Simultaneous Determination of Different Antibiotic Residues in Bovine and in Porcine Kidneys by Solid-Phase Fluorescence Immunoassay

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Parallax®, a solid-phase fluorescence immunoassay (SPFIA) developed for antibiotic residue detection in milk, was used for analysis of bovine and porcine kidney tissue. Four tetracyclines, 2 broad-spectrum cephalosporins, 3 beta-lactam antibiotics, and cepharin were detected in one run after minimal sample preparation. This commercially available test system is designed as cartridges, each with a combination of 1–4 tests. One cartridge can be used to detect 4 analytes in the same sample, or 1 or 2 analytes in different samples. The cartridge with the combination tetracyclines—ceftiofur—penicillin—cephapirin was selected because tetracyclines, beta-lactam antibiotics as well as cephalosporins, are registered for oral or parenteral use in bovines and pigs in Europe. The test is qualitative and is recommended only for screening. Tetracycline, oxytetracycline, chlorotetracycline, and doxycycline were easily detected at 300 ppb with the tetracyclines channel; ceftiofur at 1000 ppb and cefquinome at 200 ppb with the ceftiofur channel; penicillin G, ampicillin, and amoxicillin at 50 ppb with the penicillin channel; and cepharin at 100 ppb with the cepharin channel. These levels are equal to or lower than the corresponding maximal residue limits in kidney tissue. Cephalexin was not detected. The SPFIA test can be used as an alternative to classical inhibition tests and for post-screening inhibitor-positive kidneys, because it detects 3 specific groups of antibiotics, which enables selection of specific confirmatory methods for identification and quantification.

Such tests are very useful in the milk and meat industries, because the capacity of inhibiting bacterial growth may cause failures in the production of fermented foods such as cheese, yogurt, or dry sausages.

Inhibition tests, however, do not guarantee the safety of foodstuffs of animal origin for consumers; therefore, safe levels or maximal residue limits (MRL) have been established, based primarily on toxicological grounds. Food that contains a residue in a concentration equal to or lower than the MRL or safe level is considered harmless for human health.

Until now, bioassays have been used for residue monitoring programs, but it is unlikely that these traditional methods detect all residue levels of any antibiotic above the MRL. Limits of detection (LOD) are often higher than the MRL, and as sample preparation is minimal with such tests, the matrix may influence bacterial inhibition so that even high residue levels may not be detected (4, 5). On the other hand, despite a positive outcome of a simple inhibition test, no conclusions may be drawn about the identity of the antibiotic or its concentration. The question arises whether traditional bioassays should be replaced by other, more specific methods. The simplicity and low cost of inhibition tests make it difficult to find a suitable alternative, because of the large number of samples analyzed in monitoring programs. Confirmatory methods, such as liquid chromatography and gas chromatography coupled to mass spectrometry (LC/MS and GC/MS), are not considered as alternatives because they are highly specific and require sophisticated equipment and skilled laboratory personnel.

Receptor tests and immunological methods may be used for antibiotic residue screening of slaughter animals when sample preparation is minimal; when the tests are simple to perform, and large numbers of samples can be analyzed in a short time; and when screening tests can detect as many members of an antibiogram family as possible, with optimal cross-reactivity (i.e., comparable LOD for each antibiotic detected).

Parallax® (IDEXX Laboratories, Westbrook, ME) is a solid-phase fluorescence immunoassay (SPFIA)-based test, designed for milk analysis, which is very easy to perform and yields results within 5 min. Different types of test cartridges are available, each containing 4 capillary channels for detection of 1–4 different analytes, irrespective of cross-reactions. The test itself, including mixing of the sample with the anti-
Table 1. Detection of 3 penicillins with the penicillin channel

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Concentration, µg/kg</th>
<th>Nc. of observations</th>
<th>Average</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin</td>
<td>50</td>
<td>13 (8B + 5P)</td>
<td>2.11 ± 0.17</td>
<td>1.83–2.37</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>50</td>
<td>20 (8B + 11P)</td>
<td>2.57 ± 0.19</td>
<td>2.32–2.81</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>50</td>
<td>25 (12B + 13P)</td>
<td>2.29 ± 0.24</td>
<td>1.77–2.48</td>
</tr>
<tr>
<td>Blank</td>
<td>--</td>
<td>32 (14B + 16P)</td>
<td>0.34 ± 0.45</td>
<td>–0.55–1.19</td>
</tr>
</tbody>
</table>

* Ratio obtained with blank tissue and with tissue spiked with amoxicillin, ampicillin, or penicillin G.

* B = Bovine kidneys, P = pork kidneys.

body, immunological binding and reading, takes <5 min. One of the possible combinations is tetracyclines, cephalosporins, and cephalixin. Many pharmaceutical specialties intended for systemic treatment of cattle and pigs contain beta-lactam antibiotics (penicillins as well as cephalosporins), or tetracyclines. Therefore, we investigated whether the method was able to detect those antibiotics in spiked bovine and porcine kidney, at levels equal to or lower than the corresponding MRL established by European Union regulations.

**Experimental**

**Preparation of Samples**

Kidneys were collected from healthy slaughter pigs and bovines and transferred to the laboratory within 1 day. Each kidney was sampled for a 1-plate inhibition assay, i.e., the agar diffusion test that is routinely applied in Belgium for monitoring (6). Thereafter, 1 or 2 lobes of the bovine kidneys and ca half of the pig kidneys were blended in a kitchen blender (Rondo 1000, SEB, Beaune, France). Samples were then frozen and stored at -20°C until analysis. Kidneys not containing inhibiting substances were selected and used as blanks and for preparing spiked samples.

Blank samples were prepared from 16 animals (8 bovines and 8 pigs). Samples were thawed partially and 5 g tissue was weighed in a stomacher bag. Ten mL Sørensen phosphate buffer, pH 7.6, prepared with 13.2 mL KH₂PO₄ (9.08 g/L) and 86.8 mL Na₂HPO₄2H₂O (11.88 g/L) were added, and the contents were mixed for 1 min in a stomacher. Approximately 2 mL was placed in Eppendorf tubes and centrifuged for 5 min at 20 000 × g. The supernatants were transferred into another tube, carefully avoiding floating fat particles.

Positive samples were prepared from blanks, which were spiked with one of the following antibiotics: penicillin G, ampicillin, amoxicillin, tetracycline, oxytetracycline, doxycycline, chlorotetracycline, cefotaxime, cefquinome, cephalixin, and cephalexin. Antibiotic standards originated from Sigma (St. Louis, MO), except cefquinome and cefotaxime, which were obtained from Hoechst Roessle Vet Untersheehisheim, Germany) and Pharmacia & Upjohn Puurs, Belgium, respectively. Stock solutions of 1 mg/mL were prepared in methanol (tetracyclines) or distilled water beta-lactam antibiotics), and stored at -18°C or less for a maximum of 6 months. Just before use, the stock solutions were diluted in distilled water. Positive samples were prepared by adding 100 µL of one of the following antibiotic solutions to 5 g blended tissue in a stomacher bag: 2.5 mg/mL for penicillin G, ampicillin, and amoxicillin; 15 µg/mL for the tetracyclines; 10 µg/mL for cefotaxime; 5 µg/mL for cephalixin; and 50 µg/mL for cephalexin. The levels obtained in the fortified samples were equal to or lower than the corresponding MRL values: 50 µg/kg for penicillin G, ampicillin, and amoxicillin (MRL in kidney = 50 µg/kg); 300 µg/kg for the tetracyclines (MRL = 600 µg/kg); 200 µg/kg for cefquinome (MRL = 200 µg/kg); 1000 µg/kg for cefotaxime (MRL = 6000 µg/kg) and cephalexin (MRL = 1000 µg/kg); and 100 µg/kg for cephalixin. The fortified samples were further treated as the blanks.

**Solid-Phase Fluorescence Immunoassay Test**

During analysis, all extracts from blank and fortified samples were kept on an ice bath.

The Paralux system is an SPFIA test, which consists of a processor and specific kits, both commercialized by IDEXX. Each kit contains a reagent tray, used to mix samples and reagents, and a cartridge with 4 capillaries. The reagent tray is fitted into its place in the preparation station of the Paralux processor. Before starting the analyses, the processor must be calibrated with a negative milk control, delivered with the test kits. The test is then performed as prescribed by the manufacturer. All manipulations are guided by instructions on the screen of the Paralux processor.

After the wells in the reagent tray are filled with 100 µL sample, the contents are mixed with labeled antibodies already present in the wells; thereafter, the mixtures are allowed to react with the solid phase in the capillary tubes. Detailed instructions are given in the instruction manual of the apparatus. When samples do not contain any analyte reacting with the antibodies, a large amount of labeled antibody remains free to bind to the solid phase. After capillaries are washed and dried, a laser source excites fluorescence, and the result is given as the ratio of the sample inhibition value/cutoff inhibition value, while the sample inhibition value = (1 – (sample signal/negative control signal)) × 100. The cutoff inhibition value is lot- and capillary-specific and is included in the bar code that must
be scanned before each test. A ratio >1 is recorded as positive by the processor.

Results and Discussion

No difference in ratio was observed between blank kidneys from the 2 animal species (data not shown) and, therefore, no distinction was made between results from blanks or fortified samples. The numbers of fortified samples from both species were nearly equal for most analytes, except for those more likely to be found in one of the species. Cefquinome, for example, is registered for use in bovines, and more fortified samples from the target species were analyzed than from the other. Other antibiotics such as ceftiofur are recommended for bovines as well as for porcines, and still others such as doxycycline are incorporated in medicated feed and, in practice, are not given to adult cattle.

The range of ratios obtained with blank and fortified kidney tissue is shown in Tables 1–3. Table 1 lists results of tests in the penicillin channel, Table 2 in the tetracyclines channel, Table 3 in the cefquinome channel, and Table 4 in the cephalapirin channel. Kidneys with no inhibiting substances were considered as blanks and tested with the 4 channels. The ratios obtained were <1.00 for tetracyclines, ceftiofur, and cephalapirin, but in the penicillin channel, ranges were sometimes >1.00. The ratios obtained with blank kidney samples ranged from 0.86 to 0.84 in the cefquinome channel (n = 34), from 0.09 to 0.80 in the tetracyclines channel (n = 19), from 0.55 to 1.19 in the penicillin channel (n = 32), and from 0.79 to 0.36 in the cephalapirin channel (n = 30; Tables 1–4).

All antibiotics tested, except cephalaxin, were detected with the SPPIA at concentrations equal to (penicillin, ampicillin, amoxicillin, cephalapirin, and cephalaxin) or lower (tetracycline and ceftiofur) than the MRL. Only 3 pork kidneys spiked with 1000 μg/kg cephalaxin were tested, and the ratios were as follows: -0.62 to 0.03 in the ceftiofur channel; -0.16 to 0.61 in the penicillin channel; and -0.25 to -0.27 in the cephalapirin channel. The ranges of ratios from spiked samples spiked with other antibiotics, obtained in the respective channels, are shown in Tables 1–4. They differed in all cases from those obtained with the blanks. The ratios obtained with penicillin, amoxicillin, or ampicillin were always >1.70. The average ratio minus 3 times the standard deviation was 1.59, 2.00, and 1.56 for amoxicillin, ampicillin, and penicillin G, respectively. The average ratio of the blanks plus 2 times the standard deviation in the penicillin channel was 1.24. Thus, theoretically, a ratio of 1.50 should give <25% false positives and <50% false negatives.

Group-specific tests, such as immunological or receptor assays, have been recommended often as an alternative to inhibition tests for detection of antibiotic residues (7). Such methods can also be used to identify an antibiotic or antibiotic family in a food sample containing an inhibitor, but not for quantification when they detect more than one substance. Therefore, group-specific tests are considered as post-screening tests: identification of an antibiotic group enables the analyst to select the appropriate chromatographic technique for final identification and quantification of the substance involved, which is necessary to prove that the food is unfit for human consumption.

For various reasons, immunological techniques have not commonly been used as post-screening methods in monitoring programs. Commercial enzyme immunoassay (EIA) kits are expensive and have a limited shelf life, especially from the moment that the reagents have been reconstituted. After the first microbiological screening, only a few samples remain for further examination, while EIA tests are only profitable when a large number of samples have to be investigated. Sample preparation can be laborious, differing from test to test, making it impossible to screen for more than one antibiotic or antibiotic family with the same extract. Until now, the only commercial system able to detect several antibiotic groups in one extract has been the Charm II system, a receptor-based technique (7). The tracers used with this system are marked with radioisotopes, and many laboratories avoid such methods because of their implications on the environment and the stringent safety measures required.

The SPPIA test described in this study has several advantages over other immunoassays and receptor tests: 4 different tests are performed in 1 run, which takes only 5 min, the kit does not contain toxic reagents, and the shelf life of the kit components is relatively long. Each cartridge is separately
Table 3. Detection of 2 broad-spectrum cephalosporins with the ceftiofur channel

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Concentration, µg/kg</th>
<th>No. of observationsb</th>
<th>Average</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cefotiofur</td>
<td>1000</td>
<td>22 (15B + 9P)</td>
<td>2.02 ± 0.11</td>
<td>1.81–2.16</td>
</tr>
<tr>
<td>Cefquinome</td>
<td>200</td>
<td>35 (30B + 5P)</td>
<td>1.74 ± 0.17</td>
<td>1.35–1.97</td>
</tr>
<tr>
<td>Blank</td>
<td>—</td>
<td>34 (14B + 16P)</td>
<td>0.17 ± 0.28</td>
<td>−0.86–0.84</td>
</tr>
</tbody>
</table>

* Ratio obtained with blank tissue and with tissue spiked with ceftiofur or cefquinome.

b B = Bovine kidneys, P = pork kidneys.

packed and sealed and, subsequently, there is no loss of reagents when only a few samples have to be tested. Therefore, it is a very interesting method for post-screening as well as for screening slaughter animals.

Although the SPFIA test has been designed for milk analysis, we validated it for detection of 10 different antibiotics, belonging to 3 antibiotic families, in bovine and porcine kidney tissue. A screening method can be validated by comparing results obtained with blanks and with samples spiked at the concentration that should be detected to guarantee that the food is fit for consumption (8). There was a clear distinction between all spiked and blank samples for the following antibiotics: tetracycline, doxycycline, oxytetracycline, and chlorotetracycline; ceftiofur and cefquinome; penicillin G, amoxicillin, and ampicillin; and cepahpin. For the penicillin group, however, the cutoff ratio should be 1.50 and not 1.00, as proposed by the manufacturers for milk analysis.

The kidneys were fortified with tetracyclines at 300 ppb; the MRL of all members of this group is 600 ppb in kidneys. The MRL of tetracyclines in muscle tissue is 100 ppb and, although residue levels are usually higher in kidney than in muscle tissue, levels <500 ppb in kidney do not always guarantee that the meat is safe. This is especially the case for doxycycline (S. Croubels, Ghent University, and J.M Degroodt and P. Batjoens, IPH<, Brussels, Belgium, personal communication, 2000). A level <300 ppb is more accurate for that purpose. Indeed, kidney is tested in order to evaluate all edible parts of the animal; therefore, it is not sufficient to quantify the residues in kidneys and declare the carcass safe when residue concentrations are lower than the MRL in kidney without testing the more valuable parts.

The MRL of ceftiofur is very high for bovine and porcine kidneys (6000 ppb). The method was validated with kidneys spiked with 1000 ppb ceftiofur. As a consequence, it is probable that slaughter animals will screen positive when ceftiofur levels in their kidney tissue are much lower than the MRL. In practice, this will not be a substantial problem, because ceftiofur is administered by injection and not often used shortly before slaughter. Cefquinome is a cephalosporin closely related to ceftiofur and, to our knowledge, this report is the first to mention that both cephalosporins cross-react in an immunological assay.

When considering the suitability of a method for residue analysis, the evaluation of differences in response between blanks and spiked tissues is only a first, but necessary, step. Indeed, detection of residues in incurred samples can be more difficult when analytes are not free but are bound to lipid components. Furthermore, it is not clear whether possible metabolites are detected as easily as the parent drugs. Preparing incurred samples with 10 different antibiotics, at different concentrations, is time-consuming and expensive, and beyond the purpose of the present study. Until now, all our positive results of naturally contaminated kidneys with the tetracycline channel have been confirmed with LC. Most samples contained doxycycline or oxytetracycline at levels ≤300 ppb (unpublished data).

Immunological methods and receptor tests are generally considered as the most suitable methods to determine an antibi-
oic family in an inhibitor-positive sample. Other authors prefer to use different inhibition tests with varying media and test bacteria. Myllyniemi et al. (9) identified antimicrobial drugs in kidney and muscle samples of bovines and pigs using 18 different combinations of 8 test bacteria, varying medium pH and substances blocking the action of certain antibacterials. They found fully consistent results between

Table 4. Detection of cepahpin with the cepahpin channel

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Concentration, ppb</th>
<th>No. of observationsb</th>
<th>Average</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cephahpin</td>
<td>100</td>
<td>30 (15B + 15P)</td>
<td>1.88 ± 0.043</td>
<td>1.80–1.06</td>
</tr>
<tr>
<td>Blank</td>
<td>—</td>
<td>30 (15B + 50P)</td>
<td>−0.11 ± 0.34</td>
<td>−0.79–0.36</td>
</tr>
</tbody>
</table>

* Ratio obtained with blank tissue and with tissue spiked with cepahpin.

b B = Bovine kidneys, P = pork kidneys.
chemical and microbiological identification of tetracyclines and fluoroquinolones in kidneys. Afterwards, they simplified the method and used only 6 plates for microbiological confirmation, 2 of them supplemented with penicillinase in order to distinguish between penicillinase-sensitive and penicillinase-resistant beta-lactam antibiotics (10). However, post-screening with microbiological techniques has a serious disadvantage: the presence of residues belonging to more than one antibiotic group can lead to erroneous conclusions, especially when blocking substances such as penicillinase or paraaminobenzoic acid are used. In addition, the SPFIA post-screening method is simpler, less laborious, and much faster.

The cartridge that was tested for the present report contained 4 different channels. The first 3 channels each detect more than one related antibiotic. The fourth channel detects only cephalaxin at a level equal to the MRL. Cephalaxin is a narrow spectrum cephalosporin with an MRL established for meat and kidney tissue, does not cross-react with cephalaxin or with any other capillary in the SPFIA test described. Other commercial Parallux combinations include a capillary for ciprofloxacin and dicloxacin, which are beta-lactam antibiotics used for local treatment of mastitis and thus unlikely to occur in meat or kidneys. Another combination includes 3 channels for 3 different sulfonamides. We have not yet validated the SPFIA test for sulfonamides in meat or kidney. Parallux does not contain cartridges intended for detection of macrolides or fluoroquinolones. Thus, the system cannot completely replace the inhibition tests, although it is not clear whether classical kidney tests detect all samples that are not compliant with the antibiotic residue legislation.

It can be concluded that the SPFIA test Parallux is a valuable tool for identification of 3 antibiotic groups in bovine or porcine kidneys that contain inhibiting substances. It is also useful as a first step in a screening procedure, when fast results are necessary, for example, in slaughter houses. It is clear that the cartridge tetracyclines/beta-lactam combination does not detect residues of other important groups, such as sulfonamides or macrolide antibiotics. The results of the tests are given as ratios, and a ratio >1.00 is read by the processor as positive. We recommend this value for the tetracyclines, ceftiofur, and cephalaxin channels. For the penicillin channel, a ratio of 1.50 should detect nearly all kidney samples with penicillin G, ampicillin, or amoxicillin levels equal to the MRL, while the risk of false positives is <2.5%.

References