Acta vet. scand. 1979, *20,* 477—491.

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A MODIFIED METHOD FOR THE DETECTION OF ANTIBIOTIC* RESIDUES IN SLAUGHTER ANIMALS**

By

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FABIANSSON, S. and Å. RUTEGÅRD: *A modified method for the detection of antibiotic residues in slaughter animals.* Acta vet. scand. 1979, *20,* 477—491. — Biological methods in current use for the detection of antibiotic residues in slaughter animals are reviewed. A modified method is suggested in which the conditions for the control have been standardized. By the use of a semi-defined medium, the batch-to-batch variations are minimized. In order to facilitate the detection of sulfonamides the medium is supplemented with trimethoprim. The standardized conditions included the use of a sporulating organism, Bacillus subtilis, an inoculum size of 0.5×10^5 spores per ml medium, and 5 ml medium of pH 6.0 per plate. A preincubation-diffusion time of 1 h in room temperature is recommended before incubation.

The modified method was compared with the currently prescribed Swedish method. The new method was easier to perform and showed a more uniform sensitivity to most of the antibiotics used.

antibiotic residues; chemotherapeutic residues; residues; slaughter animals; Bacillus subtilis; trimethoprim.

Over the past few years there has been increasing concern over the extensive administration of antibiotics to farm animals either therapeutically or as feed additives (*Swann* 1969, *Anon.* 1977). In Sweden, a total amount of 40,200 kg of antibiotic substances were administered to farm animals in 1976. Of this

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* Throughout used so as to include antibiotics and chemotherapeutics.

** This study was supported by grants from the National Swedish Food Administration.

amount 20,200 kg were used therapeutically, 7,500 kg administered prophylactically by medicated feed, and 12,500 kg used as feed additives for growth promoting purposes. The corresponding farm animal stock can roughly be estimated at 1.8 million head of cattle, 2.5 million pigs, 0.05 million horses, 0.4 million sheep and goats and 7.6 million fowl.

Depending on the mode and rate of excretion of the compounds, antibiotic residues can be present when slaughtering meat producing animals. In recent years, the potential hazards of the presence of antibiotic residues in food products have been emphasized (*Pichnarcik et al.* 1969, *FAO/WHO* 1969, *Mol* 1975) and various methods have been developed for the detection of such residues. There is no generally accepted method, because the demands vary between countries in the antibiotics most commonly used. Surveys for antibiotic residues have been carried out in several countries: in Denmark (*Lange & Madelung* 1959), USA (*Huber et al.* 1969), Hungary (*Takács & Kovács* 1969), the Netherlands (*Schothorst & Peelen-Knol* 1970), France (*Fréres et al.* 1971), Italy (*Gasparini et al.* 1972), USSR (*Fellegiova & Hustavova* 1972), Switzerland (*Schuler* 1972), the German Federal Republic (*Götze & Djalili-Afchar* 1973), Poland (*Kulezakiewicz* 1973), Czechoslovakia (*Malíková et al.* 1974), Norway (*Yndestad & Sørheim* 1975), Canada (*Tittiger et al.* 1975) and Ireland (*McCracken & O'Brien* 1976). Results of these studies vary widely reclecting regional differences.

Officially approved methods have been introduced in several countries. In Denmark a method using Micrococcus luteus as the test organism was described in 1959 (*Lange & Madelung*) and introduced in 1962 (*Anon.* 1961). A similar method was introduced in Sweden in 1966 (*Anon.* 1966). The same strain of Micrococcus luteus used in those countries is also used in the Netherlands in a modified method (*Schothorst* 1969), while in the German Federal Republic Bacillus subtilis is used as the test organism in a method introduced in 1974 (*Rieve et al.* 1974).

Research has been carried out particularly in Germany (*Lorenzen* 1967, *Pichnarcik et al., Bartels et al.* 1972) and the Netherlands (*Schothorst, Schothorst et al.* 1973, *Nouws & Smulder* 1974, *Mol*) to develop and standardize methods for the detection of antibiotic residues in meat or other food products. During the experimental work the test organisms most commonly used have been Bacillus subtilis BGA, Bacillus subtilis

ATCC 6633, Bacillus stearothermophilus var. calidolactis or Micrococcus luteus ATCC 9341 (*Schothorst, Bartels et al., Kundrat* 1972). The most important improvements in media composition are the enhancement of the sulfonamide sensitivity by the removal of antagonistic substances (*Gudding* 1974, *Huckerby* 1976) and the introduction of synergistic acting compounds (*Gudding* 1976).

Most of the officially approved methods have been criticized in some way or another. Already in 1967 the official Swedish method had been recognized as being too unspecific (*Ekstam* 1967). However, more important drawbacks in using this method are the poor degree of standardization prescribed and the insufficient sensitivity for tetracyclines, sulfonamides (*Thorén* 1967, *Pakkala et al.* 1976) and chloramphenicol (*Fabiansson & Rutegård* 1976).

The aim of the present study was to improve and standardize the most influential methodological parameters involved in the biological detection of antibiotics and chemotherapeutics and to design a method suitable for the purpose of detecting these residues in slaughter animals. The work has been restricted to substances and conditions valid for Scandinavian circumstances.

MATERIALS AND METHODS

Test organisms

Three different strains, Micrococcus luteus ATCC 9341, Bacillus subtilis BGA (No. 10649 E. Merck) and Bacillus stearothermophilus var. calidolactis (Orion Diagnostica) were used. M. luteus was stored as a broth culture at 4°C. When used, 1 ml of the stock culture was inoculated into 9 ml of broth, incubated for 18 h at 30°C and then used directly or after dilution 1:5 with physiological saline. B. subtilis and B. stearothermophilus were delivered by the manufacturer as spore suspensions. The suspensions were inoculated directly into the medium.

Culture media

The media used were Mueller Hinton Agar (CM 337, Oxoid Ltd), Standard II Nutrient Agar (No. 7883, E. Merck), Iso-Sensitest Agar (CM 471, Oxoid Ltd) and Peptone free Agar according to the official Swedish regulations (*Anon.* 1966)*.

The pH was adjusted with 0.1 M-HCl or NaOH to the desired value. The media were sterilized in autoclave according to the re-

* Meat juice (1 part of meat, 2 parts of water) 1 l, NaCl 5 g, $NaNH_4HPO_4 \cdot 4\ H_2O$ 1 g, agar 10 g.

commendations given by the manufacturer. In some instances 15 or 25 μg trimethoprim (AB Kabi) was added to 100 ml of the medium previously cooled to about 55°C.

Antibiotics

Different water dilutions of the following antibiotics were prepared: benzylpenicillin, dihydrostreptomycin, neomycin, oxytetracycline, chlortetracycline, tetracycline, oleandomycin, chloramphenicol, sulfanilamid (all Sigma Chem. Co.), spiramycin (AB Leo), tylosin (Eli Lilly Int. Corp.), sulfadoxin/trimethoprim, 5:1 (Hoechst AG) and sulfaphenazole (Pfizer Inc.). Preimpregnated discs (Ø 5 mm) with the following substances were purchased (the concentration per disc is given within brackets): sulfaisodimidin (250 μg), sulfamethoxazole/trimethoprim (23.8/1.2 μg), benzylpenicillin (1 μg), phenoxymethylpenicillin (10 μg), ampicillin (10 μg), streptomycin (30 μg), chloramphenicol (30 μg), erythromycin (15 μg), oxytetracycline (30 μg), tetracycline (30 μg), neomycin (30 μg), bacitracin (10 i.u.) and oleandomycin (50 μg) (all AB Biodisk).

Experiments

Unless otherwise stated, pour plates were used containing 5 ml of the medium with an inoculum of 0.5×10^5 spores (bacteria)/ml in disposable Petri dishes (Ø 9 cm). Antibiotics were allowed to diffuse from preimpregnated discs into the medium for a period of 4 h at 4°C. The plates were then incubated for 18 h at 30°C (B. subtilis, M. luteus) or at 50°C (B. stearothermophilus). The diameter of the resulting inhibition zones was measured using a vernier caliper.

The media compared

Two media, Mueller Hinton agar and Iso-Sensitest agar, were compared with the media officially prescribed in Sweden and the German Federal Republic. In accordance with the official Swedish regulations, M. luteus was used as the test organism and the pH was adjusted to 7.4. The resulting inhibition zones on Mueller Hinton agar, Iso-Sensitest agar and Peptone free agar were compared.

In accordance with the German regulations, B. subtilis was used as test organism and the pH was adjusted to 6.0. The resulting inhibition zones on Mueller Hinton agar, Iso-Sensitest agar and Standard II Nutrient agar were compared.

MIC-values

The minimal inhibitory concentrations (MIC) for the combinations of antibiotics and test organisms used were determined in tube dilution series. In the tubes 0.9 ml Iso-Sensitest agar with 10^5 bacteria (or spores) per ml was mixed with 0.1 ml of different dilutions of the water solutions of antibiotics. To one dilution series using B. subtilis, 0.25 μg trimethoprim was added to each of the media. For comparison, the official Swedish method was transformed

to a tube dilution method. The tubes were incubated for 18 h at 30°C (M. luteus, B. subtilis) or at 50°C (B. stearothermophilus). Growth/no growth in the tubes was noted as "visible" to the naked eye.

The pH of the medium

Using Iso-Sensitest agar, the resulting inhibition zones at pH 6.0, 6.5, 7.0, 7.5 and 8.0 were compared. The pH was adjusted as previously mentioned.

Thickness of medium, inoculum size and preincubation-diffusion conditions

Pour plates using B. subtilis in Iso-Sensitest agar with a pH of 6.0 were prepared. The thickness of the plates was varied by using 5, 10 or 15 ml of the medium per plate, giving medium thicknesses of about 1, 2 and 3 mm, respectively. The inoculum size was varied between a colony count of 1.0×10^4 and 1.0×10^5 per ml medium. The preincubation-diffusion times used varied between 0 and 4 h at a temperature of 4°C or 20°C.

Pour plates or spread plates

A comparison of the pour plate and spread plate technique was made using M. luteus and Iso-Sensitest agar. A broth culture of M. luteus prepared as previously described was diluted 1:5 in physiological saline and flooded into the surface of the plate or mixed with the medium (0.5 ml/100 ml).

Preliminary comparison of combinations

The official Swedish method using a pH of 7.4 was compared with three methods designed according to the results in the previous sections: B. subtilis in Iso-Sensitest agar at a pH of 6.0, B. subtilis in Iso-Sensitest agar with 0.25 μg trimethoprim added per ml and B. stearothermophilus in Iso-Sensitest agar at a pH of 7.4. Five ml of the medium containing 0.5×10^5 spores per ml were poured into each plate. The samples to be examined consisted of kidney tissue of about 1 cm in diameter taken from the margin between the cortex and the medulla. These were removed just after slaughter from 123 emergency-slaughtered animals (cattle, pigs and horses). Between the sample and the medium on the B. stearothermophilus plate a 4 cm diameter dialysis membrane (No. 130885, Kebo Grave) was placed. Before use, the membranes were boiled in distilled water for 1 h in order to remove inhibiting substances. The size of the inhibition zones was measured from the edge of the tissue sample to the edge of the inhibition zone.

Statistical methods

Conventional statistical methods were used (*Gore* 1952). Student's paired t-test was used for statistical comparison between the values of the resulting inhibition zones.

RESULTS AND DISCUSSION

Comparison of media

When compared to three common media used in the control of antibiotics, Iso-Sensitest agar showed the largest inhibition zones both at a pH of 6.0 and of 7.4 (Table 1). The differences were significant at pH 7.4 ($P < 0.01$).

Table 1. Comparison of the diameter of inhibition zones on different media at pH 6.0 with B. subtilis and at pH 7.4 with M. luteus.

Micrococcus luteus			Bacillus subtilis		
	Diameter of inhibition zone in mm				
Medium at pH 7.4	mean (n=12)	mean of the paired difference ± s		mean (n=12)	Medium at pH 6.0
Peptone free agar	11.3			15.4	Standard II Nutrient agar
		3.75±3.60 (P<0.01)	0.75±3.33 (P>0.05)		
Iso-Sensitest agar	15.1			16.2	Iso-Sensitest agar
		2.17±1.64 (P<0.01)	1.50±2.39 (P>0.05)		
Mueller Hinton agar	12.9			14.7	Mueller Hinton agar
		1.58±3.09 (P>0.05)	0.75±2.34 (P>0.05)		
Peptone free agar	11.3			15.4	Standard II Nutrient agar

Iso-Sensitest agar is a representative of semi-defined media devoid of substances antagonistic to sulfonamides or tetracyclines. Besides showing larger inhibition zones than the other media tested, the batch-to-batch variation can also be supposed to be lower because of the better definition of the ingredients. For these reasons Iso-Sensitest agar was preferred for the rest of the investigations following.

Test organisms and MIC-values

In Table 2 the minimal inhibitory concentrations of the antibiotics used are shown for the different strains. Differences in the patterns of sensitivity between the tested strains were particularly obvious for tetracyclines where B. subtilis showed the best sensitivity. The sensitivity to sulfonamides was very low for all the strains tested.

Table 2. Minimal inhibitory concentrations (MIC) for different antibiotics as determined by tube dilutions in Iso-Sensitest agar and Peptone free agar.

Antibiotics	Iso-Sensitest agar			
	B. subtilis		M. luteus	
	pH 6	pH 7.4	pH 6	pH 7.4
Dihydrostreptomycin	0.8[2]	0.2	>10	1
Benzylpenicillin	0.002	0.004	0.004	0.004
Sulfanilamid	>100	>100	10—40*	1—4*
Sulfadimidin	>100	>100	10—40*	2
Sulfaphenazol	10	40	1—6*	8—10*
Sulfadoxin-trimethoprim (5:1)	1	1	2	0.6
Tylosin	0.2	0.1	0.6	0.1
Tetracycline	0.06	0.1	0.4	1
Oxytetracycline	0.08	0.2	0.6	2
Chlortetracycline	0.02	0.06	0.2	0.4
Chloramphenicol	0.6	0.8	2	0.8
Spiramycin	1—10	0.2	8	0.2
Neomycin	0.1—1	0.4	4	0.6

Table 2 (continued).

Antibiotics	Iso-Sensitest agar			Peptone free agar
	B. stearothermophilus		B. subtilis + trimethoprim[1]	M. luteus
	pH 6	pH 7.4	pH 6	pH 7.4
Dihydrostreptomycin	6	0.6	<0.4	0.2
Benzylpenicillin	0.001	0.002	<0.002	0.004
Sulfanilamid	>100	10—100	2	1—4*
Sulfadimidin	10—100	10—100	2	1—4*
Sulfaphenazol	10	4—8*	<0.1	1—4*
Sulfadoxin-trimethoprim (5:1)	0.4	0.4	0.4	0.4—0.8*
Tylosin	0.06	0.06	<0.2	0.4
Tetracycline	0.08	0.08	<0.04	0.6
Oxytetracycline	6	6	0.08	0.6
Chlortetracycline	0.06	2	<0.02	0.4
Chloramphenicol	1—10	4	0.4	0.8
Spiramycin	2	0.2	2	0.4
Neomycin	1	0.1	0.2	0.8

* Diffuse border between growth/no growth.

[1] 0.25 µg trimethoprim per ml medium.

[2] MIC in µg/ml.

Besides the importance of the sensitivity of the strains, other practical implications must also be considered. In this respect all the different strains used showed advantages and disadvantages that made them more or less suitable for a general inhibitor test. With M. luteus it was difficult to standardize the inoculum size. The reproducibility of the method can thus vary from time to time and from laboratory to laboratory, since one factor controlling the size of the inhibition zone is the size of the inoculum (*Kavanagh* 1972).

The two Bacillus strains are available as standardized spore suspensions which give a more accurate inoculum size. B. stearothermophilus also has the additional advantage of high optimum temperature growth, 50—65°C, and of being a true thermophile (minimum growth temperature 40°C). This facilitates prediffusion which with this organism can be performed at room temperature without the test organisms growing and preventing the growth of a contaminating flora during incubation. B. stearothermophilus is sensitive to most of the antibiotics tested but unfortunately also to many other substances.

Forschner & Seidler (1976) reported that by using a semipermeable membrane most of the "unspecific reactions" shown in the German method could be avoided. In the present study it was also found that by placing a semipermeable membrane between the sample and the B. stearothermophilus test plate most of the unspecific reactions could be avoided. However, this was time-consuming and sometimes caused problems due to uneven growth underneath the membrane.

The greatest disadvantage of B. subtilis was the poor sulfonamide sensitivity. Small amounts of trimethoprim in the medium substantially improved the sensitivity because of a synergistic action with sulfonamides. The sensitivity was enhanced 50 times by this procedure. The same principle can be applied to M. luteus or B. stearothermophilus, but with the latter there is a certain risk of obtaining a complete growth inhibition.

pH of the medium

The significance of alterations in the pH of the medium on the inhibitory effect of antibiotics is shown in Tables 2 and 3.

The pH of the medium had the greatest implications for certain groups of antibiotics. Some antibiotics, e.g. tetracyclines

Table 3. The significance of the pH of the medium for the size of the antibiotic inhibition zones.

Antibiotics	pH 6.0	pH 6.5	pH 7.0	pH 7.5	pH 8.0
Sulfaisodimidin	12.0[1]	12.0	12.5	12.5	13.0
Sulfamethoxazole/trimethoprim	14.5	12.5	12.5	13.0	14.0
Benzylpenicillin	21.0	19.5	18.5	19.0	19.0
Phenoxymethylpenicillin	21.5	19.0	18.0	19.0	18.5
Ampicillin	20.5	17.5	17.0	17.5	18.0
Streptomycin	11.0	11.5	12.0	12.5	12.5
Chloramphenicol	16.5	14.5	13.0	13.5	14.5
Erythromycin	12.5	12.5	13.0	13.0	15.0
Oxytetracycline	17.0	14.0	11.5	11.5	9.5
Tetracycline	17.5	15.5	13.0	12.0	11.0
Neomycin	11.5	11.5	12.0	13.0	14.0
Bacitracin	10.0	6.0	8.0	8.0	5.0
Oleandomycin	13.0	12.0	13.0	14.0	15.0

[1] Diameter of inhibition zone in mm.

and bacitracin, had their optima of action at a low pH, while others, e.g. neomycin, streptomycin and erythromycin, had their optima near pH 8.0. Changes in the pH of the medium in some instances altered the sensitivity of detection by 10 times or more.

In the German Federal Republic, the official method prescribes the use of two different pH values (pH 6 and 8) to assure a good sensitivity for most of the antibiotics. At pH 8, however, it seems as if a greater number of unspecific reactions appear (*Forschner & Seidler, Korkeala et al.* 1976). A low pH value therefore seems to be more suitable for a routine method, while higher pH values can be used in specific methods for certain antibiotics. Independent of the pH level chosen a careful adjustment is important because of the variations in inhibitory action of antibiotics that occur even with small changes in the pH.

Thickness of medium and inoculum size

The results show that a small number of colonies in the medium gives larger inhibitory zones. The difference in the size of the inhibition zones (mean for the difference $\pm$ s, $n = 24$) between a colony count of 1.0×10^4 and 0.5×10^5 per ml medium was 1.25 ± 0.87 mm which was significant ($P < 0.01$), and

between a colony count of 0.5×10^5 and 1.0×10^5 it was an almost significant difference ($P < 0.05$) of 0.67 ± 0.89 mm.

The quantity of medium per plate also plays a significant role. The smaller the quantity of medium the larger the inhibition zone. The difference between inhibition zones with 5 ml medium and 10 ml medium per plate was 0.83 ± 0.72 mm (mean for the difference $\pm$ s, $n = 24$, $P < 0.01$) and between 10 ml and 15 ml medium 0.75 ± 1.14 mm ($P < 0.05$).

The smaller the inoculum size and the less the quantity of medium per plate the better the sensitivity. However, to allow for the plate to be read the microbial growth must be confluent. For this reason it seemed to be necessary to have at least 0.5×10^5 colonies per ml of medium. Five ml of medium in a 9 cm Petri dish was sufficient to avoid drying out during incubation.

Preincubation-diffusion conditions

The importance of the preincubation-diffusion time is illustrated in Fig. 1. At very short preincubation times inhibition

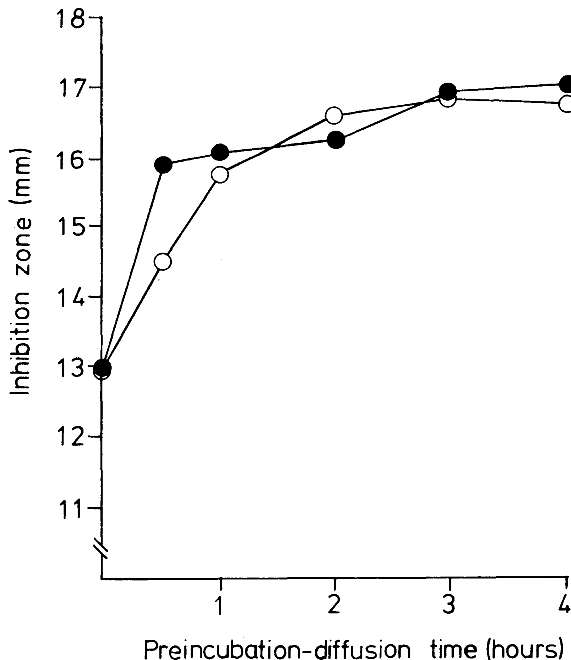

Figure 1. Inhibition zone as a function of preincubation-diffusion time at 4°C and 20°C. Values are means of 12 individual measurements. O—O 4°C, ●—● 20°C.

zones were significantly larger when room temperature was used than at 4°C. After ½ h the difference in zone size was 1.42 ± 1.08 mm (mean for the difference ± s, $n = 24$, $P < 0.001$). However, at 1 h of preincubation the difference in zone sizes between the different temperatures was only 0.33 ± 0.65 mm which was insignificant ($P > 0.05$).

The largest increase in the size of inhibition zones, about 3 mm, was shown between the test start and 1 h's diffusion. Further increase was less than 1 mm during the remaining test time. The diffusion speed is temperature dependent, but an elevated temperature also gives rise to an increased growth rate in the microorganisms used. The width of the zone of inhibition is thus determined from the speed of the diffusion of the antibiotic and the growth rate of the test organism. When the bacterial colonies have reached a critical size they are not influenced by the antibiotic any more and the border of the inhibition zone is established.

A closer study of the influence of the time of prediffusion at different temperatures, but with small quantities of antibiotics, will be published.

Pour plates or spread plates

The total difference between pour plates (larger zones) and spread plates (smaller zones) was on average 2.45 ± 1.92 mm ($n = 24$). The difference between pour plates and spread plates was significant ($P < 0.01$).

Results reported in literature comparing pour plates and spread plates are contradictory (*Schothorst* 1969, *Mol* 1975). Because of the better standardization of the inoculum size with the pour plate technique it was preferred in the comparison of combinations.

Comparison of combinations

After studying the parameters involved in the standardizing of the methodology three test methods were designed and compared with the official Swedish method. The purpose of this comparison was to get a rough estimation of the usefulness of the methods and an idea of practical difficulties that could arise. The results are shown in Table 4. Out of 123 samples examined seven showed inhibition zones of varying size on the test plates.

Table 4. Comparison of methods on an emergency slaughter material.

Species	Diagnosis	Treatment	Time between the last treatment and slaughter	Diameter of inhibition zones in mm			
				MLP	BSS	BSS+T	BStS
Pig	Mastitis	Sulfadoxin+trimethoprim	24 h	4	12	18	20
Pig	Leg weakness	Penicillin+DHS	10 d	0	4	4	10
Pig	Muscle injury	Penicillin+DHS	8 d	9	10	10	15
Cow	Mastitis, paresis puerp.	No information	—	22	22	25	40
Cow	Mastitis, metritis	No information	—	16	19	21	30
Horse	Intoxication	Several antibiotics	?	22	20	22	40
Cow	Traum. peritonitis	Sulfanilamid	12 h	0	0	24	—

MLP = Micrococcus luteus in Peptone free agar.
BSS = Bacillus subtilis in Iso-Sensitest agar.
BSS+T = Bacillus subtilis in Iso-Sensitest agar + 0.25 μg trimethoprim/ml.
BStS = Bacillus stearothermophilus in Iso-Sensitest agar.

The largest inhibition zones appeared on the B. stearothermophilus plates, but these plates were difficult to handle in practice mainly because of the necessary membrane between the sample and the plate. This combination therefore needs further development before it can be adopted as a method for a routine control.

The other two methods using B. subtilis were easy to read with sharp edges around the inhibition zones. Compared to the M. luteus plate both the B. subtilis plates were easier to prepare. The trimethoprim supplemented plate was preferred mainly because of the better sulfonamide sensitivity seen in the sulfadoxin/trimethoprim and sulfanilamid treated animals. An obvious advantage of this plate was the uniform sensitivity to most of the antibiotics used.

The method of choice according to this study, therefore seemed to be B. subtilis in a semi-defined medium supplemented with trimethoprim. To prove this assertion the proposed method will be compared with the official Swedish method in the normal abattoir work.

ACKNOWLEDGEMENTS

We want to express our sincere thanks to Miss Kerstin Karlsson, the Department of Food Hygiene, for technical assistance.

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SAMMANFATTNING

En modifierad metod för påvisande av kemoterapeutika i slaktkroppar.

Rester av kemoterapeutika kan förekomma i muskulaturen hos köttproducerande djur efter nutritiv eller terapeutisk behandling med sådana preparat. Vid köttkontrollen används i olika länder olika biologiska metoder för att påvisa rester av kemoterapeutika. Den från 1966 i Sverige föreskrivna metoden uppvisar flera svagheter. En förändrad och bättre standardiserad metod föreslås. Genom användning av ett väldefinierat substrat, Iso-Sensitest agar, och en sporulerande mikroorganism, Bacillus subtilis, uppnås en mindre variation mellan dagar och mellan laboratorier. Genom tillsats av trimethoprim förbättras sulfonamidkänsligheten. Lämplig substrat- och inokulationsmängd är 5 ml substrat med $0,5 \times 10^5$ sporer per ml. Efter provapplicering förinkuberas plattorna en timme i rumstemperatur. Med den nya metoden kan flera av den tidigare föreskrivna metodens nackdelar undvikas.

(Received February 16, 1979).

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