Detection of Antibiotic/ Drug Residues in Milk & Dairy Products

Introduction:

Common management practice for dairy animals worldwide includes antibiotic therapy. Residues of these antibiotics—whether infused, injected, or added to the diet—may enter the milk supply from the treated animals. Regulations for use of antibiotics require that milk from treated animals be withdrawn from sale for a prescribed time. Antibiotic residues may induce allergic reactions in sensitive individuals, slow starter culture growth, and may create an environment favorable to resistant bacteria. To prevent antibiotics from entering the milk supply, each tanker of milk is tested prior to acceptance at the processing plant. Milk that is contaminated with antibiotics must be discarded. In the last several years, the number of tests available to detect penicillin and other common antibiotics has multiplied both qualitative and quantitative tests exist; some are applicable for dairy farmer use to prevent antibiotics from entering the milk supply at the source.

Bacterial Growth Inhibition Methods:

Growth inhibition tests were the first routinely and widely used antibiotic tests. The earlier common, official test (Sarcina lutea) was time consuming and not easily performed by some laboratories. Later, growth inhibition tests were developed that were easy to do, relatively inexpensive, and, therefore, adopted for routine use on raw milk as well as selected finished and intermediate dairy products. For large-volume testing, these tests are still the most cost effective choice. The time to obtain results is longer than for rapid screening tests, and some rapid screening tests are sensitive to lower residue levels than the growth inhibition tests. Growth inhibition tests have an added benefit of some sensitivity to multiple drug families and are required for some regulatory testing in the U.S. for finished products. The first methods for detection of antibiotic residues in milk were based on the inhibition of growth of susceptible microorganisms. A cylinder plate assay method and a filter paper disc method were described in the early 1940s. Initially, Bacillus
*subtilis* was the organism of choice but in recent years, assays have been developed that rely on *Bacillus stearothermophilus* inhibition. Growth inhibition can be both qualitative and quantitative. The basis for microbial inhibition procedures is the presence of clear zones on an agar plate medium to which bacterial spores have been seeded. The sample to be assayed is placed on the surface of the agar, either on filter paper disks or in stainless steel cylinders. After incubation for the appropriate time, zones are measured (to the nearest 0.1 mm) with calipers and compared with standards. Several samples are tested on the same agar plate. The depth of the agar is important to the sensitivity and reproducibility of the method. A thin layer is more sensitive than a thick layer. Penicillinase is an enzyme that specifically inactivates penicillin. It is added to the sample to confirm the presence of penicillin. If a zone of inhibition is present after the milk is heated to 82°C for 2 min and treated with penicillinase, another inhibitor and not penicillin is present. In the qualitative *B. stearothermophilus var. calidolactis* disc assay method a control containing 0.008 IU penicillin/ml is tested on each agar plate, varying the location on the plate. This reference gives a zone of inhibition of 16 to 20 mm. Plates are incubated at 64 ± 2°C for about 2.5 h. If the zone of inhibition around the disc containing untreated milk is <12.7 mm, the sample is presumed to be free of inhibitory substances. If the heated milk has a zone of > 12.7 mm but the penicillinase treated milk has no zone, the milk is positive for penicillin. If the zone sizes are equivalent from all sample treatments, inhibitors other than penicillin are present. If penicillinase treatment reduces the zone of inhibition but does not reduce it to zero, penicillin and other inhibitors are present. If there is no zone around the heat-treated milk but a zone was present initially, a heat-labile inhibitor may be present. This disk assay method has been used to successfully detect minimum penicillin G residues of 0.005-0.008 U/ml, as well as ampicillin, cephapirin, and cloxacillin.

The fact that *B. stearothermophilus var. calidolactis* produces acid during growth is utilized in a commercially available procedure (Delvotest®P). Bromcresol purple dye changes from purple to yellow in the absence of β-lactam inhibitors. If inhibitors are present, the bacteria do not grow and produce acid; there is no change in the indicator. Test kits are available for individual samples or for multiple sample analyses. In the multiple test kit, one plate contains 96 test wells. A plate
can be subdivided by the analyst into six blocks each with 16 cups. Positive (0.008 or 0.010 IU/ml) and negative controls are prepared with inhibitor-free milk. Samples and controls are added to the ampule or block of cups and incubated at $64 \pm 2^\circ C$ for exactly 2 h and 45 min. Colors are read through the agar for individual ampules or from the bottom for multitest units. Samples giving a purple color to all or part of the solid medium should be confirmed to contain penicillin by heat-treating and penicillinase treatment. The multitest procedure does not work well with chocolate milk because chocolate interferes with color reading. A host of inhibitory substances may be detected with a commercially available test kit called the BR TEST AS®. This method combines agar diffusion and color reduction techniques, utilizing $B. stearothermophilus$ var. calidolactis spores. Drug residues in excess of the detection limit of the method inhibit metabolism of bacteria during incubation. When inhibitors are present, test color remains blue. During incubation of inhibitor-free milk, oxidation-reduction reactions within the mixture cause a change from blue to yellow. The test is useful for raw or pasteurized fluid milks.

**Competitive Binding Methods:**

Charm Sciences, Inc. (Maiden, MA) has developed a variety of test procedures to detect inhibitory substances in milk. The original test developed by the company, the Charm® Test, prove its sensitivity, accuracy, and expand its selectivity. The basis for this procedure is that $\beta$-lactam residues have a specific, irreversible affinity for enzyme sites on the cell wall of microorganisms. In the test procedure, 14C-labeled penicillin and Bacillus stearothermophilus vegetative cells are combined with the sample. If penicillin is present in the sample, it competes for binding sites on the bacterial cell wall and more 14C-label is free in solution. If no penicillin is present in the sample, the labeled penicillin binds with the cell wall and is removed from solution with centrifugation. The supernatant fluid is decanted and the bacterial cells containing the bound penicillin are resuspended and transferred to a metal planchet. The planchet is dried and radioactivity determined in an isotope counting device. Positive and negative controls are prepared and the results from the sample compared to the controls. Results are available within 15 min and the test is applicable to levels of 0.01 IU penicillin/ml. Many dairy
laboratories have converted to the Charm II® procedure as a screening procedure for seven families of antimicrobial drugs. Two different microorganisms are used to provide necessary binding sites for the seven drug families. Antimicrobial families detected are β-lactam, tetracyclines, macrolides, streptomycin, novobiocin, sulfonamides, and chloramphenicol. The method detects biologically active drugs in about 8 min for one or two families or 15 min for all seven families. The Charm II® procedure uses a liquid scintillation counting device rather than a dry sample counter to detect the labeled compound. The procedure is sensitive to β-lactam antibiotics and all sulfa drugs in raw milk, milk powder, and pasteurized milk. The equipment is contained within a case for portability and operates on a 12-V battery.

Another competitive binding method involves the binding of an enzyme DD-carboxypeptidase to β-lactam antibiotics. This test is available in a kit as the Penzyme® and Penzyme® III procedures. Enzyme and sample are incubated 5 min at 47 ± 1°C, and then substrate [(R)-D-Ala-D-Ala)] is added. Any unbound enzyme is free to react with this substrate. The substrate is contained in a tablet that produces a yellow color on dissolving. The mixture is incubated for 15 min at 47 ± 1°C. Also contained in the tablet are reagents necessary to cause the conversion of free D-alanine to pyruvate and H2O2 and produce a color reaction when H2O2 is oxidized. A pink color indicates a negative test, a yellow color indicates an inhibitor residue is present; an orange/yellow color suggests the possibility of β-lactam residues and the sample should be retested to verify the result. The test detects β-lactam residues at 0.01 IU/ml in raw milk. Positive and negative controls should be run along with all samples.

Other Methods:

The Spot® test is an immunological agglutination technique. Latex beads coated with specific inhibitory molecules (penicillin-G, cephalirin, or cloxacillin) and antibodies to these inhibitory molecules are mixed with the milk sample. If inhibitors are present in the milk, the antibody and inhibitor coated latex beads do not agglutinate. If no inhibitor is present in the milk, visible graininess is present in the mixture. The test is performed on a glass slide which is rotated during the reaction. As with all inhibitor tests, positive and negative controls are
performed. Sulfamethazine can be detected in milk at 1 to 2 ppb using a HPLC technique. Enzyme-linked immunosorbent assays (ELISA) are rapidly becoming available for detection of specific antibiotics in foods. Although each method is unique, they are similarly antibody-antigen reactions which are visualized by linking with an enzyme reaction that produces a color. Color indicates the presence or absence of antibiotic or drug residues. Methods that are being applied to milk at this time include: ELISA, LacTek® screening kit, CITE® probe kit, SIGNAL® detection test, EZ-SCREEN®, Agri-Screen® and SNAP® test. Each has specific advantages and disadvantages and must be evaluated on an individual basis depending on specific requirements for the analysis.