Microbiological Procedures

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Methods for detecting tylosin and streptomycin in animal tissues are given. Problems such as test variation, animal-to-animal variation, limits of sensitivity, background activity, and zone differentiation are discussed. Along with the description of the traditional microbiological method, some new ideas are offered which are relevant to both microbiological and chemical methods. This new approach will supply a method for interpreting residue data.

Microbiological assay procedures offer a convenient and very sensitive tool for the detection of residual antibiotics. Often, however, problems of interpretation arise which require very careful evaluation.

In the literature on pesticides there is very little information concerning antibiotic residues, particularly the newer antibiotics. Pensac *et al.* (*9*) studied the tetracyclines and penicillin in swine. Poultry was included by Broquist and Kohler (*2*) in their study of chlortetracycline. Oxytetracycline residues in poultry and swine were studied by Luther *et al.* (*7*). Durbin *et al.* (*1*) assayed chlortetracycline residues in chicken tissue. More attention has been directed toward milk residues after mastitis treatment. Albright, Tuckey, and Woods (*1*) and Marth (*8*) have published reviews of available information.

The following experiments were performed to study the traditional microbiological approach and offer a means of analyzing data with reference to test sensitivity.

Materials and Methods

Samples of tissues (fat, lean, kidney, and liver) were taken from animals after slaughter and frozen until extractions could be made. At that time samples were thawed, ground in a suitable food grinder, and mixed. Aliquots of the mixed sample were used for extraction purposes.

Streptomycin. To each 30-gram sample of lean, liver, fat, and kidney were added 21 ml. of methanol. A slurry was prepared in a Hamilton Beach homogenizer and the pH adjusted to 4.5 with hydrochloric acid. The suspension was centrifuged for 1 hour, and the supernatant was collected and adjusted to pH 8.0 with sodium hydroxide. This extract was used for microbiological assay. An additional 0.65 ml. of 0.1*M* phosphate buffer, pH 8.0, was added for each gram of fat tissue after centrifugation.

Tylosin. To each 30-gram sample of lean, liver, and kidney were added 30 ml. of water. A slurry was prepared as above and centrifuged. The sediment was discarded and the supernatant used for the assay. Fat samples were extracted with 1.5 ml. of distilled water per gram of sample.

Samples were tested on the plate systems outlined in Table I.

Inocula. A stock cell suspension of *Sarcina lutea* was prepared by inoculating 300 ml. of penicillin seed agar in a Roux bottle from a 24-hour agar slant. After incubation at 30° C. for 16 to 18 hours, the growth was washed off the agar with 25 ml. of nutrient broth and adjusted so that a 1 to 100 dilution of the suspension gave 25% transmittance at 530 mμ on a Spectronic 20. The uninoculated broth was used as the blank. A 1 to 10 dilution of the stock suspension was used to make the inoculation. The suspension was held at 5° C. for approximately 2 weeks.

A spore suspension of *Bacillus subtilis* was prepared by inoculating 300 ml. of penicillin seed agar in a Roux bottle and incubating at 37° C. for 1 week. The growth was washed off the agar with 25 ml. of sterile distilled water and the cells were collected by centrifugation. Following the initial wash, the cells were resuspended in sterile distilled water and heated for 30 minutes at 65° C. Two additional washes with intermittent heat shocking were performed. The final concentration was adjusted to give satisfactory sensitivity and zone definition.

Inhibitory zone diameters were measured with a Fisher-Lilly zone reader.

Table I. Assay Systems

Organism	Streptomycin *Bacillus subtilis*, A.T.C.C. 6633	Tylosin *Sarcina lutea*, A.T.C.C. 9341
Media	Medium 5 (*5*)	Medium 1 (pH 8.5) (*5*)
Base, ml.	6	...
Seed, ml.	4	5
Inoculum	0.05% from spore suspension	0.05% from cell suspension
Incubation, °C.	37	30
Sample container	Penicylinder	Penicylinder

Results and Discussion

Although the dilution factor is dependent upon the over-all antibiotic activity, small volumes of extracting solvents were used to reduce this factor. Grove and Randall (*5*) recommend 5 ml. of extracting solvent to 1.0 gram of tissue for penicillin and streptomycin. Quantities of solvent used in this study are considerably smaller, to allow greater sensitivity.

Sensitivity may be defined as the smallest detectable quantity of antibiotic per gram of sample. It is well established in analytical-micro-

biological laboratories that the depth of agar affects the over-all sensitivity of the antibiotic test. To illustrate, a sample of chicken lean was extracted according to the method outlined for tylosin. This extract was used to dilute the various levels of the standard curve. These levels were replicated on the different agar plate systems as shown in Table II.

Each seeded system contained the same per cent inoculum. Results of this study are given in Figure 1. Straight lines were drawn according to the simplified least squares method outlined by Deutschberger and Kirshbaum (*3*).

It is evident that agar depth can be altered to increase test sensitivity. In fact, it would require more than three times the quantity of antibiotic to produce a 13-mm. zone in system 2 as in system 4.

A point of diminishing returns is reached in using the small volume, single agar layer system. Plates incubated at 37° C. for 16 to 18 hours tend to dry, leaving film with irregular zones. Single layers require more care in distributing the agar over the plate surface.

Variation between days and between animals is evident in Figures 2 and 3. These were prepared by extracting negative control swine livers by the streptomycin method and using the extract as a diluent for the various levels.

Figure 2 represents the response in three animals. Tissues from two animals were re-extracted to show day-to-day variation. Differences in slopes are evident.

Figure 3 shows a wider range of levels over a period of 3 days in one sample extract.

Employing the tissue-compensated standard curve one may take the lowest active level in units per milliliter or micrograms per milliliter and convert it to units per gram or micrograms per gram (parts per million), provided a no-response level is included, and estimate the test sensitivity. This is, however, subject to some criticism because loss of antibiotic through extraction is not included. The extraction is made prior to the addition of various concentrations of the antibiotic. To correct for this, "standard recoveries" may be assayed with each group of samples.

Recoveries are prepared by taking a sample of control tissue, adding a given quantity of antibiotic, and extracting by the recommended method. From these samples, the per cent recovery can be calculated. Problems arise when these data are used to correct for all unknown samples. As shown on the scatter diagram of Figure 4, these are not linear responses and may introduce errors.

Points on Figure 4 represent responses obtained over 7 days with six animals.

It is not unusual to see a recovery or a sample read below the detectable level of a standard curve. Reasons for such occurrences cannot be explained, unless they represent inherent variation.

Another factor to be considered in developing these tests is variability in reading, which is directly related to zone definition. Figure 5 represents a "recovery curve" of streptomycin in swine liver.

Zone 1 is a negative control extract. Zones 2, 3, and 4 are recoveries of 0.2, 0.3, and 0.4 μg. per gram (p.p.m.) respectively. The question arises as to whether the limit of the zone should be measured inside or outside the rim of stimulated growth. It requires a decision on the part of the analyst. Experience, too, is necessary to be able to read all plates in the same manner. Sometimes these problems can be resolved by reducing the inoculum concentration and/or reducing incubation time.

For quantitative purposes only the linear or nearly linear portions of the compensated standard curves were used. This, however, does not include a zero response level as suggested to define test sensitivity. Extending standard curves beyond this range may result in an asymptotic response, as shown in Figure 3. These tests were performed with one tissue sample over a period of 3 days including six levels for each curve. Three levels were read on day I, six on day II, and five on day III, with the smallest zones measuring between 9.2 and 9.6 mm. These data show that the sensitivity range for streptomycin in swine liver would be 0.025 to 0.2 μg. per ml. or converted to parts per million, 0.07 to 2.7 μg. of streptomycin per gram of tissue.

Table II. Agar Plate Systems

System	Base Agar, Ml.	Seed Agar, Ml.
1	...	5
2	6	4
3	12	4
4	20	4

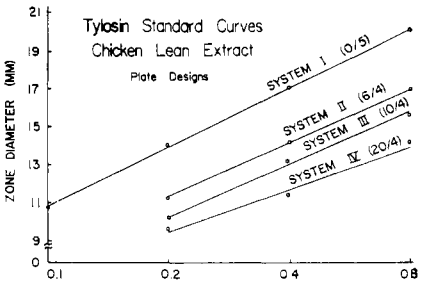

Figure 1. *Sarcina lutea* response to various concentrations of tylosin diluted in an extract of chicken lean

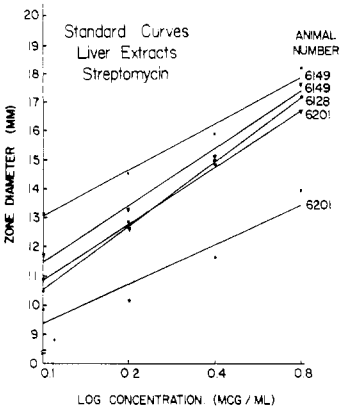

Figure 2. *Bacillus subtilis* response to various concentrations of streptomycin diluted in liver extracts

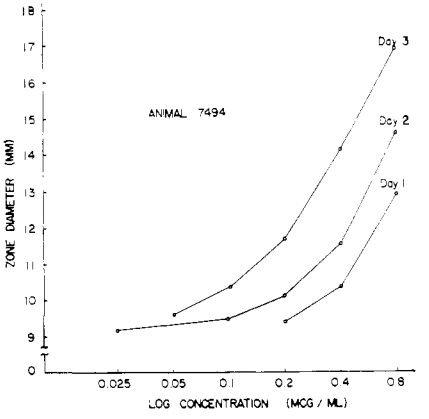

Figure 3. Extended standard curves of streptomycin in swine liver extracts

Bacillus subtilis

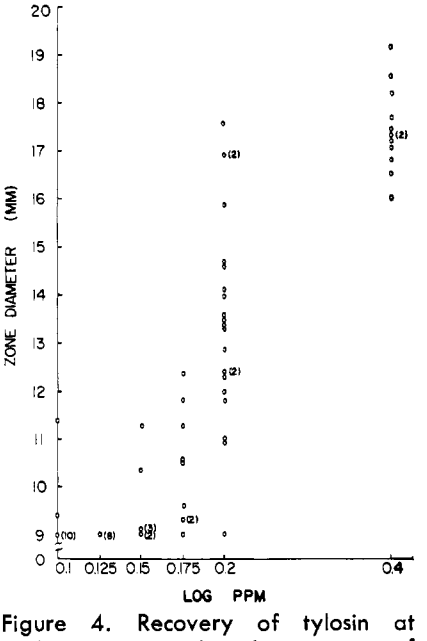

Figure 4. Recovery of tylosin at various concentrations in presence of chicken lean

Values in parentheses, No. of observations at that point. Negative controls (21). 9.0 mm.

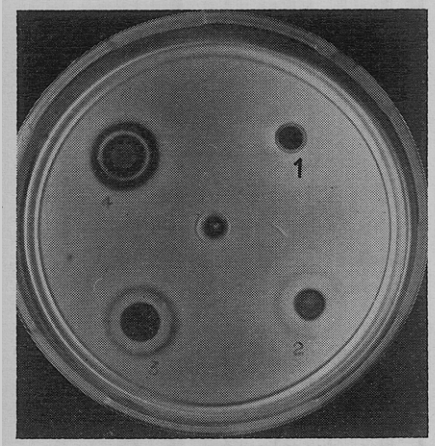

Figure 5. Streptomycin recovery in swine liver

Table III. Test Design to Establish Sensitivity of Microbiological Assay Procedure

		Experimental Design			
		Negative control,	Recoveries		
Day	Animal	C	C + K_1[a]	C + K_2	C + K_3
I	A	×	×	×	×
	B	×	×	×	×
	C	×	×	×	×
II	A	×	×	×	×
	B	×	×	×	×
	C	×	×	×	×
III	A	×	×	×	×
	B	×	×	×	×
	C	×	×	×	×

[a] μg./g. standard added.

Rather than use this method of establishing test sensitivity, Kennedy (*6*) recommended that a range be used to define the sensitivity of the method. "Relative zero" under these conditions would fall at the upper limit of this range. This more realistic approach would allow for some variation in the method and would not require pinpoint titrations which are not resolvable by microbiological techniques. It would also allow the investigator to establish a sensitivity with some degree of confidence.

New tissue residue analyses should be designed to include a thorough study of the system. It is proposed that the design employ a decision rule for the establishment of residues or no residues. It would be impossible to eliminate all the problems associated with these sensitive tests, but certain standards can be established, giving the investigator some degree of confidence that a sample contains or does not contain a residual antibiotic. A technique for evaluating test data has been attempted in our laboratories and represents a change from what is considered the traditional microbiological approach.

Three animals were chosen to evaluate test sensitivity. The basic outline is included in Table III.

Three standard "recoveries" are made on each animal. Each negative control and series of recoveries are extracted by the routine procedure and replicated on ten agar plates. Zone diameters in millimeters are recorded. This is repeated for each animal over a period of 3 days. After completion, zone diameters are averaged and negative values—i.e., no zones of inhibition—are replaced by the value 9.0 mm. for penicylinders. These values are the smallest practical diameters to read. Averages are calculated on the basis of ten individual zones, except where cause can be assigned, such as "leakers."

Statistical analysis (*10*) is then used to determine a point of significant difference between the negative control and the control plus antibiotic. The level where the difference is significant is used to establish the test sensitivity range. If necessary, titrations are made to establish the point of insignificant difference. A value denoting significant difference on a single day's test in terms of millimeters of diameter ("delta") is calculated and used for all future tests which employ that specific antibiotic in that particular tissue.

For testing purposes, negative control samples from three animals receiving no antibiotic are submitted with test samples. Five test animals are used for each treatment. Where possible, each test animal is assayed individually against a pool of three negative controls.

Again each sample and control is replicated 10 times. Averages are calculated and compared to the negative control. If the sample has an average zone diameter greater than that shown by the negative control plus the significant "delta," that sample is considered to have a residual antibiotic.

Further developments are now being studied to control these tests. These include the addition of a "recovery" at some point in the upper range of test sensitivity with each group of samples. In this manner one will be able to describe the test performance.

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