Determination of Antibiotic Residues in Canadian Slaughter Animals by Thin-Layer Chromatography–Bioautography

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A thin-layer chromatography–bioautography method used for detection and identification of antibiotic residues in animal tissues is presented. Three extracts were prepared from each sample. If present, tetracyclines were concentrated in extracts I and III, penicillin in extracts I and II, chloramphenicol, monensin, novobiocin, and the macrolides erythromycin, oleandomycin, and tylosin in extract II, and the aminoglycosides streptomycin, dihydrostreptomycin, neomycin, and gentamycin in extract III. Bioautogram parameters for detection of antibiotics in each extract were investigated. This technique was used to confirm swab test on premises and calf antibiotic and sulfa test positive samples from Canadian abattoirs. Results of the national surveillance program are presented. Penicillin was most often found, followed by tetracyclines, streptomycin, and chloramphenicol. Comparative data between thin-layer chromatography–bioautography and standard microbial inhibitor tests used at the abattoirs indicated that kidney or injection site tissue should be used as the target sample for these tests.

Slaughter animals are tested for antibiotic residues in Canadian abattoirs by the swab test on premises (STOP) (Johnston et al., 1981) or the calf antibiotic and sulfa test (CAST) (USDA, 1984). These bioassay tests detect inhibitory substances but do not identify them. Tissues from animals found positive by these tests, and tissues from animals deemed suspect after physical examination, are forwarded to our laboratory for confirmation and identification of any antibiotics present. The thin-layer chromatography–bioautography (TLCB) method used for these analyses, which tests three extracts from each sample, is based on that recently described by Neidert et al. (1987). This report describes our further investigations into detection parameters and outlines the methodology as applied to a routine analytical program. This TLCB method is currently capable of detecting residues of the following antibiotics: penicillin G (PEN); the tetracyclines (TCS) tetracycline (TTC), oxytetracycline (OTC), and chlortetracycline (CTC); chloramphenicol (CHL); monensin (MON); novobiocin (NOV); the macrolides erythromycin (ERY), tylosin (TYL), and oleandomycin (OLE); and the aminoglycosides streptomycin (STR), dihydrostreptomycin (DIH), gentamycin (GEN), and neomycin (NEO).

Bovine, porcine, avian, and equine samples submitted to our laboratory between March 1984 and April 1987 were analyzed by the described method. Bovine samples submitted as CAST positive were also analyzed for sulfonamides by TLC (Thomas et al., 1983). Antibiotic residues were identified in 54.5% of the 1379 animals tested. Penicillin was most frequently found (55%), followed by tetracyclines (35%), streptomycin (19%), and chloramphenicol (5%).

MATERIALS AND METHODS

Standards, Solvents, and TLC Plates. Chlortetracycline hydrochloride, oxytetracycline dihydrate, tetracycline, sodium penicillin G, erythromycin, tylosin tartrate, chloramphenicol, sodium monensin, novobiocin, gentamycin sulfate, streptomycin sulfate, and dihydrostreptomycin sulfate were purchased from Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178. Oleandomycin chloroform adduct was obtained from Pfizer Inc., Eastern Point Road, Groton, CT 06340. Standard solutions were prepared in deionized water (STR, DIH, GEN, NEO) to contain 1000 μg/mL or in methanol (all others) to contain 100 μg/mL. All standard solutions were stored at −20 °C. The solvents used were distilled in glass or reagent grade. Channeled Whatman silica gel (LK6D) and cellulose (LK2D) TLC plates (20 × 20 cm) with preadsorbent spotting zones were obtained from Terochem Laboratories Ltd., P.O. Box 8188, Station F, Edmonton, Alberta T4H 4P1.

Bioautography Media. Antibiotic medium 1, antibiotic medium 5, and other media ingredients were manufactured by Difco and obtained from BDH Chemicals Canada Ltd., 501 45th St. W, Saskatoon, Saskatchewan S7L 5Z9. Saskatoon antibiotic medium (SAM) was prepared at our laboratory and contained (g/L) beef extract (1.5), yeast extract (3.0), tryptone (4.0), peptone (6.0), dextrose (1.0), and Noble agar (10.0). For SAM-3 (pH 6.7), 5.0 mL of triple salt solution (2.5 g/L each of $MgCl_2{\cdot}6H_2O$, $CaCl_2{\cdot}2H_2O$, and $MnSO_4{\cdot}H_2O$) was added to 1000 mL of SAM. SAM-3-8 (pH 8.0) contained 12.1 g of Tris and 26.0 mL of 1.0 M HCl in 1000 mL of SAM-3. C-SAM (pH 5.6) contained 0.84 g of citric acid monohydrate and 4.7 g of sodium citrate dihydrate in 1000 mL of SAM. All media were prepared in aliquots of 100 mL, autoclaved at 121 °C for 20 min, and stored at 4 °C.

Survey Samples. Submissions from each animal consisted of samples of one or more of kidney, liver, injection site, and muscle tissue. Samples were shipped frozen to the laboratory, and most arrived thawed but cool. Samples deemed unfit upon receipt were not analyzed. The samples were stored at −20 °C until analyzed.

Extraction. Ten milliliters of methanol was added to 10 g of diced tissue in a 50-mL polypropylene centrifuge tube, and the tissue was homogenized for 30 s at medium speed with a Polytron blender. The probe was rinsed into the tube with 3 × 2 mL of methanol. The tubes were shaken for 30 min at high speed and then centrifuged at 1300*g* for 5 min. The supernatant was decanted into a second 50-mL centrifuge tube and the extraction repeated twice, but shaken for only 10 min. The methanol extracts were combined and centrifuged at 1300*g* for 5 min. The supernatant was transferred to a 250-mL round-bottom flask (RBF) containing 200 mg of NaCl and rotoevaporated at 50 °C to about 4 mL. It was necessary to bleed air into the system during the initial evaporation to control foaming. The residue was transferred to a glass 50-mL cen-

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Table I. Spotting Guide for Sample Extracts and Standard Solutions

channel	plate 1	plate 2	plate 3
1	MON, CHL, PEN, TTC	CTC	blank
2	sample 1, extract 1	sample 1, extract 1	GEN
3	blank	blank	blank
4	sample 2, extract 1	sample 2, extract 1	sample 1, extract 3
5	blank	blank	blank
6	sample 3, extract 1	sample 3, extract 1	sample 2, extract 3
7	blank	blank	blank
8	sample 4, extract 1	sample 4, extract 1	NEO
9	NOV, ERY, CTC	OTC	blank
10	blank	blank	sample 3, extract 3
11	sample 1, extract 2	sample 1, extract 3	blank
12	blank	blank	sample 4, extract 3
13	sample 2, extract 2	sample 2, extract 3	blank
14	blank	blank	blank
15	sample 3, extract 2	sample 3, extract 3	STR
16	blank	blank	blank
17	sample 4, extract 2	sample 4, extract 3	blank
18	blank	blank	DIH
19	TYL, OLE, OTC	TTC	blank

trifuge tube with a 25-mL $CHCl_3$ rinse. The tube was capped, shaken for 30 s, and then centrifuged at 500*g* for 5 min. The aqueous (top) layer was pipetted to a second glass tube and reextracted with 25 mL of $CHCl_3$. The top layer was then pipetted to a 4-mL sample vial (extract I). The $CHCl_3$ extracts were combined in a 100-mL RBF, rotoevaporated to dryness at 50 °C, and reconstituted in 1 mL of methanol (extract II). Ten milliliters of methanol–concentrated HCl (98:2) was added to the tissue residue in the first centrifuge tube, and the contents were vortexed to break up the plug, shaken for 10 min, and centrifuged at 1300*g* for 5 min. The supernatant was decanted into a third polypropylene 50-mL centrifuge tube and the extraction repeated once. The methanol–HCl extracts were combined in the third tube and centrifuged at 1300*g* for 5 min, and the supernatant was transferred to a 100-mL RBF. The methanol–HCl extract was rotoevaporated to dryness at 50 °C and reconstituted in 1 mL of methanol (extract III).

Thin-Layer Chromatography. Standard solutions and sample extracts were spotted onto channeled TLC plates as indicated in Table I. The preadsorbent zones of the TLC plates were warmed over a heating strip at 50 °C during spotting. Plate 1 (Whatman LK6D silica gel) was spotted with 15 μL of each standard and 30 μL of extracts I and II of each sample and developed in a paper-lined saturated developing tank containing 200 mL of solvent [chloroform–methanol–acetone–glycerine (49:30:20:1)]. The plate was developed to within 2–3 cm of the top (approximately 45 min), dried with a blow dryer for 20 min in a fume hood, and then air-dried for 3 h. Plate 2 (Whatman LK2D cellulose) was spotted with 5 μL of CTC standard, 10 μL each of OTC and TTC standards, and 15 μL of extracts I and III from each sample and developed with 218 mL of solvent [acetone–chloroform–1-propanol–0.01 N phthalate buffer (pH 3.75)–glycerine (16:20:57:15.2:0.8)] to within 2–3 cm of the top (approximately 90 min). The plate was dried as described above. Plate 3 (Whatman LK6D silica gel) was spotted with 10 μL of each standard and 30 μL of extract III of each sample and developed with 180 mL of solvent [1-butanol–methanol–acetic acid–water (15:30:9:36)] to within 10 cm of the top (approximately 90 min). The plate was dried with a blow dryer for 20 min, redeveloped to within 2–3 cm from the top (2.5 h), blow-dried for 20 min, and then placed in a drying oven at 85 °C for 2 h. Failure to completely remove the solvent from this plate results in total organism growth inhibition on the bioautogram.

Bioautography. One hundred milliliters of molten medium cooled to 48 °C was inoculated with 0.5 mL of *Bacillus subtilis* ATCC 6633 spore suspension (Difco, Western Scientific Ltd., 100 Paramount Rd., Winnipeg, Manitoba R2X 2W3), gently mixed, poured into a leveled sterile 245 × 245 mm plastic NUNC bioassay dish (Gibco/BRL, 2270 Industrial St., Burlington, Ontario L7P 1A1), and allowed to solidify. The test TLC plate was placed face down on the surface of the agar, left for 15 min to allow diffusion of antibiotics into the agar, and removed. SAM-3 medium was used for plate 1, C-SAM for plate 2 and SAM-3-8 for plate 3. The agar plates were incubated overnight at 37 °C and examined the next morning for the presence, size, and location of zones of inhibition. The visualization was enhanced by spraying a 0.2% 2,3,5-triphenyltetrazolium chloride solution onto the surface of the medium and incubating for a further 10 min. The retardation factors (R_f) were calculated, and the bioautograms were photocopied to provide a permanent record.

Determination of Bioautogram Conditions. Bioautograms were produced from cellulose or silica TLC plates on which 10-μL volumes of antibiotic dilutions had been spotted in triplicate and allowed to dry. After overnight incubation the diameters of the zones of inhibition were measured with calipers and the means, standard errors, correlation coefficients, and dose–response lines calculated. The bioautogram conditions, test organism, and medium producing straight dose–response lines of moderate slope, correlation coefficients >0.999, and reproducible results on different days were determined for each antibiotic.

Determination of Sensitivity. The minimum detectable level (MDL) for each antibiotic was determined for standard solutions and spiked tissues. The MDL was defined as the lowest amount of antibiotic producing a zone of inhibition in four out of four replicates in one run. Antibiotic standard solutions were spotted onto TLC plates, the plates developed, and bioautograms prepared as described above. As well, bovine and porcine liver and kidney samples, previously analyzed and found negative for antibiotic residues, were spiked with antibiotic standard solutions and carried through the TLCB procedure. The spiking levels were decreased following a 100–50–20–10–5 ppm pattern. Some additional testing was done at levels between the increments near the MDL.

Confirmation of Extract II Antibiotics. Extract II was evaporated to dryness on an N-Evap and reconstituted in 1 mL of $CHCl_3$. Four hundred microliters of the reconstituted extract was added to 1 mL of 1 N HCl and 400 μL to 1 mL of 1 N NaOH, and the remainder was saved. After vortexing, the mixtures were centrifuged, the aqueous layers were aspirated off, and 30 μL of the three $CHCl_3$ extracts was spotted on Whatman LK6D silica gel plates. The plates were developed and bioautograms produced as described for plate 1. The results were interpreted as shown in Table II.

RESULTS AND DISCUSSION

Methodology. Difco antibiotic medium 1 and antibiotic medium 5 were used initially for bioautography. However, with both of these media, growth of *B. subtilis* was in-

Table II. Acid-Base Confirmation Test for Antibiotic Identification

antibiotic	inhib act. after treatment	
	HCl	NaOH
CHL	—[a]	—
ERY	—	++[b]
MON	++	++
NOV	++	—
OLE	—	++
PEN	++	—
TYL	+[c]	++

[a] No inhibition zone. [b] Inhibition zone clearly present. [c] Inhibition present, but diffuse.

hibited after contact with cellulose TLC plates, possibly due to substances in the cellulose reacting with medium components to form inhibitory compounds. SAM media, which contained more highly purified peptones and agar, were developed to overcome this problem.

Preliminary experiments compared the sensitivity in TLCB of other bacteria used for antibiotic testing, including *Bacillus stearothermophilus* and *Bacillus cereus* spore suspensions (Difco), *Staphylococcus aureus* ATCC 29213, *S. aureus* ATCC 25923, *Escherichia coli* ATCC 25922, and *Micrococcus luteus* ATCC 9341. Under our test conditions, *B. subtilis* was most sensitive to all antibiotics except ERY, OLE, NOV, and TYL, which produced larger zones with *M. luteus*, and therefore *B. subtilis* was retained as the test organism. Some trade-offs with optimum bioautogram conditions for individual antibiotics were necessary, or the procedure would be too cumbersome to be practical.

Table III lists the MDL and R_f data for standard solutions of each of the 14 antibiotics and for spiked bovine and porcine muscle and kidney. The MDLs for standard solutions were comparable to those reported for similar techniques (Neidert et al., 1987; Frerichs and Chandler, 1982; Smither and Vaughan, 1978). However, some of the MDLs for antibiotics spiked in tissues were much higher than those for standard solutions, due in part to our stringent acceptance criterion (four of four replicates producing inhibition in one run) and to what was believed to be a large variation in the recovery of these antibiotics in the presence of the tissue matrix. In practice, antibiotic activity was often detected in one or more replicates of tissues spiked with as little as one-fifth the MDL, suggesting that the 95–100% recoveries reported by Neidert et al. (1987) may not be consistently attainable.

During the MDL study, there was considerable variation in the R_f values for the aminoglycosides and the tetracyclines in spiked tissue samples. Coefficients of variation (CV) for the R_f values for these two groups were 5–50% and 2–20%, respectively. The CV for extract 2 antibiotic R_f values ranged from 1 to 10%. The presence of residual hydrochloric acid in extract 3 was suspected to be partly responsible for this variation, and further testing is under way to verify this. The major contributor to the high variability of R_f values is the nature of the extracts themselves. The limited cleanup results in very crude extracts that are thick and viscous in nature. Coextracted tissue components exert a significant effect on the mobilities of most of the antibiotics. Because of this, it was not possible to identify antibiotics on the basis of their R_f values alone.

On plate 1, only PEN was readily identifiable by its R_f and its characteristic serrated-edged zone of inhibition. Additional confirmation procedures were required to distinguish between ERY and OLE, and between NOV, TYL, CHL, and MON. The acid–base procedure (Table II) was useful but did not give clear-cut results if the antibiotics were present in high concentrations (as, for example, in injection sites). With experience, the analyst can use the size of the original zone of inhibition to estimate a suitable dilution of extract 2 prior to performing the

Table III. Minimum Detectable Levels (μg/g) and Retardation Factors of Antibiotics in Standards and in Fortified Tissue

	std[a]	R_f	beef kidney	R_f	beef muscle	R_f	pork kidney	R_f	pork muscle	R_f
CHL[b]	0.33	0.76	2	0.65	1	0.69	2	0.79	2	0.80
ERY[b]	0.03	0.24	1	0.40	1	0.41	1	0.35	1	0.36
MON[b]	0.33	0.85	5	0.87	5	0.88	10	0.83	10	0.83
NOV[b]	0.33	0.70	2	0.66	2	0.66	2	0.71	2	0.73
OLE[b]	0.33	0.25	5	0.33	5	0.30	10	0.32	10	0.33
PEN[b]	0.03	0.35	0.1	0.26	0.1	0.29	0.2	0.29	0.05	0.32
TYL[b]	0.33	0.72	10	0.29	10	0.66	20	0.26	20	0.67
CTC[c]	0.03	0.51	0.5	0.55	0.5	0.50	0.5	0.50	0.5	0.60
OTC[c]	0.07	0.45	3	0.50	3	0.45	5	0.49	5	0.49
TTC[c]	0.07	0.52	15	0.51	10	0.41	10	0.51	10	0.55
DIH[c]	0.67	0.44	200	0.53	200	0.26	150	0.54	150	0.30
GEN[c,d]	0.33	0.0+	30	0.0+	30	0.0+	30	0.0+	50	0.0+
		0.10		0.42		0.23		0.48		0.23
NEO[c,d]	3.3	0.0+	150	0.0+	150	0.0+	200	0.0+	200	0.0+
		0.13		0.44		0.15		0.47		0.32
STR[c]	0.67	0.49	75	0.50	75	0.23	75	0.54	100	0.28

[a] Converted to micrograms per gram of tissue equivalency for comparison. [b] Extract 2. [c] Extract 3. [d] Two zones of inhibition present.

Table IV. TLCB Results for Submissions Received March 1, 1984, to March 31, 1987

species	no. of animals tested	no. of animals positive	antibiotics identified[a]							
			PEN	TCS	STR	CHL	NOV	ERY	TYL	other[b]
bovine	915	534	289	181	109	35	3	1	1	5
porcine	401	200	115	79	32	0	0	0	0	0
avian	50[c]	8	4	4	0	0	0	0	0	0
equine	13	9	4	2	1	0	0	0	0	3
total	1379	751	412	266	142	35	3	1	1	8

[a] Some animals contained more than one antibiotic residue. [b] An inhibitory substance was detected but not identifiable. [c] Some submissions consisted of samples pooled from several birds.

Table V. Comparison of STOP and CAST Results with TLCB Results for Specific Tissues

species	no. of samples			
	kidney	muscle	injection site	liver
		Bovine		
STOP+, TLCB+	131	52	35	11
STOP+, TLCB−	38	60	5	11
CAST+, TLCB+	34	14	32	1
CAST+, TLCB−	14	14	15	0
		Porcine		
STOP+, TLCB+	47	3	1	0
STOP+, TLCB−	17	5	1	0

acid–base test. The gas chromatography procedure of Simpson and Pili (1981) with a detection limit of 5 ppb was used to confirm and quantitate CHL in samples suspected of containing CHL.

On plate 2, all TCS produced an elongated zone of inhibition, making differentiation by R_f impossible. During the second half of the survey, TCS were confirmed and identified by the HPLC method of Oka et al. (1985), capable of detecting TTC and OTC at 50 ppb and CTC at 100 ppb.

On plate 3 the aminoglycosides were only partially resolved; NEO and GEN could not be distinguished by R_f values, nor could STR and DIH. STR/DIH-positive samples were reported as STR during the survey.

Survey. Results of our national antibiotic residue testing survey are presented in Table IV. Penicillin and tetracyclines were most frequently found.

Of the 1379 animals tested, samples from 822 were submitted as STOP or CAST positive, although specific information such as the tissues tested was not always included. At least one tissue from 357 of 501 (71%) STOP-positive bovine animals and 119 of 217 (55%) STOP-positive porcine animals were confirmed as having antibiotic residues by TLCB. Of the 104 CAST-positive bovine animals 76 (73%) were confirmed as having antibiotic residues by TLCB or sulfonamide residues by TLC. STOP information was not provided with avian and equine submissions.

Of the 822 STOP- and CAST-positive submissions, 541 were accompanied by sufficient information to allow direct comparison of the plant tests with the TLCB results for specific tissues, as shown in Table V. The presence of antibiotics was not confirmed in 69 of 281 (25%) kidney, 11 of 23 (48%) liver, 21 of 89 (24%) injection site, and 79 of 148 (53%) muscle specimens. The number of porcine specimens was too small to permit any conclusions on the effect of animal species. The high percentage of false-positive STOP and CAST results, especially in muscle and liver, was not unexpected, as microbial inhibitors present in tissues may include substances such as growth hormones, antiprotozoals, or products of bacterial growth (Smither et al., 1980). Both STOP and CAST tests were originally developed for use with kidney tissue, and on the basis of our results, we recommend that plant inspectors concentrate their testing efforts and resources on kidney and injection site tissues.

Conclusion. The TLCB method, used in conjunction with the STOP and CAST tests, provides antibiotic detection and identification, which is a major improvement over previous simple tests for microbial inhibitors. In conjunction with specific confirmatory tests for CHL and TCS, it has proven suitable for our national surveillance program. However, experience with both spiked tissue samples and with actual field specimens has shown that TLCB sensitivity is not as great as initially predicted on the basis of work with standard solutions (Neidert et al., 1987) and that further work is necessary to improve its sensitivity, reproducibility, and ability to discriminate between related antibiotics.

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Registry No. PEN, 61-33-6; TTC, 60-54-8; OTC, 79-57-2; CTC, 57-62-5; CHL, 56-75-7; MON, 17090-79-8; NOV, 303-81-1; ERY, 114-07-8; TYL, 1401-69-0; OLE, 3922-90-5; STR, 57-92-1; DIH, 128-46-1; GEN, 1403-66-3; NEO, 1404-04-2.

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