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AN IMPROVED MICROBIOLOGICAL PROCEDURE FOR DETECTION OF STREPTOMYCIN RESIDUES IN MILK AND ANIMAL TISSUES

By

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SØGAARD, HENRY: *An improved microbiological procedure for detection of streptomycin residues in milk and animal tissues.* Acta vet. scand. 1979, *20*, 38—44. — A method is described which allows detection of 0.025 µg streptomycin sulfate per ml. This represents an improvement of sensitivity by 8 times when compared with the currently used method. By adding penicillin to the assay medium in subinhibitory concentrations, a synergistic effect of streptomycin and penicillin is exerted towards the test organism, Bacillus subtilis, resulting in an increased sensitivity to streptomycin.

synergism; penicillin; streptomycin; antibiotic residues.

For detection of residues of antibiotics in milk and organs from slaughtered animals, microbiological agar diffusion tests are widely used. Compared to chemical methods, microbiological assays are advantageous being cheap and less laborious. A critical point, however, is to obtain satisfactorily sensitive test organisms and media which support diffusion of the drugs to be tested for.

In Denmark, Bacillus subtilis is used as test organism in detection of streptomycin residues in kidneys from slaughtered animals. The lowest concentration of streptomycin sulfate which can be detected by the currently used test is approx. 0.4 µg/ml. Milk and milk products are routinely examined for antibiotic residues by using B. stearothermophilus var. calidolactis as test organism. This strain is sensitive to approx. 1.0 µg/ml streptomycin sulfate.

Recently, microbiological methods have been developed in which improved sensitivity to drugs is obtained by adding substances to the test media which enhance inhibition by synergism: *Gudding* (1976) found that addition of trimethoprim to Mueller-Hinton medium improved the sensitivity of the test bacteria to sulphonamides by 20—50 times. *Kornfeld* (1977) used addition of egg-white lysozyme which improved the sensitivity of Sarcina lutea to penicillin from 0.0125 to 0.0075 i.u./ml. Synergism between penicillin and aminoglycosides has been recognized for many years (*Jawetz et al.* 1950). When sensitive bacteria are simultaneously exposed to penicillin and streptomycin, the cell walls are damaged by penicillin and the streptomycin is able to gain access and exert its lethal effect (*Moellering & Weinberg* 1971).

The method presented in this paper is based on this synergistic effect. By adding penicillin to the test medium at a subinhibitory concentration the sensitivity of Bacillus subtilis to streptomycin is increased.

MATERIALS AND METHODS

Media

As assay medium, Bacto Antibiotic Medium 5 (Difco 0277) was used, whereas for propagation and sporulation of the test bacteria, Bacto Antibiotic Medium 1 (Difco 0263) was used.

Test organism

A spore suspension of Bacillus subtilis ATCC 6633 was prepared by growing the strain for 10 days at 37°C in Roux bottles containing 200 ml of sporulation medium. The bacterial growth was suspended in 50 ml of sterile physiological saline and centrifuged. The supernatant was decanted and the sediment was washed 3 times in saline. The cells were resuspended in 50 ml of saline, and the suspension was heat-treated for 5 min. at 80°C. The number of spores in the suspension was determined by plate count on blood agar to be 10^9/ml.

Procedure for determination of the size of inoculum and concentration of penicillin

In order to obtain maximum synergistic effect an optimum combination of inoculum size and concentration of penicillin had

to be determined. To aliquots of the melted assay medium, cooled to 70°C, spore suspension was added at 3 different concentrations per ml: 0.5×10^7, 1.0×10^7 and 2.0×10^7. To the seeded media 4 concentrations of K benzylpenicillin were added (i.u./ml): 0, 0.1, 0.2 and 0.4 which produced assay media with 12 combinations of bacteria and penicillin. Twenty ml of these media were spread in duplicate into Petri dishes (140 mm). The plates were allowed to cool. On each plate a streptomycin-containing tablet (Neo-sensitab®, Rosco) with a streptomycin content of 1 mg was placed. The plates were incubated at 37° for 20 hrs. and examined for bacterial growth and inhibition zones.

The results are summarized in Table 1. It appears that inhibition zones on plates without penicillin were 23—26 mm. When penicillin was added, complete growth inhibition occurred with 0.5×10^7 spores per ml. At a concentration of spores of 1.0×10^7 per ml growth occurred with 0. 1 and 0.2 i.u./ml of penicillin and the inhibition zones were considerably enlarged compared to the plates without penicillin. The medium supplemented with 0.1 i.u./ml yielded the maximum inhibition zone, 53 mm. By increasing the concentration of spores to 2.0×10^7/ml the sensitivity of the system was not further improved. For the following experiments, the combination of 1.0×10^7 spores and 0.1 i.u. of penicillin per ml was used.

Table 1. Inhibition zones and bacterial growth at different combinations of penicillin and B. subtilis spores using Neo-sensitabs containing streptomycin.

B. subtilis spores per ml $\times 10^7$	Inhibition zones, mm, and bacterial growth			
	Penicillin, μg per ml			
	0	0.1	0.2	0.4
0.5	26	No growth	No growth	No growth
1.0	24	53	49	No growth
2.0	23	35	39	46

Preparation of dose response curves

A master standard solution was prepared by dissolving 16 mg of streptomycin sulfate in 100 ml phosphate buffer, pH 8.0. From this solution, which can be stored at 5°C for 4 weeks, the fol-

lowing concentrations were prepared by dilution in phosphate buffer: 1.6, 0.8, 0.4, 0.2, 0.1, 0.05, 0.025 and 0.0125 µg/ml. Standard response curves were prepared from inoculated assay medium without and with penicillin. The medium without penicillin was inoculated with 10^6 spores per ml.

The standard solutions in 0.1 ml aliquots were transferred by pipettes to 12.7 mm filter paper discs (Schleicher & Scheull Inc.) which were placed on the media. The plates were kept at 5°C for 2 hrs. After incubation at 37°C for 20 hrs., zones of inhibition were measured by using a slide gauge.

A similar procedure was followed in preparing dose response curves where steel cylinders (8×18 mm) were used instead of paper discs.

RESULTS AND DISCUSSION

The lowest concentration of streptomycin sulfate which could be detected in the test system without adding penicillin was 0.2 µg per ml when steel cylinders were used (Fig. 1 and Table 2) and 0.4 µg per ml when using paper discs (Table 3).

When penicillin was added to the assay medium the lowest concentrations of streptomycin sulfate which were certain to produce inhibition zones were 0.025 and 0.05 µg per ml, respectively. This means that the sensitivity of the method is improved 8 times.

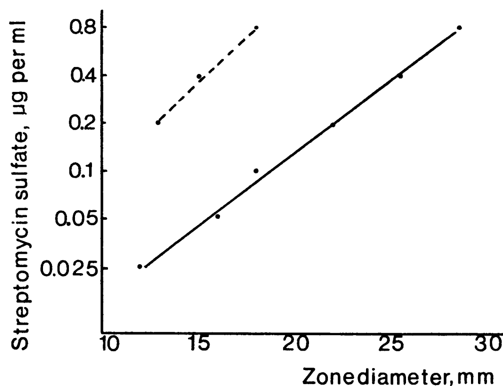

Figure 1. Inhibition zones plotted against the concentration of streptomycin sulfate in Bacto Antibiotic Medium 5 (o- - -o) and in the same medium supplemented with 0.1 i.u. penicillin per ml (o——o). Cylinder plate procedure.

Table 2. Inhibition zone diameters as function of streptomycin sulfate concentrations. Cylinder plate procedure.

Streptomycin sulfate concentration, µg/ml	Inhibition zones in Bacto Antibiotic Medium, mm	
	10^6 B. subtilis spores/ml	10^7 B. subtilis spores + 0.1 i.u. penicillin/ml
1.6	20.5	32.5
0.8	18.0	28.5
0.4	15.0	25.5
0.2	13.0	22.0
0.1	0	18.0
0.05	0	16.0
0.025	0	12.0
0.0125	0	trace

Streptomycins are widely-used antimicrobial drugs in veterinary medicine. According to Swedish statistics on antibiotic consumption in animal husbandry, dihydrostreptomycin, in terms of treated animals, is the most commonly used antimicrobial drug (*Fabiansson et al.* 1977).

Streptomycin may induce allergy in humans. It is therefore an important responsibility of public heath laboratories to ensure animal food products free of residues of this drug. Sensitive methods which can reveal such residues at sufficiently low concentrations combined with legal sanctions in cases of positive findings are the fundamental measures to be taken.

Table 3. Inhibition zone diameters as function of streptomycin sulfate concentrations. 12.7 mm paper discs.

Streptomycin sulfate concentration, µg/ml	Inhibition zones in Bacto Antibiotic Medium, mm	
	10^6 B. subtilis spores/ml	10^7 B. subtilis spores + 0.1 i.u. penicillin/ml
1.6	22.5	32.0
0.8	20.5	30.0
0.4	17.5	26.0
0.2	trace	19.0
0.1	0	17.5
0.05	0	16.0
0.025	0	trace
0.0125	0	0

Inglis & Katz (1978) developed a more sensitive procedure for detection of dihydrostreptomycin residues in milk and dairy products. The method includes precipitation of milk proteins by acidification and centrifugation, thereby removing physical barriers to diffusion in the cylinder plate assay. In this way 0.06 μg/ml dihydrostreptomycin in milk could be detected.

The method described in this paper allows detection of even lower concentrations without a rather laborious clean-up procedure. Besides, the method is applicable also to organs like kidneys, which are usually used for examination of slaughtered animals. One disadvantage, however, should be mentioned. Addition of penicillin to the assay medium makes it necessary to increase the inoculum of test bacterium by 10 times. The size of the inoculum is rather critical and therefore the procedure has to be standardized very accurately.

In laboratory routines for detection of antibiotics it is desirable to have at one's disposal methods to identify the type of drug which inhibits the test organism. Penicillin can be identified specifically with penicillinase and sulfonamides with p-aminobenzoic acid.

Other aminoglycosides, e.g. neomycin will exert a similar synergistic effect on microbial cells when combined with penicillin. Streptomycin drugs, however, constitute the predominant part of the aminoglycosides used in veterinary medicine. Therefore an enhancement of inhibition zones when penicillin is added to the assay medium will provide a significant indication of presence of streptomycin residues in samples of milk or animal tissues.

The method has already been used in our laboratory routine together with the officially prescribed method without penicillin. In 4 cases it has been clearly demonstrated that inhibition zones of kidneys are considerably enlarged when using the new method.

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SAMMENDRAG

En forbedret mikrobiologisk metode til påvisning af streptomycinrester i mælk og animalt væv.

Der beskrives en metode, der tillader påvisning af 0,025 μg streptomycin sulfat pr. ml. Dette er en forbedring af følsomheden på 8 gange sammenlignet med den for tiden anvendte metode. Ved at sætte penicillin til substratet i subinhibitive koncentrationer opnås en synergistisk virkning af streptomycin og penicillin over for testorganismen, Bacillus subtilis, hvilket resulterer i en forøget følsomhed for streptomycin.

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