The NAT test – screening for antibiotic residues in the tissues of food-producing animals

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Abstract

Antibiotics are commonly used to treat infectious diseases not only in humans, but also in animals. As a result of the use of antibiotics in food animals, however, the presence of residues of these drugs in animal tissues is inevitable and poses a potential threat to public health. In order to protect the public health, it is necessary to ensure compliance with maximum residue limits, which are stipulated for each product, and to screen for residues of veterinary drugs in animal products. Screening for residues of veterinary drugs in animal products was introduced in practice soon after the introduction of the antibiotic treatment of infectious diseases in animals. Microbial inhibition tests (MITs) are now commonly used in practice and are the first choice in the context of the monitoring of antibiotic residues in animal products. They are based on inhibition of the growth of bacterial strains in the presence of an inhibitory substance. The Nouws Antibiotic Test (NAT) is, in principle, consistent with other MITs and represents an alternative to the MITs currently used for the detection of residues of antibiotics in the tissues of food-producing animals.

Antibiotics, residues, NAT, food producing animals

Introduction

According to Commission Decision 2002/657/EC as amended, screening methods mean methods that are used to detect the presence of a substance or class of substances at a given level of interest. The level of interest is defined as the concentration of a substance in a sample that is significant in determining its compliance with the legislation, i.e. the established maximum residue limit. Screening methods should have the capability of high sample throughput, and should be used to screen large numbers of samples for potential non-compliant results.

Screening methods for the determination of antibiotic residues

In order to protect public health, Commission Regulation 37/2010 as amended established maximum limits for residues of pharmacologically active substances in foodstuffs of animal origin, including meat, fish, milk, eggs and honey. Maximum residue limits are the maximum quantities of pharmacologically active substances that can be permitted in foodstuffs of animal origin. Maximum residue limits are reference values for the control of residues in foods of animal origin in member states and at border inspection posts (Regulation (EC) No. 470/2009 of the European Parliament and Council).

The effectiveness of the monitoring of residues of pharmacologically active substances depends largely on the method used (Pikkemaat et al. 2011). Methods used for screening for antibiotic residues in food matrices must meet the requirements set forth by Commission Decision 2002/657/EC as amended. They may be used only if it can be demonstrated that they have been validated.

In practice, antibiotic residues in foodstuffs of animal origin are monitored using microbial inhibition tests (MIT). MIT are agar diffusion tests based on the principle that the growth of an appropriate test strain will be inhibited in the presence of antibiotics and sulphonamides. They are rapid, cost effective tests with the capability for high sample throughput that makes it possible to sort a large number of samples into "negative" and "potentially positive" samples in a short period of time. Potentially positive samples are then

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subjected to confirmatory testing to confirm the presence of residues, their identification and subsequent quantification. Growth inhibition of the test strain on an agar medium manifests itself as a zone of inhibition (disk diffusion test) or, alternatively, as a change in the indicator colour caused by a drop in the pH as a result of active metabolism of the test strain in the absence of an inhibitor is not observed (tube tests) (Kožárová 2005). Reliable determination of large numbers of antibiotic residues is, however, possible only with the use of multiplate methods with a combination of bacterial test strains (Pikkemaat 2009).

Microbial inhibition plate tests

Plate tests consist of a layer of cultivation medium inoculated with spores of a sensitive test bacterium. Samples are applied either to the agar surface or into wells in the agar surface (Okerman et al. 2001). The microbial inhibition tests currently most popular include the four-plate test **(**FPT) and a screening assay for the determination of antibiotic residues using five bacterial strains – the STAR protocol.

Based on the principle of agar diffusion, the FPT uses two bacterial test strains, i.e. *Bacillus subtilis* BGA and *Micrococcus luteus* ATCC 9341, and various combinations of the pH values of the cultivation media. *Bacillus subtilis* BGA on an agar at a pH of 6.0 is sensitive to tetracycline antibiotics, *Bacillus subtilis* BGA on an agar at a pH of 8.0 to aminoglycoside antibiotics, *Bacillus subtilis* BGA on an agar at a pH of 7.2 to sulphonamides, and *Micrococcus luteus* ATCC 9341 on an agar at a pH of 8.0 to beta-lactam antibiotics. The cultivation medium for the determination of sulphonamide residues contains some trimethoprim to increase sensitivity to sulphonamides. Clear zones appear around the sample if the sample being examined contains substances that inhibit the growth of the test strain. In the case of muscle and parenchymatous organs, inhibition zones of 2 mm or larger are considered a positive finding (Bogaerst and Wolf 1980; CH 12.16.2004).

The STAR protocol is an agar diffusion test performed in five Petri dishes with an agar medium. The agar and test strain combinations are: *Bacillus subtilis* BGA on an agar at a pH of 8.0 for aminoglycoside antibiotics, *Kocuria rhizophila* ATCC 9341 on an agar at a pH of 8.0 for macrolide and beta-lactam antibiotics, *Bacillus cereus* ATCC 11788 on an agar at a pH of 6.0 for tetracycline antibiotics, *Escherichia coli* ATCC 11303 on an agar at a pH of 8.0 for quinolones, and *Bacillus stearothermophilus* var. *calidolactis* ATCC 10149 plus trimethoprim on an agar at a pH of 7.4 for beta-lactam antibiotics and sulphonamides. Clear zones appear around the sample if the sample being examined contains substances that inhibit the growth of the test strain. Samples are considered positive if the zone of inhibition exceeds 2 mm, or 1 mm in the case of milk and eggs, on at least one of the five Petri dishes. Meat samples are considered positive if the zones exceed 2 mm on plates with test strains *B. subtilis* BGA*, K. rhizophila* ATCC 9341*, B. cereus* ATCC 11778 or *E. coli* ATCC 11303. If the test strain is *B. stearothermophilus* var. *calidolactis* ATCC 10149 the zone must exceed 4 mm (CH 12.19.2006).

In addition to the MIT described above, a number of other methods of screening for antibiotic residues in products of animal origin have been developed, such as the One-Plate Test – OPT (Koenen-Dierick et al. 1995), New Dutch Kidney Test – NDKT (Nouws et al. 1988), Swab Test On Premises – STOP (Johnston et al. 1981), 6-plate microbiological method (Myllyniemi et al. 2001), combined microbiological plate method – CPMA (Ferrini et al. 1997) and others.

Microbial inhibition tube tests

The test organism used in almost all of these tests is *Bacillus stearothermophilus* var. *calidolactis* ATCC 10149. The tests consist of a cultivation medium inoculated with spores of a sensitive bacterium supplemented with a pH indicator. At an appropriate temperature,

the bacteria start to grow and produce acid, which will cause a colour change. This format has been applied mainly in screening for antibiotic residues in milk, but is also increasingly used for screening for antibiotic residues in other matrices (Pikkemaat 2009).

PREMI®TEST

One of the broad-spectrum microbial inhibition tube tests is PREMI®TEST, developed by the Dutch company DSM Food Specialties. This method is intended for the determination of antibiotic residues that may be present in meat juice (muscle, kidney and liver), fish, eggs and the urine of pigs treated with antibiotics. PREMI®TEST combines the principle of agar diffusion tests with colour change of the indicator as a result of active metabolism of the test microorganism in the absence of an inhibitor.

The test is based on the analysis of a 100 µl liquid sample. The sample tested is applied to tubes filled with a nutrient agar medium containing *Bacillus stearothermophilus* var. *calidolactis* ATCC 10149. If the test strain can grow normally during incubation, the colour of the pH indicator will change from purple to yellow. If the sample examined contains substances that inhibit the growth of the test strain, the colour of the indicator will remain purple. The test strain *B. stearothermophilus* var. *calidolactis* ATCC 10149 is incubated at 64 °C for 3 to 3.5 hours. After incubation, the colour of the lower two thirds of the agar is evaluated. Yellow-coloured agar means that there are no antibiotics in the sample. Purple-coloured agar indicates the presence of antibiotics, while a yellow-purple colour of the agar indicates that the amount of antibiotics present is at the detection limit of the test (CH 12.18.2006).

Another microbial inhibition tube test is the TOTAL ANTIBIOTICS, which comes in two forms – as a tube-based (TB) test and a microplate (MP) test. The test was developed by Euroclone S.p.A. (Italy) for rapid detection of antibiotics in milk and in the meat of food-producing animals.

The principal mechanism of the test is the growth inhibition of the test strain *Bacillus stearothermophilus* var. *calidolactis* ATCC 10149 present in the agar medium.

The testing procedure is as follows: 10 ml of extraction buffer diluted 1:10 (v/v) with distilled water is added to muscle samples. After mixing, the samples are left standing overnight at room temperature or at 37 °C for 2 hours. Clear supernatant is used for testing. Samples are incubated at 65 °C for 2.5 – 3 hours. If the sample is positive, the agar will change colour from purple to yellow. If the sample is negative, the purple colour of the agar will not change.

Nouws Antibiotic Test (NAT) – a new alternative screening for antibiotic residues in tissues of food-producing animals

The Nouws Antibiotic Test is also an agar plate diffusion test. It is based on the analysis of renal pelvis fluid, and comprises five test plates enabling the identification of a specific group of antibiotics. The test was developed by Pikkemaat et al. (2008) as a more effective alternative to the New Dutch Kidney Test (NDKT). Developed by Nouws et al. in 1988, the NDKT was a simple one-plate testing system with *Bacillus subtilis* BGA as the test strain. The test did not perform antibiotic residue screening at the required level due to the limited sensitivity of the test strain.

NAT is comprised of test plates that are inoculated with the appropriate test strain of known sensitivity: *Bacillus cereus* ATCC 11788 for tetracyclines (T-plate), *Kocuria rhizophila* ATCC 9341 for beta-lactams and macrolide antibiotics (B&M-plate), *Yersinia ruckeri* NCIM 13282 for quinolones (Q-plate), *Bacillus subtilis* BGA for aminoglycosides (A-plate) and *Bacillus pumilus* CN 607 for sulphonamides (S-plate).

The testing procedure is as follows: using a sterile corkscrew, holes of 14 mm in diameter are made in solidified agar on each plate. Samples of renal pelvis fluid are

applied to the holes in the agar, to which a plate-specific buffer solution is added. Samples should always be collected from fresh kidneys. Fluid samples are collected by absorption to filtration paper disks of 12.7 mm in diameter with an absorption capacity of 100 µl of tissue fluid. The disks are incubated at 30 °C (*Bacillus cereus* ATCC 11778 and *Yersinia ruckeri* NCIM 13282) or 37 °C (*Bacillus pumilus* CN 607, *Kocuria rhizophila* ATCC 9341*, Bacillus subtilis* BGA) for 16–18 hours. The results are evaluated by measuring the zone of inhibition around the holes with the samples tested. Samples are considered positive if the inhibition zone diameter is ≥ 15 mm.

Although renal pelvis fluid is an important indicator of residue violations (compliance/non-compliance with maximum residue levels), a system of microbial post-screening was developed for this method shortly afterwards, i.e. in the case of a positive finding, the microbial post-screening of meat juice (NAT-meat) and/or microbial post-screening of renal pelvis fluid (NAT-kidney) are performed as described below (Pikkemaat et al. 2008; Pikkemaat et al. 2009).

NAT-meat

NAT-meat is performed on the same plate that produced a positive result in the NAT. However, the plates are further supplemented with substances that are specific for the matrices examined. Supernatant obtained after centrifugation of homogenized muscle tissue after inactivation is used for the analysis. Samples have to be mixed with Tris buffer and bromelain protease when determining residues of aminoglycoside antibiotics.

NAT-meat comprises five test plates, just like NAT. However, the B&M-plate is supplemented with 0.0075 $\mu g.ml^{-1}$ tylosin and 0.2 % Tween 20, the T-plate is supplemented with 0.5 $\mu g.ml^{-1}$ chloramphenicol, the S-plate is supplemented with 0.007 $\mu g.ml^{-1}$ trimethoprim, the Q-plate is supplemented with 1 M phosphate buffer at pH 6.5, 5 % volume, and the A-plate contains no supplements. 250 µl of the samples tested (S-plate – 300 µl) is applied to the holes in the nutrient agar. The holes containing samples are furthermore supplemented with specific synergistic substances and phosphate buffer solutions. Samples are considered positive if the inhibition zones around the holes with the samples tested are ≥ 15 mm in diameter.

NAT-kidney

NAT-kidney is also performed on the same plate that produced a positive result in the NAT. Supernatant obtained after centrifugation of homogenized kidney tissue is used for the analysis. NAT-kidney comprises four test plates, but in contrast to NAT-meat, which uses an A-plate with *Bacillus subtilis* BGA as the test strain for the procedure, the A-plate uses *Bacillus pumilus* CN 607 as the test strain, while the S-plate is not used at all. Only the B&M-plate is supplemented (with 0.2 % Tween 20 and 0.0075 $\mu g.ml^{-1}$ tylosine), while the T-plate, Q-plate and A-plate are identical as for the NAT test.

250 µl of the tested samples is applied to the holes made in nutrient agar. Holes with the samples tested on the B&M-plate, Q-plate and A-plate are furthermore supplemented with specific synergistic substances and phosphate buffer solutions. Samples are considered positive if the inhibition zones around the holes with the samples tested are ≥ 15 mm in diameter, with the exception of aminoglycoside antibiotics, for which the zone of inhibition must be ≥ 20 mm for a sample to be considered positive.

Conclusions

While NAT is not presently recognised as one of the official methods of laboratory diagnostics of food designated for the screening of the residues of antimicrobial

substances in products of animal origin from food-producing animals, it does represents a new alternative to the currently used MIT in residue control processes. NAT combines the advantages of simple and effective collection of samples with high antibiotic detection capability. This testing system efficiently identifies the majority of antibiotics and sulphonamides that are used in veterinary medicine at/below their established maximum residue limits. NAT-meat and NAT-kidney serve as microbial post-screening for residues in positive samples, and allow for more accurate identification of specific groups of antibiotics. The advantages of NAT include simplicity, multiresidue character and higher efficiency in identifying antimicrobial substance residues in food matrices even during screening for residues.

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