

Meeting maximum residue limits: an improved screening technique for the rapid detection of antimicrobial residues in animal food products

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A rapid, high-throughput antimicrobial screening assay was developed using either a physical fluid extraction or a solvent extraction technique coupled to the commercially available Premi[®]Test. The solvent extraction approach was fully validated for a wide range of tissues and the fluid extraction approach partially validated for porcine muscle. Both procedures can detect a wide range of antimicrobial compounds at or below maximum residue limit concentrations. The use of a solvent extraction provides an enhanced test capable of detecting a wider range of drugs than the fluid extraction approach at or below half maximum residue limit levels in a variety of matrices. Biochemical methods for the class-specific identification of β -lactams and sulphonamides following initial screening were developed and validated. The approach is a significant improvement on existing methodologies as a tool for residues monitoring in surveillance programmes.

Keywords: antimicrobial, screening, Premi[®]Test, food safety

Introduction

There is a requirement under Directive EC 96/23 (1996) to screen animal products for residues of antimicrobial drugs. A large number of such drugs

(including β -lactams, tetracyclines, sulphonamides, macrolides, fluoroquinolones and aminoglycosides) are licensed for use in food-producing animals and now have established maximum residue limits (MRLs) (EC 1990).

An ideal antimicrobial screening method would detect all licensed antimicrobials at or below their MRLs. Because of the large number of sample analyses involved in statutory and non-statutory surveillance, the method should be rapid, simple (preferably automated) and applicable to a wide range of food types. Such a method should form part of an integrated strategy that enables rapid identification and confirmation of antimicrobials present in screen-positive samples.

The majority of the antimicrobial screening in the UK and elsewhere is currently carried out using plate-based microbial inhibition assays of which the four-plate test (FPT) or the modified four-plate test (MFPT) have been most widely used (Chang *et al.* 2000). However, the FPT and similar assays are far from ideal. While they are simple, they require long incubation times and many of the antimicrobials listed above are not detected effectively, e.g. sulphonamides (Okerman *et al.* 1998a,b). Furthermore, there is a lack of meaningful quality assurance (QC) data presented in the literature (e.g. using fortified samples) and there are wide variations in the performance of the same test between laboratories. Also, the potential for automating the final test measurement is limited. There is therefore a need for the development of an improved antimicrobial screening methodology for application in surveillance programmes.

A new product in the form of an ampoule-based test kit, called the Premi[®]Test recently became available. The Premi[®]Test is based on inhibition of growth of *Bacillus stearothermophilus*, a thermophilic bacterium sensitive to many antibiotics. A standardized number of spores are embedded in an agar medium with

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selected nutrients. On heating to 64°C, the spores germinate and in the absence of inhibitory substances will multiply, producing an acid. This is visualized as a colour change from purple to yellow using the acid-based indicator Bromocresol purple present in the agar medium.

The present paper describes the development and validation of methodology based on the Premi® Test for the detection and post-screening identification of antimicrobial residues in animal food products.

Materials and methods

Chemicals and reagents

Premi® Test kits were supplied by DSM (DSM Food Specialities R&D, Delft, the Netherlands). Lab Lemco broth and β -lactamase were from Oxoid (Basingstoke, UK). All other solvents and solid reagents were from Sigma-Aldrich (Poole, UK). Analytical standards were purchased from Sigma-Aldrich with the exception of enrofloxacin, which was obtained from Bayer (Leverkusen, Germany).

Sample extraction

Method A: Fluid expression (DSM, test kit instructions). Sample, 2 g, was squeezed in a garlic press and the expressed fluid collected. The variation of fluid volume expressed was quite significant between samples. A total of 100 μ l was collected for application to the test ampoule.

Method B: Solvent extraction. Multiresidue extraction solvent, 5 ml acetonitrile/acetone (70:30, v/v), was added to 2 g sample and homogenized for 45 s. The extract was centrifuged (4500 rpm, 4°C, 10 min) and the supernatant removed and evaporated to near dryness under a stream of nitrogen at 40–45°C. The residue was suspended in 250 μ l Lab Lemco broth (8 g l⁻¹).

Fortification procedure

Analyte fortification was carried by applying a known concentration of drug to known blank tissue before

extraction. For example, 100 μ l for a 1 μ g ml⁻¹ solution were added to 2 g tissue to give an equivalent tissue concentration of 50 μ g kg⁻¹. Following fortification, the tissue was allowed to equilibrate for 15 min before extraction.

Premi® Test

Performance of the inhibition assay was carried out as per the test kit instructions. A total of 100 μ l extract 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).

Post-screening detection of β -lactams

β -lactamase solution (20 μ l) was added to an extract (reconstituted in 230 μ l Lab Lemco broth) and the extract applied to the Premi® Test.

Post-screening detection of sulphonamides

p-Aminobenzoic acid solution (50 μ l, 5 mg ml⁻¹) was added to an extract (reconstituted in 200 μ l Lab Lemco broth) and the extract applied to the Premi® Test.

Validation

Validation was carried out by spiked addition of analyte at appropriate concentrations (e.g. MRL, ½ MRL, etc.) to known blank samples (typically $n = 4$ for each analyte/matrix combination). Samples containing residues of incurred drugs, for which the drug concentration had been determined by chromatographic methods, were also used (data not shown).

Results and discussion

Initial experiments were undertaken to compare fluid expression with solvent extraction. The results are summarized in table 1. Both techniques are clearly capable of detecting a range of analytes at or below the MRL concentration. In general, the limit of detection (LOD) is lower for the solvent extraction method. This becomes important where the LOD for

Table 1. Comparison of fluid expression (garlic press) versus solvent extraction for a range of analytes using tissue fortified with antimicrobial compounds.

Compound	Limit of detection (LOD) for pork muscle ($\mu\text{g kg}^{-1}$)		MRL in muscle ($\mu\text{g kg}^{-1}$)
	Fluid extraction	Solvent extraction	
<i>β-lactams</i>			
Penicillin G	< 2.5	< 2.5	50
Ampicillin	5	< 2.5	50
Amoxicillin	> 5	< 2.5	50
Oxacillin	100	< 50	300
Cloxacillin	> 100	< 50	300
<i>Tetracyclines</i>			
Chlortetracycline	100–200	50	100*
Oxytetracycline	100	50	100*
Tetracycline	50	< 25	100*
Doxycycline	50	< 25	100*
Demeclocycline	50	25	–
<i>Sulphonamides</i>			
Sulphaguanidine	150	< 150	100**
Sulphadimethoxine	25–50	< 25	100**
Sulphapyridine	50	100	100**
Sulphamethizole	50–100	< 50	100**
Sulphamethoxypyridine	25	< 50	100**
Sulphisoxazole	25	< 25	100**
Sulphathiazole	25	< 25	100**
Sulphadiazine	50	< 25	100**
Sulphachloropyridazine	25	< 50	100**
Sulphamerazine	25	< 50	100**
Sulphanilamide	150	100	100**
Sulphaquinoxaline	50	< 50	100**
Sulphamethazine	50–100	50–75	100**
<i>Others</i>			
Tylosin	< 25	12.5	100

* Sum of parent drug and its epimer.

** Combined total residues of all substances within the sulphonamide group should not exceed $100 \mu\text{g kg}^{-1}$.

'<' indicates a strong positive response was recorded at the denoted concentration. The actual limit of detection will be, by definition lower than this value.

the fluid extraction procedure is above the MRL, e.g. chlortetracycline. However, the fluid expression procedure offers the advantage of simplicity, the only additional equipment or reagents required being a garlic press. This is appropriate to an 'on-site' test, such as at an abattoir.

It was considered that an alternative method based on solvent extraction was more appropriate for an 'in-laboratory' test for a number of reasons. Chemically and mechanically denaturing the tissue assists with the release of drug residues from tissue. This provides a known sample weight that can be taken through the sample preparation to a known volume enabling the result to be more easily related

to the MRL legislation. Use of a solvent extraction step enables effective concentration of the extract to provide increased sensitivity to reach $\frac{1}{2}$ MRL limits that otherwise would not be achievable. In the laboratory, screening of samples is generally performed to $\frac{1}{2}$ MRL, and samples showing a positive response are subjected to further investigations based on chromatographic methods with specific detectors. For these reasons, the solvent extraction procedure was more extensively investigated.

During the initial evaluation, the test incubation time was seen to increase when used for matrices containing high levels of lysozyme, an endogenous

compound exhibiting microbial inhibition and found in kidney, liver, egg and fish tissue. The incubation time increased from approximately 3.5 to 4.5 h. To counteract the increase in incubation time, the manufacturer of the Premi[®]Test suggested that the ampoules were subjected to a brief heat shock for 10 min at 80°C to degrade the lysozyme before the incubation period. The brief heat shock has been found not to affect the test response to various antibiotics used in the study.

A variety of solvent conditions were evaluated by using various mixtures of acetone and acetonitrile. A solvent ratio of 70:30 v/v acetonitrile/acetone was found experimentally to be the optimum for extracting the widest range of analytes. In addition, dissolution of the final extract in a microbiological growth medium (i.e. Lab Lemco broth) was also found to improve the results. It is thought that the presence of nutrients within the extract facilitates the bacterial growth cycle. The test incubation times were therefore found to reduce when the extract was applied to the ampoules in Lab Lemco broth compared with when using phosphate-buffered saline or sterilized water.

The optimized solvent-based procedure was then evaluated for sensitivity towards a wide range of analytes (table 2). With the exception of cephalixin in pig muscle, LODs are well below the ½MRL. For the sulphonamide class, the majority of individual LODs fall significantly below the MRL. Since the MRL is expressed as 'total' sulphonamides (as parent compounds), this indicates that the developed methodology is capable of detecting reliably multiple sulphonamide residues present in the range one-half to one times the MRL. For the tetracyclines, data indicate that it is possible to detect sub-MRL levels of tetracyclines in the majority of tissue types, the exceptions being oxytetracycline and chlortetracycline in fish and egg which are detectable at or slightly above the MRL. These are both tissues that contain lysozyme and it may be that either this or the thermal sample treatment required to remove the compound interfere with the sensitivity of the final determination.

The developed methodology was also evaluated against a range of other compounds. The microorganism employed in the Premi[®]Test, *B. stearothermophilus*, shows a higher sensitivity towards Gram-positive selective antimicrobial compounds compared with Gram-negative ones. For this reason, test sensitivity towards the aminoglycoside compounds

(gentamicin, lincomycin, neomycin, streptomycin) and the phenicol type (chloramphenicol, thiamphenicol) is poor. The technique does show sufficient sensitivity towards the macrolide class (i.e. Tylosin). The sensitivity towards the Annex IV compound dapsone (EC 1990) is excellent at $< 2.5 \mu\text{g kg}^{-1}$.

Because the methodology is sensitive towards a range of compounds, it becomes increasingly important to find ways of distinguishing the classes of compounds as a first step to confirmatory analysis. As part of the ongoing work to develop an integrated strategy for the detection of antimicrobial substances in food products, investigations into post-screening tests were undertaken.

The use of β -lactamases is well established for the detection of the presence of β -lactam compounds (Rang and Dale 1994). The efficacy of this route was again demonstrated during the course of the present work. By adding β -lactamase to an extract, the presence or absence of a β -lactam can quickly be determined.

A novel approach (Braham *et al.* 2001) to the detection of sulphonamides as a class was also evaluated using the solvent-based extraction coupled to the Premi[®]Test. The mode of action of the sulphonamide class of drugs is the inhibition of dihydropteroate synthetase in folic acid synthetic pathway of prokaryotic cells. *Para*-aminobenzoic acid (*p*-ABA) is the natural agonist of the dihydropteroate synthetase enzyme (Gale *et al.* 1972, Rang and Dale 1994). By establishing a competition for agonist binding to the enzyme active site between the sulphonamide and *p*-ABA, it is possible to reverse the bacteriostatic action of sulphonamides on the microbial cell. Using this technique, selective inhibition of the effect of sulphonamides on the Premi[®]Test response can be achieved. This was successfully demonstrated using chicken muscle incurred with sulphadiazine and a mixed sulphonamide solution containing equal proportions of sulphamethazine, sulphamethoxypyridine, sulphaquinoxaline, sulphadiazine and sulphadimethoxine spiked into porcine kidney.

Conclusions

Improved methodology for the detection of antimicrobial compounds in food matrices using the commercially available Premi[®]Test has been developed.

Table 2. Limit of detection of a range of antimicrobials in a variety of matrices following acetonitrile/acetone extraction using tissue fortified with antimicrobial compounds.

Compound	Limit of detection (LOD) for each matrix ($\mu\text{g kg}^{-1}$)									
	Chicken muscle	Pig muscle	Pig kidney	Salmon muscle	Chicken egg	Bacon	Liver pate	Cows milk	White fish	Honey
β-lactams										
Pencillin G	< 2.5	< 2.5	2.5	< 5	< 2.5	< 5	> 5	< 2.5	< 12.5	< 12.5
Amoxicillin	2.5	< 2.5	5	< 5	2.5–5	< 5	> 5	< 2.5	< 12.5	< 12.5
Ampicillin	2.5	< 2.5	2.5–5	< 5	2.5–5	< 5	> 5	< 2.5	< 12.5	25
Oxacillin	< 50	< 50	< 50	< 50	< 50	< 50	100	< 10	< 75	75
Cloxacillin	< 50	< 50	50	< 50	< 50	< 50	100	< 10	< 75	100
Dicloxacillin	–	–	–	–	–	–	–	–	< 75	< 75
Ceftiofur	–	< 100	< 250	–	–	–	–	< 25	–	–
Cefazolin	–	< 100	< 250	–	–	–	–	< 12.5	–	–
Cefacetile	–	< 100	< 250	–	–	–	–	< 30	–	–
Cephalonium	–	< 100	< 250	–	–	–	–	2.5	–	–
Cefoperazone	–	< 100	< 250	–	–	–	–	< 12.5	–	–
Cephapirin	–	< 100	< 250	–	–	–	–	< 15	–	–
Cephalexin	–	< 100	< 250	–	–	–	–	25–30	–	–
Sulphonamides										
Sulphaguanidine	75	< 150	< 150	75	75	–	–	< 25	< 50	75
Sulphadimethoxine	12.5	< 25	< 25	25	25	–	–	25–50	< 50	75
Sulphapyridine	30–50	100	< 75	< 50	25–50	–	–	25	< 50	75
Sulphamethizole	< 50	< 50	< 100	< 50	> 50	–	–	50	< 50	75
Sulphamethoxypyridine	12.5	< 50	< 50	< 50	< 25	–	–	< 25	< 50	75
Sulphisoxazole	< 30	< 25	75	< 50	25–50	–	–	25–50	< 50	50–75
Sulphathiazole	12.5–25	< 25	< 75	< 50	< 25	–	–	< 25	< 50	< 50
Sulphadiazine	12.5	< 50	< 75	< 50	< 25	–	–	50	< 50	75
Sulphachloropyridazine	< 12.5	< 50	< 50	< 50	< 25	–	–	25–50	< 50	< 50
Sulphamerazine	25	< 50	< 75	50	< 25	–	–	50	< 50	75
Sulphanilamide	50–75	100	100–200	75	> 75	–	–	> 50	< 50	75
Sulphaquinoxaline	< 12.5	< 50	50	< 50	< 25	–	–	< 25	< 50	75
Sulphamethazine	< 30	50–75	75	< 75	25–50	–	–	< 25	< 50	75
Tetracyclines										
Chlortetracycline	50	50	400	100	200–250	75	200	> 100	150	80–90
Oxytetracycline	50	50	400	100	200–250	75	200	100	150	75–80
Tetracycline	25–50	25–50	< 200	75	200	50	200	100	50–75	50–60
Doxycycline	25–50	25–50	< 200	75–100	200	50	150	100	25–50	50–60
Demecocycline	25–50	25–50	200	75–100	–	50	300	–	–	–
Other										
Erythromycin	–	< 100	–	–	–	–	–	< 100	–	–
Spiramycin	–	125	–	–	–	–	–	< 125	–	–
Tylosin	–	25	–	–	–	–	–	< 25	–	–
Dapsone	–	< 2.5	–	–	–	–	–	< 2.5	–	–
Thiamphenicol	–	500	–	–	–	–	–	500	–	–
Chloramphenicol	–	500–1000	–	–	–	–	–	500–1000	–	–
Trimethoprim	–	50	–	–	–	–	–	25	–	–
Neomycin	–	–	2500	–	–	–	–	–	–	–
Lincomycin	–	–	< 300	–	–	–	–	–	–	–
Gentamycin	–	–	> 500	–	–	–	–	–	–	–
Streptomycin	–	–	> 500	–	–	–	–	–	–	–

Data are expressed as $\mu\text{g kg}^{-1}$.

–, Not tested.

Two sample extraction procedures have been evaluated. A fluid expression technique using a garlic press provides the basis of a simple procedure capable of detecting a range of compounds at MRL levels or better. Of greater efficacy is a procedure based on extraction using 70:30 v/v acetonitrile/acetone. This procedure provides improved sensitivity, particularly for the tetracyclines and sulphonamides, compared with existing microbial inhibition assays. This approach also offers the advantage of not requiring specialist microbiology facilities or expertise in order to perform the analysis. The approach described here is technically simple and can be performed in a rudimentary laboratory. Results for up to 40 samples can be generated within the working day. There is also scope for automation of this methodology to improve further the high sample throughput. Sub-MRL detection limits have been demonstrated for the β -lactam, sulphonamide and tetracycline classes and also the macrolide class. This procedure covers the greater part of antimicrobial agents currently in use.

Post-screening tests to distinguish the β -lactams and sulphonamides have also been demonstrated successfully.

Further work is currently being undertaken in a number of areas. As part of the development of an integrated approach to antimicrobial analysis, improvements are being sought to the post-screening recognition of the remaining classes, which are active in the Premi[®]Test, such as the tetracyclines.

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