DEVELOPMENT OF AN IMMUNOASSAY FOR THE DETECTION OF SULFAMETHAZINE IN MILK USING AN OPTICAL BIOSENSOR: INFLUENCE OF FLOW RATE AND ANTIBODY ON THE PERFORMANCE OF THE ASSAY

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Contexte

La sufaméthazine est un antibiotique de la famille des sulfamides. Ce principe actif, retrouvé dans de nombreux médicaments vétérinaires, est utilisé chez les animaux de rente et en particulier chez les vaches laitières. Des résidus de sufaméthazine peuvent être retrouvés dans le lait de vache. La Limite Maximale de Résidus (LMR) pour la somme des sulfamides dans le lait a été fixée à 100 µg/kg. Les méthodes de dépistage doivent donc être capables de détecter cet antibiotique dans le lait, au niveau ou en dessous de la LMR. Il existe différents types de méthodes de dépistage pouvant détecter les sulfamides dans le lait : des méthodes microbiologiques [22, 29, 302], immunologiques (type ELISA) [66, 70, 159, 303, 304], physico-chimiques, telles que la CCM [305], la CLHP [306] ou CL-SM/SM [307]. Cependant, les méthodes microbiologiques présentent le plus souvent des limites de détection supérieures aux LMR pour les sulfamides (LMR = 100 µg/kg).

La technologie « Biomolecular Interaction Analysis » (BIA) permet l'étude en temps réel des interactions entre biomolécules, sans marquage. Au départ, la principale application était la détermination de l'affinité entre biomolécules et de la cinétique des interactions [308]. Les applications se sont ensuite diversifiées et ont touché de nombreux domaines des sciences de la vie. Le système Biacore X (Biacore AB, Suède) est un biocapteur optique, dont la détection est basée sur la résonance plasmonique de surface (SPR) [309]. Le principe de la méthode est de fixer de manière covalente un ligand (anticorps) sur une surface appelée *sensor chip* **(Figure 44**) et d'introduire un analyte dans un débit de tampon. Les deux molécules vont alors former des complexes. Ces interactions entre biomolécules, sont visualisées en temps réels grâce à un détecteur optique. Le sensorgramme permet de visualiser les variations de l'angle de résonance sous l'effet de l'association et de la dissociation des analytes sur la surface (**Figure 45**).

Les premières publications de développement de méthodes pour détecter les résidus d'antibiotiques avec ce système sont parues entre 1995 et 2001 [162, 168, 310-312].

Méthodologie/Principaux résultats

Nous avons développé, optimisé et validé une méthode de dépistage de la sufaméthazine dans le lait, en utilisant deux anticorps polyclonaux anti-sufaméthazine. La première étape du développement consistait à optimiser l'immobilisation de la sufaméthazine à la surface de la sensor chip (la biopuce). Ensuite, nous avons optimisé la concentration active d'anticorps primaire qui va être mélangée avec l'échantillon, ainsi que la concentration d'anticorps secondaire. Ce type d'essai est compétitif : en cas d'absence de sufaméthazine dans l'échantillon, l'anticorps primaire en excès pourra se lier totalement à la surface de la puce, donc le signal sera maximum (détection du changement de masse). En cas de présence de sufaméthazine dans l'échantillon, elle entrera en compétition avec la sufaméthazine immobilisée à la surface, pour se lier à l'anticorps primaire ; donc la quantité d'anticorps à la surface diminuera et le signal aussi. Le signal est donc inversement proportionnel à la quantité de sufaméthazine dans l'échantillon. Un anticorps secondaire anti-anticorps de lapin a été utilisé pour augmenter le signal. De plus, le débit du flux à travers le système a été optimisé.

Figure 44. La *sensor chip.*

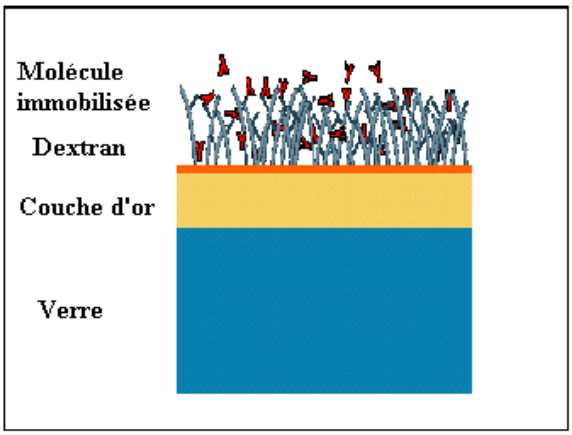
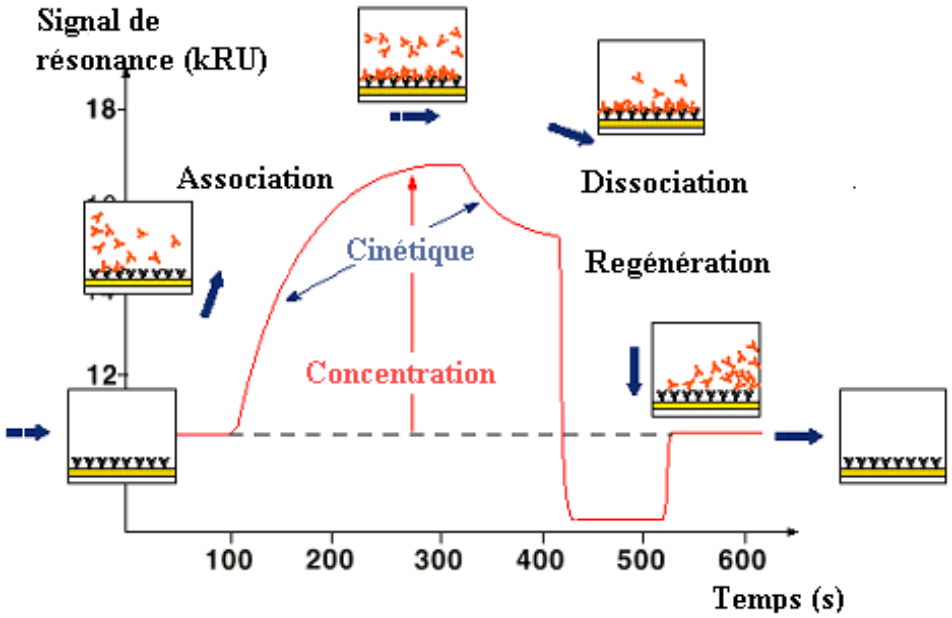


Figure 45. Le sensorgramme.



Au moment de cette validation, la réglementation européenne pour la validation des méthodes analytiques pour les résidus et contaminants était la décision 93/256/CE [313], en vigueur jusqu'en 2002 (abrogée par la décision 2002/657/CE) [13]. Selon cette décision, les caractéristiques de performance à déterminer pour une méthode de dépistage étaient : la spécificité, la limite de détection, la praticabilité (grand nombre d'échantillons, couts limités) pour les méthodes qualitatives et de surcroit pour les méthodes quantitatives, la fidélité et l'exactitude. La limite de détection était définie comme la concentration la plus basse qui pouvait être déterminée comme statistiquement différente (3 écart-type) d'un lait de contrôle négatif ou de tampon (réponse moyenne de 20 contrôles négatifs). Dans cette étude, la validation de la méthode a consisté à déterminer la concentration minimale inhibitrice à 50% (IC50), la spécificité, la limite de détection et la fidélité. L'IC50, qui correspond à la concentration d'analyte qui inhibe 50 % du signal, est une caractéristique très utilisée pour caractériser les tests ELISA et pour comparer les performances de tests ELISA entre eux, dans le domaine médical par exemple. En effet, un test ELISA avec une IC50 inférieure à celle d'un autre test est un test ELISA qui aura une limite de détection plus basse, donc il sera plus performant. Cette caractéristique est aussi parfois utilisée pour les tests ELISA pour le dépistage des résidus d'antibiotiques [314-316]. Etant donné que l'approche Biacore est très proche du principe de l'ELISA, l'IC50 a aussi été déterminée lors de cette validation, à partir des courbes de calibration, même s'il ne s'agit pas d'une exigence de la décision 93/256/CE. La répétabilité du signal a été mesurée sur les points de la courbe d'étalonnage, en répétant chaque point de la courbe 3 fois par jour, pendant 3 jours, soit 9 réplicats par point de la gamme.

Lors du développement de la méthode, les concentrations actives des anticorps primaires et de l'anticorps secondaires ont été optimisées. L'influence du débit de l'échantillon sur les limites de détection et sur l'IC50 dans le lait et dans le tampon a été montrée avec les 2 anticorps. La limite de détection dans le lait avec les deux anticorps étaient plus élevée à 20 µl/min qu'à 5 µl/min. Cependant, une limite de détection à 8 µg/kg restait bien inférieure à la LMR (100 µg/kg). Etant donné le gain de temps pour l'analyse à 20 µl/min (8 minutes contre 30 minutes à 5 µl/min), le débit de 20 µl/min a été conservé. Les limites de détection obtenues en tampon (2.9 µg/kg) et dans le lait (1.7 µg/kg) avec l'anticorps 2 étaient plus basses que celles obtenues avec l'anticorps 1 (10 µg/kg dans le tampon et 8 µg/kg dans le lait). Aucune réaction croisée n'a été observée pour les deux anticorps primaires avec 6 sulfamides testés (sulfadiméthoxine, sulfaméthoxypyridazine, sulfamonométhoxine, sulfadoxine, sulfadiazine, sulfaphénazole), ni avec des molécules des autres grandes familles d'antibiotiques (pénicilline, céphalosporine, aminoside, tétracycline, chloramphénicol, quinolone). La répétabilité des résultats était un peu plus élevée avec l'anticorps 2 qu'avec l'anticorps 1, mais encore très satisfaisante (< 13%), par rapport aux critères de performance de la décision 93/256/CE (11,5 à 15 % en conditions de répétabilité à 100 µg/kg).

Conclusions/Importance

En conclusion, nous avons démontré l'applicabilité du Biacore au développement de méthodes pour le dépistage de résidus d'antibiotiques dans les aliments d'origine animale. Deux méthodes ont été développées, utilisant deux anticorps différents, capables de détecter la sufaméthazine dans le lait, largement en dessous de la LMR. Les deux méthodes sont rapides (8 minutes par échantillon) et nécessitent une faible préparation de l'échantillon (pasteurisation, écrémage, dilution). En fonction de l'objectif, la spécificité de la méthode pour la sufaméthazine peut représenter un problème car la LMR des sulfamides est fixée pour la somme des sulfamides. Donc, dans le cadre du développement d'une méthode de dépistage, l'idéal serait de détecter tous les principes actifs de la famille des sulfamides avec la même méthode. Toutefois, dans le cadre d'une application ciblée sur la sufaméthazine (cinétique de déplétion), la méthode est adaptée. Cette première validation d'une méthode immunologique suivant la décision 93/256/CE a été réalisée en considérant la méthode développée comme une méthode quantitative. C'est pourquoi la fidélité (répétabilité) a été déterminée.

Lors des validations réalisées suivant le protocole recommandé dans la décision européenne 93/256/CE pour des méthodes immunologiques (*eg.* ELISA, Biacore), la limite de détection était calculée à partir de l'analyse de 20 échantillons blancs et du bruit de fond associé. Cette approche pouvait donner des limites de détection, qui n'étaient pas toujours représentatives de la performance réelle de la méthode, étant donné la variabilité associée aux mesures avec ce type de méthodes, surtout en ELISA. En général, la fidélité des méthodes développées sur le système Biacore étaient meilleures que celles obtenues avec des kits ELISA. Toutefois, nous pouvons dire à présent que l'approche du CCβ apporté par la décision européenne 2002/657/CE, est plus adaptée et apporte plus de confiance dans le résultat. En effet, le CCβ est déterminé à partir d'échantillons supplémentés à une concentration cible, et non à partir d'échantillons blancs. Enfin, nous pouvons ajouter qu'avec l'expérience, la plupart du temps, les méthodes immunologiques sont validées uniquement comme des méthodes qualitatives et non plus comme des méthodes quantitatives, sauf si leur utilisation l'exige.

Par la suite, nous avons pu développer d'autres applications du système Biacore, telles que le dépistage d'une substance interdite avec une limite de détection inférieure à la Limite Minimale de Performance (LMPR) [317], d'une famille d'antibiotiques (*eg.* sulfamides [163], pénicillines [162]). Les limites de détection basses et la préparation d'échantillons réduite étaient prometteuses pour une mise en place plus large de ce système de biocapteur dans le contrôle Européen. Le système Biacore présentait d'autres avantages, dont celui de pouvoir réaliser faire son propre développement de méthodes (préparation des biopuces et optimisation de la méthode). De plus, la technologie ne nécessitait pas de marquage de l'analyte ou du biorécepteur, puisque le système détecte les changements de masse à la surface). De plus, le Biacore X™ possédait 4 canaux en parallèle (le Biacore 2000™ avait 8 canaux), ce qui permettait en théorie de combiner plusieurs essais pour plusieurs sulfamides ou d'autres familles d'antibiotiques.

Determination of Sulfamethazine in Milk by Biosensor Immunoassay

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A biosensor based on surface plasmon resonance (SPR) measurement was developed for use in an immunoassay for detection of sulfamethazine (SMZ) in milk. The biospecific surface was a carboxymethyl dextran-modified gold-surface sensor chip to which SMZ was covalently bound. The assay was based on inhibition of the binding of polyclonal antibodies to immobilized SMZ by SMZ in the sample. The SPR response changed inversely in relation to the antibiotic concentration in the sample. Calibration curves were constructed for SMZ in buffer and in milk at a concentration which included the maximum residue limit (0 to 200 µg/kg). The analysis time per sample varied from 8 to 30 min. Different flow rates and antibodies were modified alternatively during the study to assess their influence on the performance of the assay. The active antibody concentration was calculated at approximately 1880 and 180 nM for the antibody anti-SMZ 1 and the antibody anti-SMZ 2, respectively. No cross-reactivity of antibodies with other antibiotics was found. Under optimal conditions, the detection limits in milk for SMZ were 8 and 1.7 µg/kg, respectively, for antibody 1 and antibody 2, at a flow rate of 20 µL/min.

Sulfamethazine is a member of the sulfonamide family, the oldest and largest group of synthetic antimicrobial agents. Sulfonamides have a broad spectrum of antibacterial activity and are commonly used for prophylactic or therapeutic purposes in veterinary medicine. Therefore, maximum residue limits (MRLs) were established according to European Union (EU) regulations for the protection of consumers of animal products. The MRL for sulfonamides in milk and meat has been set at 100 µg/kg (1).

Analytical methods are needed to detect drug residues in foods of animal origin. Microbiological and immunological methods are used routinely for screening, and physicochemical methods (e.g., liquid chromatography [LC] and mass spectrometry) are used for identification and quantitation of antibiotic residues. However, microbiological methods lack sensitivity and specificity, and physicochemical methods require qualified staff, sample pretreatment, and high purity chemicals. Although immunological methods such as the ELISA are specific and generally sensitive, they are time-consuming. Therefore, there is a need for new screening methods that are fast, sensitive, and automated, with high sample throughput and minimum preparation of samples. The BIACORE™ system is being evaluated for detection of antibiotics in our laboratory. Analogous methods using this system for SMZ in milk (2, 3) and enrofloxacine/ciprofloxacine (4) were recently described. The published assay reported that sulfamethazine (SMZ) had a concentration of 1 to 10 µg/kg, which did not include the MRL, and an analysis time of 50 min (30 min incubation and 20 min injection). This report describes the development of a faster assay for SMZ in milk with a larger concentration range and underlines the influence of flow rate and quality of the antibody on method performance.

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Experimental

Instrumentation

A BIACORE X optical sensor (Biacore AB, Uppsala, Sweden) was used to perform the assay. The use of BIA technology (biospecific interaction analysis) enabled us to study label-free molecular interactions in real time. The biospecific surface was a carboxymethyl dextran-modified gold-surface sensor chip to which one interactant was covalently bound. Other molecules were injected by the microfluidic cartridge. Using the detection principle known as surface plasmon resonance (SPR), we detected changes of mass on the surface which represented association or dissociation of complexes (5).

Reagents

(a) *CM5 sensor chips (research grade).*

(b) *HBS buffer.*—pH 7.4, as eluent, consisting of 10 mM Hepes (4-[2-hydroxyethyl]piperazine-1-ethane-sulfonic acid), 150 mM NaCl, 3.4 mM EDTA, and 0.005% (v/v) Surfactant P 20.

(c) *Amine coupling kit.*—Containing NHS (100 mM *N*-hydroxysuccinimide in water), EDC [400 mM *N*-ethyl-*N'*-(3-diethylaminopropyl)carbodiimide in water]. and ethanolamine hydrochloride, pH 8.5 (Biacore AB). SMZ and all other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

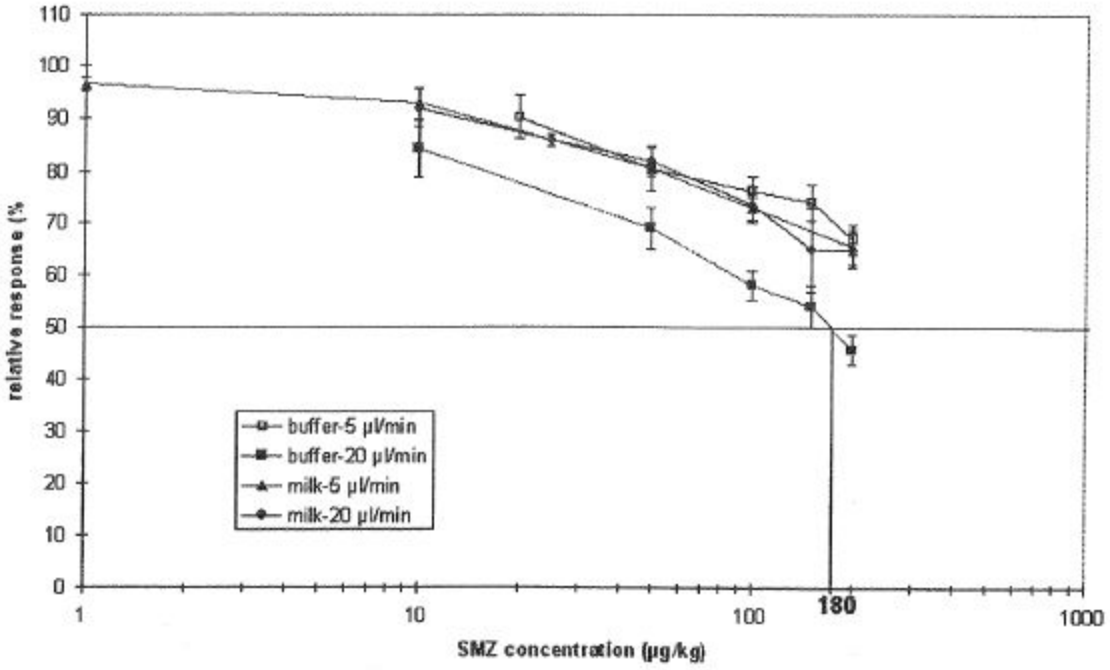

Figure 1. Calibration curves for sulfamethazine in buffer and skim milk. Comparison of 2 flow rates (antibody 1 [2.8 nM], secondary antibody [33.3 nM]). Each point represents the mean of 9 replicates; the error bars indicate the coefficients of variation (CV). The absolute response readings corresponding to the 100% relative response at flow rates of 5 and 20 µL/min were 1483.08 ± 109.30 and 588.40 ± 33.47 RU in assay buffer, and 2251.90 ± 28.68 and 712.26 ± 53.96 RU in milk, respectively. Mean IC_{50} values derived from standard curves at flow rates of 5 and 20 µL/min were 1000 and 180 µg/kg for assay buffer, respectively, and 1000 µg/kg for the 2 flow rates for milk.

Antibodies

The first polyclonal antibody, anti-SMZ (antibody 1, produced in rabbits), was obtained from Eurodiagnostica SA (Gentilly, France). The second polyclonal antibody, anti-SMZ (antibody 2, produced in rabbits), was obtained from Philipp Hammer (Institute für Hygiene, Kiel, Germany). The secondary antibody was an IgG fraction of a goat anti-rabbit antiserum obtained from Sigma Chemical Co.

Immobilization of SMZ on Sensor Chip Surface

SMZ was covalently immobilized to the surface of one of 2 channels of the sensor chip by amine coupling (6). All injections were made at a constant flow rate of 5 µL/min.

A solution of 5 mg SMZ/mL was prepared in 10 mM HCl with 10% dimethylformamide (DMF), pH adjusted to 3.0.

The surface was activated by injecting a mixture of NHS–EDC (1 + 1; 2 × 45 µL). SMZ was then immobilized by injecting the SMZ solution (8 × 45 µL). Finally, the surface was deactivated by injecting ethanolamine HCl, pH 8.5 (2 × 45 µL). Immediately after immobilization, the surface was conditioned by injecting 100 mM NaOH + 20% DMF (10 µL) and 100 mM HCl + 20% DMF (10 µL).

Determination of Active Antibody Concentration

If mass transport of the analyte to the surface is much slower than association, binding will be limited. Under these conditions, the binding rate at the beginning is directly proportional to analyte concentration and independent of association kinetics. To obtain mass transport conditions, the concentration of surface binding sites must be increased.

A calibration curve in which the binding rate has been plotted against active antibody concentration from 0.5 to 160 nM is valid for a large variety of antibodies (7).

Serial 2-fold dilutions of polyclonal antibodies were prepared in HBS (dilution factors from 20 to 2560×) to find a dilution for which the binding rate in resonance units per second (RU/s) was constant over time. The observed binding rate was used to determine active antibody concentration by using the calibration curve.

Preparation of Standards

Standard solutions of SMZ were used to prepare known concentrations of SMZ in HBS or milk. Antibiotic-free milk served as control milk and was also used to prepare spiked samples. SMZ was dissolved at 1 mg/mL in 50 mM borate buffer, pH 8.5, and working solutions were prepared by dilu-

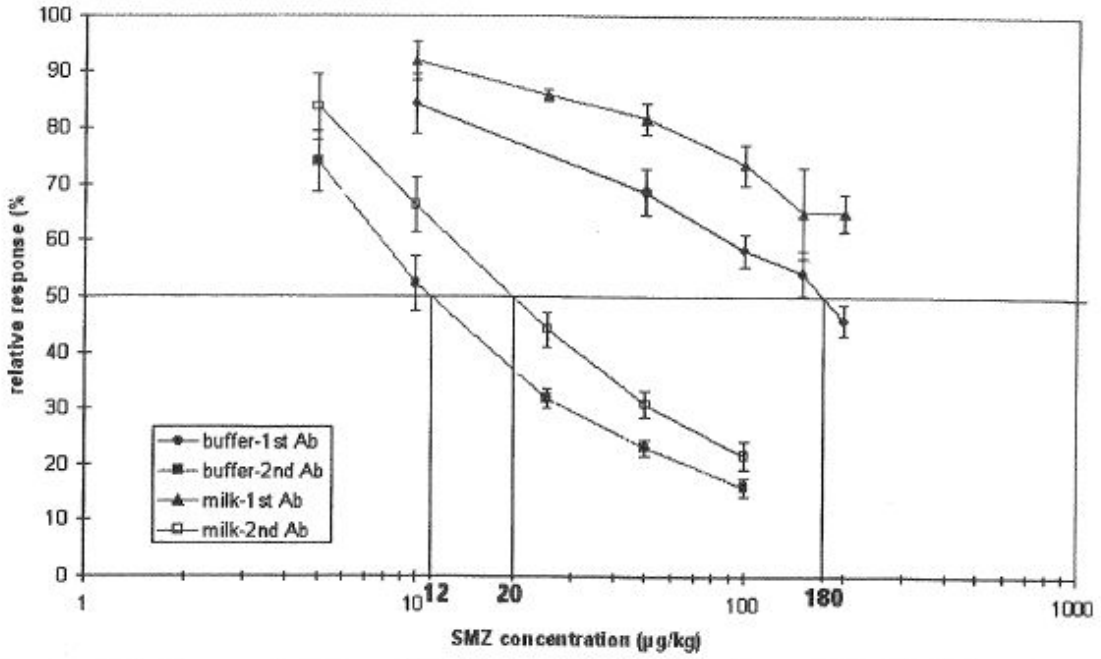

Figure 2. Calibration curves in buffer and skim milk: comparison of 2 antibodies anti-SMZ at 20 µL/min. Antibody 1, 2.8 nM; secondary antibody, 33.3 nM; antibody 2, 2.1 nM; secondary antibody, 33.3 nM. Each point represents the mean of 9 replicates; the error bars indicate CVs. The absolute response readings corresponding to the 100% relative response in assay buffer and in milk at 20 µL/min with antibody 2 were 1288.08 ± 68.20 and 1115.73 ± 147.42 RU, respectively. Mean IC_{50} values derived from standard curves made in assay buffer for antibodies 1 and 2 were 180 and 12 µg/kg and in milk 1000 and 20 µg/kg, respectively.

tion in HBS. These solutions were finally diluted (1:100) in HBS or milk.

Sample Preparation

Milk samples were pasteurized by heating 15 min at 80°C, and then centrifuged (Jouan GR 4.11) for 10 min at 1000 × *g*. The fat on top was discarded.

Assay Principle

The assay was based on inhibition of the binding of polyclonal antibodies to immobilized SMZ by SMZ in the sample. The SPR response changed inversely in relation to the antibiotic concentration in the sample. The primary antibody was mixed (1 + 1) with HBS or milk; 35 µL of this mixture was injected at a constant flow rate, and 35 µL of secondary antibody was then injected to enhance the signal from the primary antibody.

After each measurement, the surface was regenerated by consecutive injections of 15 µL 50 mM NaOH, pH 12.2, and 15 µL 75 mM HCl, pH 3.3.

Optimization of SMZ Antibody Concentrations

Different concentrations of 2 antibodies anti-SMZ (1.4–11.2 nM and 1.05–8.4 nM, respectively) were tested to determine the optimal concentration for best sensitivity of the assay. Similar tests were done with the secondary antibody.

Assay Validation

The concentration range for SMZ standard curves was 0–200 µg/kg. Calibration curves were prepared by plotting log (SMZ concentration) against the relative response, as follows:

$$\text{Relative response, \%} = A/Ao \times 100$$

where *A* was the response of one sample in RU and *Ao* was the response of a negative control sample in RU.

The IC_{50} was defined as the concentration of the inhibitor (antibiotic) required to reduce the signal in RU to one-half the signal measured when no inhibitor was present.

The precision of the assay was estimated by repeating standard curves 3 times per day for 3 days. Coefficients of variation (CVs) were calculated ($n = 9$) for each concentration.

The limit of detection was defined as the lowest concentration that could be determined as statistically different (3 standard deviations) from a negative control milk or HBS (mean response of 20 negative controls).

In order to determine the specificity of the assay, 6 different sulfonamides (sulfadimethoxine, sulfamethoxypyridazine, sulfamonomethoxine, sulfadoxine, sulfadiazine, sulfaphenazole)

and other commonly used antibiotics from major classes (ceftiofur, spiramycin, dihydrostreptomycin, oxytetracycline, penicillin, ampicillin, trimethoprim, chloramphenicol, thiamphenicol, florfenicol, enrofloxacine) were tested at a concentration of 1000 µg/kg. The response of each antibiotic was compared with a standard curve of SMZ to determine the corresponding SMZ concentration (equivalent SMZ) and expressed as a percentage:

$$\text{Cross reactivities, \%} = \text{equivalent SMZ}/1000 \times 100$$

Results and Discussion

Primary Antibodies

The active antibody concentrations were calculated at approximately 1880 and 180 nM for antibody 1 and antibody 2 anti-SMZ, respectively. Optimum concentrations which gave the best sensitivity for antibodies 1 and 2 were, respectively, 2.8 and 2.1 nM (data not shown).

Secondary Antibody

Optimum concentrations were those that gave the best sensitivity with maximum reduction of primary antibody concentration and sufficient enhancement of the signal. For the secondary antibody, optimum concentration was 33.3 nM (data not shown), whatever the primary antibody.

Because the secondary antibody enhanced the signal, use of the primary antibody was limited (in this assay, the reduction factor was 1.5–3). However, there were 2 disadvantages when the secondary antibody was used: a decrease of sensitivity (5–20%) of the assay and increase of analysis time.

Specificity and Selectivity

For the 2 primary antibodies, no cross-reactions were observed in milk with either the 6 tested sulfonamides or with molecules of the main families of antibiotics (penicillin, cephalosporin, aminoglycoside, tetracycline, chloramphenicol, quinolone). It was essential that these antibodies were selective for the sulfonamide family because that was the purpose of developing this screening test.

However, the specificity for SMZ was a problem because the MRL has been set for the sum of sulfonamides. It would be interesting, therefore, to find an antibody that would cross-react with the main sulfonamides. Production of this kind of antibody and new technologies in antibody engineering may eventually solve this problem. In the meantime, several antibodies may be used in the assay, each antibody being specific for one sulfonamide; the sum of the results would thus be closer to the real concentration of sulfonamides in the sample. The multichannel system of the BIACORE would permit testing of several sulfonamides in parallel, one by flow cell.

Effect of Flow Rate

The inhibition curves for SMZ in buffer and in milk at 2 different flow rates using antibody 1 are shown in Figure 1. The IC_{50} was estimated in buffer at about 1000 µg/kg at 5 µL/min and 180 µg/kg at 20 µL/min. In milk, the IC_{50} was 1000 µg/kg for the 2 flow rates. Some values of IC_{50} (1000 µg/kg) were determined approximately by extrapolation because they were at the end of the standard curve. Because the IC_{50} was lower in buffer than in milk at 20 µL/min, the effect of the milk matrix was observed at this flow rate and not at 5 µL/min.

For antibody 1, the biosensor immunoassay in buffer had a detection limit of 20 and 10 µg/kg at 5 and 20 µL/min, respectively. In milk, the detection limits were 2.2 and 8 µg/kg at 5 and 20 µL/min, respectively.

The IC_{50} and detection limits in buffer showed better results at 20 µL/min than at 5 µL/min. In milk, the IC_{50} was equivalent for the 2 flow rates and the limit of detection was lower at 5 µL/min. However, we decided to work at 20 µL/min to study antibody 2 anti-SMZ because of the rapidity of the assay (8 min per sample at 20 µL/min instead of 30 min at 5 µL/min) and because a limit of detection of 8 µg/kg in milk was sufficient to detect SMZ near the MRL.

The CVs for each point of the calibration curve were similar in buffer and in milk for both flow rates, between 1.3 and 7.4%. The precision of the assay, therefore, was satisfactory.

Effect of Antibody

The inhibition curves for SMZ in buffer and in milk for antibodies 1 and 2 at 20 µL/min are shown in Figure 2. The estimated IC_{50} was lower in buffer (12 µg/kg) than in milk (20 µg/kg) at 20 µL/min for antibody 2. The effect of the milk matrix was similar to that for antibody 1.

Values for antibody 2 were more precise than those for antibody 1 because the IC_{50} values were included in the middle of the standard curve.

The biosensor immunoassay had a detection limit of about 2.9 and 1.7 µg/kg in buffer and in milk, respectively, with antibody 2 at 20 µL/min. Therefore, whatever the flow rate or antibody, the detection limits were well below the MRL of 100 µg/kg.

Both in buffer and in milk, the assay had a lower IC_{50} and a better limit of detection with antibody 2 anti-SMZ than with antibody 1. The CVs were a little higher with antibody 2, between 5.3 and 12.7%. The precision of the assay was also satisfactory with this antibody.

Conclusions

The development of this biosensor assay led to 2 possible screening tests because 2 antibodies could actually be used. The optimum conditions have been determined at 20 µL/min for the 2 antibodies. Antibody 2 was more sensitive than antibody 1, with a lower detection limit; however, the linear range of the curve did not include the MRL. Moreover, the detection limit of antibody 1 was sufficient to detect SMZ near the MRL. Therefore, antibody 1 could be used directly in the assay or antibody 2 could be used with diluted milk. The advantage of using antibody 2 would be that this dilution step could replace the pasteurization step.

This study demonstrates the impact of flow rate and quality of the antibody on the detection of drug residues by the

immunosensor technique, and would thus be useful for comparing antibodies from different suppliers.

The assay presented here is fast (8 min per sample), sensitive, specific, and repeatable, with minimum preparation of samples, and the concentration range includes the MRL. Therefore, this assay could be used as a routine method for screening of SMZ in milk. Moreover, because the BIACORE system is a multichannel device, it would be possible to measure several different analytes in parallel, either several sulfonamides with very specific antibodies or several families of antibiotics with selective antibodies. For further experiments, a less specific antibody is needed, which would be directed against major molecules of the sulfonamide family, because the MRL was set for the sum of sulfonamides.

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II. ARTICLE 2

AFNOR VALIDATION OF PREMI®TEST, A MICROBIOLOGICAL-BASED SCREENING TUBE TEST FOR THE DETECTION OF ANTIMICROBIAL RESIDUES, IN MUSCLES FROM DIFFERENT ANIMAL ORIGINS

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Food Additives and Contaminants: Part A

Contexte

Les animaux d'élevage peuvent être traités avec des antibiotiques, soit de façon préventive, soit de façon curative. Donc des résidus d'antibiotiques peuvent être retrouvés dans le muscle de ces animaux, soit sous la forme de la molécule mère, soit sous une forme métabolisée. Dans ce cadre, des Limites Maximales de Résidus (LMR) ont été fixées pour tous les antibiotiques autorisés. Les méthodes de dépistage qui sont la première étape du contrôle doivent permettre de détecter ces antibiotiques en dessous ou au moins au niveau de ces LMR. Le kit commercial appelé Premi®Test (commercialisé initialement par la société DSM, Pays-Bas) permet une détection large spectre de plusieurs grandes familles d'antibiotiques, dans le muscle de différentes espèces animales. Ce test microbiologique est basé sur la détection de l'inhibition de la croissance d'une souche bactérienne (*Bacillus stearothermophilus*) pré-ensemencée dans un milieu gélosé prêt à l'emploi. La détection de l'inhibition de la croissance bactérienne en présence de résidus d'antibiotiques dans l'échantillon se fait par un changement de couleur, liée à la présence d'un indicateur de pH, en moins de 4 heures. En effet, cette souche bactérienne possède la propriété d'acidifier le milieu quand elle se développe. En 2008, la société DSM a déposé un dossier de certification AFNOR. En tant que laboratoire expert, nous avons réalisé les études de validation et présenté les résultats au Bureau Technique agro-alimentaire de l'AFNOR.

Méthodologie/Principaux résultats

La validation selon le référentiel AFNOR, spécifique des kits de dépistage des résidus d'antibiotiques, est constituée de deux étapes : l'étude préliminaire et l'étude collaborative. L'un des grands principes de l'étude préliminaire est la comparaison d'une méthode alternative (le kit commercial) avec une méthode de référence. Toute l'étude préliminaire est réalisée avec les 2 méthodes en parallèle ; par contre, l'étude collaborative ne nécessite la pratique des 2 méthodes que chez le laboratoire expert, alors que les laboratoires participants réalisent seulement les analyses avec la méthode alternative. Dans le domaine du dépistage des résidus d'antibiotiques, la méthode de référence est en principe la méthode officielle utilisée pour la réalisation des plans de contrôle et plans de surveillance français. En effet, il n'existe pas de méthodes normalisées dans ce domaine, contrairement au domaine de la microbiologie alimentaire. Dans le cas du Premi®Test, la méthode officielle française, appelée Méthode des 4 Boites (M4B), a été choisie comme méthode de référence. La comparaison s'avérait complexe étant donné que les 2 méthodes divergeaient en de nombreux points :

- La préparation des échantillons de muscle est très différente. En effet, pour la M4B, des carottes sont prélevées dans des muscles entiers (non broyés) et congelés. Puis, des rondelles d'une épaisseur de 2 mm sont découpées dans ces carottes et déposées sur les boites de milieu gélosé préalablement ensemencé. Dans le cas du Premi®Test, les muscles doivent être entiers, puis pressés pour en obtenir le jus. Ensuite, le jus est déposé sur les ampoules de Premi®Test contenant le milieu gélosé pré-ensemencé. Le spectre de détection des 2 méthodes est différent. En effet, la M4B a un spectre plus large que la méthode alternative, puisque plusieurs germes sont ensemencés dans

différents milieux pour la M4B. alors qu'un seul germe ensemencé dans un seul milieu est utilisé dans la méthode alternative.

- Un problème méthodologique se posait donc pour la supplémentation des muscles. En effet, pour déterminer les limites de détection des méthodes, les matrices sont usuellement supplémentées, avec des quantités connues d'antibiotiques. Dans le cas de la M4B, il est impossible de supplémenter des muscles intacts puisque l'homogénéité de la répartition ne peut pas être garantie. De même, dans le cas du kit, supplémenter du muscle intact puis récupérer le jus ne constituait pas un protocole fiable. De plus, pour déterminer les limites de détection des 2 méthodes, il faut tester de nombreux antibiotiques, chacun à 3 concentrations. Il est impossible de trouver les matériaux naturellement chargés (non broyés), contenant les antibiotiques qui nous intéressent, et de surcroit à 3 concentrations différentes. C'est pourquoi cette étude préliminaire a été décomposée en 3 étapes (**Figure 46**).

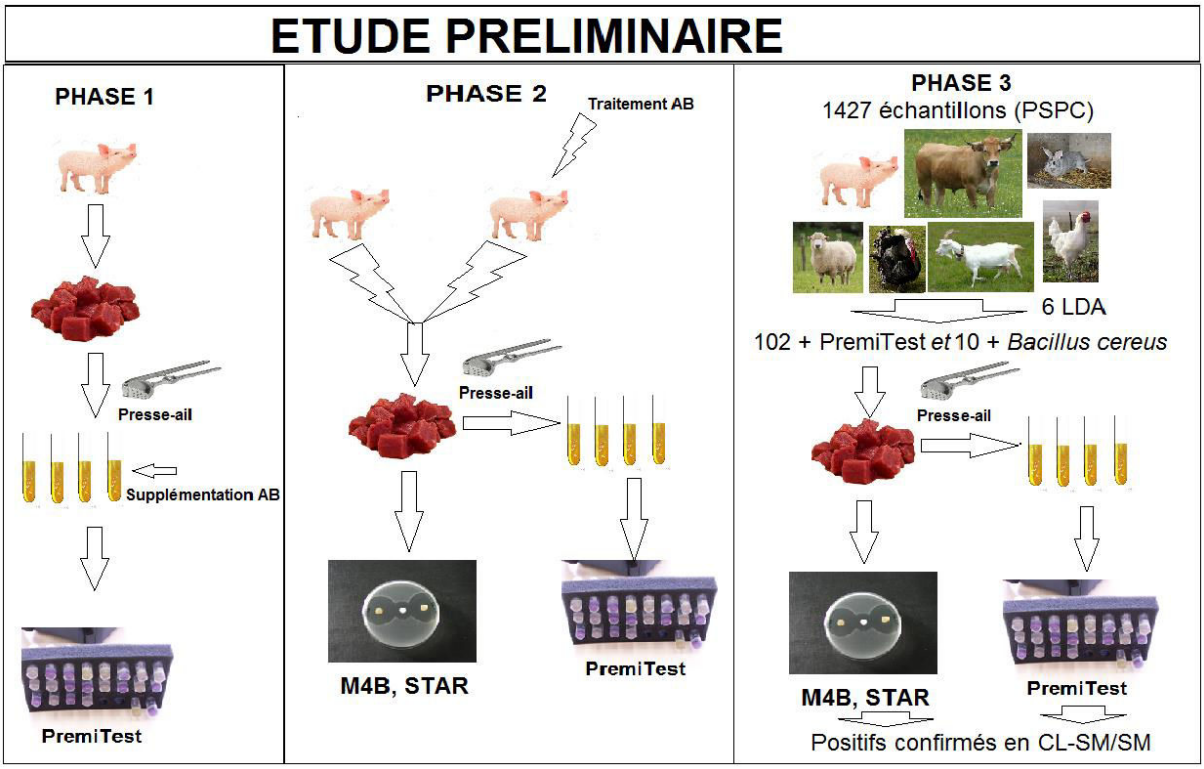
Suite à l'étude préliminaire, une étude collaborative impliquant la participation de 10 laboratoires, a été organisée. Des échantillons de jus de viande supplémentés ont été envoyés aux participants, puis analysés. Chaque matériau a été préparé, codifié et envoyé en double aveugle, soit 32 échantillons à analysés par laboratoire. En plus des échantillons, l'Anses a préparé et fourni à tous les laboratoires collaborateurs 3 témoins négatifs (porc, bœuf, poulet) et un témoin positif bœuf (contenant de la pénicilline G à 10 µg/kg). Les laboratoires participants devaient réaliser les analyses en double aveugle (2 séries distinctes d'analyses).

L'étape 1 a permis de déterminer les limites de détection du Premi®Test pour 6 molécules de 5 familles différentes d'antibiotiques à partir de jus de viande de porc supplémentés. Excepté pour la gentamicine, les limites de détection du Premi®Test se situent au niveau des Limites Maximales de Résidus (LMR). Ces résultats sont en accord avec les performances du test annoncées par le fabricant.

L'étape 2 a permis de déterminer un taux de faux-positifs et un taux de faux-négatifs de 0% pour le Premi®Test. Les taux de faux-négatifs et de faux-positifs étaient meilleurs pour le Premi®Test (0%) que pour la M4B (57% et 15% respectivement). En effet, les limites de détection de la M4B sont insuffisantes pour certains antibiotiques (*eg*. aminosides). De plus, cette phase de l'étude a montré que la M4B et le Premi®Test donnent des résultats concordants (« les 2 méthodes ne sont pas différentes »).

L'étape 3 a permis de montrer que le Premi®Test était applicable aux muscles de différentes espèces animales (porcins, bovins, volaille, ovin, lapin, …). La comparaison entre le Premi®Test et la M4B réalisée sur les seuls échantillons analysés au niveau de l'Anses a fait apparaître une différence entre les 2 méthodes. Toutefois, l'intégration des résultats négatifs dans les LVD permet de constater que les 2 méthodes ne sont pas différentes. La capacité de chaque test à détecter les différentes familles d'antibiotiques a été étudiée. Le Premi®Test détecte mieux les antibiotiques de certaines familles (*eg*. béta-lactamines) que la M4B et moins bien certaines comme les tétracyclines.

Figure 46. Protocole expérimental de l'étude préliminaire.



Les résultats des laboratoires participants ont été analysés afin de déterminer différents paramètres de validation. Le référentiel AFNOR n'indique pas de limites minimales à atteindre pour les caractéristiques de performance déterminées. La spécificité et la sensibilité au niveau L_3 ont été déterminées respectivement à 95,3 % et 72,5 %. La sensibilité au niveau L_3 est de 100 % pour la sufaméthazine à 400 µg/kg (4*LMR) dans le muscle de bovin et pour la tylosine à 200 µg/kg (2*LMR) dans le muscle de poulet. Elle est diminuée par un taux de faux-négatifs plus élevé pour le ceftiofur à 800 µg/kg (0,8*LMR) et l'oxytétracycline à 400 µg/kg (4*LMR) dans le muscle de porc. Ces résultats sont satisfaisants en regard des résultats obtenus lors de l'étude préliminaire. Les capacités de détection obtenues lors de l'étude préliminaire (étape 1) par le laboratoire expert étaient plus basses (sufaméthazine à 2*LMR, tylosine à la LMR, ceftiofur à 0,5*LMR et oxytétracycline à 2*LMR). C'est logique car les résultats d'un seul laboratoire, de surcroît expert dans ce genre de validation, sont le plus souvent meilleurs que les résultats globaux d'une étude collaborative, impliquant des participants plus ou moins habitués à utiliser la méthode alternative. Les résultats des laboratoires participants en termes de répétabilité et de reproductibilité étaient très satisfaisants, avec respectivement un pourcentage moyen de 94,8% pour un même échantillon et de 92,7 % pour 2 échantillons identiques (paire) en répétabilité et un pourcentage moyen de 89,1 en reproductibilité.

Conclusions/Importance

En conclusion, le kit commercial Premi®Test a obtenu la certification AFNOR en 2006 pour le dépistage des résidus d'antibiotiques dans le muscle porcin, bovin et de volaille (hors viande hachée). Cette certification a été reconduite en 2010 et 2014, sur présentation d'un dossier par le laboratoire expert. Le kit est actuellement certifié jusqu'en 2018. La certification AFNOR possède une reconnaissance nationale et européenne, voire internationale. Les études de validation qui conduisent à la certification sont complétées par des audits des sites de production. Ce type de validation permet aux fournisseurs de tests d'obtenir une reconnaissance des performances de leurs tests, de la qualité et de la traçabilité de leur production. Toutefois, nous avons pu voir dans le cadre de cette certification que le principe de base de la certification AFNOR qui repose sur la comparaison de la méthode alternative avec une méthode de référence pose de nombreux problèmes méthodologiques :

- D'une part pour la mise en place de l'étude préliminaire : la préparation des échantillons pour les deux méthodes peut être très différents. La supplémentation de matrices entières, telles que du muscle, ne permettent pas de garantir l'homogénéité de la répartition des résidus. Le spectre de détection des deux méthodes peut être différent. De plus, les méthodes auparavant utilisées comme méthodes de référence sont de moins en moins utilisées dans les laboratoires et ne seront donc plus disponibles dans l'avenir pour réaliser les comparaisons.
- D'autre part pour l'exploitation des résultats de cette l'étude préliminaire : la méthode alternative présente le plus souvent des limites de détection inférieures à celles de la méthode de référence. Donc on ne peut conclure à l'équivalence des deux méthodes, ce qui pose problème dans le cadre de la validation AFNOR. De plus, comme les spectres de détection peuvent être différents, cela renforce la non équivalence des méthodes.

Cependant, le point fort principal de la validation selon le protocole AFNOR est l'organisation d'une étude collaborative. Cette validation inter-laboratoires est très importante car elle permet de déterminer la répétabilité et la reproductibilité de la méthode alternative, mais aussi de vérifier la robustesse de la méthode. Pour toutes ces raisons, l'Anses en tant que laboratoire expert a proposé à l'AFNOR de réviser le référentiel spécifique des kits antibiotiques. Un groupe de travail réunissant l'Anses, l'AFNOR et d'autres experts est en cours de rédaction d'un nouveau référentiel, qui sera officialisé en 2016. Ce nouveau référentiel conserve les 2 études (préliminaire et collaborative). Par contre, l'étude préliminaire ne sera plus basée sur la comparaison de méthodes, mais sur une approche critère par critère.

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AFNOR validation of Premi®Test, a microbiological-based screening tube-test for the detection of antimicrobial residues in animal muscle tissue

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Premi®Test contains viable spores of a strain of *Bacillus stearothermophilus* which is sensitive to antimicrobial residues, such as beta-lactams, tetracyclines, macrolides and sulphonamides. The growth of the strain is inhibited by the presence of antimicrobial residues in muscle tissue samples. Premi®Test was validated according to AFNOR rules (French Association for Normalisation). The AFNOR validation was based on the comparison of reference methods (French Official method, i.e. four plate test (FPT) and the STAR protocol (five plate test)) with the alternative method (Premi®Test). A preliminary study was conducted in an expert laboratory (Community Reference Laboratory, CRL) on both spiked and incurred samples (field samples). Several method performance criteria (sensitivity, specificity, relative accuracy) were estimated and are discussed, in addition to detection capabilities. Adequate agreement was found between the alternative method and the reference methods. However, Premi®Test was more sensitive to beta-lactams and sulphonamides than the FPT. Subsequently, a collaborative study with 11 laboratories was organised by the CRL. Blank and spiked meat juice samples were sent to participants. The expert laboratory (CRL) statistically analysed the results. It was concluded that Premi® Test could be used for the routine determination of antimicrobial residues in muscle of different animal origin with acceptable analytical performance. The detection capabilities of Premi®Test for beta-lactams (amoxicillin, ceftiofur), one macrolide (tylosin) and tetracycline were at the level of the respective maximum residue limits (MRL) in muscle samples or even lower.

Keywords: validation; Premi®test; screening; antimicrobial residues; muscle; collaborative study; routine analysis

Introduction

Animal treatment can lead to the presence of residues in food of animal origin. The presence of antimicrobial residues could lead to human health and safety risks, such as allergies or toxicity, when foodstuffs containing residues enter the food chain. Different families of antimicrobial residues are concerned: beta-lactams, sulfonamides, macrolides, tetracyclines, quinolones, etc. For these reasons, maximum residue limits (MRL) were set for many antimicrobial residues to protect consumer safety (EEC Directive 2377/90 and amendments). Different methods have been developed for the detection of antimicrobial residues in food of animal origin. These screening methods are usually microbiological, based on the inhibition of bacterial growth by antimicrobial residues. Microbiological plate tests generally give results in 18–24 h; however, they require skilled technicians with specific training. Microbiological-based tests are an interesting screening step because they are capable of detecting a wide range of antimicrobial residues (several families of antimicrobials are detectable with one single method). The levels of detection are also generally satisfactory with respect to European regulations (EEC, 1990) for beta-lactams, tetracyclines and macrolides (detection capabilities lower or equal to the MRL). The best detected class of compound is generally the beta-lactam family. The detection capabilities of sulphonamides are very variable, depending on the sulphonamide structure. Using microbiological-based tests, the least detected antibiotics are generally some sulphonamides, aminoglycosides and quinolones. None of the existing microbiological screening methods are capable of detecting all MRL substances in animal matrices. Therefore, some countries have implemented other methods in parallel to specifically detect these antimicrobials. In France, the official method for the control of muscle samples is the four plate test (FPT) (Bogaerts and Wolf 1980). In the past, some commercial tube-tests were available but only for the analysis of milk, such as Delvotest®, or, more recently, the COPAN® test. These tests are based on the detection of growth by production of acid, visible by a colour change in the test medium (pH indicator). More recently, the Premi®Test, a commercial growth-inhibitor test, was developed by DSM Food

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Specialities R&D (Delft, The Netherlands) for the detection of antimicrobial residues in muscle. The test is based on inhibition of the *Bacillus stearothermophilus* strain and allows the detection of antimicrobial residues in muscle in less than 4 h (Fabre 2003a,b; Fabre et al. 2004; Stead 2004).

Over the last few years, several evaluations of the Premi®Test have been published, based on spiked meat juice samples (Reybroeck 2000a) or incurred poultry muscles (Reybroeck 2000b). Premi®Test has generally been compared to a reference method, focussing on a family of antimicrobials, such as beta-lactams, using spiked meat juice samples and incurred poultry muscle samples (Popelka et al. 2005) or tetracyclines (Okerman et al. 2004). The detection limits for various antimicrobials, calculated in spiked juice samples, were similar in the different studies. The overall conclusion of these studies was that Premi®Test is unsuited to the detection of tetracyclines at MRL level in spiked and incurred samples. Premi®Test was very sensitive for the screening of beta-lactams, more than the usual microbiological plate tests. Premi®Test was suitable for the detection of beta-lactams and sulphonamides at MRL level or even below.

The AFNOR validation is based on a comparison between a reference and the alternative method. In the present study, the Premi®Test, i.e. the alternative method, was compared with two other microbiological methods: the French Official method (four plate test, FPT) which was the reference method, and the STAR protocol (the CRL protocol) (Gaudin et al. 2004). Thereafter, an interlaboratory study was organised, in which only the Premi®Test was used to analyse spiked meat juice samples. This paper presents the results of preliminary and collaborative studies.

Materials and methods

Presentation of the methods

Principle of the kit

The Premi®Test allows the detection of antimicrobial residues in fresh meat, kidneys, fish and eggs (Arts et al. 2000). The Premi®Test is based on the growth inhibition of *Bacillus stearothermophilus*, in which standardised spores are included in a medium with selected nutrients. The meat juice I placed in ready-to-use tubes and, after 20 min of pre-diffusion at room temperature, the meat juice was removed by three washing steps. Finally, the ampoule was incubated for approximately 3 h at 64°C. The reading of the "yes/no" result was based on a colour comparison. Without antimicrobials, the spores germinate and develop, involving acidification of the medium and a change of colour (yellow). Conversely, in the presence of antimicrobials, bacterial growth is inhibited and a purple colour indicates the presence of antibiotics at or above the detection limit of the test. Doubtful and positive samples were confirmed by the multi-residue LC/MS–MS method described below. This broad-spectrum test makes it possible to detect a large number of antimicrobials in less than 4 h in muscle juice samples (extracted by pressing a piece of meat).

Principle of the four plate test (FPT)

The four plate test is the French Official method for monitoring muscle samples (Bogaerts and Wolf 1980). A microorganism, sensitive to antibacterial substances, is inoculated into an agar medium in a Petri dish. The following test organisms are used: *Bacillus subtilis BGA* (reference 10649; Merck, Nogent-sur-Marne, France) (in test agar pH 6.0, Merck), *Bacillus subtilis BGA* (reference 10649, Merck) (in test agar pH 8.0, Merck), *Bacillus subtilis BGA* (reference 10649, Merck) (in agar ASS pH 7.4, Merck) and *Kocuria varians* (ATCC 9341, Pasteur Institute) (in test agar pH 8.0, Merck). Slices of frozen muscle were placed on the surface of the inoculated medium and incubated at the optimal temperature for growth of the test organism. After diffusion, the antibacterial substance should produce an inhibition zone around the sample by inhibiting the growth of the test organism.

Principle of the STAR protocol and Bacillus cereus

The STAR protocol is the CRL method (Gaudin et al. 2004), which was developed to improve the performance of the FPT, and the detection principle is the same. The following test organisms are used: *Bacillus subtilis BGA* (reference 10649, Merck) (Antibiotic medium II at pH 8.0, Difco, Detroit, MI, USA), *Kocuria varians* ex. *Micrococcus luteus* (ATCC 9341, Pasteur Institute) (in test agar at pH 8.0, Merck), *Bacillus cereus* Bc6 (ATCC 11778, Pasteur Institute) (in test agar at pH 6.0, Merck), *Escherichia coli* (ATCC 11303, Pasteur Institute) (in test agar at pH 8.0, Merck), *Bacillus stearothermophilus* (ATCC 10149, Merck) (in DST (Diagnostic Sensitive Test) medium; Oxoid, Basingstoke, UK). The *Bacillus cereus* used by field laboratories at the 3rd step of the preliminary study is plate Bc6 of the STAR protocol, which is selective for the detection of the tetracycline family. Muscle sample slices of 2 mm in thickness were cut from frozen muscle and placed on the plates.

LC/MS–MS method

A multi-residue LC/MS–MS method was developed for the screening of antimicrobials in meat. The principle is based on two different extractions: one with trichloroacetic acid (TCA), allowing the detection of tetracyclines, aminoglycosides and quinolones; the second with acetonitrile (ACN), allowing the detection of

penicillins, cephalosporins, macrolides and sulfonamides. TCA extracts are directly injected after ultracentrifugation and filtration. ACN extracts are evaporated and the residue is then dissolved in 0.6 ml of ammonium acetate before filtration and injection. Two different gradients with pentafluoropropionic acid and ACN are used for the LC analyses. LC/MS–MS is used in multi-reaction monitoring mode (MRM) and two MRM transitions are monitored for each compound. Identification of the detected compounds is based on retention time and the presence of the two specific transitions. Quantitative determination was carried out using calibration curves obtained with spiked samples at 0.5, 1 and 1.5 MRL levels. Internal standard were used for the quantification. All 50 monitored compounds, except some aminoglycosides, were detected at a level below the MRL.

Principle of AFNOR (French Association for Normalisation) validation

Any alternative method should be compared with a reference method. A reference method could be a standardised method, if it exists, an official method or a widely known and used method, taken in reference. An alternative method is a commercial test allowing analysis of, for a category of products given, the same analytes as that measured by the reference method, but presenting one or more of the following criteria: speed of analysis, ease of execution and/or automation, analytical performances (limit of detection, specificity, etc.). A specific guide for validation of alternative methods for the detection of antimicrobial residues in foodstuffs of animal origin is available (Anon. 2005a). It defined the requirements relating to the organisation of preliminary and collaborative studies carried out by one expert laboratory. This document establishes the general principle as well as the technical protocol for the validation of alternative methods for the detection of antimicrobial residues in foodstuffs of animal origin.

Preliminary study

The preliminary step are divided into three parts.

Step 1. Analysis of blank and spiked meat juice samples

Porcine muscles was used from practical experience. The absence of antimicrobial substances in these pig samples, assumed as blank materials, was checked via FPT and the STAR protocol. The negative results confirmed that these pig samples could be used to prepare blank meat juices.

Muscle samples were pressed through a garlic press. Thereafter, meat juice samples were spiked with known concentrations of six different antimicrobials: sulfamethazine (sulphonamides), oxytetracycline (tetracyclines), tylosin (macrolides), amoxicillin (penicillins), ceftiofur (cephalosporins) and gentamicin (aminoglycosides). Each antimicrobial was spiked at three concentrations: sulfamethazine (50, 100, 200 $\mu g\,kg^{-1}$), oxytetracycline (50, 100, 200 $\mu g\,kg^{-1}$), tylosin (50, 100, 200 $\mu g\,kg^{-1}$), amoxicillin (25, 50, 100 $\mu g\,kg^{-1}$), ceftiofur (100, 200, 400 $\mu g\,kg^{-1}$) and gentamicin (50, 100, 200 $\mu g\,kg^{-1}$).

Five aliquots (five combinations of sample/antimicrobial/concentration) were prepared "blind to the technician" and analysed in duplicate only with the Premi®Test. The aliquots were stored in a freezer (approximately −20°C) before analyses. Blank meat juice and spiked meat juice samples were analysed in duplicate only with the Premi®Test.

Step 2. Treatment of animals and analysis of incurred porcine muscle samples

Two untreated pigs were slaughtered before the treatments of three other pigs. These pigs were supplied by a farm guaranteeing that the pigs did not receive prior antimicrobial treatments. Moreover, the absence of antimicrobials in muscle samples was checked with the FPT and STAR protocol.

Three pigs were treated, one each with tylosin, amoxicillin and a oxytetracycline/sulfadimethoxine mixture, and slaughtered. Each material was analysed by a multi-residue LC/MS–MS method to quantify antimicrobials. The following concentrations were obtained: tylosin $750.9 \pm 76.1\,\mu g\,kg^{-1}$ (material 1), amoxicillin $269.5 \pm 18.0\,\mu g\,kg^{-1}$ (material 2), oxytetracycline $764.9 \pm 44.8\,\mu g\,kg^{-1}$ and sulfadimethoxine $151.1 \pm 13.2\,\mu g\,kg^{-1}$ (material 3).

Afterwards, five pieces of each material (blank and treated animals) of approximately 20 g were (five combinations of sample/antimicrobial/concentration) prepared "blind to the technician" and analysed in duplicate using three methods: Premi®Test, STAR protocol and FPT. These samples were stored in freezer at approximately −20°C before analyses.

Step 3. Analysis of field samples

This step allowed a comparison of the Premi®Test, STAR protocol and FPT in "naturally" incurred samples for a wide number of antimicrobials and matrices of different origins (e.g. different species). The samples came from a pilot study on the implementation of a new screening method for antimicrobial residues in meat (Anon. 2005b). The samples were sent regularly to laboratories from the French veterinary services (official control). Six field veterinary laboratories (LVD or "Laboratoire Vétérinaire Départemental" in French) were trained in the

implementation of the Premi®Test and one plate *Bacillus cereus* (for detection of tetracyclines) (STAR protocol), analyzing 1427 field samples over a period of 4 months. All their positive samples (with Premi®Test and/or *Bacillus cereus*) were sent frozen to the CRL every 15 days. All sample characteristics (species, reception date, analysis date, dispatch date, etc.), as well as the results obtained with Premi®Test and *Bacillus cereus*, were also sent to the CRL. All samples from the field laboratories where stored in the freezer upon arrival in our laboratory. Blind analyses were carried out at the CRL with the Premi®Test, FPT and STAR protocol, including the *Bacillus cereus* plate. Finally, the positive samples from at least one of these methods were analyzed by the multi-residue LC/MS–MS method (systematic screening, then identification and quantification). Doubtful Premi®Test samples were also tested by LC/MS–MS.

Interlaboratory study

Eleven laboratories were contacted to participate to this inter-laboratory study, including the six field laboratories which had already participated to step 3 of the preliminary study. When an AFNOR validation is implemented, an expert laboratory is designated by the AFNOR Technical Office. The expert laboratory performs the intra-laboratory validation and organised the inter-laboratory study. Moreover, during the inter-laboratory study, the results of the expert laboratory are not included with the participants' results, but these results are considered as the reference results (to be obtained by the participants). In this case, the Community Reference Laboratory (CRL) was considered the expert laboratory.

Spiked muscle juice samples were used for the interlaboratory study instead of incurred samples, for several reasons. Firstly, it is very difficult to prepare raw muscle with a target concentration of each antimicrobial owing to the individual variability of animal pharmacokinetic (depletion of the antibiotic in tissues). Moreover, the use of raw pieces of meat did not allow mincing of meat to adjust the concentration. Secondly, the study was based on several animal species and, therefore, treatment would need to be performed in each species. This would have been very expensive, time-consuming and impossible to implement in our laboratory. Thirdly, when producing incurred muscles and sending raw muscle samples (pieces of meat), it is very difficult to ensure homogeneity of the samples, which is basic when organising a collaborative study.

Preparation of the materials

Porcine, bovine and chicken muscles were pressed. Blank meat juice samples (negative controls and blank unknown samples) were prepared. Furthermore, spiked samples were prepared with four different antibiotics at three concentrations. Oxytetracycline and ceftiofur were added to porcine meat juice samples, sulfamethazine to bovine meat juice samples and, finally, tylosin to chicken meat juice samples. Table 1 presents the content of the 16 combinations.

A stability study was carried out on materials before sending the samples and over the period of analyses by the participants. The samples were analysed with the Premi®Test at the CRL. Material stability was proven over the period of analyses. A random codification of the materials was performed and each laboratory was identified by a code (from A to P).

Dispatch of materials

One negative control from each species was sent and one positive control containing penicillin G at 10 $\mu g\,kg^{-1}$. A total of 32 different frozen meat juice samples (16 materials in blind duplicate: spiked and blank samples) were sent under frozen conditions (dried ice) to the participants to ensure stability of matrix and analyte. The samples were stored in a freezer upon arrival. Eleven laboratories received parcel instructions and a results form. The participants had no information about the antibiotics contained in the materials.

Because it is recommended to test one negative control from each analysed species, each laboratory received a table indicating the species origin of each sample, according to a sample code (1–32), to compare

Table 1. Interlaboratory study: Content of the 16 materials sent in blind duplicates.

		Porcine	Bovine	Porcine	Chicken
Antimicrobial	Level of contamination	Oxytetracycline	Sulfamethazine	Ceftiofur	Tylosin
MRL	–	100	100	1000	100
Spiked concentrations ($\mu g\,kg^{-1}$)	L0	Blank	Blank	Blank	Blank
	L1	20	20	40	10
	L2	200	200	400	100
	L3	400	400	800	200

the results of each sample with the negative control of the corresponding species. All analyses were carried out in blind duplicate (two different series of analyses) with Premi®Test only. Analyses were performed within 1 week maximum after receiving samples. A negative control for each species, a positive sample (spiked with penicillin G at 10 $\mu g\,kg^{-1}$) and coded materials were analysed. All results were returned rapidly and compiled at the CRL.

Results and discussion

Preliminary study

Step 1. Analysis of blank and spiked meat juice samples

Among the 20 blank meat juice samples analysed in duplicate, only one meat juice was positive twice and four other meat juices were "doubtful" in one of the two replicates and negative in the other. A total of 15 meat juices were negative twice. The false positive rate is the number of positive results for blank samples (free of antibiotic substances) divided by the total number of positive samples (the same number plus the number of contaminated samples showing positive results) and multiplied by 100: 18% (=6/(6+27)100) of results were false positive. The result was satisfactory because the false positive rate should be minimal for a screening method, since the samples declared positive should be confirmed by a physicochemical method for identification and quantification.

The detection limit corresponded to the lowest concentration which gave a positive or a doubtful result for each of the five replicates. Table 2 presents the determination of limits of detection of Premi®Test for the six antimicrobials tested.

The study of spiked pig meat juice samples showed that, for five molecules belonging to four different classes of antibiotics, the detection limit of Premi®Test was at a level of one or two MRL maximum. For only one antibiotic (gentamicin), the detection limit was two times higher than the MRL (40% of positive results at 2 × MRL). The false negative rate corresponds to the number of negative results obtained for contaminated samples (spiked samples) divided by the total number of negative samples (the same number plus the number of blank samples giving a negative result) and multiplied by 100. The false negative rate calculated at 1 × MRL was 22% (=10/(10+34)100). However, the false negative rate calculated at twice the MRL was 8% (=3/(3+34)100).

Step 2. Treatment of animals and analysis of incurred porcine muscle samples

Concentrations in naturally incurred samples were much higher than the respective MRL of the four antimicrobials (from 1.5 to 7.6 × MRL). However, the analyses of incurred materials with known antimicrobial concentrations was of significant interest. Table 3 summarises the results of step 2.

Data exploitation was carried out according to the reference document of AFNOR validation (Anon. 2005a). Exploitation is based on a comparison of two methods: the reference method and the alternative method. The FPT was set as the reference method and the Premi®Test as the alternative method.

Three different parameters were calculated which allowed a comparison of reference and alternative method: relative accuracy (AC = (PA + NA)/N × 100%), relative specificity (SP = (NA/N−) × 100%), relative sensitivity (SE = (PA/N+) × 100%), where NA is the negative agreement (negative result obtained with both methods), PA is the positive agreement (positive result obtained with both methods), ND id the negative discrepancy (positive result obtained with reference method and negative result with alternative test), PD is the positive discrepancy (negative result obtained with reference method and positive result with alternative test); N = NA + PA + PD + ND is total number of samples; N− is the total number of negative samples obtained with the reference method (NA + PD); N+ is the total number of positive samples obtained with the reference method (PA + ND).

Relative accuracy, relative specificity and relative sensitivity were calculated as 70, 42.9 and 84.6%, respectively. Relative accuracy and specificity were satisfactory, but the low relative specificity value could be explained because the sensitivity of the FPT was sometimes insufficient for certain antibiotics.

In conclusion, FPT and Premi®Test gave similar results for performance characteristics (relative accuracy, relative specificity and relative sensitivity). However, at this step, the false negative and the false positive rates were lower for the Premi®Test (both 0%) than for FPT (33 and 40%, respectively).

Step 3. Analysis of field samples

A total of 1427 field incurred samples, from six French field veterinary laboratories, were analyzed via Premi®Test and *Bacillus cereus* plate, of which, 1325 were negative, and 102 samples doubtful (36) and positive (66) with the Premi®Test.

Among the 1325 negative results, 10 samples were positive with the *Bacillus cereus* plate. Therefore, 112 muscle samples were sent to the CRL for confirmation. The correlation between the Premi®Test results from LVD and AFSSA was examined (Table 4). A total of 76% of the samples (78/102) found positive or doubtful with Premi®Test in the LVD were found positive or doubtful with Premi®Test at the CRL. A total of 24 samples were positive or doubtful at the

Table 2. Results of Premi®Test analyses on spiked juice samples (step 1).

Antibiotic family	Sulphonamide	Tetracycline	Macrolide	Beta–lactam	Aminoglycoside	Beta–lactam	
Antibiotic	Sulfamethazine	Oxytetracycline	Tylosin	Amoxicillin	Gentamicine	Ceftiofur	Global
MRL (muscle, $\mu g\,kg^{-1}$)	100	100	100	50	50	1000	–
Tested concentrations ($\mu g\,kg^{-1}$)	50/**100**/200	50/**100**/200	50/**100**/200	25/**50**/100	**50**/100/200	100/200/400	–
Detection rate at 0.5 MRL	0%	60%	80%	100%	(0%)*	–	48%
Detection rate at MRL	20%	80%	100%	100%	0%	(100%)	67%
Detection rate at 2 × MRL	100%	100%	100%	100%	40%	(100%)	90%
Detection capability	2 × MRL	2 × MRL	MRL	0.5 × MRL	> 2 × MRL	0.5 × MRL	–

*MRL in bold character in tested concentrations.
Percentage is between brackets when no were analyses performed at this concentration, i.e. all the tested concentrations for ceftiofur were below MRL and even 0.5 MRL. At 400 $\mu g\,kg^{-1}$, 100% of the results were positive or doubtful.

LVD and negative at AFSSA. This could be due either to false positive results from the field laboratories or to antibiotic instability between the two analyses, although the recommended storage and transport conditions were strictly respected. Among the 10 samples negative with Premi®Test and positive with *Bacillus cereus* in the field laboratories, six were positive or doubtful with Premi®Test at the CRL and confirmed positive with *Bacillus cereus*. The four other samples were positive only on plate Bc6. Finally, the presence of a tetracycline was confirmed by LC/MS–MS in nine of these samples. Therefore, *Bacillus cereus* plate (one plate of the STAR protocol, which is selective for the detection of tetracyclines) is more sensitive for tetracyclines than Premi®Test.

The false positive rate in the field laboratories was occasionally high, mainly at the beginning of the study. Thereafter, with reading experience, most of the positive results with Premi®Test at the field laboratories were confirmed positive with Premi®Test at the CRL.

Many animal species were studied in step 3 because samples were from field laboratories, while the first two steps were based only on pig muscle. The distribution of species samples is presented in Table 4.

The confirmatory rate of the LC/MS–MS method (number of samples really containing antimicrobial residues divided by the number of tested samples (positive screening) multiplied by 100) was 41% (25/61). The confirmatory rate varied between species and there were more false positive results in some species (37% for bovine samples compared to 75% for poultry samples).

The results of the comparative analysis between the alternative method (Premi®Test) and the reference method (FPT) are presented in Table 5.

When data analysis was based only on the 112 samples reanalysed in the expert laboratory, relative accuracy (33.9%) and relative specificity (33.4%) were very low. Only relative sensitivity (70%) was satisfactory, as in the first step of the validation study. However, in this case, Premi®Test negative samples (1315 negative/1427 analyzed samples) obtained in the field laboratories were not taken into account. Only the positive samples in the field laboratories were reanalysed at the CRL with Premi®Test and FPT. None of the 1315 negative samples was analysed with the FPT. From experience (a previous study in 2005; data not shown), all samples which were declared negative with Premi®Test and negative with

Table 3. Analyses of naturally incurred samples with the three screening methods (step 2).

Antibiotic	OTC/SDMX	Amoxicillin	Tylosin	Global results of incurred samples	Blank	Fp+ (%)	Fp– (%)
MRL (pig muscle) ($\mu g kg^{-1}$)	100/100	50	100	–	–	–	–
Concentrations by LC/MS–MS ($\mu g kg^{-1}$)	760/150	270	750	–	–	–	–
Number of positive results with Premi®Test	5/5	5/5	5/5	15/15	0/5	0	0
Number of positive results with STAR	5/5	5/5	5/5	15/15	3/5	60	0
Number of positive results with FPT	5/5	4/5	1/5	10/15	2/5	40	33

Fp+: false positive rate; Fp–: false negative rate; OTC: oxytetracycline; SDMX: sulfadimethoxine.

Table 4. Correlation between field laboratories and AFSSA: comparison of the three screening tests and LC/MS–MS method in relation to the species (step 3).

	Species	Bovine	Porcine	Poultry	Others	Unknown	Total
LVD	Number of samples analysed at LVD[b]	379	671	205	26	146	1427
	Number of positive and doubtful results at LVD Premi®Test	63	26	1	5	7	102
AFSSA	Number of samples analysed at AFSSA	65	31	4	5	7	112[a]
	Number of positive and doubtful results with Premi®Test	45	19	2	5	7	78
	Correlation LVD/AFSSA (%)	69	61	50	100	100	76
	Number of positive samples with FPT	3	5	2	0	0	10
	Number of positive samples with STAR	13	13	4	0	2	32
AFSSA	Number of tested samples	38	18	4	1	0	61
LC/MS–MS	Number of positive samples (identified molecule)	14	8	3	0	0	25
	Rate of positive confirmation (%)	37	44	75	0	–	41

[a]Total of samples sent to AFSSA for confirmation.
[b]LVD is Laboratoire Vétérinaire Départemental in French, which is a field veterinary laboratory.

Bacillus cereus were also all negative with the FPT (28 samples). Therefore, it was assumed that the 1315 samples, found negative with both Premi®Test and *Bacillus cereus*, would have also been negative with the FPT. In this case, the agreement (94.8% relative accuracy and 95% relative specificity) between the results of Premi®Test and FPT was very satisfactory. The two methods were different by a statistical test owing to a high rate of positive deviation (Premi®Test+/FPT−), which may be caused partially by false positive results of the Premi®Test and/or by the lack of sensitivity of the reference method towards some antimicrobial residues.

The agreement (relative accuracy) between the results of Premi®Test and STAR was 42.99%, relative specificity 31.3% and relative sensitivity 71.9%. Therefore, it is similar to the comparison of the FPT with Premi®Test. Agreement between the FPT and STAR protocol was higher (70%; data not shown). Only one sample was negative with the STAR protocol and positive with the FPT. The absence of antimicrobial residues in this sample was confirmed by LC/MS–MS.

The results obtained in the field laboratories, and then at the CRL, with the Premi®Test, FPT, STAR protocol and the confirmatory method by LC/MS–MS are compared in Table 6. The number of positive results with the Premi®Test (78) was much higher than the number of positives with the multi-plate tests (FPT: 10; STAR: 31 samples). Among the 112 samples analysed at the CRL, only the positive or doubtful samples with one of the screening methods (Premi®Test, FPT or STAR) were confirmed by LC/MS–MS (88 samples).

Table 5. Correlation between Premi®Test and FPT (step 3).

	1st analysis (112 samples)	2nd analysis (1427 samples)
Relative accuracy AC (%)	33.9	94.8
Relative specificity SP (%)	30.4	95.0
Relative sensitivity SE (%)	70.0	70.0

Relative accuracy: AC = (PA + NA)/N × 100%.
Relative specificity: SP = (NA/N−) × 100%.
Relative sensitivity: SE = (PA/N+) × 100%.
NA is the negative agreement; PA is the positive agreement; ND is the negative discrepancy; PD is the positive discrepancy.
N = NA + Pa + PD + ND is total number of samples.
N− is the total number of negative samples obtained with the reference method (NA + PD).
N+ is the total number of positive samples obtained with the reference method (PA + ND).

Table 6. Identification of positive samples with LC/MS–MS at AFSSA: comparison of Premi®Test, FPTand STAR protocol in 2005 (step 3).

			Premi®Test results			FPT results		STAR results	
Identified AB family	Quantification/MRL	Number of analysed samples	−	D	+	−	+	−	+
MACRO	<MRL	1		1			1		1
SULFA	<MRL	1		1		1		1	
TTC	<MRL	10	4	3	3	6	4	2	8
TTC	>MRL	2	1		1		2		2
BL	<MRL	10		2	8	10		7	3
BL	>MRL	2			2	1	1		2
TTC + SULFA	<MRL	1			1		1		1
BL + sulfa	>MRL	1			1	1		1	
MACRO + TTC + BL	<MRL	1			1	1		1	
TTC + BL	<MRL	1			1	1		1	
AMINO + BL	<MRL	1			1	1		1	
Global	<LMR	26	4	7	15	20	6	13	13
	>LMR	5	1	0	4	2	3	1	4
	Total	31	5	7	19	22	9	14	17
Absence		57	5	8	44	56	1	43	14
Not analysed		24	24			24		24	
Total		112	34	15	63	102	10	81	31

D: doubtful.

After confirmatory analyses, 31 samples really contained antimicrobial residues (35% of the confirmed samples) at different levels (26 samples at concentrations lower than the respective MRLs and five only at concentrations higher than the respective MRLs).

False positive rates for the FPT, Premi®Test and STAR protocol were 3% ((1/(1+31)) × 100), 62% ((52/(52+31)) ×100) and 31% ((14/(14+31))×100), respectively. The false positive (rate of each method was calculated as the number of positive results for blank samples (free of antibiotic substances) divided by the total number of positive samples (the same number plus the number of contaminated samples showing positive results) and multiplied by 100.

A total of 52 samples were Premi®Test positive or doubtful, but no antibiotic residue could be identified when analyzed by LC/MS–MS. However, a false-positive result could be due to degradation of the antimicrobials initially contained in the sample (too long a delay before analysis by LC/MS–MS, very unstable molecules, etc.). Furthermore, it could be false compliant results of the confirmatory method. The antimicrobial present in the sample may not be detected and identified by the multi-residue LC/MS–MS method if the molecule is not present in the spectrum of detection of the method (metabolites, etc.), or sensitivity may be insufficient.

After a positive result with Premi®Test, the presence of antimicrobial residues was confirmed in 26 samples (33%), of which four samples contained antimicrobials at concentrations higher than the MRL. Three of the 10 positive FPT samples and four of the STAR positive samples were at concentrations greater than the MRL. Premi®Test and STAR screened positive 22 and 13 samples, respectively, which actually contained antimicrobial compounds at concentrations lower than the MRL. The FPT detected only six of these samples. Therefore, the sensitivity of Premi®Test and STAR protocol was lower than the FPT.

The highest confirmatory rate was obtained for the FPT (90%). The false negative rates of the FPT, Premi®Test and the STAR protocol were 8, 28 and 20%, respectively. The false negative rate was calculated as the number of false negative results divided by the total of the true negative samples plus the number of false negative results for each method.

The most common antimicrobial classes detected during this pilot study were beta-lactams (penicillin and amoxicillin) (15 samples) and tetracyclines (doxycycline, tetracycline, oxytetracycline and chlortetracycline) (15 samples). Premi®Test (12 positive samples) was much more sensitive for the detection of beta-lactams than the FPT (one positive sample) and the STAR protocol (five positive samples), even too sensitive in some cases (10 positive samples confirmed at concentrations lower than MRL). Premi®Test was also more sensitive for the screening of sulphonamides than the two other methods (FPT and STAR failed to detect one sample at a concentration higher than the MRL). Finally, Premi®Test was less sensitive than the FPT, and especially the STAR protocol, for the detection of tetracyclines. Therefore, this study has demonstrated the capability of Premi®Test to detect samples at the MRL level for sulphonamides and beta-lactams, but not for tetracyclines. These conclusions have confirmed the results of different teams over recent years (Reybroeck 2000a,b; Okerman et al. 2004; Popelka et al. 2005).

Table 7 presented the combined results of a previous study carried out in 2003 and 2004 (64 samples actually containing antimicrobial residues) with the pilot study.

After the confirmatory analyses of positive samples, 31 samples contained antimicrobial residues at concentrations lower than the respective MRLs and 33 samples at concentrations higher than the respective MRLs. The total number of positive samples reported with Premi®Test (52) was higher than with FPT (38). However, the number of samples detected positive with Premi®Test (26) or FPT (25), which really contained antimicrobial compounds at concentrations higher than MRL, was identical. Moreover, six false negative results were obtained with Premi®Test and seven with FPT.

The presence of beta-lactams was confirmed in 17 samples by the LC/MS–MS method. All 17 samples were positive or doubtful with Premi®Test, while only seven samples were detected positive with the FPT (1 <MRL and 6> MRL). Therefore, the detection capability of Premi®Test for beta-lactams was better than FPT. Sensitivity was also lower for sulphonamides (four positive Premi®Test samples, four negative FPT samples). Two of these samples actually contained sulphonamides at concentrations higher than the MRL, which the FPT failed to detect but Premi®Test succeeded in detecting. However, the sensitivity of FPT for tetracyclines was better (15 doubtful or positive samples with Premi®Test compared to 19 positive samples with FPT).

The most detected antimicrobials were tetracyclines (25 samples) and beta-lactams (17 samples), followed by macrolides (6), sulphonamides (4), quinolones (3) or a mix of antimicrobials (8).

Collaborative study

Results of the expert laboratory

The expert laboratory obtained only one false positive result for the two series of analyses on white chicken sample (code 10), whereas the same sample (code 27) was found negative for the two series. The L1 concentration was selected to give negative results

Table 7. Identification of positive samples with LC/MS–MS at AFSSA: comparison of alternative (Premi®Test) and reference method (FPT) from 2003 to 2005 (step 3).

			Premi®Test results			FPT results	
Identified AB family	Quantification/MRL	Number of analysed samples	–	D	+	–	+
Beta–lactams	< MRL	9		1	8	8	1
	> MRL	8			8	2	6
	Sum	17					
Tetracyclines	< MRL	13	5	4	4	6	8
	> MRL	12	5	1	6		11
	Sum	25					
Sulphonamides	< MRL	2		1	1	2	
	> MRL	2			2	2	
	Sum	4					
Quinolones	> MRL	3	1		2		3
BL + Sulfa	< MRL	2			2	1	1
	> MRL	2			2	2	
	Sum	4					
Tetra + Sulfa	< MRL	1			1		1
	> MRL	3			3		3
	Sum	4					
Tetra + BL	< MRL	1			1	1	
Macrolides	< MRL	3	1	1	1	1	2
	> MRL	3			3	1	2
	Sum	6					
Global	< MRL	31	6	7	18	19	13
	> MRL	33	6	1	26	7	25
Total		64	12	8	44	26	38

D: Doubtful.
MRL: Maximum Residue Limit.
Sum: sum of samples confirmed lower and higher than respective MRLs.

(Table 1). The expert laboratory did not obtain any false negative result because the L3 concentration was designed to give positive results, whereas the L2 concentration was at the limit of sensitivity of the test.

It should be noted that later analyses were carried out assuming doubtful results as positive results, as applied at the time for routine analyses.

The results of the expert laboratory were very satisfactory (Table 8): 14 blank samples of 16 were negative (only two positive results for chicken samples) (L0). Below the assumed limit of detection, 14 samples of 16 were negative (L1). At the assumed limit of detection, all samples were positive (L2). Above the assumed limit of detection, all samples were positive (L3).

Results of the participants

Before the study, the expert laboratory stated that the results of one laboratory would be ignored if the negative control was detected positive, the positive control was detected negative, if samples were in bad condition at reception, or if a storage problem was established. Two laboratories were eliminated from analysis of the results: Laboratory L for several reasons: parcel delivery was delayed, samples were defrosted and chicken negative controls were detected positive for the two series of analyses; Laboratory H because chicken negative controls were detected positive for the 1st series of analyses. The raw data of the participants for the 16 combinations are presented in Table 9. Each participant received 32 samples (16 materials in blind duplicate) that they analysed in two different series.

The results were homogeneous between laboratories, as analysed and summarised in Table 10. Concerning negative samples (L0) (16 by laboratories corresponding to three different species), four laboratories reported 100% negative results, five laboratories found one or two samples doubtful or positive, sometimes in only one series of analyses (laboratories M and N).

For the samples containing antibiotics at a concentration below the detection limit (L1), six laboratories found 100% negative results; three laboratories found one or two samples doubtful or positive. When the concentration was considered near the detection limits (L2), tylosin was generally detected (83% of positive results), whereas the other antibiotics were less detected (oxytetracycline 11%; ceftiofur 11%; sulfamethazine 17%).

The average rate of positive samples for the L3 concentration was 81%. The rate of detection at the L3 concentration was 100% for four antibiotics and for

Table 8. Number of positive results from the expert laboratory (AFSSA) during interlaboratory study.

Level of contamination	Oxytetracycline Porcine	Sulfamethazine Porcine	Ceftiofur Bovine	Tylosin Chicken
L0	0	0	0	2
L1	0	0	0	2
L2	4	4	4	4
L3	4	4	4	4

three laboratories (B, D, N). This rate was 100% for the totality of the laboratories concerning tylosin and ceftiofur.

The results of the participating laboratories were analyzed to calculate different validation parameters. A laboratory was removed from the analysis due to a delay in transport and samples arriving in defrosted conditions.

Table 10 also presents analysis of the results of all the participating laboratories in term of reproducibility, by material (combination animal/antibiotic species) and globally. The reproducibility, expressed as a percentage, is the ratio of the number of identical results, the most common type (e.g. negative results for blank samples or positive result for samples contaminated with an antimicrobial concentration exceeding the detection limit), to the total number analysed.

The results of the participating laboratories in term of reproducibility are very satisfactory, with an average of 89.1%. The worse reproducibility was observed for the bovine/sulfamethazine combination. A slightly higher concentration of sulfamethazine would undoubtedly have given better results in term of reproducibility.

The percentage specificity (SP) for levels L0 and L1 was calculated as: $SP = [1\ (FP/N-) \times 100\%]$, where N−: is total number of tests L0 and L1, and FP is number of false positive results. The percentage sensitivity (SE) for each positive contamination level L2 and L3 was calculated using the following equation: $SP = (TP/N+)) \times 100\%$, where: N+ is total number of tests L2 or L3, respectively, and TP is number of true positives.

Specificity (953%) and sensitivity (72.5%) of the Premi®Test to the L3 level were very satisfactory.

Repeatability was estimated for each laboratory by (1) comparing the results of the two tests performed on each sample (two different sets of analyses), where knowledge of the initial result can influence the reading at the second analysis, and (2) comparing the results obtained with the two samples of each pair. Repeatability, expressed as a percentage, is the ratio of the number of identical results per couple of analyses on the total number of couples. Table 11 presents total analysis of the results of all the participants obtained with the Premi®Test in term of repeatability.

The results of the participating laboratories in term of repeatability are very satisfactory with an average of 94.8% for the same sample and 92.7% per two identical samples (pair). The limits of detection of the Premi®Test during the preliminary study were confirmed by the collaborative study.

Conclusions

This paper presents a significant dataset on the performance of the Premi®Test for different samples: spiked meat juice samples, incurred samples and routine field samples (confirmed by a LC/MS–MS method). The detection capabilities of Premi®Test for beta-lactams (amoxicillin, ceftiofur), one macrolide (tylosin) and tetracycline were at the respective maximum residue limits (MRL) in muscle samples or even lower. Applicability of the test to different animal species was proven. Moreover, applicability of Premi®Test to routine analysis of samples was demonstrated. The FPT (reference method) and the Premi®Test showed comparable performances in term of sensitivity and specificity. The false negative rate of Premi®Test was always lower than that of the FPT. In a screening method for antibiotic residues, this is the most important parameter to minimise. On the other hand, the false positive rate of Premi®Test was, in step 3, higher than that of the FPT. This means that the number of samples requiring confirmation by physico-chemical methods would be higher if the laboratories used the Premi®Test alone. However, it also means that more actual positive samples would be detected because Premi®Test was more sensitive than FPT for some antimicrobials (beta-lactams and sulphonamides). Beta-lactams and some sulphonamides were satisfactorily detected at the MRL level by the Premi®Test.

To the best of our knowledge, this is the first time that an interlaboratory study, organised for the detection of antibiotics with Premi®Test, has been described. The results of the collaborative study were very satisfactory: nine laboratories were finally analyzed plus the expert laboratory. Specificity was

Table 9. Raw data of the participants for each material and 16 combinations.

	Porcine oxytetracycline															
	L0 (0)				L1 (20*)				L2 (200)				L3 (400)			
	Mat 1		Mat 5		Mat 2		Mat 6		Mat 3		Mat 7		Mat 4		Mat 8	
Lab	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2
A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	D
B	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+
C	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+
D	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+
E	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
F	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+
G	–	–	–	–	–	–	–	–	–	–	–	–	D	D	D	D
M	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
N	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+

	Porcine ceftiofur															
	L0 (0)				L1 (40)				L2 (400)				L3 (800)			
	Mat 9		Mat 13		Mat 10		Mat 14		Mat 11		Mat 15		Mat 12		Mat 16	
Lab	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2
A	–	–	–	–	–	–	–	–	–	D	–	–	+	D	+	+
B	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+
C	–	–	+	+	–	–	–	–	–	–	–	–	+	+	+	+
D	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+
E	–	–	–	–	–	–	–	–	–	–	–	–	D	D	+	+
F	D	D	D	D	–	–	D	D	–	D	+	+	+	+	+	+
G	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+
M	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+
N	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+

	Bovine sulfamethazine															
	L0 (0)				L1 (20)				L2 (200)				L3 (400)			
	Mat 17		Mat 21		Mat 18		Mat 22		Mat 19		Mat 23		Mat 20		Mat 24	
Lab	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2
A	–	–	–	–	–	–	–	–	–	–	–	–	D	–	–	–
B	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+
C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
D	–	–	–	–	–	–	–	–	–	D	–	D	+	+	+	+
E	–	+	–	+	–	+	–	+	–	+	–	+	–	+	–	+
F	–	–	–	–	–	–	–	–	–	–	+	+	–	–	D	D
G	–	–	–	–	–	–	–	–	–	–	–	–	–	–	D	D
M	–	D	–	–	–	–	–	D	–	–	–	–	–	–	–	–
N	+	–	–	–	–	–	–	–	–	–	–	–	D	+	D	+

	Chicken tylosin															
	L0 (0)				L1 (10)				L2 (100)				L3 (200)			
	Mat 25		Mat 29		Mat 26		Mat 30		Mat 27		Mat 31		Mat 28		Mat 32	
Lab	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2
A	–	–	–	–	–	–	–	–	+	D	–	D	D	+	D	+
B	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+
C	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+
D	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+
E	–	–	–	–	–	–	–	–	–	–	–	D	+	+	+	+
F	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+
G	–	–	–	–	–	–	–	–	+	+	–	D	+	+	+	+
M	–	–	–	–	–	–	–	–	D	–	D	D	+	+	+	+
N	–	–	–	–	–	–	–	–	D	+	+	+	+	+	+	+

A1: first analysis; A2: second analysis; Mat: material; L0, etc.: concentrations (ng ml^{-1}).

Table 10. Results of all participating laboratories in term of% positive results for each material and in term of reproducibility by material (combination animal/antibiotic species) and global reproducibility.

Material	Number of sample (number of the pair)	Level of contamination	False positive and true positive rates	Positive results for each material (%)	Reproducibility (%)
Porcine OTC	1	**L0**	FP0 a)*	0	100.0
	2	**L1**	FP1 b)	0	100.0
	3	**L2**	TP2 c)	11**	88.9***
	4	**L3**	**TP3 d)**	**69**	69.4
Porcine ceftiofur	5	**L0**	FP0 a)	11	83.3
	6	**L1**	FP1 b)	8	94.4
	7	**L2**	TP2 c)	17	88.9
	8	**L3**	**TP3 d)**	**53**	100.0
Bovine sulfamethazine	9	**L0**	FP0 a)	17	91.7
	10	**L1**	FP1 b)	6	91.7
	11	**L2**	TP2 c)	11	83.3
	12	**L3**	**TP3 d)**	**100**	52.8
Chicken Tylosin	13	**L0**	FP0 a)	0	100.0
	14	**L1**	FP1 b)	0	100.0
	15	**L2**	TP2 c)	83	83.3
	16	**L3**	**TP3 d)**	**100**	100.0
	Total				**89.1**

FP: false positive rate.
TP: true positive rate.
a) False positive at level L0.
b) False positive at level L1.
c) True positive at level L2.
d) True positive at level L3.
*At each level of contamination, nine laboratories reported four results for two materials in blind duplicates. The total number of samples per level is $9 \times 4 = 36$ samples.
**TP2 c) = 4 TP/36 samples at level L2 $\times 100 = 11\%$.
***Reproducibility at L2 = 32 negative results divided by 36 samples $\times 100 = 88.9\%$.

Table 11. Global analysis of the results of all participants in term of repeatability with the Premi®Test.

Lab	(Number of identical results for the same sample/N) $\times 100\%$	(Number of identical results for 2 identical samples (pair)/N) $\times 100\%$
A	87.5	87.5
B	100.0	100.0
C	100.0	93.8
D	93.8	100.0
E	71.9	96.9
F	96.9	78.1
G	96.9	90.6
M	90.6	90.6
N	96.9	96.9
Total	94.8	92.7

N: total number of samples (32).

estimated at 95.3% and sensitivity of the test to the L3 level was 72.5%. These results were similar to other validation results for kits for the detection of antibiotic residues in milk. The results, in term of repeatability and reproducibility, are very satisfactory, with averages of 94.8 and 92.7% for repeatability and 89.1% for reproducibility.

In conclusion, Premi®Test is easy to perform. It is ideal for ''on site'' use (slaughterhouses, test laboratories), as no special laboratory equipment is needed to perform the test. The rapidly response "yes/no" result is simply read by colour comparison. Premi®Test is applicable to muscle tissue of various species (porcine, bovine, ovine), using a "blank'' muscle of each analyzed species as negative control to optimise reading time.

Finally, since 2006, field laboratories in France are authorised to use the Premi®Test as for pre-screening.

All positive samples with Premi®Test are then subjected to mandatory analysis by the FPT (Anon. 2006). All positive samples with the FPT are sent to our laboratory (CPL) for confirmation as usual.

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