Adaptation of a microbiological method to detect antibiotic residues in shrimp tissues from Vietnam

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Summary

The basic principle of the Belgian "Kidney Test" has been adapted and validated to detect antibiotic residues in shrimps. The aim of the present study was to adapt the "Kidney Test" to detect three groups of antibiotics (quinolones, tetracyclines and sulfonamids) widely used in shrimp production in Vietnam.

The method is a microbiological assay based on the use of Bacillus subtilis. The sensibility of this method was established by analysing 13 antibiotics standard solutions and blank shrimp samples spiked with antibiotics at different concentrations.

The results obtained with shrimps spiked with antibiotics indicate that the method can detect a wide range of compounds at or below MRL (Maximum Residue Limit).

Most of the quinolones and tetracyclines are detected at lower concentrations than the MRL level (enrofloxacine and flumequin at 0.5 x MRL, tetracycline, chlortetracycline, danofloxacine, difloxacine and ciprofloxacine at 0.75 x MRL) with the exception of two antibiotics (oxytetracycline and oxolinic acid) and sulfonamids, which were detected at MRL. The norfloxacine (with no fixed MRL by EU) is detected at 100 ng/g.

These preliminary results are promising and will be the basis for a future research. Shrimp contamination experiments will be realized to optimize and evaluate the method with incurred samples before to use it routinely in control laboratory in Vietnam.

Keywords: *antibiotic detection, residue, shrimp, microbiological inhibition test.*

Introduction

In Viet Nam, the sectors of fisheries and aquaculture is rapidly increasing and play an importance role in the economic growth, which is of 7-8% per year. Most of the production in this country is exported, generating large amounts of foreign exchange. The fisheries and aquaculture export values in 2007 were USD 3.7 billions in total, which corresponds to an increase of 9 % over 2006, with more than 40 % coming from shrimp product (FICEN, 2007). The total fisheries and aquaculture export value of period from 2001 to 2007 is more than USD 16 billions, with an average growth rate of more than 10% per year. However, Vietnam has been faced to difficulties such as trade competition, anti-drumping and overall, food safety requests of importers and local consumers. Therefore, this problem has been discussed many times in recent regular meetings of the Vietnam National Assembly.

Recent decades have seen significant progress in the development of quantitative confirmatory methods for the detection of antimicrobial residues, but for the reason of their complication this orientation would lead to drastic reduction of the number of analyses. Therefore, microbiological methods retain a vital role in antimicrobial residue analytics because of their broad-spectrum characteristics, with make them the most suitable (and so far, the only feasible) option for a screening step. Furthermore, they are simple and inexpensive to perform.

Microbiological inhibition screening test are widely used and play an important role to detect residues of antibiotic of many countries in the World (Ferrini et al. 1997; Myllyniemi et al. 2000; Popelka et al. 2005). Many microbiological tests are investigated, developed and adapted for the detection of antimicrobial residues in the different animal food products (Cooper et al. 1998) such as Four Plate Test (FPT) (Bogaerts & Wolf 1980), Belgian Kidney

Test (BKT) or One Plate Test (Koenen-Dierick et al. 1995), Three Plate (Okerman et al. 2001) and New Dutch Kidney Test (NDKT) (Nouws et al. 1988).

Presently, the Belgian Kidney Test is used to determine the presence of antibacterial substances in the residue official control programme of Belgium. This "pre-screening" microbiological test is applied on kidneys of slaughtered animals (Anonymous, 1995). Advantages of this method are that the test is simple, easy-to-use, inexpensive and allows a broad-spectrum antimicrobial screening. In practice, many microbiological methods are developed, validated and adapted to detect antimicrobial residues in different matrices of different animal products, but there are very few available for the aquaculture products in general, and for shrimps in particular.

The aim of the present study was to adapt the "Kidney Test" to detect three groups of antibiotics (quinolones, tetracyclines and sulfamides) widely used in shrimp production in Vietnam, and to validate this test according to criteria of European Commission.

Materials and methods

- All of antibiotic standards used in this study were provided by Sigma-Aldrich (St Louis, MO, USA), except danofloxacin which was from Pfizer (Groton, CT, USA).

Stock solutions (1mg/ml) were prepared in methanol, except for three sulfonamids (sulfamethoxazole, sulfadiazin, sulfadimethoxin) and 7 (fluoro)quinolones (oxolinic acid, danofloxacin, enrofloxacin, difloxacin, flumequin, ciprofloxacin, norfloxacin), which were dissolved in a small volume of HCl 2N for sulfonamids and in NH_4OH 2M for quinolones before methanol addition.

*Standard working solution*s: Standard working solutions were prepared by diluting stock solutions in purified sterile water.

- *Culture media:* Culture media used were Standard II Nutrient Agar for microbiology (Merck 1.07883, Darmstadt, Germany). Media were prepared as recommended by the Ministry of public health and environment of Belgium (Anonymous, 1995) with two modifications (0,6% dextrose and 0.4 µg TMP/ml culture).

- *Bacterial strain: Bacillus subtilis* strain BGA spore suspension was commercially available in standardized concentration of 10^7 spores/ml (Merck 1.10649, Darmstadt, Germany).

- *"Blank" samples* were shrimp samples confirmated to not contain antibiotic by LC/MSMS method and were provided by CART (Centre d'Analyse des Résidus en Traces) of the University of Liège, Belgium

- PABA (para-aminobenzoïc acid) and trimethoprime (TMP) were provided by Sigma-Aldrich (Steinheim, Germany)

- *Sample extraction*: the extraction method was adapted from the Premi-Test as described for other matrices (Stead et al. 2004). Three grams of shrimp homogenate was extracted in Acetonitrile/Acetone (70/30 v/v) under rotative shaking during 10 minutes. The mixture was then centrifuged at 3000 rpm for 10 minutes at 15°C. The supernatant was transferred into a clean conical tube and evaporated to dryness under N_2 at 40 °C. The dry residue was dissolved in 200 µl methanol.

- Microbiological test: The extract was centrifuged again and the 50 µL of the supernatant was applied on paper disc on the seeded agar plates with *Bacillus subtilis*. Plates were then incubated for 24h at 30°C, before to measure inhibitions zones.

Result interpretation: according to other microbiological test (FPT, NDKT), we considered a results as positive or suspect if the diameter of the inhibition zone (including the paper disc) was equal or higher than 16 mm, or if the size of the inhibition zone around the paper disc was equal or higher than 2 mm.

Method optimization: Standard quality control were performed with 50 µl of each of the 13 standard solutions at a concentration of 20 µg/ml (=1 µg of antibiotic per disc).

Then MIQ (Minimum Inhibitory Quantity) is the minimum quantity of antibiotic able to produce an inhibition zone which is equal or bigger than 2 mm. The MIQ was determined by using 13 standard solutions in the range of concentration from 625 to 3500 ng/ml. On the basis of the MIQ and the MRL (maximum residue limit) of each antibiotic, the minimal shrimp quantity (MSQ) to be used for the analysis was determined using the following formula:

$$\mathbf{MSQ\ (g) = MIQ\ (ng) * MRL^{-1}}\ (g/ng)$$

Then LODs (Limit of detection) for each antibiotic was determined by analysing 20 spiked samples.

The method was validated following the "Guide for analytical validation of screening methods" written by the CRL (Community Reference Laboratory), AFSSA, Fougères, France. The accuracy, the sensitivity and the selectivity was determined by analysing 20 "blank" samples and 20 spiked samples.

Identification of the family of antibiotics: the sulfonamid group was identified by adding, together with the sample extract, 10 µl of PABA solution (100µg/ml) on the paper disc.

3. Results and discussion

3.1. Standard Quality Controls

To evaluate the plate quality and the sensitivity of *B. subtilis* to tested antibiotics, 50 µl of each of the 13 standard solutions at the concentration of 20 µg/ml were analyzed on 12 mm diameter paper discs.

Each antibiotic was tested with 12 repetitions (3 repetitions in 4 independent series of disk preparation) on 4 different days (4 independent preparations of medium).

All the 13 antibiotics of the 3 tested groups were able to induce an inhibition zone. The mean diameters of inhibition zones of the majority of tested antibiotics were ≥ 28mm with coefficient of variation (CV) of 3 – 6% (Table 1).

Table 1. Diameters of inhibition zones generated by antibiotic standards (1 µg op standard/disc)

Group	Antibiotic	Diameters of inhibition zones (n=12) (mm)				
		$\overline{X_a} \pm s_a$	Max	Min	CV (%)	$\overline{X_g} \pm s_g$
Tetracyclins	Tetracycline	29 ± 1	30	26	5	30 ± 2
	Oxytetracycline	28 ± 1	31	26	5	
	Chlortetracycline	32 ± 2	35	27	6	
Sulfonamids	Sulfadiazine	23 ± 1	25	22	5	24 ± 1
	Sulfadimethoxine	24 ± 1	26	22	5	
	Sulfamethoxazole	25 ± 1	27	23	4	
Quinolones	Oxolinic Acid	33 ± 2	36	31	5	33 ± 3
	Danofloxacine	35 ± 2	38	33	4	
	Difloxacine	34 ± 1	37	32	4	
	Ciprofloxacine	33 ± 2	36	30	5	
	Norfloxacine	28 ± 1	30	26	5	
	Flumequine	32 ± 2	35	29	5	
	Enrofloxacine	36 ± 1	38	34	3	

$\overline{X_a}$, s_a = mean diameter of inhibition zone and standard deviation for each antibiotic

$\overline{X_g}$, s_g = mean diameter of inhibition zone and standard deviation for each antibiotic group

The mean of the inhibition zones induced by sulfonamids was lower than 26 mm (Sulfadiazine: 23 ± 1mm, CV=5 %; Sulfadimethoxine: 24 ± 1mm, CV=5 %; Sulfamethoxazole: 25 ± 1mm, CV= 4 %).

The means of inhibition zones obtained for quinolones were higher than 32 mm, except for Norfloxacine (28 ± 1 mm and CV = 5 %). Among them, Enrofloxacine induced the est inhibition zone (36 ± 1 mm) with a CV = 3 %. For Danofloxacine, Difloxacine, Ciprofloxacine, Oxolinic Acid and Flumequine, the diameters of the inhibition zones were respectively of 35 ± 2 mm, 34 ± 1 mm ; 33 ± 2 mm ; 33 ± 2 mm and 32 ± 2 mm.

The means of inhibition zones generated by Tetracycline was 29 ± 1 mm, by Oxytetracycline was 28 ± 1 mm and by Chlotetracycline was 32 ± 2 mm, with CV of 5 and 6% respectively.

The results in Table 1 also showed that the means of inhibition zones for the 3 tested antibiotic groups were statistically different with $p < 0.05$ (24 ± 1 mm for sulfonamids, 30 ± 2 mm for tetracyclines and 33 ± 3 mm for quinolones).

These results showed that the *Bacillus subtilis* strain chosen and the gelose composition were fully adapted to the detection of the 3 antibiotic groups of interest.

In the aim to improve the sensitivity of the test to tetracyclines and sulfonamids, we increased the dextrose and TMP concentrations respectively from 0.4 to 0.6 % and from 0.2 to 0.4 %. The diameters of the inhibition zones of all the tested antibiotics in control tests (1µg /disk) were equal or higher than 23 mm, the lower sensitivity was for Sulfadiazine (diameter mean of inhibition zones was 23 mm, varying between 22 and 25 mm). These results are accepted for the Four-Plate Test (Bogaerts & Wolf 1980).

According to the recommendations for BKT, the diameters of inhibition zones in control tests (1µg /disk) have to be at least 17 mm for sulfamidine and 18 mm for Oxytetracycline (Anonymous, 1995). So, we can consider that the sensitivity of our method is adapted to detect the 3 antibiotic groups tested.

3.2. Test evaluation with standard antibiotic solutions

3.2.1. Determination of the minimal inhibitory quantity (MIQ)

the MIQ is very necessary for establishing and adjusting the sample extraction procedure. Based on measured MIQ and on MRL of each antibiotic (fixed by regulation 2377/90/CEE), it is possible to determine the minimal shrimp quantity to be used for the analysis.

Thirteen standard solutions, of concentrations varying from 625 to 3.500 ng/ml, were used for the evaluation of the MIQ. Fifty µl of standard solution was dripped on the paper disks laid on the Petri plate. Each standard solution at each concentration was tested in 8 repetitions with 4 series of Petri plate, on 4 different days.

As expected, the width of the inhibition zones around the disks loaded with 50 µl of standard solution varied in function of concentrations. The dose-response curves of the 3 tested antibiotic groups were different. At the same concentration, quinolones could create the larger inhibition zone, followed by tetracyclins, and then, sulfonamids (Figure 1). Concretely, Danofloxacine and Enrofloxacine produced an inhibition zone higher than 2 mm at concentrations between 37.50 and 50 ng/disk, while the other 5 quinolones as well as the 3 tetracyclines produced the same inhibition zones at 50 – 62.5 ng/disk. Meanwhile, sulfonamids induced inhibition zones ≥ 2 mm on the disk only with the minimal concentration of 75 ng of antibiotic per disc.

Thus, the *B. subtilis* strain in the above-mentioned adjusted medium was the most sensie to Danofloxacine and Enrofloxacine (MIQ varied from 37.5 to 50 ng/disk), followed by the other quinolones and the 3 tetracyclines (MIQ varied from 50 to 62.5 ng/disk). The lower sensitivity was for tested sulfonamids with MIQ varying from 62.5 to 75 ng/disk (Figure 1). These results were in full concordance with those obtained by Currie and co-workers (Currie et al, 1998) in their study on the evaluation of the FPT method.

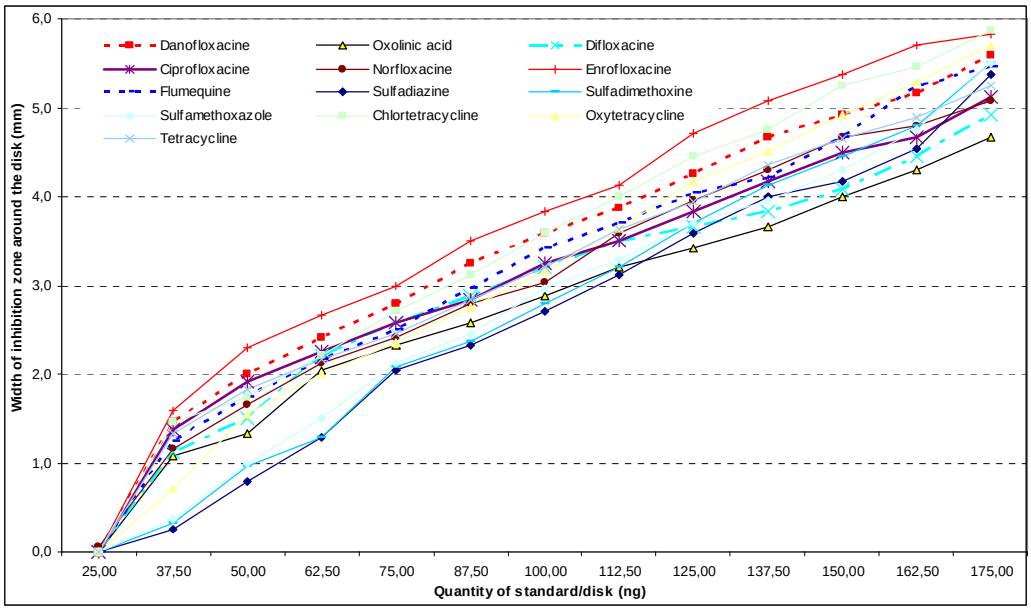

Figure 1. Width of inhibition zones around the disk depending on quinolone, sulfonamid and tetracycline quantity in 50 µl of standard solution (or per disc)

3.2.2. Determination of the minimal quantity of shrimps to be sampled for an extraction

In order to detect antibiotics in samples, an extraction procedure is applied. Based on the extraction procedure, the maximum residue limit (MRL) fixed by EU and the MIQ (ng in 50 µl/disk), we calculated the minimal shrimp quantity (MSQ) to be taken for an extraction using the formula mentioned in the material and methods section.

Table 2. Determining minimal shrimp quantity to be sampled for an extraction

Antibiotics	**MRLs**(*) (ng/g)	**MIQ** (ng/disk)	***Minimal shrimp quantity to be sampled for extraction*** (in 50µl of the final extraction solution) (g)	***Minimal shrimp quantity to be sampled for an extraction*** (***) (g)
Tetracycline	100	50 - 62,5	0,6250	2,0 - 2,5
Oxytetracycline	100	62,5	0,6250	2,5
Chlortetracycline	100	50 - 62,5	0,6250	2,0 - 2,5
Sulfadiazine	100	75,0	0,7500	3,0
Sulfadimethoxine	100	62,5 - 75,0	0,7500	2,5 - 3,0
Sulfamethoxazole	100	62,5 - 75,0	0,7500	2,5 - 3,0
Oxolinic acid	100	62,5	0,6250	2,5
Danofloxacine	100	37,5 - 50,0	0,5000	1,5 - 2,0
Difloxacine	100	50 - 62,5	0,6250	2,0 - 2,5
Ciprofloxacine	100	50 - 62,5	0,6250	2,0 - 2,5
Norfloxacine	**	62,5	0,6250	2,5
Flumequine	200	62,5	0,3125	1,25
Enrofloxacine	100	37,5 - 50,0	0,5000	1,5 - 2,0

*(*): MRLs fixed by regulation 2377/90/CEE (CE, 1990) **: without fixed MRL*
(***): if dry residue after the last evaporation step is dissolved in 200 µl of methanol

If the dry residue after the evaporation step following the extraction is recovered in 200 µl of methanol, and if 50 µl are applied on a disk, the minimal shrimp quantity for an extraction will be 4 times the MSQ.

The results in table 2 show that it is necessary to sample at least 3 grams of shrimp tissue for an extraction to detect all antibiotic groups tested at a concentration equal or lower MRL.

3.3. Validation

3.3.1. Detection threshold

Validation of residue screening methods is most often done using samples spiked with the analyte at the required concentration, because it is impossible to produce incurred samples from different animal species with a specified concentration of residue. Nevertheless, this poses a problem when intact meat has to be analysed, as it is prescribed for the FPT (Heitzman 1994). To avoid the difficulty of producing spiked undiluted samples, the meat fluid spiked was used in some investigates. For practical reasons, it was supposed that the antibacterial substance concentration in the fluid was approximately equal to the antibacterial substance in the whole tissue (Okerman et al. 2004).

According to the decision of the European Commission 2002/657/CE, the detection capability can be investigated with fortified blank material at the decision limit (MRL). The aim is to find the concentration level where false compliant results are less than 5% (maximum 1 false compliant out of 20 fortified samples). Therefore, at least 20 investigations for at least one concentration level have to be carried out in order to ensure a reliable basis for this determination.

This method was validated by using ground and fortified blank samples with standard antibiotics of the 3 tested groups. After fortification, the samples were kept for one night at 4^0C before extraction.

Table 3. Detection threshold of the method

Groups	Standards	MRLs(*) (ng/g)	First Assay		Second Assay		LOD (ng/g)
			Tested concentration (ng/g)	Number of positive samples (inhibition zone ≥ 2 mm)	Tested concentration (ng/g)	Number of positive samples (inhibition zone ≥ 2 mm)	
Tetracyclin	Tetracycline	100	75	19/20	100	20/20	**75**
	Oxytetracycline	100	75	1/20	100	19/20	**100**
	Chlortetracycline	100	75	20/20	100	20/20	**75**
Sulfonamid	Sulfadiazine	100	100	1/20	125	19/20	**125**
	Sulfadimethoxine	100	100	2/20	125	19/20	**125**
	Sulfamethoxazole	100	100	0/20	125	19/20	**125**
Quinolone	Oxolinic acid	100	75	0/20	100	19/20	**100**
	Danofloxacine	100	50	0/20	75	20/20	**75**
	Difloxacine	100	75	20/20	100	20/20	**75**
	Ciprofloxacine	100	75	20/20	100	20/20	**75**
	Norfloxacine	(**)	75	0/20	100	19/20	**100**
	Flumequine	200	100	19/20	150	20/20	**100**
	Enrofloxacine	100	50	20/20	75	20/20	**50**

*(*) : MRLs fixed by regulation 2377/90/EC ** : without fixed MRL*

The LOD of the method for each antibiotic was identified by analyzing 20 fortified samples at interesting concentrations. The results showed that the method is capable of detecting antibiotics tested at concentrations very close to MRLs. All the tested antibiotics were detected at concentrations ≤ MRL , except for sulfonamids, which were detected at

levels equal to 1.25 x MRL. Two antibiotics were detected at 0.75 x MRL: Enrofloxacine and Flumequine. Oxytetracycline and Oxolinic acid were detected at levels just equal to MRL. Norfloxacine which hasn't got MRL was detected at 100 µg/kg.

The detection threshold is the fortified concentration, at which 5% or less of the samples aren't detected. In this study, among 20 samples analyzed at a fortified concentration, a unique sample had negative results. The percentage of 5% was chosen from critical concentration beta (CC_β or *Detection capability*) described in the Decision of the European Commision N°2002/657/EC (European Commission, 2002). The beta error (rate of « false positive ») has to be 5% or less for those compounds having a MRL, and 1% or less for those compounds fully prohibited. Based on MIQ of each antibiotic and its MRL, we have fortified the blank samples at concentrations calculated to take into account of loss of analytes during the extraction.

For each antibiotic, we analyzed 20 fortified samples at different concentrations. For quinolones and tetracyclines, blank samples fortified at 2 different concentrations were analyzed (MRL and 0.75 x MRL, for Norfloxacine which haven't got MRL, at 75 and 100 ng/g). Chlotetracycline, Enrofloxacine and Danofloxacine were tested at 0.5 x MRL and 0.75 x MRL. For sulfonamids, the samples were fortified at higher concentrations (MRL and 1.25 x MRL)

The results indicated in Table 3 indicated that among the 13 antibiotics of the 3 tested groups, 7 antibiotics were detected at the concentrations lower than MRL, concretely:

- Enrofloxacine and Flumequine at 0.5 x MRL
- Tetracycline, Chlotetracycline, Danofloxacine, Difloxacine and Ciprofloxacine at 0.75xMRL.

Two antibiotics were detected at their MRLs: Oxytetracycline and Oxolinic acid (Norfloxacine was detected at 100 ng/g)

At last, 3 antibiotics of the sulfonamid group were detected at a threshold slightly higher than MRL (125 ng/g or 1.25 x MRL).

3.3.2. Sensitivity, Specificity and Accuracy

According to the recommendations of CRL, in order to reduce costs and labour of the evaluation of parameters related to the method performance, it is possible to choose some representative antibiotics of the groups (Gaudin & Sanders 2005). The chosen representative antibiotics are those which have similar antibacterial activities and are the most frequently used in the shrimp production.

For each group, 2 representative standard antibiotics were chosen. For each representative antibiotic, we analyzed 20 "Blank" samples (considered as « True Negatives ») and 20 fortified "bank" samples at the concentration at the detection threshold (« True Positives »). Obtained data are calculated and presented in Table 4.

Table 4. Performance parameters of the method for the 3 tested antibiotic groups

Group	**Representative antimicrobial**	**MRLs(*)** (ng/g)	**Test concentration**		**Performance characteristics**	
			(ng/g)	(MRL)	Accuracy (%)	Sensitivity (%)
Tetracyclin	Tetracycline	100	75	0,75	97,5	95
	Chlotetracycline	100	75	0,75	100	100
Sulfamid	Sulfadiazine	100	125	1,25	97,5	95
	Sulfadimethoxine	100	125	1,25	97,5	95
	Sulfadiazine**	100	100	1,00	97,5	95
	Sulfadimethoxine**	100	100	1,00	97,5	95
Quinolone	Enrofloxacin	100	50	0,5	100	100
	Flumequin	200	100	0,5	97,5	95

*(*) : MRLs fixed by regulation 2377/90/EC ** : loading of 63 µl of extraction solution/disk*

The results in Table 4 show that the method was able to detect the 3 antibiotic groups tested at a concentration very close to their MRL with acceptable accuracy and sensitivity. The accuracy and sensitivity of the method were 100% for Chlotetracycline at 0.75 x MRL and for Enrofloxacine at 0.5 x MRL. As for other antibiotics, the method can detect antibiotics at 0.75 x MRL (for Tetracycline), at 0.5 x MRL (for Flumequine) and at 1.25 x MRL (for 2 sulfonamids) with an accuracy and a sensitivity of 97.5% and 95% respectively. The specificity of the method (assessed by the percentage of really negative samples after the screening) is 100%.

The accuracy and the sensitivity of the method for the 2 sulfonamids fortified at MRL when loading 63 µl of sample extract on a disk, was the same at the fortified concentration of 1.25 x MRL than when loading 50 µl/disk.

For tetracyclines and quinolones, the accuracy and the sensitivity of the method are 100% when analyzing fortified samples at MRL.

According to European Commission 2002/657/EC, reports of residue tests should not mention positive and negative results, but the terms of «non-compliant» and «compliant» should be used. A screening test result can be either compliant or suspect. However, the result can only be considered as compliant when the detection capability of the screening test is below the MRL for a given analyte. The actual multiresidue test relying on inhibiting characteristics of antibiotics do not detect all antibiotics at MRL levels, and as long as the test is not validated for a given antibiotic or group of antibiotics, it is not known if the result is compliant or not. For example, a negative Premi® Test result does not allow to decide that the sample is compliant for tetracyclines, and a negative FPT result does not mean that the sample is compliant for sulfonamid (Korsrud et al., 1998), although both tests are intended as general screening tests for antibiotics. Indeed, they do not detect samples contaminated with the respective analytes at MRL levels. Therefore, as the terms “suspect”, “compliant” and “not compliant” are to be considered as juridical rather than scientific.

Therefore, through the analyses of “blank” samples and fortified samples with 6 representative antibiotics of the 3 groups, the accuracy and the sensitivity of the method are established. The method ensures the detection capability of these 3 groups with acceptable accuracy and sensitivity. The accuracy of the method is higher or equal to 95% for all the 6 representative antibiotics at concentrations equal to LOD. These results are satisfying and meet minimal demands of Decision N^0 2002/657/EC.

3.4. Results of identification tests of antibiotics

An ideal antimicrobial multiresidue method would detect and identify all licensed antimicrobials at or below their MRLs.

A multi-residue analysis method will be ideal if it is able of detecting and identifying all chemicals at or below their MRLs. These methods have been developed and adapted in order to detect residues of several antibiotic groups in different matrices. Typically, the FPT method, the Premi® Test and other microorganism tests were developed, based on combining many plates, many pH levels, different media and strain of microorganisms sensitive to different antibiotic groups (Calderon et al. 1996). These methods were used to screen positive samples before formatting and confirmatory analyses by means of other accurate methods..

The mechanism of action of sulfonamids is the inhibition of the synthesis of the dihydrofolic acid in the biosynthesis of folic acid in prokaryote cells (Rang & Dale 1994). During the synthesis of folic acid, there is a competition between sulfonamids and PABA. By adding an excess of PABA, sufonamids can not compete any more, and they loss their inhibitory properties. By using this technique (addition of PABA), we have successfully identified shrimp samples fortified with sulfonamids. This technique was also successfully applied in other tests such as Premi® Test (Stead et al. 2004), CPMA (Combined Plates Microbial Assay) (Ferrini et al. 1997).

Based on obtained results, in order to detect sulfonamids at their MRL, it is necessary to load 63 µl of sample extract after extraction.

The strategy to detect the 3 antibiotic groups of interest at least their MRL is the following : 3 paper disks numbered 1, 2 and 3, were laid on each Petri box, we load 50 µl of sample extract on disk 1, sixty three µl on disks 2 and 3. The third disk is for the detection of sulfonamids and is added with 10 µl PABA (100µg/ml).

Table 5. Results of antibiotic group identification

Samples	**Number of positive samples** (Width of inhibition zone >= 2 mm)		
	Disc N°1 (50 µl of solution after extraction)	**Disc N°2** (63 µl of solution after extraction)	**Disc N°3** (63 µl of solution after extraction + 10 µl of PABA)
« Blank sample » (n = 5)	0/5	0/5	0/5
Spiked samples with Sulfadiazine (125 ppb) (n = 5)	5/5	5/5	0/5
Spiked samples with Sulfadiazine (100 ppb) (n = 5)	0/5	5/5	0/5
Spiked samples with Sulfadiazine (50 ppb) (n = 5)	0/5	0/5	0/5
Spiked samples with Enrofloxacine (50 ppb) (n = 5)	5/5	5/5	5/5
Spiked samples with Enrofloxacine (25 ppb) (n = 5)	0/5	2/5	2/5
Spiked samples with Tetracycline (100 ppb) (n = 5)	5/5	5/5	5/5
Spiked samples with Tetracycline (50 ppb) (n = 5)	0/5	0/5	0/5

In order to confirm the inhibition capability of PABA on sulfonamids, we used standard antibiotic solutions. We have loaded 50 µl of the standard solution at a concentration of 20 µg/ml on the disk (1 µg/disk is equivalent to 13 times of QMI) in presence or absence of 10 µl of PABA (100 µg/ml). In case of absence of PABA, all tested sulfonamids were able to produce inhibition zones, on the contrary, in presence of PABA, no inhibition zones appeared.

Table 6. Interpretation of resulsta and identification of sulfonamids

Disc N°1 (50 µl of solution after extraction)	**Disc N°2** (63 µl of solution after extraction)	**Disc N°3** (63 µl of solution after extraction + 10 µl PABA)	**Identification**
-	-	-	Negative
-	+	-	Sulfonamid (100 to 125 ppb)
+	+	-	Sulfamid (>= 125 ppb)
+	+	+	Quinolone or tetracycline or antibiotic of other groups (> = LOD)
-	+	+	Quinolone or tetracycline or antibiotic of other groups (< LOD)

We carried out a test to confirm the ability to identify the sulfonamid group by analyzing blank and fortified samples with different standard antibiotics as described in Table

6. This table indicates that the identification of sulfonamid groups is completely done by this method.

Concretely, if the inhibition zone width of all the 3 disks is ≥ 2 mm, it is possible to conclude that these samples aren't contaminated with sulfonamids, (or they may be contaminated at concentration smaller than MRL), but well with antibiotics from the other groups. In this case, it is possible to use specific methods in order to identify antibiotic groups, for example, Tetra-sensor for tetracycline and ELISA for quinolone before reconfirmation by other accurate physico-chemical methods.

For other cases, Table 6 (in which the sign « + » corresponds to an inhibition zone ≥ 2 mm, and the signs « - » is the contrary) indicate how to interprete the results.

CONCLUSION

Owing to 2 modifications of medium compositions in comparison with the Belgian Kidney Test (dextrose 6%, TMP 0.4%) and an extraction procedure by the mixture of acetonitrile/acetone (70 :30 v/v), we succeeded to improve the sensitivity of the microbiological method described here. The initial results of our study showed that the method was able to detect the 3 antibiotic groups at concentrations very close to their MRL, with an accuracy and a sensitivity which are satisfying and meet demands of the Decision of the European Commission N^0 2002/657/CE.

The identification of sulfonamids, in a post-screening step, was also successfully tested.

These preliminary results are promising and will be the basis for a future research. Shrimp contamination experiments will be realized to optimize and evaluate the method with incurred samples before to use it routinely in control laboratory in Vietnam.

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