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Short communication: Antimicrobial susceptibility and frequency of resistance genes in *Escherichia coli* isolated from bovine mastitis

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ABSTRACT

Escherichia coli isolated from bovine milk samples submitted to the Ohio Agricultural Research and Development Center Mastitis Laboratory (Wooster) in 1985 to 1987 and in 2009 were compared for antimicrobial susceptibility and prevalence of antimicrobial resistance genes. Forty-four isolates from 1985 to 1987 and 55 isolates from 2009 were tested. Minimum inhibitory concentrations of 15 antimicrobials were determined using a commercially available broth microdilution system. Multiplex polymerase chain reaction was performed for gene detection. The percentage of isolates susceptible to trimethoprim/sulfamethoxazole, ampicillin, and kanamycin was lower in those collected in 1985 to 1987 than in isolates collected in 2009. Susceptibility did not differ between isolates from 1985 to 1987 and isolates from 2009 for the 12 other antimicrobials tested. A trimethoprim/sulfamethoxazole resistance gene was detected more frequently in isolates from 1985 to 1987 than in isolates from 2009. Gene frequencies for streptomycin resistance and tetracycline resistance were similar among 1985 to 1987 isolates and 2009 isolates. Resistance to most antimicrobials did not differ between isolates submitted to a mastitis diagnostic laboratory in 1985 to 1987 and those submitted in 2009. Changes observed indicated an increase in frequency of susceptibility in isolates to trimethoprim/sulfamethoxazole, ampicillin, and kanamycin in 2009 isolates compared with 1985 to 1987 isolates.
Key words: mastitis, antimicrobial susceptibility, *Escherichia coli*

Short Communication

Antimicrobials generally have little effect on the clinical outcome of coliform mastitis because spontaneous cures often occur regardless of therapy (Hogan and Smith, 2003). In addition, many gram-negative bacteria are resistant to β-lactam drugs used in mastitis treatment (Watts et al., 1995; Salmon et al., 1998). The use of antimicrobials in agriculture has led to concerns about the potential for antimicrobial resistance to increase on farms and spread to human populations. However, Erskine et al. (2002) reported that susceptibility to several antimicrobials increased in many common mastitis pathogens from 1994 to 2000. Another study from 1994 to 2001 by Makovec and Ruegg (2003) reported similar trends in increasing susceptibility. Antimicrobial susceptibility of gram-negative mastitis pathogens did not change in South Korea from 2003 to 2008 (Nam et al., 2009). All 3 studies obtained bacteria from mastitic milk samples that had been submitted to a diagnostic laboratory for testing. The National Mastitis Council (Verona, WI) conducted a review of antimicrobial resistance studies and concluded that no evidence existed of increasing antimicrobial resistance among mastitis pathogens (Erskine et al., 2004). The purpose of the current trial was to compare phenotypic antimicrobial susceptibility and prevalence of antimicrobial resistance genes in *Escherichia coli* isolated from milk samples submitted to a mastitis diagnostic laboratory in 1985 to 1987 and 2009.

Escherichia coli (n = 99) isolates tested were from aseptically collected quarter-milk samples submitted to the Ohio Agricultural Research and Development Center Mastitis Laboratory (Wooster). Samples were submitted from 1985 to 1987 (n = 44) or in 2009 (n = 55). Primary culture for all quarter-milk samples was surface plating 10 μL of milk on Trypticase soy agar (Becton, Dickinson and Co., Sparks, MD) with 5% bovine blood and 0.1% esculin and 100 μL of milk on MacConkey agar (Becton, Dickinson and Co.). Primary cultures were incubated aerobically at 37°C and plates were examined at 24 and 48 h. *Escherichia coli* were presumptively identified by colony morphology, cellular morphology, Gram reaction, motility, triple sugar iron, and citrate utilization tests (Hogan et al., 1999). *Escherichia coli* were identified biochemically using the API 20E system (bioMérieux, Marcy-l'Étoile, France). *Escherichia coli* ATCC 25922 was used as a positive control isolate in biochemical testing. Confirmed isolates were stored in Trypticase soy broth plus 20% glycerin at −80°C until testing. Isolates collected from 1985 to

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Table 1. Primer sets used for detection of antimicrobial genes from *Escherichia coli* isolated from milk samples submitted to a mastitis diagnostic laboratory from 1985 to 1987 and 2009

Primer set	Gene	Primer		Annealing temperature (°C)	Fragment size (bp)
		Direction	Sequence		
1	*aadA*	Forward	GTGGATGGCGGCCTGAAGCC	68	525
		Reverse	AATGCCCAGTCGGCAGCG		
	sulI	Forward	GTGACGGTGTTCGGCATTCT	68	779
		Reverse	CCTGGTGATAACGGCAATTC		
	strA	Forward	CCTGGTGATAACGGCAATTC	55	546
		Reverse	CCAATCGATAACGGCAATTC		
2	*strB*	Forward	ATCGTCAAGGGATTGAAACC	55	509
		Reverse	GGATCGTAGAACATATTGGC		
	tetA	Forward	GGATCGTAGAACATATTGGC	64	502
		Reverse	CGGCAGGCAGAGCAAGTAGA		
3	*tetB*	Forward	CATTAATAGGCGCATCGCTG	64	930
		Reverse	TGAAGGTCATCGATAGCAGG		
	tetC	Forward	GCTGTAGGCATAGGCTTGGT	64	888
		Reverse	GCCGGAAGCGAGAAGAATCA		

1987 were tested every 36 to 48 mo for ability to grow aerobically on MacConkey agar until the current trial was initiated in September, 2009.

Minimum inhibitory concentrations of antimicrobials were determined by microdilutions in commercially available plates (Sensititre gram-negative NARMS; Trek Diagnostic Systems Inc., Cleveland, OH). Microdilution plates included amikacin, ampicillin, amoxicillin/clavulanic acid, ceftriaxone, chloramphenicol, cefoxitin, trimethoprim/sulfamethoxazole, gentamicin, kanamycin, nalidixic acid, sulfisoxazole, streptomycin, tetracycline, and ceftiofur. Isolates were inoculated on esculin blood agar and incubated at 37°C for 24 h. After incubation, several colonies of each isolate were emulsified in distilled water and diluted in Mueller-Hinton broth (Trek Diagnostic Systems Inc.) to approximately 1×10^5 cfu/mL. Microdilution plates were inoculated with the bacterial culture, covered with an adhesive seal, and incubated at 37°C for 24 h. After 24 h of incubation, plates were read with a manual plate reader (Sensititre Manual Viewer; Trek Diagnostic Systems Inc.). Isolates were classified as susceptible, intermediate, or resistant according to National Committee on Clinical Laboratory Standards (NCCLS) standards (CLSI, 2008). *Escherichia coli* ATCC 25922 was used as a control.

Deoxyribonucleic acid was purified from *E. coli* (Generation Capture Column; Qiagen Inc., Valencia, CA) and antimicrobial resistance genes were amplified (Qiagen Multiplex PCR Kit; Qiagen Inc.) in a thermal cycler (Hybrid Sprint; Thermo Electron Corp., Milford, MA.) The initial activation step was 15 min at 95°C; DNA was then denatured at 94°C for 30 s, annealed for 90 s at the given annealing temperature for each primer set (Lanz et al., 2003; Table 1), and extended at 72°C for 90 s. This cycle was performed 34 times before the final extension step at 72°C for 15 min. The PCR products were stored at −28°C. Samples were loaded (GelPilot DNA Loading Dye; Qiagen Inc.), electrophoresed in 2.0% agarose (NuSieve GTG Agarose; Lonza Rockland Inc., Rockland, ME) gels at 100 V and 200 mA for 2 h, and photographed (Gel Doc XR; Bio-Rad Laboratories Inc., Hercules, CA).

The proportion of isolates that were susceptible to each antimicrobial was calculated for both time periods. The proportion of isolates with each gene combination was calculated for both time periods. Differences in frequencies of antimicrobial susceptibility or genes were compared using a Fisher exact test when an outcome had fewer than 5 results. A χ^2 test was used when an outcome had more than 5 results (Sokal and Rohlf, 1981). Results were considered significant at $P < 0.05$.

Distributions of MIC were similar between *E. coli* isolated from milk samples submitted from 1985 to 1987 (Table 2) and those from 2009 (Table 3) for most antimicrobials. Changes observed indicated an increase ($P < 0.05$) in frequency of susceptibility in isolates to ampicillin, trimethoprim/sulfamethoxazole, and kanamycin (Table 4) in those from 2009 compared with isolates from 1985 to 1987. The percentage of isolates susceptible did not decrease in isolates from 2009 compared with isolates from 1985 to 1987 for any of the antimicrobials tested. The percentage of isolates susceptible to tetracycline, streptomycin, chloramphenicol, nalidixic acid, amoxicillin/clavulanic acid, sulfisoxazole, gentamicin, cefoxitin, and ceftiofur did not differ between isolates from 1985 to 1987 and isolates from 2009. All 99 *Escherichia coli* isolates from both 1985 to 1987 and 2009 were susceptible to amikacin, ceftriaxone, and ciprofloxacin.

The percentage of isolates susceptible to ampicillin increased from 79.5% of isolates in 1985 to 1987 to

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