SPECIAL REPORT

Bacterial Inhibition Tests Used To Screen for Antimicrobial Veterinary Drug Residues in Slaughtered Animals

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Bacterial inhibition tests used to screen milk, tissues, blood, and urine for antimicrobial veterinary drug residues must be high volume, quick, rugged, inexpensive, and sensitive. Bacterial inhibition tests—such as the Swab Test on Premises (STOP), the Calf Antibiotic and Sulfa Test (CAST), the Fast Antibiotic Screen Test (FAST), the Charm Farm Test (CFT), the Antimicrobial Inhibition Monitor 96 (AIM-96) assay, the German Three Plate Test, the European Union Four Plate Test and the New Dutch Kidney Test—have been used to screen tissues for antimicrobial activity. The CFT and the Brilliant Black Reduction Test (BBRT) also have been used to screen plasma. The Live Animal Swab Test (LAST) was developed to screen urine. This review examines the use and limitations of these screening tests for regulatory control and avoidance of veterinary drug residues in meat. The ideal bacterial inhibition test for screening antimicrobial residues in slaughtered animals does not exist. Each of the current and potential tests has limitations.

A screening method is "a rapid, relatively inexpensive, and rugged field method, used for testing for a specific substance or closely related group of substances, which is sufficiently selective and sensitive to allow at least semiquantitative detection of residues in contents in accordance with the established maximum limit" (1). This review focuses on bacterial inhibition methods for screening tissues and body fluids from food animals, excluding milk. For information on milk screening tests, refer to the proceedings of the symposium held in Kiel, Germany (2).

Screening methods are classified as level III methods by the Codex Alimentarius Commission (1). Such methods should identify samples that contain a residue concentration at the level of interest, usually the maximum residue limit (MRL); be high volume, quick, and sensitive; and have the ability to detect classes of compounds and be portable to nonlaboratory environments. The number of false negatives must be low at the levels of interest. Some false positives are acceptable because screening tests are generally followed by confirmatory analyses, which will eliminate such samples.

Screening tests are used at slaughter plants to identify animals with violative levels of antimicrobial residues in edible tissues. Further testing of the edible tissue is required to identify and confirm the residue and to determine the level of contamination (3, 4). In Canada a violative antimicrobial concentration must be found in muscle tissue before a carcass can be condemned.

Bacterial Inhibition Tests Currently Used To Screen for Antimicrobial Veterinary Drug Residues at Packing Plants in Canada and the United States

Both Swab Test On Premises (STOP) and Calf Antibiotic and Sulfa Test (CAST) are used in slaughter plants in Canada and the United States. The United States recently implemented a new test, the Fast Antimicrobial Screen Test (FAST), in some of its bob veal and cattle slaughter plants and has plans to implement the test in pig slaughter plants. FAST is not yet used in Canadian meat inspection. The 3 tests were developed by scientists at the U.S. Department of Agriculture Food Safety and Inspection Services to screen kidneys from food animals for antimicrobial drug residues at

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slaughter (5–7). For STOP, the tissue is macerated with the shaft of the swab. For CAST and FAST, an incision is made with a knife. For all 3 tests, cotton swabs are inserted into kidney tissue to soak up fluids. Swabs are then incubated on inoculated medium with a disk containing an antibiotic standard used to monitor the viability of the organism and its growth inhibition.

STOP uses *Bacillus subtilis*, Antibiotic Medium No. 5, and a 16–24 h incubation at 27°–29°C. CAST uses *B. megaterium*, Mueller–Hinton Medium, and a 16–24 h incubation at 44°–45°C. A zone of inhibition around the swab suggests the presence of a microbial inhibitor in the sample. In the United States, bob veal calf carcasses are condemned on the basis of a positive CAST without further confirmation.

For FAST, the organism and the temperature are the same as those for the CAST, but the CAST medium is supplemented with dextrose and bromcresol purple. The faster growth rate of bacteria with FAST allows reduction of minimum incubation from 16 to 6 h.

STOP has proven to be high volume, quick, rugged, and inexpensive for field use. By replacing Antibiotic Medium No. 5 in STOP with Saskatoon Antibiotic Medium 3 (i.e., by decreasing the pH of the medium from 8 to 6.7 and adding appropriate nutrients), the sensitivity of STOP for standard solutions was increased for 15 of 22 antibiotics (8).

All 3 tests lack sensitivity to detect chloramphenicol and sulfa drugs. The CAST is more sensitive to sulfa drugs than STOP, but at 1 ppm, it lacks the sensitivity to enforce an MRL of 100 μg/kg. CAST and FAST are less rugged than STOP, requiring a higher temperature and more precise temperature control. The advantages of FAST compared with CAST are minimal, unless the slaughter plant is running 2 shifts per day. When the slaughter plant is operating with one 8 h shift per day, the 6 h time required for FAST allows only same-day results for samples collected during the first 2 h of the shift.

Bacterial Inhibition Tests Currently Used To Screen for Antimicrobial Veterinary Drug Residues at Packing Plants in the European Union

European Union (EU) countries are required to test for antibiotics under a program known as the National Plan (9). Under this plan, 0.1% of slaughtered livestock must be sampled at the slaughter plant for antibiotic residue testing. Annually, 285 million farm animals are slaughtered in Europe, requiring 285 000 samples to be tested. The EU does not recommend an official method for routine testing but provides certified reference materials that can be used to check the effectiveness of method being used. The New Dutch Kidney Test, the German Three Plate Test, and the EU Four Plate Test currently are used in various EU countries to screen tissues for antimicrobial residues.

The New Dutch Kidney Test (10) has been used officially in The Netherlands since April 1, 1988, and it replaced the *Micrococcus luteus* kidney test developed by Van Schothorst (11). The total number of tests conducted per year in The Netherlands is 110 000 for monitoring and 220 000 for suspect animals. The New Dutch Kidney Test is a one-plate test based on examination of renal pelvis fluid (preurine) for antimicrobial residues. The organism is *B. subtilus*; the growth medium is pH 7.0 standard II Nähragar supplemented with 1% dextrose, phosphate buffers, and trimethoprim (0.12 μg/mL agar); and incubation is 13–18 h at 37°C. The kidney is incised, and four 12.7 mm diameter paper disks are inserted and left there for 30 min. Two paper disks are placed diagonally on the surface of the test plate, and 2 paper disks are deep-frozen for reexamination, if required. Three control disks containing 0.5 μg oxytetracycline, 0.05 μg sulfamethazine, and 0.5 μg tylosin are placed in the middle of the plate. To each paper disk, including the standards, 25 μL 10% NaCl solution containing 2 μg trimethoprim/mL is added. If both sample inhibition diameters are ≥20 mm, the whole carcass is condemned. The test detects residues of β-lactams, tetracyclines, and macrolides. It is more sensitive to sulfonamides than the German Three Plate Test or the EU Four Plate Test, but it is relatively insensitive to aminoglycosides. With respect to MRLs set for organ tissues (liver or kidney), the New Dutch Kidney Test is too insensitive for aminoglycosides, sulfonamides, and macrolides. One analyst routinely can complete 150–200 tests per day.

The German Three Plate Test uses *B. subtilis*. Test plates are set up at pH 6.0, 8.0, and 7.2, with the last plate incorporating trimethoprim as a potentiator for sulfonamide residues. Incubation is 18–24 h at 30°C. Both muscle and kidneys are tested. Slices 2 mm thick are prepared from frozen muscle samples and from fresh or frozen kidney samples. Pieces of muscle and kidney, 9 mm in diameter, are punched and placed in duplicate on prepared agar plates. The test is considered positive if, starting from the edge of the tissue specimen, clear growth inhibition zones ≥2.0 mm are seen around both tissue specimens. A positive muscle test condemns the whole carcass, whereas a positive kidney test rejects only the organs. With frozen pig and horse kidney samples, placing a semipermeable membrane on the agar surface prior to application of the kidney disk greatly reduces the incidence of false positives (9), which are believed to be due to release of lysozymes from ruptured cellular tissue. Residues of tetracyclines, β-lactams, quinolones, aminoglycosides, and trimethoprim/sulfa drug combinations can be detected, but the sensitivity does not meet established EU tolerance levels for some of these antibiotics (e.g., oxytetracycline, ampicillin, amoxicillin, sulfa drugs, and trimethoprim). For macrolides, polymyxins, enrofloxacin and trimethoprim/sulfa drug combinations,

the test system is not sensitive enough. The test is more laborious and more expensive than the New Dutch Kidney Test.

The EU Four Plate Test is used routinely or incidentally in Denmark, Spain, the United Kingdom, Switzerland, France, Italy, and Greece to detect antimicrobial residues in slaughter animals. The test is essentially the German Three Plate Test with an additional *M. luteus* test plate at pH 8, which is incubated 18–24 h at 37°C. The performance of the Four Plate Test EU is the same as that described for the German Three Plate Test. In principle, only deep-frozen muscle samples are examined with this method. By taking precautions like use of dialysis membranes and postscreening confirmation techniques, the test can be performed on kidneys as well. This test detects macrolide residues at lower levels than does the German Three Plate Test, but it has the same shortcomings as the others. In addition, false positives are recorded for the *M. luteus* test plate because of bacterial contamination. This test is the most laborious and most expensive. Test comparisons are summarized in Table 1.

Potential Tissue Screening Methods

Potential tissue screening methods include the Cube Inhibition Test (CIT), the Charm Farm Test (CFT), and the Antimicrobial Inhibition Monitor (AIM-96).

CIT was adapted by the Centre for Veterinary Drug Residues, Saskatoon, Canada and has been used to screen muscle samples from imported carcasses. Tissue preparation is similar to that in the German Three Plate Test in that tissue cubes are placed on seeded media. The organism is *B. subtilus*. Two agar plates—Saskatoon Antibiotic Medium (SAM-3) and P-SAM (12)—are used, and incubation is 18–24 h at 37°C.

CFT is conducted on tissue extract in a test tube with *B. stearothermophilus* and tableted reagents. Incubation is 2.5 h at 64°C. Acid production in the presence of microbial activity is measured with bromcresol purple. During incubation, positive samples remain blue, and negative samples turn yellow. When CFT was evaluated with incurred and diagnostic samples, agreement between CFT results and liquid chromatographic results were good for muscle and kidney samples from healthy market pigs injected with procaine penicillin G (13). Some false-positive results were observed for muscle and kidney samples from pigs fed rations containing sulfamethazine, chlortetracycline, and penicillin G (14). A low incidence of false-positive kidney results and a high incidence of false-positive muscle results were observed for cattle and pig diagnostic samples, with false-negative kidney results more frequent than false-negative muscle results (15).

Recommended MRLs for cattle and pig kidneys and CFT limits of detection (LODs) for pig kidney are presented in Table 2. LODs are acceptable for benzylpenicillin, ceftiofur sodium, gentamicin, neomycin, and tetracycline; borderline for chlortetracycline, oxytetracycline, and streptomycin; and too high for sulfamethazine. Comparative field testing with fresh tissues at the packing plants is required. Since this testing was done, CFT has been modified and has not been reevaluated by the authors.

Comparisons of STOP, CAST, FAST, and CFT are summarized in Table 3. These tests have the required sensitivity for β-lactams, but none has the required sensitivity for sulfonamides.

AIM-96, an adaptation of CFT for use with 96-well microtiter plates, also has been evaluated in the authors' laboratory (Saskatoon) for screening of muscle samples. A high proportion of such samples are expected to be negative. Sensitivities for most antimicrobials, with the exception of penicillin G, were not sufficient to monitor at MRLs. Because of variability in

Table 1. Comparisons of bacterial inhibition tests[a]

Criterion	New Dutch Kidney Test	German Three Plate Test	European Union Four Plate Test
Cost	A	B	C
Convenience	A	B	C
Labor	A	B	C
β-lactam screening	A	A	A
Tetracycline screening	A	A	A
Sulfonamide screening	F	F	F
Macrolide screening	F	F	D
Aminoglycoside screening	F	A	A

[a] A = best, B = better, C = acceptable, D = poor, F = unacceptable.

Table 2. Maximum residue limits for cattle and pig kidney and Charm Farm Test limits of detection for fortified pig kidney

Antimicrobial	Maximum residue limit, μg/kg[a]	Charm Farm Test limit of detection, μg/kg[b]
Benzylpenicillin	50	10
Ceftiofur sodium	4000	400
Chlortetracycline	600 TE	800
Gentamicin	1000 TE	200
Neomycin	5000 TE	100
Oxytetracycline	600	500
Spectinomycin	5000 TE	NE
Spiramycin	200 and 300 TE	NE
Streptomycin	1000 TE	900
Sulfamethazine	100	400
Tetracycline	600 TE	300

[a] Data are from reference 16; TE, MRL is temporary.

[b] Data are from reference 15; NE, LOD has not been established.

Table 3. Comparisons of tissue screening methods[a]

Criterion	STOP	CAST	FAST	CFT
Cost	A	A	A	A
Convenience	A	A	A	C
Labor	A	A	A	C
β-lactam screening	A	A	A	A
Sulfonamide screening	F	F	F	D

[a] STOP = Swab Test On Premises, CAST = Calf Antibiotic and Sulfa Test, FAST = Fast Antibiotic Screen Test, CFT = Charm Farm Test. Key: A = best, B = better, C = acceptable, D = poor, F = unacceptable.

performance of test kit lots, the manufacturer is continuing developmental work.

Urine and Plasma Tests

The Live Animal Swab Test (LAST) was adapted from STOP to screen urine from live animals. To reduce incidence of false positives, a higher concentration of *B. subtilus* spores is used in LAST compared with STOP. A high percentage of false-positive results was observed when LAST was used to screen urine from cull dairy cows (17).

The Brilliant Black Reduction Test (BBRT) incorporates a redox indicator in the medium in place of an acid–base indicator. In the absence of inhibitors in the test sample, the redox indicator, Brilliant Black, is reduced from purple (oxidized) to yellow (reduced) by the growth of the test organism, which indicates a negative test. The organism is *B. stearothermophilus*, and incubation is 3 h at 65°C.

CFT and BBRT were used to screen penicillin G residues in plasma from healthy steers (18). Both tests gave positive results for samples containing residues at concentrations greater than the test's detection sensitivity (5 ng/mL for BBRT and 30 ng/mL for CFT). Neither test gave false-positive results. CFT was used to screen plasma and urine from healthy market pigs injected with procaine penicillin G or fed rations containing sulfamethazine, chlortetracycline, and penicillin G (13, 14). For the injection study, tests were positive for all plasma and urine samples from pigs with penicillin G in their muscles. For the feeding study, 16% of results were false positive (6 kidney, 4 muscle, and 6 urine samples) and 1% were false negative (urine).

Conclusion

The ideal bacterial inhibition test for detection in slaughtered animals of all antimicrobial veterinary drug residues of concern at currently established MRLs does not exist. Each of the current and potential tests has limitations in sensitivity, specificity, ruggedness, or other performance attributes.

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