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PROBLEMS WITH INDIVIDUAL ANIMAL ANTIBIOTIC RESIDUE TESTING
[SOLUTIONS AND RECOMMENDATIONS]

James S. Cullor DVM, Ph.D.
UC Davis School of Veterinary Medicine
Davis, CA 95616-8739

SUMMARY:

The continued regulatory emphasis on food safety issues has initiated a series of programs that increase the possibility that antibiotic residue violations can be detected when present in milk or meat. This necessitates that dairy producers and their veterinarians continue to evaluate new approaches to antibiotic use on the dairy. This series of initiatives has also matured the premise that Food Safety Begins On The Dairy, an effort that is now an integral part of the daily approach for dairy production.

The emphasis on Preharvest Food Safety has magnified the effort to detect the presence of violative antibiotic residues in the milk of individual animals before they are permitted to go back into production. Therefore, a principal goal of individual animal or cowside testing for various types of residues is to serve as a beneficial aid in the production of milk free of violative levels of antibiotic or other categories of residues.

However, a serious problem has arisen in that many of the antibiotic residue test kits currently on the market yield an enormous number of false-positive test results on individual animal milk samples. These test kits have been validated under an old in vitro model validation protocol that has little, if any biological relevance to the dairy cow.

- The scenario: It has been well documented that several antibiotic residue assays currently on the market incorrectly identify antibiotic-free individual animal milk samples as "assay positive" for the presence of antibiotic residues, while at the same time, FDA bulk tank assays in certified laboratories identify the milk as safe (i.e. not containing violative levels of antibiotic residues). If we pull together a consumer advocate, an investigative reporter, and one of these test systems, the group can hire a certified laboratory to perform the assay on individual cows and on the bulk tank sample from these cows. Even though the treatment records reveal that none of the animals being milked have not been treated, it is possible for the assay results to indicate that 30% of the cows in the milking string are contributing antibiotics to the bulk tank; however, the "validated" regulatory bulk tank test, indicates that no antibiotic residues are present. It becomes suspected by the group now that this recommended individual animal test kit has revealed that the government's bulk tank test is not "sensitive" enough to pick up this serious problem. It is easy to predict what will happen next:

a) The media picks up "the fact" that antibiotics are in our milk supply, and the group judges that the regulatory test cannot detect them.
b) They also learn that mastitis occurs on every dairy and they can document a $2 billion dollar/year antibiotic sales to animal agriculture.

c) It is thought that the regulatory agencies test the bulk tank or tanker truck because this allows for a "dilution factor" that will hide the real story concerning antibiotics in milk.

- The result will be an enormous, and perhaps irreparable blow to the level of trust between the consumer and FDA, dairy producers, and veterinarians. There already seems to be a moderate level of distrust between the consumer and pharmaceutical companies. The consumer will no longer trust any of these groups, and will lose confidence in the ability of these groups to provide a safe, and wholesome product for their children. The 10-point Milk and Dairy Beef Residue Avoidance Program will lose acceptance in the dairy industry because of the poor test kit performance. And finally, the welfare of the individual dairy cow is at risk because multiple positive assay outcomes after recommended antibiotic withdrawal times will result in her being sent to the slaughterhouse. In this case, the false-positive assay outcomes result in the untimely deaths of thousands of dairy cows.

Recommendations:

- Separate residue validation protocols must be developed for the following assay categories:
  
  a) Tanker truck
  b) Bulk tank
  c) Individual animal [this includes cowside assays]

- It is suggested that a more complete and informative label be required for each category above.

- Regulatory and laboratory groups should implement residue validation protocols that have been suggested by university research scientists and the Research Committee of the National Mastitis Council

* Author's note: Productive meetings concerning these recommendations have been carried out between CVM/FDA and NMC research scientists.

FALSE-POSITIVE ASSAY OUTCOMES: "WE'RE ERRING ON THE SAFE SIDE?"

- The current Pasteurized Milk Ordinances have created an increased probability that an antibiotic residue violation can be detected when present in the milk. This necessitates that dairy producers and their veterinarians take more deliberate approaches to antibiotic use on the dairy.
• One goal of individual animal testing or cowside tests for antibiotic residues, is to assist in the production of nonviolative, or antibiotic residue-free milk.

• The reliability of a "antibiotic residue assay positive" outcome is important to the dairy industry in assessing appropriate management decisions to assure a safe product is being delivered to the processing plant.

• Diagnostic tools employed in medical practice are regarded as a means of reducing the uncertainty in diagnosis. In this discussion, the antibiotic residue test assays are employed to make decisions concerning food safety issues and recommendations. False-positive test outcomes increase the level of apprehension surrounding these recommendations, and put the veterinarian at risk for litigation.

• False-positive assay outcomes: What is their true impact?

a) They lead to unwarranted waste of milk and economic loss for the producer and consumer

b) The socioeconomic impact can harm the dairy industry if antibiotic tests with inadequate biomedical specificity, the ability to correctly identify an untreated cow, are indiscriminately used to test individual cow samples. The false-positive outcomes create a mistrust between the consumer and the producer, veterinarian, and regulatory personnel because they are interpreted that the safety of the milk is not being adequately monitored at the level of the bulk tank.

c) Any number of false-positive results detected could lead to the inaccurate conclusion that a significant proportion of "normal" dairy cows are delivering residues into our milk supply each day.

d) Regardless of the efforts made by the dairy and medical industries to produce a safe and wholesome dairy product, negative reports based upon inappropriately validated and applied technologies will be the reports that the consuming public remember.

e) The welfare of the individual dairy cow is at risk because too many positive assay outcomes after recommended withdrawal times have been followed, will result in her being sent to the slaughterhouse. In this case, the false-positive assay outcomes result in the untimely death of the dairy cow, and will occur thousands of times across the country each month.

ANTIBIOTIC RESIDUE TESTS FOR MILK AND OTHER BIOLOGICAL SAMPLES

• It is essential that the dairy industry and veterinary profession demand that validation protocols, based on good scientific practices, be implemented before the residue test assays be allowed for sale.
Two essential definitions that must be applied to residue assays in field conditions:

1) **Sensitivity**: The assay measures the proportion of those cows with antibiotics in their milk who are correctly identified by the test. In other words, it measures how accurate the test is in correctly identifying cows with antibiotic in their milk.

2) **Specificity**: The assay measures the proportion of those cows without antibiotic residues in their milk who are correctly identified by the test. Again, this measures how accurate the test is at authenticating that the milk is antibiotic-free.

Some salient questions concerning current and proposed antibiotic residue test validation procedures are as follows:

a) Does the sample set evaluated include an appropriate spectrum of mild and severe, treated and untreated disease, plus individuals with different, but commonly confused disorders? Can the test tell the difference between a "normal and healthy cow" or can it correctly identify a patient undergoing antibiotic therapy vs one that is not receiving antibiotics (i.e. treated or untreated)?

b) Does the test possess the capability to differentiate between natural host defense mechanisms and antibiotic therapy?

c) Does the validation protocol employ the appropriate biomedical negative control (e.g. assessing the residue status of a pretreatment sample)?

d) Is the reproducibility of the test result (precision) and its interpretation (observer variation) determined?

e) Is the term "normal" or "range of normal" defined sensibly? (Gaussian, percentile, risk factor, diagnostic, or therapeutic?)

f) Is the "utility" of the test documented? Is the consumer, producer, veterinarian, and dairy cow better off after having this residue test performed on the sample?

g) If the test is "positive" before the cow is treated, then the patient undergoes antibiotic therapy with appropriate milk/meat withdrawal times observed, and the test is still "positive for the presence of antibiotic residues", is this an accurate test? How can sound medical advice be derived from such an assay outcome?

h) What is the biological relevance in field conditions of a residue test that is "validated" based upon assay results obtained from milk in the laboratory that has been "spiked" with antibiotic?

Comments on Product Labeling:

- It may be, that within current labeling guidelines, regulatory agencies could restrict use of the antibiotic residue test kits to the following categories: 1) Tanker truck loads, 2) Bulk
tank milk, and 3) individual animals. The individual animal category is a better designation than "cowside." Some test kits may actually be employed at "cowside," because this will be a significant market opportunity. However, what must be controlled is the "real world" situation where the producer or veterinarian will take the sample to the processing plant and request that the sample be evaluated. In this case, as already happens, the plant will use assays approved for bulk tank milk evaluation on the individual animal sample. The result—we have not solved the original problem of inaccurate test performance on this sample set. The "off-label" use of the test kit cannot be permitted.

- It is obvious that most, if not all of the individual animal test results will not go on to be confirmed, and there is little or no capability to do internal validation processes at the level of the dairy.

- It seems somewhat trivial at this point, but it is apparent that regulatory agencies and kit companies must clearly understand what constitutes a "residue test." Many of the assays are not measuring the component (antibiotic) that they state on the label. In fact, many cannot distinguish between normal host antimicrobial activities and the presence of antibiotics in the milk.

- The assay must perform better than the results of flipping a coin. The documented Epidemiological Sensitivity (Se) + the Epidemiological Specificity (Sp) should = 1.8 or better. The lower limit of either value should be 0.85.

- The label must also include the following information: "false-positive range with the 95% confidence interval (CI) is from X-X/100 samples.

- No "false violatives" should be allowed

- Detection limit of the assay for each antibiotic claimed must be stated

- The FDA established Safe and Tolerance for each antibiotic claim for the assay must be placed on the label.

- The label must be complete and informative:

  - Explain the nature of the test (i.e. assay format)
  - It must be clearly stated when the assay format cannot differentiate between antibiotics and natural host defense mechanisms

- Potential summary statement on the label:

  "This assay detects growth inhibiting substances, but does not differentiate between the presence of antibiotics and natural inhibitory substances in milk."

- Sufficient data should be obtained to permit the calculation of epidemiological sensitivity, specificity, and positive/negative predictive values for varying prevalences of residues.
These values should be required to be placed either on the label or in the package insert.

- Any other label claim than this will require experiments documenting that the assay can differentiate between natural inhibitors in milk and the presence of antibiotics in the milk. This can be accomplished via various phases of the protocol presented previously in this document.

- There already exists sufficient evidence that misbranding of a marketed device has taken place (i.e. failure of the device to perform as labeled or advertised)

There is a window of opportunity to correct past mistakes or misdirected compromises. It should be noted that these same principles apply to residue detection systems for all potential chemical contaminants (e.g. pesticides, herbicides, etc.). The protocol has had input from experts in epidemiology, statistics, diagnostic assay development/evaluation, infectious diseases of veterinary species, animal science and husbandry, mastitis, microbiology, immunology, pathology, internal medicine, pharmacology, pharmacokinetics, toxicology, and mammary gland defense mechanisms. This represents a rather decisive amount of scientific expertise that does not exist in regulatory agencies or the residue test kit manufacturing companies that has been brought forth to address this question. It is reasonable to consider that support for the scientific merit of the principles contained in this protocol and need for practical implementation can be gathered from the following groups:

- 7-State Food Animal Consortium on Preharvest Food Safety
- National Mastitis Research Workers
- NE-112 Regional Research Project: Resistance to Mastitis in Dairy Cattle
- NCR-176 Regional Research Project: Preharvest Food Safety
- FARAD
- Many, if not all, of the veterinary colleges
- American Association of Bovine Practitioners
- Academy of Veterinary Consultants
- Several producer and processing plant organizations

WHY DO ALL OF THESE STEPS IN "VALIDATING" A RESIDUE TEST KIT?

Phase I: Fortifying bulk tank milk with: a) varying levels of normal bovine plasma, b) specific levels of bovine milk lactoferrin, and c) varying concentrations of β-lactamase or organisms that produce β-lactamase.

- This is an easy method to initially determine the potential of the assay to successfully complete the remainder of the protocol; thus, minimizing corporate expenditures in test kit development. This phase allows a rapid series of experiments that will determine the propensity of the test to yield both false-positive and false-negative results.
Phase II: Evaluating test kit performance in milk obtained from lactating cows identified as producing "saleable milk" and had not received any treatment for at least 30 days.

- Common criticism of this phase: "No one is going to use these tests on normal animals!"

- This phase was included for the following reasons:

a) This is an established data set that has been identified by medical epidemiologists as both a scientifically and medically correct procedure to require for a screening test. The test must correctly identify that the milk samples obtained from these animals do not contain antibiotic residues. We presented substantial evidence that several test kits yield false-positive test results under these conditions.

b) The unwillingness to accept this principle of diagnostic medicine unveils a serious flaw in the critical thinking of many kit manufacturers and regulatory agencies. This is a long-established and scientifically sound requirement for a diagnostic screening assay. Additionally, the adverse regulatory consequences are enormous. By rejecting this phase and the pretreatment sample set required by the protocol, regulatory agencies set up themselves, veterinarians, and the dairy industry for a devastating breach of trust with the consumer and other regulatory agencies.

Phase III: This series of experiments involves challenging cows in one quarter with a mild dose of intramammary endotoxin and measuring the kit performance in various categories (e.g., pretreatment, after antibiotic treatment, and with no antibiotic treatment).

This is a classic cross-over study for a challenge/treatment investigation. It allows for documenting assay performance under experimentally-induced disease processes. This is a common biomedical technique for evaluating screening assays.

Phase IV: This is a field investigation that includes examining the performance of the assay(s) on samples obtained from cows with clinical mastitis. It requires sample collections that will allow for scientifically determining the epidemiological sensitivity, specificity, and predictive values (+/-) for the test(s).

- This phase based upon scientific appropriateness. The protocol allows for the evaluation of the test under field conditions, and provides for a final look at the capability of the assay to correctly identify treated and untreated cows. Additionally, this will determine if the "test" can tell the difference between normal host defense and the presence of antibiotics in the milk. The last critical feature of this phase is that it again provides for the assessment of the test's propensity to allow false-negative assay outcomes.

The major disagreement between many test kit manufacturers and this protocol is concerning the appropriateness of the "pretreatment" sample of mammary gland secretion.
The common complaint is: "It's not "saleable milk", therefore not an appropriate sample to evaluate."

Sound medical doctrine follows that this is an appropriate sample set......If the test is positive before you treat the patient, then you treat her with antibiotics, withhold milk for the appropriate time, perform the antibiotic residue assay, and it is "positive" once again, what is the correct diagnostic interpretation? Does she have antibiotics in her milk or not? Is this really a "test"? Can the veterinarian advise that her milk production can go into the bulk tank? How much economic loss must the producer endure before he decides the test is not accurate? Keep in mind that we have followed these false-positive results for up to 3 weeks after the initial infection had taken place. How many animals will go to slaughter because the "test" indicates a "prolonged drug withdrawal" time? There is little or no capability to perform internal validation procedures at the level of the individual dairy.

Good Laboratory Procedures

Section A) All certified laboratory protocols provide for the inclusion of "positive" and "negative" control samples to be evaluated along with the other milk samples under investigation. For example, if the negative control sample is "positive", then the entire set of assay outcomes run with that control are disregarded, and the problem is identified and corrected. This is a scientifically appropriate approach for laboratory diagnostic or screening procedures.

Section B) Now consider the appropriate course of action when the "test" is used outside the laboratory for medical evaluation purposes. Remember, we are attempting to apply a "validated test" in determining if the milk from a patient contains violative antibiotic residues. The established, scientifically correct course of action is quite simple. The biomedical "negative" control is the pretreatment sample. If the pretreatment sample is "assay positive" for antibiotics in the milk and the veterinarian in charge of the case knows this animal has not been treated, then the "antibiotic residue test" has a major flaw and is useless. Just as in section A above when the sample outcomes are disregarded, the test outcome under this "real world" circumstance must be categorized as unreliable. In fact, by all medical standards, this is not a "test" that can determine the antibiotic residue status of this milk and cannot be allowed to be available for sale. Afterall, the test has just identified the milk of an untreated cow as containing antibiotics. The position of disallowing the appropriate performance of the assay on the pretreatment sample set is indefensible under any medical criteria.

In summary, many regulatory agencies and test kit manufacturers have taken the position of not requiring that the test system can correctly identify the antibiotic residue status of nonantibiotic treated animal. In addition, they have also denied the scientifically appropriate biomedical negative control, the pretreatment sample, to be included as a regulatory criteria for validation of the residue test kit. By taking this position, they allow the kit companies to sell an "antibiotic residue detection assay" that cannot correctly differentiate between normal host defense mechanisms and the presence of antibiotics in milk. The fundamental doctrines contained in medical application of screening or
diagnostic procedures cannot be brushed aside in developing validation protocols for residue detection assays applied to milk or meat.

- A common position taken by regulatory agencies and companies selling antibiotic residue test assays: "Should these alleged false-positive results you are reporting be true, it's not that troublesome. Afterall, we're erring on the safe side".

I think that sufficient evidence has been published in refereed journals, past and present, to document the serious inadequacies of past residue test kit validation procedures. It is time to stop, take a breath, and recognize that these flaws exist. It should be clear now that "fortifying" milk with parent compound of antibiotic and then verifying the test outcome possesses little, if any, biological relevance. The NMC-RC residue test kit validation protocol addresses many of the problems that currently exist.

HOW ACCURATE ARE THE CURRENT ASSAYS ON THE MARKET?

A Brief Literature Review


Egan and Meaney used three microbial growth inhibition assays, Bacillus stearothermophilus var. calidolactis, Bacillus subtilis, and Streptococcus thermophilus Y, to evaluate milk samples from mastitic cows and heifers, and colostrum samples from heifers. The samples assayed were not obtained from any animal treated with an antibiotic within the previous 21 days. The mammary gland secretions in this study were not heat-treated prior to performing any of the assays. The outcome of microbiological assays included isolates of Staphylococcus aureus, Streptococci, Coliforms, and no growth. This study documented the presence of natural bacterial growth inhibitors in that the false-positive test outcomes ranged from 53.6% to 0.8%, depending upon the assay and the type of sample examined.


Okada reported in a study that included the milk from 540 normal lactating cows, that a false-positive rate of 10.2% was observed with the paper disk test. The false-positive rate increased to 19.7% when the somatic cell count was >300,000/ml. The investigation also documented that the milk lactoferrin levels were 0.49±0.14 mg/ml in the assay positive milk samples. The author concluded that lactoferrin in milk is to be considered as one of the natural inhibitors in milk that resulted in a clear zone similar to that produced by the addition of antibiotic in the paper disk assay.

Tyler et al. employed several antibiotic residue assay formats in examining the mammary gland secretions from 8 lactating cows with experimentally-induced endotoxin mastitis. The intramammary endotoxin challenge produced a systemic mediator shock and intramammary inflammation. Mammary gland secretions were collected prechallenge and on a scheduled basis for 288 hours after the endotoxin infusion. The proportion of false-positive assay results varied from 0 to 1.00 among combinations of sampling time and mammary secretion evaluated (endotoxin-infused quarter vs a composite sample from the noninfused quarters). The LacTek β-lactam had no false-positive assay outcomes in this investigation, while the Charm Farm assay yielded the highest proportion of false-positive results (0.86). The other two commonly-used residue assays, the Delvotest P and the CITE Probe β-lactam also yielded a high proportion of false-positive assay outcomes at 0.45 and 0.48 respectively. The authors concluded that the ability of some of these assays to correctly identify a patient that has not received antibiotics (test specificity) varies greatly among assay kits, and that intramammary inflammation may increase the proportion of false-positive assay outcomes.


Carlsson et al. examined the performance of the Delvotest P assay in mammary gland secretions collected from cows subjected to acute experimental mastitis by intramammary infusion of Salmonella typhimurium endotoxin. No antibiotics were administered to the study subjects. They documented a positive correlation between false-positive Delvotest P outcomes and an increase in lactoferrin and lysozyme concentrations in the secretions during various sampling time points. There was some biological variation between cows, yet the secretions yielded false-positive results at multiple time points in all animals. This study demonstrates that concentrations of lysozyme and lactoferrin obtained during host defense of the mammary gland can produce an incorrect assay outcome that is interpreted as indicating the presence of antibiotic in the milk.


Seymour, Jones and McGilliard conducted a study to determine the effectiveness of on-farm screening assays (BsDA, Delvotest P, Penzyme) for the detection of antibiotics in milk and urine. Composite milk samples were obtained from 58 lactating cows that had received a single antibiotic treatment by any route of administration. Samples were obtained 72 hours post-treatment, and sampling continued every 24 h until all residue tests indicated assay negative. Although statistical analysis did not indicate that the three milk antibiotic residue tests should yield different test outcomes, a Chi-square analysis was employed to determine the likeness of the Delvo or Penzyme to differ from the disc assay (BsDA).
• Delvotest: Only 78% of the Delvotest results were the same as the BsDA; 5% of the Delvo tests were negative when the BsDA was positive; and 17% were positive when the BsDA outcomes were negative.

• Penzyme: Again, only 79% of the Penzyme assay outcomes were the same as the BsDA; 4% were negative when the disc assay was positive, and 17% were positive when the BsDA was negative. Cows treated with cephalolin, penicillin, and liquamycin produced those results not in agreement with the BsDA results. Although the Penzyme is reported to detect the presence of β-lactam antibiotics in milk, 19 of the 58 animals were treated with non-β-lactam antibiotics.

• It is noteworthy that this study did not test mammary gland secretions prior to antibiotic therapy. Therefore, it is unknown what influence the natural inhibitory host defense substances might have had on this study. The pretreatment assay outcomes are necessary in providing the appropriate medical "negative control" for evaluating the true assay specificity.

Their study also included an initial investigation into the accuracy of the Live Animal Swab Test (LAST). This on-farm screening assay is used to detect potential antibiotic residues in meat before the animal is processed. Urine was obtained from 39 culled dairy cows prior to slaughter and the LAST assay was performed on this set of biological samples. Treatment records from these study subjects were studied to determine their treatment status and if appropriate withdrawal times had been observed. The assay results indicated that 27 of the 39 cows (69%) contained violative residues in their urine, despite the fact that all animals had completed the recommended withholding period specified for each antibiotic administered. It is clear that this test is not specific enough for detecting the presence of antibiotics, as 75% (15 of 20) of the untreated animals in the study were assay positive for antibiotic residues.


Macauley and Packard evaluated several antibiotic residue detection assays on raw comingled milk that was fortified with various levels of penicillin, erythromycin and chloramphenicol. The authors reported the Delvotest P yielded 11% false-positive outcomes on negative control samples.


Van Der Leek et al. reported that the use of diflubenzuron controlled-release insecticide boluses in dairy cattle had no effect on the Delvotest P milk antibiotic residue test. Although cows were eliminated from the study if they developed mastitis, if they were treated locally or parenterally with antibiotics, or if they were not sampled at the correct time, the test falsely-indicated that the cows (n=96) had antibiotics in their milk up to 5 milkings after calving.

Cullor et al. performed milk antibiotic residue assays on mammary gland secretions from individual cows. The assays were performed on: a) mammary gland secretions, AM/PM for 14 days, from three cows with experimentally-induced coliform mastitis, b) mammary gland secretions from seven cows with naturally-occurring coliform mastitis, and c) bulk tank milk that was fortified with bovine serum or plasma from antibiotic-free donors.

- **Experimentally-induced coliform mastitis:** All but one of the assays identified the normal mammary gland defense as "antibiotic positive." The patients were not treated with antibiotics. The number of correct assay outcomes are as follows: Charm Farm (10/72), CITE Probe β-lactam (11/72), Delvotest P (10/72), BsDA (50/72), and the LacTek β-lactam (72/72). The data sets from the challenge and control quarters document similar poor performance from all assays. However, the LacTek β-lactam assay correctly identified these samples as not containing antibiotic residues.

- **Naturally-occurring coliform mastitis:** The LacTek was the only assay that correctly identified the pretreatment quarter samples as not containing β-lactam antibiotics. The **per cent false-positive** for the other assays are as follows: Charm Farm (100%), CITE Probe (100%), Delvotest P (83%), and the BsDA (33%).

- **Bulk tank milk fortified with bovine plasma:** Both the Charm Farm and the CITE Probe assay incorrectly identified the serum/plasma fortified milk as being contaminated with β-lactam antibiotic.


Sischo and Burns obtained milk samples from either the weigh jar or from a sampling meter in the milk line. All of the assays were reported to be performed as recommended by the manufacturer, but none of the tests were performed in actual field conditions. The assays were run under more controlled conditions in the university laboratory setting. None of the 199 study subjects were reported to have clinical mastitis that initiated antibiotic therapy on that sample collection day. The individual animal records indicated that none of these subjects had received medications for at least 30 days prior to being included in the investigation. The study excluded some cows from the analyses predicting false-positive test results even though it was documented that they were well beyond recommended antibiotic withholding times. However, the investigation revealed that the assays did yield false-positive outcomes. Depending upon the test, 5-22% of untreated cows were inappropriately identified as having antibiotic positive milk. When the confounding of cofactors was controlled in their logistic model, there was a positive effect of somatic cell count on the probability of a false-positive result for the Charm Farm, CITE Probe β-lactam, and the Delvo Mini P. Essentially, as the somatic cell count of...
individual animal milk samples increased, so did the likelihood for false-positive antibiotic residue test outcomes.


Van Eenennaam et al. (submitted 1993) performed antibiotic residue assays mammary gland secretions from 172 commercial dairy cows and heifers with cases of mild to moderate clinical mastitis. False-positive assay results were recorded on pretreatment samples, non-treated animals, and samples obtained 21 days after the first treatments had been administered. The percentage of false-positive results was 43.6% (n=839) for the β-lactam CITE Probe, 37.7% (n=839) for the Delvotest P, 81.7% (n=387) for the Charm Farm assay, 2.6% (n=836) for the LacTek β-lactam test, and 18.8% (n=819) for the disc assay (BsDA). The study also documented apparent problems with false-negative outcomes for some of the test kits. One example of mention is at milking quarter sample 4, the CITE Probe β-lactam had a false-negative rate of 15.3%.


Carlsson and Bjorck examined bulk tank milk samples that were suspected of containing violative residues of inhibitory substances. All samples analyzed yielded assay positive results in the determination of tetracyclines and macrolides by the Charm II microbial receptor tests. Two agar free assays were evaluated in this investigation. The Arla microtest (SMR, Malmo, Sweden) employs a freeze-dried culture of Bacillus subtilis and the Valio T101 assay (Valio, Helsinki, Finland) uses growth inhibition of Streptococcus thermophilus as the indicator system. After incubation with the test sample, growth of the organism is indicated by a color change of a pH or redox indicator. The Delvotest SP is based upon the agar diffusion principle and uses growth inhibition of Bacillus stearothermophilus as the indicator system. The investigators initially employed a liquid chromatography technique as a confirmatory test for the presence of tetracyclines in the test samples. In the course of the study, it became apparent that the inhibitory substance being detected by many of the assays was not tetracycline antibiotic. The samples were found to be false-positive for the presence of tetracyclines and macrolides by the Charm II assay. The study found that as little as 2-5% by volume of serum in the negative control milk resulted in immediate count reductions in the Charm II assays. Thus, indicating the “antibiotic receptors” were binding “antibiotic” when indeed none was present.

Their investigation demonstrated that lipolysis of milk fat can give rise to false-positive antibiotic residue indications in the Arla microtest and the Valio T101 assay. Free Fatty Acids can also interfere with the Charm II determination of tetracyclines and macrolides by the microbial receptor tests. The authors determined that their earlier speculations of increased tetracycline usage by dairy producers that were based upon results indicated with these antibiotic residue tests, now appears to be less probable because of thse
false-positive results. The lipolysis that occurred in the herd milk could have been stimulated by spontaneous action during transportation or stimulated by physical treatment, e.g. agitation and foaming. The outcomes of the assays were not due to the examination of "bad milk".


A high proportion of false-positive assay results were obtained in both the pretreatment milk samples from cows with cases of clinical mastitis, and those obtained 21 days after initial non-antibiotic and antibiotic therapy for the treatment of mastitis. Additionally, a high rate false-positive assay outcomes was obtained from the milk of clinically normal individuals that had not received medications of any description for at least 30 days prior to the evaluation of the test sample. The results of these studies indicate a serious problem in using some antibiotic residue detection assays that were designed to evaluate bulk tank milk samples for individual cow samples. This error in assay specificity results in the unjustifiable discarding of milk that meets regulatory standards and may be misused to accuse the producer or veterinarian of not adhering to regulatory guidelines.

Producer, Practitioner: "Test the Tests Yourself!"

Some Practical Ways to "Test the Tests": The following is a modified version of the four phase program suggested in other publications. We'll call them "Phase I-P, etc." to designate the practitioner or practical phase of the test kit evaluation. This is an easy set of experiments that can be performed by an AHT in cooperation with the veterinarian, producer, processing plant personnel, or Extension Specialist.

• Phase I-P of the suggestions could be easily accomplished by the practitioner in the following manner:

a) Obtain 25 ml of plasma from each of 5 cows that they can certify: 1) are in normal physical condition, and 2) has not received any therapy for at least 30 days prior to collection time.

b) Pool the plasma from these animals and use it to spike the bulk tank milk.

c) Bulk tank milk: must have a SCC below 1 million/ml and the veterinarian can document that no treated animals went into the bulk tank that day. This sample must be fresh each day that they use it to "test" the tests, because some assays cannot be used on frozen milk samples.

d) Make up the following sample sets to test β-lactam residue assays:

1) Zero control (100% v/v bulk tank milk): [v/v = volume/volume]
2) 10% v/v plasma and 90% v/v bulk tank milk
3) 20% v/v plasma and 80% v/v bulk tank milk
4) 40% v/v plasma and 60% v/v bulk tank milk
5) Positive control: mix 1.0 ml of a β-lactam antibiotic in 3 ml of bulk tank milk

e) "Test" the test kit by running it in triplicate on each sample set according to manufacturer's recommendations.

f) The residue kit should yield a "assay negative" outcome on the zero control milk and an "assay positive" outcome on the positive control sample.

g) An "assay positive" outcome on any one of the other sample sets is suggestive that the test kit possesses an inappropriate assay specificity, and it may be unable to correctly identify that a sample does not contain β-lactam antibiotics.

• Phase II-P (Clinical Mastitis Cases) of the test kit evaluation may be accomplished as follows:

a) Collect pretreatment mammary gland secretions from 30 individual animals that have been diagnosed as having clinical mastitis in one quarter. The procedure for the sample collection is provided below.

b) Sample 1: Is composed of premilking mammary gland secretions from the mastitic quarter (5 ml).

c) Sample 2: Is made from 5.0 ml aliquots of premilking mammary gland secretions from each of the three remaining normal quarters.

d) "Test" the test by running it in triplicate on each sample set according to manufacturer's recommendations on the following sample sets:

1) Zero control (100% v/v bulk tank milk)
2) Positive control: mix 1.0 ml of a β-lactam antibiotic in 3 ml of bulk tank milk
3) Sample 1: pretreatment milk from the infected quarter
4) Sample 2: pretreatment milk from the composite sample of the 3 normal quarters

e) The residue kit should yield a "assay negative" outcome on the zero control milk and an "assay positive" outcome on the positive control sample.

f) An "assay positive" outcome on any one of the other sample sets is suggestive that the test kit possesses an inappropriate assay specificity, and it may be unable to correctly identify that this clinical case of mastitis has not been treated with β-lactam antibiotics.
Discussion: [Epidemiological specificity]—the probability of correctly identifying a non-treated animal is the first measure that field screening tests must satisfy

Mastitis is the single most common disease syndrome in dairy cows. Any residue test that does not account for mammary gland inflammation and other host defense mechanisms in its assay format contains a serious scientific and practical flaw. If the test cannot differentiate between normal host defense and the presence of antibiotics in the milk, it is indefensible as either a screening or diagnostic assay under any circumstances. It has been previously documented that false-positive antibiotic residue assay outcomes are a serious problem (e.g. Carlsson et al. 1989, 1992, Macaulay and Packard 1981, Eagen et al. 1984, Tyler et al. 1992, Cullor et al. 1991, 1992; Van Eenennam, 1992). These data sets clearly demonstrate that several antibiotic residue assays that yield false-positive outcomes are on the market today and can create unwarranted concerns for regulatory personnel, veterinarians, consumers and dairy producers. In addition, they cause milk to be discarded unjustifiably far beyond present regulatory withdrawal times and adversely affect the way the producer and veterinarian may employ necessary medications for the welfare of their patient. It is clear that, under these circumstances, both the producer and the veterinarian could be falsely accused of not following regulatory guidelines.

The scenario presented in the Summary introduces a genuine concern that can arise when individual animal residue assays are not properly validated before being made available for sale to the market place. In this scenario, the tanker truck and bulk tank monitoring systems would be falsely identified as not protecting the welfare of the public. This can occur because the assay cannot correctly differentiate between normal and sick animals or treated and untreated patients. The ability of the assay to correctly differentiate between normal host defense and the presence of violative residues in milk or meat is a fundamental medical principle that must remain in place in regulatory issues as well as in clinical medicine.

The definitions of applicable terms presented in this report are based upon accepted scientific epidemiological and biomedical standards. There will be some resistance in willingness to apply these terms in assay development and validation (i.e., epidemiological sensitivity, specificity, positive/negative predictive value, etc.). Another instance that may arise is the issue of "false-positive" vs "false-violative" assay outcomes. The false-positive outcome is considered to be a positive test outcome for a milk sample when there is no antibiotic present. A false-violative is defined as a positive test outcome for a milk sample that contains antibiotic residues below the safe/tolerance level but is above the limit of detection of the test. This last definition allows the kit manufacturer to allow or set their detection limit below the established safe/tolerance level. Thus, indicating that there is a residue problem, when, in fact, there is no residue problem. The inclusion of the concept of false-violative results unnecessarily complicates the issue of food safety. How can the veterinarian or producer interpret if the assay positive is a true positive, false-positive, or false-violative result? The only way is to send the sample in for analysis by a confirmatory assay. This approach would involve a prolonged lag time in receiving a final answer and is not feasible for individual animal testing.
Screening tests designed to detect residues in milk are considered veterinary devices under the Federal Food, Drug, and Cosmetic Act and thus are regulated by the Food and Drug Administration. However, the Act does not require the manufacturer to obtain approval of veterinary devices prior to marketing. This allows manufacturers of residue screening tests to market such assays without FDA approval. Currently, the only regulatory impact that can be implemented by CVM/FDA is in cases of misbranding of a marketed device (i.e., failure of the device to perform as labeled or advertised). In these circumstances, the burden of proof is on the FDA to demonstrate that the veterinary device is not in compliance with the labeling provisions of the ACT.

A thorough evaluation of all data available on appropriate research and development of the individual animal or cowside test must be sought before recommendations are put forth. Most kit manufacturers do not understand that the performance of an assay in spiked samples of normal milk with parent compound of an antibiotic is not the same as treating an active case of mastitis or other form of systemic disease and then determining when the patient may safely go back into production. Both the kit manufacturers and agencies responsible for establishing validation protocols must realize that fortifying milk samples with parent compounds of antibiotics and then measuring the detection limits of the assay has little, if any biological relevance. Therefore, either the residue validation protocol presented herein or the one proposed by the National Mastitis Council Research Committee can provide appropriate scientific guidelines for evaluating the ability of a proposed assay to function accurately in field conditions.

The 10-point Milk and Dairy Beef Quality Assurance Program will continue to develop into a valuable tool to aid in assuring the consumer that the dairy industry is maintaining appropriate safeguards in producing a safe and wholesome product. The producer and veterinarian can maintain appropriate on-the-farm controls over the use of medications by employing current regulatory guidelines and by supplementing them with the other portions of the 10-point plan. Dairy producers, milk processors and those who advise them need more specific tests to help them assure consumers of a safe, residue-free milk supply.

- I have tried to supply information in this document that will aid in making informed decisions concerning residue test kits and their appropriate place in residue prevention strategies.

- If the assay cannot differentiate between normal host defense and antibiotics in the milk, it is not a test!

**Study 1 [Mastitis field cases]:** The data presented in Table 1 demonstrate the level of false-positive assay outcomes from pretreatment milk samples of clinical mastitis field cases \((n = 148)\). *Note that we present the results from the clinical composite milk sample*
from the three clinically normal mammary gland quarters; therefore, the mammary gland secretion from the inflamed quarter is not part of this test sample. The false-positive rate when evaluating the mammary gland secretions from the infected quarter is much higher for all assays except the LacTek β-lactam (data not shown).

Table 1: Bovine Mastitis Clinical Cases [Pretreatment Quarter Composite Sample]*

<table>
<thead>
<tr>
<th>Residue Assay</th>
<th>False (+) Outcomes</th>
<th>Statistical Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CITE Probe (β-lactam)</td>
<td>51/148</td>
<td>0.655</td>
</tr>
<tr>
<td>Delvotest P</td>
<td>37/148</td>
<td>0.750</td>
</tr>
<tr>
<td>LacTek (β-lactam)</td>
<td>4/148</td>
<td>0.973</td>
</tr>
<tr>
<td>BsDA</td>
<td>18/148</td>
<td>0.946</td>
</tr>
</tbody>
</table>

* Composite sample of the 3 clinically normal mammary gland quarters of cows with clinical mastitis in one quarter

b BsDA results if ≥16 mm zone of inhibition is reported as a (+) test outcome
c BsDA results if any zone of inhibition is reported as a (+) test outcome

Study 2 [Mastitis field cases]: Mammary gland secretions from cows with mild to moderate clinical cases of mastitis were examined using the residue test kit assays. The secretions examined (n = ranged from 387-839) were comprised of pretreatment samples, non-treated animals, and secretions from patients 21 days after the first of 3 consecutive treatments had been administered (Table 2). Once again, the statistical specificity [the ability of the test kit to correctly identify a non-treated animal] of many of the test systems was less than acceptable scientific performance to be employed as a reliable screening assay.

Table 2: Mastitis Field Case Treatment Groups

<table>
<thead>
<tr>
<th>Residue Assay</th>
<th>False-Positive Outcomes</th>
<th>Number of Samples Tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Charm Farm</td>
<td>81.7%</td>
<td>n=387*</td>
</tr>
<tr>
<td>CITE Probe (β-lactam)</td>
<td>43.6%</td>
<td>n=839</td>
</tr>
<tr>
<td>Delvotest P</td>
<td>37.7%</td>
<td>n=839</td>
</tr>
<tr>
<td>LacTek (β-lactam)</td>
<td>2.6%</td>
<td>n=836</td>
</tr>
<tr>
<td>BsDA</td>
<td>18.8%</td>
<td>n=819</td>
</tr>
</tbody>
</table>

* This table depicts the percentage of positive assay outcomes recorded for each of the 3 treatment groups. Based on treatment protocols and records, all pretreatment samples, 21-day samples, and oxytocin therapy group samples were free of external antibiotic residues. Any sample with an assay positive result in this data set must be considered false (+).

* The number of samples examined in this data set is reduced due to a corporate recall of the loaned equipment.