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Suzanne Noel Gibbons-Burgener, DVM

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AN EPIDEMIOLOGICAL STUDY OF ANTIMICROBIAL RESIDUES DETECTED IN MICHIGAN COWS' MILK

By

Suzanne Noel Gibbons-Burgener, DVM

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ABSTRACT

AN EPIDEMIOLOGICAL STUDY OF ANTIMICROBIAL RESIDUES DETECTED IN MICHIGAN COWS' MILK

By

Suzanne Noel Gibbons-Burgener, DVM

Following widely publicized accounts of undetected antimicrobial residues in milk making it to market in the late 1980s, the Milk and Dairy Beef Quality Assurance Program (QAP) was developed. Though designed to prevent drug residues, the impact of the QAP on residue occurrence has yet to be determined. A commonly promoted and adopted preventative practice has been the unapproved use of residue detection assays to test for antimicrobial residues in milk from treated individual cows. The reliability of these assays in testing individual cow milk has yet to be established.

The epidemiological research presented here was achieved through two main studies that addressed six objectives. The first study was a retrospective study of Michigan dairy farms evaluating the Milk and Dairy Beef Quality Assurance Program (QAP) and its role in the prevention of violative antimicrobial residues in milk and the adoption of prudent drug management practices. The first and second objectives were to determine if QAP certification and specific management factors were associated with a reduced risk of having antimicrobial residues in milk. Certification in the QAP was associated with a tendency toward reduced risk (OR=0.3 [0.07-1.32]) of having experienced a violative residue in bulk-tank milk. The risk of having had a residue was reduced on farms treating >10% of their herd for metritis, and having their milk processor perform residue testing. However, on-farm residue testing and maintaining written

identification records of treated cows was associated with an increased risk of having had a residue. In a separate set of analyses the associations between QAP certification and the use of prudent drug management practices were evaluated (Objective 3). Involuntary certification was associated with maintenance of good written treatment records and performance of on-farm residue testing. Voluntary certification was weakly associated with use of refrigerated drug storage. These results suggest that farms adopted specific management practices, irrespective of certification.

The second study was a longitudinal experimental study evaluating the reliability of 3 on-farm assays when used to test individual cow milk for antimicrobial residues following treatment for mild clinical mastitis. Methods were developed (Objective 4) to improve the high performance liquid chromatography (HPLC) analyses for detection of ampicillin and pirlimycin in milk. The reliability of the assays was expressed as sensitivity, specificity, and positive and negative predictive values (Objective 5). Ranging from 32.14 to 73.68%, the positive predictive values were poor for all three assays when using the assays' detection limits. Additional statistical analyses were used to determine whether somatic cell count, IgG_1, bacterial isolates or specific antimicrobial treatments were associated with false-positive results (Objective 6). Milk IgG_1 concentrations were positively associated with false positive results from the all 3 assays.

The tendency of the QAP to prevent violative residues provides encouraging information for the continued promotion and implementation of the Program. Dairy producers and veterinarians can use the findings to target their residue prevention efforts. Producers should reconsider their reliance on screening assays for testing individual cows' milk on-farm as a primary tool for residue prevention.

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RATIONALE

Since the introduction of penicillin nearly half a century ago, the use of antimicrobials to prevent and treat diseases in cattle has increased tremendously. With the use of antimicrobials in livestock came the potential risk of residues these medications could pose. Harmful effects ascribed to antimicrobial residues found in meat and milk include: manufacturing difficulties in products requiring fermentation or live cultures, allergic reactions following consumption of residues in food, and potential contributions to the development of antimicrobial resistant bacterial strains. Additionally, consumer perceptions can have adverse effects on the demand for implicated foods. The dairy industry has long recognized the need to be proactive in requiring quality raw milk from farms. Following widely publicized accounts of undetected antimicrobial residues in milk making it to market in the late 1980s, the National Milk Producers Federation and the American Veterinary Medical Association developed the Milk and Dairy Beef Quality Assurance Program (QAP). The QAP provides 10 critical control points meant to reduce the incidence of individual farms experiencing violative residues. There have been limited epidemiological studies determining risk factors for farms experiencing residues. Many of the critical control points address risk factors identified by these studies, however much of the QAP appears to be based more on common sense and intuition than on scientific studies. The eighth critical control point emphasizes the use of residue screening assays in the prevention of residues in the bulk tank. Many in the industry, including veterinarians, producers, and

dairy co-operatives, have interpreted this recommendation to imply testing individual cows' milk is the best prevention. Several studies have indicated that when testing individual cow milk, false-positive and false-violative results were obtained using the currently available assays that were validated for testing commingled milk. Therefore, there is a need for field trials to adequately evaluate the use of residue detection assays and other components of the QAP in the pursuit of on-farm residue prevention.

PROBLEM STATEMENT

Consumer, industry and dairy producer concerns about potential drug residues in milk continues to drive the dairy industry toward investing more resources into the monitoring and prevention of residues. Though designed to prevent drug residues, the impact of the QAP on residue occurrence has yet to be determined. Specifically, it is important to evaluate the Program's influence in changing producers' drug use management practices. One of the most promoted and commonly adopted preventative practice has been the use of residue detection assays to test for antimicrobial residues in milk from treated individual cows prior to keeping and marketing its milk. However, the assays are only approved for use in testing commingled (tanker) milk. The reliability of these same assays in testing individual cow milk has yet to be established.

OBJECTIVES

This dissertation focuses on the epidemiological evaluation of the prevention of antimicrobial residues in milk. The objectives of the two main studies were to:

1. Determine if QAP certification was associated with a reduced risk of having antimicrobial residues in milk.
2. Define specific management factors that may have predisposed dairy farms to having violative antimicrobial residues in milk.
3. Determine if QAP certification was associated with the use of prudent drug management practices.
4. Develop robust gold standard methods for use in determining the reliability of the Delvo-SP, Penzyme Milk Test and SNAP β-lactam assays in the detection of ampicillin, cephapirin and pirlimycin in raw milk.
5. Determine the reliability of the Delvo-SP, Penzyme Farm Milk Test and SNAP β-lactam residue test assays when used to test individual cow milk.
6. Identify risk factors that may be associated with false assay results.

HYPOTHESES

Chapters 2, 3, 5 and 6 each contain specific hypotheses.

OVERVIEW

Chapter 1 is a literature review of the epidemiology and detection of antimicrobial residues in milk. The remaining chapters have been written in a format suitable for independent publication. Two main studies were conducted to accomplish the six objectives. The second and third chapters present findings from a retrospective study evaluating the effect of the QAP, while chapters 4-6 contain the findings from a prospective study evaluating the reliability of residue detection assays used to test

individual cow milk. Chapter 2 evaluates the impact of QAP certification in the prevention of antimicrobial residues. Chapter 3 evaluates the potential associations between the use of specific drug management practices and a dairy farm's QAP certification status (non-, involuntarily and voluntarily QAP-certified). Chapter 4 describes the development of two high pressure liquid chromatography methods used as gold standards in the detection of trace amounts of ampicillin, cephapirin and pirlimycin. Chapter 5 evaluates the reliability of the Delvo-SP, Penzyme Farm Milk Test and SNAP β-lactam assays when used to test milk from individual cows diagnosed and treated for mild clinical mastitis. Chapter 6 then identifies risk factors that may contribute to the occurrence of false-results when using the residue test assays on individual cow milk. The contribution of both main studies to the understanding of the epidemiology of residue prevention is presented in the overall summary.

CHAPTER 1

ANTIMICROBIAL RESIDUES IN MILK - A REVIEW

Introduction

Residues in food products of animal origin are the presence of foreign substances that can be parent compounds, their metabolites or other substances produced as a consequence of administering the parent compound. There are a variety of chemicals that can result in residues in meat and milk, including antimicrobials, insecticides, antihelminthics, hormones, heavy metals and pesticides. Most chemicals are excreted in the urine, feces and milk of the exposed animal. Veterinary pharmaceuticals approved for use in food producing animals include meat, and possibly milk, withholding periods on their labels. These withholding periods are based on the pharmacokinetics of the drug and provide a timeline within which the animal will excrete enough of the drug and its metabolites to allow any remaining residue to fall below the established tolerance or safe level. A violative residue occurs when the chemical is detected in milk or tissues at a level exceeding the tolerance limit. Condemnation of the carcass or load of milk is the usual course of action.

Concerns regarding residues in meat and milk

Processors of dairy products were some of the first to note the adverse effect of antimicrobial residues on the production of products requiring live cultures and fermentation (Stoltz and Hankinson, 1953; Albright et al, 1961). Public health concerns

regarding residues in food products have evolved as more pharmaceuticals have been utilized in animal production and the testing methods have become more sensitive (Engel, 1980). Often the difficulty is deciphering whether the concerns are based on reality or perception. Consumers are particularly concerned when the apparent hazard isn't visible and avoidance is, therefore, perceived as out of their control. Research has shown that consumers rarely list antimicrobials or hormones as a major concern. Yet, when directly asked, half the people believed that those chemicals could pose a serious hazard (Bruhn, 1996). Does the consumer have reason to worry? The most significant hazard centers on the link between antimicrobial use in food-producing animals and the development of microorganisms that are resistant to those antimicrobials (Franco, et al., 1990; Brady, et al., 1993). Though it's the actual use of antimicrobials that have come under scrutiny, residues may be seen as evidence of possible misuse of the drugs. In addition, there is the remote possibility of an allergic reaction to some drugs, especially β-lactams (Huber, 1986; Kindred and Hubbert, 1993).

Acknowledging the potential negative impact of press articles (Ingersoll, 1989, 1990) publicizing alleged antimicrobial residues in retail dairy products, the National Conference on Interstate Milk Shippers strengthened the grade A Pasteurized Milk Ordinance by mandating increased testing of marketed milk (Center for Veterinary Medicine, 1996). With active surveillance of milk at the creamery in place, it quickly became apparent that prevention of antimicrobial residues should begin at the farm level. Though not essential, knowing the causes of violative residues could aid the development of preventative practices. Because total elimination of pharmaceutical use in the

treatment of diseased cows isn't a realistic option, other preventative measures should be explored.

Risk factors for antimicrobial residues in milk

Few studies have scientifically identified risk factors for residues in milk. A case-control study by Kaneene and Ahl reported larger herd size, more hired employees, and use of pre-medicated feeds were associated with an increased risk of a farm having experienced a residue during the prior 5 years (Kaneene and Ahl, 1987). Those farms with residues were also more likely to acknowledge the importance of adhering to withdrawal periods and have residue testing equipment available. Another case-control study (McEwen et al, 1991 a) of farms with violative residues in their milk concluded that the employment of part-time labor to milk cows was associated with an increased risk of residues. Management factors that reduced the risk were pipeline milking in tie stalls, use of antimicrobial test assays, use of separate equipment when milking treated cows and the belief that increasing the withholding period was necessary when increasing the drug dose. With the addition of increased frequency of intramammary antibiotic treatments associated with an increased risk of having had a residue, an earlier dairy farm survey by the same group had similar findings (McEwen et al, 1991 b). To date, the research evaluating potential causes of residues has been restricted to retrospective studies, because violative residues are rare occurrences. A study (Kaneene and Willeberg, 1989) cautioned that results of these retrospective studies may exhibit significant recall and information biases.

Preventing antimicrobial residues

In an effort to reaffirm the commitment of the US dairy industry to maintaining the quality and safety of the nation's milk supply, the National Milk Producers Federation and American Veterinary Medical Association completed the development of the Milk and Dairy Beef Quality Assurance Program (QAP) in 1992. Official certification is given when the producer espouses the principles of providing a high quality product by preventing residues in milk and dairy beef. Certification can be voluntarily pursued by producers, or it can be involuntarily implemented (i.e., required) in instances of a residue violation in milk.

The QAP is based on the hazard analysis critical control point (HACCP) principles. The residue prevention protocol comprises 10 critical control points (Table 1-1), or good management practices, focusing on drug use protocol, herd health management practices, record-keeping and employee education (Boeckman and Carlson, 1997). Many of the critical control points address risk factors that were identified in studies for the increased risk of drug residue occurrence (Kaneene and Ahl, 1987; McEwen et al, 1991 a & b). Other components of the critical control points, such as maintaining treatment records and properly storing medications, appear to have been incorporated into the QAP because of anecdotal and intuitive reasons (Day, 1993). Does the QAP accomplish its goal of reducing the incidence of violative drug residues? That question has yet to be answered.

Table 1-1 Ten critical control points of the QAP Residue Prevention Protocol.

Critical control point	Description of critical control point
1	Practice healthy herd management
2	Establish and maintain a valid veterinarian/client/patient relationship
3	Use only approved drugs (Rx and OTC)
4	Ensure all drugs used on the farm have labels that comply with state and/or federal labeling requirements
5	Store all drugs correctly
6	Administer all drugs properly and visibly identify all treated animals
7	Maintain and use proper treatment records on all treated animals
8	Use drug residue screening assays to test animals receiving drugs in an extra-label manner.
9	Implement employee/family awareness (education) of proper drug use to avoid marketing adulterated products
10	Review farm plan for residue prevention annually and recertify every 2 years.

In a study that evaluated the use of an on-farm risk assessment tool (Sischo et al, 1997), the authors expressed concern that although the QAP does a good job of articulating the hazards of residues, the program is deficient in three necessary components of any HACCP program. Specifically, the Program doesn't provide adequate motivation and tools to allow farm owners to assess their own risk of illegal residues, develop a plan to reduce their risk, or monitor their progress toward residue prevention. In their study, the treatment and control herds received a copy of the QAP booklet and were evaluated by use of the risk assessment tool. The treatment group received additional information and guidance that led to a farm plan to reduce their risk of

residues. Although the overall risk of antibiotic residues was reduced by approximately 19%, there was no significant difference between the groups. It is difficult to ascertain whether the risk assessment tool, the QAP booklet, or the combination of these two factors had the greatest impact on risk reduction. Nevertheless, their study is one of the few that have addressed the challenge of evaluating the QAP.

Ideally, the adoption of all the good management practices would significantly reduce a farm's risk of residue occurrence. Some farms may be more interested in making only some management changes and want to know which of the critical control points are most beneficial. Even before the QAP was instituted, dairy co-operatives, veterinarians and extension personnel were promoting the use of on-farm residue screening assays, such as the Delvotest, Penzyme Milk Test, Charm Farm, SNAP, Cite Probe and LacTek assays. The assays were seen as "insurance" that a farm's bulk-tank was clear of antimicrobials prior to marketing (Jones and Seymour, 1988; Adams, 1993). The assays available for on-farm testing are relatively simple to use and give a qualitative result (yes, maybe or no drug is present). Some of the same screening assays have recently been validated for regulatory use in testing commingled milk at creameries (Center for Veterinary Medicine, 1996).

There are several reasons why the use of on-farm residue screening assays may not be the panacea they appear to be. Producers are encouraged to either submit samples or test milk on-farm from individual treated cows prior to including the cow's milk in the bulk-tank. An important point to remember is that the screening assays are approved and labeled for use in testing commingled milk only. Their reliability in testing individual cow milk has not been established. A recent study (Slenning and Gardner, 1997)

evaluating the economic risk of using on-farm residue testing programs, indicated that not testing was slightly less costly to a farm. Though small changes in milk price or assay costs will alter the dynamics of the economic models, it is evident that indiscriminate testing of treated animals is not a cost-effective recommendation as "insurance" against residues.

Since the changes to the grade A Pasteurized Milk Ordinance in 1991 mandated the screening of every tanker-load of raw milk for at least β-lactam antimicrobial residues, there has been an increased emphasis on the preharvest prevention of residues at the farm level (Adams, 1994). Unfortunately, the only tests available are approved for screening commingled milk, and have been reported to produce false-positive results when used to test individual cow milk for antimicrobial residues (Sischo and Burns, 1993; Cullor et al, 1994; Andrew et al, 1997). Regardless of the potential drawbacks of residue testing, many producers feel the benefits outweigh the negatives and opt for either on-farm or milk handler testing of individual cow milk in an effort to avoid illegal residues.

Antimicrobial Residue Testing

Although the specificity and sensitivity of the variety of assays have been established for commingled milk in controlled laboratory conditions, concern over the accuracy under field conditions exists, because to date, few studies have been conducted that validate these assays in a field setting (Cullor, 1996; Gardner et al, 1996). Approval of residue assays is dependent on the testing of milk spiked with known quantities of specific antimicrobials. In an effort to increase analytical sensitivity, many of the assays

test for antimicrobials below the US Food and Drug (FDA) established tolerance or safe levels (Mitchell et al, 1998). This creates a dilemma when discussing false-positive results. Researchers have struggled with whether to use the term false-violative when an assay result is positive, but the quantity is below the established tolerance level (Gardner et al, 1996; Mitchell et al, 1998). The picture becomes even more grey when we consider testing individual cow milk which would be diluted if included in a farm's bulk tank. The regulatory tolerance levels have been established for commingled milk being sold on the market. It is apparent that the analytical sensitivity of the current on-farm assays probably results in needless disposal of individual cow milk. Unless quantitative methods, such as high performance liquid chromatography (HPLC) are used, there is no accurate way of determining whether a sample contains a violative level of an antimicrobial.

A new European database describes many biochemistry methods developed to identify and quantitate antimicrobials in milk (Van Eeckhout et al, 1998). Mass spectrometry has been used to identify b-lactam residues at their tolerance levels (Heller and Ngoh, 1998). Beta-lactam antimicrobials comprise the majority of antimicrobials approved for use in lactating cattle and the majority of residues detected in milk. HPLC is the most commonly employed method of quantifying antimicrobials. It has been used to identify and quantify penicillins and cephalosporins (Briguglio and Lau-Cam, 1984; Dasenbrock and LaCourse, 1998; Hong et al, 1995; Moats, 1993; Moats, 1994; Moats and Harik-Khan, 1995; Moats and Romanowski, 1998), macrolides (Heller, 1997), sulfonamides (Smedley, 1994; Schwartz and Lightfield, 1995) and tetracyclines (White et al, 1993). A combination of mass spectrometry and HPLC has also been explored

(Heller, 1996; Hornish et al, 1995; Straub et al, 1994; Tyczkowska et al, 1994). Several studies have compared screening assay and liquid chromatography results (Anderson et al, 1998; Ang et al, 1997; Harik-Khan and Moats, 1995). Unlike most of the studies, Anderson et al. and Ang et al. used milk with incurred instead of spiked residues. One study used milk from only two cows (Ang et al., 1997) and the other (Anderson et al., 1998) utilized six residue screening assays to determine the qualitative status of specific HPLC fractions. None of these studies were specifically designed to determine the reliability of on-farm residue screening assays. The technical expertise, required equipment and reagents, and lengthy analysis time make the HPLC methods impractical and cost prohibitive for routine residue testing, but should be considered when gold standard detection methods are necessary.

Because violative residues in milk are a relatively rare occurrence (<0.1% of bulk tanks), the likelihood of false-negative results is negligible. Consequently, research evaluating the accuracy of on-farm residue detection assays has focused on testing individual cow milk and the possible sources of false-positive results. Possible causes of false-positive results include elevated somatic cell count (Sischo and Burns, 1993; Van Eenennaam et al, 1993); increased lactoferrin and lysozyme concentrations (Carlsson and Bjorck, 1989); lower milk production, increased parity and increased coliform counts (Andrew et al, 1997); and other inhibitory substances in the milk (Tyler et al, 1992; Cullor et al, 1994). The screening assays represent an array of detection methods, such as microbial inhibition, microbial receptor, enzymatic colourimetric, and receptor binding assays (Mitchell et al., 1998). The different detection methods may be affected differently by the potential causes of false-positive results.

Producers are still left wondering whether and how to use on-farm residue detection assays. The use of on-farm assays may have its place in investigating the source of a violative residue on a farm (Musser and Anderson, 1999). The case study by Musser and Anderson describes how a combination of detective work and cautious use of a series of assays was used to identify the probable source of a violative residue on a farm. Again the authors discouraged indiscriminate use of on-farm assays to test all cattle. Additional studies are needed to determine the reliability and usefulness of on-farm residue detection assays in the prevention of violative residues.

Areas for future study

There are few epidemiological studies of the prevention of residues in milk. Introduction of the QAP was a major industry initiative to be pro-active in the prevention of antimicrobial residues. The tremendous amount of financial and human resources invested in the QAP nationwide warrants scientific evaluation of the efficacy of the program in attaining its goals. Its impact and success in changing dairy management practices to those considered prudent in the prevention of drug residues have not been reported. By scientifically determining strengths and weaknesses of the QAP, the evaluation of the Program will elicit dialogue regarding potential improvements.

Evaluating preventative practices for antimicrobial residues hinges on the correct classification of residue occurrence. We need to know what we're preventing. Many of the practices focus on individual animal management. Because there are no residue screening assays approved or validated for use in testing individual cow milk, and there are numerous reports of false-positive results when assays approved for commingled milk

testing are utilized for individual cow milk testing, it is extremely important to determine whether on-farm residue screening assays provide reliable results for both producers and researchers. Misclassification (false positive and negative) of results can lead to unnecessary disposal of milk on farms, misleading research findings and perhaps the exposure of people to unnecessary residues. Field-based epidemiological studies will provide much needed information regarding the usefulness of on-farm assays and their appropriateness as research tools.

REFERENCES

Adams JB. Assuring a residue-free food supply: Milk. *J Am Vet Med Assn* 1993;202:1723-1725.

Albright JL, Tuckey SL, Woods GT. Antibiotics in milk - a review. *J Dairy Sci* 1961;44:779-807.

Andrew SM, Frobish RA, Paape MJ, Maturin LJ. Evaluation of selected antibiotic residue screening tests for milk from individual cows and examination of factors that affect the probability of false-positive outcomes. *J Dairy Sci* 1997;80:3050-3057.

Ang CYW, Luo W, Call VL, Righter HF. Comparison of liquid chromatography with microbial inhibition assay for detection of incurred amoxicillin and ampicillin residues in milk. *J Agric Food Chem* 1997;45:4351-4356.

Boeckman S, Carlson KR. *Milk and dairy beef quality assurance program: milk and dairy beef residue prevention protocol.* Stratford, IA: Agri-Education, Inc, 1997;1-70.

Brady MS, White N, Katz SE. Resistance development potential of antibiotic/antimicrobial residue levels designated as "safe levels." *J Food Prot* 1993;56:229-233.

Briguglio GT, Lau-Cam CA. Separation and identification of nine penicillins by reverse phase liquid chromatography. *J AOAC Int* 1984;67:228-231.

Bruhn CM. Consumer Perceptions and concerns about veterinary drug residues. In: Moats WA and Medina MB, ed. *Veterinary Drug Residues: Food Safety.* Washington, DC, American Chemical Society, 1996, pp 18-21.

Carlsson A, Bjorck L. Lactoferrin and lysozyme in milk during acute mastitis and their inhibitory effect in Delvotest P. *J Dairy Sci* 1989;72:3166-3175.

Center for Veterinary Medicine. Appendix N, Pasteurized Milk Ordinance. In *CVM Update: Milk monitor with antimicro drug screening test.* 1996, pp 5-6.

Cullor JS. Dilemmas associated with antibiotic residue testing in milk. In: Moats WA and Medina MB, ed. *Veterinary Drug Residues: Food Safety.* Washington, DC, American Chemical Society, 1996, pp 44-57.

Cullor JS, van Eenennaam A, Gardner I, et al.. Performance of various tests used to screen antibiotic residues in milk samples from individual animals. *J AOAC Int* 1994;77:862-870.

Dasenbrock CO, LaCourse WR. Assay for cephapirin and ampicillin in raw milk by high-performance liquid chromatography - integrated pulsed amperometric detection. *Anal Chem*, 1998;70:2415-2420.

Day J. A subcommittee review of the quality assurance initiative: implementation issues from the Implementation and Communication Subcommittee of the Drug Residue Committee, in *Proceedings.* 32nd Annu Meet National Mastitis Council 1993;144-146.

Engel RE. Current food safety and quality service residue control program. *J Am Vet Med Assn* 1980;176:1145-1147.

Franco DA, Webb J, Taylor CE. Antibiotic and sulfonamide residues in meat: implications for human health. *J Food Prot* 1990;53:178-185.

Gardener IA, Cullor JS, Galey FD, et al.. Alternatives for validation of diagnostic assays used to detect antibiotic residues in milk. *J Am Vet Med Assn* 1996;209:46-52.

Harik-Khan R, Moats WA. Identification and measurement of b-lactam antibiotic residues in milk: integration of screening kits with liquid chromatography. *J AOAC Int* 1995;78:978-986.

Heller, DN. Determination and confirmation of pirlimycin residue in bovine milk and liver by liquid chromatography/thermospray mass spectrometry: interlaboratory study. *J AOAC Int* 1996;79:1054-1061.

Heller, DN. Determination of Pirlimycin residue in milk by liquid chromatographic analysis of the 9-fluorenylmethyl chloroformate derivative. *J AOAC Int* 1997;80:975-981.

Heller DN, Ngoh MA. Electrospray ionization and tandem ion trap mass spectrometry for the confirmation of seven β-lactam antibiotics in bovine milk. *Rapid Commun Mass Spectrom* 1998;12:2031-2040.

Hong CC, Lin CL, Tsai CE, Kondo F. Simultaneous identification and determination of residual penicillins by use of high-performance liquid chromatography with spectrophotometric or fluorometric detectors. *Am J Vet Res* 1995;56:297-303.

Hornish RE, Cazars AR, Chester ST, Roof RD. Identification and determination of pirlimycin residue in bovine milk and liver by high-performance liquid chromatography-thermospray mass spectrometry. *J Chromatogr B* 1995;674:219-235.

Huber WG. Allergenicity of antibacterial drug residues. In: Rico AG, ed., *Drug residues in animals*. Academic Press, Toronto, ONT 1986, pp 33-49.

Ingersoll B. Milk is found tainted with a range of drugs farmers give cattle. *The Wall Street Journal*, December 29, 1989; Al, A2.

Ingersoll B. New York milk supply highly tainted, TV station says, based on own survey. *The Wall Street Journal*, February 8, 1990; A4.

Jones GM and Seymour EH. Cowside antibiotic residue testing. *J Dairy Sci,* 1988;71:1691-1699.

Kaneene JB and Ahl AS. Drug residues in dairy cattle industry: Epidemiological evaluation of factors influencing their occurrence. *J Dairy Sci*, 1987;70:2176-2180.

Kaneene JB, Willeberg P. Influence of management factors in the occurrence of antibiotic residues in milk: a case-control study of Michigan dairy herds, with examples of suspected information bias. *Acta Vet Scand,* 1989;84:473-476.

Kindred TP, Hubbert WT. Residue prevention strategies in the United States. *J Am Vet Med Assn,* 1993;202:46-49.

McEwen SA, Black WD, Meek AH. Antibiotic residue prevention methods, farm management, and occurrence of antibiotic residues in milk. *J Dairy Sci* 1991;74:2128-2137 (a).

McEwen SA, Meek AH, Black WD. A dairy farm survey of antibiotic treatment practices, residue control methods and associations with inhibitors in milk. *J Food Protect,* 1991;54:454-459 (b).

Mitchell JM, Griffiths MW, McEwen SA, et al.. Antimicrobial drug residues in milk and meat: causes, concerns, prevalence, regulations, tests, and test performance. *J Food Prot,* 1998;61:742-756.

Moats, WA. Determination of cephapirin and desacetylcephapirin in milk using automated liquid chromatographic cleanup and ion-pairing liquid chromatography. *J AOAC Int,* 1993;76:535-539.

Moats, WA. Determination of ampicillin and amoxicillin in milk with an automated liquid chromatographic cleanup. *J AOAC Int*, 1994;77:41-45.

Moats WA, Harik-Khan. Liquid chromatographic determination of β-lactam antibiotics in milk: a multiresidue approach. *J AOAC Int,* 1995;78:49-54.

Moats, WA, Romanowski, RD. Multiresidue determination of b-lactam antibiotics in milk and tissues with the aid of high-performance liquid chromatographic fractionation for clean up. *J Chromatogr A*, 1998;812:237-247.

Musser JMB, Anderson KL. Using drug residue screening tests to investigate antibiotic contamination of milk. *Vet Med,* 1999;94:474-479.

Schwartz DP, Lightfield AR. Practical screening procedures for sulfamethazine and N^4-acetylsulfamethazine in milk at low parts-per-billion levels. *J AOAC Int,* 1995;78:967-970.

Sischo WM. Quality milk and tests for antibiotic residues. *J Dairy Sci,* 1996;79:1065-1073.

Sischo WM, Burns CM. Field trial of four cowside antibiotic-residue screening tests. *J Am Vet Med Assn,* 1993;202:1249-1254.

Sischo WM, Kiernan NE, Burns CM, et al. Implementing a quality assurance program using a risk assessment tool on dairy operations. *J Dairy Sci,* 1997;80:777-787.

Slenning BD, Gardner IA. Economic evaluation of risks to producers who use milk residue testing programs. *J Am Vet Med Assn,* 1997;211:419-427.

Smedley MD. Liquid chromatographic determination of multiple sulfonamide residues in bovine milk: collaborative study. *J AOAC Int,* 1994;77:1112-1122.

Stoltz EI, Hankinson DJ. Antibiotics and lactic acid starter cultures. *Appl Microbiol,* 1953;1:21-29.

Straub R, Linder M, Voyksner RD. Determination of β-lactam residues in milk using perfusive-particle liquid chromatography combined with ultrasonic nebulization electrospray mass spectrometry. *Anal Chem,*1994;66:3651-3658.

Tyczkowska KL, Voyksner RD, Straub RF, Aronson AL. Simultaneous multiresidue analysis of b-lactam antibiotics in bovine milk by liquid chromatography with ultraviolet detection and confirmation by electrospray mass spectrometry. *J AOAC Int,* 1994;77:1122-1131.

Tyler JW, Cullor JS, Erskine RJ, et al. Milk antimicrobial residue assay results in cattle with experimental, endotoxin-induced mastitis. *J Am Vet Med Assn,* 1992;201:1378-1384.

Van Eeckhout NJ, Van Peteghem CH, Helbo VC, et al.. New database on hormone and veterinary drug residue determination in animal products. *Analyst,* 1998;123:2423-2427.

Van Eenennaam AL, Cullor JS, Perani L, et al.. Evaluation of milk antibiotic residue screening tests in cattle with naturally occurring mastitis. *J Dairy Sci* 1993;76:3041-3053.

White CR, Moats WA, Kotula KL. Optimization of a liquid chromatographic method for determination of oxytetracycline, tetracycline and chlortetracycline in milk. *J AOAC Int,* 1993;76:549-554.

CHAPTER 2

EVALUATION OF CERTIFICATION IN THE MILK AND DAIRY BEEF QUALITY ASSURANCE PROGRAM AND RELATED FACTORS ON THE RISK OF HAVING VIOLATIVE ANTIBIOTIC RESIDUES IN MILK FROM DAIRIES IN MICHIGAN

ABSTRACT

Objectives—To determine if certification in a Milk and Dairy Beef Quality Assurance Program (QAP) was associated with a reduced risk of having antibiotic residues in milk and to define specific management factors that may have predisposed dairy farms to having violative antibiotic residues in milk.

Sample Population—124 dairy farms in Michigan that had at least 1 violative residue in milk during 1993 and 248 randomly selected control farms in Michigan that did not have violative residues in milk during 1993.

Procedure—A pretested structured questionnaire was mailed to case and control farms. A conditional multivariable logistic regression model was developed to determine risk factors associated with having a violative antibiotic residue in milk.

Results—Certification in the QAP tended to reduce the risk of having a violative antibiotic residue. Annual treatment of > 10% of a herd for metritis was associated with a reduced risk of having a violative residue. Evidence suggested that a routine request for a milk processor to perform residue testing was associated with a decreased risk of having had a violative antibiotic residue, but routine on-farm residue testing was associated with an increased risk of having had a residue.

Conclusion and Clinical Relevance— QAP certification was associated with a tendency

toward reduced risk of having had a violative antibiotic residue in milk. Other risk factors associated with violative antibiotic residues are addressed by various critical control points in the QAP and may be indicators for strengths and weaknesses of a QAP.

INTRODUCTION

On Dec 29, 1989 and Feb 8, 1990, *The Wall Street Journal* (Ingersoll, 1989 & 1990) publicized alleged antimicrobial residues in retail dairy products. The negative impact that these reports had on consumer perceptions about food safety were substantiated by subsequent national surveys (Tillison, 1991). In an effort to reaffirm the commitment of the US dairy industry to maintaining the quality and safety of the nation's milk supply, the National Milk Producers Federation and American Veterinary Medical Association completed the development of the Milk and Dairy Beef Quality Assurance Program (QAP) in 1992.

The QAP is based on Hazard Analysis Critical-Control Point (HACCP) principles and is probably one of the most ambitious programs that the industry has undertaken. The QAP has 10 critical control points that producers, in conjunction with their veterinarian, should regularly monitor and evaluate in an effort to formulate their own unique plan of action to minimize the risk of violative drug residues. The 10 critical control points center around a drug-use protocol, managerial practices, personnel policies, and management strategies for maintaining cattle health (AVMA and NMPF, 1991). Many of these critical control points are associated with violative antibiotic residues in milk (Kaneene et al, 1986; Kaneene and Ahl, 1987; Kaneene and Willeberg, 1989; McEwen et al, 1991 a & b).

The National Milk Producers Federation and AVMA have conducted an extensive national campaign to encourage voluntary adoption of the QAP by dairy operations. As an additional incentive, several states have enacted legislation that reduces the penalty for having a violative residue in milk if the operation has been certified in the QAP prior to

the residue violation (Fluid Milk Act, 1996; Adulterated dairy products, 1998). The tremendous amount of financial and human resources invested in the QAP warrants scientific evaluation of the efficacy of the program in attaining its goals. Therefore, the objectives for the study reported here were to determine if QAP certification was associated with a reduced risk of having antibiotic residues in milk and to define specific management factors that may have predisposed dairy farms to having violative residues in milk. Specifically, the following hypothesis was tested: dairy operations that have had a violative drug residue in milk were less likely to have participated in the QAP.

MATERIALS AND METHODS

Design—A case-control study of Grade-A dairy herds in Michigan was conducted in 1994 to evaluate risk factors hypothesized to be associated with antibiotic residues in milk. A case was defined as a Grade-A dairy farm that had at least 1 violative antibiotic residue in milk shipped to market during 1993. A control farm was defined as a Grade-A dairy farm that did not have a violative antibiotic residue in milk shipped to market during 1993.

Sample selection—A list of 124 farms with violative antibiotic residues in milk during 1993 was obtained from the Michigan Department of Agriculture, Dairy Division, and those farms were included as case farms in the study. To control for varying degrees of general herd management and labor requirements as well as geographic differences in available veterinary services and milk marketing, case farms were stratified on the basis of geographic region (agricultural statistics district; Fig 2-1) and herd size. Two control farms were randomly selected from the records of the Michigan Department of

Agriculture, Dairy Division for each case farm located in their respective strata. Therefore, proportionately equivalent sample distributions of case and control farms across strata were generated.

Data collection—A pretested self-administered questionnaire[a] was developed to confidentially obtain data from the 372 farms in the study (124 case and 248 control farms). The questionnaire consisted of questions focusing on herd health management, drug use, record keeping, personnel management, and descriptive characteristics of each farm during 1993. Six dairy farms not included in the study were used to test the questionnaire. On the basis of that preliminary test, substantial changes were not required in the questionnaire. The questionnaire was sent to the farms in an initial mailing, and, if necessary, 2 reminder mailings were sent approximately 1 month apart. Data from completed questionnaires were recorded in a relational database program.[b]

Statistical analysis — A χ^2 goodness-of-fit test was performed to determine whether the geographic distributions of farms was comparable to those of respondents, on the basis of agricultural district as well as the overall population of Grade-A dairy farms in Michigan. Distributions of questionnaire respondents and farms included in the study were similarly evaluated on the basis of herd size. Those variables deemed to be biologically plausible risk factors for having antibiotic residues in milk were identified. Descriptive statistics were determined, including frequencies for categoric risk factors and mean ± SD for continuous risk factors. Potential correlations among risk factors were evaluated; using Pearson's and Spearman rank correlation coefficients.

The outcome of interest was the binary variable of whether a farm had a violative residue in milk during 1993. Univariate analyses between each risk factor and the

outcome variable were performed to aid the development of a conditional multivariate logistic regression model. A conditional multivariate logistic regression model, controlling for agricultural district and herd size, was developed by using a backward stepwise technique (Hosmer and Lemeshow, 1989). The initial model included those variables that had a univariate parameter estimate with a significance of $P \leq 0.30$ and those variables that were forced into the model. Reasons for forcing a variable into the model included the ability to evaluate the risk factor of greatest biological interest, the variable was a member of a categorical variable with multiple levels, or the variable was significantly correlated with a variable otherwise included in the model. Potential confounding by specific risk factors was evaluated, using methods described by Kleinbaum (Kleinbaum et al, 1988). Deviance and degrees of freedom for the initial and final regression models were compared, using the likelihood ratio statistic, to ensure that the 2 models did not differ significantly ($P > 0.05$).

RESULTS

After 3 separate mailings of the questionnaire, 45 (36%) case farms and 121 (49%) control farms responded, resulting in an overall response rate of 45%. Of the 166 returned questionnaires, 158 (95%) had relatively complete, useable responses. Analysis of χ^2 goodness-of-fit tests indicated that the 372 farms included in the study did not significantly deviate in geographic distribution from all Grade-A dairy farms in Michigan ($\chi^2 = 6.24$; $P = 0.62$) or from those who responded to the questionnaire ($\chi^2 = 5.96$; $P = 0.65$). Distribution of herd size (Table 2-1) did not differ between all 372 farms and the case-farm respondents ($\chi^2 = 6.38$; $P = 0.27$) and control-farm respondents ($\chi^2 = 5.93$; $P =$

0.31). The distribution of Grade-A dairy farms in Michigan on the basis of herd size was not available; hence, comparison to farms used in the study was not performed. The proportion of responding case farms with QAP certification was the same as that of the nonresponding case farms ($\chi^2 = 0.009$; $P = 0.92$). The same comparison was not performed for control farms, because certification status of nonresponding control farms could not be ascertained.

Frequency distributions of categoric risk factors among responding case and control farms were tabulated (Table 2-2). For example, 40 case and 114 control farms provided an answer to the question regarding whether they routinely milked treated cows last, and 22 (55%) case and 54 (47%) control farms responded that they did milk treated cows last. Distributions of continuous risk factors for case and control farms was determined (Table 2-3). Significant correlations were between routine use of on-farm test kits and a routine request that a milk processor perform antibiotic residue testing (R = -0.49; $P < 0.001$), use of another bucket when milking treated cows and use of another milking claw when milking treated cows (R = 0.51; $P < 0.001$), and number of full-time farm workers and mean herd size (R = 0.70; $P < 0.001$).

Using results of univariate analyses, 11 risk factors were identified for inclusion in the initial logistic regression model. Those risk factors included use of another milking claw when milking treated cows, use of another bucket when milking treated cows, written identification records of cows treated, routine performance of on-farm residue testing, routine request that milk processor perform antibiotic residue testing, purchase of over-the-counter drugs from nonveterinarian sources, purchase of prescription drugs from a veterinarian, annual treatment of $\leq$ 10% of herd for mastitis, annual treatment of $>$ 40%

of herd for mastitis, annual treatment of >10% of herd for metritis, and mean size of milking herd in 1993 (Tables 2-2 and 2-3). In addition, certification in the QAP prior to an antibiotic residue in milk was forced into the model because it was the risk factor of primary importance (main effect) in this study. Because of the strong correlation with mean herd size, number of full-time workers was also forced into the model. After the stepwise procedures (Hosmer and Lemeshow, 1989), the model was reduced to 8 risk factors (certification in the QAP, annual treatment of >10% of herd for metritis, annual treatment of ≤ 10% of herd for mastitis, annual treatment of >40% of herd for mastitis, routine request for milk processor to perform residue testing, routine performance of on-farm residue testing, written identification records of treated cows, and purchase of prescription drugs from a veterinarian; Table 2-4). None of the modifiers investigated made a significant contribution to the model. The final model was significantly (χ^2 = 21.54; P = 0.006) correlated with a herd having a violative antibiotic residue in milk, suggesting that the model would be sufficient to explain the odds of having an antibiotic residue in milk. The χ^2 of the log-likelihood statistic (χ^2 = 6.06; P = 0.30) between initial and final models was not significant, suggesting that the predictive ability of the model was not significantly diminished by its reduction.

Written identification records of treated cows was the only risk factor significantly associated (odds ratio [OR] = 4.78; P = 0.03) with an increased risk of having a violative antibiotic residue. Annual treatment of >10% of herd for metritis (OR = 0.20; P = 0.02) was significantly associated with a decreased risk of having had a violative antibiotic residue was. Certification in the QAP prior to having had a violative antibiotic residue tended to be associated (OR = 0.28; P = 0.11) with a reduced risk of having had a

violative antibiotic residue in milk.

During the model building process, models including on-farm residue testing or residue testing performed by milk processors indicated that these 2 variables were each significantly associated with explaining antibiotic residues. However, these variables were negatively correlated and, therefore, must be retained together in the model to avoid biased estimates. Their independent contribution to the model appears not significant, but their elimination resulted in a likelihood ratio statistic of 5.12 ($P = 0.08$) and evidence that they were significant confounders. Although separately not significant risk factors, variables for the 2 categories of mastitis treatment and purchase of prescription drugs from a veterinarian were significant confounders in the model and, consequently, were retained.

DISCUSSION

The main objective of the QAP is to reduce the incidence and risk of drug residues in beef and milk (Adams, 1993; AVMA and NMPF, 1991). To test our hypothesis that certification in the QAP reduced the risk of having had antibiotic residues in milk we forced the risk factor for prior certification into the model. Analysis of this factor indicated a tendency (OR = 0.28; $P = 0.11$) for a QAP-certified farm to have a 70% reduction in risk of having a violative antibiotic residue in milk. This association may have been weakened by measurement error regarding the degree to which a producer and their veterinarian actually participated in the QAP. It cannot be assumed that all the certified farms fully embraced the program and made major management changes. Although the analysis was restricted to farms with voluntary certification, the binary

variable for QAP certification did not discern conscientious from less-conscientious producers.

Control farms (35/109, 32.1%) were more likely to have participated in the QAP than case farms (10/41, 24.4%; Table 2-2). The study attempted to determine the duration of participation in the QAP by inquiring about the dates of initial certification and recertification. Date of certification for case farms were confirmed through analysis of records of the Michigan Department of Agriculture, Dairy Division, but information pertaining to some of the control farms participating in the program was deficient. This deficiency of information was probably the result of a lack of compliance with the request that a copy of the signed certificate be sent to the office of the Dairy Division. Five of 35 (14.3%) of certified control farms did not report the date of certification. An additional 18 of 35 (51.4%) of certified control farms reported only the year but not the day or month of initial certification. A simple comparison of operations that reported at least the year of certification did not indicate a difference in the percentage of participants in the QAP from the case or control groups (5/10, [50%] of participating case farms and 16/30 [53.3%] of participating control farms) that were certified before Jan 1, 1993 (i.e., prior to the onset of the study). Because the campaign for statewide adaptation of the QAP was initiated in 1992, it would have been critical to know the month, in addition to the year, of certification to enable us to include duration of participation in the analyses. Consequently, the effect of duration was not evaluated.

The rate for overall voluntary participation of respondents in this study in the QAP (30%) far exceeded the 1993 national participation rate and the estimated participation rate for the state of Michigan (4 and 10%, respectively[c]). On the basis of the

χ^2 analysis for comparison of certification for responding and nonresponding case farms, there was minimal chance that nonresponders biased our estimation of the rate of certification. This discrepancy might have been a result of national underreporting of certification and may indicate the need for a more reliable census of farms involved in QAP certification and participation. The issues of underreporting participation and duration need to be addressed by studies evaluating the efficacy of the QAP.

Increased drug use is believed to increase the risk of having violative residues in milk and meat. Mastitis is the most common disease of lactating dairy cows and is frequently treated with antimicrobial agents (Gardner et al, 1990; Cullor, 1993; Hady et al, 1993). Using the second mastitis category as a reference, which included the mean annual incidence of mastitis in Michigan, a tendency toward a reduced risk of residues when treating less than the mean number of cows in a herd and a tendency toward an increased risk of residues when treating more than the mean number of cows in herd were consistent with other studies (Kaneene and Ahl, 1987; McEwen et al, 1991 b). Unfortunately, results of the study reported here did not indicate a significant association among various categories of mastitis treatment and having violative residues.

In Michigan, metritis was the second most commonly reported disease of dairy cows between 1986 and 1989 (Kaneene and Hurd, 1988; Kaneene et al, 1990). Farms having treated >10% of the herd annually for metritis were associated with 80% less risk of having had a residue in milk, compared with farms having treated $\leq$10% of the herd. In Michigan, veterinarians (Kaneene and Miller, 1994) most often treat metritis. We interpreted this finding to indicate that increased presence of a veterinarian for metritis treatment possibly provides increased guidance to producers for drug use and withdrawal

periods (Kaneene and Miller, 1992). In addition, visible identification or segregation of sick and treated cows might be enhanced when treatments extend beyond otherwise-routine mastitis treatments.

Critical control point No. 8 of the QAP emphasizes the availability and use of drug residue tests. The majority of case and control respondents in this study indicated that they used residue testing during 1993. When on-farm and milk processor testing were evaluated separately, farms that routinely requested that the milk processor perform residue testing had a reduced risk of having violative residues, while on-farm residue testing was associated with increased risk of having had a violative residue. The difference between the risk associated with on-farm residue testing and that conducted by milk processors has 2 possible explanations. First, on-farm testing might have been initiated after the farm had a violative residue, as was suggested by other studies (Kaneene and Ahl, 1987; McEwen et al, 1991 b). Secondly, milk processors were more likely to have had extensive experience in performing the tests.

Milk processors performing the requested tests had more knowledge and information regarding the use of antibiotic residue test kits. It was important to have used a test that was designed to detect the compound for which the sample was being tested and that was able to detect the compound at or below the legal tolerance level. Furthermore, milk processors should have ensured that any tests were performed correctly and consistently. These criteria might not have been met when performing tests on-farm. Realizing that on-farm and milk processor testing were not mutually exclusive (a farm could perform none, one, or both) and that a small, but significant, negative correlation was detected between the 2 testing variables, the possibility of erroneous on-farm testing

should be evaluated more thoroughly in future studies. Most importantly, it would be prudent for dairies that want to reduce the risk of a violative residue in milk to entrust antibiotic testing needs with their milk processor.

Unexpectedly, keeping written identification records of treated cows was associated with a fivefold increase in the risk of having had a violative antibiotic residue. Maintenance of complete and accurate records for cattle treated on a farm is the basis of critical control point No. 7. A similar finding was previously reported (McEwen et al, 1991 b). In that retrospective study, case farms (violative residue detected) were more likely to maintain records of treatment, and significantly more farmers on case farms held the opinion that insufficient record keeping of treated cattle increased the risk for a violative residue. This paradoxic phenomenon might be the result of case farms having adopted the good management practice of recording the identification of treated cows after notification of a violative residue and mandatory completion of the QAP. As the responsibility of proving proper residue prevention practices shifts toward dairy farms, it is important that complete and accurate records be maintained prior to having a violative antibiotic residue.

Certification in the QAP had a tendency to reduce the risk of having a violative antibiotic residue in milk. Dairy operations that treated > 10% of the herd for metritis had a decreased risk of having violative antibiotic residues in milk. A positive association between maintaining written identification records of treated cows and having a violative residue was identified, but this finding probably indicates a change in management implemented after notification of having had a violative residue. Although the routine request that a milk processor perform residue testing was associated with a decreased risk

of having a violative residue, routine on-farm testing was associated with an increased risk of having had a violative residue in milk. Specific risk factors associated with having a violative residue are addressed by various critical control points in the QAP and may be indicators for some of the program's strengths and weaknesses.

FOOTNOTES

[a]Questionnaire available on request and as Appendix 1.

[b]Rbase for DOS, Microrim Inc, Redmond, Wash.

[c]McCarthy W. Michigan Department of Agriculture, Dairy Division, Lansing, MI: Personal communication, 1994.

REFERENCES

Adams JB. Assuring a residue-free food supply: milk. *J Am Vet Med Assoc* 1993;202:1723-1725.

Adulterated dairy products, Minn. Stat. 32.21, Subd. 1-4. *Minnesota statues vol. 1.* St Paul, Minn: Revisor of Statues, State of Minnesota, 1998;920-922.

American Veterinary Medical Association and National Milk Producers Federation. Milk and Dairy Beef Residue Prevention: a quality assurance program. *J Am Vet Med Assoc* 1991; 199 (Suppl):1-23.

Cullor JS. The control, treatment, and prevention of the various types of bovine mastitis. *Vet Med* 1993;88:571-579.

Fluid Milk Act of 1965, P.A. 1965, No. 233, as amended by P.A. 1993, No.5. *Michigan Compiled Laws Annotated 1996* Sections 286.1 to 288. St Paul, Minn: West Publishing Co, 1996; 417-419.

Gardner IA, Hird DW, Guterback WW, et al. Mortality, morbidity, case-fatality, and culling rates for California dairy cattle as evaluated by the National Animal Health Monitoring System, 1986-87. *Prev Vet Med* 1990;8:157-170.

Hady PJ, Lloyd JW, Kaneene JB. Antibacterial use in lactating dairy cattle. *J Am Vet Med Assoc* 1993;203:210-220.

Hosmer DW, Lemeshow S. Chapters 4-7. In: Barnett V, Bradley RA, Hunter JS, et al, eds. *Applied logistic regression.* New York: John Wiley & Sons, 1989;82-213.

Ingersoll B. Milk is found tainted with a range of drugs farmers give cattle. *The Wall Street Journal*, December 29, 1989; Al, A2.

Ingersoll B. New York milk supply highly tainted, TV station says, based on own survey. *The Wall Street Journal*, February 8, 1990; A4.

Kaneene JB, Ahl AS. Drug residues in dairy cattle industry: Epidemiological evaluation of factors influencing their occurrence. *J Dairy Sci* 1987;70:2176-2180.

Kaneene JB, Coe PH, Smith JH, et al. Drug residues in milk after intrauterine injection of oxytetracycline, lincomycin-spectinomycin, and povidone-iodine in cows with metritis. *Am J Vet Res* 1986;47:1363-1365.

Kaneene JB, Hurd HS. National Animal Health Monitoring System Round 1—Final Report. East Lansing, Mich: Michigan State University, 1988;63-74.

Kaneene JB, Hurd HS, Miller R, et al. National Animal Health Monitoring System Round 2—Final Report. East Lansing, Mich: Michigan State University, 1990;26-34.

Kaneene JB, Miller R. Epidemiological study of metritis in Michigan dairy cattle. *Vet Res* 1994;25:253-257.

Kaneene JB, Miller RA. Description and evaluation of the influence of veterinary presence on the use of antibiotics and sulfonamides in dairy herds. *J Am Vet Med Assoc* 1992;201:68-76.

Kaneene JB, Willeberg P. Influence of management factors in the occurrence of antibiotic residues in milk: a case-control study of Michigan dairy herds, with examples of suspected information bias. *Acta Vet Scand* 1989;84:473-476.

Kleinbaum DG, Kupper LL, Muller KE. Confounding and interaction in regression. In: Payne M, ed. *Applied regression analysis and other multivariable methods*. Belmont, Calif: Duxbury Press, 1988;163-180.

McEwen SA, Black WD, Meek AH. Antibiotic residue prevention methods, farm management, and occurrence of antibiotic residues in milk. *J Dairy Sci* 1991 (a);74:2128-2137.

McEwen SA, Meek AH, Black WD. A dairy farm survey of antibiotic treatment practices, residue control methods and associations with inhibitors in milk. *J. Food Prot* 1991 (b);54:454-459.

Tillison J. Consumer research review, edited by the National Dairy Promotion and Research Board. *Animal Health & Milk Quality - A Situation Analysis*. Jefferson, Wis: Morgan & Myers, 1991;10-11.

Figure 2-1 - Geographic distribution of Michigan agricultural statistics districts defined by the National and Michigan Agricultural Statistics Services. The numbers of case farms in the sample (denominator) and number that responded (numerator) in each district are indicated.

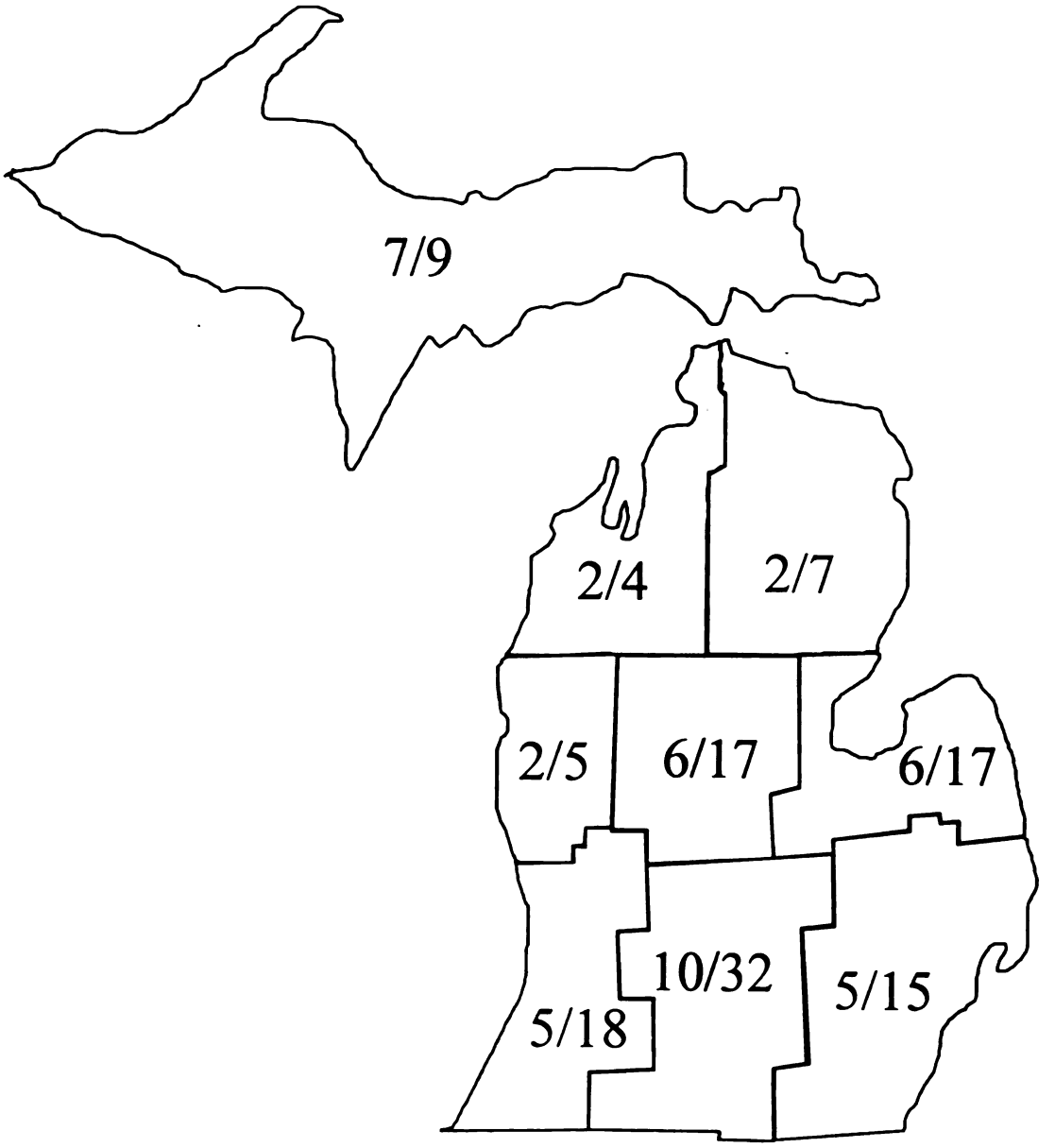


Table 2-1- Herd size categories and the number of case operations from each category.

		Case farms	
Herd size category	**No. of lactating cows**	**No. in sample**	**No. responding**
A	10-39	24	11
B	40-79	45	13
C	80-119	22	5
D	120-159	17	11
E	160-249	8	2
F	>250	8	3

Table 2-2. Distribution of categorical risk factors among case and control operations.

Risk factor	Responding operations			
	Case		Control	
	No.[1]	%[2]	No.[1]	%[2]
Certification in QAP prior to residue[3]	41	24.4	109	32.1
Use of a different milking claw for treated cows[3]	39	35.9	112	48.2
Treated cows milked last	40	55.0	114	47.4
Diverted pipeline when milking treated cows	35	34.3	105	35.2
Use of a different bucket when milking treated cows[3]	39	64.1	115	74.8
Every cow received nonlactating treatment for mastitis	41	82.9	117	84.6
All cows were routinely dewormed	41	31.7	117	35.0
Visible identification placed on treated cows	41	87.8	117	89.7
No treatment records kept	41	17.1	117	20.5
Record of treated cow identification[3]	41	73.2	116	62.9
Milkers had access to treatment records	35	94.3	93	94.6
Use of a drug test to determine milk withholding period	41	70.7	117	72.6
Use of the drug label to determine milk withholding period	41	78.0	117	71.8
Routinely perform on-farm chemical residue testing[3]	41	80.5	117	50.4
Routinely request milk processor perform residue testing[3]	41	29.3	117	53.0
Purchase over-the-counter drugs from a veterinarian	41	39.0	117	34.2
Purchase over-the-counter drugs from a nonveterinarian source[3]	41	82.9	117	70.1
Purchase prescription drugs from a veterinarian[3]	41	92.7	117	99.1
Purchase prescription drugs from a nonveterinarian source	41	9.8	117	6.8
Annual treatment of > 10% of herd for metritis[3]	41	24.4	117	43.6

Table 2-2 continued

Annual treatment ≤ 10% of herd for mastitis[3]	41	22.0	117	38.5
Annual treatment of 10.1-39.9% of herd for mastitis (reference category)	41	53.6	117	48.7
Annual treatment of > 40% of herd for mastitis[3]	41	24.4	117	12.8
Treated at least one cow in an extra-label manner during 1993	41	36.6	117	35.9

[1]Number of operations providing a response. [2]Percentage of responding operations indicating "yes." [3]Risk factors included in the initial logistic regression model. QAP = Milk and Dairy Beef Quality Assurance Program.

Table 2-3. Distribution of continuous risk factors among cases and controls.

	Responding operations					
	Case			Control		
Risk factor	**No.**[1]	**Mean**	**SD**	**No.**[1]	**Mean**	**SD**
No. of full-time workers	40	1.425	2.04	115	1.609	3.21
No. of part-time workers	39	1.333	1.53	115	1.400	1.73
Milking herd (No. of cows)[2]	41	124.95	159.84	117	108.61	90.98
Rolling herd average (lbs of milk)	36	18,836	3,350	110	19,249	3,328
Annual percentage of herd treated in an extra-label manner	38	41.55	193.92	109	9.54	34.28

[1]No. of operations providing a response. [2]Risk factors included in the initial logistic regression model. To convert lb of milk to kg, divide value by 2.2.

Table 2-4 - Results of the conditional multivariable logistic regression analysis of associations among chemical residue occurrence in milk and herd-level risk factors.

Risk Factor	**b**[1]	**SE(b)**[2]	***P***[3]	**Odds Ratio**	**95% CI (odds ratio)**
Certification in QAP prior to residue	-1.19	0.75	0.11	0.30	0.07-1.32
Annual treatment of $\geq$ 10% of herd for metritis	-1.61	0.71	0.02	0.20	0.05-0.80
Record of identification of treated cows	1.56	0.70	0.03	4.78	1.21-18.77
Routinely request that milk processor perform residue testing	-0.88	0.67	0.18	0.41	0.11-1.54
Routinely perform on-farm residue testing	0.79	0.72	0.28	2.20	0.54-9.04
Annual treatment of $\leq$ 10% of herd for mastitis	-1.08	0.72	0.13	0.34	0.08-1.39
Annual treatment of $\geq$ 40% of herd for mastitis	0.13	0.70	0.85	1.14	0.29-4.49
Purchase prescription drugs from a veterinarian	-2.01	1.68	0.23	0.13	0.01-3.61

[1]Multivariate logistic regression coefficient. [2]Standard error of b. [3]*P*-value for Wald test statistic. QAP = Milk and Dairy Beef Quality Assurance Program. 95% CI = 95% confidence interval.

Likelihood Ratio Statistic = 6.06; 5 df; $P > 0.05$.

CHAPTER 3

INFLUENCE OF THE MILK AND DAIRY BEEF QUALITY ASSURANCE PROGRAM ON MICHIGAN DAIRY FARM DRUG MANAGEMENT PRACTICES

ABSTRACT

Objective – To test the hypothesis that dairy farms certified in the Milk and Dairy Beef Quality Assurance Program (QAP) were more likely to use prudent drug management practices than farms that were not certified.

Design – Cross-sectional study.

Sample Population – 141 Michigan dairy farms, of which 74 were not certified in the QAP, 30 were involuntarily certified, and 37 were voluntarily certified.

Procedure – Dairy producers completed a self-administered questionnaire that focused on herd health management, drug use, record-keeping, personnel management, and descriptive characteristics of their farm during 1993. Separate multivariable logistic regression models were developed to determine the association of QAP certification with each of the management practices.

Results – Results suggested that farms adopted specific management practices, irrespective of certification. Large percentages of farms used visible identification and non-emergency veterinary services and discussed residue prevention with employees. Involuntary certification was associated with maintenance of good written treatment

records and performance of on-farm drug residue testing. Voluntary certification was weakly associated with use of refrigerated drug storage.

Conclusions and Clinical Relevance – QAP certification appeared to have been associated with the adoption of only a few prudent drug use practices, although QAP materials and framework were developed to assist veterinarians in the promotion of disease prevention, client communication, and residue prevention practices on farms. Veterinary care would benefit from the development and encouragement of better record keeping on farms.

INTRODUCTION

The Milk and Dairy Beef Quality Assurance Program (QAP) was jointly developed by the National Milk Producers Federation and the AVMA in 1991. The QAP is based on the hazard analysis critical control point (HACCP) principles. The residue prevention protocol comprises 10 critical control points focusing on drug use protocol, herd health management practices, record-keeping and employee education (Boeckman and Carlson, 1997). Many of the critical control points address risk factors that were identified in studies by Kaneene et al and McEwen et al of the increased risk of drug residue occurrence (Kaneene and Ahl, 1987; McEwen et al, 1991 a). Dairy farms become certified in the QAP by using the 10 critical control points to review, with their veterinarian, their farms' residue prevention practices, and define areas for improvement. Official certification is given when the producer espouses the principles of providing a high quality product by preventing residues in milk and dairy beef. Certification can be voluntarily pursued by producers, or it can be involuntarily implemented (i.e., required) in instances of a residue violation in milk.

In the report of a study that evaluated the use of an on-farm risk assessment tool (Sischo et al, 1997), the authors expressed concern that although the QAP does a good job of articulating the hazards of residues, the program is deficient in 3 necessary components of any HACCP program. Specifically, these authors pointed out that the program doesn't provide adequate motivation and tools to allow farm owners to assess their own risk of illegal residues, develop a plan to reduce their risk, or monitor their progress toward residue prevention. In their study, the treatment and control groups received a copy of the QAP booklet and were evaluated by use of the risk assessment

tool. The treatment group received additional information and guidance that led to a farm plan to reduce their risk of residues. Although the overall risk of antibiotic residues was reduced by approximately 19%, there was no significant difference between the groups. It is difficult to ascertain whether the risk assessment tool, the QAP booklet, or the combination of these 2 factors had the greatest impact on risk reduction. Nevertheless, their study is one of the few that have addressed the challenge of evaluating the QAP.

Intuitively, the QAP seems to focus on important drug residue hazards. Its impact and success in changing dairy management practices to those considered prudent in the prevention of drug residues has not been reported. The purpose of the study reported here was to test the hypothesis that dairy farms certified in the QAP were more likely to use prudent drug management practices than those farms that were not certified.

MATERIALS AND METHODS

Study design - As part of a larger study (Gibbons-Burgener et al, 1999) of Michigan dairy farms that had a violative drug residue in milk, a cross-sectional study was undertaken to test the hypothesis that QAP certification was associated with implementation of certain drug management practices.

Study population - Sample selection for the original study has been described (Gibbons-Burgener et al, 1999). Briefly, 166 of 372 (45%) farms that received the questionnaire, returned it. Results of goodness-of-fit analyses indicated that the respondents were geographically representative of dairy farms in Michigan. One hundred forty-one responding farms provided complete information regarding various drug

management practices and were included in the cross-sectional study sample. Of the 141 farms, 74 were not certified in the QAP, 30 were involuntarily certified, and 37 were voluntarily certified. Approximately a third of the study population had a residue violation in 1993. Of these farms, 73% were in involuntarily certified.

Data collection - The pretested, self-administered questionnaire[a] focused on herd health management, drug use, record keeping, personnel management, and descriptive characteristics of the farm during 1993.

Statistical analyses - Outcome variables that represented management practices for 7 of the 10 critical control points of the QAP were identified in the questionnaire. Only those management practices believed to have biologically plausible associations with QAP participation were considered. Because we suspected that the various outcomes were related, Spearman rank correlation analysis was performed with all outcome variables to test the degrees of correlation. In addition to univariate analyses, separate multivariable logistic regression models were developed to determine the association of QAP certification with each of the management practices. Because few independent variables were considered for inclusion in each model, reduction techniques were not used. Three categories were used to designate QAP certification: non-certified, involuntarily certified as a result of a drug residue violation, or voluntarily certified. Farms with a violative drug residue after becoming QAP certified were included in the voluntarily certified group. Additional variables were believed to potentially have biological relationships (primary or confounding) with each management practice. For

example, mean milking herd size and whether the producer was a college or technical school graduate were considered potential variables associated with all practices. Independent variables considered for inclusion in specific models included whether the farm utilized a milking parlor, whether the farm utilized routine, nonemergency veterinary services, and whether cows were treated with drugs in an off-label manner. Each model was estimated twice to facilitate the 3-way comparison between the categories of non-certified, involuntarily certified, and voluntarily certified. The comparison group was switched from non-certified to involuntarily certified to evaluate a difference between voluntary and involuntary certification. Seemingly unrelated 2-equation probit regression modeling was used to determine whether significant correlations between outcome variables had a substantial influence on results of logistic regression modeling (Hardin, 1996). Significance was set at $P \leq 0.05$.

RESULTS

Percentages of responding farm owners that indicated that they performed specific drug-use managerial practices were tabulated (Table 3-1). Spearman rank correlation analysis revealed significant correlations between numerous outcome variables. The correlations of most practical importance included: recorded reason for treatment and recorded type of drug used ($r = 0.51$), recorded type of drug used and recorded dose of drug used ($r = 0.63$), recorded type of drug used and recorded date(s) of treatment ($r = 0.50$), recorded identification of treated cow and recorded date(s) of treatment ($r = 0.66$), withdrawal period determined by label and withdrawal period determined by a asking a

veterinarian (r = 0.50), and routinely requested off-farm testing for residues and on-farm testing used routinely (r = -0.47).

Most of the models revealed minimal association between QAP certification and the various drug management practices; results of 3 multivariable models provided the most significant results. The first model considered the use of refrigerated drug storage as its outcome variable (Table 3-2); voluntarily certified farms were almost 3 times more likely than noncertified farms to use refrigerated drug storage, and herd size was significantly associated with refrigeration.

The second model used on-farm drug testing as the outcome variable (Table 3-3). To emphasize differences that seemed inherent in instances of involuntary certification, analysis was performed with involuntarily certified farms as the base comparison group. Compared with involuntarily certified farms, noncertified and voluntarily certified farms were less likely to have used on-farm residue testing.

The third model used good treatment records as the outcome variable (Table 3-4). The term "good" was defined as records that included the treated cow's identification, date of treatment, and drug or dose used. In this model, involuntarily certified farms were 2.5 times more likely than noncertified farms to maintain good treatment records.

Results of the seemingly unrelated 2-equation probit regression modeling indicated that correlations detected among some outcome variables did not significantly bias results of multivariable logistic regression models.

DISCUSSION

Recommendations for proper drug storage often include maintaining the drug within a certain temperature range, as well as avoiding exposure to environmental factors such as sunlight and excessive humidity. Three of the most commonly used drugs approved for use in lactating cows (procaine penicillin G, ceftiofur sodium[b] and oxytocin) are supplied with labels that recommend refrigeration (Arrioja-Dechert, 1997). Herd size may influence the type and quantity of these drugs on a given farm, which could explain its strong positive association with refrigerated storage. Independent of herd size, there was weak evidence that voluntarily certified farms were more likely (by an approximate factor of 3) than noncertified farms to use refrigerated storage. This association was not significant ($P = 0.086$) but is worthy of discussion. Of particular interest is the reduced overall effect of QAP on storage method when involuntarily and voluntarily certified farms were used together as an index for QAP certification. A true difference between involuntarily certified farms and voluntarily certified farms in the use of refrigerated storage may be related to conscientiousness or other unmeasured factors of producers voluntarily seeking certification.

Forty-five percent of the study farms requested off-farm residue testing, whereas 60% used on-farm drug tests. Although not mutually exclusive, substantial contrast among types of QAP certification was detected only for on-farm testing. Involuntarily certified farms were 5 times more likely than noncertified farms and 3.5 times more likely than voluntarily certified farms to perform on-farm residue testing. Of particular interest is the difference between involuntarily and voluntarily certified farms. This may be an indication that having a violative residue compels a farm to adopt on-farm residue testing

in an attempt to avoid additional violations. This finding is consistent with results of previous studies (Kaneene and Ahl, 1987; McEwen et al, 1991 b).

The eighth critical control point in the QAP encourages the use of antibiotic screening tests when drugs are used in an off-label manner. The use of drug screening assays for milk of individual cow's has yet to be approved; nevertheless, it is a commonly accepted practice (Sischo, 1996). There is evidence that assays designed to test commingled milk may produce false-positive or false-violative results when used on milk from an individual cow (Sischo and Burns, 1993; Tyler et al, 1992; Van Eenannaam et al, 1993), as well as pose variable economic risk (Slenning and Gardner, 1997). The QAP directs the producer to the drug's label to determine the correct withdrawal period and states that drug testing is unnecessary when using drugs according to the label (Boeckman and Carlson, 1997). Voluntarily certified farms were more likely to indicate that some of their cows were treated in an off-label manner (Table 3-1). If the QAP was indeed causing the producers to adopt drug-testing technology, we would have expected the voluntarily certified farms to have more need and, consequently, be more inclined to use individual cow testing. However, farms involuntarily certified in the QAP (all of which had a violative residue in 1993) were more likely to adopt on-farm drug testing, and noncertified farms were more likely to request off-farm drug testing. Perhaps the eighth critical control point is perceived as the most simple and reliable management change a farm can make. A broader objective of the QAP is the promotion of preventative health practices that reduce disease and the need for treatment; however, many veterinarians and producers are unsure how to determine practical and meaningful indices for herd health

improvement. As stated, the program is probably lacking the necessary tools for farm operators to evaluate their progress toward implementing the QAP (Sischo et al, 1997).

To take into account the multiple correlations among the different record types, we performed additional statistical methods, with mixed success. We decided to define a level of record keeping that wasn't necessarily optimal, but that could be considered "good." Consistent with 1996 National Animal Health Monitoring System data (USDA-APHIS-VS, 1996), 21% of the producers reported keeping no written treatment records. Maintaining complete treatment records is often thought to be one of the most important residue prevention practices (McEwen et al, 1991 b; Day, 1993). In the study reported here, farms with involuntary certification were 2.5 times more likely to keep good written records than farms that had never been certified. Although not statistically different from either of the other groups, a higher percentage of voluntarily certified farms maintained good treatment records than did noncertified farms, whereas a lower percentage of voluntarily certified farms maintained good treatment records than involuntarily certified farms.

The QAP introduces the concept of complete record keeping and provides a template for a daily treatment record (Boeckman and Carlson, 1997). Written treatment records have the potential to be used not only in a residue prevention capacity, but also epidemiologically. The incidence of treatable diseases and the efficacy of treatment may be evaluated on a herd basis. As reported (McEwen et al, 1991 b) farms that have had a violative residue may be more attuned to deficiencies in residue prevention and more motivated to alter selected practices. In the study reported here, producers who were forced to review the protocol after a violative residue was detected may have discovered

that their records were lacking and that this hindered an explanation or defense of the violation. Perhaps, it was not until the need arose that the necessity for good records became apparent. Our results do not clearly indicate that QAP alone had an impact on record-keeping practices.

We expected QAP certification to be associated with more prudent drug management practices. The use of a cross-sectional study design hindered the assessment of temporality in the adoption of management practices. Consistent with results reported by another study (Sischo et al, 1997), we found that farms may adopt management practices irrespective of certification. Large percentages of farms in each of the 3 QAP groups used visible identification and nonemergency veterinary services and chose to discuss residue prevention with their employees. Three possible explanations for these findings are that herd size may have been a better indicator for levels of herd management, farms with prior experience with residues may have already altered their drug use practices, and education level may play an important role in adopting certain management practices. With a low rate of voluntary participation in the program, the influence of involuntary certification following a residue violation needs to be addressed. The administration of the QAP alone may be insufficient to prompt producers to adopt specific drug management practices.

Involuntary certification was associated with the maintenance of good written treatment records and the performance of on-farm drug residue testing. Voluntary certification was weakly associated with the use of refrigerated drug storage. Although QAP certification appeared to have been influential in the adoption of only a few prudent drug use practices, veterinarians and producers may be using the general concepts of the

QAP to improve many of their drug residue prevention practices without formalizing QAP certification. The QAP materials and framework were developed to assist veterinarians in the promotion of disease prevention, client communication, and residue prevention practices on client farms. However, results of the study reported here could not clearly indicate the efficacy of the QAP with regard to residue prevention practices, possibly as a result of the small number of farms in the study and the cross-sectional design. A prospective study with a large sample size may overcome these shortcomings.

FOOTNOTES

[a]Questionnaire available from authors by request and in Appendix 1.

[b]Naxcel®, Pharmacia & Upjohn, Kalamazoo, MI.

REFERENCES

Arrioja-Dechert A. *Compendium of veterinary products*. 4th ed. Port Huron, MI: North American Compendiums Inc, 1997;109-1310.

Boeckman S, Carlson KR. *Milk and dairy beef quality assurance program: milk and dairy beef residue prevention protocol*. Stratford, IA: Agri-Education, Inc, 1997;1-70.

Day J. A subcommittee review of the quality assurance initiative: implementation issues from the Implementation and Communication Subcommittee of the Drug Residue Committee, in *Proceedings*. 32nd Annu Meet National Mastitis Council 1993;144-146.

Gibbons-Burgener SN, Kaneene JB, Lloyd JW, et al. Evaluation of certification in the Milk and Dairy Beef Quality Assurance Program and associated factors on the risk of having violative antibiotic residues in milk from dairy farms in Michigan. *Am J Vet Res* 1999:60;1312-1316.

Hardin JW. Bivariate probit models. *Stata Technical Bulletin* 1996;33:15-20.

Kaneene JB, Ahl AS. Drug residues in dairy cattle industry: Epidemiological evaluation of factors influencing their occurrence. *J Dairy Sci* 1987;70:2176-2180.

McEwen SA, Black WD, Meek AH. Antibiotic residue prevention methods, farm management, and occurrence of antibiotic residues in milk. *J Dairy Sci* 1991(a);74:2128-2137.

McEwen SA, Meek AH, Black WD. A dairy farm survey of antibiotic treatment practices, residue control methods and associations with inhibitors in milk. *J Food Protect* 1991(b);54:454-459.

Sischo WM. Quality milk and tests for antibiotic residues. *J Dairy Sci* 1996;79:1065-1073.

Sischo WM, Burns CM. Field trial of four cowside antibiotic-residue screening tests. *J Am Vet Med Assoc* 1993;202:1249-1254.

Sischo WM, Kiernan NE, Burns CM, et al. Implementing a quality assurance program using a risk assessment tool on dairy operations. *J Dairy Sci* 1997;80:777-787.

Slenning BD, Gardner IA. Economic evaluation of risks to producers who use milk residue testing programs. *J Am Vet Med Assoc* 1997;211:419-427.

Tyler JW, Cullor JS, Erskine RJ, et al. Milk antimicrobial residue assay results in cattle with experimental, endotoxin-induced mastitis. *J Am Vet Med Assoc* 1992;201:1378-1384.

USDA-APHIS-VS. Part III: Reference of 1996 diary health and health management National Animal Health Monitoring System. In: *NAHMS Dairy '96.* Washington, DC: USDA, 1996;17.

Van Eenannaam AL, Cullor JS, Perani L, et al. Evaluation of milk antibiotic residue screening tests in cattle with naturally occurring mastitis. *J Dairy Sci* 1993;76:3041-3053.

Table 3-1. Percentages of Michigan dairy farms that used various drug-use management practices.

Management practice	No QAP[a]	Involuntary QAP[b]	Voluntary QAP[c]
Valid veterinarian-client-patient relationship[d]			
Nonemergency veterinary care used	74	70	81
Veterinarian provided Rx drugs	100	97	92
Veterinarian provided OTC drugs	35	43	27
Use of OTC and Rx drugs[d]			
Nonveterinarian source for Rx drugs	3	7	14
Nonveterinarian source for OTC drugs	70	87	73
Cows treated in off-label manner	34	33	49
Drug storage[d]			
Drugs stored in cabinet	61	70	62
Drugs stored on an open shelf or table	36	33	32
Drugs stored in refrigerator	69	70	89
Drugs locked in storage	5	3	3
Proper drug administration and identification of treated cows[d]			
Used visible identification on treated cows	88	87	89
Milked treated cows last	50	52	47
Used different milking claw on treated cows	44	39	43
Diverted milk pipeline when milking treated cows	37	32	29
Used a special bucket when milking treated cows	71	71	69

Table 3-1 – continued

Maintain treatment records[d]			
Recorded reason for treatment	34	47	53
Recorded type of drug used	42	60	56
Recorded dose of drug used	28	33	44
Recorded identification of treated cow	59	70	78
Recorded date(s) of treatment	66	77	78
Recorded udder quarter treated	34	40	42
No written records	26	20	11
Kept records at milking location	38	53	49
Kept records where drugs are stored	15	17	22
Keeps records in cattle housing area	9	10	3
Use of drug residue screening tests[d]			
On-farm testing used routinely	49	83	62
Routinely requested off-farm (milk handler) to test for residues	50	37	43
No optional testing performed on milk	12	3	5
Withdrawal period determined by label	74	80	70
Withdrawal period determined by milk test	65	73	81
Withdrawal period determined by asking veterinarian	57	57	54
Withdrawal period determined by past experience	18	33	27
Withdrawal period determined by information from other producers	1	3	3

Table 3-1 continued

Employee education[d]			
Discussed residue avoidance with employees	68	76	76
Discussed avoidance with new employees	24	43	32
Routinely discussed avoidance throughout year	32	37	35
Discussed avoidance when problems occurred	26	27	30

[a]Farms (n = 74) were not enrolled in the Milk & Dairy Beef Quality Assurance Program (QAP). [b]Farms (n = 30) were involuntarily enrolled in the QAP. [c]Farms (n = 37) were voluntarily enrolled in the QAP.
[d]Critical control point as defined in a drug residue prevention protocol.
Rx = Prescription. OTC = Over-the-counter.

Table 3-2. Results of multivariable logistic regression analysis of associations among use of refrigerated drug storage and farm management factors.

Variable	β	*P*	Odds ratio	95% Confidence interval
QAP certification				
None	NA	NA	1.0	NA
Involuntary	0.089	0.860	1.09	0.41 – 2.93
Voluntary	1.057	0.086	2.88	0.86 – 9.63
Herd size	0.016	0.005	1.016	1.005 – 1.027
College graduate[a]	0.513	0.237	1.67	0.71 – 3.90
Parlor milking[b]	0.055	0.910	1.06	0.41 – 2.72
Intercept	-0.690	0.144	NA	NA
Model -2 log likelihood: $\chi^2 = 24.76$ (5 degrees of freedom; $P < 0.001$)				
NA = Not applicable. [a]Producer was a college or technical school graduate. [b]Farm used a milking parlor.				

Table 3-3. Results of multivariable logistic regression analysis of associations between use of on-farm drug testing and farm management factors.

Variable	β	*P*	Odds ratio	95% Confidence interval
QAP certification				
None	-1.668	0.002	0.19	0.06 – 0.55
Involuntary	NA	NA	1.0	NA
Voluntary	-1.249	0.041	0.29	0.09 – 0.95
Herd size	0.003	0.160	1.003	0.999 – 1.006
College graduate[a]	-0.263	0.473	0.77	0.37 – 1.58
Off-label drug use[b]	-0.105	0.786	0.90	0.42 – 1.92
Non-emergency veterinary care[c]	-0.019	0.965	0.98	0.42 – 2.27
Intercept	1.531	0.011	NA	NA

Model -2 log likelihood: $\chi^2 = 14.40$ (6 degrees of freedom; $P = 0.026$).

[a]Producer was a college or technical school graduate. [b]Drugs were used in an off-label manner. [c]Non-emergency veterinary care was used on farm.

Table 3-4. Results of multivariable logistic regression analysis of associations between maintenance of good* records and farm management factors.

Variable	β	*P*	Odds ratio	95% Confidence interval
QAP certification				
None	NA	NA	1.0	NA
Involuntary	0.895	0.046	2.45	1.01 – 5.91
Voluntary	0.567	0.187	1.76	0.76 – 4.09
Herd size	0.003	0.147	1.003	0.999 – 1.006
College graduate[a]	0.548	0.124	1.76	0.86 – 3.48
Intercept	-1.183	0.001	NA	NA

Model -2 log likelihood: $\chi^2 = 9.70$ (4 degrees of freedom; $P = 0.046$).
*Good records defined as records that included treated cow's identification, date(s) of treatment and drug or dose used. [a]Producer was a college or technical school graduate.

CHAPTER 4

IDENTIFICATION AND QUANTIFICATION OF AMPICILLIN, CEPHAPIRIN AND PIRLIMYCIN IN COWS' MILK USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND FLUORESCENCE DETECTION

ABSTRACT

To determine the reliability of results from three antimicrobial assays used to test individual cows' milk; it was essential to quantify the antimicrobials that were potentially present. Previously described biochemistry methods were modified to better accommodate the blinded evaluation of milk samples collected as part of a field trial. Two extraction techniques were necessary for the recovery of ampicillin, cephapirin and pirlimycin. Additionally, two derivatizations were used to elicit fluorescent products from ampicillin and pirlimycin. Naturally occurring proteins, lipids and their breakdown products hindered the clean extraction of the antimicrobials from milk. Three separate HPLC methods were necessary to obtain adequate detection sensitivity for each antimicrobial. Reversed phase HPLC with a C-18, 5m, 4.6 X 220mm column was used in all methods. Sensitivity was substantially enhanced by the use of fluorometric detection, in place of ultraviolet, to detect Fmoced pirlimycin and derivatized ampicillin. Identification and separation of cephapirin were best accomplished by premixing the B buffer (1:1 0.01M KH_2PO_4:CH_3CN) to achieve the desired gradient. The methods presented should improve the reliability of HPLC analyses used to detect ampicillin and pirlimycin in milk at or below the established FDA tolerance levels.

INTRODUCTION

Antimicrobial residues in milk present several public health and manufacturing problems (Brady et al., 1993; Mitchell et al., 1998; Waltner-Toews and McEwen, 1994). In an effort to prevent milk with residues from being marketed, the dairy creamery tests each tanker for antimicrobial residues prior to accepting the milk. The detection of antimicrobials in raw milk has been made easier with the use of various residue-screening assays. Depending on the assay, a qualitative positive or negative result is produced by an enzymatic reaction[a], receptor binding[b], or growth inhibition[c]. There has been concern that other components of raw milk, such as somatic cells (Sischo and Burns, 1993; Van Eenennaam et al., 1993) or lactoferrin (Carlsson et al.,1989) may produce false assay results. Unapproved use of the screening assays to test individual cow milk on farms has been promoted and widely adopted (Gibbons-Burgener et al., 2000). However, the reliability of the assays when used to test individual cow milk have yet to be determined.

As part of a longitudinal experimental study to determine the reliability of the SNAP β-lactam, Penzyme Milk Test and Delvo-SP assays for testing individual cow milk, gold standard methods for identification and quantification of antimicrobials used in the treatment of mastitis on the study farms were essential. Specifically, cows in the antimicrobial treatment group were treated at the producer's or veterinarian's discretion with a FDA approved intramammary preparation containing cephapirin, hetacillin or pirlimycin. Hetacillin is readily metabolized into ampicillin. Chromatographic methods are considered the most sensitive and reliable gold standard methods for evaluating the presence of antimicrobials. Limited studies have been published describing the identification and quantification of pirlimycin (Hornish et al., 1992; Hornish et al., 1995;

Heller, 1996; Heller, 1997). The detection of cephapirin and ampicillin in milk have been more widely studied (Moats and Romanowski, 1998; Moats, 1993; Moats, 1994; Dasenbrock and LaCourse, 1998; Tyczkowska et al., 1994).

Preliminary studies in our laboratory indicated that the published extraction and detection methods were inadequate for blinded screening of the large number of field samples to be tested. The objective of this part of the overall study was to determine robust gold standard methods for the identification and quantification of ampicillin, cephapirin and pirlimycin in order to evaluate the reliability of three on-farm residue detection assays when used to test individual cows' milk.

MATERIALS AND METHODS

Equipment Used

Waters 717+ autosampler

Waters Model 510 millipore pump

Waters millipore automated gradient controller

Waters 474 Scanning Fluorescence Detector

Waters 486 tunable (U.V.) absorbance detector

Perkin-Elmer, Supercosil C-18, 5m, 4.6 X 220mm column

Beckman System Gold® analog interface module 406

Reagents & Solutions Used

Reference standards for ampicillin[d], cephapirin[d] and pirlimycin[e] were used. Stock solutions of 1 mg/ml of active drug were made for each of the antimicrobials by the addition of filtered milli-Q water (MQW). Additional standard concentrations were made by diluting the stock solutions with MQW. Standards were maintained in a –20° C freezer when not in use. All acetonitrile and methanol used in solutions were either synthesis or HPLC grade.

Extraction & Derivatization Solutions

a) *26.7% Trichloroacetic (TCA) acid*: 500 g of trichloroacetic acid crystal[f] was reconstituted to 100% with 500 ml of MQW. Additional dilution with MQW made 26.7% TCA solution.

b) 2 M Sodium hydroxide (2 M NaOH): Combined 20 g of sodium hydroxide pellets[f] (FW 40.0) with 250 ml of MQW.

c) *2 N Hydrochloric acid (HCl)*: Diluted 1 part 12 M HCl with 5 parts MQW.

d) *Sorënson citrate buffer*: Dissolved 21 g of granular citric acid monohydrate[f] in 200 ml of 1 M NaOH solution. The solution was diluted to 1 liter with MQW. The addition of HCl acid is used to adjust the solution pH to 2.5.

e) *0.1% Mercury bichloride ($HgCl_2$)*: Combined 0.2 mg of dry $HgCl_2$[d] in 200 ml of Sorënson citrate buffer.

f) *0.1 M Tetraethylammonium chloride (Et_4NCL)*: 8.3 g of tetraethylammonium chloride hydrate[g] was mixed with 500 ml of MQW.

g) *pH 6.0 KH_2PO_4:Na_2HPO4 buffer*: Combined 50 ml of 0.01 M monobasic potassium phosphate and 10 ml of 0.01 M dibasic sodium phosphate. The pH was adjusted to 6.0 using glacial acetic acid.

h) *0.25 mM Sodium hydroxide (NaOH)*: Combined 2.5 ml of 10 mM sodium hydroxide (made by diluting 1 ml 1 M NaOH[f] with 99 ml MQW) with 97.5 ml of MQW..

i) *Fmoc 100 ppm solution*: Combined 10 ± 0.5 mg 9-Fluorenylmethyl chloroformate (Fmoc chloride)[h] with 10 ml of acetonitrile in a 20 ml vial. Transferred the solution to a 150-250 ml bottle using an additional 90 ml of acetonitrile to rinse the vial (Heller, 1997). The Fmoc solution was sealed and stored at 4°C. (Weigh powders on a balance in a chamber and seal the vial until adding acetonitrile in a hood.).

j) *Fmoc 10 ppm solution*: Combined 10 ml of 100 ppm Fmoc and 90 ml of acetonitrile. Sealed and stored the solution at 4°C.

k) *2 M Disodium hydrogen phosphate (2 M Na_2HPO_4):* Combined 56.8 gm of disodium hydrogen phosphate anhydrous powder[f] with 200 ml of MQW.

Buffer solutions

a) 55:45 CH_3OH:H_20 : For each liter of buffer, 550 ml of methanol was combined with 450 ml of MQW. Buffer was sparged with helium for 30 minutes.

b) 0.01 M KH_2PO_4: 1.36 g of granular potassium phosphate[f] was mixed in one liter of MQW and then filtered.

c) 1:1 0.01 M KH_2PO_4:CH_3CH : 500 ml of premixed/filtered 0.01M KH_2PO_4 was mixed with 500 ml acetonitrile.

d) 4:3:3 1% CH_3COOH:CH_3OH:CH_3CN: For each liter of buffer we combined 4 ml of glacial acetic acid[f], 396 ml of MQW, 300 ml of methanol and 300 ml of acetonitrile. The solution was mixed for 5 minutes and sparged with helium for 30 minutes.

Extraction and Derivatization of Ampicillin

Milk sample was thawed in an ice bath and briefly vortexed prior to use. One ml of milk was combined with 4 ml of MQW and 3 ml 26.7% TCA in a 15 ml centrifuge tube. Tube contents were vortexed for 10 seconds and then centrifuged 5 minutes at 1000 g. 4.5 ml of supernatant was pipetted and filtered through glass wool into a second 15 ml centrifuge tube, avoiding inclusion of the top fat layer. We added 0.5 ml of 2 M NaOH to the filtered solution and vortexed it 3 seconds and incubated for 5 minutes at room temperature. That was followed by the addition of 0.5 ml of 2 N HCl and 1 ml of 0.1% $HgCl_2$ solution. Again the solution was vortexed 3 seconds and incubated for 5 minutes at room temperature. The pH of the solution was adjusted to 6.2 by adding pre-warmed 2 M Na_2HPO_4. The solution was incubated at 38-40° C for 25 minutes. Six ml of ethyl acetate was added and the solution was vigorously shaken for 5 minutes. The solution was centrifuged for 5 minutes at 1000 g. The top, organic layer (approximately 5 ml) was decanted and retained . Liquid was evaporated in speed vac with no heat.

Extraction of Cephapirin and Pirlimycin

Milk samples were thawed in an ice bath and briefly vortexed prior to use. Combined 4 ml of milk and 0.8 ml of Et_4NCl in small beaker. Slowly added 16 ml of CH_3CH while continuously vortexing the solution. The solution was incubated for 10

minutes at 4°C. Supernatant was filtered through glass wool. 0.8 ml of 6.0 pH buffer was added and thoroughly mixed. Solution was transferred to glass tubes and the sample was dried in a speed-vac with no heat. We used 4 tubes initially and later combined 3 tubes into one resulting in one tube with residue from 1 ml of milk and one tube with residue from 3 ml of milk.

Derivatization for Pirlimycin Detection

Following the extraction and drying of the tube with residue from 1 ml of milk, a derivatization process may be undertaken 3-12 hours prior to analysis of sample in the HPLC system. One hundred ml of standard in water may also be derivatized in this manner. To the dry residue we added 0.5 ml of 0.67 NaOH (0.4 ml added to 100 µl of standard in water) and vortexed 10 seconds. Added 0.5 ml of 100 ppm Fmoc solution (use 10 ppm Fmoc with standard in water) and vortexed for 10 seconds. The mixture was incubated at room temperature for 1 hour and then vortexed 10 seconds. A minimum of two additional hours of incubation is required prior to analysis on the HPLC system.

Detection of Ampicillin Residue

The dry $HgCl_2$ derivatized residue was brought up in 100 µl of 100% methanol and vortexed for 5 seconds. The entire 100 µl was transferred to a 250 µl autosampler tube. Using an isocratic 55:45 CH_3OH:MQW buffer system with a flow rate of 0.8 ml/min, 20 µl of a sample could be injected every 30 minutes with no detected carryover from previous sample. An excitation λ = 345 nm and emission λ = 420 nm on the fluorescence detector was used for optimal detection of ampicillin residues.

Detection of Pirlimycin

Fmoced samples and standards are suspended in a 1 ml solution during the derivatization process. We transferred 200 µl of each sample to a 250 µl autosampler tube. An isocratic program using 4:3:3 1% acetic acid:CH_3OH:CH_3CN was used with an excitation λ = 260 nm and emission λ = 315 nm on the fluorescent detector to detect pirlimycin residues. 50 µl of each sample was injected into the system every 35 minutes.

Detection of Cephapirin

The dried sample containing 3 ml milk extract is eluted in 200 µl of 0.01 M KH_2PO_4. The solution is transferred to an autosampler tube and centrifuged for 4 minutes. The supernatant is decanted to another tube. A gradient program using 0.01 M KH_2PO_4 and 1:1 0.01 M KH_2PO_4:CH_3CH buffers is used with U.V. detection at λ = 290 nm. 50 µl of each sample was injected into the HPLC system every 65 minutes.

Quantification of Antimicrobials

Standard linear regression curves were developed for each drug based on the areas under the curve/peak at the specific retention time for the known blank and spiked milk samples. The areas at the same retention times measured on blinded study samples were then placed in their respective regression model to produce the estimated concentration of each drug in the sample.

RESULTS AND DISCUSSION

The ideal method would have involved one extraction protocol and one HPLC system. However, it became apparent that that was not possible and the most robust methods were sought. Several studies have used residue screening assays to test fractions collected as part of a liquid chromatography cleanup method (Harik-Khan and Moats, 1995; Moats and Romanowski, 1998; Anderson et al., 1998). Since we were determining the reliability of three of the assays it was deemed inappropriate to use the same assays in the development of the gold standards. Fraction collection and cleanup were to be avoided if possible due to time constraints.

Moats indicated difficulties in effectively clearing a LC column of ampicillin (Moats, 1994) and our preliminary investigations bore the same finding. Reports of derivatization and fluorometric detection methods used to identify <50 ppb of ampicillin in plasma, serum, kidneys and liver provided a new route to explore in detecting ampicillin in milk (Miyazaki et al., 1983; Hong et al., 1995). Slight modifications in the extraction methods used with serum and plasma samples were made to accommodate the use of milk samples. By increasing the initial TCA concentration to 26.7% we more effectively deproteinated the milk products. Another modification was the need to add greater quantities of Na_2HPO_4 at a higher molarity (2 M instead of 0.67 M) to bring the pH up to 6.2. The extraction method produced an unimpressive 11.5 – 12.3% recovery rate of ampicillin from milk. Fluorescence detection provided a peak that was clearly evident at 5.15 - 5.25 minutes in the presence of ampicillin below the lowest reported assay detection level of 7.7 ppb (Figure 4-1). By increasing the amount injected into the HPLC system from 20 µl to 40 or 50 µl one could improve the sensitivity two-fold.

Additional testing that included amoxicillin, cephapirin and pirlimycin produced no unique detectable fluorescent products. This was advantageous in verifying the specificity of this method for ampicillin identification. Conversely, it was a detriment in the pursuit of a single extraction method for the three drugs being studied.

The extraction method commonly used with LC detection of β-lactams (Moats and Romanowski, 1998) was found to be adequate for the extraction of the macrolide, pirlimycin. Recovery rates for pirlimycin were 85 – 102% down to 62 ppb. Analytical sensitivity of the HPLC method was approximately 30 ppb which was well below the 400 ppb FDA tolerance level and the 50-200 ppb detection level of the Delvo-SP assay. Identification and quantification of pirlimycin were greatly improved with the use of Fmoc derivatization (Heller, 1997). Fluorescent products from only pirlimycin were detected using an ultra-violet detector with λ = 264 nm. Detection at smaller concentrations was further enhanced by the use of a fluorescence detector (Figure 4-2). A previous study suggested that the excitation wavelength would be 275 nm and the emission wavelength would be 315 nm when detecting Fmoc bound products (Chou et al., 1989). The scanning feature on the fluorescence detector allowed us to further hone the excitation wavelength to 260 nm and maintain the 315 nm emission wavelength. Again we were able to detect only one of the drugs of interest using this derivatization method.

Cephapirin proved to be the most difficult of the three drugs to identify a clean peak using any of the proposed methods. Altering the wavelengths for fluorescence detection using either derivatization method produced negative results. Coelution of milk by-products at the retention times for desacetylcephapirin and cephapirin (20.9 and 25.32

minutes respectively) reduced the sensitivity of cephapirin detection. Without the potential benefit of fraction cleanup, the detection limit for cephapirin was 42 ppb. Unfortunately the sensitivity was inadequate for detecting cephapirin below its 20 ppb tolerance level and 5 ppb assay detection level.

In conclusion, a single method for the simultaneous identification and quantification of ampicillin, cephapirin and pirlimycin could not be found or developed. For the evaluation of 200 blinded milk samples, the use of derivatization methods and fluorescence detection provided greater detection sensitivity and specificity for ampicillin and pirlimycin than methods currently used to evaluate isolated samples.

FOOTNOTES

[a]Penzyme Milk Test, Cultor Food Science Group, New York, NY.

[b]SNAP β-lactam, IDEXX Laboratories Inc, Westbrook, ME.

[c]Delvo-SP, Gist Brocades Food Ingredients Inc, Menomonee Falls, WI.

[d] Sigma Chemical Co., St. Louis, MO.

[e] Pharmacia & Upjohn, Kalamazoo, MI.

[f] J.T. Baker, Phillipsburg, NJ.

[g] Aldrich Chemical Co., Milwaukee, WI.

[h] Pierce, Rockford, IL.

REFERENCES

Anderson KL, Moats WA, Rushing JE, O'Carroll JM. Detection of milk antibiotic residues by use of screening tests and liquid chromatography after intramammary administration of amoxicillin or penicillin G in cows with clinical mastitis. *Am J Vet Res* 1998;59:1096-1100.

Brady MS, White N, Katz SE. Resistance development potential of antibiotic/antimicrobial residue levels designated as "safe levels". *J Food Prot* 1993;56:229-233.

Carlsson A, Bjorck L, Persson K. Lactoferrin and lysozyme in milk during acute mastitis and their inhibitory effect in Delvotest P. *J Dairy Sci* 1989;72:3166-3175.

Chou TY, Gao CX, Grinberg N, Krull IS. Chiral polymeric reagents for off-line and on-line derivatizations of enantiomers in high-performance liquid chromatography with ultraviolet and fluorescence detection: an enantiomer recognition approach. *Anal Chem* 1989;61:1548-1558.

Dasenbrock CO, LaCourse WR. Assay for cephapirin and ampicillin in raw milk by high-performance liquid chromatography - integrated pulsed amperometric detection. *Anal Chem* 1998;70:2415-2420.

Gibbons-Burgener SN, Kaneene JB, Lloyd JW, Erskine RJ. Influence of the Milk and Dairy Beef Quality Assurance Program on dairy farm drug management practices. *J Am Vet Med Assn* 2000;216:1960-1964.

Harik-Khan R, Moats WA. Identification and measurement of beta-lactam antibiotic residues in milk: integration of screening kits with liquid chromatography. *J AOAC Int* 1995;78:978-986.

Heller, DN. Determination and confirmation of pirlimycin residue in bovine milk and liver by liquid chromatography/thermospray mass spectrometry: interlaboratory study. *J AOAC Int* 1996;79:1054-1061.

Heller, DN. Determination of Pirlimycin residue in milk by liquid chromatographic analysis of the 9-fluorenylmethyl chloroformate derivative. *J AOAC Int* 1997;80:975-981.

Hong CC, Lin CL, Tsai CE, Kondo F. Simultaneous identification and determination of residual penicillins by use of high-performance liquid chromatography with spectrophotometric or fluorometric detectors. *Am J Vet Res* 1995;56:297-303.

Hornish RE, Arnold TS, Bacynskyj L, et al.. Pirlimycin in the dairy cow. *In:* Hutson DH, ed. *Xenobiotics in Food producing animals - metabolism and residues, ACS symposium series 503.* American Chemical Society, Washington, DC, 1992;132-147.

Hornish RE, Cazars AR, Chester ST, Roof RD. Identification and determination of pirlimycin residue in bovine milk and liver by high-performance liquid chromatography-thermospray mass spectrometry. *J Chromatogr B* 1995;674:219-235.

Mitchell JM, Griffiths MW, McEwen, SA, et al.. Antimicrobial drug residues in milk and meat: causes, concerns, prevalence, regulations, tests, and test performance. *J Food Prot* 1998;61:742-756.

Miyazaki K, Ohtani K, Sunada K, Arita T. Determination of ampicillin, amoxicillin, cephalexin, and cephradine in plasma by high-performance liquid chromatography using fluorometric detection. *J Chromatography* 1983;276:478-482.

Moats, WA. Determination of cephapirin and desacetylcephapirin in milk using automated liquid chromatographic cleanup and ion-pairing liquid chromatography. *J AOAC Int* 1993;76:535-539.

Moats, WA. Determination of ampicillin and amoxicillin in milk with an automated liquid chromatographic cleanup. *J AOAC Int* 1994;77:41-45.

Moats, WA, Romanowski, RD. Multiresidue determination of β-lactam antibiotics in milk and tissues with the aid of high-performance liquid chromatographic fractionation for clean up. *J Chromatogr A* 1998;812:237-247.

Sischo WM, Burns CM. Field trial of four cowside antibiotic-residue screening test. *J Am Vet Med Assn* 1993;202:1249-1254.

Tyczkowska KL, Voyksner RD, Straub RF, Aronson AL. Simultaneous multiresidue analysis of b-lactam antibiotics in bovine milk by liquid chromatography with ultraviolet detection and confirmation by electrospray mass spectrometry. *J AOAC Int* 1994;77:1122-1131.

Van Eenennaam AL, Cullor JS, Perani L, et al.. Evaluation of milk antibiotic residue screening tests in cattle with naturally occurring mastitis. *J Dairy Sci* 1993;76:3041-3053.

Waltner-Toews D, McEwen SA. Residues of antibacterial and antiparasitic drugs in foods of animal origin: a risk assessment. *Prev Vet Med* 1994;20:219-234.

Figure 4-1 – Chromatogram of the fluorescent detection (em λ = 345, em λ = 420) of 20 ppb ampicillin extracted from raw milk.

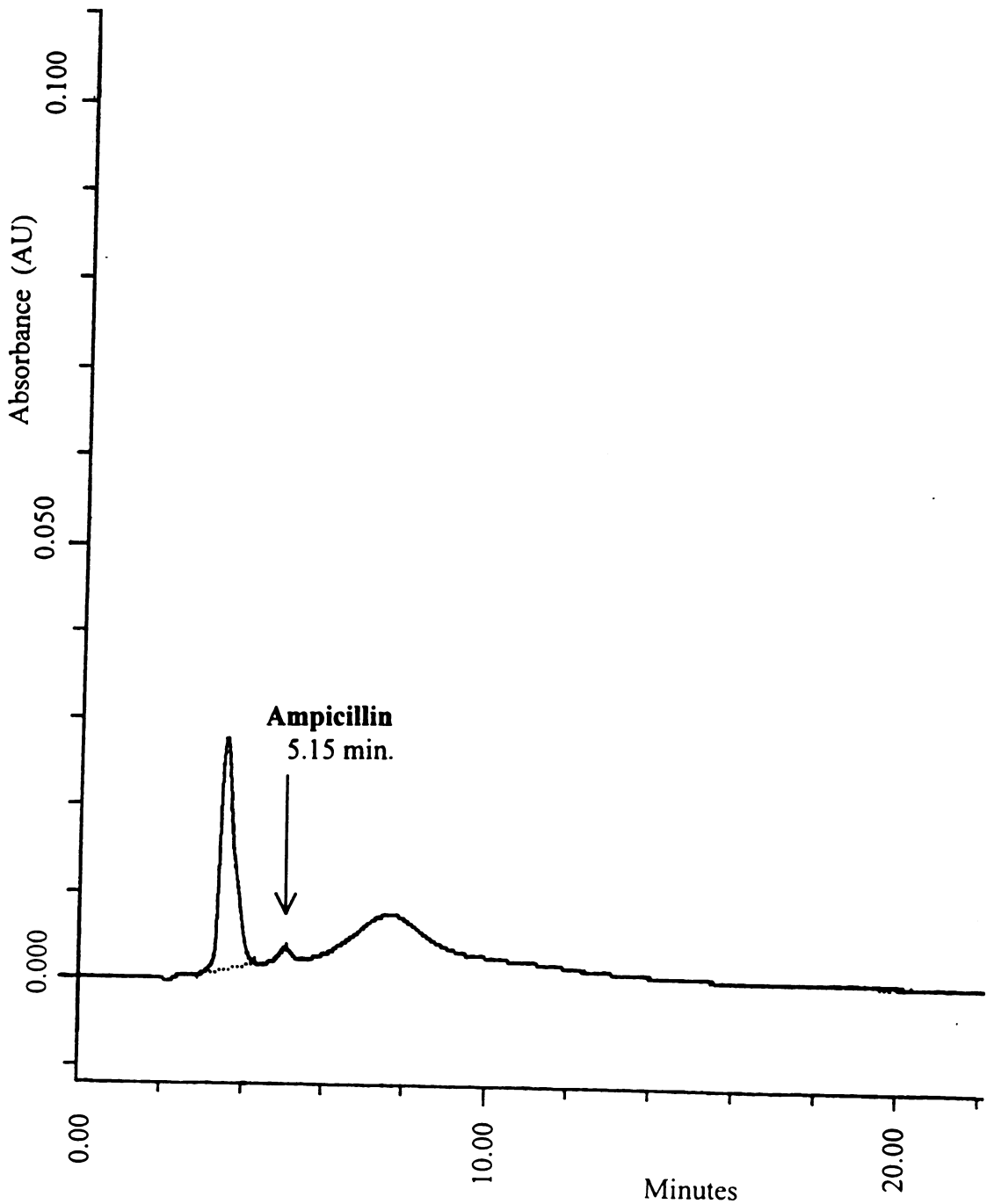
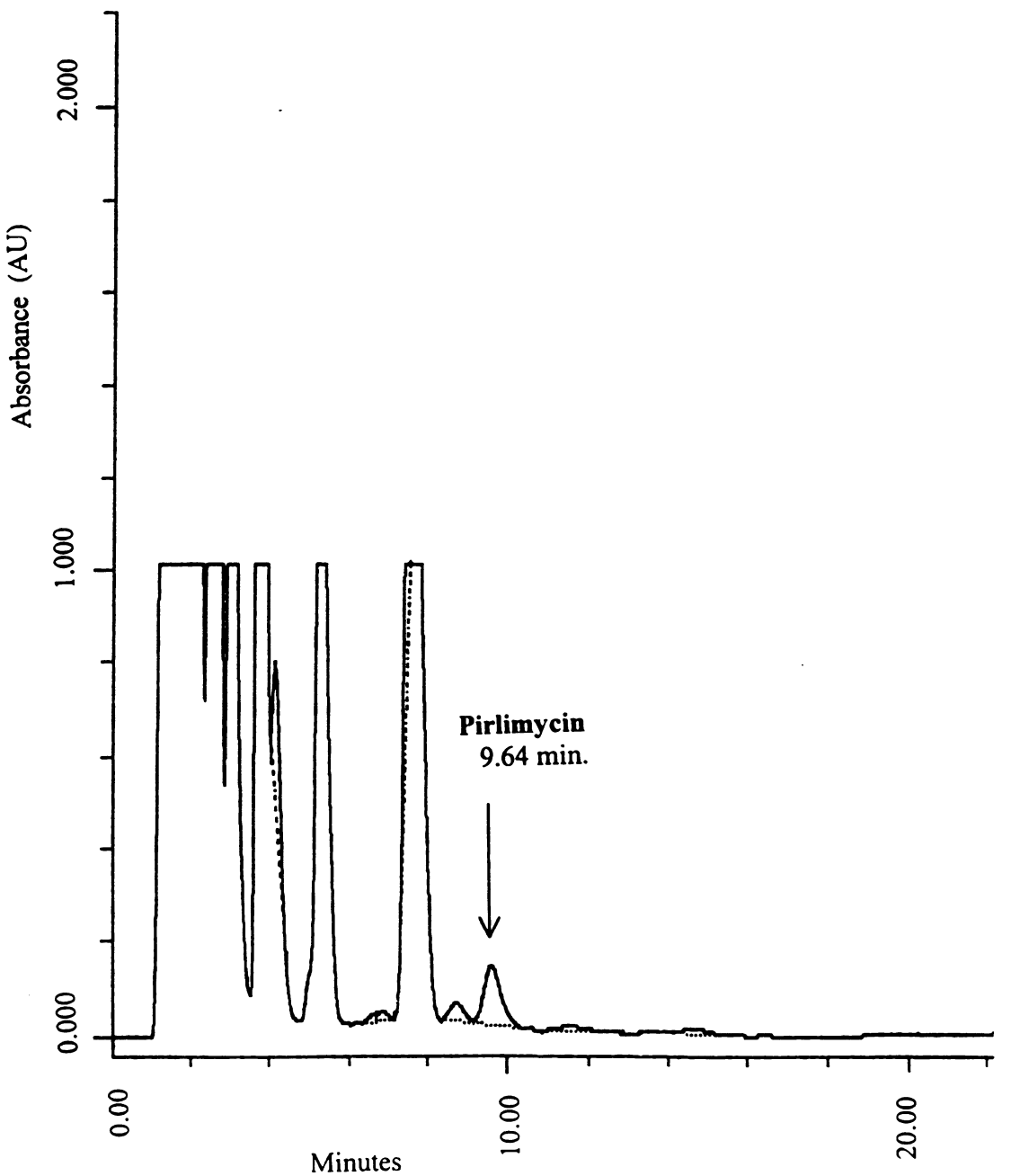


Figure 4-2 – Chromatogram of the fluorescent detection (ex λ = 260, em λ = 315) of 200 ppb pirlimycin extracted from raw milk.



CHAPTER 5

AN EPIDEMIOLOGICAL EVALUATION OF THE RELIABILITY OF BULK-TANK RESIDUE DETECTION ASSAYS USED TO TEST INDIVIDUAL COW MILK

ABSTRACT

Objectives— to determine the likelihood of false assay results when using the SNAP β-lactam, Penzyme Milk Test and Delvo-SP assays to test for antimicrobial residues in individual cows' milk.

Sample Population—111 cows diagnosed with mild clinical mastitis on one of eight participating dairy farms.

Procedure—Cows were randomly assigned to either the antimicrobial or control treatment group. Pretreatment and post-treatment milk samples were collected. Post-treatment samples were randomly tested twice using each of the 3 on-farm residue detection assays and once using high performance liquid chromatography methods. The reliability of each of the assays was determined using sensitivity, specificity, positive and negative predictive values and the kappa statistic.

Results—The Delvo-SP and SNAP β-lactam assays displayed >90% sensitivity, while the sensitivity of the Penzyme Milk Test was only 60%. Ranging from 39.29 to 73.68%, the positive predictive values were poor for all three assays. The kappa statistics for the SNAP β-lactam, Penzyme Milk Test and Delvo-SP were 0.846, 0.545 and 0.813 respectively.

Conclusion and Clinical Relevance— The kappa statistics provided strong evidence that all three assays produced good to excellent repeatability. The poor positive predictive

value of the SNAP β-lactam assay was most likely due to an undocumented cross-reactivity with pirlimycin residues. With such low positive predictive values and incidence of violative antimicrobial levels, the usefulness of the three residue detection assays in deciding the fate of milk from cows receiving treatment for mastitis is highly questionable.

INTRODUCTION

Dairy farmers, veterinarians, dairy manufacturers and researchers believe it is highly desirable to have at least one quick and reliable test for the detection of antimicrobials in milk. Prior to the approval of new drugs for use in lactating cattle, pharmaceutical companies must demonstrate that an assay or method exists that detects their drug in marketable milk. The ability to test individual cow milk for residues is essential in the determination of labeled withholding periods. However, earlier studies (McEwen, et al., 1991; Gibbons-Burgener, et al., 1999) have found that farmers depend more on residue testing than labels when deciding to withhold milk from a treated cow. The practice of testing individual cow milk off or on farms is widespread and promoted. Since there are no rapid assays labeled for use in testing individual cows' milk, it has become acceptable to use approved commingled milk testing assays.

Numerous reports have demonstrated that current on-farm testing of milk from individual cows for drug residues often yields false-positive results and that caution is warranted in their use (Andrew, et al., 1997; Cullor, 1992; Cullor, et al., 1994; Sischo and Burns, 1993; Van Eenennaam, et al., 1993). Although the specificity and sensitivity of the assays have been established for commingled milk in controlled laboratory conditions, concern over the accuracy under field conditions exists, because to date, few studies have been conducted that validate these assays in a field setting. In fact, no screening assay has been recognized by the FDA for use on milk from individual cows. The objective of this study, therefore, was to determine the likelihood of false assay results when using the SNAP β-lactam, Penzyme Milk Test and Delvo-SP assays to test for antimicrobial residues in individual cows' milk.

MATERIALS AND METHODS

Study Design - A longitudinal experimental study of cows developing mild clinical mastitis was conducted to evaluate the reliability of the SNAP β-lactam, Penzyme Milk Test and Delvo-SP assays when used to test individual cow milk for antimicrobial residues.

Case Definition - A mild clinical mastitis case was defined as 1) visibly abnormal milk stripped from the quarter, 2) quarter may be enlarged or reddened and 3) conventional therapy (intramammary antimicrobial infusions or other non-antimicrobial therapy) was believed to be an appropriate treatment. Cows were specifically excluded from the study if they 1) received antimicrobial treatment for any reason within the last 30 days, 2) were previously included in this study (a repeat case), 3) had a concurrent illness requiring antimicrobial treatment or 4) had a severe case of mastitis that required systemic (IV, IM or SQ) antimicrobial therapy.

Sample Size - Sample sizes for estimating a single proportion (i.e. False-positive results/all results) were much less than those required for evaluating potential associations with risk factors. By estimating that 10% of the population would have a positive assay result and allowing a 5% margin of error ($\alpha = 0.05$), the required sample size was 138 tests (Appendix 2).

Treatment Groups - After diagnosing a case of mild clinical mastitis and collecting the initial milk sample, cows were randomly assigned to one of two treatment groups (Figure 5-1). Cows assigned to the Antimicrobial treatment group were treated as directed on the label with a FDA approved IMM antimicrobial therapy selected by either the producer or

veterinarian. Label dose and number of treatments were followed unless the cow was removed from the study. Cows assigned to the control treatment group received appropriate treatment that did not include any form of antimicrobial therapy. Other treatments included anti-inflammatory drugs, oxytocin, saline infusions or no drug therapy.

Sample Collection - Two samples (pre and post-treatment) from each mastitis case were collected. Prior to collection of the samples, foremilk from each quarter was discarded. An aseptic collection of at least 5 ml of milk from the affected quarter was obtained for bacterial culture using standard methods (Harmon, et al., 1990). An 80-ml composite milk sample, comprised of approximately 20 ml of milk from each quarter was collected. The composite milk was then divided as follows: 1) 30 ml in a plastic vial with preservative tablet for infrared somatic cell count, 2) 10 ml in a plastic vial for IgG1 analysis, and 3) four 5-8 ml aliquots in plastic vials for antimicrobial residue analyses. The aliquots were frozen at -70°C until needed for residue analyses.

The initial sample was collected following the diagnosis of mild clinical mastitis and before initiating treatment (Figure 5-2). The second sample was collected at the milking following the completion of the labeled milk-withholding period for cows in the antimicrobial treatment group. To simulate similar potential recovery times from diagnosis to second sample, the timing for the collection of the second sample from a cow in the control treatment group was determined by using the same withholding period as the last (most recently) antimicrobial treated cow. Treatment with other drugs may have required a variety of actual withholding periods prior to shipment of milk from the farm.

The label's recommended withholding period and instructions were to be observed prior to including milk in the bulk tank.

Testing for antimicrobials - Milk samples were thawed in an ice water bath and vortexed briefly to thoroughly mix. Pre-treatment samples were randomized and tested once with each assay. Each post-treatment sample was randomized twice and tested twice. Except for the use of individual cow and thawed samples, the three assays being evaluated (Delvo-SP, SNAP β-lactam and Penzyme Milk Test) were run according to each assay's directions. The same person throughout the study performed visual interpretation of the assay results.

High performance liquid chromatography (HPLC) was used to identify and quantify the presence of potential antimicrobial residues in each of the samples. The specific extraction and detection methods have been previously described (Gibbons-Burgener, et al., 2000). By comparing the quantity of each antimicrobial found on HPLC with its FDA tolerance level and assay detection limits (Table 5-1) we were able to determine if the residue should have been detected by each assay and whether a given residue was considered violative (above the FDA tolerance level).

Statistical analysis - The reliability of each of the residue detection assays was expressed in terms of sensitivity, specificity, positive predictive value and negative predictive value using equations 1, 2, 3 and 4 respectively. The reliability statistics were calculated first using the specific assay's detection limits and second using the FDA-established tolerance levels for each of the antimicrobials.

Equation 1

$$\text{Sensitivity} = \frac{\text{No. tests with positive results on HPLC \& assay}}{\text{No. all tests with positive results on HPLC (+/- assay)}} \times 100$$

Equation 2

$$\text{Specificity} = \frac{\text{No. tests with negative results on HPLC \& assay}}{\text{No. all tests with negative results on HPLC (+/- assay)}} \times 100$$

Equation 3

$$\text{Positive Predictive Value} = \frac{\text{No. tests with positive results on HPLC \& assay}}{\text{No. all tests with positive results on assay (+/- HPLC)}} \times 100$$

Equation 4

$$\text{Negative Predictive Value} = \frac{\text{No. tests with negative results on HPLC \& assay}}{\text{No. all tests with negative results on assay (+/- HPLC)}} \times 100$$

Calculating the kappa statistic for each of the three commercial assays compared the concordance of the first and second tests run on the post-treatment samples (Equation 5).

Equation 5 (Rosner, 1995)

$$\text{Kappa} = (P_o - P_e) / (1 - P_e)$$

P_o = observed probability of concordance between 2 testings

P_e = expected probability of concordance between 2 testings

RESULTS

Eight farms participated in the study and collected data. Of the 111 cows that developed clinical mastitis and were enrolled as cases, 92 remained in the study through their post-treatment sample collection (83% case retention rate). About half (45/92) of the cows were in the antimicrobial treatment group and received either pirlimycin (26/45,

57.8%), hetacillin (9/45, 20%) or cephapirin (10/45, 22.2%) IMM therapy. We were unable to interpret the assay results from 3 of the cows. Occasionally a milk sample would produce no visual result (dud) on a given assay. Consequently, of the potential 178 post-treatment tests run on each assay, the evaluation of the SNAP β-lactam, Penzyme Milk Test and Delvo-SP assays included 168, 175 and 177 tests respectively.

The frequency distributions of the assay results (Figure 5-3 and 5-4) indicate relatively low numbers of positive results. Sensitivity, specificity and predictive values for the 3 assays are in Tables 5-2 and 5-3. Twenty-three cows had levels of an antimicrobial that were detectable by at least one of the commercial assays. Only 6 of those 23 animals had violative levels. The statistical sensitivities of the SNAP β-lactam, Penzyme Milk Test and Delvo-SP assays to detect the violative samples were 83.33, 62.5 and 91.67% respectively (Table 5-3).

The kappa statistics for the pairs of tests run on the SNAP β-lactam (κ = 0.846) and Delvo-SP (κ = 0.813) were similar. The Penzyme Milk Test had a lower statistic (κ = 0.545).

DISCUSSION

Unlike some earlier studies (Harik-Khan and Moats, 1995; Halbert, et al., 1996; Andrew, et al., 1997) this study was an experimental field trial designed to simulate the collection and testing of milk as commonly performed on farms. This meant samples were collected from diseased cows requiring their milk to be withheld from the bulk-tank, the samples weren't spiked with known quantities of antimicrobials and withholding periods were observed to best simulate the return of milk to the bulk tank. Another

consequence of the study design was that the enrollment of farms willing and capable of following the study protocol included farms with relatively low incidence rates of mild clinical mastitis. These study requirements limited the number of accessible cases. However, the obtained sample sizes provided adequate precision in the calculation of the reliability statistics.

The testing done on the pre-treatment samples was performed only to ensure the absence of pre-existing residues or abnormalities that could have interfered with the interpretation of the post-treatment samples. Because all the study cows were initially diagnosed with clinical mastitis and abnormal looking milk is a manufacturer contraindication for the use of the Penzyme and Delvo assays, it was inappropriate to use the pre-treatment test results to evaluate the reliability of the tests. In addition, testing for residues prior to treatment on a farm is a rare request. By blindly testing the post-treatment samples twice and comparing the results with the kappa statistic we found strong evidence that all three assays produced good to excellent outcome repeatability. This is consistent with the report of a discussion forum which also recommended the use of two assays in series instead of simply repeating the same assay on presumptive positive samples (Gardner, et al., 1996). However, in this study the tests couldn't be evaluated in series. Different assays may test for different antimicrobials at different detection limits, which can make interpreting series tests more difficult. This was particularly evident in this study where only the Delvo-SP assay was reported to be able to detect pirlimycin. As recommended by the discussion forum, it would be most beneficial to have a sensitive initial assay and a more specific second assay. The three assays we evaluated had similar

detection limits for ampicillin and cephapirin, and provided little improvement of specificity to distinguish false positive results.

The SNAP β-lactam and Delvo-SP had comparable statistical sensitivities. The Penzyme Milk Test had a few more false-negative results, which (combined with a low prevalence of detectable residues) had a profound effect on lowering the sensitivity reported by this study. Specificities for all three assays were comparable. The predictive values for the assays are better indicators of what the dairy farmer encounters in deciding to discard or sell the milk from a tested cow. The positive predictive value is the likelihood that a positive assay result has truly detected an antimicrobial residue within its detection limits or above the FDA-established tolerance level. Each of the assays had lower positive predictive values than one might have expected with fairly good specificities. This phenomenon is particularly evident when the disease (detectable residue) prevalence is low. The SNAP β-lactam assay had a poor positive predictive value, which is most likely due to previously undocumented cross-reactivity with pirlimycin residues. Six of the 17 false-positive results were recorded for samples with HPLC-detected pirlimycin residues. If the assay had been approved for pirlimycin detection the positive predictive value would have improved to almost 61%. With only a rare false-negative result, each of the assays exhibited an excellent negative predictive value. Assay users should feel very confident in a negative result if they run an appropriate assay developed to detect the administered antimicrobial.

This study found that 6 of the 45 cows treated according to the label with an antimicrobial approved for use in lactating cattle had violative levels in their milk after observing the labeled milk-withholding period. It is difficult to discuss violative levels in terms of individual cow milk, because the standards are set for commingled or bulk-tank milk where an individual cow's milk is usually diluted. As at the creamery, the farmer can not quantify the amount of antimicrobial in the milk following a positive assay result. The conservative approach to a positive assay test on an individual cow's milk is to discard the milk and retest at a later milking. If this practice had been followed with the study cows, milk from 17 of the cows may have been unnecessarily discarded. With such low positive predictive values and prevalence of violative antimicrobial levels, the usefulness of the three residue detection assays in deciding the fate of milk from cows receiving treatment for mastitis is highly questionable if farms want to minimize the quantity of unnecessarily discarded milk.

REFERENCES

Andrew SM, Frobish RA, Paape MJ, Maturin LJ. Evaluation of selected antibiotic residue screening tests for milk from individual cows and examination of factors that affect the probability of false-positive outcomes. *J Dairy Sci* 1997;80:3050-3057.

Cullor JS. Tests for identifying antibiotic residues in milk: how well do they work? *Vet Med* 1992;87:1235-1241.

Cullor JS, van Eenennaam A, Gardner I, et al.. Performance of various tests used to screen antibiotic residues in milk samples from individual animals. *J AOAC* 1994;77:862-870.

Gardener IA, Cullor JS, Galey FD, et al.. Alternatives for validation of diagnostic assays used to detect antibiotic residues in milk. *J Am Vet Med Assn* 1996;209:46-52.

Gibbons-Burgener SN, Kaneene JB, Lloyd JW, Erskine RJ. Evaluation of certification in the Milk and Dairy Beef Quality Assurance Program and associated factors on the risk of having violative antibiotic residues in milk from dairy farms in Michigan. *Am J Vet Res* 1999;60:1312-1316.

Gibbons-Burgener SN, Leykam JF, Kaneene JB. Identification and quantification of ampicillin, cephapirin and pirlimycin in cows' milk using high performance liquid chromatography and fluorescence detection. *J Vet Diagn Invest*, 2000(manuscript submitted).

Halbert LW, Erskine RJ, Bartlett PC, Johnson GL. Incidence of false-positive results for assays used to detect antibiotics in milk. *J Food Prot* 1996;59:886-888.

Harmon RJ, Eberhart RJ, Jasper DE, et al.. Microbiological procedures for the diagnosis of bovine udder infections, ed 3. Arlington, VA, National Mastitis Council, 1990.

Harik-Khan R, Moats WA. Identification and measurement of β-lactam antibiotic residues in milk: integration of screening kits with liquid chromatography. *J AOAC Intl* 1995;78:978-986.

McEwen SA, Meek AH, Black WD. A dairy farm survey of antibiotic treatment practices, residue control methods and associations with inhibitors in milk. *J Food Prot* 1991;54:454-459.

Rosner BA. Chapter 10 Hypothesis testing: Categorical data. *In Fundamentals of Biostatistics,* 4th ed. London, England, International Thomson Publishing, 1995;423-426.

Sischo WM and Burns CM. Field trial of four cowside antibiotic-residue screening tests. *J Am Vet Med Assn* 1993;202:1249-1254.

Van Eenennaam AL, Cullor JS, Perani L, et al.. Evaluation of milk antibiotic residue screening tests in cattle with naturally occurring mastitis. *J Dairy Sci* 1993;76:3041-3053.

Figure 5-1 Assignment and treatment options of cases in the antimicrobial and control treatment groups.

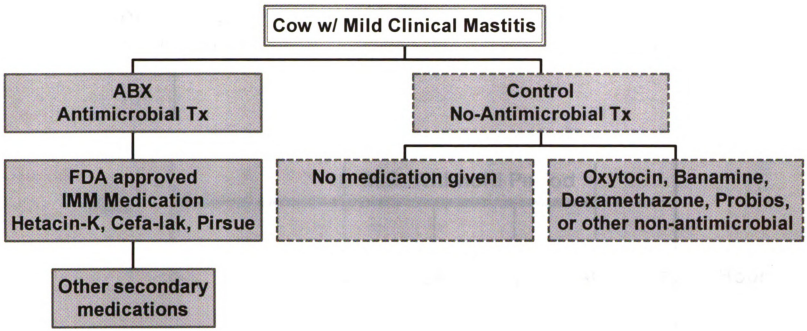


Figure 5-2 – An example of how sampling frequency was determined for the collection of the pre and post-treatment samples using the most prior antimicrobial (ABX) treated cow's sampling interval for the control group cow.

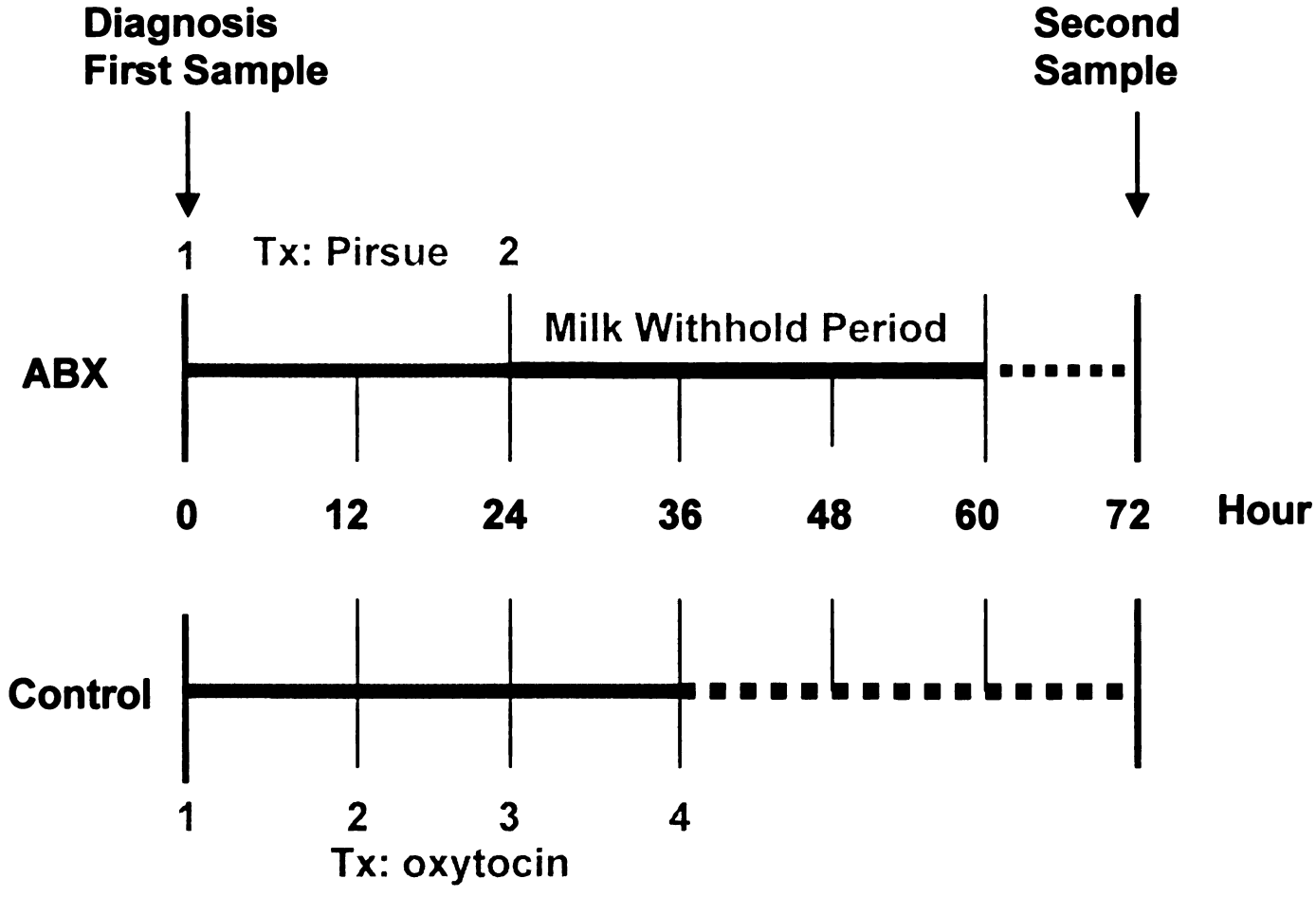


Figure 5-3 – 2 x 2 charts for the distribution of assay results using the reported antimicrobial detection limits for each assay.

Delvo-SP Assay

	HPLC +	HPLC -	
+	28	10	38
-	3	136	139
	31	146	177

Penzyme Milk Test Assay

	HPLC +	HPLC -	
+	6	4	10
-	4	161	165
	10	165	175

SNAP β-lactam Assay

	HPLC +	HPLC -	
+	11	17	28
-	1	139	140
	12	156	168

Figure 5-4 – 2 x 2 charts for the distribution of assay results using FDA-established antimicrobial tolerance levels.

HPLC

Delvo-SP Assay	+	-	
+	11	25	36
-	1	138	139
	12	163	175

HPLC

Penzyme Milk Test Assay	+	-	
+	5	4	9
-	3	161	164
	8	165	173

HPLC

SNAP β-lactam Assay	+	-	
+	5	19	24
-	1	139	140
	6	158	164

Table 5-1. FDA tolerance levels for specific antimicrobials in marketable milk and the visual minimal detection limits of 3 on-farm residue detection assays.

Antimicrobial	**FDA tolerance level** (ppb)	**SNAP b-lactam d.l.[a]** (ppb)	**Penzyme Milk Test d.l.[a]** (ppb)	**Delvo-SP d.l.[a]** (ppb)
Ampicillin[b]	10	4-6	4-6	4
Cephapirin	20	2	4-8	5
Pirlimycin	400	NA[c]	NA[c]	50-200

[a]d.l.=minimal detection limits / analytical sensitivity, [b]Ampicillin is the immediate product of hetacillin metabolism, [c]NA = no information available regarding ability to detect residue or the potential for cross-reactivity.

Table 5-2. Sensitivity, specificity and predictive values for the detection of antimicrobials at or above the reported assay detection limit by three on-farm residue detection assays evaluated in a field trial.

Assay	**Sensitivity** (95% CI)[a] %	**Specificity** (95% CI)[a] %	**Positive P.V.**[b] (95% CI)[a] %	**Negative P.V.**[b] (95% CI)[a] %	**n**[c]
SNAP β-lactam	91.67 (61.52 – 99.79)	89.10 (83.12 – 93.52)	39.29 (21.50 – 59.42)	99.29 (96.08 – 99.98)	168
Penzyme Milk Test	60.0 (26.24 – 87.84)	97.58 (93.91 – 99.34)	60.0 (26.24 – 87.84)	97.58 (93.91 – 99.34)	175
Delvo-SP	90.32 (74.25 – 97.96)	93.15 (87.76 – 96.67)	73.68 (56.90 – 86.60)	97.84 (93.82 – 99.55)	177

[a] lower and upper limits of the 95% confidence interval , [b] P.V. = predictive value, [c] n=number of tests

Table 5-3. Sensitivity, specificity and predictive values for the detection of antimicrobials above the FDA-established tolerance levels by three on-farm residue detection assays evaluated in a field trial.

Assay	**Sensitivity** (95% CI)[a] %	**Specificity** (95% CI)[a] %	**Positive P.V.**[b] (95% CI)[a] %	**Negative P.V.**[b] (95% CI)[a] %	**n**[c]
SNAP β-lactam	83.33 (35.88 – 99.58)	87.97 (81.86 – 92.60)	20.83 (7.13 – 42.15)	99.29 (96.08 – 99.98)	164
Penzyme Milk Test	62.5 (24.49 – 91.48)	97.58 (93.91 – 99.34)	55.56 (21.20 – 86.30)	98.17 (94.74 – 99.62)	173
Delvo-SP	91.67 (61.52 – 99.79)	84.66 (78.20 – 89.82)	30.56 (16.35 – 48.11)	99.28 (96.06 – 99.98)	175

[a] lower and upper limits of the 95% confidence interval , [b] P.V. = predictive value, [c] n=number of tests

CHAPTER 6

RISK FACTORS ASSOCIATED WITH FALSE-POSITIVE RESULTS USING THREE ON-FARM BULK TANK RESIDUE DETECTION ASSAYS ON INDIVIDUAL COW MILK

ABSTRACT

Objectives— To determine whether SCC, IgG_1, microbiologic isolates or specific antimicrobial treatments were associated with false-positive results when using the SNAP β-lactam, Penzyme Milk Test and Delvo-SP assays to test milk from cows treated for naturally occurring mastitis.

Sample Population—111 cows diagnosed with mild clinical mastitis on one of eight participating dairy farms.

Procedure—Cows were randomly assigned to either the antimicrobial or control treatment group. Post-treatment samples were randomly tested twice using each of the 3 on-farm residue detection assays and once using HPLC methods. Microbiologic culture was performed using milk from the affected quarter. Composite milk samples were also tested for somatic cell count and bovine IgG_1 concentration. Logistic regression was used to determine associations between risk factors and false-positive assay results.

Results—Inflammatory-related milk proteins, represented by bovine IgG_1 concentrations, were positively associated with false-positive results from the SNAP β-lactam, Penzyme Milk Test and Delvo-SP assays. The SNAP β-lactam assay was less likely to have a false-positive result when the initial bacteriologic culture result was negative. Low numbers of

false-positive results precluded multivariable statistical analysis and warrants caution in over-interpreting the apparent associations.

Conclusion and Clinical Relevance— This study demonstrated that components in milk other than antimicrobials were associated with positive assay results, which sheds more doubt on the use of on-farm antimicrobial residue screening assays to test milk from individual cows.

INTRODUCTION

Having reliable testing methods for antimicrobials in milk is essential to the dairy industry. Since the changes to the grade A Pasteurized Milk Ordinance in 1991 mandated the screening of every tanker-load of raw milk for at least β-lactam antimicrobial residues, there has been an increased emphasis on the preharvest prevention of residues at the farm level (Adams, 1994). Producers are encouraged to either submit samples or test milk on-farm from individual treated cows prior to including the cow's milk in the bulk-tank. Unfortunately, the only tests available are approved for screening commingled milk, and have been reported to produce false-positive results when used to test individual cow milk for antimicrobial residues (Cullor et al, 1994; Sischo and Burns, 1993; Andrew et al, 1997; Gibbons-Burgener et al, 2000).

Because violative residues in milk are a relatively rare occurrence (<0.1% of bulk tanks), the likelihood of false-negative results is negligible. Consequently, research evaluating the accuracy of on-farm residue detection assays has focused on the possible sources of false-positive results. Possible causes of false-positive results include elevated somatic cell count (Sischo and Burns, 1993; Van Eenennaam et al, 1993); increased lactoferrin and lysozyme concentrations (Carlsson and Bjorck, 1989); lower milk production, increased parity and increased coliform counts (Andrew et al, 1997); and other inhibitory substances in the milk (Tyler et al, 1992; Cullor et al, 1994). The objective of this study was to determine whether SCC, IgG_1, microbiologic isolates or specific antimicrobial treatments were associated with false-positive results when using the SNAP β-lactam, Penzyme Milk Test and Delvo-SP assays to test milk from cows treated for naturally occurring mastitis.

MATERIALS AND METHODS

Study Design - A longitudinal experimental study of cows developing mild clinical mastitis was conducted to evaluate the reliability of the SNAP β-lactam, Penzyme Milk Test and Delvo-SP assays when used to test individual cow milk for antimicrobial residues.

Case Definition - A mild clinical mastitis case was defined as 1) visibly abnormal milk stripped from the quarter, 2) quarter may be enlarged or reddened and 3) conventional therapy (intramammary antimicrobial infusions or other non-antimicrobial therapy) was believed to be an appropriate treatment. Cows were specifically excluded from the study if they 1) received antimicrobial treatment for any reason within the last 30 days, 2) were previously included in this study (a repeat case), 3) had a concurrent illness requiring antimicrobial treatment or 4) had a severe case of mastitis that required systemic (IV, IM or SQ) antimicrobial therapy.

Sample Size - The initial sample size calculations (Appendix 2) indicated that 199 cows were needed in each treatment group (400 total) to evaluate the most conservative estimate for risk factor exposure (antimicrobial treatment group).

Treatment Groups - After diagnosing a case of mild clinical mastitis and collecting the initial milk sample, cows were randomly assigned to one of two treatment groups. Cows assigned to the Antimicrobial treatment group were treated as directed on the label with a FDA approved IMM antimicrobial therapy selected by either the producer or veterinarian. Label dose and number of treatments were followed unless the cow was removed from the study. Cows assigned to the control treatment group received appropriate treatment

that did not include any form of antimicrobial therapy. Other treatments included anti-inflammatory drugs, oxytocin, saline infusions or no drug therapy.

Sample Collection - As previously described (Gibbons-Burgener et al, 2000 b), pretreatment and post-treatment samples from each mastitis case were collected. Prior to collection of the samples, foremilk from each quarter was discarded. An aseptic collection of at least 5 ml of milk from the affected quarter was obtained for bacterial culture using standard methods (Harmon et al, 1990). An 80-ml composite milk sample was divided as follows: 1) 30 ml in a plastic vial with preservative tablet for infrared somatic cell count (SCC), 2) 10 ml in a plastic vial for IgG_1 analysis, and 3) four 5-8 ml aliquots in plastic vials for antimicrobial residue analyses. The aliquots were frozen at -70°C until needed for residue analyses.

Testing for somatic cell count - Raw milk with the preservative was stored in the refrigerator 4-48 hours prior to delivering the sample to the Michigan Dairy Health Improvement Association laboratory. The laboratory utilizes infrared instrumentation to determine SCC.

Quantification of bovine IgG_1 - A technique described by Guidry, et al. as modified (Erskine et al, 1989) was used to analyze the milk for bovine IgG_1. Proteins in the 10 ml sample of composite milk were stabilized by the addition of 10 µl of 1 M benzamidine hydrochloric acid and maintained at -10°C. Samples were thawed in an ice bath and vortexed in preparation for the various steps of centrifugation and dilution (Guidry et al, 1980) that precede the application of a commercial radioimmunodiffusion assay[a].

Diffusion diameters were measured and interpreted according to the manufacturer's directions.

Bacteriologic culture of milk - Frozen aseptically-collected samples were thawed and vortexed. Sheep blood agar was inoculated with 10 μl of milk and aerobically incubated at 37°C for up to 48 hours. When necessary, additional testing was performed to determine the catalase status of streptococcus species and coagulase status of staphylococcus species. Specific culturing techniques for the identification of Mycoplasma infection were not performed.

Testing for antimicrobial residues - Performance of the SNAP b-lactam, Penzyme Milk Test and Delvo-SP assays and high performance liquid chromatography (HPLC) have been previously described by the authors (Gibbons-Burgener et al, 2000 a & b). Briefly, post-treatment samples were randomly tested twice using each of the assays. Assay results were compared to the HPLC identification and quantification of potential residues to determine the true positive or negative status of the sample. A positive assay result was considered false-positive when HPLC results indicated that antimicrobials were not present at or above the assay detection limit. A false-negative was the product of a negative assay result and the HPLC detection of an antimicrobial at or above the reported assay detection limit.

Statistical analysis - Descriptive statistics were calculated for the false-positive, true-positive and negative assay results. Logistic regression modeling was used to evaluate potential associations between risk factors and false-positive results obtained with each of the three residue assays. A false-positive assay result on one or both of the repeated tests for each assay was the outcome for all of the models. Independent variables considered

in the models were somatic cell count (x 10^6 cells/ml), IgG_1 concentration (mg/ml), negative initial bacteriologic culture (1, 0), and antimicrobial mastitis therapy (none, pirlimycin, hetacillin or cephapirin). When multivariable modeling was possible backward reduction techniques that considered confounding and effect modification (Hosmer and Lemeshow, 1989) were utilized.

RESULTS

Eighty-nine cases of mild clinical mastitis were included in the analyses. Because some assay results were uninterpretable, the SNAP β-lactam and Penzyme Milk Test had 86 and 88 cases respectively available for analysis. Means and standard deviations are presented for the continuous variables and frequencies for the categorical variables in tables 6-1, 6-2 and 6-3. Specific bacteria that were isolated from the samples were *E. coli*, *Streptococcus* spp. (catalase negative), *Staphylococcus* spp. (coagulase negative), *Klebsiella* spp., *S. aureus, C. bovis* and *A. pyogenes*.

The univariate logistic regression results (Table 6-4) excluded those cases that had true positive results and computed the relative risk of having a false positive result in a sample population of known negatives. Since there were only 3 false-positive Penzyme results, the univariate results are of questionable value and multivariable modeling was impossible. The 6 false-positive Delvo-SP results also restricted the possibility of multivariable modeling with data for that assay. The reduced logistic model for the SNAP β-lactam assay retained two variables that confounded each other (Table 6-5).

DISCUSSION

The achieved sample size (n=89) was substantially less than the predetermined size of approximately 400 cases. Statistical analyses were restricted by the limited number of cows that had false-positive assay results and the relatively small sample size. However, there was evidence that inflammatory-related milk proteins, represented by bovine IgG_1 concentrations, were positively associated (Tables 6-4 and 6-5) with false-positive results by all three residue detection assays. It is important to remember that a cow with mastitis will experience an increased influx of many different proteins including serum albumin, IgG_1, IgG_2, lactoferrin and lysozyme into her milk. This study only quantified the milk IgG_1 levels, because its levels are correlated with those of serum albumin and IgG_2 (Erskine et al, 1989; Li-Chan and Kummer, 1997). Because of the close association of the various inflammatory proteins and their increased presence in milk from mastitis cases we feel confident to conclude that an inflammatory-related protein in the milk is associated with the occurrence of false-positive results, but cannot definitively ascribe the results to IgG_1 concentrations.

Each of the assays we evaluated had a different mechanism of antimicrobial detection. The SNAP β-lactam is a receptor binding assay. The Penzyme Milk Test is an enzymatic colourimetric assay and involves the inactivation of an enzyme by antimicrobials. The Delvo-SP is a traditional microbial inhibition assay with a color indicator. It is unlikely that a single inflammatory protein profoundly interferes with all 3 of the assays. Additional laboratory studies controlling the levels of each protein would be beneficial in resolving which of the proteins are culpable in altering the results of the

screening assays. Until such studies are conducted it is advisable to consider the diagnostic specificity of the assays to be compromised by inflammatory proteins.

The SNAP β-lactam assay was less likely to have a false-positive result when the initial bacteriologic culture result was negative. This finding can also be interpreted as an increased risk of false-positive results when bacteria have been isolated prior to treatment of a cow with mastitis. Other studies have reported similar rates of negative cultures in the diagnosis of clinical mastitis (Anderson et al, 1982; Smith et al, 1985; White and Montgomery, 1987). In addition to not being present in the milk, bacteria may be present in very low numbers not detected by standard culturing methods (Dinsmore et al, 1992). The culture results from this study were from pre-treatment samples and the antimicrobial detection assay results are from post-treatment samples, which raises speculation as to whether the live bacteria were present in the post-treatment samples. The presence of bacteria usually precedes the elevation of SCC. We found that initial culturing of bacteria was significantly associated ($p=0.005$) with pre-treatment SCC, but only marginally ($p=0.087$) with post-treatment SCC. The lack of association between SCC and false-positive results indicates that another aspect of bacterial presence may need to be considered. A possibility could be the bacterial production of an antibiotic-like substance that is bound by the conjugated enzyme in the SNAP assay. Again, the actual mechanism of the association is unknown, but should be considered when testing individual cow samples for antimicrobial residues.

The authors previously suggested that the SNAP β-lactam assay may experience an undocumented cross-reactivity with pirlimycin (Gibbons-Burgener et al, 2000 b). It was surprising that having received pirlimycin IMM therapy was not a

significant risk factor for false-positive results. We were also unable to confirm the association of SCC with false-positive results reported by other studies (Sischo and Burns, 1993; Van Eenennaam et al, 1993). There appeared to be a trend toward higher SCCs in samples testing positive on the SNAP and Delvo assays (Table 6-1), yet the insignificant regression model findings may be the result of large standard errors and insufficient statistical power.

The smaller than desired sample size that was achieved and the relatively low occurrence of false-positive results by the 3 residue detection assays prevented the application of more extensive modeling techniques and compromised the statistical precision and power of the simpler models. However, this study was able to demonstrate that components in milk other than antimicrobials were associated with positive assay results, which sheds more doubt on the use of on-farm antimicrobial residue screening assays to test milk from individual cows.

FOOTNOTES

[a] VMRD Inc., Pullman, Wash.

REFERENCES

Adams JB. Results of drug screening from a producer's view. *J Dairy Sci,* 1994;77:1933-1935.

Anderson KL, Smith AR, Gustafsson BK, et al.. Diagnosis and treatment of acute mastitis in a large dairy herd. *J Am Vet Med Assn,* 1982;181:690-693.

Andrew SM, Frobish RA, Paape MJ, Maturin LJ. Evaluation of selected antibiotic residue screening tests for milk from individual cows and examination of factors that affect the probability of false-positive outcomes. *J Dairy Sci* 1997;80:3050-3057.

Carlsson A, Bjorck L. Lactoferrin and lysozyme in milk during acute mastitis and their inhibitory effect in Delvotest P. *J Dairy Sci,* 1989;72:3166-3175.

Cullor JS. Tests for identifying antibiotic residues in milk: how well do they work? *Vet Med* 1992;87:1235-1241.

Cullor JS, van Eenennaam A, Gardner I, et al.. Performance of various tests used to screen antibiotic residues in milk samples from individual animals. *J AOAC,* 1994;77:862-870.

Dinsmore RP, English PB, Gonzalez RN, Sears PM. Use of augmented cultural techniques in the diagnosis of the bacterial cause of clinical bovine mastitis. *J Dairy Sci,* 1992;75:2706-2712.

Erskine RJ, Eberhart RJ, Grasso PJ, Scholz RW. Induction of *Escherichia coli* mastitis in cows fed selenium-deficient or selenium-supplemented diets. *Am J Vet Res,* 1989;50:2093-2100.

Gardener IA, Cullor JS, Galey FD, et al.. Alternatives for validation of diagnostic assays used to detect antibiotic residues in milk. *J Am Vet Med Assn* 1996;209:46-52.

Gibbons-Burgener SN, Leykam JF, Kaneene JB. Identification and quantification of ampicillin, cephapirin and pirlimycin in cows' milk using high performance liquid chromatography and fluorescence detection. *J Vet Diagn Invest*, (manuscript submitted 2000). (a)

Gibbons-Burgener SN, Kaneene JB, Leykam JF, et al.. An epidemiological evaluation of the reliability of bulk-tank residue detection assays used to test individual cow milk. *Am J Vet Res,* (manuscript submitted 2000). (b)

Guidry AJ, Paape MJ, Pearson RE. Effect of udder inflammation on milk immunoglobulins and phagocytosis. *Am J Vet Res,* 1980;41:751-753.

Harmon RJ, Eberhart RJ, Jasper DE, et al., Microbiological procedures for the diagnosis of bovine udder infections, ed. 3. Arlington, VA, National Mastitis Council, 1990.

Hosmer DW, Lemeshow S. Chapters 4-7. *In*: Barnett V, Bradley RA, Hunter JS, et al, eds. *Applied logistic regression.* New York: John Wiley & Sons, 1989;82-213.

Li-Chan ECY, Kummer A. Influence of standards and antibodies in immunochemical assays for quantitation of immunoglobulin G in bovine milk. *J Dairy Sci*, 1997;80:1038-1046.

Sischo WM, Burns CM. Field trial of four cowside antibiotic-residue screening tests. *J Am Vet Med Assn,* 1993;202:1249-1254.

Smith KL, Todhunter DA, Shoenberger PS. Environmental mastitis: cause, prevalence, prevention. *J Dairy Sci*, 1985;68:1531-1553.

Tyler JW, Cullor JS, Erskine RJ, et al.. Milk antimicrobial drug residue assay results in cattle with experimental, endotoxin-induced mastitis. *J Am Vet Med Assn,* 1992;201:13781384.

Van Eenennaam AL, Cullor JS, Perani L, et al.. Evaluation of milk antibiotic residue screening tests in cattle with naturally occurring mastitis. *J Dairy Sci,* 1993;76:3041-3053.

White ME, Montgomery ME. The resemblance of clinical attributes between mastitis cows with no growth on bacterial milk cultures and those with gram-positive bacteria cultured. *Can J Vet Res,* 1987;51:181-184.

Table 6-1 - Means and standard deviations of somatic cell count (SCC) and milk IgG_1 concentration for false-positive residue detection assay results.

Assay		Result[a]	n	Mean	std. dev.
Overall	**SCC**		91	3396.81	2948.57
	log_{10} SCC		91	7.490	1.495
	IgG_1		89	122.0	107.49
SNAP b-lactam	**SCC**	TN	68	2928.78	2679.28
		FP	11	4235.82	3593.65
		TP	6	3735.17	3370.04
	log_{10} SCC	TN	68	7.30	1.556
		FP	11	7.828	1.272
		TP	6	7.722	1.282
	IgG_1	TN	68	103.28	74.88
		FP	10	169.70	126.17
		TP	6	202.83	253.66
Penzyme Milk Test	**SCC**	TN	79	3222.53	2903.65
		FP	3	2904.0	2137.78
		TP	4	3463.5	2122.42
	log_{10} SCC	TN	79	7.411	1.530
		FP	3	7.612	1.222
		TP	4	7.967	0.745
	IgG_1	TN	78	104.96	59.48
		FP	3	278.67	317.26
		TP	4	181.75	144.88
Delvo-SP	**SCC**	TN	66	3007.73	2938.88
		FP	6	3853.0	2069.95
		TP	16	4111.25	2995.62
	log_{10} SCC	TN	66	7.282	1.582
		FP	6	7.964	1.083
		TP	16	7.907	1.150
	IgG_1	TN	66	96.23	38.45
		FP	5	252.0	225.49
		TP	15	189.93	188.22

[a] Results for the assay when compared to the HPLC identification and quantification of antimicrobials. TN = true negative, FP = false positive, TP = true positive

Table 6-2 - Frequency distribution of antimicrobial therapy as a risk factor for false-positive residue detection assay results.

		Antimicrobial Therapy			
Assay		**None**	**Pirlimycin**	**Hetacillin**	**Cephapirin**
Overall		45	26	9	9
SNAP β-lactam	TN[a]	39	19	6	5
	FP	4	5	1	1
	TP	2	1	0	3
Penzyme Milk					
Test	TN	41	25	7	7
	FP	3	0	0	0
	TP	1	1	1	1
Delvo-SP	TN	40	14	6	7
	FP	2	3	1	0
	TP	3	9	2	2

[a] Results for the assay when compared to the HPLC identification and quantification of antimicrobials. TN = true negative, FP = false positive, TP = true positive

Table 6-3 - Frequency distribution of bacteriologic culture results as risk factors for false-positive residue detection assay results.

		Bacteriologic Culture				
Assay		**Neg.**	**Coliform**	**Noncontagious gram + cocci**	**Gram + pleomorphic rods**	***S. aureus***
Overall		33	21	21	3	2
SNAP β-lactam	TN[a]	29	11	16	2	2
	FP	1	5	4	1	0
	TP	2	3	1	0	0
Penzyme Milk Test	TN	31	15	20	3	2
	FP	2	1	0	0	0
	TP	0	4	0	0	0
Delvo-SP	TN	26	15	14	3	1
	FP	3	1	2	0	0
	TP	4	5	5	0	1

[a] Results for the assay when compared to the HPLC identification and quantification of antimicrobials. TN = true negative, FP = false positive, TP = true positive

Table 6-4 - Univariate logistic regression results used to estimate the relative risk of possible milk components producing a false-positive result. All samples included were HPLC confirmed negative for antimicrobials the specific assay was designed to detect.

Assay Risk Factor	β[a]	S.E.[b]	p[c]	OR[d]
SNAP β-lactam				
Neg. bacterial culture	-2.269	1.081	0.036	0.103
SSC (10^6 cells/ml)	0.0001	0.0001	0.162	1.0
$\log_{10}$ SCC (10^6 cells/ml)	0.298	0.277	0.283	1.347
Milk bovine IgG_1 (mg/ml)	0.0058	0.003	0.066	1.006
Antimicrobial IMM therapy				
None	reference	NA	NA	1.0
Pirlimycin	0.942	0.727	0.195	2.566
Hetacillin	0.486	1.201	0.686	1.625
Cephapirin	0.668	1.215	0.583	1.950
Penzyme Milk Test				
Neg. bacterial culture	0.948	1.248	0.447	2.581
SSC (10^6 cells/ml)	-0.00004	0.0002	0.866	1.0
$\log_{10}$ SCC (10^6 cells/ml)	0.111	0.439	0.80	1.117
Milk bovine IgG_1 (mg/ml)	0.0055	0.0027	0.0412	1.006
Delvo-SP				
Neg. bacterial culture	0.208	0.858	0.809	1.231
SSC (10^6 cells/ml)	0.0001	0.0001	0.491	1.0
$\log_{10}$ SCC (10^6 cells/ml)	0.414	0.394	0.294	1.513
Milk bovine IgG_1 (mg/ml)	0.021	0.009	0.021	1.021

[a] Parameter estimate; [b] standard error of β; [c] p-value for the Wald χ^2; [d] Odds ratio

Table 6-5- Multivariable logistic model of risk factors associated with false-positive SNAP β-lactam assay results (n=78).

Risk Factor	**β[a]**	**S.E.[b]**	***p*[c]**	**OR[d] (95% CI)**
Intercept	-2.495	0.721	0.0005	
Neg. bacteriologic culture	-4.314	2.413	0.074	0.013 (0.000-1.515)
Milk bovine IgG_1 (mg/ml)	0.012	0.005	0.026	1.012 (1.002-1.022)
-2 Log likelihood $\chi^2 = 13.342$, 2 df, p=0.0013				
[a] Parameter estimate; [b] standard error of β; [c] p-value for the Wald χ^2; [d] Odds ratio and the 95% confidence interval for OR.				

SUMMARY AND CONCLUSIONS

SUMMARY

The epidemiological research presented in this dissertation was achieved through two main studies. The first study was a retrospective study of Michigan dairy farms evaluating the Milk and Dairy Beef Quality Assurance Program (QAP) and its role in the prevention of violative antimicrobial residues in milk and the adoption of prudent drug management practices. The initial study (case-control) population consisted of case farms having experienced a violative residue in their milk during 1993, and control farms not having experienced a residue during the same period. In the second part (cross-sectional) of the first study, the distinction between voluntary and involuntary participation in the Program was included in the analyses. 1993 was the first full year of QAP participation in Michigan and offered a unique opportunity to evaluate the initial impact of the Program. Though the temporal relationships between some management practices and residue occurrence and QAP participation were unclear, the overall study represents one of the first scientific evaluations of the QAP.

The use of on-farm antimicrobial screening assays has consistently emerged as a management tool believed to aid the prevention of residues. The second study, therefore, was a longitudinal experimental study evaluating the reliability of 3 on-farm assays when used to test individual cow milk. The development of novel biochemistry methods for gold standard antimicrobial detection was necessary to the completion of the second study. The study population for the experimental study consisted of cows diagnosed with mild clinical mastitis. The cases were randomly assigned to either the antimicrobial or

control treatment group. The study was designed to simulate the collection and testing of milk samples as commonly performed on farms. The reliability of 3 on-farm residue screening assays was expressed as sensitivity, specificity, positive predictive value and negative predictive value. Additional statistical analyses were used to determine whether somatic cell count, IgG_1, bacterial isolates or specific antimicrobial treatments were associated with false-positive results when using the 3 assays to test individual cow milk.

CONCLUSIONS

There were six objectives addressed by the two main studies of this dissertation. The first objective was to determine if QAP certification was associated with a reduced risk of having antimicrobial residues in milk. Certification in the QAP was associated with a tendency toward reduced risk (OR=0.3 [0.07-1.32]) of having experienced a violative residue in bulk-tank milk. Though it was not statistically significant, the potential protective effect of the QAP was encouraging. Additional research may demonstrate a stronger association if the distinction between conscientious and less conscientious QAP participants is made.

The second objective was closely related to the first and was to define specific management factors that may have predisposed dairy farms to having violative antimicrobial residues in milk. The risk of having had a violative residue was reduced on farms treating >10% of their herd for metritis, and having their milk processor perform residue testing. However, on-farm residue testing was associated with an increased risk of having had a residue. A positive association between maintaining written identification records of treated cows and having a violative residue was identified, but

this finding probably indicates a change in management implemented after notification of having a violative residue. Specific risk factors associated with having a violative residue are addressed by various critical control points in the QAP and may be indicators for some of the program's strengths and weaknesses.

In a separate set of analyses the associations between QAP certification and the use of prudent drug management practices were evaluated (Objective 3). Results suggested that farms adopted specific management practices, irrespective of certification. Large percentages of farms used visible identification and non-emergency veterinary services and discussed residue prevention with employees. Involuntary certification was associated with maintenance of good written treatment records and performance of on-farm drug residue testing. Voluntary certification was weakly associated with use of refrigerated drug storage. QAP certification appeared to have been associated with the adoption of only a few prudent drug use practices, although QAP materials and framework were developed to assist veterinarians in the promotion of disease prevention, client communication, and residue prevention practices on farms.

Results from the first main study evaluating the QAP indicated strong associations between the use of on-farm residue testing and violative residue occurrence and involuntary QAP certification. These were strong indications that farms were adopting on-farm individual cow testing to avoid additional violative residues. This unapproved and unvalidated use of antimicrobial screening assays led to the second main study designed to determine the reliability of three commonly used on-farm commercial assays when testing individual cow milk.

The residue assay study was dependent on the gold standard methods for detection and quantification of antimicrobials. The fourth objective was to develop robust gold standard methods for use in determining the reliability of the Delvo-SP, Penzyme Milk Test and SNAP β-lactam assays in the detection of ampicillin, cephapirin and pirlimycin in raw milk. Previously described biochemistry methods were modified to better accommodate the blinded evaluation of milk samples collected as part of the field trial. The methods developed should improve the high performance liquid chromatography (HPLC) analyses used to detect ampicillin and pirlimycin in milk.

The fifth objective was to determine the reliability of the Delvo-SP, Penzyme Farm Milk Test and SNAP β-lactam residue test assays when used to test individual cow milk. Comparing the post-treatment assay results to the HPLC results, the Delvo-SP and SNAP β-lactam assays displayed >90% sensitivity, while the sensitivity of the Penzyme Milk Test was only 60%. Ranging from 32.14 to 73.68%, the positive predictive values were poor for all three assays when using the assays' detection limits. The positive predictive values were even less when the FDA tolerance levels were used as the cut-offs. The poor positive predictive value of the SNAP β-lactam assay was most likely due to an undocumented cross-reactivity with pirlimycin residues. The kappa statistics provided strong evidence that all three assays produced good to excellent repeatability. With such low positive predictive values and incidence of violative antimicrobial levels, the usefulness of the three residue detection assays in deciding the fate of milk from cows receiving treatment for mastitis is highly questionable.

Another objective of the residue assay study (overall Objective 6) was to ascertain possible associations between specific milk components and false-positive assay results.

Inflammatory-related milk proteins, represented by bovine IgG_1 concentrations, were positively associated with false-positive results from the SNAP β-lactam, Penzyme Milk Test and Delvo-SP assays. The SNAP β-lactam assay was less likely to have a false-positive result when the initial bacteriologic culture result was negative. Low numbers of false-positive results and a relatively small sample size compromised the statistical power of the study and warrants caution in over-interpreting the apparent associations.

Even with the possible limitations, both studies make significant contributions to the epidemiology of antimicrobial residues in milk. The tendency of the QAP to prevent violative residues provides encouraging information for the continued promotion and implementation of the Program. Dairy producers and veterinarians can use the findings to target their residue prevention efforts. To reduce the likelihood of recall bias and allow for the better establishment of temporal relationships, questions regarding QAP participation and management practices should be administered at the initiation of a residue inquiry. Producers may reconsider their reliance on screening assays for testing individual cows' milk on-farm as a primary tool for residue prevention. Researchers and regulatory personnel may use these findings to improve the accurate detection and investigation of violative residues. Future studies evaluating the reliability of on-farm residue assays should include further assessment of inflammatory-related milk proteins that may be interfering or cross-reacting with the assays.

APPENDIX 1

Dairy Production Management Study

If you would prefer to answer the questions during a telephone conversation, please mark the box below and provide your phone number so we can contact you. Return the entire questionnaire in the enclosed envelope and we will contact you soon.

___ I would prefer to be contacted by telephone
My telephone number is _____
The best time to call is _____

1. Please provide the following information on your farm's principal operator:

   a. Age ___ (yrs.)

   b. Education level
   _____ Did not complete high school
   _____ High school diploma
   _____ Some college
   _____ Technical school graduate
   _____ Bachelor's degree
   _____ Some graduate school
   _____ Graduate degree

2. Have you completed the Milk and Dairy Beef Quality Assurance Program? **Yes** or **No**

   If yes, when were you initially certified? _____
   If you have been recertified, when? _____

Please use the period January 1993 - December 1993 to answer the following questions.

3. How many workers did you employ full-time? _____
   How many workers did you employ part-time? _____

4. How many people on your farm were allowed to administer drugs with withdrawal time requirements to your dairy herd? _____

5. Which of the following management tool(s) did your farm utilize in 1993? (check all that apply)
   _____ DHIA
   _____ Dairy Comp 305 or other individual farm computer package
   _____ Daily milk weights for each cow
   _____ Other (specify) _____
   _____ None

6. What was your average herd size (lactating plus dry cows) during the period from January 1, 1993 through December 31, 1993? _____ cows

7. What was the predominant breed of dairy cows on your farm? _____

8. What was your herd's rolling average (average milk production per cow per year) from January 1993 - December 1993? _____ lbs per cow per year

9. Which of the following best describes the milking operation of your farm (check only one)?

_____ Milking Parlor
_____ Stanchion/Comfort Stalls with Milk Pipeline
_____ Stanchion/Comfort Stalls with Bucket Milker
_____ Other (describe): _____

10. Please describe the protocol used to milk treated cows. (circle **Yes** or **No** after every part, a-d.)

a. Were treated cows milked last? **Yes** or **No**
b. Was a different milker claw used when milking treated cows? **Yes** or **No**
c. Was a milk pipeline (with milk diverted at end) utilized when milking treated cows? **Yes** or **No**
d. Was a special Bucket used when milking treated cows? **Yes** or **No**

11. What veterinary services did you use during 1993. Please check the **one best** answer:

_____ a. Emergency, problem, and sick cow work only
_____ b. Emergencies plus regularly scheduled visits
_____ c. Regularly scheduled visits only
_____ d. Other - please specify: _____

12. If you had regularly scheduled veterinary visits, how often did they occur? Please check the **one best** answer:

_____ a. Every other month
_____ b. Once per month
_____ c. Twice per month
_____ d. Weekly
_____ e. Other - please specify: _____

13. Approximately how many cases of the following diseases were treated each month (or year) in your adult herd?

Mastitis	___ cases/month	or	___ cases/yr
Retained placenta	___ cases/month	or	___ cases/yr
Metritis (uterine infection)	___ cases/month	or	___ cases/yr
Respiratory disease	___ cases/month	or	___ cases/yr
Lameness	___ cases/month	or	___ cases/yr
Digestive problems (eg. Hardware diarrhea, DA)	___ cases/month	or	___ cases/yr

14. Did the above case numbers come from your farm records? **Yes** or **No**

15. Please indicate which of the following preventative procedures were included in your herd health management (check all that apply):

_____ Dry-cow intramammary treatment for every cow
_____ Dry-cow intramammary treatment only for selected individual cows
_____ Vaccination program [other than Brucellosis (BANGS) vaccination]
_____ All cows routinely given a magnet
_____ Use of a foot bath by all cows
_____ All cows routinely dewormed

16. How did you identify lactating cows treated with antibiotics? Please check all that apply and circle the primary type of identification used.

_____ Leg band
_____ Paint
_____ Tail tape
_____ Special tags
_____ Other - please specify: _____

17. Which of the following records did you maintain for treatments administered to cows? (check all that apply)

_____ Reason for treatment (disease or condition)
_____ Type of drug used
_____ Dosage given
_____ ID or name of cow(s) treated
_____ Date the treatment was given
_____ Quarter(s) treated (if appropriate)
_____ No records were kept

18. If you kept records, where were they kept? (check all that apply)

__ In milking parlor
__ In drug storage area
__ In Stanchion/Comfort Stall Area
__ In barn where cows are housed
__ Other location: _____

19. Did the people that milk your cows have access to these records? **Yes or No**

20. How did you determine how long to withhold a cow's milk after treatment? Please check all of the following that apply and circle your primary source of information.

_____ Past experience
_____ Drug residue testing
_____ Information from other producers
_____ Read the drug's label
_____ Ask the veterinarian
_____ Other - please specify: _____

21. Which of the following best describes your use of available residue testing for milk in 1993? (check all that apply)

_____ Bulk Tank Testing was available and used routinely on the farm
_____ Individual Cow Testing was available and used on the farm
_____ Testing was performed by milk handler or off the farm
_____ No optional residue testing was done on milk
_____ Other - please specify: _____

22. Which of the following describe how you stored drugs (check all that apply)?

_____ In locked cabinets
_____ In unlocked closed cabinets
_____ On non-enclosed shelves or table
_____ In a locked refrigerator
_____ In an unlocked refrigerator
_____ Other (describe): _____

**Please use the following definitions when answering question 23.

Over the Counter (OTC) Drugs are those drugs that can be purchased anywhere without a veterinarian's prescription or supervision.

Prescription (Rx) Drugs are those drugs that require a veterinarian's prescription and supervision. (For example: Lutalyse, Naxcel, Oxytocin, Gentocin, Quartermaster, Dari-clox and others.)

23. What was your primary source of OTC and Rx drugs for your dairy herd? Please check the one best answer in each column:

OTC Drugs	Rx Drugs	
____	____	a. Veterinarian
____	____	b. Local feed or livestock supply store
____	____	c. Mail-order catalog
____	____	d. Other - please specify: _____

**Please use the following definition when answering question 24.

Extra-label or Off-label Use is the use of a drug in a manner that is different than what the manufacturer's label specifies. [For example: administering a higher dose of penicillin than what the manufacturer recommends, or administering a drug to a lactating cow that the manufacturer states is only approved for non-lactating cattle.]

24. In your herd, approximately how many cows were treated with drugs used in an off-label manner per month? _____ cows/month

25. Did you discuss ways of avoiding drug residues in milk with your employees during 1993? **Yes or No**

If you did, when did you discuss residue avoidance with employees?

_____ When employees were new
_____ Routinely - _____ times a year
_____ When problems occurred
_____ Other - please describe: _____

Please add any comments you may have regarding dairy management or the Dairy Quality Assurance Program:

_____

_____

_____

_____

_____

_____

APPENDIX 2

Sample Size Calculations

A. Sample size required per group when using the z statistic to compare proportions of dichotomous variables.

$$n = \frac{\left(Z_\alpha \sqrt{2\bar{p}(1-\bar{p})} + Z_\beta \sqrt{p_c(1-P_c) + p_i(1-p_i)}\right)^2}{(p_c - p_i)^2}$$

Assumptions:

$\alpha = 0.05$, two-tailed test
$\beta = 0.20$ (80% power)
$p_c = 0.20$ (proportion of samples from non-exposed cows that produce false-positive results)
$p_i = 0.10$ (proportion of samples from exposed (antibiotic treatment) cows that produce false-positive results)
$\bar{p} = 0.15$

$$n = \frac{\left(1.96\sqrt{2(.15)(.85)} + .84\sqrt{.2(.8) + .1(.9)}\right)^2}{(.2 - .1)^2} = 198.7 = 199 \text{ each group}$$

Adjustments to sample size:

The sample size will be increased by approximately 15% to adjust for the possibilities of 1) a cow suffering a second disease problem between the pre-treatment and post-withholding period sample collection, or 2) a cow dying or being culled prior to collection of post-withholding period sample.

Adjusted sample size = 460 — 230 in antimicrobial treatment group
230 in non-antimicrobial treatment group

Sample Size Calculations

B. Total sample size required when estimating a single proportion (Kelsey et al, 1996).

$$n = Z^2 p (1-p) / L^2$$

Assumptions:

$Z = 1.96$ ($\alpha = 0.05$)
p = estimated proportion of population having a particular exposure
L = margin of error of estimated proportion

p	L	n
0.01	0.01	380
	0.02	95
	0.05	15
0.02	0.01	753
	0.02	188
	0.05	30
0.05	0.01	1824
	0.02	456
	0.05	73
0.1	0.01	3456
	0.02	864
	0.05	138
0.9	0.05	138
0.95	0.05	73

REFERENCES

Adams JB. Assuring a residue-free food supply: Milk. *J Am Vet Med Assn* 1993;202:1723-1725.

Adams JB. Results of drug screening from a producer's view. *J Dairy Sci,* 1994;77:1933-1935.

Adulterated dairy products, Minn. Stat. 32.21, Subd. 1-4. *Minnesota statues vol. 1.* St Paul, Minn: Revisor of Statues, State of Minnesota, 1998;920-922.

Albright JL, Tuckey SL, Woods GT. Antibiotics in milk - a review. *J Dairy Sci,* 1961;44:779-807.

American Veterinary Medical Association and National Milk Producers Federation. Milk and Dairy Beef Residue Prevention: a quality assurance program. *J Am Vet Med Assoc* 1991;199 (Suppl):1-23.

Anderson KL, Moats WA, Rushing JE, O'Carroll JM. Detection of milk antibiotic residues by use of screening tests and liquid chromatography after antramammary administration of amoxicillin or penicillin G in cows with clinical mastitis. *Am J Vet Res* 1998;59:1096-1100.

Anderson KL, Smith AR, Gustafsson BK, et al.. Diagnosis and treatment of acute mastitis in a large dairy herd. *J Am Vet Med Assn,* 1982;181:690-693.

Andrew SM, Frobish RA, Paape MJ, Maturin LJ. Evaluation of selected antibiotic residue screening tests for milk from individual cows and examination of factors that affect the probability of false-positive outcomes. *J Dairy Sci* 1997;80:3050-3057.

Ang CYW, Luo W, Call VL, Righter HF. Comparison of liquid chromatography with microbial inhibition assay for detection of incurred amoxicillin and ampicillin residues in milk. *J Agric Food Chem,* 1997;45:4351-4356.

Arrioja-Dechert A. *Compendium of veterinary products.* 4th ed. Port Huron, MI: North American Compendiums Inc, 1997;109-1310.

Boeckman S, Carlson KR. *Milk and dairy beef quality assurance program: milk and dairy beef residue prevention protocol.* Stratford, IA: Agri-Education, Inc, 1997;1-70.

Brady MS, White N, Katz SE. Resistance development potential of antibiotic/antimicrobial residue levels designated as "safe levels." *J Food Prot,* 1993;56:229-233.

Briguglio GT, Lau-Cam CA. Separation and identification of nine penicillins by reverse phase liquid chromatography. *J AOAC Int* 1984;67:228-231.

Bruhn CM. Consumer Perceptions and concerns about veterinary drug residues. In: Moats WA and Medina MB, ed. *Veterinary Drug Residues: Food Safety.* Washington, DC, American Chemical Society, 1996, pp 18-21.

Carlsson A, Bjorck L. Lactoferrin and lysozyme in milk during acute mastitis and their inhibitory effect in Delvotest P. *J Dairy Sci,* 1989;72:3166-3175.

Center for Veterinary Medicine. Appendix N, Pasteurized Milk Ordinance. In *CVM Update: Milk monitor with antimicro drug screening test.* 1996, pp 5-6.

Chou TY, Gao CX, Grinberg N, Krull IS. Chiral polymeric reagents for off-line and on-line derivatizations of enantiomers in high-performance liquid chromatography with ultraviolet and fluorescence detection: an enantiomer recognition approach. *Anal Chem* 1989;61:1548-1558.

Cullor JS. Tests for identifying antibiotic residues in milk: how well do they work? *Vet Med* 1992;87:1235-1241.

Cullor JS. The control, treatment, and prevention of the various types of bovine mastitis. *Vet Med* 1993;88:571-579.

Cullor JS. Dilemmas associated with antibiotic residue testing in milk. In: Moats WA and Medina MB, ed. *Veterinary Drug Residues: Food Safety.* Washington, DC, American Chemical Society, 1996, pp 44-57.

Cullor JS, Van Eenennaam A, Gardner I, et al.. Performance of various tests used to screen antibiotic residues in milk samples from individual animals. *J AOAC Int* 1994;77:862-870.

Dasenbrock CO, LaCourse WR. Assay for cephapirin and ampicillin in raw milk by high-performance liquid chromatography - integrated pulsed amperometric detection. *Anal Chem*, 1998;70:2415-2420.

Day J. A subcommittee review of the quality assurance initiative: implementation issues from the Implementation and Communication Subcommittee of the Drug Residue Committee, in *Proceedings.* 32nd Annu Meet National Mastitis Council 1993;144-146.

Dinsmore RP, English PB, Gonzalez RN, Sears PM. Use of augmented cultural techniques in the diagnosis of the bacterial cause of clinical bovine mastitis. *J Dairy Sci,* 1992;75:2706-2712.

Engel RE. Current food safety and quality service residue control program. *J Am Vet Med Assn* 1980;176:1145-1147.

Erskine RJ, Eberhart RJ, Grasso PJ, Scholz RW. Induction of *Escherichia coli* mastitis in cows fed selenium-deficient or selenium-supplemented diets. *Am J Vet Res* 1989;50:2093-2100.

Fluid Milk Act of 1965, P.A. 1965, No. 233, as amended by P.A. 1993, No.5. *Michigan Compiled Laws Annotated 1996* Sections 286.1 to 288. St Paul, Minn: West Publishing Co, 1996; 417-419.

Franco DA, Webb J, Taylor CE. Antibiotic and sulfonamide residues in meat: implications for human health. *J Food Prot,* 1990;53:178-185.

Gardener IA, Cullor JS, Galey FD, et al.. Alternatives for validation of diagnostic assays used to detect antibiotic residues in milk. *J Am Vet Med Assn* 1996;209:46-52.

Gardner IA, Hird DW, Guterback WW, et al. Mortality, morbidity, case-fatality, and culling rates for California dairy cattle as evaluated by the National Animal Health Monitoring System, 1986-87. *Prev Vet Med* 1990;8:157-170.

Gibbons-Burgener SN, Kaneene JB, Lloyd JW, Erskine RJ. Influence of the Milk and Dairy Beef Quality Assurance Program on dairy farm drug management practices. *J Am Vet Med Assn* 2000;216:1960-1964.

Gibbons-Burgener SN, Kaneene JB, Leykam JF, et al.. An epidemiological evaluation of the reliability of bulk-tank residue detection assays used to test individual cow milk. *Am J Vet Res,* (manuscript submitted 2000). (b)

Gibbons-Burgener SN, Kaneene JB, Lloyd JW, et al. Associations of milk and dairy beef quality assurance program certification and related risk factors with the occurrence of antibiotic residues in Michigan milk. *Am J Vet Res* 1999:60;1312-1316.

Gibbons-Burgener SN, Leykam JF, Kaneene JB. Identification and quantification of ampicillin, cephapirin and pirlimycin in cows' milk using high performance liquid chromatography and fluorescence detection. *J Vet Diagn Invest*, (manuscript submitted 2000). (a)

Guidry AJ, Paape MJ, Pearson RE. Effect of udder inflammation on milk immunoglobulins and phagocytosis. *Am J Vet Res,* 1980;41:751-753.

Hady PJ, Lloyd JW, Kaneene JB. Antibacterial use in lactating dairy cattle. *J Am Vet Med Assoc* 1993;203:210-220.

Halbert LW, Erskine RJ, Bartlett PC, Johnson GL. Incidence of false-positive results for assays used to detect antibiotics in milk. *J Food Prot* 1996;59:886-888.

Hardin JW. Bivariate probit models. *Stata Technical Bulletin* 1996;33:15-20.

Harik-Khan R, Moats WA. Identification and measurement of β-lactam antibiotic residues in milk: integration of screening kits with liquid chromatography. *J AOAC Int.* 1995;78:978-986.

Harmon RJ, Eberhart RJ, Jasper DE, et al.. Microbiological procedures for the diagnosis of bovine udder infections, ed 3. Arlington, VA, National Mastitis Council, 1990.

Heller, DN. Determination and confirmation of pirlimycin residue in bovine milk and liver by liquid chromatography/thermospray mass spectrometry: interlaboratory study. *J AOAC Int,* 1996;79:1054-1061.

Heller, DN. Determination of Pirlimycin residue in milk by liquid chromatographic analysis of the 9-fluorenylmethyl chloroformate derivative. *J AOAC Int,* 1997;80:975-981.

Heller DN, Ngoh MA. Electrospray ionization and tandem ion trap mass spectrometry for the confirmation of seven β-lactam antibiotics in bovine milk. *Rapid Commun Mass Spectrom,* 1998;12:2031-2040.

Hong CC, Lin CL, Tsai CE, Kondo F. Simultaneous identification and determination of residual penicillins by use of high-performance liquid chromatography with spectrophotometric or fluorometric detectors. *Am J Vet Res,* 1995;56:297-303.

Hornish RE, Arnold TS, Bacynskyj L, et al.. Pirlimycin in the dairy cow. *In:* Hudson DH, ed. *Xenobiotics in Food producing animals - metabolism and residues, ACS symposium series 503,* American Chemical Society, Washington, DC, 1992; pp. 132-147.

Hornish RE, Cazars AR, Chester ST, Roof RD. Identification and determination of pirlimycin residue in bovine milk and liver by high-performance liquid chromatography-thermospray mass spectrometry. *J Chromatogr B,* 1995;674:219-235.

Hosmer DW, Lemeshow S. Chapters 4-7. In: Barnett V, Bradley RA, Hunter JS, et al, eds. *Applied logistic regression.* New York: John Wiley & Sons, 1989;82-213.

Huber WG. Allergenicity of antibacterial drug residues. In: Rico AG, ed., *Drug residues in animals.* Academic Press, Toronto, ONT 1986, pp 33-49.

Ingersoll B. Milk is found tainted with a range of drugs farmers give cattle. *The Wall Street Journal,* December 29, 1989; Al, A2.

Ingersoll B. New York milk supply highly tainted, TV station says, based on own survey. *The Wall Street Journal,* February 8, 1990; A4.

Jones GM and Seymour EH. Cowside antibiotic residue testing. *J Dairy Sci,* 1988;71:1691-1699.

Kaneene JB and Ahl AS. Drug residues in dairy cattle industry: Epidemiological evaluation of factors influencing their occurrence. *J Dairy Sci*, 1987;70:2176-2180.

Kaneene JB, Coe PH, Smith JH, et al. Drug residues in milk after intrauterine injection of oxytetracycline, lincomycin-spectinomycin, and povidone-iodine in cows with metritis. *Am J Vet Res* 1986;47:1363-1365.

Kaneene JB, Hurd HS. National Animal Health Monitoring System Round 1—Final Report. East Lansing, Mich: Michigan State University, 1988;63-74.

Kaneene JB, Hurd HS, Miller R, et al. National Animal Health Monitoring System Round 2—Final Report. East Lansing, Mich: Michigan State University, 1990;26-34.

Kaneene JB, Miller R. Epidemiological study of metritis in Michigan dairy cattle. *Vet Res* 1994;25:253-257.

Kaneene JB, Miller RA. Description and evaluation of the influence of veterinary presence on the use of antibiotics and sulfonamides in dairy herds. *J Am Vet Med Assn* 1992;201:68-76.

Kaneene JB, Willeberg P. Influence of management factors in the occurrence of antibiotic residues in milk: a case-control study of Michigan dairy herds, with examples of suspected information bias. *Acta Vet Scand,* 1989;84:473-476.

Kelsey JL, Whittemore AS, Evans AS, Thompson WD. Methods of sampling and estimation of sample size. *In:Methods in observational epidemiology 2nd ed.* New York, NY: Oxford University Press, 1996:337-338.

Kindred TP, Hubbert WT. Residue prevention strategies in the United States. *J Am Vet Med Assn,* 1993;202:46-49.

Kleinbaum DG, Kupper LL, Muller KE. Confounding and interaction in regression. In: Payne M, ed. *Applied regression analysis and other multivariable methods*. Belmont, Calif: Duxbury Press, 1988;163-180.

Li-Chan ECY, Kummer A. Influence of standards and antibodies in immunochemical assays for quantitation of immunoglobulin G in bovine milk. *J Dairy Sci*, 1997;80:1038-1046.

McEwen SA, Black WD, Meek AH. Antibiotic residue prevention methods, farm management, and occurrence of antibiotic residues in milk. *J Dairy Sci*, 1991;74:2128-2137. (a)

McEwen SA, Meek AH, Black WD. A dairy farm survey of antibiotic treatment practices, residue control methods and associations with inhibitors in milk. *J Food Protect,* 1991;54:454-459. (b)

Mitchell JM, Griffiths MW, McEwen SA, et al.. Antimicrobial drug residues in milk and meat: causes, concerns, prevalence, regulations, tests, and test performance. *J Food Prot,* 1998;61:742-7568.

Miyazaki K, Ohtani K, Sunada K, Arita T. Determination of ampicillin, amoxicillin, cephalexin, and cephradine in plasma by high-performance liquid chromatography using fluorometric detection. *J Chromatography* 1983;276:478-482.

Moats, WA. Determination of cephapirin and desacetylcephapirin in milk using automated liquid chromatographic cleanup and ion-pairing liquid chromatography. *J AOAC Int,* 1993;76:535-539.

Moats, WA. Determination of ampicillin and amoxicillin in milk with an automated liquid chromatographic cleanup. *J AOAC Int*, 1994;77:41-45.

Moats WA, Harik-Khan. Liquid chromatographic determination of β-lactam antibiotics in milk: a multiresidue approach. *J AOAC Int,* 1995;78:49-54.

Moats, WA, Romanowski, RD. Multiresidue determination of b-lactam antibiotics in milk and tissues with the aid of high-performance liquid chromatographic fractionation for clean up. *J Chromatogr A*, 1998;812:237-247.

Musser JMB, Anderson KL. Using drug residue screening tests to investigate antibiotic contamination of milk. *Vet Med,* 1999;94:474-479.

Schwartz DP, Lightfield AR. Practical screening procedures for sulfamethazine and N^4-acetylsulfamethazine in milk at low parts-per-billion levels. *J AOAC Int,* 1995;78:967-970.

Sischo WM. Quality milk and tests for antibiotic residues. *J Dairy Sci,* 1996;79:1065-1073.

Sischo WM, Burns CM. Field trial of four cowside antibiotic-residue screening tests. *J Am Vet Med Assn,* 1993;202:1249-1254.

Sischo WM, Kiernan NE, Burns CM, et al. Implementing a quality assurance program using a risk assessment tool on dairy operations. *J Dairy Sci,* 1996;80:777-787.

Slenning BD, Gardner IA. Economic evaluation of risks to producers who use milk residue testing programs. *J Am Vet Med Assn,* 1997;211:419-427.

Smedley MD. Liquid chromatographic determination of multiple sulfonamide residues in bovine milk: collaborative study. *J AOAC Int,* 1994;77:1112-1122.

Smith KL, Todhunter DA, Shoenberger PS. Environmental mastitis: cause, prevalence, prevention. *J Dairy Sci*, 1985;68:1531-1553.

Stoltz EI, Hankinson DJ. Antibiotics and lactic acid starter cultures. *Appl Microbiol*, 1953;1:21-29.

Straub R, Linder M, Voyksner RD. Determination of β-lactam residues in milk using perfusive-particle liquid chromatography combined with ultrasonic nebulization electrospray mass spectrometry. *Anal Chem,* 1994;66:3651-3658.

Tillison J. Consumer research review, edited by the National Dairy Promotion and Research Board. *Animal Health & Milk Quality - A Situation Analysis*. Jefferson, Wis: Morgan & Myers, 1991;10-11.

Tyczkowska KL, Voyksner RD, Straub RF, Aronson AL. Simultaneous multiresidue analysis of β-lactam antibiotics in bovine milk by liquid chromatography with ultraviolet detection and confirmation by electrospray mass spectrometry. *J AOAC Int*, 1994;77:1122-1131.

Tyler JW, Cullor JS, Erskine RJ, et al. Milk antimicrobial residue assay results in cattle with experimental, endotoxin-induced mastitis. *J Am Vet Med Assn,* 1992;201:1378-1384.

USDA-APHIS-VS. Part III: Reference of 1996 diary health and health management National Animal Health Monitoring System. In: *NAHMS Dairy '96.* Washington, DC: USDA, 1996;17.

Van Eeckhout NJ, Van Peteghem CH, Helbo VC, et al.. New database on hormone and veterinary drug residue determination in animal products. *Analyst*, 1998;123:2423-2427.

Van Eenennaam AL, Cullor JS, Perani L, et al.. Evaluation of milk antibiotic residue screening tests in cattle with naturally occurring mastitis. *J Dairy Sci* 1993;76:3041-3053.

Waltner-Toews D, McEwen SA. Residues of antibacterial and antiparasitic drugs in foods of animal origin: a risk assessment. *Prev Vet Med* 1994;20:219-234.

White CR, Moats WA, Kotula KL. Optimization of a liquid chromatographic method for determination of oxytetracycline, tetracycline and chlortetracycline in milk. *J AOAC Int,* 1993;76:549-554.

White ME, Montgomery ME. The resemblance of clinical attributes between mastitis cows with no growth on bacterial milk cultures and those with gram-positive bacteria cultured. *Can J Vet Res,* 1987;51:181-184.