

micro-organisms (Okerman & Hoof, 1997). The pH of the test media is an important factor influencing the detection limits of most antibiotics. It is therefore possible that tissue components, such as proteins, change the composition of the medium and influence the inhibitory zone produced by an antibiotic residue present in the sample (Okerman *et al.*, 1998). The antibiotic residues were determined in animal food products (Stead *et al.*, 2004), meat samples (Okerman & Hoof, 1997), pork (Chang *et al.*, 2000), chloramphenicol residues were determined in the tissues (Lynas *et al.*, 1998).

The other microbiological method for determining antimicrobial agents in meats is the 'Premi test'. Antimicrobial agents are used in aquaculture and are notorious for their apparent accumulation and persistence in fish. Safety assurance and inspection of edible products from fish need screening methods to show, in a fast and cost-effective way, the absence or the presence of this class of compounds at acceptable legislative levels. Recently, the 'Premi test' was introduced as an easy-to-use broad-spectrum microbial screening test for detection of antibacterial compounds in meat and meat products from cattle, pigs and chicken; thus from terrestrial animals (Geijp *et al.*, 1998; Reybroek, 2000).

The aim of the present study was to first measure the inhibition zones for penicillin, sulfadimidine and streptomycin for checking the test plates and then preparing paper discs with different concentrations of antibiotics for determining the detection limits. The next step was the determination of antibiotics in trout which were fed with antibiotics (sulfadiazine/trimethoprim) by using EEC FPT and 'Premi Test'.

Materials and methods

EEC four-plate test

Media for residue testing

Test agar pH 6.0 (Merck, 10663), test agar pH 7.2 (Merck, 15787) and test agar pH 8.0 (Merck, 10664) were prepared and autoclaved. After cooling to 45–50 °C, the bacterial suspensions and the supplement for pH 7.2 test agar were added and the plates were poured.

Four different inoculated media were used for antibiotic detection:

medium I – test agar pH 6.0, seeded with *B. subtilis* (10^4 CFU mL⁻¹); medium II – test agar pH 7.2, with trimethoprim (Riedel-de Haen, 46984) added to a final concentration of 50 µg L⁻¹ medium and seeded with *B. subtilis* (10^4 CFU mL⁻¹); medium III – test agar pH 8.0, seeded with *B. subtilis* (10^4 CFU mL⁻¹); medium IV – test agar pH 8.0, seeded with *M. luteus* (10^4 CFU mL⁻¹). Sterile petri dishes (diameter, 90 mm) were filled with 12 mL of the prepared and seeded media.

The antibiotic standards that were used included the following: penicillin G Sodium Salt (Fluka, 13752),

sulfadimidin (Riedel-de Haen, 46802), streptomycin (Fluka, 02586), sulfadiazin (Riedel-de Haen, 35033). Stock solutions of 0.0062 µg per 10 µL were prepared for penicillin, 0.5 µg per 10 µL for sulfadimidin, streptomycin and sulfadiazine, respectively. Twofold dilutions were prepared from these stock solutions. A total of five dilutions were prepared for each antibiotic. Testing detection limits of antibiotics used was as follows: three plates from one batch of the medium were used for each concentration tested. At a distance of 10 mm from the edge of the plate, 0.01 mL of these dilutions was applied to 6.5-mm diameter paper discs and four paper discs were laid on each plate. Each dilution was tested four times on the medium considered to be most sensitive for the antibiotic tested.

Fish samples and diet preparation

Trout were obtained from the Ahrensburg Station of the Institute of Fishery Ecology. Five trout were used as the untreated control group, 55 trout were fed with Aquavet S/T which contains 40% of powder Tribissen including 6.67% of Trimethoprim and 33.33% of sulfadiazine. The use of 75 mg of powder which is 30 mg of combined active ingredients per kilogram body weight per day was used according to the recommendations of the producer, 'Biomar' of the medical food Aquavet S/T to use 0.5% Aquavet per total body weight per day. The total uptake of the food was guaranteed by personal observation. The medicated feed was applied for a period of 7 days. From day 10 onwards, the remaining trout were fed with commercial pellets (Trouvit) without antibiotics. During the first seven days, five trout were taken daily; after break off of the diet five trout were taken every ninth day 5. All trout were slaughtered and stored at –24 °C until testing.

Sample preparation

Frozen trout were thawed at 4 °C overnight. The trout fillets were homogenised by using Ultra Turrax T25. About 10 g of the trout homogenate was taken for centrifugation. Trout homogenate was centrifuged at 19 500 r.p.m. for 15 min. Then 10 µL of the supernatant for each trout was applied to paper discs (Mast Diagnostics, BD0638W) and the discs were dried at 40 °C for 10 min. These dried paper discs were put on the seeded agar plates which were prepared before. Then 10 µL of the supernatant was applied to four plates of agar media. Plates containing media I, II and III were incubated overnight at 30 °C; while plates with medium IV were incubated for 24 h at 37 °C. Each of the sixty trout was analysed four times with each medium.

Premi test

'Premi test' is a broad-spectrum microbial screening test especially developed for the detection of antimicrobial compounds, such as antibiotics and sulfonamide

residues in meat or meat products at or below most Maximum Residue Limit (MRL) levels. 'Premi test' is based on the inhibition of the growth of *Bacillus stearothermophilus*, a bacterium very sensitive to many antibiotics and sulfa compounds. A standardised number of spores are imbedded in an agar medium with selected nutrients. When meat fluid is added to the 'Premi test' and heated at 64 °C, the spores will germinate. The germinated spores will multiply and form an acid when no inhibitory substances are present. This will be made visible by a colour change from purple to yellow of the indicator. When antimicrobial compounds are present in sufficient amount (above the detection level), no growth will occur and the colour will remain purple (DSM Food Specialties, R & D, Delft, the Netherlands).

For the 'Premi test', performance of the inhibition assay was carried out as per the test kit instructions. A total of 100 µL of the fish supernatants was applied to an ampoule. The ampoule was then incubated at 64 °C until the yellow end point was visible in the negative control (3–4 h).

Results and discussion

Detection limits for different antibiotics are shown in Table 1. Six observations were obtained with each concentration of antibiotics. Inhibition zones were measured and correlated to antibiotic concentrations. Detection limits were as follows: penicillin G on medium I (sodium salt), 0.4 ng; sulfadimidin on medium II, 62.5 ng; streptomycin on medium III, 62.5 ng; streptomycin on medium IV, 250 ng. It was reported by Okerman *et al.* (1998) that the detection limit of penicillin on medium I was 0.5 ng; sulfadimidin on medium II was 50 ng; and streptomycin on medium III was 20 ng, respectively. In another report, on media I, II, III and IV, the inhibition zones were found to be 6-, 6-, 8- and 6-mm wide for 0.01 IU of penicillin G-Na, 0.5 µg of sulfadimidin and 0.5 µg of streptomycin, respectively (Bogaerts & Brussels, 1980).

Sulfadiazine/trimethoprim was detected on media I, II and III, but detection limits on plates I and III were lower than that on plate II. A positive result was indicated by the complete inhibition of growth on the surface of the agar around discs on one or more test plates, in an inhibition zone not less than 2-mm wide. Results on plate II were as follows: at 500 ng, the inhibition zones were 14.5 mm. Detection limit was 1.95 ng for sulfadiazine/trimethoprim, which was not less than 2-mm wide.

Chloramphenicol was also checked for EEC FPT. Chloramphenicol was detected on media II, III and IV, but the zones were too small. At 500 ng, inhibition zones on media II, III and IV were 6.33 ± 0.52 -, 2.67 ± 0.52 - and 3.17 ± 0.98 -mm wide, respectively.

Table 1 Detection limits for different antibiotics

| Medium | Antibiotics (and detection limits) | Antibiotic (ng)/ paper disc tested | Diameter of zones without fish supernatant (range of six observations) per mm | | |
|--------|---|------------------------------------|---|-------|-------|
| I | Penicillin G (0.4 ng) | 6.2 | 10-8 | | |
| | | 3.1 | 8-7 | | |
| | | 1.6 | 6-5 | | |
| | | 0.8 | 5-3 | | |
| | | 0.4* | 2-0 | | |
| | | 0.2 | 0 | | |
| | | II | Sulfadimidine (62.5 ng) | 500 | 8-7 |
| | | | | 250 | 6 |
| | | | | 125 | 5-4 |
| | | | | 62.5* | 4-1 |
| 31.2 | 1-0 | | | | |
| 15.6 | 0 | | | | |
| II | Sulfadiazine/ Trimethoprim (1.95 ng) | | | 500 | 15-14 |
| | | | | 250 | 12 |
| | | | | 125 | 11-10 |
| | | | | 62.5 | 10-9 |
| | | 31.2 | 7 | | |
| | | 15.6 | 5-4 | | |
| | | 7.8 | 4-3 | | |
| | | 3.9 | 3-2 | | |
| | | 1.95* | 2-1 | | |
| | | 0.98 | 0 | | |
| III | Streptomycin (62.5 ng) | 500 | 7-6 | | |
| | | 250 | 6-5 | | |
| | | 125 | 4-3 | | |
| | | 62.5* | 2-1 | | |
| | | 31.5 | 0 | | |
| | | 15.6 | 0 | | |
| IV | Streptomycin (250 ng) | 500 | 6-5 | | |
| | | 250* | 4-3 | | |
| | | 125 | 1 | | |
| | | 62.5 | 0 | | |
| | | 31.2 | 0 | | |
| | | 15.6 | 0 | | |

*Detection limit.

Chloramphenicol detected in aqueous solutions did not produce an inhibitory zone of 12 mm. According to our findings the determination of chloramphenicol by using FPT method was not suitable.

Detection of antibiotic residues in trout fed with Aquavet S/T is shown in Fig. 1.

In Fig. 1, inhibition zones on the media I, II III were detected, but on medium II the zones were the widest, meaning that this medium was the most sensitive to residues of sulfadiazine/trimethoprim. The method used in this test is based on the use of different pH as this test is measures an effect of generally unknown substance which can be active at different pH. Different activities occurred and different inactivation occurred at different pH. In the control group (day 0), no inhibition zones

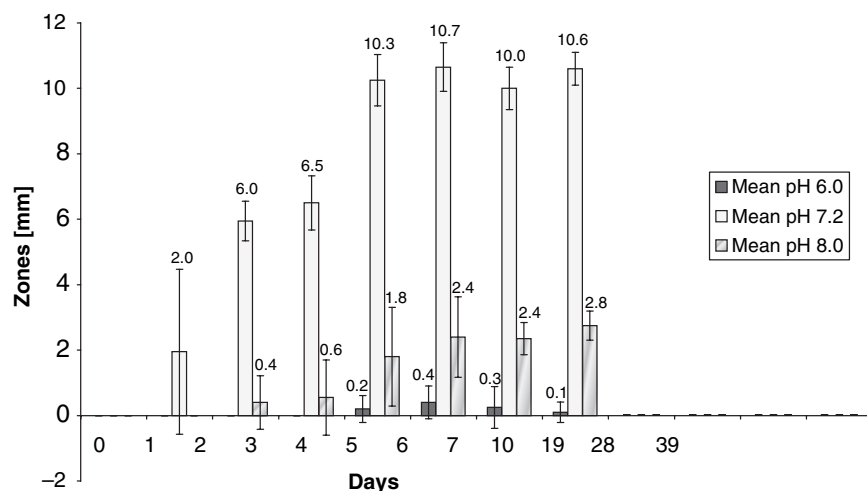


Figure 1 Detection of antibiotic residues in trout fed with Aquavet S/T.

Table 2 Detection of residues in trout by 'Premi test'

| | Number of trout | Presence/absence of the antibiotic |
|---------------|-----------------|------------------------------------|
| Control group | 1–5 | Negative |
| Day 1 | 5–10 | Positive |
| Day 2 | 11–15 | Positive |
| Day 3 | 16–20 | Positive |
| Day 4 | 21–25 | Positive |
| Day 5 | 26–30 | Positive |
| Day 6 | 31–35 | Positive |
| Day 7 | 36–40 | Positive |
| Day 10 | 41–45 | Positive |
| Day 19 | 46–50 | Negative |
| Day 28 | 51–55 | Negative |
| Day 39 | 56–60 | Negative |

Detection limit for sulfadiazine/trimethoprim is 1.95 ng.

were detected. Trout fed with pellets containing antibiotics on day 1 exhibited an inhibition zone of about 2-mm wide; the inhibition zones increased according to the duration of feed application. On day 7, the zones were increased to 10.6-mm wide. The concentration of the residues accumulated and reached a plateau after five days. A day after discontinuing the medicated diet, the antibiotics were detectable in the same concentration as on day 7, but three days later (on day 10) they were no longer detectable in the trout.

Detection of residues in trout by 'Premi test' is shown in Table 2. According to the 'Premi test', the results of trout from days 1 to 10 were found to be positive and after day 10, they were found to be negative.

In the present study, the results showed us that the 'Premi test' was more sensitive than the EEC FPT. In contrast to the 'FPT', three days after discontinuing the medicated diet, there were still residues detectable by 'Premi test' (on day 10). Data is presented that shows that the FPT and the Premi test methods can be used for the determination of sulfadiazine/trimethoprim residues in trout.

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