

EFFECTIVENESS OF ON-FARM SCREENING TESTS FOR DETECTION OF
ANTIBIOTIC RESIDUES AND CONTROL OF MASTITIS THROUGH
ANTIBIOTIC THERAPY BASED ON SOMATIC CELL COUNT

by

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(ABSTRACT)

The effectiveness of on-farm tests for the detection of antibiotic residues in milk and urine was examined using the Virginia Tech dairy herd. Milk samples from treated cows were collected 72 hours post treatment and tested for residues by the Bacillus stearothermophilus Difco disc assay, the Delvotest-P, and the Penzyme test. Urine of cull cows was collected and tested with the Live Animal Swab Test (LAST). Results showed 21% to 24% of milk samples contained antibiotic residues past the recommended withholding period. In a comparison of the two on-farm tests to the officially recognized B. stearothermophilus disc assay, no significant difference was seen between the tests. An inordinately high (62.5%) level of urine samples from cull cows exhibited antibiotic contamination with the LAST, although all animals had completed the recommended withholding period specified for each antibiotic.

In the second phase of this study, cows in the Virginia Tech dairy herd were assigned to experimental or control groups. Milk from cows in either group whose linear somatic cell count score (SCC) ≥ 5 for the first time during that lactation was cultured on blood agar plates to detect mastitis pathogens, and the SCC determined. Experimental group cows were treated in those quarters with a SCC ≥ 5 while control cows had antibiotic therapy administered based only on clinical symptoms. Treatment group had no significant effect on milk production or SCC, however treatment of elevated SCC quarters did result in trends for decreased SCC and reduced milk yield loss. Treatment group significantly altered infection status of the cow. Of the quarters cured of infection, a greater percentage (63%) belonged to the experimental group. Results from this study indicate that the practice of administering antibiotic treatment based on elevated SCC should not be adopted until further studies on herds of higher infection rates are conducted.

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INTRODUCTION

Antibiotic preparations have been infused into the mammary glands of dairy cattle for the treatment of mastitis for nearly thirty years (29). During the past few years, concern has increased over the presence of antibiotic residues in milk. Although there are divergent opinions concerning the significance of such residues, they are in fact illegal (26). Strict financial penalties imposed by milk processors, as well as lost markets and damaged reputations are some of the consequences associated with these residues. The problem is of concern because of the effect on people who are allergic to antibiotics, the possible buildup of antibiotic-resistant organisms in humans and its impact on human health, and the inhibition of dairy starter cultures used to produce cultured milk products such as yogurt and cheeses.

Jones (19) reported antibiotic residues in bulk tank milk in Virginia of .06% to .2% which can translate to a loss of approximately \$580,000 annually to the Virginia dairy industry. A strict residue avoidance program is essential to the prevention of residues in milk. One phase of such a program should include the use of an on-farm antibiotic test. The most common and economically feasible tests available at this time are the Delvotest P, and the Penzyme test. One objective of this study was to determine how frequently

antibiotic residues occur in cows' milk, and the accuracy of the two on-farm residue tests, the Delvotest P and the Penzyme test, in comparison to the officially recognized Bacillus stearothermophilus disc assay. A further objective was to examine the merit of the Live Animal Swab Test (LAST) on the urine of cull dairy cattle as an antibiotic residue detection device for slaughter animals.

Mastitis is recognized worldwide as the most costly disease afflicting dairy cattle. Major losses from mastitis are attributed to: a) realized (clinical) and unrealized (sub-clinical) reduced milk production; b) milk discarded because it contained antibiotics or was of abnormal composition; c) cost of veterinary services to treat acute or chronic mastitis; d) cost of antibiotics or other drugs purchased by dairy farmers for intramammary infusion; e) cost of increased labor to care for mastitic cows; f) decreased sale value of cows sold for dairy purposes; and g) increased herd replacement costs when cows are culled due to mastitis (6). Dodd et al. (11) stated that clinical mastitis is nearly always preceded by subclinical infection, and further suggested that treatment of these subclinical infections may reduce both the rate of new infections and their duration. The objective of this phase of the study was to determine the benefits of treating subclinically infected quarters

which had elevated somatic cell counts.

REVIEW OF LITERATURE

EXPERIMENT 1

ANTIBIOTIC RESIDUES

Antibiotics are not destroyed during milk processing, and therefore may end up as contaminating residues in dairy products. Penicillin residues are the most common. DeWeck, as reported by Vilim (55), stated that they are of particular concern because they may cause allergic reactions in individuals sensitized to penicillin or may bring about sensitization of those previously not allergic to the antibiotic. It has been estimated that between 5% to 10% of all American adults are hypersensitive to antibiotics (14). As little as .003 units of penicillin / ml of milk may cause allergic responses after ingestion (30). Responses to drug residues in sensitive humans may include skin rashes, asthma, anaphylactic shock, or even death (30).

It has been suggested (35) that minute levels of drug residues may even play a role at the molecular level (i.e., carcinogenic, teratogenic, mutogenic, enzyme induction and inhibition, and interaction with other environmental chemicals). Further work must be conducted in this area to more accurately define this relationship.

The growth of drug resistant microorganisms in the enteric flora of humans is another area for concern. This may occur as a result of ingestion of minute amounts of antimicrobial agents. Mercer (35) stated that small residual amounts of certain antimicrobial agents in food have been shown to significantly shift the resistance patterns in the enteric flora of man. Further, it has been suggested (1) that dairy foods made from milk containing antibiotics may sometimes contain antibiotic-resistant strains of infectious bacteria.

Lastly, antibiotics inhibit the growth of dairy starter cultures used to make yogurt and cheeses. As little as .0017 IU of penicillin per ml. can cause partial inhibition of starter activity (3), .005 IU per ml. can cause significant inhibition (23), and .007 IU per ml. can cause 50% or greater inhibition of dairy starter cultures (3).

Even farms with the most diligent antibiotic residue avoidance programs have been plagued by unexplained drug residues in milk. Larocque and Neville (29) theorize that most of the currently available antibiotic intramammary products were developed and introduced to the market many years ago, before methods for comprehensive and specific testing were developed. Mercer (35) stated that most food animal drugs approved since 1962 have undergone strict Food and Drug

Administration (FDA) requirements, but many drugs in use prior to that time have not had quality residue data developed. This category of drugs includes certain of the sulfonamides, the nitrofurans, the tetracyclines, the penicillins, streptomycin, dihydrostreptomycin, neomycin, erythromycin, tylosin, bacitracin, hygromycin B, nystatin, lincomycin, spectinomycin, novobiocin, and chloramphenicol.

Properties which may affect the excretion rates of antibiotics include the solubility of the drug as well as the base in which the drug is suspended. For example, sodium salts of products are readily soluble and may be used for lactating cows. Conversely, benzathine salts have a very low solubility in water causing a slower excretion rate, and therefore are only suitable for dry cow products. Many intramammary products are formulated using an oil base to give the product a longer shelf-life. The less hydrophobic oils tend to be emulsified in milk at a faster rate, and are thus excreted from the udder more quickly (7). Mineral oils are more hydrophobic than vegetable oils, therefore antibiotics with a mineral oil base would tend to have a longer retention time than those in a vegetable oil base.

Problems with residues may also arise when products are not used according to label directions. Insufficient milk discard times (16), multiple dosing with the same or another

product (16), careless use of pesticides, and improper management of treated and dry cows (21) are some of the conditions that may lead to drug residues in dairy products.

ROUTES OF ADMINISTRATION

Intramammary

There are four general methods of antibiotic administration to dairy cattle : a) infusion into the udder for both the cure and prevention of mastitis; b) injection (intramuscular, intravenous, or subcutaneous) for the treatment of numerous diseases; c) orally or as a dietary supplement; and d) reproductive "flush" for uterine, cervical, and vaginal infections (4). Albright et al. (1) stated that wide variations exist between these different routes concerning the relative persistence of the amount of antibiotics found in milk.

According to surveys conducted by the FDA, the improper use of antibiotics to control bovine mastitis is largely responsible for the adulteration of the milk supply by antibiotic residues (42). In 1960, 6% of random milk samples from the USA and 4.5% to 5% of samples from England and Wales contained penicillin residues (30). Reports from research conducted on antibiotic residues from intramammary infusion

products are conflicting.

Larocque and Neville (29) treated six cows with a bovine infusion product containing penicillin G, streptomycin, neomycin, and polymixin B sulfate. They demonstrated the persistence of penicillin G potassium, streptomycin sulfate, and neomycin sulfate residues beyond the recommended milk withholding time of 96 hours indicated on the product label. No residues of polymixin B sulfate were detected. Streptomycin residues persisted beyond 348 hours or 14.5 days after the last infusion.

Egan and Meaney (13) administered one of two commercial antibiotic preparations containing either 500,000 IU sodium penicillin, or 500 mg. sodium cloxacillin to 30 cows. Results showed penicillin residues were detected for a maximum of 72 hours after treatment, while cloxacillin residues were present for a maximum of 64 hours.

Similarly, Coulson and Rochford (10) found no antibiotic residues in seven cows at 72 hours after the last intramammary infusion of a multi-ingredient mastitis preparation containing procaine penicillin, neomycin, dihydrostreptomycin, novobiocin, and prednisolone.

Long et al. (31) found no antibiotic residues in the milk of

10 goats by the end of the recommended bovine milk-discard period with one exception. The mastitis preparations tested contained, as the single active ingredient, either erythromycin, oxytetracycline, penicillin, or cephalixin, and milk-discard times specified on approved labels for the products were 36, 96, 60, and 96 hours respectively. Rapid elimination occurred although the dose given to the goats, relative to body mass, was five times the dose recommended for cattle. Penicillin residues were detected in the milk of one goat at 72 hours after the last treatment.

Intrauterine

The most common means of treating uterine infections in dairy cattle is by uterine infusion of antibiotics or antibacterial solutions. However, little information is available on residues and milk withdrawal times for such infusions.

Kendrick, as reported by Caudle et al. (9), suggested that intrauterine infusions of low concentrations of penicillin or oxytetracycline do not cause antibiotic residues in milk.

Similarly, Caudle et al. (9) found no antibiotic residues in the milk of four cows given 2 g of ampicillin trihydrate by intrauterine infusion. They obtained the same results from

all residue assay procedures used: a) Delvotest P; b) Sarcina lutea cylinder plate method; c) Bacillus stearothermophilus disk assay; and d) Bacillus subtilis swab test.

Studies conducted by Kendrick and Pier, as reported by Black et al. (5), found no antibiotic residues in milk when penicillin (1,000,000 units), streptomycin (1 g), and oxytetracycline (100 mg) were administered by intrauterine infusion. Conversely, Gedek et al., as reported by Black et al. (5), found evidence of antibiotic residues in the milk of cows treated with intrauterine antibiotic tablets containing tetracycline, dihydrostreptomycin, and benzyl penicillin. Righter et al., as reported by Black et al. (5), infused cows with sulfamethazine (30 mg/lb), oxytetracycline (500 mg), or penicillin streptomycin (2,000,000 units and 2.5 g respectively). They observed contaminating residues in the milk of cows treated with each of the antibiotics.

Black et al. (5) found that of 165 cows given intrauterine treatments, detection of drug residues occurred in 11 first, 5 second, 3 fourth, and 6 sixth post-treatment milk samples. They concluded that when such milk is diluted in a bulk tank with milk from untreated cows it would, in all likelihood, be undetected using our present detection methods. However, treatment of several cows in the herd and improvements in these detection methods could result in the finding of inhi-

bitory substances in the bulk tank milk.

A study carried out by Miller and Bergt (37) showed contaminating residues in milk for 24 hours post intrauterine infusion of 4 mg/kg body weight of nonchelated oxytetracycline.

Kaneene et al. (25) administered either oxytetracycline (3 g), lincomycin-spectinomycin (2 g of one-third lincomycin and two-thirds spectinomycin), or povidone-iodine (6 g) by intrauterine infusion to 123 cows. Thirty of the 61 cows treated with oxytetracycline showed detectable residues in their post-injection milk for variable periods (range 12.5 to 44.0 hours). Seventeen of the 47 cows treated with lincomycin-spectinomycin showed residues for 14.5 to 24 hours. Povidone-iodine was not detected in any milk samples. Kaneene et al. (25) suggested that milk from animals treated with these antibiotics should probably be tested until it is negative for antibiotic residues.

Other Routes

As stated, antibiotics may be administered by many routes. Information regarding injection and oral administration of antibiotics and the possibility of contaminating residues from these is scarce.

Ziv and Rasmussen (56) determined that antibiotic uptake by cream separated from whole milk after intramuscular injection was higher than after intramammary infusion.

It has been suggested (1) that wide variations exist between the different routes of administration of antibiotics and their vehicles concerning the relative persistence of the amount of antibiotics found in milk. This is supported by work conducted by Cannon et al. (8). They detected residues in milk of test animals for a maximum of 96 hours following intramuscular injection of penicillin in oil, 72 hours following intramuscular injection of aqueous penicillin, 44 hours after intravenous injection of aqueous penicillin, 27 to 31 hours following intrauterine infusion of aqueous penicillin, and 86 hours post-feeding of penicillin in oil. Further, they reported that all correlations between duration of penicillin in milk and average daily milk production, fat percentage in milk, and body weight were low, indicating that none of these variables contributed significantly to the variation in duration of secretion of penicillin in milk.

RESIDUES ASSOCIATED WITH DRY COW TREATMENT

Dry cow therapy has become an important weapon in the fight against bovine mastitis. The reasons for the intramammary

antibiotic treatment of cows at the start of the dry period are twofold: to eliminate subclinical and chronic infections present at the end of lactation, and to protect the udder against the introduction of bacterial infections during the dry period. The manufacturers of these dry cow preparations generally recommend that the product not be used immediately prior to the expected calving date, along with a specific period of time for withholding the milk after parturition. Ideally, the farmer would like to believe that the antibiotic is present in the udder throughout the dry period to obtain the above objectives, but fully excreted by the specified withholding time after calving. Questions have been raised (18) as to the course of action should a miscalculation of the calving date or the birth of a premature calf occur.

A study was conducted on 150 cows in seven commercial herds to determine antibiotic excretion rates of a long-acting dry cow preparation. Pugh et al. (49) infused all four quarters with a single dose of a long-acting intramammary preparation containing 1 g procaine penicillin G and .5 g dihydrostreptomycin in an oil base. Dry cow secretion samples showed that penicillin persisted in the dry udder for at least three weeks post infusion, while streptomycin was present for a maximum of five weeks. Further, all milk samples taken more than four days post calving were free of antibiotic

residues. In 412 samples taken within four days of calving, only two were positive. Pugh et al. (49) concluded that, provided all milk is discarded for the recommended four days after calving, no antibiotic residues should be observed.

Oliver et al. (40) infused 186 cows in five dairy herds with one of five treatments to determine the persistence of antibiotic residues in colostrum and milk following dry cow therapy. Treatments were: 1) one million units procaine penicillin G plus 1 g dihydrostreptomycin; 2) 400 mg novobiocin; 3) 500 mg benzathine cloxacillin; 4) 300 mg cephalixin benzathine; and 5) no antibiotic. Composite colostrum samples collected from the first milking after parturition showed four samples were positive for antibiotic residues by the Delvotest. Only one of these four was confirmed positive by the Bacillus stearothermophilus disc assay. Further, samples were also collected of first marketable milk from 141 cows treated at drying off and over 96% of these were negative for antibiotic residues by the Delvotest. Oliver et al. (40) concluded that if manufacturer's recommendations are followed, antibiotic residues in colostrum and milk from cows treated at drying off should not be a significant problem even though 4% of first marketable milk samples were positive for antibiotic residues.

The question still remains as to the state of milk from cows

whose calving date has been miscalculated or who have calved prematurely. Johnson et al. (18) conducted a study to determine if antibiotics in dry cow treatments would persist longer than the manufacturer's recommended time for withholding milk from the market supply if cows were to calve earlier than expected. Nineteen dry Holstein cows were infused approximately 10 to 12 days prior to calving with either 500 mg benzathine cloxacillin or 1,000,000 units of procaine penicillin G and 1 g of dihydrostreptomycin sulfate in an oil base. The manufacturer of the cloxacillin preparation recommends that the product not be used during the four weeks prior to the expected date of calving. A milk withholding time of 72 hours post calving is also recommended. The recommendation for the penicillin preparation states that the product should not be used during the six weeks prior to the expected calving date with a milk withholding time of 96 hours post calving. Cloxacillin was detected in the milk of two cows after calving, but for one milking only. Penicillin residues were not detected in milk samples collected from cows treated more than nine days prior to calving, but seven of nine cows treated with the penicillin preparation up to nine days prior to calving had detectable penicillin residues in the milk from treated quarters after calving. Only one of these cows showed detectable residues beyond the 96 hour milk withholding period. Results from this study indicate that no antibiotic residues should be

detected when manufacturer's recommendations are followed for either dry cow preparation. Johnson et al. (18) did recommend that milk of cows calving within 10 days after infusion of a dry cow antibiotic preparation be tested for residues before their milk enters the market supply.

OFFICIAL ANTIBIOTIC RESIDUE TESTS

The Grade A Pasteurized Milk Ordinance (PMO) is the basic standard used in the voluntary Cooperative State - Public Health Service Program for Certification of Interstate Milk Shippers, a program involving 49 states. It is incorporated by reference in Federal specifications for procurement of milk and milk products; is used as the sanitary regulation for milk and milk products served on interstate carriers; and is recognized by the public health agencies, the milk industry, and many others as a national standard for milk sanitation (54). In other words, the PMO is the final word in official regulatory action. The PMO (54) states that all sampling procedures and required laboratory examinations shall be in substantial compliance with the Edition of Standard Methods for the Examination of Dairy Products of the American Public Health Administration, and the Edition of Official Analytical Chemists (AOAC). This leads to some confusion due to the fact that the AOAC may adopt an antibiotic test procedure which the PMO will not accept. The PMO

will not officially accept a test, though, unless it is first accepted by the AOAC. For example, both the Delvotest P and the Bacillus stearothermophilus disc assay have received official first action by the AOAC, but only the B. stearothermophilus assay is currently accepted by the PMO. Therefore, the only officially recognized antibiotic residue detection test at this time is the B. stearothermophilus disc assay. Milk cooperatives and laboratories may choose to conduct the Delvotest, but any milk samples testing positive must be confirmed by the official assay.

ANTIBIOTIC RESIDUE TESTS USED IN THIS STUDY

Bacillus Stearothermophilus Difco Disc Assay

The B. stearothermophilus Difco disc assay using PM Indicator Agar was officially recognized by the AOAC in 1982. As previously stated, the B. stearothermophilus assay is also the only antibiotic residue test which is officially recognized by the U.S. Department of Health and Human Services in their Grade A Pasteurized Milk Ordinance (54). Early methods of residue detection utilized Bacillus subtilis, with a sensitivity of only .05 U of penicillin /ml of milk, as the test organism. The adoption of Sarcina lutea as the test organism enhanced sensitivity, but was a slow, difficult test to perform. The B. stearothermophilus disc assay has

gained popularity since its adoption because the test organism is highly sensitive to many antibiotics and non-antibiotic chemical inhibitors likely to be found in milk, and also because of its relatively short incubation time (2 hours and 50 minutes at 65° C, or 3 hours and 50 minutes at 56° C). A further advantage of this procedure is that the use of a spore suspension creates a readily available source of inoculum for smaller laboratories (the spore suspension is available in sealed ampules).

As noted, one of the benefits of the B. stearothermophilus disc assay is its relatively short incubation time. Kabay (24) noted that of 14 clinically active antibiotics and 6 chemical preservatives tested, no significant differences in accuracy were found between the conventional 16-18 hour agar diffusion assay and the four hour B. stearothermophilus assay. Further, this study demonstrated the test organism's sensitivity to many antibiotics and chemical preservatives.

Researchers have observed differing minimum detection levels with the B. stearothermophilus disc assay, but most have been considerably lower than previous assays. Kaufmann's data (27) showed the B. stearothermophilus disc assay able to detect .004 U of penicillin /ml using plates which had been stored for 8 days. When fresh plates and fresh standard solutions were employed levels of .002 U/ml were observed.

Vilim et al. (55) found minimum detectable levels of penicillin G, ampicillin, and cloxacillin to be .002 U/ml, .0014 µg/ml, and .021 µg/ml, respectively, using this assay. Work by Ouderkirk (42) in 1979 found that of six collaborating laboratories, four were able to detect levels of .005 units or µg/ml for penicillin G, ampicillin, and cephalixin, and .05 µg/ml for cloxacillin. The other two collaborators reported detectable levels of .008 unit and .08 µg/ml respectively. Ouderkirk (42) concluded that the B. stearothermophilus method was rapid, simple to perform, and more sensitive than either the Sarcina lutea or the Bacillus subtilis assays, the official methods of that time. The method was adopted official first action for the detection of penicillin in milk.

The B. stearothermophilus Difco disc assay has a slight modification from the version accepted by the AOAC in 1979. This version of the assay uses Difco PM Indicator Agar in place of Antibiotic Medium No. 4. Messer et al. (36) demonstrated that results from the disc assay using PM Indicator Agar did not differ significantly at the $\alpha=.05$ level from those using Antibiotic Medium No. 4; therefore either medium is suitable for use in this assay. In addition, no false positive results were reported. Messer's study (36) demonstrated that this B. stearothermophilus disc assay method could be used to detect and identify a beta-lactam inhibitor

rapidly, precisely, and accurately. The Associate Referee recommended adoption as official first action at a level of .008 IU/ml, and the General Referee concurred (36).

Peeler et al.(45) stated that all AOAC methods are collaboratively studied and reported before official acceptance, but are infrequently reported after widespread acceptance and use. They therefore conducted a collaborative study in 1983 to reevaluate the results of the AOAC B. stearotheophilus disc procedure for antibiotics in milk (36). Results showed the disc assay using PM Indicator Agar at 64° C was able to detect a beta-lactam concentration of .008 U/ml 98% of the time, and a concentration of .005 U/ml 89% of the time. Results of this study confirmed the detectability observed by Messer et al. (36) in the AOAC collaborative study report, as well as the speed, precision, and accuracy of this method of antibiotic residue detection.

Delvotest P

The Delvotest P antibiotic residue test appeared on the market in 1974 and was officially recognized by the AOAC in 1982. The Delvotest provides several advantages over other tests as it requires a relatively short incubation time, 2 and 1/2 hours, and it can be used routinely by a well equipped laboratory as well as for occasional checks of milk

samples on the farm. The manufacturer, GB Fermentation Industries Inc., states a sensitivity of .004 IU of penicillin /ml of milk, but the AOAC recognizes a detection limit of .007 IU/ml (26).

Bishop et al. (4) conducted a study to compare the results of the Delvotest P and the B. stearothermophilus Difco disc assay. Identical results were obtained at the .050 U/ml, .010 U/ml, and .005 U/ml levels with both tests reading positive 100% of the time. Further, all control samples for both tests showed negative results. They concluded that the Delvotest P and Difco disc assay were equal in sensitivity.

A collaborative study was conducted by Pater (44) with the co-operation of 17 milk-receiving stations. He found 99.5% correct readings of negative control samples, 100% at .004 U of penicillin/ml, 100% at .005 U/ml, 100% at .006 U/ml, 85% at .007 U/ml and 100% at .008 U/ml. The conclusion reached from this study was that the Delvotest P has proven to be suitable under field conditions in every respect.

Kelly (28) observed similar results in a 24 collaborator study to determine the ability of the Delvotest P to detect low levels of penicillin G in seven types of fluid milk products. All 24 collaborators reported negative results with the .002 IU/ml penicillin G standard and the negative

control samples. Results of the .004 and .006 IU/ml standards were reported as 72.3% and 98.8% positive respectively. All analysts reported .008 and .010 IU/ml standards as positive. He concluded that the Delvotest P Ampule method is a rapid, reliable, sensitive and portable method for detecting antibiotics in large numbers of milk samples at the farm and receiving station levels. It was recommended that this technique be adopted official first action by the AOAC. The General Referee concurred, but recommended that the lower limit of detection be .007 U/ml; this level was detected consistently in a wide variety of products (28).

Conflicting results (10,32,40) regarding the accuracy of the Delvotest have been observed by some researchers. Macaulay and Packard (32) found 11% false positive results on negative control samples. A known antibiotic-free milk sample which shows a positive test result is termed a false positive. The Delvotest P was able to detect penicillin 100% of the time at a concentration of .025 U/ml. There was a further inconsistency seen where, in one set of samples, the method gave positive readings at .0025 U/ml, but was negative at .005 and .01 U/ml. The manufacturer of the Delvotest P lists the test's sensitivity to erythromycin at 1.75 µg/ml, but Macaulay and Packard found positive results at this level occurred only 56% of the time.

Similarly, Oliver et al. (40) encountered problems with false positive results with the Delvotest P method. They found only one of four colostrum samples and one of three milk samples positive by Delvotest were confirmed positive by B. stearothermophilus disc assay. Delvotest false positive readings were in 1.6% of colostrum samples and 1.4% of milk samples. Oliver et al. (40) theorized that in some instances natural inhibitors in colostrum and milk may produce false positive Delvotest reactions.

Another possible explanation for false positive results with the Delvotest P method of residue detection was suggested by Coulson and Rochford (10). They observed that changes in the pH of milk samples due to storage and transport may influence the test result. They concluded that it is important to realize that false positive results do occur in samples screened by the Delvotest P, and that this should be kept in mind when unexpected positive results are reported by laboratories during routine screening with this test.

Penzyme Test

The Penzyme test is an enzymatic, colorimetric method for rapid determination of beta-lactam antibiotics used extensively in the prevention and treatment of dairy cattle diseases. The manufacturer, Smithkline Beckman Corporation,

states that it is a quick, simple, economical, reliable screen test to determine antibiotic residue levels in raw milk. The test requires only 20 minutes to run, and thus, it could be an excellent tool for the dairy farmer. According to the manufacturer, the detection limits are between .004 IU/ml to .012 IU/ml. Unfortunately, very little research information is available as to the accuracy of this on-farm residue detection test.

Amodio et al. (2) found detection limits of .009, .009 and .050 $\mu\text{g/ml}$ for ampicillin, cephalothin, and oxacillin respectively, and .008 IU/ml for penicillin with the penzyme test. The test was not sensitive to streptomycin. Corresponding detection limits for the B. stearoothermophilus disc assay were .001, .001, .006 $\mu\text{g/ml}$ and .0004 IU/ml for penicillin. The detection limit for streptomycin with the disc assay was found to be .100 $\mu\text{g/ml}$. They concluded that, although the enzyme test is quicker, it is less accurate than the microbiological method.

LIVE ANIMAL SWAB TEST (LAST)

The Live Animal Swab Test (LAST) was the first test available for on-the-farm use in screening animals for potential antibiotic residues before they are slaughtered. It was developed by scientists in the U.S. Department of Agricul-

ture's Food Safety and Inspection Service. The LAST should be used on animals which have been treated with antibiotics but are ready for slaughter, and should be performed only after the antibiotics have been withdrawn for the prescribed period. When the urine is free of antibiotics, the tissue levels should also be reduced, and the animal can be safely marketed. Animals testing positive with the LAST should be withheld from slaughter, as the carcass may be positive for residues.

Oliver et al. (41) screened cull dairy cows for antibiotic residues with the LAST on urine. Results showed 23% of urine samples were positive using the LAST. Residues in the kidneys of these cows, using the Swab Test on Premises (STOP) were found in only 5.6% of samples, and residues in milk, using the Delvotest P, were found in only 3.4%. Only one cow was positive in both kidney and urine samples, indicating the possibility of false positive results with the LAST. Oliver et al. (41) concluded that there was no clear association between residues in kidney and urine.

REVIEW OF LITERATURE

EXPERIMENT 2

LOSSES FROM MASTITIS

Mastitis is recognized worldwide as the most costly disease afflicting dairy cattle. Major losses from mastitis are attributed to: a) realized (clinical) and unrealized (sub-clinical) reduced milk production; b) milk discarded because it contained antibiotics or was of abnormal composition; c) cost of veterinary services to treat acute and chronic mastitis; d) cost of antibiotics purchased by dairymen for intramammary infusion; e) cost of increased labor to care for mastitic cows; f) decreased sale value of cows sold for dairy purposes; and g) increased herd replacement costs when cows were culled due to mastitis (6).

The largest of the losses associated with mastitis is due to decreased milk production. Janzen (17) reported losses in total milk production due to mastitis of 5% to 25% or more, in fat of .1% to .45%, in solids not fat of .1% to .57%, in lactose of .1% to .77%, and in total solids of 1.07%. These alterations in composition also represent important losses as they significantly affect the yield and quality of dairy products. Miller, as reported by Blosser (6), determined

that clinical mastitis reduced milk production by more than 20% for the total lactation yield. Losses of milk yields caused by mastitis in 1976 were estimated to be 386 kg/cow per year, and losses of discarded milk 62 kg/cow per year (6). Further, in 1976, for the eleven million cows in the United States, total losses from mastitis were estimated at 1.294 billion dollars (6). Blosser (6) calculated that if economic losses were reduced 2% per year for ten years by research, and expenditures for mastitis research remained the same, the benefit to cost ratio from mastitis research would be approximately 9.6 to 1. Jones et al. (22) estimated the cost of mastitis in Virginia in 1984 at \$300 dollars/cow per year for the average herd, and \$200 dollars for well managed herds. It is clear from this information that mastitis is a costly disease to dairymen. Any practice which might aid in its reduction should be studied thoroughly.

SUBCLINICAL MASTITIS

Mastitis is a disease of the mammary gland causing inflammation in response to injury of mammary secretory tissue. This inflammatory response is designed to neutralize or destroy the irritant, and to initiate repair of the tissue and resumption of normal function. Bacteria and their toxins are the most common and most serious causes of mastitis. Staphylococci (Staphylococcus aureus, and non coagulase staphylo-

coccus), streptococci (Streptococcus agalactiae, Streptococcus dysgalactiae, and Streptococcus uberis), and coliforms (Escherichia coli, and Klebsiella pneumoniae) are the most common microorganisms involved (39).

Subclinical mastitis, or "hidden mastitis" is a form of the disease which is characterized by an elevated milk somatic cell count (SCC). No apparent physical signs, such as swelling, fever, or abnormal milk are present, but changes in milk composition and somatic cell concentration may be detected by the use of a cowside mastitis test such as the California Mastitis Test (CMT). Philpot (46) stated that subclinical mastitis almost always causes a general herd problem of one degree or another, whereas clinical mastitis causes an individual cow problem. It is generally believed that subclinical mastitis is the most common form of the disease, and accounts for the greatest economic losses.

Subclinical mastitis may cause tremendous financial losses through unrealized milk production. Blosser (6) concluded that reduced milk production from cows with subclinical mastitis was responsible for the largest economic losses from mastitis. In an effort to gain more information on mastitis losses, Blosser (6) sent a questionnaire to one dairy specialist in each of the 50 states, requesting that he, or a person whom the questionnaire recipient considered more

qualified, respond. Responses from reporting states substantiated the view that milk production losses from cows with subclinical mastitis were responsible for the largest dollar loss per cow.

Dodd et al. (11) stated that clinical mastitis is nearly always preceded by subclinical infection, and further, suggested that treatment of these subclinical infections may reduce both the rate of new infections, and their duration. Similarly, Philpot (46) stated that logically, many cases of clinical mastitis will be prevented if the subclinical form of the disease is controlled effectively. He concluded that the progressive pathology of subclinical mastitis emphasizes the need for initiating treatment before the organisms become established in inaccessible areas of the udder.

THE USE OF THE SOMATIC CELL COUNT SCORE TO DETECT MASTITIS

Numerous screening tests have been developed for the detection of subclinical mastitis. One test in common use is the electronic SCC. Dohoo (12) stated that the use of electronic particle counters to determine the total SCC of milk is a well established and reliable procedure. Somatic cells include two cell types: a) sloughed off epithelial cells; and b) polymorphonuclear leukocytes. By measuring the numbers of leukocytes (white blood cells which fight infec-

tions, and are present in the milk of mastitic cows in greater numbers than in uninfected cows) in a milk sample, the probability of mastitis may be predicted (33). Research has shown that the probability of infection increases as SCC rises, and milk production decreases with increasing SCC (33). Paape et al. (43) found that SCC, which are 90% polymorphonuclear leukocytes, in subclinically infected quarters averaged 700,000 cells/ml of milk. Similarly, it was stated in Current Concepts of Bovine Mastitis (39) that the presence of more than 500,000 leukocytes per ml of mixed herd milk strongly suggests a significant incidence of mastitis in a given herd. Postle, as reported by Postle et al. (48), studying four herds, concluded that within each herd there was a significant relationship between the presence of both medium and high leukocyte contents and the isolation of pathogenic bacteria. Further, he determined that herds classified as having low bulk milk cell counts (<500,000 cells/ml) produced more milk than herds with high bulk milk cell counts (>1,000,000 cells/ml).

Blosser (6) stated that investigators have attempted to quantify the losses associated with mastitis by establishing the milk production of cows, and concurrently measuring the SCC of their milk. He concluded that, after a sizeable number of cows and quarters have been compared, decreased milk production can be estimated by SCC. King, as reported by

Blosser (6), cited a Milk Marketing Board study in Great Britain in 1971 which attributed losses of 164, 289, 661, and 770 kg of milk per cow per year to SCC ranges of 250,000 to 499,000; 500,000 to 749,000; 750,000 to 999,000; and 1,000,000 or more cells/ml of milk respectively.

Dairy Herd Improvement Associations (DHIA) offer the dairyman the option of a monthly SCC profile for the entire herd. The use of this SCC profile has gained popularity as a management tool for monitoring both herd and individual cow mammary health status, as well as a means of identifying subclinical mastitis (52).

LINEAR SOMATIC CELL COUNT SCORING SYSTEM

Until 1984, SCC were not reported in a uniform manner by DHIA computing centers. Prior to that time, one method was to report SCC as 1000 cells/ml of milk. Late in 1984, most DHIA computing centers adopted the $\log_2$ or linear SCC scoring system. The $\log_2$ method of reporting SCC was presented to the National Mastitis Council in 1982 by Shook (51). The reason for adopting this system, based on a 0 to 9 SCC score, was to find a common way of reporting all cell counts in all regions of the country which would improve communication with dairymen and reduce confusion. See table 1 for conversion of SCC scores from actual counts (000's/ml) to

Table 1. Conversion of somatic cell count scores (SCC) from 000's/ml to the new linear DHIA score (20).

Somatic cell count range 000's/ml	DHIA score $\log_2$ SCC
0 - 17	0
18 - 34	1
35 - 70	2
71 - 140	3
141 - 282	4
283 - 565	5
566 - 1130	6
1131 - 2262	7
2263 - 4525	8
4526 & above	9

the new linear DHIA score.

TREATING MASTITIS BASED ON SOMATIC CELL COUNT

One study has reported that dairymen who receive SCC reports are able to decrease their percentage of cows with elevated cell counts (15). One explanation for this may be that SCC programs are an indirect aid in decreasing mastitis in that they prompt action to improve mastitis control practices. One question is whether the individual cow SCC be used as an aid in making decisions on whether to treat lactating dairy cows.

There is speculation that treating udder infections as soon as possible (ie., as subclinical mastitis) after they are detected may prevent enough tissue damage, and hence loss of milk production, to make this type of lactation therapy cost effective. Plastringe (47), observed a recovery in milk yield following the successful treatment of some cows infected with Streptococcus agalactiae. Similarly, Timms and Schultz (52) observed that milk yield returned almost back to normal levels after treatment of cows with clinical mastitis. Conversely, Crossman et al., as reported by Janzen (17), observed that treated quarters of clinical mastitis cases did not recover their proportionate yield until the next lactation. Wheelock et al., as cited by Janzen (17),

noted that losses in milk yield were not completely recovered even during the following lactation, although the infection had been eliminated. The literature on the recovery of milk production after antibiotic therapy for mastitis is conflicting. However, as Dodd et al. (11) stated, clinical mastitis is nearly always preceded by subclinical infection. It is possible that initiating treatment before the organisms become established may avert the onset of clinical mastitis, and hence prevent the loss of milk production before it occurs.

One question often raised by dairymen is whether a cow with elevated SCC, but without clinical mastitis symptoms, should be a candidate for intramammary antibiotic therapy during lactation. Timms and Schultz (52) administered intramammary treatment with a broad spectrum antibiotic to cows with a single high monthly SCC, and to cows with clinical cases of mastitis. Cows with cell counts above 400,000 cells/ml of milk on the monthly DHIA report were quarter sampled at the regular milking for bacteriological analysis. A California Mastitis Test (CMT) was performed on milk from each quarter. Quarters with a CMT reaction of 1+ or greater were treated with an over-the-counter broad spectrum antibiotic (cephapirin). Cows showing clinical signs of mastitis were also quarter sampled and treated in those quarters with a CMT score of 1+ or greater. Results from this study showed no

significant decrease of SCC on successive DHIA test days as a result of treatment for high SCC. Bacteriological cure rates were low for those cows treated based on SCC (23.3%), and for cows treated based on clinical symptoms of mastitis (21.6%). Although treatment efficacy for cows treated for elevated SCC was poor, the investigators felt that elevated SCC was still a useful tool for detecting subclinical mastitis as bacterial isolates were found in 70% of the quarters treated based on SCC. For those cows treated for clinical mastitis, SCC decreased significantly and posttreatment milk yields returned almost back to normal. One problem noted in this experiment was a lag time of five to fourteen days between the time of DHIA sampling and receipt of the SCC results . Based on these findings, Timms and Schultz (52) supported the usual recommendation of treatment during lactation for cows affected with clinical mastitis.

McDermott et al. (34) conducted a similar study on lactating cows from five commercial herds. The objective of the experiment was to study the benefits from lactation therapy for cows with subclinical mastitis. Cows in the experimental group were infused in all four quarters and for two consecutive milkings with cephalixin if their SCC rose above 400,000 cells/ml of milk. Statistical analysis showed that treatment group did not contribute significantly to variation in milk production. Further analysis conducted on true

positive cows (cows that were both > 400,000 cells/ml and infected with a mastitis causing pathogen) in both groups, showed that the experimental cows' SCC-based intramammary infusions did not alter milk production. McDermott et al. (34) recommended that SCC above 400,000 cells/ml not be used as an indication for intramammary therapy during lactation.

Heider and Bashandy (15) suggested a further use for individual cow SCC. They indicated that milk from cows with elevated SCC might be regularly (monthly) cultured on blood agar plates to determine infection status. This information could then be used to recommend that cows be culled, dried off and treated, or treated during lactation. They were conducting a study where all cows with SCC over 250,000 cells/ml were routinely cultured. Thus far, they have treated and evaluated 13 cows. Eight of these 13 (62%) had SCC less than 250,000 cells/ml at the next test day. Further work must be conducted in the area of culturing milk based on elevated SCC to determine its merit as a tool for decreasing mastitis.

MATERIALS AND METHODS

EXPERIMENT 1

The Virginia Tech dairy herd, consisting of approximately 200 milking cows, was used to carry out this study. Composite milk samples (equal amounts from each quarter) were taken from cows receiving antibiotic treatment for any reason and by any route of administration. Antibiotics used and their recommended withholding periods are shown in table 2. Milk samples were obtained, during the afternoon milking, 72 hours $\pm$ 2 hours post-treatment and continued every 24 hours until all tests showed a negative reading for antibiotics. Samples also were obtained at the nearest milking to 72 hours postpartum from all cows which had been dry-treated at the end of the previous lactation to determine if the long-acting dry cow treatment was present in milk. Cows testing positive were retested every 24 hours until their milk was free detectable of antibiotic contamination. All dry cows received 300 mg cephapirin benzathine (trade name Cefa-Dri¹, see table 2 for recommended withholding period). Milk samples were transported to the laboratory after collection for analysis by three different antibiotic residue detection tests: 1) the Bacillus stearothermophilus Difco disk assay²; 2) the Delvotest-P³; and 3) the Penzyme test⁴. The urine

¹ Bristol Laboratories, Syracuse, New York

² Difco Laboratories, Detroit, Michigan

³ GB Fermentation Industries, Charlotte, North Carolina

⁴ Smithkline Beckman Corporation, West Chester, Pennsylvania

Table 2. Antibiotics administered singly or as combinations during this study and their recommended withholding times.

<u>Drug</u>	<u>Withholding period</u>	
	(days)	(hours)
Cephapirin = C	4	96
Gentamicin = G	7	168
Liquimycin (Oxytetracycline) = L	4	96
Naquasone(Trichlormethazide + Dexamethasone)=N	3	72
Penicillin G = P	3	72
Triple sulfa = S	4	96
Tetracycline = T	4	96

Drug combinations used.

G + C + L	7	168
L + T	4	96
G + L	7	168
C + L	4	96

<u>Dry cow treatment</u>	<u>Withholding period (days)</u>
Cephapirin benzathine	30

of cull cows was collected and tested with the Live Animal Swab Test (LAST) as an indication of antibiotic residues present in slaughter carcasses.

BACILLUS STEAROTHERMOPHILUS DIFCO DISC ASSAY

The B. stearothermophilus Difco disc assay is not available as a kit, as are the other antibiotic residue tests used in this study. Materials required to conduct this assay included: a) Bacto-PM Indicator Agar; b) Bacto-Thermospore Suspension PM; c) Bacto-Concentration Discs 1/2" Penicillin .05 unit; d) Bacto-PM Negative Control; e) Bacto-Concentration Discs Sterile Blanks 1/2"; and f) 100 mm flat-bottom Petri dishes. Bacto-PM Indicator Agar is specially formulated to produce rapid growth and acid formation by the Bacillus stearothermophilus organism. Bacto-Thermospore Suspension PM is a standardized suspension of this B. stearothermophilus organism stored in an ampule.

The B. stearothermophilus disc assay is carried out on PM Indicator Agar plates. To prepare these plates, 3.2 g of Bacto-PM Indicator Agar, in a powder form, was added to 100 ml distilled or deionized water and heated to boiling to aid in the dissolving of the indicator powder. The dissolved medium was then sterilized in the autoclave for 15 minutes at 15 pounds pressure (121° C) and cooled to 55° C in a

water bath for inoculation and dispensing into plates. Each ampule of Bacto-Thermospore Suspension PM was then shaken to obtain a uniform suspension, and added to each 100 ml of Bacto-PM Indicator Agar at 55° C. Swirling of the inoculated medium was performed to obtain a uniform suspension. Six ml amounts of this inoculated agar were dispensed into sterile 100 mm flat-bottom Petri dishes. These were immediately rotated to obtain a uniform layer of medium covering the bottom of the plates. The plates were allowed to solidify on a flat surface for 15 minutes. The dispensed and solidified plates were then placed inverted in an incubator at 55° C to 65° C for 30 minutes. After the incubation period the plates were inverted in plastic bags and stored in the refrigerator at 2° C to 8° C. These plates are satisfactory for use up to seven days.

Milk samples should be tested with the B. stearothermophilus disc assay as soon as possible, but preferably within ten hours of sampling. Samples not tested immediately should be refrigerated at 5° C. Samples not tested within ten hours should be stored frozen at -15° C to -30° C and tested as soon as possible within one week. Using clean, flamed forceps, a disc was removed from the vial of Bacto-Concentration Discs Sterile Blanks 1/2", and touched to the surface of the milk sample to become saturated by capillary action. Forceps must be rinsed in distilled or deionized

water, flamed and cooled between samples. All discs were placed on the agar surface about 9 mm from the outer rim of the plate, and at least 10 mm apart. Disks were pressed gently to obtain contact with the agar surface. Forceps were again rinsed, flamed, and cooled before another blank disc was removed from the vial, touched to the surface of the reconstituted PM Negative Control, and applied to the agar surface. This disc served as the negative control for the assay. A Concentration Disc 1/2" Penicillin .05 unit was touched to the surface of distilled water until the disc became saturated. This disc served as the positive control for the assay. In other words, these discs were a check to show that the plates were properly prepared and in working order. To determine whether an inhibition zone was due to penicillin or some other inhibitor, a Concentration Disc Penase 1/2" was touched to the test milk sample and applied to the agar surface. Penase is an enzyme which inactivates penicillin. The plates were then incubated in an inverted position for 2 hours and 50 minutes at $65^{\circ}\text{C} \pm 1^{\circ}\text{C}$.

Plates were observed for clear zones of inhibition surrounding the test sample discs, and, with the plates inverted, observed for a change in color of the medium under the sample discs (purple to yellow). The manufacturer, Difco Laboratories, states that zones will appear if penicillin is present at a level of .004 to .005 units/ml or above, and

the medium under the disc will remain purple. A clear zone of >14 mm indicates the presence of inhibitory substances. Zones of ≤14 mm are considered negative. If no zone appears, and the medium under the disc changes to yellow, no inhibitory substance is present. If the response obtained is due to penicillin, no zone will appear around the Concentration Disc Penase 1/2". If a smaller zone diameter appears around the penase disc than around the blank milk sample disc, the sample contains penicillin and some other inhibitory substance. If the same zone diameter appears around both the penase disc and the milk sample disc, the sample does not contain penicillin, but contains some other inhibitor.

DELVOTEST-P

The Delvotest P ampule method of antibiotic residue detection utilizes a culture medium containing the microorganism Bacillus stearothermophilus in sporulated form. The test was carried out according to manufacturer directions (see appendix table 1). Under optimum conditions, when extra nutrients are added and the temperature is raised to 63° C to 66° C, the microorganisms will multiply rapidly. These nutrients are added in the form of a glucose nutrient tablet containing a pH indicator. After two and one half hours, sufficient acid is produced from this rapid growth to change the color of the pH-indicator from purple to yellow, indicating a

sample free from antibiotic contamination. If the introduced milk sample contains residues of antibiotics in a sufficiently high concentration, these residues will inhibit the growth of the microorganisms. Hence, no acid will be formed, and the indicator will remain purple, indicating a sample contaminated by antibiotics.

PENZYME TEST

The Penzyme milk test was conducted according to manufacturer directions (see appendix table 2). The test relies on an enzyme activation process which causes a color change in the absence of beta-lactam antibiotics. The enzyme, carboxypeptidase, rapidly forms a stable complex with these commonly used beta-lactam antibiotics. Carboxypeptidase is an enzyme which reacts with proteins. Incubation of a specific amount of enzyme with milk containing sufficient (.004 U/ml) antibiotic, results in the inactivation of the enzyme. If this enzyme inactivation occurs, no further reaction will take place when the substrate tablet, alanine, is added. In this case the contents of the vial will remain yellow, indicating the presence of beta-lactam antibiotics. If there is no antibiotic, or an insufficient amount of antibiotic in the milk sample, the enzyme will remain active and will react with the substrate. The colorless indicators will then be oxidized and changed into a pink colored compound, indi-

cating that no beta-lactam antibiotics are present. Thus, if the milk sample is free of beta-lactam residues, the enzyme will be fully active after the first incubation, and the maximum color indicator will be formed during the second incubation; hence a definite pink color will result.

LIVE ANIMAL SWAB TEST (LAST)

The LAST was conducted according to the U.S. Department of Agriculture guidelines (53) (see appendix table 3). Cotton swabs were dipped in urine and placed on a specially prepared test plate. During a period of incubation, bacterial growth will occur on the plate. The extent of this growth indicates whether antibiotics are present in the animals carcass. Antibiotics in urine will inhibit bacterial growth on the plate. The presence of a clear zone of inhibition around the swab tips is evidence of antibiotics. A positive test indicates the presence of contaminating residues in the kidneys and meat. When antibiotics are not present in the animal, the bacterial growth will not be inhibited and will completely surround the cotton swabs on the test plate.

MATERIALS AND METHODS

EXPERIMENT 2

In the second phase of this project, the Virginia Tech dairy herd was again utilized. All cows lactating at the beginning of the experiment were aseptically sampled (composite milk samples) and the milk cultured on blood agar plates to determine the presence or absence of mastitis pathogens (existing pathogens shown in table 3). All cows were assigned to experimental or control groups based on current Dairy Herd Improvement Association (DHIA) SCC, SCC during the previous lactation, estimated relative producing ability (ERPA) for milk production, and month of lactation during the current lactation (group averages shown in table 4). Cows were grouped in this fashion to avoid differences in the variables to be measured caused by these factors, rather than by the treatment groups. First calf heifers were assigned to treatment groups based on month of lactation and estimated transmitting ability (ETA) (group averages shown in table 5).

Dairy Herd Improvement Association (DHIA) records were obtained after each monthly herd test day from the Virginia DHIA laboratory located on the Virginia Tech campus. The SCC of cows in both groups were studied, and all cows whose

Table 3. Mastitis pathogens isolated from lactating dairy cows in the Virginia Tech dairy herd at the beginning of this study.

	<u>Treatment group</u>	
	Control	Experimental
Number of cows	54	59
<u>S. aureus</u> ¹	--	1
non coagulase staph. ²	17	21
<u>Strep. spp.</u> ³	1	2
<u>Strep. ag.</u> ⁴	--	--
<u>E. coli</u> ⁵	1	1
Total	19 (17%)	25 (23%)

¹Staphylococcus aureus

²non coagulase staphylococcus

³Streptococcus (dysgalactiae, uberis)

⁴Streptococcus agalactiae

⁵Escherichia coli

Table 4. Current lactation somatic cell count (SCC), previous lactation somatic cell count, estimated relative producing ability (ERPA), and month of lactation averages of lactating cows for the two treatment groups.

Variable	<u>Treatment group</u>	
	<u>Control</u>	<u>Experimental</u>
Current SCC ¹	.22	.23
Previous SCC ¹	.08	.06
ERPA (pounds)	934.66	920.05
Month of lactation	7.59	7.98

¹ Cows coded 0 if SCC < 5, coded 1 if SCC ≥ 5 on any DHIA test day.

Table 5. Month of lactation and estimated transmitting ability (ETA) averages of primiparous cows for the two treatment groups.

	<u>Treatment group</u>	
	<u>Control</u>	<u>Experimental</u>
Month of lactation	2.00	1.91
ETA (pounds)	205.33	202.44

linear SCC $\geq$ 5 for the first time during that lactation were aseptically quarter sampled within two weeks of the DHIA test date. The linear SCC scoring system has been nationally adopted by the DHIA. A score of 5 represents those animals with counts between 283,000 and 565,000 cells/ml of milk (table 1). Cows with a score of $\geq$ 5 should be considered as having subclinical mastitis (20). The quarter samples obtained were cultured on blood agar plates to detect mastitis pathogens, then submitted to the Virginia DHIA laboratory for electronic SCC determination. Experimental group cows were infused with a commercial cephalixin preparation¹ only in those quarters with a SCC $\geq$ 5, as determined by DHIA quarter sample SCC analysis. Quarters above this threshold were treated for two milkings, beginning on the day the quarter SCC results were obtained from the DHIA laboratory. This infusion, based on elevated SCC, was conducted only once per lactation. Control group cows had antibiotic therapy administered based on clinical symptoms, as was the normal practice. Cows in either group whose quarters became swollen or hard, who had an elevated temperature, or who had other symptoms of coliform mastitis were treated accordingly by the herdsman. Three weeks after the first quarter sampling

¹ Cefa-Lak, Na cephalixin, 200 mg, Bristol Laboratories.
Syracuse, NY

the same cows were again aseptically quarter sampled. These quarter samples were again cultured on blood agar plates, and sent to the Virginia DHIA laboratory for electronic SCC analysis to determine the effectiveness of the experimental procedure in eradicating mastitis causing organisms or reducing SCC.

LABORATORY PROCEDURES

FOR THE ISOLATION OF MASTITIS PATHOGENS

PREPARATION OF AESCULIN BLOOD AGAR PLATES

Aesculin blood agar plates were prepared from BBL Trypticase Soy Agar to which ferric citrate, aesculin, distilled water, and citrated calf blood were added. One gram aesculin, .1 g ferric citrate, and 40 g BBL Trypticase Soy Agar were added to one liter distilled water and heated to boiling to facilitate the dissolving of the agar powder. This solution was then poured into 500 ml flasks, capped, autoclaved for 15 minutes at 15 pounds pressure (121° C), and cooled in a water bath to 45° C to 48° C. After cooling, 5 ml of citrated calf blood was added to each 100 ml of sterile, melted base medium. Ten to 12 ml of this medium was added to each 10 cm diameter Petri plate. Plates were immediately rotated to obtain a uniform layer of medium covering the bottom of the plate. Plates were allowed to solidify, then

were placed inverted in plastic bags, and stored at 2° C to 8° C.

ROUTINE PLATING OF MILK SAMPLES

Aseptic samples to be tested were shaken thoroughly 25 times and the foam allowed to disperse. The inoculum was transferred to the medium surface with a standard .01 ml wire loop. Each Petri plate was divided into quadrants and the milk from the four quarters of a cow were spread on a single plate. Plates were then incubated at 35° C to 37° C and examined after 18 to 24 hours for colonies of streptococci, staphylococci, coliforms or other organisms. Further procedures for the identification of specific groups or species of mastitis organisms were conducted and include: a) the CAMP-esculin test (38), used for separating Streptococcus agalactiae (*S. agalactiae*) from the other streptococcal species; b) the coagulase test (38), will distinguish Staphylococcus aureus (*S. aureus*) from non coagulase staphylococcus; and c) the MacConkey Agar Plate test (38), which distinguishes the different species of coliform bacteria from one another.

STATISTICAL ANALYSIS

EXPERIMENT 1

Three major areas were analyzed by analysis of variance procedure using the general linear models procedure of the Statistical Analysis System (50). The first two areas of interest utilized only the B. stearothermophilus Difco disc assay results, as that test is officially recognized as a standard procedure for antibiotic residue determination, whereas the Delvotest-P and Penzyme tests are not considered official tests. The first analysis examined the accuracy of the recommended drug withholding periods when cows were treated with a single antibiotic. Secondly, the drug withholding recommendations of cows treated with either one single antibiotic or any combination of antibiotics were analyzed. Lastly, a new variable, test, was brought into the model to determine if the antibiotic test utilized (either the B. stearothermophilus disc assay, the Delvotest-P, or the Penzyme Test) produced significantly different results.

Three hundred and three milk samples were collected and examined for antibiotic contamination. Data for the first analysis were obtained by editing this data set so that it included only those cows which had been treated with a single antibiotic. The set was then reedited to include only

one record per cow or per case (i.e., the day the disc assay showed no antibiotic contamination). The model $Y_{ijklmno} = \mu + D_i + R_j + C_k + L_l + B_m + N_n + M_o + E_{ijklmno}$ was used, where:

$Y_{ijklmno}$ = days until negative test result relative to the withholding period

μ = mean disc assay days

D_i = effect of drug

R_j = effect of route of administration

C_k = effect of case number (for cows treated for more than one episode)

L_l = effect of length of time treated (days)

B_m = effect of body weight

N_n = effect of lactation number

M_o = effect of milk production

$E_{ijklmno}$ = random error

The dependent variable ($Y_{ijklmno}$), was the difference between the test day after treatment on which the disc assay showed no antibiotic contamination, and the antibiotic's stated withholding time, or in other words, days relative to the stated withholding period of the antibiotic. A negative value or zero would indicate that the milk in question was free of antibiotic contamination beyond the stated withhold-

ing period, while a positive value for the days variable would indicate that the milk was contaminated with antibiotic past the stated withholding time.

The second analysis was again edited so that it contained only one record per cow or per case (for cows treated for more than one episode). This data set contained not only those cows treated with one antibiotic, but also those treated with any combination of antibiotics. The model $Y_{ijkl} = \mu + DR_i + C_j + L_k + B_l + E_{ijkl}$ was used where:

Y_{ijkl} = days relative to the withholding period

μ = mean disc assay days

DR_i = effect of drug and route

C_j = effect of case number (for cows treated
for more than one episode)

L_k = effect of length of time treated (days)

B_l = effect of body weight

E_{ijkl} = random error

The dependent variable (Y_{ijkl}) was again days relative to the antibiotic's stated withholding period. The variables drug and route were combined to form one variable, as those cows treated with more than one antibiotic usually had them administered by more than one route of administration (i.e.,

intramuscular plus intramammary) It was therefore impossible to separate combination drugs from their routes of administration.

For the third analysis, a new variable, test, was brought into the model to determine if the three antibiotic residue tests, the B. stearoothermophilus disc assay, the Delvotest-P, and the Penzyme Test, produced significantly different results. The data set included three records per cow (ie., the three days that the disk assay, the Delvotest-P, and the Penzyme Test showed no antibiotic contamination). The model $Y_{ijklm} = \mu + DR_i + T_j + C_k + L_l + B_m + E_{ijklm}$ was used where:

Y_{ijklm} = days relative to the withholding period

μ = mean days for all three tests

DR_i = effect of drug and route

T_j = effect of test

C_k = effect of case number (for cows treated
for more than one episode)

L_l = effect of length of time treated (days)

B_m = effect of body weight

E_{ijklm} = random error

The dependent variable was again days relative to the stated withholding time. The test variable was used to compare the

results of the two on-farm antibiotic residue detection tests, the Delvotest-P and the Penzyme Test, to results from the standard B. stearothermophilus disk assay.

EXPERIMENT 2

In the second phase of this study, there were again three areas of interest. The first two areas were analyzed by analysis of variance using the general linear models procedure of the Statistical Analysis System (50). The third area was analyzed by a two-way chi square, again using the Statistical Analysis System. The first analysis examined how changes in milk production were affected by cow's treatment group (either control or experimental). The second analysis was concerned with how treatment group affected the somatic cell count of each quarter within the mammary gland. Lastly, a two-way chi square was utilized to determine how treatment group affected infection status.

The first analysis determined which variables affected change in milk production from test 0 to test 2. Test 0 represented the production on DHIA test day when a cow reached the threshold of $SCC \geq 5$ for the first time during that lactation. Test 2 milk production was that same cow's production on the second test day after she reached this threshold of $SCC \geq 5$. The model for this analysis was Yijk

$= \mu + T_i + S_j + L_k + T_i * L_k + E_{ijk}$ where:

Y_{ijk} = difference in milk production between test 2 and test 0

μ = mean milk change

T_i = effect of treatment group

S_j = effect of stage of lactation

S_1 = 115 days

S_2 = 115 to 225 days

S_3 = >225 days

L_k = effect of lactation number

$T_i * L_k$ = effect of treatment group and lactation number interaction

E_{ijk} = random error

The dependent variable (Y_{ijk}) was the difference in milk production between test 2 and test 0. The same model and variables were also used to analyze the change in milk production using a multivariate analysis of variance (MANOVA). A MANOVA is used when there is more than one dependent variable. In this case, the dependent variables were the change in milk production from test 0 to test 1, and the change from test 1 to test 2. This analysis was included because the use of one dependent variable from test 0 to test 2 could mask differences which might occur between tests 0 and 1 and tests 1 and 2.

The second analysis determined which variables affected the change in SCC of each quarter within the mammary gland from the first to the second sampling. The first quarter sampling took place from five to fourteen days after the DHIA test day on which the cow obtained the threshold of SCC ≥ 5 . The second sampling was conducted three weeks after the first sample collection. The model used for the analysis was $Y_{ijk} = \mu + T_i + C(i)j + Q_k + E_{ijk}$ where:

Y_{ijk} = difference in SCC between sample 2 and sample 1 for each quarter within the mammary gland

μ = mean quarter SCC change

T_i = effect of treatment group

$C(i)j$ = effect of cow within treatment group

Q_k = effect of quarter

E_{ijk} = random error

The dependent variable (Y_{ijk}) was the difference in SCC between the first and second samplings for each quarter within the mammary gland.

A two-way chi square, with treatment group and final quarter infection status as the areas of interest, was undertaken for the third analysis. A quarter was diagnosed as infected if at least one of the following organisms was isolated: 1)

Streptococcus agalactiae, 2) Streptococcus spp. other than agalactiae, 3) Staphylococcus aureus, 4) non coagulase staphylococcus, or 5) coliforms. No other pathogens were isolated from our samples. The first quarter sampling took place from five to fourteen days after the DHIA test day on which the cow obtained the threshold of SCC $\geq$ 5. The second sampling was conducted three weeks after the first sample collection. This analysis determined whether intramammary treatment of subclinical mastitis (DHIA SCC $\geq$ 5) had an effect on infection status from sample 1 to sample 2.

RESULTS AND DISCUSSION

EXPERIMENT 1

The first analysis sought to determine which variables contributed significant variation to the dependent variable, days relative to the withholding period. For those cows treated with a single antibiotic (n=58), the drug administered to the animal was the only significant ($p<.05$) variable which affected days relative to the recommended withholding period. Table 6 presents least squares means and standard error values for each drug. Neither route of administration, case number, number of times the cow was treated, body weight, lactation number, or milk production contributed significant variation to the model ($R^2=.58$, $p<.05$).

Non orthogonal contrasts, using the Bonferroni F test, conducted on certain antibiotics are shown in table 6. Cephapi-
rin had a significantly higher ($p<.05$) value for the variable, days, than gentamicin; gentamicin had a significantly lower value for days than penicillin; gentamicin had a significantly lower value for days than the average of the other drugs; penicillin had a significantly higher days value than the average for the other drugs; and liquimycin had a significantly lower value than penicillin. Mercer (35) stated that most food animal drugs approved before 1962 have

Table 6. Least squares means and non-orthogonal contrasts of the Y variable, days in relation to the recommended withholding time, for cows treated with a single drug.

<u>Drug</u>	<u>n</u> ¹	<u>LSMEANS Days</u>	<u>SE</u>	<u>Raw mean</u>
Polymyxin B	1	-1.89	1.68	-1.00
Cephapirin	24	-0.20	0.66	0.08
Gentamicin	3	-4.29	1.15	-3.67
Liquimycin	6	-1.65	0.92	-1.00
Naquasone	4	0.79	1.22	0.00
Penicillin	15	0.50	0.67	0.80
Triple sulfa	4	-1.51	1.03	-1.00
Tetracycline	1	-1.35	1.61	-1.00

Non-orthogonal contrasts

<u>Drugs</u>	<u>F value</u>
C vs G	18.69 *
G vs P	22.04 *
L vs P	7.56 *
G vs average of all drugs	14.32 *
P vs average of all drugs	11.62 *

* Significant $P < .05$, Bonferroni $F_{.05,5,40} = 7.27$

¹Number of cows treated with each drug

not had definitive data on residue withdrawal times developed; this includes the penicillins. Results from these contrasts, indicating that penicillin is the antibiotic which most often exceeded its withholding time, support this theory.

It is interesting to note that route of administration did not contribute significant variation to the model (routes screened are shown in table 7). Different routes of administration of antibiotics have caused wide variation in the relative persistence of the amount of antibiotic found in milk (1). This theory was supported by Cannon et al. (8) who found route of administration contributed significantly to variation in the secretion of penicillin in milk. Cannon et al. (8) did observe that average daily milk production, fat percentage in milk, and body weight did not contribute significantly to the model, which supports the results obtained in this study.

A chi square analysis was conducted to determine the percentage of milk samples which were free of antibiotic contamination on or before the recommended withholding period for each antibiotic (figure 1). Day 0 represents the percentage of cows free of antibiotic contamination on the last day of the recommended withholding period. A negative value for days represents those cows which were free of antibiotic

Table 7. Least squares means of the Y variable, days in relation to the recommended withholding time, for cows treated with a single drug or any drug combinations, and their routes of administration.

<u>Drug</u>	<u>route</u>	<u>n</u>	<u>LSMEANS Days</u>	<u>SE</u>	<u>Raw mean</u>
Gentamicin (G)	Mam ¹	3	-4.46	1.54	-3.66
Cephapirin (C)	Mam	24	-0.24	0.87	0.08
Penicillin (P)	IU ²	2	0.09	2.01	0.00
Penicillin (P)	IM ³	13	0.27	0.91	0.92
Tetracycline (T)	IV ⁴	1	-1.39	2.29	-1.00
Liquimycin (L)	IV	6	-1.78	1.21	-1.00
Triple sulfa (S)	Mou ⁵	3	-1.19	1.50	-1.00
Naquasone (N)	Mou	4	0.09	1.73	0.00

<u>Drug combinations</u>					
GL	Mam, IV	6	-4.28	1.27	-3.66
TL	IU, IV	6	-1.13	1.25	-0.50
GCL	Mam, Mam, IV	4	-2.74	1.39	-2.00
All other combinations		14	0.43	.90	1.07

- ¹ intramammary
² intrauterine
³ intramuscular
⁴ intravenous
⁵ mouth

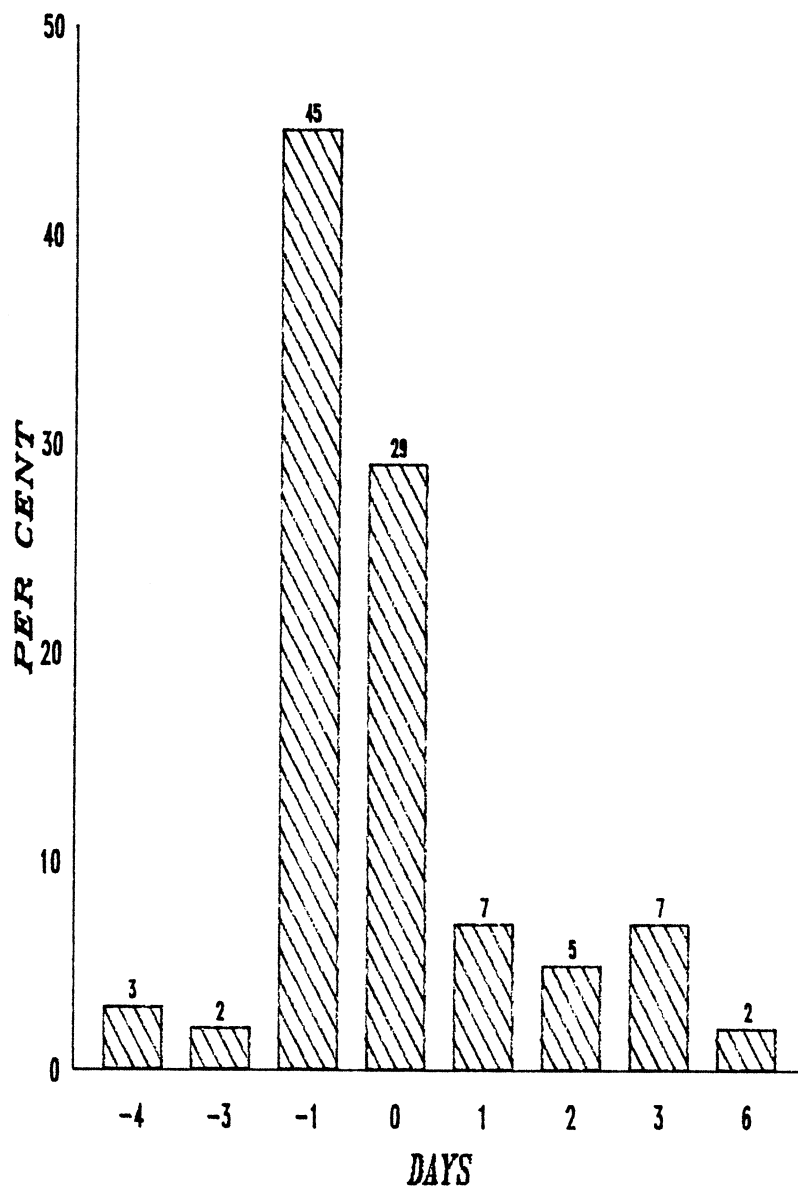


Figure 1. Distribution of the Y variable "days" in relation to the recommended withholding time for cows treated with a single drug.

contamination prior to their specified withholding period, while a positive value for days represents those cows which exceeded their recommended withdrawal periods. Results indicate that 79% of those cows treated with only one type of antibiotic showed no contamination of their milk beyond the drug's recommended withholding period. Conversely, 21% of these cows did show contamination of their milk for varying periods of time past their recommended withdrawal time. Table 8 presents the distribution of days in relation to the recommended withholding time for each drug administered singly during this study. Although the results of the two on-farm antibiotic residue tests, the Delvotest-P and the Penzyme Test, were not included in this analysis, interest was aroused as to how the antibiotic retention time of milk samples tested by these on-farm tests would compare to those of the B. stearothersophilus disc assay. Interestingly, these results were very similar to those of the disc assay: 74% and 71% respectively, as opposed to 79% for the B. stearothersophilus assay, showed no antibiotic contamination past the recommended withholding period with the Delvotest-P and the Penzyme Test respectively. Conversely, 26% and 29% exceeded the recommended period.

These findings support the conclusion by Larocque and Neville (29) that even farms with the most diligent antibiotic residue avoidance programs may be plagued by unexplained

Table 8. Distribution of the Y variable, days in relation to the recommended withholding time, for each drug administered singly during this study.

Drug	Withholding period (days)	Days	Percent
Polymyxin B	4	-1	100.0
Cephapirin	4	-1	58.3
		0	8.3
		1	12.5
		2	8.3
		3	12.5
Gentamicin	7	-4	66.7
		-3	33.3
Liquimycin	4	-1	100.0
Naquasone	3	0	100.0
Penicillin	3	0	73.3
		1	6.7
		2	6.7
		3	6.7
		6	6.7

drug residues in milk. Their theory, that most of the currently available antibiotic intramammary products were developed and introduced to the market many years ago, before comprehensive and specific testing methods were developed, may be one explanation for this high (21%) percentage of cows which exceeded their recommended withholding periods.

Milk samples were also obtained 72 hours postpartum from all cows which had been dry-treated at the end of the previous lactation to determine if the long-acting dry cow treatment was present in milk. Results showed that 3 of 62 cows (5%) showed antibiotic contamination of milk at three days postpartum using the B. stearothermophilus disc assay. All cows were free of contamination by day four postpartum. Results from the Delvotest-P showed that 6 of 62 cows (10%) showed antibiotic contamination at three days postpartum, and 8% (5 cows) still exhibited contamination on day four postpartum. Penzyme Test results showed 4 of 62 cows (6%) positive for antibiotics three days postpartum, and 5% (3 cows) were still positive on day four postpartum. Results from the B. stearothermophilus disc assay in this study support the conclusion of Pugh et al. (49) that, provided all milk is discarded for the recommended four days after calving, no antibiotic residues should be observed. Oliver et al. (40) also concluded that if manufacturer's recommendations are

followed, antibiotic residues in colostrum and milk from cows treated at drying off should not be a significant problem. Oliver et al. (40) did, however, report false positive readings in 1.6% of colostrum samples and 1.4% of milk samples with the Delvotest-P. None of these false positive readings were obtained from cows with dry periods less than 46 days. The authors theorized that, in some instances, natural inhibitors in colostrum and milk may produce false positive Delvotest-P reactions. Results of this study show similar problems with positive results from both the Delvotest-P and the Penzyme Test which were negative for the B. stearothermophilus disc assay. Such positive results from these on-farm antibiotic residue tests may question the value of testing cows postpartum unless the dry period is shorter than the recommended withholding period.

The accuracy of the recommended drug withholding periods for cows treated with either a single drug, or any combination of drugs were examined (n=86). The only significant ($p<.05$) variable was drug-route ($R^2=.43$). Table 7 shows least squares means and standard error values for each drug-route. As in the analysis conducted on cows treated with a single antibiotic, neither case number, length of time treated, nor body weight contributed significant variation to the model.

Chi square analysis showed that the percentage of milk

samples which were free of antibiotic contamination on or before the end of the recommended withholding period for combination treatments were similar to that for cows receiving only one antibiotic. Figure 2 shows that of those cows receiving either one antibiotic or any antibiotic combination, 76% showed a negative value or zero for the variable, days, indicating that milk from these cows was free of antibiotic contamination on or before the end of the recommended withholding period. Conversely, 24% of these cows showed a positive value for the days variable, indicating that they exceeded the recommended withholding period. Table 9 shows the distribution of days in relation to the recommended withholding time for those drug combinations administered during this study.

The results of the B. stearothersophilus Difco disc assay were compared to those of the two on-farm antibiotic residue detection tests, the Delvotest-P and the Penzyme Test. The B. stearothersophilus disc assay is the only officially recognized residue detection test at this time, and it was of interest to determine the accuracy of the two on-farm tests as compared to this assay. Those cows treated with only one antibiotic were again analyzed, with the addition to the model of the variable, test. If this variable was significant, then it could be concluded that the three test results, the B. stearothersophilus, the Delvotest, and the

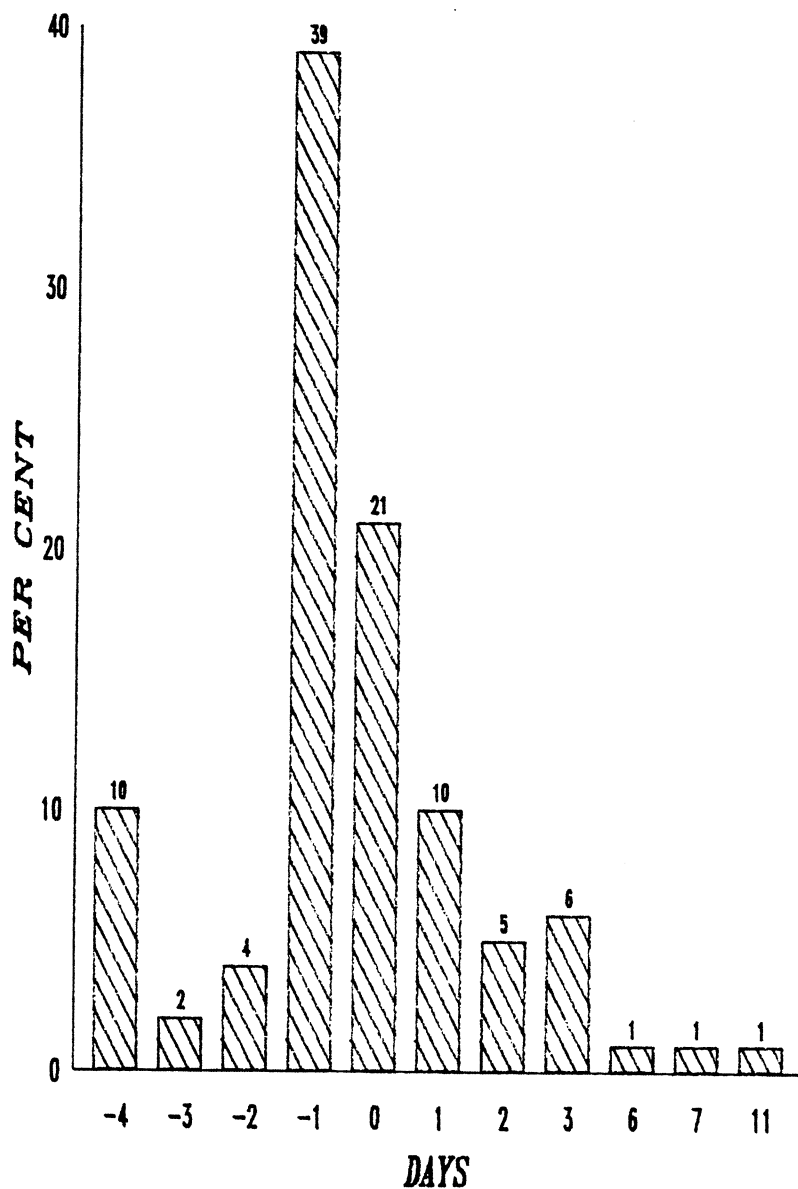


Figure 2. Distribution of the Y variable "days" in relation to the recommended withholding time for cows treated with either a single drug or any combination of drugs.

Table 9. Distribution of the Y variable, days in relation to the recommended withholding time, for each antibiotic combination.

Drug	Withholding time (days)	Days	Percent
Gent + Liqu ¹	7	-4	83.3
		-2	16.7
Liqu + Tet ²	4	-1	66.7
		0	16.7
		1	16.7
Gent + Liqu + Ceph ³	7	-4	25.0
		-3	25.0
		-2	25.0
		1	25.0
All other combos		-4	7.1
		-2	7.1
		-1	35.7
		1	21.4
		2	7.1
		3	7.1
		7	7.1
		11	7.1

¹ Gentamicin + liquimycin combination

² Liquimycin + tetracycline combination

³ Gentamicin + liquimycin + cephapirin combination

Penzyme, were significantly different. Conversely, if this variable was not significant, it could be concluded that the three test results were not statistically different. The variable, test, was not significant ($p < .05$). Least squares means for the three tests are presented in table 10. Contrasts between the B. stearothersophilus assay and Delvotest, and the B. stearothersophilus and Penzyme, supported the analysis of variance findings that there was no significant difference between test results.

Although the test variable was not statistically significant, a chi square analysis was conducted to determine the percentage of Delvotest and Penzyme test results which were the same as the B. stearothersophilus assay results. Only 78% of Delvotest-P results were exactly the same as B. stearothersophilus results; 5% showed differences in negative results; and 17% showed differences in positive results (Figure 3). Similarly, in a comparison of the Penzyme test results to those of the B. stearothersophilus (Figure 4) 79% of Penzyme test results were exactly the same as those of the B. stearothersophilus, 4% showed differences in negative results, and 17% showed differences in positive results.

The same statistical analyses were carried out on results from those cows treated with any combination of antibiotics. The variable test was not significant, indicating that

Table 10. Least squares means and non-orthogonal contrasts of the Y variable, days in relation to the recommended withholding time, of the three antibiotic residue tests for cows treated with a single drug.

Test	Days	SE
<u>B. stearothermophilus</u>	-0.96	0.43
Delvotest P	-0.77	0.43
Penzyme	-0.75	0.43

Test	Non-orthogonal contrasts	F value
<u>B. stearothermophilus</u> vs Delvotest P		.59 *
<u>B. stearothermophilus</u> vs Penzyme		.70 *

* non significant, $p > .05$, Bonferroni $F .05, 2, 150 = 5.00$

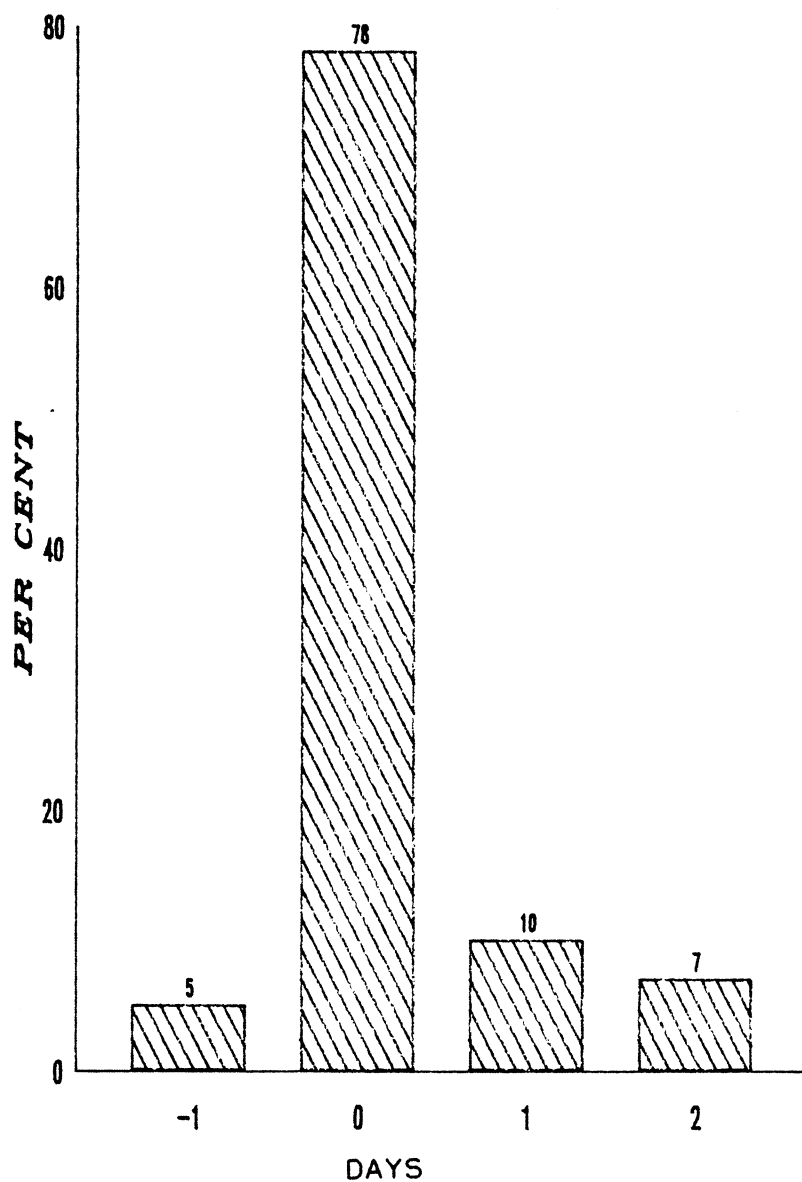


Figure 3. Comparison of differences between the Delvotest-P and the Bacillus stearothermophilus disc assay for number of days until a negative test result was observed when cows were treated with a single drug.

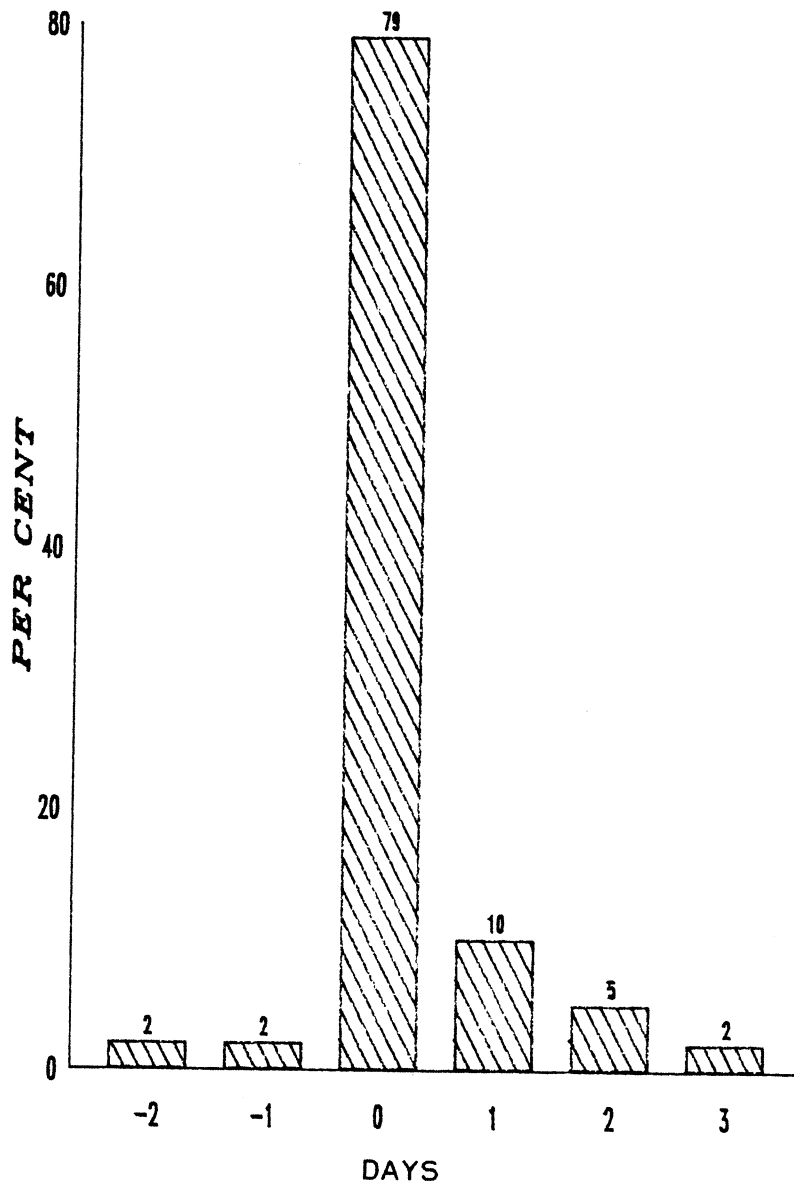


Figure 4. Comparison of differences between the Pezyme test and the Bacillus stearothermophilus disc assay for number of days until a negative test result was observed when cows were cows treated with a single drug.

results from the three tests were not statistically different. Least squares means were generated and contrasts conducted between the B. stearothermophilus and Delvotest, and the B. stearothermophilus and Penzyme test results (table 11). Results of chi square analyses, conducted to determine the percentage of on-farm residue tests having the same results as the official B. stearothermophilus disc assay, are presented in figures 5 and 6. Figure 5 shows 79% of Delvotest results to be the same as B. stearothermophilus results, 3% to show differences in negative results, and 18% to show differences in positive results. Figure 6 indicates that 81% of Penzyme results were the same as those of B. stearothermophilus, 3% showed differences in negative results, and 16% showed differences in positive results.

Although literature on the accuracy of the Penzyme Test is scarce, results from this study support the conclusion of Amodio et al. (2) indicating that, although the Penzyme Test is quick, it was less accurate than the B. stearothermophilus disc assay. One further problem observed with the Penzyme Test in this study was the difficulty in reading the color results of the test. Resulting colors are very light pastel shades of pink and yellow. People have reported difficulty in reporting the results of the test. Those with any degree of color blindness may be completely unable to decipher the test results. The Penzyme Test is very rapid

Table 11. Least squares means and non-orthogonal contrasts of the Y variable, days in relation to the recommended withholding time, of the three antibiotic residue tests for cows treated with either a single drug or any drug combinations.

Test	Days	SE
<u>Bacillus stearothermophilus</u>	-1.30	0.54
Delvotest P	-1.09	0.54
Penzyme	-1.10	0.54

Test	Non-orthogonal contrasts	F value
<u>B. stearothermophilus</u> vs Delvotest P		.45 *
<u>B. stearothermophilus</u> vs Penzyme		.41 *

* non significant, $p > .05$, Bonferroni $F .05, 2, 233 = 5.00$

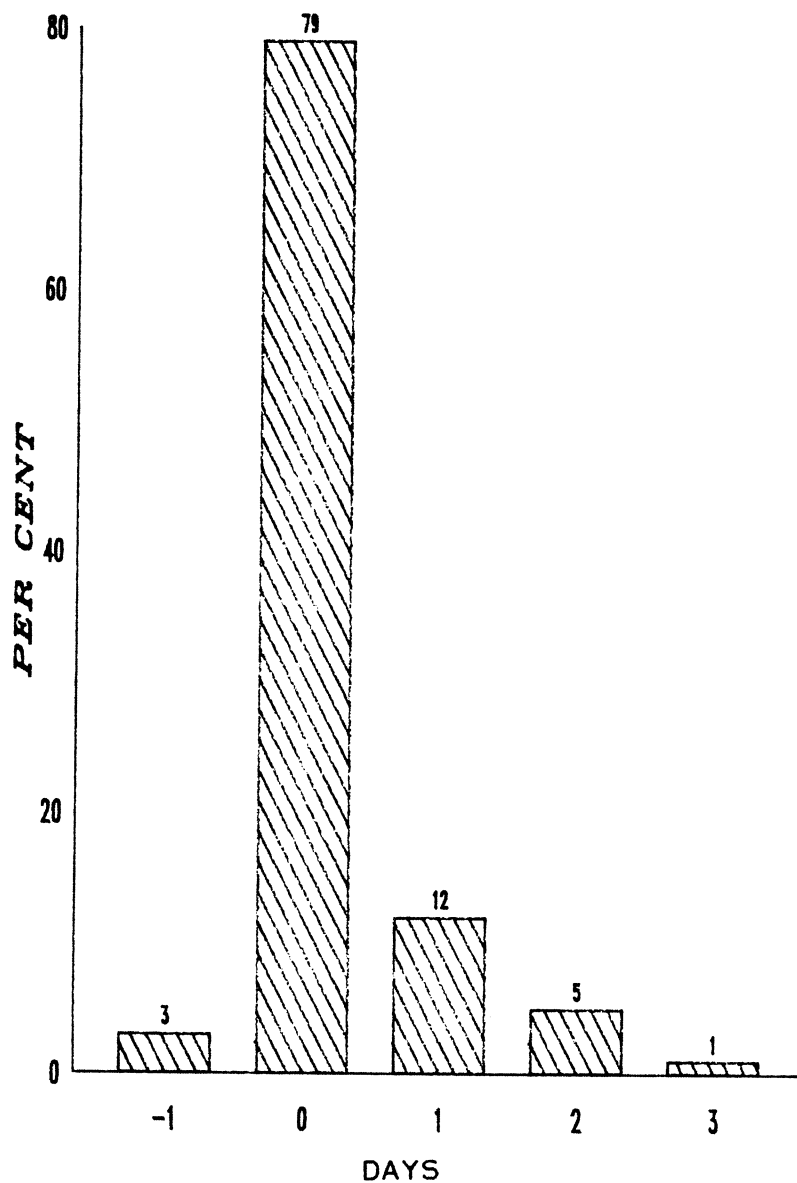


Figure 5. Comparison of differences between the Delvotest-P and the Bacillus stearothermophilus disc assay for number of days until a negative test result was observed when cows were treated with either a single drug or any combination of drugs.

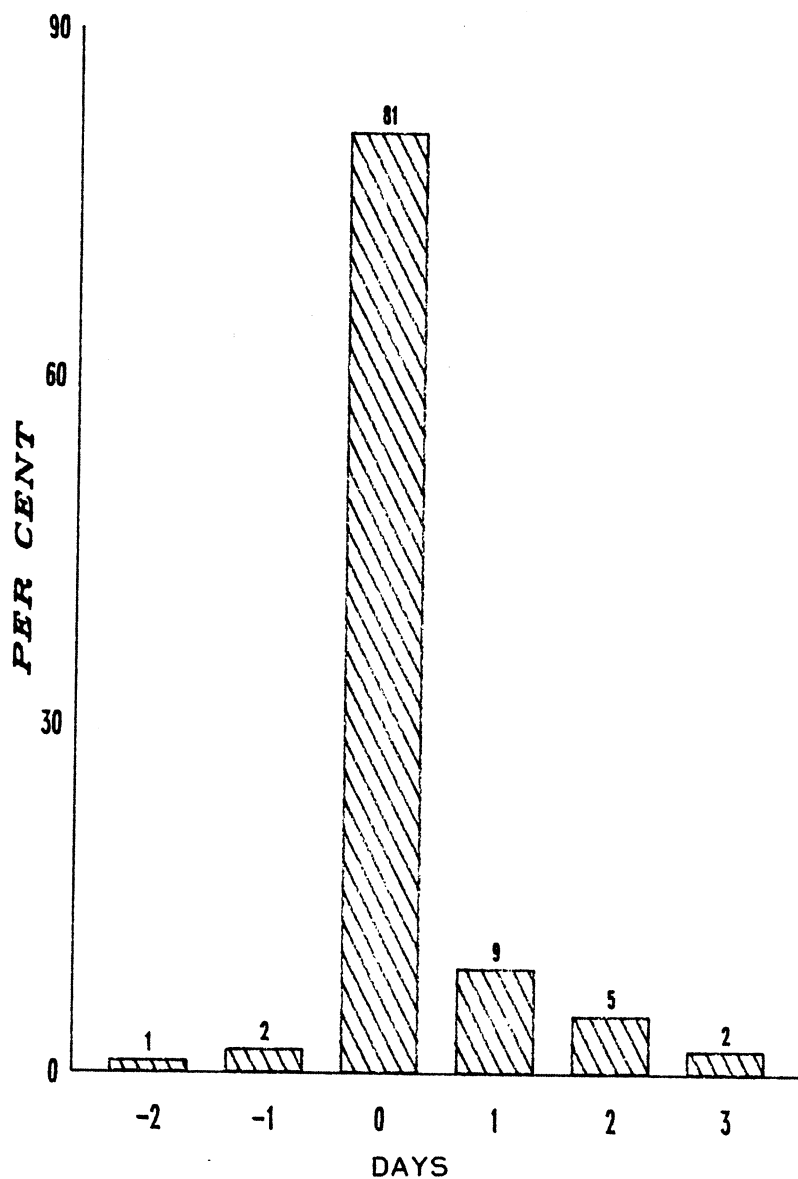


Figure 6. Comparison of differences between the Penzyme test and the Bacillus stearothermophilus disc assay for number of days until a negative test result was observed when cows were treated with either a single drug or any combination of drugs.

(20 minutes), thus, if this color problem were corrected, it might increase this test's marketability.

Although results from the Delvotest-P were not significantly different from those of the B. stearothermophilus, there was a considerable difference in positive readings. In other words, the Delvotest-P indicated a positive result, while the B. stearothermophilus assay showed a negative reading for the same sample. There are two possible explanation for this discrepancy in results. One is that the Delvotest-P is more sensitive than the B. stearothermophilus assay. This is a possibility, as the AOAC recognizes a detection level of .007 IU/ml for the Delvotest (28), and a level of .008 IU/ml for the B. stearothermophilus disc assay (36). Whether this is the case or not, it is the B. stearothermophilus which is recognized in the PMO, and therefore it is considered the standard in regards to antibiotic contamination of milk. Another possible explanation for the discrepancy is that the Delvotest may sometimes deliver a positive reading even though no antibiotic residues are present. Macaulay and Packard (32) found 11% false positive results on negative control samples (milk containing no antibiotics) with the Delvotest-P. Coulson and Rochford (10) observed that changes in the pH of milk samples due to transport and storage may influence Delvotest-P results. They concluded that it is important to realize that false positive results do occur in

samples screened by the Delvotest-P, and that this should be kept in mind when unexpected positive results are reported by laboratories during routine screening with this test. Samples obtained during this study were transported one mile to the laboratory, and analyzed within three hours. As previously reported, Oliver et al. (40) theorized that natural inhibitors in milk and colostrum may produce false positive Delvotest reactions.

Results from this study indicate that there is no significant difference between the two on-farm residue tests, the Delvotest-P and the Penzyme Test, and the official B. stearothermophilus disc assay. It should be noted, though, that differences in positive results may occur with either of these tests. The number of differences in negative test results obtained from both on-farm tests was almost negligible, however, indicating that these tests seldom miss positive samples.

Urine samples were collected from cull dairy cows prior to slaughter, and tested with the LAST to detect antibiotic residues. Results indicated that 15 of 24 cows (62.5%) showed antibiotic contamination of urine regardless of the fact that all animals had completed the recommended withholding period specified for each antibiotic administered. Three of these positive results were obtained from cows

which had never been treated with any antibiotic. No false negative results were observed.

Findings in this study support those of Oliver et al. (41) that there appears to be an inordinately high percentage of positive results with the LAST. Treatment records were available on the cows in this experiment, and indicate that all drug withholding times had been fulfilled. Kidney or other tissues were not sampled at slaughter.

EXPERIMENT 2

Neither treatment group (control or experimental), stage of lactation, lactation number, or treatment group * lactation number interaction contributed significantly to the change in milk production from test 0 to test 2 ($R^2=.59$, $n=23$). Least squares means and standard errors for the two treatment groups are shown in table 12. Similarly, MANOVA test criteria showed no significance with the Wilk's Criterion for the variables: treatment group, stage of lactation, lactation number, or treatment * lactation number interaction. This test allows the use of two dependent variables: the change in milk production from tests 0 to 1, and tests 1 to 2. The use of one dependent variable, the change from tests 0 to 2, could mask changes between tests 0 and 1, and tests 1 and 2. Results indicate that this did not occur.

Table 12. Least squares means of the difference in DHIA milk yield between the DHIA test day of threshold somatic cell count and the second succeeding DHIA test day.

Treatment group	Milk difference(kg)	SE
Control	-5.03	1.49
Experimental	-3.56	1.58

Clinical mastitis is nearly always preceded by subclinical infection (11). Further, Blosser (6) concluded that reduced milk production from cows with subclinical mastitis was responsible for the largest economic losses from mastitis. There is speculation that treating udder infections as soon as possible (ie., as subclinical mastitis) after they are detected may prevent extensive tissue damage, and hence loss of milk production, to make this type of lactation therapy cost effective. McDermott et al.(34) administered antibiotic therapy to cows with subclinical mastitis, based on elevated SCC, and found no increase in milk yield. They did report a higher number of new quarter infections during the study in the experimental group (480), as opposed to the control group (353). This higher incidence of infected quarters by major pathogens could have contributed to the lack of response in milk production in the experimental group. Whether this disparity in infected quarters was a result of the treatment of the experimental cows, or that, by chance, cows in the experimental group were more susceptible to infection, is unclear. Possibly, cows with a high SCC in the previous lactation were not divided evenly between the two study groups which would have avoided having a greater number of susceptible cows in one group. One of the criteria for assigning cows to either control or experimental groups in this study was SCC in both the current and previous lac-

tations. Even though this precaution was taken, results from this study support those of McDermott et al. (34). Treating cows for subclinical mastitis based on elevated SCC did not significantly affect differences in milk production. Cow within treatment group was the only variable which approached significance ($p < .07$) and affected the change in SCC of each quarter within the mammary gland from sample 1 to sample 2 ($R^2 = .80$). Neither treatment group nor quarter (ie., right front, right rear etc.) contributed significant variation to the model. Least squares means and standard error values for the treatment groups are shown in table 13. Since this variable was not significant, it can be concluded that treating lactating cows for mastitis based on elevated SCC does not significantly lower SCC from sample 1 to sample 2.

Research has shown that the probability of infection increases as SCC rises, and milk secretion decreases with increasing SCC (33). As in this study, Timms and Schultz (52) found no significant decrease in SCC on successive DHIA test days in relation to treatment for high SCC. The authors did, however, observe a significant decrease in SCC, and post-treatment milk yields which returned almost back to normal for those cows treated for clinical mastitis. This was not the case in this study. Since the variable treatment group was not significant, it was shown that there was no

Table 13. Least squares means of the difference in quarter somatic cell count between samples prior to treatment and samples obtained three weeks posttreatment.

Treatment group	SCC difference($\log_2$)	SE
Control	-1.27	0.49
Experimental	-1.98	0.43

difference in SCC between cows treated based on elevated SCC and those treated for clinical symptoms of mastitis.

Two way chi square analysis showed treatment group to approach significance ($p < .1$) on change in infection status (infected to cured) from sample 1 to sample 2. After the first sampling, prior to any treatment, there were found to be a total of 40 infected quarters; 20 of these were in the control group and 20 were in the experimental group. Cows were then either treated based on clinical signs of mastitis (control group) or treated in those quarters with elevated SCC (experimental group). After the second sampling, three weeks later, there were 19 quarters which had been cured and 21 quarters still infected. Of the 19 cured quarters, 7 (37%) belonged to the control group, while 12 (63%) belonged to the experimental group (Figure 7). Conversely, of the 21 quarters still infected, 13 (62%) belonged to the control group, while 8 (38%) belonged to the experimental group (Figure 8).

Postle , as reported by Postle et al. (48), conducted a study of four herds and concluded that within each herd, there was a significant relationship between the presence of both medium and high leukocyte concentrations and the isolation of pathogenic bacteria. Further, it has been suggested (11,34) that treatment of subclinical infections may reduce

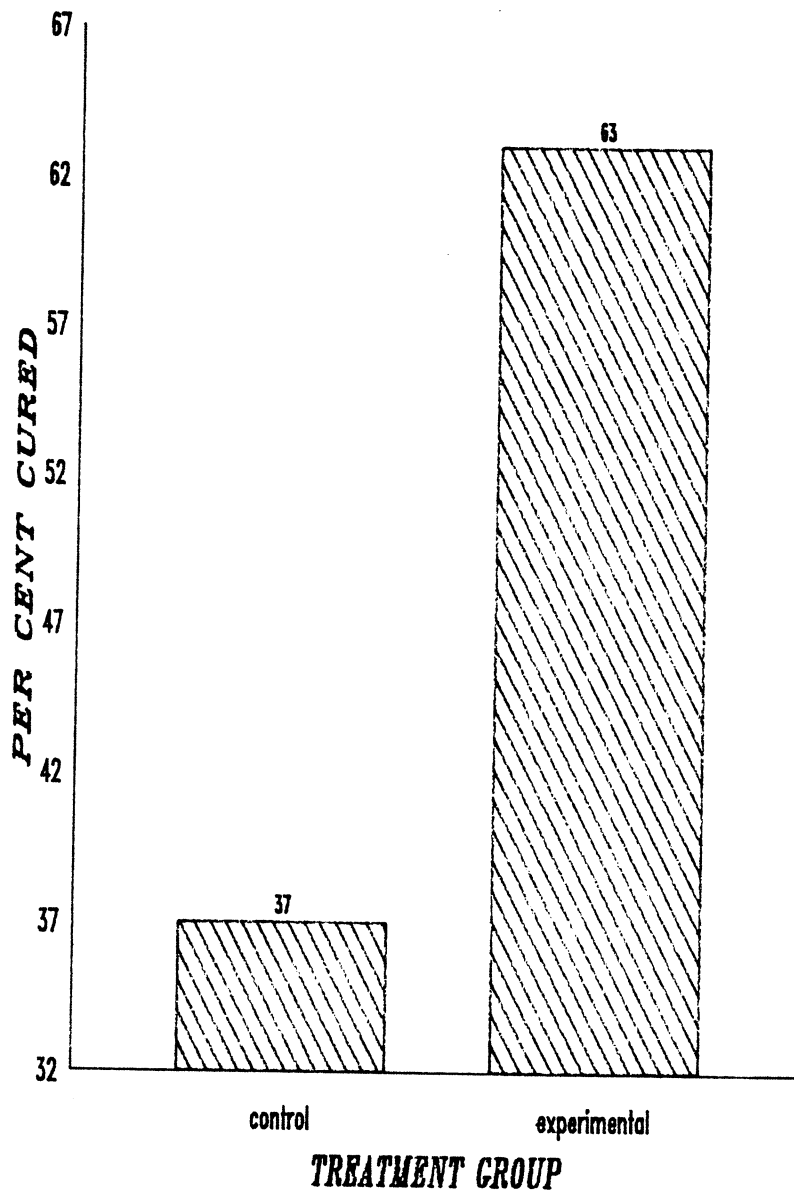


Figure 7. Distribution of cured quarters in the two treatment groups.

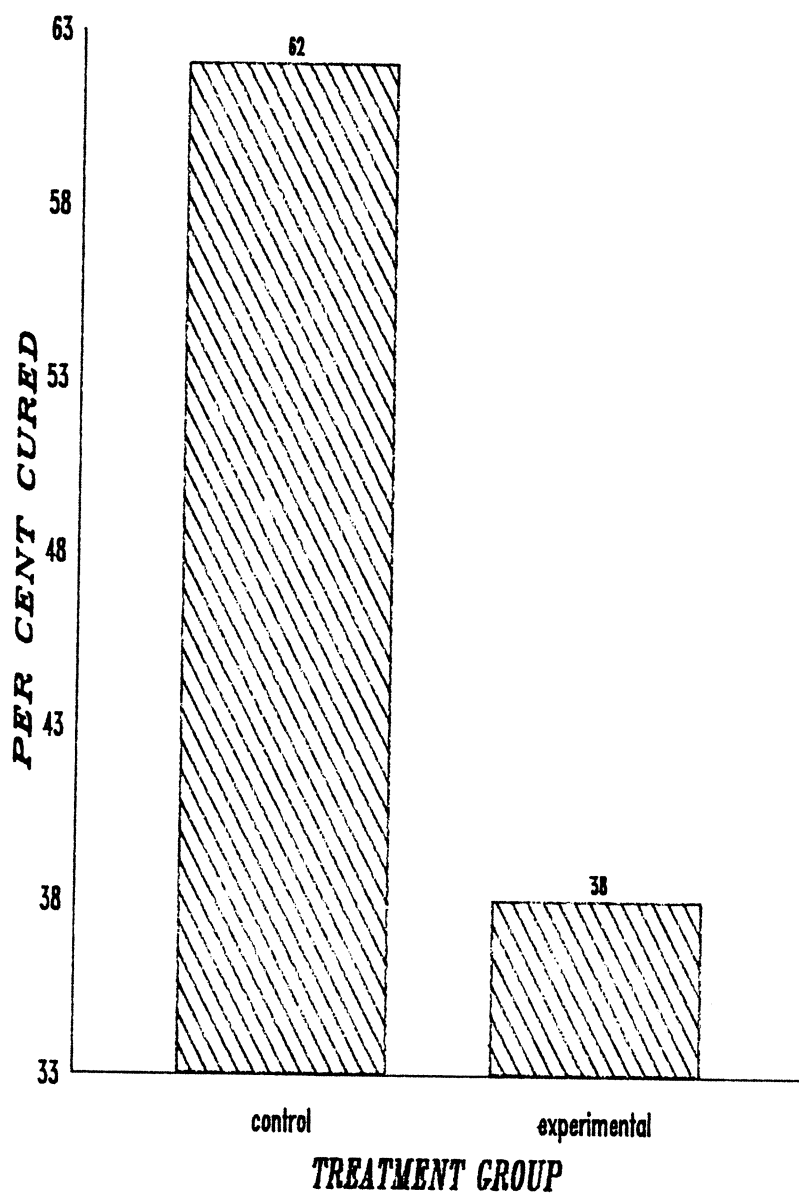


Figure 8. Distribution of infected quarters in the two treatment groups.

both the rate of new infections and their duration. If this is the case, then treating quarters with high SCC scores should reduce the number of quarters infected with pathogenic bacteria. Results from this study support this theory. Treating those quarters with elevated SCC did significantly ($p < .1$) decrease the number of infected quarters. These results are not contradictory of the results from the analysis conducted on milk production. Wheelock et al., as cited by Janzen (17), noted that losses in milk yield from mastitis were not recovered even during the following lactation, although the infection had been eliminated. Eliminating infection does not necessarily mean the recovery of normal milk production. Heider and Bashandy (15) suggested a use for the DHIA SCC which may be more effective than simply treating all cows with an elevated SCC. Milk from these cows might be regularly (monthly) cultured on blood agar plates to determine infection status. This information could then be used to recommend that cows be culled, dried off and treated, or treated during lactation. More work must be conducted in this area, but this may be a more effective use of the DHIA SCC than treating all cows with subclinical mastitis.

SUMMARY AND CONCLUSIONS

EXPERIMENT 1

The objectives of this study were to determine with what frequency antibiotic residues occur in milk, and to compare the accuracy of two on-farm antibiotic residue tests, the Delvotest P and the Penzyme test, to that of the officially recognized Bacillus stearothermophilus disc assay.

The Virginia Tech dairy herd, consisting of approximately 200 milking cows, was used to carry out this study. Composite milk samples were taken 72 hours post-treatment from all cows receiving antibiotic treatment for any reason and by any route of administration. Samples were also obtained 72 hours postpartum from all cows which had been dry-treated at the end of the previous lactation. All milk samples were transported to the laboratory after collection for analysis by the three different antibiotic residue detection tests.

Analysis revealed that the type of antibiotic administered to the cow was a major factor in the actual drug withdrawal period. Penicillin was found to be the antibiotic which most often exceeded its recommended withholding period. Route of administration was not a significant factor in residues detected at the end of the withholding period. A surprising

21% of cows treated with only one antibiotic and 24% of all cows treated, including those treated with any combinations of antibiotics, exceeded their recommended withholding times with the B. stearothersophilus Difco disc assay.

In a comparison of the two on-farm residue detection tests, the Delvotest P and the Penzyme test, to the official B. stearothersophilus disc assay, no significant difference in test results was observed. Statistically, the two on-farm tests provided comparable results to the B. stearothersophilus assay. Further analysis did show, however, a considerable difference in positive results with both the Delvotest P (17%) and the Penzyme test (16%). The number of differences in negative results was very low, however, indicating that these tests seldom miss antibiotic contaminated milk samples.

In the study using the Live Animal Swab Test to detect antibiotic residues in the urine of cull dairy cows prior to slaughter, results showed an inordinately high (62.5%) level of positive results. It would appear that this test is not specific enough for antibiotics, as three of the cows testing positive had never been treated, and all cows testing positive had completed the recommended withholding period specified for each antibiotic administered. No false negative results were observed, but due to the high percentage

of positive results with the LAST (62.5%), this test is not recommended for on-farm use.

The need for a reliable on-farm antibiotic residue test has been demonstrated by this study. Results show that both the Delvotest P and the Penzyme test provided reliable information on the antibiotic status of milk. Although both tests showed a considerably higher level of positive results, as compared to the B. stearothermophilus disc assay, the number of differences in negative samples was negligible. A difficulty in deciphering the Penzyme test due to light colored results was the one problem encountered with this on-farm residue test. Results from the LAST on urine revealed its inadequacy, at this time, for on-farm use.

EXPERIMENT 2

The benefits of treating subclinical mastitis, by treating those specific quarters with elevated SCC, as opposed to treating cows based on clinical signs of mastitis were explored. Specific objectives sought to determine the effect of this practice on milk production, SCC, and infection status.

The Virginia Tech dairy herd was utilized to conduct this study. Cows in the herd were assigned to experimental or

control groups based on current DHIA SCC, SCC during the previous lactation, ERPA for milk production, and months into lactation. All cows in the experimental group with a SCC score of ≥ 5 for the first time during that lactation were treated with a commercial cephalosporin preparation in those quarters with elevated SCC. This infusion, based on elevated SCC, was conducted only once per lactation. Control group cows had antibiotic therapy administered based on clinical symptoms, as was the normal practise.

Treatment group had no significant effect on milk production or SCC. It may therefore be concluded that treating mastitis based on elevated SCC did not significantly increase milk production or decrease SCC, although trends for decreased SCC and decreased loss in milk yield were evident. Treatment group did, however, significantly alter infection status. Of the quarters cured of infection, a greater percentage (63%) belonged to the treatment based on elevated SCC (experimental) group than to the treatment based on clinical symptoms (control) group. Conversely, of the quarters which remained infected, a far greater percentage (62%) belonged to the control or treatment based on clinical symptoms group than to the experimental group.

Results from this study indicate that even though milk production and SCC were not significantly affected by treatment

group, the number of quarters infected by major pathogens was significantly reduced in the experimental group. One problem noted in this experiment was a lag time of five to fourteen days between the time of DHIA sampling and receipt of the SCC results. Timms and Schultz (52) noted this problem in a similar experiment. An alternative to treating all cows with elevated SCC was suggested by Heider and Bashandy (15). They indicated that milk from cows with elevated SCC might be regularly (monthly) cultured on blood agar plates to determine infection status. This information could then be used to recommend that cows be culled, dried off and treated,, or treated during lactation. At the present time, this would appear to be a more effective use of the DHIA SCC than simply treating all cows with elevated SCC.

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APPENDIX A. MANUFACTURER DIRECTIONS FOR THE DELVOTEST-P

The Delvotest-P ampule test came with 100 ampules containing *Bacillus stearothermophilus* spores in solid medium, 100 glucose based nutrient tablets with a pH indicator incorporated, a dosing syringe for sampling milk, 100 disposable pipette tips, and one set of tweezers. In addition to these, a Delvotest block heater was purchased for sample incubation.

Ampules and unopened bottles of nutrient tablets were kept refrigerated until use. Twenty minutes prior to testing, the block heater was plugged in to obtain the proper temperature of $65^{\circ}\text{C} \pm 1^{\circ}\text{C}$. To begin the test, an ampule tip was snapped off and one nutrient tablet was added to the ampule with the supplied tweezers. The nutrient tablets should not be touched by hand. A fresh pipette tip was then placed on the syringe, and the prescribed amount of milk (.1 ml) was withdrawn. Each sample of milk must be placed in a separate ampule. This ampule was placed in the block heater and incubated for two hours and thirty minutes. Upon completion of this incubation time, the color of the solid medium was observed: A yellow color of the medium indicates a negative result; a purple color indicates the presence of antibiotics; and a yellow/purple color indicates a doubtful result.

APPENDIX B. MANUFACTURER DIRECTIONS FOR THE PENZYME TEST

The Penzyme milk test came with 25 enzyme vials in a plastic tray, 25 reagent tablets in a strip pack, and a spring syringe with 25 disposable tips. In addition to these, an incubator was required to conduct this test. An incubator specially designed for the Penzyme milk test was purchased from Smithkline Beckman Corporation, the manufacturer of this test.

To begin the test, the incubator was plugged in and allowed to preheat for approximately 20 minutes to the desired temperature of 47° centigrade. The tear-off seal and rubber cap were carefully removed from the enzyme vial. A fresh disposable tip was placed firmly on the spring syringe. The syringe plunger was then completely depressed, and the end of the disposable tip placed one-half inch into the milk sample. The plunger was then allowed to gradually return to its original position under pressure of the spring. The correct sample size is .2 ml of milk, and this level should almost fill the disposable tip. The milk sample was then transferred to the enzyme vial by slowly depressing the syringe plunger. After placing the recapped vial into the preheated incubator, the sample was incubated for five minutes. After the five minute incubation, one reagent tablet was added to the vial, and the contents gently rotated to

facilitate tablet disintegration. Incubation of the vial with the reagent tablet was then continued for an additional 15 minutes. Five minutes into the incubation, the vial was removed and gently swirled again to aid tablet disintegration. After the entire 15 minute incubation was complete, the vial was removed from the incubator, and the results determined from the color of the contents of the vial. A pink color indicates a negative result (no antibiotics present), a yellow color indicates a positive result (antibiotics present), and intermediate shades indicate residue levels of approximately .004 IU/ml to .012 IU/ml. The test should be read immediately after the last 15 minute incubation as colors will change and darken with time.

APPENDIX C. DIRECTIONS FOR THE LIVE ANIMAL SWAB TEST

The LAST was developed by the United States Department of Agriculture, but two companies are currently handling complete kits for the test^{1,2}. One kit may be used for ten tests, and includes ten agar plates which support bacterial growth, 24 cotton tipped swabs, 20 containers for urine samples, One vial of neomycin control discs, one vial of bacterial spores, one ruler, one marking pen, and one pair of forceps. An incubator is also required, and may either be purchased from the companies listed, or assembled at home.

One hour prior to testing, the incubator was plugged in to obtain the required temperature of 82° to 86° F (29° to 31° C). To begin the test, the LAST plate was prepared by completely streaking the plate with bacterial suspension using cotton swabs. The neomycin disc was then positioned on the test plate using the supplied tweezers. After that step the preparation of the test plate was complete and the actual testing of the urine sample began. Two fresh swabs were removed from their paper wrappers and dipped into the urine sample. Excess urine was gently shaken off, and the shafts of the swabs were broken as close to the cotton tip as

¹ Northeast Laboratory Services, China Road, Waterville, ME

² J & L Analytical Services, 2068 Laphan Drive, Modesto, CA

possible without touching the tips. The swabs were then placed next to the neomycin disc in rabbit ear configuration.

Plates were incubated for 18 to 24 hours. Upon completion of this required incubation time, the plates were observed for zones of inhibition around the cotton swab tips. The warmth of the incubator allows the bacteria to grow, but the presence of antibiotics interferes with bacterial growth and causes clear zones of inhibition. A urine sample containing antibiotic residues is characterized by clear zones around the swab tips as well as a clear zone around the neomycin disc between 10/16" and 15/16" (16 to 24 mm). Animals should not be marketed until the test produces negative results.

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