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COMPARISON OF DIFFERENT AGAR DIFFUSION METHODS FOR THE DETECTION OF ANTIMICROBIAL RESIDUES IN SLAUGHTER ANIMALS

By

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KORKEALA, H., O. SORVETTULA, O. MÄKI-PETÄYS and J. HIRN: *Comparison of different agar diffusion methods for the detection of antimicrobial residues in slaughter animals.* Acta vet. scand. 1982, *23*, 407—415. — The different agar diffusion methods were compared using antibiotic and sulphonamide-impregnated filter-paper discs and the kidneys of healthy and emergency-slaughtered pigs and cows after slaughter.

No method used seemed to be sensitive to all antimicrobial drugs preimpregnated onto discs. Tetracycline yielded a greater zone of inhibition at pH 6 than at pH 8 and aminoglycosides, erythromycin, polymyxin B and lincomycin at pH 8 than at pH 6. It therefore seems necessary to use different pHs (6 and 8). The addition of trimethoprim to the medium is necessary for the detection of sulphonamides. Bacillus subtilis BGA used as the test organism was more sensitive to sulphonamides on the "Test agar for the inhibitor test" containing trimethoprim than on the "Iso-Sensitest agar" also containing trimethoprim. The addition of trimethoprim to "Test agar for the inhibitor test" is recommended at pH 8 but not at pH 6 because false-positive cases (with inhibition zones > 2 mm) were observed at pH 6 with trimethoprim on the kidneys of healthy pigs.

agar diffusion method; antimicrobial residues; Bacillus subtilis; Micrococcus luteus; slaughter animals; trimethoprim.

During recent years several attempts have been made to increase the sensitivity of agar diffusion methods in the detection of antimicrobial residues in slaughtered animals. The use of 2 different pHs of the agar media (pH 6 and 8) has increased the sensitivity of the method in the detection of tetracyclines, amino-

glycosides and macrolide antibiotics (*Levetzow & Weise* 1974). *Gudding* (1976) introduced the idea of adding trimethoprim to the medium to increase the sensitivity of the test organism to sulphonamides. The studies of *Gudding* (1974) and *Bogaerts et al.* (1981) underlined the importance of the medium in the procedure.

The purpose of the present work was to compare different agar diffusion methods using antibiotic and sulphonamide-impregnated filter-paper discs, and to analyse the kidneys of healthy and emergency-slaughtered pigs and cows with these methods.

MATERIALS AND METHODS

Antimicrobial agents

Discs (⌀ 5 mm) preimpregnated with the following substances were used (the content per disc is given within brackets): ampicillin (10 μg), cefalexin (30 μg), chloramphenicol (30 μg), erythromycin (15 μg), penicillin G (10 μg), polymyxin B (10 μg), neomycin (30 μg), streptomycin (30 μg), sulfaisodimidin (250 μg), sulfamethoxazole + trimethoprim (23.8 + 1.2 μg) and tetracycline (30 μg) (AB Biodisk, Solna, Sweden). Lincomycin (Upjohn S.A. Puurs, Puurs, Belgium) was impregnated onto filter-paper discs (Schleicher & Schüll, Dassel, Federal Republic of Germany, ⌀ 12.7 mm). The content of lincomycin was 10 μg per disc.

Kidney samples

The kidneys of 22 healthy and 6 emergency-slaughtered cows and of 35 healthy and 13 emergency-slaughtered pigs were analysed for antimicrobial residues with the agar diffusion technique. The samples were taken from the margin between the kidney cortex and the kidney medulla.

Agar diffusion

Test organisms. Micrococcus luteus ATCC 9341 and Bacillus subtilis BGA were used. The M. luteus was incubated in a nutrient broth (Orion Diagnostica, Espoo, Finland) 24 h at 30 °C and diluted 1:10 with sterile physiological saline. After dilution, 3 ml of M. luteus suspension was spread on the medium and after diffusion for 5 min the rest of the M. luteus suspension

was pipetted away. The medium was then allowed to dry. 0.1 ml of B. subtilis BGA spore suspension (E. Merck, Darmstadt, Federal Republic of Germany) was added to 100 ml of the medium.

Trimethoprim (Tr) solution. 100 mg of Tr (Sigma Chemical Co., St. Louis, Mo., USA) was dissolved in 20 ml of ethanol and 80 ml of sterile distilled water was added. The Tr-solution was further diluted with distilled water.

Agar-media. M. luteus was seeded on "Pen assay seed agar" (Orion Diagnostica) (denoted M. luteus method below). B. subtilis BGA was added to the following media: "Iso-Sensitest agar" (Oxoid, England) (denoted Iso-Sensitest method below) containing 0.15 µg Tr/ml, "Test agar pH 6.0 for the inhibitor test" (E. Merck) (denoted Test agar 6 method below), "Test agar pH 6.0 for the inhibitor test" containing 0.15 µg of Tr/ml, "Test agar pH 8.0 for the inhibitor test" (E. Merck) (denoted Test agar 8 method below) and "Test agar pH 8.0 for the inhibitor test" containing 0.03 or 0.06 µg of Tr/ml. The pH of "Iso-Sensitest agar" was adjusted to 6.0 with 1 mol/l HCl. Ten ml of each medium was poured onto a Petri dish (⌀ 9 cm). Thus the effect of the thickness of the plates on the size of the inhibition zones was avoided (*Fabiansson & Rutegård* 1979).

Test procedure. The discs or the kidney samples of 9 mm diameter (thickness about 2 mm) were laid on the media. The prediffusion period was 2 h at 4 °C for M. luteus and 1 h at 20 °C for B. subtilis. The prediffusion time of 2 h for the M. luteus method was used because it is part of the official Finnish method. For the other methods a prediffusion time of 1 h was used according to the recommendations of *Fabiansson & Rutegård*. After prediffusion the plates were incubated for 20 h at 30 °C. In the case of the Test agar 6 method, with or without Tr, a disc containing 5000 units of penicillinase (BBL, Cockeysville, Md., USA) was laid near the antibiotic disc or kidney sample. In the case of the Test agar 8 method with Tr, the disc containing 5 µg of p-aminobenzoic acid (PABA) (E. Merck) was laid near the disc or kidney sample.

If a zone of complete inhibition of growth was surrounded by a zone of clearly distinguishable partially inhibited microbial growth, the partial inhibition zone was included in the inhibition zone. This was done because pig kidneys sometimes show a par-

tial inhibition zone, with or without a complete inhibition zone within it, after treatment with antimicrobial drugs. A zone of partial inhibition of growth without a zone of complete inhibition, however, was considered as negative, since the concentration of the drug possibly present in the kidney in these cases must be relatively low and the possibility of a false-positive reaction is apparent.

RESULTS AND DISCUSSION

The zones of inhibition caused by discs containing antimicrobial agents using the various methods are presented in Table 1. None of the methods used seemed to be sensitive to all the antimicrobial drugs studied. The Test agar 6 method and the Test agar 8 method, both with Tr, were found to be the best. However, the Test agar 6 method with Tr yielded quite small zones of inhibition with aminoglycosides, erythromycin, polymyxin B and lincomycin and the Test agar 8 method with Tr similarly with tetracycline. It therefore seems necessary to use 2 different pH's (6 and 8) and to add Tr to the medium. The Finnish official method, the M. luteus method, yielded large and distinct zones of inhibition with penicillin G, cefalexin, lincomycin and especially ampicillin but was less sensitive to the other antimicrobial drugs studied. Sulfamethoxazole + trimethoprim yielded zones of partial inhibition with this method. It is to be noticed, however, that the M. luteus method is more laborius to perform and difficult to standardize than the other methods.

The addition of 0.06 μg of Tr to the medium decreased the density of B. subtilis BGA but the size of the zone of inhibition could still be measured. The addition of 0.03 μg of Tr did not seem to decrease the density of B. subtilis BGA to the naked eye but was also not enough to increase the sensitivity of the test microbe to sulphonamides.

The discs containing penicillinase inhibited the effect of penicillin G and ampicillin, and the discs containing PABA inhibited the effect of sulphonamides. Discs like these can be used for the identification of antimicrobial residues (*Fabiansson et al.* 1981).

Table 2 shows the results when the kidneys of healthy cows and pigs were analysed by different agar diffusion methods used for the examination of antimicrobial residues. No method yielded false-positive cases when the kidneys of cows were examined.

Table 1. Comparison of different agar diffusion methods in sensitivity testing of antimicrobial agents.

Antimicrobial agent	µg/ disc	Mean diameter of inhibition zones of 2 experiments (in mm including the 5 mm disc)						
		M. luteus ATCC 9341 + Pen assay seed agar	B. subtilis BGA + Iso-Sensi-test agar pH 6.0 with 0.15 µg Tr[a]/ml	B. subtilis BGA + Test agar pH 6.0	B. subtilis BGA + Test agar pH 6.0 with 0.15 µg Tr/ml	B. subtilis BGA + Test agar pH 8.0	B. subtilis BGA + Test agar pH 8.0 with 0.03 µg Tr/ml	B. subtilis + Test agar pH 8.0 with 0.06 µg Tr/ml
Ampicillin	10	49.4	42.0	45.9	46.7	38.5	43.6	45.0
Cefalexin	30	48.4	48.6	48.6	50.3	43.7	50.0	47.0
Chloramphenicol	30	39.3	39.7	40.9	40.7	39.3	38.2	40.8
Erythromycin	15	37.5	34.4	34.1	36.2	41.6	44.0	43.7
Penicillin G	10	52.8	46.7	50.8	51.3	41.5	46.9	46.9
Polymyxin B	10	5.0	9.8	13.4	13.6	15.1	15.5	21.9
Neomycin	30	15.0	26.5	27.4	27.7	40.2	42.8	43.1
Streptomycin	30	23.8	25.4	35.8	34.3	39.8	41.5	42.3
Sulfaisodimidin	250	5.0	48.5	41.6	55.3	30.7	48.0	54.4
Sulfamethoxazole	23.8							
+ trimethoprim	1.2	5.0	53.0	47.0	56.7	37.9	46.3	53.1
Tetracycline	30	33.6	43.1	50.4	50.7	34.3	38.5	38.4
Lincomycin[b]	10	40.4	29.0	12.7	12.7	23.0	31.8	35.9

[a] Tr = trimethoprim

[b] The diameter of the discs was 12.7 mm

Table 2. Mean inhibition zone[a] and false-positive cases[b] with different agar diffusion methods of testing kidneys of healthy animals.

Species	Number of animals		M. luteus ATCC 9341 + Pen assay seed agar	B. subtilis BGA + Iso-Sensitest agar pH 6.0 with 0.15 μg Tr[c]/ml	B. subtilis BGA + Test agar pH 6.0	B. subtilis BGA + Test agar pH 6.0 with 0.15 μg Tr/ml	B. subtilis BGA + Test agar pH 8.0	B. subtilis BGA + Test agar pH 8.0 with 0.06 μg Tr/ml
Cow	22	Inhibition zone[a]	0	0	0	0.1	0	0
		False-positive cases[b]	0	0	0	0	0	0
Pig	35	Inhibition zone	0	0	0.1	0.2	0.2	0.4
		False-positive cases	0	0	0	2	0	0

[a] Inhibition zones in mm from edge of the kidney sample to outer egde of the zone
[b] Cases with inhibition zones greater than 2 mm
[c] Tr = trimethoprim

Two bovine kidneys yielded small zones of inhibition with the Test agar 6 method with Tr. Zones of inhibition were observed with the kidneys of pigs with some methods. Two false-positive cases (with inhibition zone > 2 mm) were recorded with the Test agar 6 method with Tr. Almost every pig kidney yielded zones of partial inhibition with this method. The largest zone of partial inhibition was 3.7 mm. Zones of complete inhibition were present for few kidneys of pigs, and the zones larger than 2 mm were always composed of both complete and partial inhibition. In some cases the zones of complete inhibition were present with the Test agar 8 method with Tr. The largest zone was 1.8 mm. The results with pig kidneys were more difficult to interpret reliably with the Test agar 6 method with Tr than with the Test agar 8 method. The Test agar 8 method with Tr is therefore recommended.

Table 3. Comparison of different agar diffusion methods with emergency slaughter material[a].

Species	Diagnosis	Inhibition zones in mm from edge of the kidney sample to outer edge of the zone					
		MLP	BSI	BST6	BST6+Tr	BST8	BST8+Tr
Pig	Metritis	0	9.0	3.5	13.0	0	2.3
Pig	Cachexia, abscesses	0	0	0	5.8	0	0
Pig	Endocarditis	0	0	0	2.6	0	0
Pig	Polyarthritis	0	0	0	2.2	0	1.0
Pig	Pyelonephritis	0	9.0	0	11.3	0	10.0
Pig	Hernia umbilicalis, enteritis, peritonitis	0	0	0	4.0	0.7	1.2
Cow	Pneumonia	5.5	8.3	8.7	11.3	8.5	15.0
Cow	Prolapsus uteri	0	0	1.9	3.9	1.6	0

MLP = Micrococcus luteus ATCC 9341 in Pen assay seed agar
BSI = Bacillus subtilis BGA in Iso-Sensitest agar (pH 6.0) + 0.15 µg trimethoprim/ml
BST6 = B. subtilis BGA in Test agar pH 6.0
BST6+Tr = B. subtilis BGA in Test agar pH 6.0 + 0.15 µg trimethoprim/ml
BST8 = B. subtilis BGA in Test agar pH 8.0
BST8+Tr = B. subtilis BGA in Test agar pH 8.0 + 0.06 µg trimethoprim/ml

[a] Only those cases are included where the zone of inhibition with any of the methods was > 2 mm

Those cases where the zones of inhibition for the kidneys from emergency-slaughtered animals are larger than 2 mm with any method used are presented in Table 3. Analysing the kidneys of 13 pigs, 6 positive cases were observed. The PABA discs reduced the zones of inhibition for the kidneys of sows with metritis and pyelonephritis. The sows had obviously been treated with drugs containing sulphonamides. The rest of the positive cases yielded zones of inhibition only for the Test agar 6 method with Tr. The reason for these zones remained unknown; the possibility of false-positive reactions must be considered.

With the kidneys of 6 emergency-slaughtered cows 2 positive zones of inhibition were observed (Table 3). The PABA discs decreased the zones of inhibition slightly for a cow with pneumonia caused by aspiration. The cow had probably been treated with several drugs, also containing sulphonamides. The reason for the other positive case remained unknown.

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SAMMANFATTNING

Jämförelse mellan olika agar-diffusions metoder för påvisning av antimikrobiella rester i slaktkroppar.

Olika agar-diffusions metoder jämfördes genom att använda filterpapperdisker innehållande antimikrobiella läkemedel, och njurar från friska och nödslaktade slaktsvin.

Ingen använd metod syntes vara känslig för alla antimikrobiella läkemedel, som hade impregnerats på diskerna. Tetracyklin förorsakade en större hämningszon vid pH 6 än vid pH 8 och aminoglycosider, erytromycin, polymyxin B och lincomycin vid pH 8 än vid pH 6. Det synes därför nödvändigt att använda två olika pH-värden (6 och 8). Genom tillsats av trimetoprim var det möjligt att bestämma sulfonamider. Bacillus subtilis BGA var känsligare för sulfonamider på „Test agar for the inhibitor test", som innehöll trimetoprim, än på „Iso-Sensitest agar", som också innehöll trimetoprim. Tillsats av trimetoprim rekommenderas vid pH 8 men icke vid pH 6. Falskapositiva fall (hämningszoner > 2 mm) observerades vid pH 6 med trimetoprim, när njurar av svin underssöktes.

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