract. Many methods are used to detect antibiotic resistance genes in samples. The objective of the study reported here was to compare polymerase chain reaction (PCR) analysis of community DNA with fecal culturing for detecting antibiotic resistance genes in cattle samples. In the laboratory-based portion of this study, known concentrations of an *Escherichia coli* strain with 3 antibiotic resistance genes (*cmy*-2, *flo*, and *cat*) were added to feces from dairy cattle. These genes were used to assess the effect of various primer pairs, chromosomally versus plasmid-encoded genes, and gene copy number on the sensitivity of PCR amplification. Gene-specific PCR amplification was performed on the community DNA extracted from the feces. Feces were cultured for the inoculated strain. In the field-based portion of the study, 80 cattle fecal samples of unknown gene status were compared by use of similar methods. Culture and PCR amplification from community DNA extractions produced variable results, and this variability was most noticeable at dilutions that approached the detection limit of the assay. Typically, PCR amplification had a higher sensitivity than did culture for detecting the gene of interest. However, the sensitivity of culture was improved by plating on selective media containing antibiotics. The community DNA approach enables assessment of bacterial communities in complex samples such as feces, a task that can be prohibitive by budget or time constraints associated with culture methods. Through a strategic combination of culture and community DNA approaches, the relationship between specific selection pressures and the persistence and dissemination of specific resistance genes can be elucidated.

Key words: Antibiotic resistance; community DNA; DNA extraction.

Introduction

With concerns of increasing rates of antibiotic resistance, numerous studies have focused on the spread of resistance genes. In studying the factors that influence the acquisition, dissemination, and persistence of resistance, work has been performed in a variety of settings, including human hospitals and human communities,[5,21] animal and plant agricultural systems,[9,18] processing plants and retail meats,[35,48] and the environment.[6,33] Many methods assess the type and quantity of antimicrobial resistance genes that are present in this diversity of samples. However, many of these methods can be difficult and expensive to perform, especially if multiple samples must be tested over time.

Most studies and surveillance systems for antibiotic resistance rely on culturing of bacteria to obtain isolates that can be subsequently tested for resistance. Many studies screen for phenotypic resistance through direct plating or by determining the minimum inhibitory concentration (MIC) of various antimicrobials. Other studies look for the presence of specific genes that confer this phenotypic resistance. All of these approaches, which rely on the culturing of specific bacteria, can be time consuming and expensive. More importantly, these isolation techniques are often highly selective,[17,23,29,39] and may miss most of the bacteria in a sample that are unculturable.[12,15,44] Studies using metagenomics and environmental samples to investigate unculturable bacteria also have been published.[8,10,31] Currently, this approach involves the screening of shotgun DNA libraries from environmental sources. This approach also can be time consuming and expensive.

Another approach to antibiotic resistance detection and monitoring is to analyze the total DNA (community DNA) extracted from a sample for the presence of a specific resistance gene. This approach makes the assumption that the resistance gene is the unit of interest because of the potential for this gene to move among bacteria of variable relatedness. In theory, the extracted community DNA should contain the genetic information from all of the bacteria present within the sample. Several studies have used community DNA as a template for 16S

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rRNA amplification analysis of the bacterial genetic diversity within a sample,[13,34,36] and occasionally, for the detection of antibiotic resistance genes.[1,6] In other studies, the conserved sequences found in integrons have been used to assess the presence and diversity of gene cassettes within the integrons from environmental DNA.[32,38] Whereas culturing approaches must focus on a specific set of bacteria, then must select specific isolates from each cultivated sample, the use of community DNA as a template for gene-specific polymerase chain reaction (PCR) amplification may allow screening of most of the bacteria in a sample. This approach could potentially identify antibiotic resistance gene reservoirs with increased accuracy and reduced cost. Not only can the presence or absence of a particular resistance gene be assessed, but this technique can enable assessment of the diversity among the resistance genes identified.[1,6] A major failing of this approach, however, is that it is typically impossible to ascertain any information about the specific bacteria that are harboring the detected resistance gene.

Polymerase chain reaction amplification is a sensitive method for detection of pathogens in clinical samples.[14,40] However, DNA extractions from fecal material contain numerous inhibitors that can hinder PCR amplification. These include bile salts, hemoglobin degradation products, complex polysaccharides, and polyphenolic substances from plant tissues.[16,24,26,43] The sensitivity of PCR amplification can be affected by the DNA extraction method and types of enhancers used in each reaction.[11,24] The sensitivity of the community DNA approach can also be affected by the amount of sample used and the number of replicate extractions made from each sample.[28,30]

The use of community DNA from a sample as a means for detecting antibiotic resistance genes and for identifying potential gene reservoirs is becoming more common.[1,27] However, little work has been done on the validation of this approach. The objective of the study reported here was to determine the specific level at which antibiotic resistance genes can be detected within community DNA prepared from cattle fecal samples. The goal was to better understand the performance of community DNA PCR detection of antibiotic resistance genes in fecal samples and to compare these results with standard culturing approaches.

Materials and methods

Study design

An experimental laboratory trial, in which known concentrations of an *Escherichia coli* strain containing various antibiotic resistance genes were added to feces from dairy cattle. Second, a field study was conducted in which fecal samples of unknown status were obtained from dairy cattle. These samples were subsequently tested for the presence of various resistance genes using fecal culturing techniques and PCR analysis of community DNA.

Bacteria strains and growth conditions

Escherichia coli DH5-α[a] was used to construct the antibiotic resistance gene-containing strain added to fecal samples. Cultures were incubated at 37°C with shaking in Luria-Bertani (LB) broth.[b] MacConkey agar was prepared as per manufacturer's directions.[c] When required, antibiotics were added to agar and broth at the following concentrations: chloramphenicol, 25 μg/ml; nalidixic acid, 15 μg/ml; florfenicol, 16 μg/ml; and ceftriaxone, 2 μg/ml.

Resistance gene selection

We focused on 2 specific antibiotic resistance genes. The first was the *cmy*-2 family of AmpC-like β-lactamase genes. These genes confer resistance to the cephalosporin class of antibiotics, and of particular concern, the third-generation cephalosporins such as ceftriaxone, cefotaxime, and ceftiofur.[45] These genes are typically located on plasmids. The second focus was the *flo* gene, which confers resistance to florfenicol and chloramphenicol. This gene has been found on plasmids and in the chromosome of Enterobacteriaceae.[7]

Strain construction

Escherichia coli strain DH5-α was mutagenized with a mini-*Tn10*-chloramphenicol transposon (mini-*Tn10*-*cam*) using a specialized λ phage according to published protocols.[19,41] Phage vehicle λNK1323 was used. Mutants were selected that were resistant to chloramphenicol. Southern blot hybridization was performed using a *cat*-specific probe to ensure that only 1 insert was present in the strain. The *cat*-specific probe was constructed through PCR amplification of plasmid pACYC184 with the primers: catF: 5′CAG ACC GTT CAG CTG GAT AT-3′ and catR: 5′GAC GGC ATG ATG AAC CTG AA-3′. The primers were designed using the *cat* gene from plasmid pACYC184 (GenBank/EMBL sequence accession number X06403) and the MacDNAsis program.[d] The *cat* PCR product was agarose gel-purified using a Qiagen gel purification kit.[e] Genomic DNA from the chloramphenicol-resistant strain was digested with *Sal*1,[f] and was blotted to Hybond-N.[g] Southern blot hybridization[3] was performed using a DNA probe labeled with the Amersham ECL Random Prime kit.[g] A single band from the genomic DNA hybridized to the *cat* probe on the Southern blot. This strain was designated DH5-α/*Tn10*-*cat*. Growth curve assays were obtained by diluting (1:100) broth cultures, grown overnight in LB broth, into fresh LB broth. Cultures were grown at 37°C with shaking. Every 30 min, turbidity was measured spectrophotometrically at 660 nm. Growth differences were not observed between the mutant strains and wild-type DH5-α strain.

An *E. coli* isolate that contains the *cmy*-2 and *flo* genes on a conjugatable plasmid was previously isolated in the

authors' laboratory (unpublished data). Strain DH5-α/*Tn10-cat* was conjugated with this strain by streaking both strains on nonselective semisolid agar media. After overnight incubation at 37°C, the intersection of growth from the 2 strains was streaked to agar plates containing antibiotics. Both strains also were streaked individually to the antibiotic plates as negative controls. Colonies that were resistant to nalidixic acid, chloramphenicol, ceftriaxone, and florfenicol were isolated. The resultant strain contained the *flo* and *cmy-2* genes on a large plasmid and a single-copy mini-*Tn10-cat* chromosomal insert. This strain was designated AR-940/CFT.

Extraction of DNA

Total community DNA was extracted from fecal samples using the QIAamp DNA Stool Mini Kit.[e] Initial extractions were performed according to manufacturer's instructions. Various changes were subsequently made to the extraction methods to determine whether sensitivity could be improved.

Experimental spiking of fecal samples

Fecal samples were taken rectally from cattle. One-gram aliquots of feces were suspended in 9 ml of LB broth. Two additional 10-fold serial dilutions were made. One-hundred microliters of each dilution was plated on MacConkey agar and MacConkey agar containing ceftriaxone or florfenicol. A 0.1-g aliquot of each fecal sample was used for DNA extraction with the QIAamp DNA Stool Mini Kit.[e] Polymerase chain reaction amplification was performed on each DNA preparation using primers specific for *cmy*-2, *flo* and *cat*. The PCR products were electrophoresed on a 1.5% agarose gel. Only samples that had negative results of PCR amplification and did not have any growth on the antibiotic-containing agar plates were used in the experimental spiking study.

The following day, fecal samples were taken from the cattle that had negative PCR amplification results for the 3 genes and had no growth on MacConkey agar containing antibiotics. Although the new samples may have contained the target antimicrobial resistance genes, it was imperative to have fresh samples for this experiment. Fecal samples from 3 cattle were used in this experiment.

An overnight broth culture of AR-940/CFT was serially 10-fold diluted in LB broth, and 10 dilutions were made. One-hundred microliters of each dilution was plated on MacConkey agar and Tryptic Soy Agar[b] (TSA) and was incubated at 37°C for 24 hr for enumeration of *E. coli*. Three milliliters of each dilution was mixed with 3 g of feces and vortexed vigorously. The DNA was extracted from three 0.1-g aliquots of each spiked mixture using the modified QIAamp DNA Stool Mini Kit protocol.

One gram of each of the spiked fecal samples was mixed with 9 ml of LB broth. Two additional 10-fold serial dilutions were made. One-hundred microliters of each dilution was plated on MacConkey agar and MacConkey agar containing chloramphenicol in duplicate, and plates were incubated overnight at 37°C. The following day, 48 colonies from the MacConkey agar plates were picked to MacConkey agar in a grid pattern. These colonies were picked in approximate proportion to the distribution of various colony morphologic features on the plate. Lactose-positive and lactose-negative colonies were picked from plates containing both types, because AR-940/CFT is lactose negative, whereas wild-type commensal *E. coli* were assumed to be lactose positive. The dilution at which there were no lactose-negative colonies was recorded. The number of colonies on the MacConkey plates containing chloramphenicol was recorded as well as the dilution at which no more chloramphenicol-resistant colonies were recovered.

After growth overnight, the colonies picked onto MacConkey agar were replica-plated to MacConkey agar containing the following antibiotics: ceftriaxone, florfenicol, chloramphenicol, tetracycline, and agar without antibiotic. The spiked *E. coli* was recovered and confirmed to be the AR-940/CFT strain, as it was lactose negative and resistant to chloramphenicol, florfenicol, ceftriaxone, and tetracycline.

For each of the 3 fecal samples that were spiked, two 0.1-g aliquots were removed before bacteria were added. These fecal samples were processed as described previously, and were considered additional negative controls. These 6 unspiked samples (2 for each of the 3 cattle fecal samples) were included to ensure that the negative resistance gene status of the samples from the previous day had not changed. Community DNA was extracted from these negative samples and was processed with the spiked samples. Results of PCR amplification of these community samples were consistently negative. After plating, no colonies that were resistant to nalidixic acid, chloramphenicol, florfenicol, or ceftriaxone were isolated.

Polymerase chain reaction amplification

Aliquots of each community DNA extraction were electrophoresed on 1.5% agarose gels to ensure that approximately the same amount of DNA was used in each amplification reaction. All extractions were estimated to be approximately the same concentration by visual inspection. Each reaction used 2 µl of template DNA. Each gene-specific PCR amplification was found to have different optimal conditions. All PCR amplicons were resolved by gel electrophoresis on 1.5% agarose gels, and were visualized by staining with ethidium bromide.

Polymerase chain reaction amplification of the cat gene. Primers used were catF and catR, as described previously. These amplify a 456-bp amplicon. The PCR conditions in a final reaction volume of 25 µl using 2 µl of template DNA were as follows: 0.625 U of *Taq* polymerase[f] in 1× buffer, as supplied by the manufacturer, with 0.25 mM deoxynucleoside triphosphates,[f] 2.0 mM $MgCl_2$, 0.1 mg of bovine serum albumin[a] (BSA), and 15.625 pmol each primer. Cycling conditions were as follows: 94°C for 2 min, followed by 29 cycles of 94°C for 30 sec, 60°C for 45 sec, and 72°C for 1 min. A final extension of 72°C was carried out for 7 min. Samples were then kept at 4°C until processed further.

Polymerase chain reaction amplification of the flo gene. Primers used were: flo 1: 5′CGC CGT CAT TCT TCA

CCT TC-3′, and flo 2: 5′AAT CAC GGG CCA CGC TGT ATC-3′.[4] These primers amplify a 215-bp product. The PCR conditions were identical to those of the *cat* gene PCR, except that 25 pmol of each primer was used. The PCR cycling parameters were: 94°C for 2 min, followed by 29 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min. A final extension of 72°C was carried out for 7 min. Samples were then kept at 4°C until processed further.

Polymerase chain reaction analysis of the cmy-2 gene. The *cmy-2* gene PCR amplifications were performed using 2 primer sets in a nested PCR design. Samples were amplified by 1 set of primers, and the product of that reaction was diluted and used as the template for a second PCR amplification. The second primer pair was designed to amplify an internal region of the first PCR product and to amplify at a higher annealing temperature. The first primer pair is unable to amplify at the higher annealing temperature. Primers used in the first PCR amplification were: cmy-F: 5′GAC AGC CTC TTT CTC CAC A-3′, and cmy-R: 5′TGG AAC GAA GGC TAC GTA-3′.[47] This primer pair amplifies a 1,100-bp product. The PCR conditions were the same as those for the *flo* gene, except that acetamide[h] was added to a final concentration of 2.5%. The PCR cycling parameters were the same as those for the *cat* gene PCR amplification. The second (nested) PCR used 2 µl of 1:10 diluted template from the first PCR amplification. Primers used in the nested PCR were: cmy F2: 5′CTC AGG AAT GAG TTA CGA AGA GG-3′, and cmy R2: 5′AAT CCA CCA GTG GAG CCC 3′. Primers were designed in MacVector[i] using *Citrobacter freundii ampC* gene (GenBank: AJ011291), *Salmonella seftenberg cmy-2* gene (GenBank: U77414), and *Klebsiella pneumoniae cmy-2* gene (GenBank: Y16784). These primers amplify a product of 550 bp. Reaction conditions were the same as those for the first PCR amplification. Cycling conditions were as follows: 94°C for 2 min, followed by 29 cycles of 94°C for 30 sec, 65.6°C for 45 sec, and 72°C for 1 min. A final extension of 72°C was carried out for 7 min. Samples were then kept at 4°C until processed further.

Field samples

Fecal samples from 80 dairy cattle were collected from the rectum and were processed on the day of collection. Age of animals located on 3 farms ranged from 3 to 42 months. Two of the farms had approximately 200 milking cows each, and the third farm had approximately 100 milking cows. All farms used medicated milk replacer for the calves, therapeutic antibiotics for the cows (including ceftiofur), and routine medicated dry-cow therapy. The samples that were used in this study, therefore, included calves, heifers, and lactating and nonlactating cows. The specific timing of antibiotic administrations to the individual animals was not incorporated into the analysis.

One gram of feces from each sample was weighed and suspended in 9 ml of LB broth. Two 10-fold dilutions were made, and the tubes were mixed vigorously. One hundred microliters of each dilution was plated on MacConkey agar. After incubation overnight at 37°C, 48 flat, pink colonies in a grid pattern were picked to MacConkey agar. After growth, these colonies were replica-plated to MacConkey agar containing ceftriaxone, florfenicol, and agar without antibiotics. Phenotypic resistance for each colony was recorded. Colonies also were tested on Simmons citrate agar and triple sugar iron (TSI) agar. Colonies that were citrate negative and had acid/acid reaction on TSI agar were presumed to be *E. coli*. These colonies were preserved and were used as a template for colony PCR amplification of specific genes. In lieu of a DNA extraction step, a single colony was scraped into 100 µl of sterile 5% Chelex-100 resin.[j] After vigorous vortexing, the samples were heated at 95°C for 10 min. Two microliters of this template was then used in the gene-specific PCR amplifications of *flo* and *cmy*-2.

Sequencing of DNA

Samples for DNA sequencing were prepared by PCR amplification with appropriate primers. The PCR amplification products were agarose gel purified using a gel purification kit according to manufacturer's instructions.[e] Samples were sent to the W. M. Keck Center for Comparative and Functional Genomics, Urbana, IL. Sequencing primers were the same as those used in the PCR amplification. The DNA sequences were analyzed by searching the GenBank database of the National Center for Biotechnology Information via the BLAST network service.

Statistical analysis

In the field study portion of this study, each of the samples had an unknown antibiotic resistance gene status. The results of the resistance gene PCR amplification from community DNA and from the culturing methods for each sample were dichotomized into present/absent for the *flo* and *cmy-2* resistance genes. The PCR and culturing data were then compared by calculating the proportional agreement between the methods using a statistical software package.[k] The 95% confidence intervals (CI) also were calculated.

Results

Construction of the *E. coli* strain

To determine the differing conditions that affect PCR amplification from a fecal sample, an *E. coli* strain (AR-940/CFT) containing 3 antibiotic resistance genes was constructed. Two of these genes (*cmy-2* and *flo*) are encoded on a large conjugatable plasmid. The plasmid is large (approx. 150 kb), so it is assumed that it is a fairly low copy number,[22] but the exact copy number or the exact location of the genes on the plasmid has not been determined. The strain also contains a mini-*Tn10* transposon insertion in single copy in the chromosome. To ensure that the mutant strain was not defective in growth due to the transposon insertion, growth curves were performed. The growth of strain AR-940/CFT was identical to the growth of strain DH5-α, thus ensuring that the

mutant strain had no growth defects due to the insertion mutation (data not shown).

Optimization of the DNA extraction method

Known numbers of bacterial cells spiked into cattle fecal samples were used to determine the analytical sensitivity of the PCR amplification. On the basis of previous studies, the stool kit[e] has been documented to adequately extract purified DNA for use in PCR amplification.[11,24] However using DNA that was purified following strict adherence to the manufacturer's directions only resulted in amplification of samples with fairly high numbers of cells present (5×10^6 colony-forming units [CFU]/g). Also the amount of inhibitors present in DNA extractions required the use of 50-µl PCR reaction volumes for amplification. This can become cost prohibitive if screening a large number of samples. To optimize DNA extraction from fecal samples, the following modifications were made to the manufacturer's recommended protocol[e]: using 0.1 g instead of 0.2 g of sample, all centrifugation steps were increased by 30 seconds; samples were incubated at 95°C instead of 70°C during the lysis step (protocol step 3); the amount of proteinase K was increased to 20 µl[11]; the incubation step (protocol step 12) was changed from 70°C for 10 minutes to 55°C for 30 minutes[11]; the 2 wash steps (protocol steps 15 and 16) were repeated. Using a lesser sample amount allowed an extraction with less carry-through of inhibitors, although a lesser amount of DNA was ultimately recovered. Diluting the DNA sample allowed use of a smaller reaction volume, but sensitivity was lost. It was found that using less original starting material allowed a cleaner preparation without loss of sensitivity. These modifications resulted in a 10-fold increase in the sensitivity of the PCR amplification and enabled the reliable use of a 25-µl PCR amplification volume.

Gene-specific optimization

The 3 antibiotic resistance genes in the strain were found to require different conditions for optimal amplification. Each PCR amplification was performed on triplicate DNA extractions from 3 cattle samples. Each PCR amplification was performed in duplicate.

Polymerase chain reaction amplification of the cat gene. The *cat* gene is present in single copy in the chromosome of the test strain. The PCR amplification was found to be more sensitive with added BSA as an enhancer. The *cat*-specific PCR amplification was able to detect between 5×10^5 and 5×10^3 CFU/g of feces (Fig. 1). A second PCR amplification was also performed on template diluted (1:10) from the first PCR amplification. The same primer pair was used for this secondary amplification. This occasionally resulted in a 10-fold increase in the amplification sensitivity, but this increase was not reliable.

Polymerase chain reaction amplification of the flo gene. The *flo* gene is encoded on a large conjugatable plasmid in the experimentally constructed strain. The exact copy number of this plasmid is unknown. A plasmid-encoded gene was used because plasmid supercoiling may negatively affect PCR efficiency.[20] The *flo*-specific PCR amplification also had increased sensitivity when BSA was added. The amplification was able to detect 5×10^5 to 5×10^4 CFU/g of feces. A second amplification step was performed using diluted template from the primary PCR amplification. This second PCR amplification appeared to increase the sensitivity; however, a number of nonspecific bands of approximately the same size as those of the *flo* amplicon made interpretation difficult and unreliable (data not shown).

Polymerase chain reaction amplification of the cmy-2 gene. The *cmy-2* gene is encoded on a large conjugatable plasmid of unknown copy number. By use of Southern blot hybridization, the *flo* and *cmy-2* genes are encoded on the same plasmid (data not shown). The *cmy-2* amplification had increased sensitivity when BSA and acetamide were added to the PCR amplicfication. The primary PCR was able to amplify product at a concentration of 5×10^4 cfu/g. Using a second nested set of primers that were specific to sequences within the first amplification product enabled an increase in the sensitivity to 5×10^3 CFU/g and, occasionally, to 5×10^2 CFU/g (Fig. 1). The nested primer set also had an annealing temperature 5°C higher than that for the first primer pair. The primary primer pair is unable to amplify product at the higher annealing temperature. This allowed the second PCR reaction to be done without having to purify product from the primary PCR amplification.

Citrobacter fruendii also encodes a chromosomal *ampC* β-lactamase gene. A laboratory isolate of *C. freundii* was tested to ensure that the primers did not amplify this gene because this study was principally focused on plasmid-encoded β-lactamase genes. The primary primer pair cmyF/R does not amplify *C. freundii*. The nested pair can amplify an appropriately sized product from *C. fruendii*, but not at the annealing temperature used (65.6°C). To ensure that the use of 2 different primer pairs did not lead to nonspecific product, amplicons from the control strain and from 2 field samples from the primary and nested PCR amplifications were sequenced. Both products had 95–99% similarity to the *cmy-2* genes of *Klebsiella pneumoniae* (GenBank accession number

Animal		cfu / g	$5x10^7$	$5x10^6$	$5x10^5$	$5x10^4$	$5x10^3$	$5x10^2$
6731	Culture		42	41	26	7	0	0
	PCR	cat-a	■■■	■■■	■■■	⊟⊟□	□□□	□□□
		cat-b	■■■	■■■	■■■	□□□	□□□	□□□
		flo-a	■■■	■■■	■■□	□□□	□□□	□□□
		flo-b	■■■	■■■	■■■	□□□	□□□	□□□
		cmy-a	■■■	■■■	■■■	■■⊟	⊟⊟□	□□□
		cmy-b	■■■	■■■	■■■	■■□	⊟⊟□	⊟□□
6869	Culture		28	24	9	0	0	0
	PCR	cat-a	■■■	■■■	■■■	■■■	■□□	□□□
		cat-b	■■■	■■■	■■■	■■□	□□□	□□□
		flo-a	■■■	■■■	■■■	■■□	□□□	□□□
		flo-b	■■■	■■■	■■■	■□□	□□□	□□□
		cmy-a	■■■	■■■	■■■	■■■	⊟⊟□	⊟□□
		cmy-b	■■■	■■■	■■■	■■■	⊟⊟⊟	□□□
7139	Culture		28	24	14	0	0	0
	PCR	cat-a	■■■	■■■	■■■	■■⊟	⊟□□	□□□
		cat-b	■■■	■■■	■■■	■■■	□□□	□□□
		flo-a	■■■	■■■	■■■	□□□	□□□	□□□
		flo-b	■■■	■■■	■■□	■□□	□□□	□□□
		cmy-a	■■■	■■■	■■■	■■□	⊟⊟⊟	□□□
		cmy-b	■■■	■■■	■■■	■■■	⊟□□	⊟□□

Figure 1. Gene-specific community DNA polymerase chain reaction analysis (PCR), compared with culturing in the laboratory study. CFU/g indicates how much experimental *Escherichia coli* was added to each sample. Culture results indicate the number of spiked antibiotic-resistant *E. coli* colonies recovered from the 48 picked colonies. The PCR amplification for each DNA extraction was performed in duplicate (rows labeled a and b for each gene). DNA extractions were performed in triplicate for each dilution. ■ represents a PCR-positive result, ⊟ represents a PCR-positive result on the second round of amplification, and □ represents a PCR-negative result.

X91840) and *S. enterica* serotype Senftenberg (GenBank accession number U77414). These were the sequences used to design the 2 primer pairs.[47]

Isolation of *E. coli* in culture

The spiked fecal samples from the 3 animals also were cultured for *E. coli*. Lactose-negative, antibiotic-resistant colonies were not recovered from the samples prior to spiking. In addition, lactose-positive colonies that were resistant to florfenicol or ceftiaxone were not recovered from the samples prior to spiking.

After spiking the fecal samples with AR-940/CFT, the spiked *E. coli* were recovered from 1 fecal sample (animal 6731) at a concentration of 5×10^4 cfu/g and at 5×10^5 cfu/g from the other 2 animals (Fig. 1). As stated in the ''Materials and methods'' section, fecal samples were plated on MacConkey agar, and random colonies were picked before identification as commensal or spiked bacteria. However since spiked *E. coli* is lactose negative, these colonies were light colored on MacConkey agar. Therefore, it was simple to determine at what level the experimentally spiked *E. coli* was recovered. Pink, lactose-positive colonies (commensal *E. coli*) were recovered from all dilution tubes (10^0–10^{-10}) for all the animals.

The fecal samples were plated on MacConkey agar with and without added chloramphenicol (25 μg/ml). The use of selective media provided at least a 10-fold better recovery than that for media without added antibiotics (data not shown).

Gene-specific amplification

In the laboratory portion of this study, the 3 resistance genes that were targeted had differing PCR results (Fig. 1). The chloramphenicol resistance gene,

cat, was detected at a10-fold lower concentration than that from culture (5×10^4 cfu/g) in 5 of the 6 extractions from 2 separate animals. However, in animal 6731, only the second PCR amplification detected the gene at this concentration, but not in all replicates. The method of diluting the PCR product and using it as template in a second reaction with the same set of primers increased the number of non-specific bands and made interpretation of the results difficult. Samples that were faint by electrophoresis in the first PCR amplification had stronger band intensities (data not shown), and this method may be considered a way to identify weak-positive results conclusively.

The *flo*-specific PCR amplification was at least as sensitive as culture for 2 of the 3 animals tested, but again, was not as sensitive as culture for animal 6731. The *flo*-specific PCR amplification had fewer positive replicates than did the *cat*-specific PCR amplification at the higher dilutions. The design of the *flo* primers may have contributed to this lower sensitivity. The primers amplified a small product; thus, it was impossible to design a nested set of primers for use with this reaction. In future studies, redesigning the primers so that the primary PCR amplifies a larger product may increase sensitivity.

The *cmy–2*-specific PCR amplification, which was a nested PCR scheme, yielded the most sensitive results. The primary PCR amplification was able to amplify product at the same dilution from which colonies were recovered (5×10^5 cfu/g) for animal 6731 and was 10-fold better for the other 2 animals (5×10^4 cfu/g). However, when the nested PCR protocol was used, product was amplified at 5×10^3 cfu/g, and rarely at 5×10^2 cfu/g; dilutions that were 100- to 1,000-fold more sensitive than results of culturing. Use of a nested PCR amplification resulted in the most sensitive method for detection of resistance genes within the community DNA extraction.

Polymerase chain reaction amplification of field samples

As part of an ongoing study of antibiotic resistance, fecal samples have been collected over time from dairies in Illinois. *Escherichia coli* has been isolated from these samples and tested for phenotypic resistance to ceftriaxone and florfenicol. Gene-specific PCR amplification has been performed on the isolates as well. Community DNA was extracted from 80 samples selected randomly from the ongoing studies. The *cmy-2* and *flo* PCR amplifications were performed on the total DNA extractions. The results of culture and PCR amplification for these are shown in Table 1. The *cmy-2* PCR on the first-pass amplification and the *flo* PCR had high agreement with the culture results. The *cmy-2* first-pass PCR amplification and culture indicated a proportional agreement of 0.850 (95% CI: 0.753, 0.920) whereas the *flo* PCR amplification and culture had a proportional agreement of 0.925 (95% CI: 0.844, 0.972). The second pass of the *cmy-2* PCR amplification had a lower proportional agreement with culture (0.650, 95% CI: 0.535, 0.753). This reduction in agreement was entirely due to the identification of an additional 19 *cmy–2*-positive samples by community DNA PCR analysis. The test-positive samples came from animals of all ages (data not shown).

Table 1. Field sample comparison of culturing versus gene-specific polymerase chain reaction (PCR) analysis in the detection of specific resistance genes. The count in each cell represents the number of samples out of 80 with that specific result.

		PCR	
Gene	Culture	Positive	Negative
*cmy-2**	Positive	5	3
	Negative	9	63
cmy-2†	Positive	8	0
	Negative	28	44
flo	Positive	11	5
	Negative	1	63

* Comparison of culturing with the first round of *cmy-2* PCR amplification.

† Comparison of culturing with the added nested *cmy-2* PCR amplification.

Discussion

This study was performed to explore the feasibility of using community DNA as a method to detect antibiotic resistance genes in complex samples such as cattle fecal samples. Many studies are beginning to incorporate community DNA methods, but rarely have these methods been compared with traditional culturing. In the experimental portion of this study, the use of laboratory-spiked samples helped identify optimal DNA extraction methods as well as optimal PCR primers and amplification protocols. For each type of sample used, the DNA extraction methods and PCR protocols should be evaluated. Obtaining high-quality DNA from a complex matrix such as feces is a major issue in these types of studies, and consequently, the quality of this step will directly influence the sensitivity of the assay and the potential variability in results.

The field study represented a larger challenge because the true status of each sample was unknown. In the experimental portion of the study, evaluation of the gels from the PCR amplification could have been biased because it was known that the samples had been spiked, and thus, the culture results were already known. When using PCR analysis on samples of unknown status, one way to remove potential

subjectivity in the reading of the gels from PCR analysis would be to perform nucleotide sequencing on any PCR product to confirm that the sample was truly positive for the target gene. Although this would be a conservative approach to identifying positive samples, the added time and cost would likely make this strategy intractable.

In the analysis of the data from the field study, the second pass of the nested *cmy-2* PCR amplification had poor agreement with culture because of the high number of PCR-positive, culture-negative samples. This finding should not be unexpected, as there are several possible explanations. One plausible explanation for this discordance is that PCR amplification is detecting the gene in bacteria other than *E. coli*. Because *cmy-2* and *flo* genes have been detected in a number of different bacteria such as *Salmonella*, *Klebsiella*, *Citrobacter*, and *Proteus* species,[2,25,42,46] the community DNA PCR amplification might be more likely to identify a sample as positive. As the diversity of bacteria with the same resistance gene increases, the probability of correctly detecting a positive sample should increase. In addition, this probability should increase with increasing gene copy number per cell and prevalence of bacteria with the target gene. A second plausible explanation is that the discordant data represent false-negative culture results. In general, PCR analysis would be expected to yield a higher sensitivity for gene detection than would culturing, but there is also the possibility that the PCR analysis is detecting the gene in viable unculturable organisms. The community DNA approach cannot differentiate the bacterium from which the gene is amplified or whether the bacterium is viable.

Given the ease of a simple DNA extraction and PCR amplification, community DNA approaches can have many uses in antibiotic resistance studies and surveillance systems. First, many resistances are mediated principally by a small subset of resistance genes. In the case of florfenicol resistance, the responsible gene is most commonly the *flo* gene. Consequently, detection of *flo* in a sample can indicate whether colonies are likely to be florfenicol resistant.[37] In large epidemiologic studies, many samples are evaluated for the presence of resistant organisms. In addition to screening many different bacteria for specific resistances, community DNA approaches could quickly indicate whether specific resistance genes are present within any of the bacteria in the community of the sample. Furthermore, this approach is more time and cost efficient than is the laborious process of culturing a variety of bacteria. Of course, specific bacterial isolates are required to identify the location of these resistance genes within the cell and to evaluate their ability to move among different bacteria. Finally, community DNA approaches also could be used in diagnostic settings. Because certain resistance genes are often indicative of treatment failure if found within certain bacteria, community DNA PCR analysis could be used to quickly identify whether these resistance genes are present in the sample. Long before culture and antibiotic susceptibility testing are complete, results from the community DNA PCR analysis would already be available. In the diagnostic setting, community DNA PCR analysis has applications beyond antibiotic resistance. For example, these methods could enable the screening of entire herds or populations for particular pathogens, as long as appropriate primers can be designed.

This study focused entirely on resistance genes that are known to exist within *E. coli*. In a field study in which resistance genes might be present in a diversity of bacteria, culturing would likely miss a number of organisms with the resistance genes, even within the few organisms typically cultured. Community DNA approaches enable assessment of entire bacterial communities in complex samples such as feces, a task that would be practically intractable with culture methods. Because community DNA extractions do not allow determination of the specific bacterium carrying a particular gene, the use of community DNA PCR analysis for gene detection assumes that the gene is the unit of interest. This assumption may be warranted given the frequency with which certain genes have moved among distantly related bacteria via horizontal gene transfer. This approach makes it possible to assess the distribution of the gene among heterogeneous samples to make more accurate assessments of the means by which these genes are disseminated. Even if the bacterium harboring the gene is a viable unculturable organism, the presence of the resistance gene can still be considered an important finding, depending on the goals of the study or the surveillance system.

In the field study component of this project, numerous *cmy–2*-positive samples were identified in the absence of cultured *E. coli* with the gene. This high prevalence of samples with the resistance gene could represent a low background level of this resistance gene among many animals on the farm and among many bacteria in the sample. A strategic combination of culturing and community DNA approaches may be the optimal approach for clearly elucidating the relationship between specific selection pressures and the persistence and dissemination of specific resistance genes. Including a quantitative approach through methods such as real-time PCR analysis might be an additional enhancement because it would potentially allow assessment of the amount

of the antibiotic resistance gene in the total community DNA. Selection pressures such as antibiotic use act on all of the bacteria in the sample, not just those that are cultured. Consequently, a quantitative measure of total resistance gene copy in a sample might enable a more accurate assessment of the effect of specific selection pressures than do studies that rely on the prevalence of cultured colonies that are antibiotic resistant.

Acknowledgements

The research for this project has complied with all relevant animal use federal guidelines and institutional policies. This project was supported by USDA National Research Initiative Competitive Grant 00-35212-9398 (Singer). The authors thank Dr. Carol Maddox for advice on the study.

Sources and manufacturers

a. Promega, Madison, WI.
b. Difco, Detroit, MI.
c. Becton Dickinson, Franklin Lakes, NJ.
d. Molecular Biology Insights, Cascade, CO.
e. Qiagen Inc., Valencia, CA.
f. Invitrogen, Carlsbad, CA.
g. AmershamBiosciences, Piscataway, NJ.
h. Sigma, St. Louis, MO.
i. Accelrys, San Diego, CA.
j. Bio-Rad, Hercules, CA.
k. Stata 7.0, Stata Corporation, College Station, TX.

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