Primary validation of Charm II tests for the detection of antimicrobial residues in a range of aquaculture fish

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Abstract

The study carried out a primary validation of Charm II tests for the detection of antimicrobial residues in aquaculture fish. The Validation was performed according to European Commission Decision 2002/657/*EC and the* parameters determined included: detection capability, repeatability, reproducibility, specificity and robustness for the detection of antimicrobial residues in fish. Fish materials from different species including cat fish, trout, salmon, sea bass, tilapia, lingue and pangasius, were spiked with varying concentrations of selected antibiotics including sulfonamides, beta-lactams, macrolides, tetracyclines and aminoglycosides to determine the detection capabilities and other validation parameters of the Charm II tests.

Results of the validation showed that the detection capabilities for the tetracyclines ranged from 25 µg/kg to 100 µg/kg, while the sulfonamides and aminoglycosides were detected at 25 µg/kg for all species under study. The detection capabilities for the beta-lactams ranged from 25 µg/kg to 300 µg/kg; and was 100 µg/kg for the tested macrolides. Results of the study showed that there was no significant difference between counts for samples read immediately after addition of the scintillation fluid and those read fourteen hours after addition of the scintillation fluid, provided that there was good vortexing before analysis. There was also no significant difference between counts for the same samples analyzed in different runs under repeatability and reproducibility conditions at the same spiking concentrations for the different fish species analyzed. The relative standard deviation for both repeatability and reproducibility ranged from 1.2 to 15.1%.

The Charm II tests were found to be 100% group specific, as none of antimicrobials kits, gave false positive results when testing non-target antimicrobial drugs. Results of this study prove the specificity and precision of the Charm II assay in the detection of various antimicrobial residuals in fish. The results also demonstrate the suitability of the Charm II technique as a rapid screening tool for detection of antimicrobial residues in a variety of fish species at Maximum Residue Limits established in the EU guidelines, and its applicability for the rapid evaluation of the quality of aquaculture fish for safety and trade purposes.

Introduction

Fish farming is a fast emerging industry that besides creating employment, is a source of good quality animal protein and essential macronutrients in the diet. Fish and fish related products provide income and livelihoods for numerous communities across the world besides playing a crucial role in assuring sufficient availability of safe and healthy food [1, 2]. The increased demand for fish for the growing international population, especially in the developing world, has continued to deplete the sustainable yields from lakes, rivers, swamps, seas and other natural water bodies. Aquaculture is growing rapidly

and is seen as a remedy to address and supplement the dwindling quantities and shortfall in wild catch [3]. However, big numbers of fish in a confined volume of space tend to increase incidences of bacterial infections and other diseases; which greatly affects yield in the aquaculture business. Productivity in aquaculture may be enhanced by use of antimicrobials such as tetracyclines, macrolides, beta-lactams, sulfonamides, and streptomycins, for the prevention and treatment of opportunistic infections in fish [4, 5].

Antimicrobials are used to control ectoparasitic, fungal and bacterial diseases of the body and gills of fish [6, 7, 8]. Tetracyclines in particular are frequently employed in aquaculture due to their broad spectrum of activity as well as their low cost, compared to other antibiotics. The tetracyclines are used to combat bacterial *hemorrhagic septicemia* in catfish as well as diseases caused by pseudomonas liquifaciens [9]. Currently, there are over 20 tetracyclines available; although, tetracycline, chlortetracycline, oxytetracycline, and doxycycline are the most common ones in veterinary medicine and aquaculture [10, 11]. The aforementioned antibiotics are the only tetracyclines with registration within the European Union (EU) for use as veterinary medicinal products in food producing animals with established maximum residue limits (MRLs) in different food matrices [12]. Other antimicrobials such as sulfonamides, beta-lactams, macrolides and aminoglycosides also have a wide spectrum of activities against most Gram positive and Gram negative organisms and are used for the prevention and treatment of bacterial infections in livestock and aquaculture. The antibiotics are typically administered in the water, often as components of fish feed, and are occasionally injected [13, 14].

The extensive use and misuse of antimicrobials in farm animals as growth promoters or as nonspecific means of infection prevention has been reported to lead to accumulation of residues in edible tissue [7, 15, 16]; which may cause allergic and toxic effects in consumers as well as contributing to the development of antibiotic resistant bacteria [17 - 20]. In this respect, residues in foodstuffs create public health concerns, consumer perception problems and trade disputes that have enormous negative impacts on the food industry. In order to protect human health, regulatory authorities like the EU, established maximum residue limits (MRLs) for some pharmaceutical compounds in fish and other foodstuffs of animal origin [12]. The safety concerns regarding drug residues in various food products, calls for development and validation of rapid and reliable techniques for detection of these compounds. Such rapid techniques can facilitate fast decision making to minimize technical barriers for trade and also enhance routine monitoring in order to protect consumer health.

The Charm II radio receptor assay technique developed by Charm Sciences Inc, is one of the rapid screening techniques for detection of residues of antimicrobials such as beta-lactams, sulfonamides, tetracyclines, chloramphenicol, quinolones, macrolides and aminoglycosides in various food products including fish, meat, tissue, eggs, honey, and milk, as well as non-food matrices including water, feed and urine. This technique utilizes a microbial cell with receptor sites that bind the specific antimicrobial drug. The analytical process involves a binder being added to a sample extract along with an amount of ^{3}H or ^{14}C labeled antimicrobial tracer. Any antimicrobial in the sample extract competes for the binding sites with the tracer. The amount of tracer that binds to the receptor sites is measured and compared to a

previously determined control point. Therefore, the more radiolabelled antibiotic detected in the mixture, the lower the concentration of antibiotic in the sample. The smaller the amount of tracer measured, the greater the drug concentration in the sample [21, 22]. The Charm II technique has very limited validation data for the detection of antimicrobials in different fish species. Thus, this study conducted a primary validation of the Charm II tests in order to generate comprehensive analytical data to prove the validity, applicability and also address potential limitations of the Cham II assays particularly for the screening of antimicrobials in different aquaculture fish species.

Materials And Methods

3.1 Reagents, materials and equipment

The antimicrobial test assay kit was obtained from Charm Sciences Inc., Lawrence, MA; and included items for the detection of beta-lactams (PMSU-050A); sulfonamides (SMMSU-022C), macrolides (EMSU-023A); tetracyclines (TMSU-025); and streptomycins (STMSU-023A). Consumables and equipment used for the tests included: M2 Buffer, zero and positive control standards, MSU extraction buffer, and radioactive labelled tablets; scintillation fluid (Opti-Fluor O, PerkinElmer), Intronic incubator (Charm Sciences Inc), Wallac 1409 scintillator counter, refrigerated centrifuge Sigma 4K15c (Sigma-Aldrich), R2 blender (Robot-Coupe) and a water bath (Julabo MB13). In addition, scintillation vials, AES mix masticator stomacher and IEC Centra CL-3 centrifuge were also used.

3.2 Preparation of standard reference material and stock solutions

The multi antimicrobial concentrate standard (MSU, Charm Sciences Inc.) was prepared fresh on the day of use and diluted with 10 ml of deionized water, shaken well and allowed to stand on ice for 15 min. The reconstituted stock solution contained; penicillin G, 1000 µg/kg; erythromycin, 10,000 µg/kg; sulfamethazine,1000 µg/kg; chlortetracycline, 4,000 µg/kg; and streptomycin, 10,000µg/kg. Other analytical standards were purchased from Sigma Aldrich, Pfizer Inc. US Pharmacopeia Convention and Acros Organics (Supporting information, Table 1a). These standards were appropriately diluted with deionized water to make working standard solutions of the respective antimicrobial, and kept below 4°C. The working standards were used for spiking fish samples at different concentration levels ranging from 25 µg/kg to 300 µg/kg.

3.3 Methods

The study carried out a primary validation of the Charm II tests for the detection of antimicrobial residues in aquaculture fish. The Validation was performed according to Commission Decision 2002/657/*EC [23] and all* methods of analysis used were adopted from the general Charm II protocols [21].

3.4 Fish samples selected for the study

The fish materials used in the study were obtained from dead fish purchased from Melle and Ghensh shops and supermarkets in Belgium. Aquaculture fish species including cat fish (*Siluriformes)*, trout

(*Oncorhynchus mykiss)*, salmon, (*Salmo salar)*, seabass (*Dicentrarchus labrax)*, tilapia (*Oreochromis niloticus),* lingue (*Molva molva)*, dorade (*Sparus aurata)* and pangasius (*Pangasius bocourti)*, were selected for the study. Fish sample materials were taken by carefully removing the muscle tissue from the side of each fish taking precaution to exclude scales and skin. The fish samples that were not used immediately were stored below -18°C for a maximum of 2 months.

3.5 Sample preparation

The fresh fish sample was weighed in a centrifuge tube and stored at -18°C until further processing. The frozen fish samples were thawed at 4°C overnight and cut into small pieces before blending in a high speed blender. The blended fish material (10 g) was transferred into a polypropylene centrifuge tube and used immediately.

3.5.1 Preparation of control samples

All fish samples were first tested with the different Charm II kits and only used in case no veterinary drug residues was detected. Absence of residual antibiotics in the fish samples was confirmed though evaluation of their counts per minute in comparison with results obtained using the negative control extraction buffers supplied with the Charm II kits. The control buffers are contaminant free and are used to qualify the matrix as negative when a known negative is not available. The tolerance considered for the fish matrix to qualify as negative and selected for use in subsequent test was for counts within ± 20% of the average result obtained with the respective negative control extraction buffer. Samples with counts beyond the tolerance limits were discarded while those meeting the criteria were selected for the study. The selected blank fish materials after blending, were spiked with antimicrobial standards of known concentrations and used as control samples for the establishment of the control point counts per minute (cpm). A list of control standards used is shown in the supporting information (Table 1a).

- **Extraction of drugs from the fish materials**

The MSU extraction buffer (30 ml) was added to blended fish material (10 g) in a polypropylene centrifuge tube. The mixture was homogenized using a stomacher for 2 min and returned to the centrifuge tube. The homogenate was incubated in water bath at 80°C for 30 min, during the determination of streptomycin, macrolides, or beta- lactams; and 45 min, when determining tetracyclines or sulfa drugs. After incubation, the tube was cooled on ice water for 10 min and then centrifuged at 3300 rpm for 10 min, using a refrigerated centrifuge 4K15C (Sigma-Aldrich). The resulting supernatant solution was collected and used for the required tests. The pH of the supernatant was where necessary adjusted to pH 7.5 using reconstituted Charm II kit M2 buffer for low pH, or 0.1M hydrochloric acid for high pH.

3.7 Determination of tetracycline in the fish samples

In the detection of tetracycline, the white tablet from the kit containing the binding reagent (TMSU-025) was introduced into a test tube, and water (300 µl) was added. The contents of the tube were mixed for at least 10 s to ensure breakup of the tablet. The sample extract or control sample (4 ml) was added to the

tube, followed by addition of the orange tablet containing the tracer reagent from the kit (TMSU-025). The resultant was mixed for about 10 s and the mixture was incubated at 35 ^{O}C for 5 min; and then centrifuged for another 5 min on a IEC Centra CL-3 centrifuge. The supernatant was poured off carefully, avoiding the formed pellet from sliding out of test tube. Deionized water (300 µl) was added to the tube and the contents mixed thoroughly to break up the pellet. After suspension of the pellet in water, the scintillation fluid (3.0 ml) was added and test tube capped. The tube was shaken until the mixture had a uniform cloudy appearance. The glass tube contents were transferred completely into a scintillation vial and the mixture counted using a Wallac liquid scintillation counter for 60 s on the [^{3}H] channel. The results for the sample was compared with the control point counts per minute.

3.8 Determination of macrolides in the fish samples

During the detection of macrolides, the white tablet from the Charm II kit containing the binding reagent (EMSU-023A) was introduced into test tube, and water (300 µl) was added. The contents of the tube were mixed for at least 10 s to ensure breakup of the tablet. The sample extract or control sample (4 ml) was added to the tube and the contents mixed on a vortex for 10 s. The resultant was incubated at 55 ^{O}C for 2 min, followed by addition of a green tablet containing the tracer reagent from the kit (EMSU-023A). The resultant was mixed on a vortex for 10 s. The mixture was incubated at 55 ^{O}C for 2 min, and then centrifuged for 5 min. The supernatant was poured off carefully and the edge of tube blotted on absorbent paper. Deionized water (300 µl) was added to the tube and the contents mixed thoroughly to break up pellet. After suspension of the pellet in water, the scintillation fluid (3.0 ml) was added and the test tube capped. The contents were mixed on a vortex until the mixture had a uniform cloudy appearance. The content of the glass tube was transferred completely into a scintillation vial and the mixture counted using a Wallac liquid scintillation counter for 60 s on the [^{14}C] channel. The counts per minute (cpm) of the sample was compared with the control point.

3.9 Determination of sulfa drugs in the fish samples

In the detection of sulfa drugs, the white tablet from the Charm II kit containing the binding reagent (SMMSU-022C) was introduced into a test tube, and water (300 µl) added. The contents of the tube were mixed for at least 10 s to ensure breakup of the tablet. The sample extract or control sample (4 ml) was added to the tube, followed by addition of the pink tablet containing the tracer reagent (SMMSU-022C) from the kit. The resultant was mixed by swirling the contents up and down for about 15 s. The mixture was incubated at 65 ^{O}C for 3 min, and then centrifuged for another 3 min. The supernatant was poured off carefully, avoiding the formed pellet from sliding out of test tube; and the edge of tube was blotted on absorbent paper. Deionized water (300 µl) was added to the tube and the contents mixed thoroughly to break up the pellet. After suspension of the pellet in water, the scintillation fluid (3.0 ml) was added and test tube capped. The tube was shaken until the mixture had a uniform cloudy appearance. The glass tube contents were transferred completely into a scintillation vial and the mixture counted using a Wallac liquid scintillation counter for 60 s on the [^{3}H] channel. The cpm results of the sample were compared with the control point.

3.10 Determination ofaminoglycoside- streptomycins in the fish samples

In the determination of streptomycins, the white tablet from the kit containing the binding reagent (STMSU-023A) was introduced into a test tube, and water (300 µl) added. The contents of the tube were mixed for at least 10 s to ensure breakup of the tablet. The sample extract or control sample (2 ml) was added to the tube and mixed. This was followed by addition of the green tablet containing the tracer reagent (STMSU-023A). The resultant was mixed by swirling the contents up and down for about 10 s. The mixture was incubated at 35 ^{O}C for 2 min, and then centrifuged for another 3 min. The supernatant was poured off carefully and the edge of tube was blotted with absorbent paper. Deionized water (300 µl) was added to the tube and the contents mixed thoroughly. After suspension of the pellet in water, the scintillation fluid (3.0 ml) was added and test tube capped. The tube was shaken until the mixture had a uniform cloudy appearance. The glass tube contents were transferred completely into a scintillation vial and the mixture counted using a Wallac liquid scintillation counter for 60 s on the [^{3}H] channel. The cpm results for the sample were compared with the control point.

3.11 Determination of beta-lactams in the fish samples

In the determination of macrolides, the green tablet from the Charm II kit containing the binding reagent (PMSU-050A) was introduced into test tube, and water (300 µl) was added. The contents of the tube were mixed to ensure breakup of the tablet. The sample extract or control (2 ml) was added to the tube and the contents mixed on a vortex for 10 s. The resultant was incubated at 55 ^{O}C for 2 min, followed by addition of a yellow tablet containing the tracer reagent (PMSU-050A) from the kit. The resultant was mixed on a vortex for 10 s. The mixture was incubated at 55 ^{O}C for 2 min, and then centrifuged for 5 min at 1750 G. The supernatant was poured off carefully and the edge of tube blotted on absorbent paper. Deionized water (300 µl) was added to the tube and the contents mixed thoroughly to break up the pellet. After suspension of the pellet in water, the scintillation fluid (3.0 ml) was added and test tube capped. The contents were mixed on a vortex until the mixture had a uniform cloudy appearance. The mixture was transferred completely into a scintillation vial and counted using a Wallac liquid scintillation counter for 60 s on the [^{14}C] channel. The cpm of the sample was compared with the control point.

3.12 Method validation

The method validation was done according to the criteria of the European Commission Decision 2002/657/EC [23]. The validation parameters performed included; detection capability (CCβ), repeatability, reproducibility, robustness and cross reaction activity.

3.12.1 Detection capability

The detection capability (CCβ) was examined by spiking blank fish matrices with different antimicrobials including tetracyclines, macrolides, β-lactam, aminoglycosides, and sulfonamides. The number of samples analyzed for each individual antimicrobial agent ranged from 20 to 30 as indicated in Table 3.

The spiking concentrations varied around the recommended maximum residue limit (MRL), including 0.05MRL, 0.25MRL, 0.5MRL, 0.75MRL and MRL, for the respective antimicrobial.

3.12.2 Repeatability

The repeatability of the technique was studied by analysis of selected fish samples spiked with different antimicrobials including tetracyclines, macrolides, β-lactam, aminoglycosides, and sulfonamides. The total number of samples analyzed for each individual antimicrobial compound ranged from 20 to 30 samples, and $n \geq 6$ for the same fish species. The spiking concentrations varied around the recommended maximum residue limit (MRL), including 0.05MRL, 0.25MRL, 0.5MRL, 0.75MRL and MRL, for the respective antimicrobial. The analysis was performed within a short interval, by a single researcher using the same method and scintillation fluid counter equipment.

3.12.3 Reproducibility

The reproducibility of the method was studied by repeat analysis of selected fish samples spiked with different antimicrobials including tetracyclines, macrolides, β-lactam, aminoglycosides, and sulfonamides. The number of samples analyzed for each individual antimicrobial ranged from 20 to 30 samples, with $n \geq 6$ for the same fish species. The spiking concentrations varied around the recommended maximum residue limit (MRL), including 0.05MRL, 0.25MRL, 0.5MRL, 0.75MRL and MRL, for the respective antimicrobial. The analysis was performed on different days by two different researchers using the same method and a scintillation fluid counter equipment.

3.12.4 Robustness

The robustness of the techniques was tested by deliberately varying the experimental time indicated in the Charm II analytical protocol. This was intended to study the effect of variation in reading time interval for a large batch of processed samples. Reading of the cpm for the samples spiked with 50 µg/kg amoxicillin was done immediately after the addition of the scintillation fluid and then after 14 hours on the same batch of extracted sample. The samples after the first reading were stored overnight in the fridge at 4 ^{0}C, removed and allowed to attain room temperature, and then read the second time after vortexing.

3.12.5 Cross reaction activity

Cross reactivity was investigated by spiking residue-free blank fish samples with high concentrations, above 100 MRLs of the respective antimicrobial belonging to other antimicrobials groups and the samples run on targeted channels to investigate false identification.

3.13 Data Analysis

All data generated was statistically analyzed using one-way analysis of variance (ANOVA) to examine any significant differences between the observed results under different experimental setups.

Results And Discussion

4.1 Counts per minute for blank samples

The blank samples used in the study were those fish tissue matrices which were carried through the complete analytical procedure, and no antibiotic residues were detected in them using the respective Charm II assay kits [21]. The blank fish samples to which the binder and tracer had been added but without addition of an antimicrobial agent were extracted with the different kits and read on the respective channels. The results of the counts per minute (cpm) for the blank fish samples are summarized in Table 1.

From Table 1, the cpm for tilapia, trout, salmon, pangasius, seabass, dorate, catfish, and lingue, fish species were statistically evaluated using ANOVA and it was found that the overall F-calculated (0.22) was less than F-Critical (2.5), which implied that there was no significant difference between results for the blank fish samples of the aforementioned species when using antimicrobial test kits for β-lactams, tetracyclines, macrolides and streptomycins. However significant difference in cpm values was observed with the sulfonamides extraction kit while testing catfish, lingue and pangasius. The cpm for these species were almost double those of the other types of fish and their F-calculated (15.1) was greater than F-critical (2.4). The big variation in cpm for the catfish, lingue and pangasius fish species as compared to the rest could be attributed to the high fish fat content extracted by the sulfonamide kit protocol. In this respect, the three fish species (catfish, lingue and pangasius) cpm blank values were handled separately when calculating control points to minimize chances of getting false negative or false positive results. For the rest of the fish species, the blank cpm results were used to derive the respective control points for the different residues.

4.2 Evaluation of the Control Points for the different drug residues

The control point (CP) of a sample is the cut-off point between a negative or positive result. Any antimicrobial agent present in the sample extract competes for the binding sites with the tracer, thus, the greater the cpm measured, the lower the antimicrobial drug concentration in the sample and vice versa. Samples with high counts are considered negative (tracer antimicrobials are largely bound to the binder) while those with low counts are considered positive (tracer antimicrobials are largely free in solution). The CP for the different antimicrobials were determined independently; and with the exception of tetracyclines, the MRL value for each drug was spiked to the respective blank fish sample. In order to cater for the deviations in the different fish matrices, a percentage tolerance was added to or subtracted from the obtained average cpm value of the spiked blank fish sample. The CP evaluation was performed according to the Charm II protocol, and the percentages added to the mean value of spiked samples at detection capability or subtracted from the mean value of blanks serve to minimise occurrence of false positive or negative readings [21, 24, 25].

In this respect, the CP for the beta-lactams was evaluated from averaging the results of 6 negative samples spiked with benzyl penicillin at 25 µg/kg (0.5 MRL) and adding 20% of the obtained average

cpm value. Whereas, for the sulfonamides, the CP was evaluated by averaging results of negative samples spiked at 50 µg/kg with sulfamethazine and adding 30% of the average obtained cpm value. In this respect, a control point of 1530 was calculated for the beta-lactam (Table 2). On the other hand, the CP for tetracyclines was calculated by averaging cpm results of negative control standards provided in the tetracycline test kit and subtracting 40% of the obtained average cpm value.

For macrolides, the CP was derived from averaging the results of 6 negative samples spiked with erythromycin A at 100 µg/kg (0.5 MRL) and adding 20% of the obtained average cpm value. Using a similar approach, the CP for streptomycins was derived from averaging results of negative samples spiked at 25 µg/kg with streptomycin and adding 30% of the average obtained cpm value. The results of the calculated CP for the different antimicrobials involved in the study are presented in Table 2. During the analysis of antimicrobial residues in different fish samples, results less than or equal to each respective CP were interpreted as positive while those greater than the CP as negative.

A brief comparison of the control points (CP) for the different antibiotics obtained using the Charm II assay kits with the corresponding cut-off points (Fm), calculated following Annex II of the EU guideline, for Community Reference Laboratories Residues 20/1/2010 [26], is presented in Table 3.

The Fm value refers to the response or signal from a screening test which indicates that a sample contains an analyte at or above the screening target concentration [26]. From the results presented in Table 3, the Fm values obtained using the EU guideline and the respective calculated CP according to the Charm II protocol are comparable. However, in all cases the CP value for a particular family of antibiotics is slightly higher the corresponding Fm readings, with the exception of sulfathiazole; this implies that there will be less incidences of false negative readings in the detection of the different antimicrobial compounds in fish matrix.

4.3 Detection capability for the different antimicrobials in selected fish species

The detection capability (CCβ) is the lowest concentration of the analyte that could be detected in the sample giving at least a 95% positive result. In these studies, blank negative fish tissue samples were spiked with different antimicrobials and a summary of the CCβ for the selected drugs is presented in Table 4. Results of detection capability show that the Charm II analytical technique can detect tetracycline and chlortetracycline at 25 µg/kg (0.25 MRL) and oxytetracycline at 100 µg/kg (MRL) for the different fish species (cat fish, trout, salmon, seabass, tilapia, lingue, dorade, and pangassius) with 100% detection. However, the batch of the multi antimicrobial standard, provided in the Charm II kit was not sensitive enough for chlortetracycline to be detected at 100 µg/kg (MRL) level. This could be attributed to the deterioration of the chlortetracycline in the standard due to poor handling, probably during transportation. In this respect, a Sigma Aldrich standard was used and chlortetracycline detected at a concentration as low as 0.25MRL. Interestingly, it was observed that the technique is also capable of detecting other antimicrobials belonging to the tetracycline family (tetracycline, oxytetracycline) and not limited to the chlortetracycline provided for in the Charm II test kit.

The sulfa drugs including, sulfadimethoxine, sulfadiazine, sulfathiazole were detected at 25 µg/kg (0.25 MRL) for the different fish species (cat fish, trout, salmon, seabass, tilapia, lingue, dorade, and pangassius) at 100% detection, and sulfamethazine, 25 µg/kg (0.25 MRL) at 96.6% detection (3.4% false negatives). It should be noted that the catfish, pangassius, and lingue fish species gave high counts for blank samples in respect to sulfonamide drug residues as compared to other fish species and their control points were established separately. The results also show that the technique can detect other antimicrobials belonging to the sulfonamides group (sulfamethazine, sulfadimethoxine, sulfamerazine, sulfadiazine, sulfathiazole) which are not in the MSU multi antimicrobial standard mix, provided in the Charm II test kit. For the macrolides; erythromycin A, tilmicosin, and Tylosin A were detected at 100 µg/kg, for the different fish species (cat fish, trout, salmon, seabass, tilapia, lingue, dorade, and pangassius) with 100% detection. Whereas, results for the beta-lactams show that benzyl penicillin, ampicillin, amoxicillin, oxacillin, dicloxacillin and cloxacillin were detected at 25 µg/kg, 50 µg/kg, 50 µg/kg, 300 µg/kg, 300 µg/kg and 300 µg/kg respectively for the different fish species (cat fish, trout, salmon, seabass, tilapia, lingue, dorade, and pangassius). Thus, benzylpenicillin is detected at 0.5MRL, whereas ampicillin, amoxicillin, oxacillin, dicloxacillin and cloxacillin are all detected at their respective MRL. However, 4.5 and 5% of the results for dicloxacillin and cloxacillin respectively, were false negatives (Table 4). Further on, the Charm II technique is capable of detecting streptomycin at 25 µg/kg (0.05MRL) for the different fish species (cat fish, trout, salmon, seabass, tilapia, lingue, dorade, and pangassius) at 100% detection.

A comparison of the detection capabilities (CCβ) and maximum residue limits (MRL) for the different antimicrobials is shown in Figure 1. The results show that, CCβ for the validated antimicrobials were below or equal to the MRL for all drug residues in this study, with the exception of tilmcosin which was detected at 2 MRL. Most of the drug residues exhibited detection capabilities in the range 0.05 MRL to 0.5 MRL, with 100% accuracy. The Charm II technique exhibited better detection capability for tetracyclines at 25 ppb (0.25 MRL) compared to other rapid screening techniques such as the ELISA kit of R-Biopharm for screening tetracycline antibiotic residues in the muscle of chicken, beef, and shrimp, which detected the same at 100 ppb (MRL) [27]. In another study, results of the revolutionary Biochip Array Technology detected tylosin and oxytetracycline at 0.10 and 0.5 of the respective MRL in samples [28].

The limits of detection (LOD) obtained using the Charm Test II assays, and the limits of quantitation (LOQ) for selected literature chemical methods are presented in Table 1b (Supporting information). The LOD results for fish matrix obtained in this validation using the Charm II kits, are comparable to the manufacturer's claims for the tissue matrix. However, some antimicrobial compounds could be detected in fish tissue at levels lower than the manufacturer's claim (Table 1b, Supporting Information). The LOD results were also compared with the LC-MS/MS analysis of sulfadimethoxine [29], HPLC-MS/MS analyses of tetracyclines, chlortetracycline, oxytetracycline, sulfadimethoxine, sulfamerasine and sulfadiazine [30]; and LC-ESI-MS/MS analyses of a range of tetracyclines, β-lactams, aminoglycosides and sulfonamides [31]. Generally, the rigorous chemical techniques, as expected, offer lower LOQ values compared to the respective LOD obtained with the Charm II tests. However, the Charm II test demonstrated ability to detect a wider range of antibiotics belonging to different classes including tetracyclines, macrolides, β-lactams, aminoglycosides and sulfonamides at MRL or lower levels.

4.4 Repeatability of the method

Repeatability analysis was performed using the same Charm II protocol for a specific antimicrobial on different fish species performed by the same researcher. This was evaluated by means of the intraday coefficient of variations and the results are presented in Table 5. Results of the repeatability study characterized by the relative standard deviation (%RSD) was satisfactory with a precision of less than 12% for the different antimicrobial drugs including tetracyclines, macrolides, beta-lactam, aminoglycosides, and sulfonamides; spiked in blank fish samples at MRL, 0.5MRL or concentration less than 0.5MRL and analysed under repeatability conditions ($n \geq 6$). The coefficient of variation expressed as percentage relative standard deviation (RSD_r) ranged from 7.8 to 9.8% for tetracyclines (chlortetracycline and oxytetracycline), 2.8 to 6.3% for macrolides (erythromycin), 6.9 to 9.7% for beta-lactams (penicillin G), 10.01 to 11.5% for aminoglycosides (streptomycin); and for sulfonamides (sulfathiazole) it was from 1.2 to 8.7%. These results, ably demonstrate the protocol's repeatability when used for testing different antimicrobial residues in fish tissue matrix.

A closer look at results obtained under repeatability conditions in the analysis of different fish samples spiked with 25 µg/kg sulfathiazole is presented in Table 6. The results showed that there was no significant difference in cpm readings for the same fish species, and amongst different fish species including dorade, salmon and seabass, spiked with sulfathiazole at the same concentration level (ANOVA, overall F-critical 3.35 > F-calculated 1.99) with RSD < 10%. Similar observations were made for the other antimicrobial agents, whose summarized results are presented in Table 5.

4.5 Reproducibility of the method

The reproducibility studies were performed by two different researchers following the same Charm II protocol on selected fish species, spiked with different antimicrobial agents and evaluated by means of intra-day and inter-day coefficient of variations. The reproducibility study characterized by the relative standard deviation (%RSD) was satisfactory with a precision of less than 15.3% for the different antimicrobial drugs (tetracyclines, macrolides, beta-lactam, aminoglycosides, and sulfonamides) spiked in blank fish samples at MRL, 0.5MRL or concentration less than 0.5MRL and studied under reproducibility conditions ($n \geq 6$). The coefficient of variation calculated as percentage relative standard deviation (%RSD) for tetracyclines (chlortetracycline and oxytetracycline) was 7.2 to 11.4%; macrolides (erythromycin) ranged from 5.8 to 8.9%; beta-lactam (penicillin G) from 10.4 to 11.2%; aminoglycosides from 8.9 to 15.1% and sulfonamides (sulfathiazole) from 2.8 to 8.3% as indicated in Table 7.

An elaborate presentation of some results of the reproducibility studies performed by two different researchers following the same Charm II protocol on selected fish species, spiked with oxytetracycline at a concentration level of 100 µg/kg, is presented in Table 8. A comparison of the results obtained by the two researchers for the same fish species showed no significant difference; and the overall analysis showed no significant difference in the cpm results for the different fish species including seabas, pangasius and salmon (ANOVA, F-critical 4.1 > F-calculated 0.64), with RSD < 10%, which further

demonstrates the technique's reproducibility with little matrices interference. Similar observations were made for the other antimicrobial compounds, whose summarized results are presented in Table 7.

4.6 Robustness of the method

Analysis of batches of many samples often require a couple of hours before completion; and there is likely to be a time interval between the first and last analysis of the processed samples. In the robustness testing of the Charm II assay, the effect of variation in reading time interval for processed samples was studied. Robustness testing was performed on samples spiked with 50 µg/kg amoxicillin and analysed on the beta-lactam channel immediately after mixing (0 hour) and after 14 hours. The control point for beta-lactam was set at 1530, and the robustness results are presented in Table 9.

4.7 Specificity and cross reactivity of the technique

The specificity and cross reactivity analysis was carried out in order to determine whether the presence of non-target drugs may lead to false identification of the target drug; or whether the identification of the target analyte may be hindered by the presence of one or more interferences. Representative blank fish samples were spiked with different antimicrobial drugs at known concentration levels higher than those likely to interfere with the identification of the analyte of interest, and then analysed using the respective Charm II protocol for the target drug. The aminoglycosides (spectinomycin, neomycin and paromomycin) were analysed using a macrolide channel meant for (erythromycin, tilmicosin and tylosin) and the results of this study are presented in Table 10. In these studies, standard mix containing the respective antimicrobials (aminoglycosides) at 150, 300 and 500 µg/kg concentration were used to spike the samples.

Results show that although the macrolides which were the targeted antimicrobials tested positive, the non-target aminoglycosides intentionally analyzed on the same channel, tested negative since in all cases the observed cpm were above the set control point of the macrolides of 2118. In similar studies, cross reactivity was investigated by spiking residue-free, blank fish samples with high level concentrations (10 MRL) of antimicrobial substances belonging to other groups (sulfonamides, beta-lactams, macrolides, and tetracyclines) were analysed on the aminoglycosides channel; and the results are presented in Table 11.

The results of these studies also showed that no residues of the non-target drugs (tetracycline, penicillin G, sulfamethazine and tilmicosin) could be detected using the aminoglycosides channel as shown in Table 11. All spiked samples tested negative and the non-target compounds could not be detected even at the high concentration of 10 MRLs. Similar observations were made utilizing the Biochip Array Technology technique, none of the tested six antibiotics could be detected under cross-reactivity studies [27].

Conclusions

The Charm II radio receptor assay technique was successfully validated for the screening residues of tetracyclines, sulfonamides, beta-lactams, aminoglycosides and macrolides in different aquaculture fish species. The Charm II technique can detect tetracycline and chlortetracycline at 25 µg/kg (0.25 MRL) and oxytetracycline at 100 µg/kg (MRL) for different fish species including, cat fish, trout, salmon, seabass, tilapia, lingue, dorade, and pangassius, with 100% detection. The sulfonamides including sulfadimethoxine, sulfamerasine, sulfadiazine, sulfathiazole could be detected at 25 µg/kg (0.25 MRL) for all fish species involved in the study, with the exception of catfish, pangassius, and lingue, which gave high counts for the blank samples. Results for the macrolides analysis, showed that erythromycin, tilmicosin, and tylosin, could be detected at 100 µg/kg (0.5 MRL), 100 µg/kg (2 MRL) and 100 µg/kg (MRL), respectively, for the different fish species. Whereas, the beta-lactams including benzyl penicillin, ampicillin, amoxicillin, oxacillin, dicloxacillin and cloxacillin were detected at 25 µg/kg (0.5MRL), 50 µg/kg (MRL), 50 µg/kg (MRL), 300 µg/kg (MRL), 300 µg/kg (MRL) and 300 µg/kg (MRL), respectively, for different fish species. Under the aminoglycosides analysis, streptomycin was detected at 25 µg/kg (0.05 MRL) for all fish species involved in the study. Interestingly, the technique can detect a broader range of antimicrobials other than only the compounds specified in the Charm II assay kit. All antimicrobial compounds involved in the study could be successfully detected using the Charm II assay at 100% rate, with the exception of dicloxacillin, cloxacillin and sulfamethazine that exhibited false negative rates of 4.5, 5.0 and 3.4%, respectively.

Robustness studies showed that there was no significant difference between results for counts of the same samples read immediately or after 14 hours of addition of the scintillation fluid. In addition, no evidence of *cross-reactivity* was observed among the targeted antimicrobial compounds on interchanging the antimicrobial analysis channels. The results of this validation study prove the robustness, specificity, reliability and accuracy of the Charm II radio receptor assay technique in the detection of various antibiotic residues in different fish species. The study confirms the suitability of the Charm II technique as a valuable screening tool for detection of antimicrobial residues in a variety of fish species; and its applicability for the rapid evaluation of the quality of aquaculture products for safety and trade purposes.

Declarations

Authors' contributions

AKM, WR, MFKG and JS participated in experimental design and coordination of laboratory activities. AKM, MFKG and WR carried out the sampling, sample preparation and analysis. WR, JJTT, AKM and ET participated in data analysis. AKM, ET, MH and SAN wrote the manuscript. All authors participated in manuscript review and approval for submission.

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Availability of data and materials

All supporting information including table of results and detailed methods is available upon request.

Competing interests

The authors declare that they have no competing interests.

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Abbreviations

ANOVA: one-way analysis of variance; CCβ: detection capability; CP: control point; CPM: counts per minute; EU: European Union; FAO: Food and Agriculture Organization; IAEA: International Atomic Energy Agency; ILVO: Flanders Research Institute for Agriculture, Fisheries and Food; MRL: maximum residue limit; MSU: multi antimicrobial standard; RSD: relative standard deviation; SD: standard deviation; UNBS: Uganda National Bureau of Standards.

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Tables

able 1: Blank counts per minute for the different fish species obtained using the Charm II echnique

Scintillation counter results (cpm)										
Charm II test	β-lactams kit		Sulfonamides kit		Tetracyclines kit		Macrolides kit		Streptomycins kit	
Fish sp.	Mean	SD	Mean	SD	mean	SD	Mean	SD	mean	SD
Tilapia	2704	0.9	2596	736	3027	0.7	2448	191.1	5103	346
Trout	2506	192.0	2332	1184	2830	260	2799	87.1	4799	259.9
Salmon	2571	207.4	2472	541.2	2939	165.0	2110	117.2	3085	133.4
Pangasius	2469	195.7	5625	1254	2931	221.4	2893	110	4796	437.7
Seabass	2432	232.1	2144	672.1	2971	252.2	2700	153.6	4805	594.7
Dorate	2512	171.1	1977	621.4	2864	93.4	2803	167.3	4967	485.8
Catfish	2493	312.7	5872	774.3						
Lingue			4454	650.1						

cpm: counts per minute; SD: standard deviation

able 2: Control points for the different antimicrobials in blank fish samples

Antimicrobial Drug	Level of Analyte spiking (µg/kg)	Mean cpm of Spiked Sample	Variation allowance for matrix effect	Control point cpm	Mean blank cpm
β-lactams	25 µg/kg Benzyl Penicillin	1275	Spiked cpm +20%	1530	2502
Sulfonamides	50 µg/kg Sulfamethazine	1096	Spiked cpm +30%	1424	3162
Tetracyclines	0 µg/kg Tetracycline at	2524	Blank cpm - 40%	1514	2524
Macrolides	100 µg/kg Erythromycin A	1765	Spiked cpm +20%	2118	2587
Streptomycins	25 µg/kg Streptomycin	2574	Spiked cpm +30%	3346	4605

able 3: Comparison of control points by Charm II protocol and calculated EU guideline cut-ff

points [26]

Family	Compound	Spiked concentration (µg kg^{-1})	Calculated Fm value as per EU guideline Fm = M + 1.64 * SD, [26]	Calculated control point CP as per Charm II assay
Tetracyclines (CP= 1514 cpm)	Tetracycline	25	816	1514
	Chlortetracycline	25	1417	
	Oxytetracycline	100	1427	
Macrolides (CP= 2118 cpm)	Erythromycin	100	1904	2118
	Tilmicosin	100	2002	
	Tylosin	100	1740	
β-lactam (CP= 1530 cpm)	Penicillin G	25	1438	1530
	Ampicillin	50	1341	
	Amoxicillin	50	1487	
	Oxacillin	300	1478	
	Dicloxacillin	300	1489	
	Cloxacillin	300	1413	
Aminoglycosides	Streptomycin	25	2592	3346
Sulfonamides (CP= 1424 cpm)	Sulfamethazine	25	1379	1424
	Sulfadimethoxine	25	972	
	Sulfamerasine	25	930	
	Sulfadiazine	25	1184	
	Sulfathiazole	25	1485	

ut-off factor (Fm) = M + 1.64 * SD

/here, M = mean response of spiked sample, SD = standard deviation for the spiked sample eadings

able 4: Detection capability for the selected antibiotics

Family	Compound	EU-MRL (µg kg^{-1})	CCβ (µg kg^{-1})	No of samples	No of positive samples	Counter results (cpm)			% Detection of each antibiotic
						Mean	Min	Max	
Tetracyclines (CP= 1514 cpm)	Tetracycline	100	25	20	20	724	650	825	100
	Chlortetracycline	100	25	21	21	1200	942	1421	100
	Oxytetracycline	100	100	31	31	1269	1074	1460	100
Macrolides (CP= 2118 cpm)	Erythromycin	200	100	30	30	1669	954	1955	100
	Tilmicosin	50	100	21	21	1565	1221	2078	100
	Tylosin	100	100	21	21	1440	1103	1742	100
β-lactams (CP= 1530 cpm)	Penicillin G	50	25	22	22	1175	921	1421	100
	Ampicillin	50	50	21	21	1055	837	1451	100
	Amoxicillin	50	50	22	22	1132	908	1409	100
	Oxacillin	300	300	24	24	1286	1082	1459	100
	Dicloxacillin	300	300	22	21	1186	827	1839	95.5
	Cloxacillin	300	300	20	19	1143	681	1547	95.0
Aminoglycosides (CP= 3346cpm)	Streptomycin	500	25	22	22	2424	1642	3074	100
Sulfonamides (CP= 1424 cpm)	Sulfamethazine	100	25	29	28	1240	813	1831	96.6
	Sulfadimethoxine	100	25	20	20	968	737	923	100
	Sulfamerasine	100	25	21	21	842	716	960	100
	Sulfadiazine	100	25	20	20	948	735	1361	100
	Sulfathiazole	100	25	20	20	989	698	1782	100

able 5: Repeatability study at MRL, 0.5MRL or Concentration of < 0.5MRL

Family	Compound	Spiking concentration (µg/kg)	Mean CPM	SD_r	RSD_r
Tetracyclines	Chlortetracycline	25µg/kg(0.25MRL)	1207.0	118.2	9.8%
	Oxytetracycline	100 µg/kg (MRL)	1270.06	98.41	7.75%
Macrolides	Erythromycin	100 µg/kg (0.5MRL)	1762.4	110.4	6.3%
		200 µg/kg (MRL)	1478.1	41.2	2.8%
β-lactam	Penicillin G	25 µg/kg(0.5MRL)	1285.6	89.3	6.9%
		50 µg/kg (MRL)	648.5	62.7	9.7%
Aminoglycosides	Streptomycin	250 µg/kg (0.5MRL)	1125.8	112.7	10.01%
		500 µg/kg (MRL)	1110.5	127.2	11.5%
Sulfonamides	Sulfathiazole	25 µg/kg(0.25MRL)	922.2	80.1	8.7%
		100 µg/kg(MRL)	706.5	8.6	1.2%

D_r - Standard deviation under repeatability conditions

SD_r - Relative standard deviation under repeatability conditions

lean CPM - Average of counts per minute under reproducibility conditions

able 6: Repeatability in the detection of sulfathiazole at 25 µg/kg for selected fish samples

Parameter	Dorade -25 µg/kg cpm	Salmon - 25 µg/kg cpm	Seabass-25 µg/kg cpm
	989	914	735
	815	969	780
	862	886	976
	876	1015	896
	782	932	890
	934	1075	978
	985	930	976
	877	1070	898
	863	935	975
	986	890	976
Average	896.9	961.6	908
SD	73.6	69.3	88.3
RSD	0.082	0.07	0.097

able 7: Reproducibility study at MRL, 0.5MRL or Concentration of < 0.5MRL

Family	Compound	Spiking concentration (µg/kg)	Average CPM	SD_R	RSD_R
Tetracyclines	Chlortetracycline	25µg/kg(0.25MRL)	1224.5	139.2	11.4%
	Oxytetracycline	100 µg/kg (MRL)	1277	92.6	7.2%
Macrolides	Erythromycin	100 µg/kg (0.5MRL)	1748.5	156.1	8.9%
		200 µg/kg (MRL)	1456.5	83.9	5.8%
β-lactam	Penicillin G	25 µg/kg(0.5MRL)	1204.9	135.0	11.2%
		50 µg/kg (MRL)	702.1	73.0	10.4%
Aminoglycosides	Streptomycin	250 µg/kg (0.5MRL)	1110.6	98.7	8.9%
		500 µg/kg (MRL)	1132.6	171.4	15.1%
Sulfonamides	Sulfathiazole	25 µg/kg(0.25MRL)	943.7	78.1	8.3%
		100 µg/kg(MRL)	647.1	18.1	2.8%

D_r - Standard deviation under reproducibility conditions

SD_r - Relative standard deviation under reproducibility conditions

verage CPM - Average of counts per minute under reproducibility conditions

able 8: Reproducibility in the detection of oxytetracycline at 100 µg/kg for selected fish amples

Fish sp.	Researcher 1 cpm	Researcher 2 cpm
Seabass	1176	1296
	1177	1265
	1295	1177
	1334	1281
	1127	1166
	1341	1417
	1170	1460
	1261	1074
	1371	1361
	1201	1335
Pangassius	1225	1185
	1166	1223
	1094	1224
	1408	1407
	1408	1405
Salmon	1307	1307
	1378	1299
	1298	1310
	1274	1311
	1313	1279
	1266.2	1289.1
Average	1176	1296
Standard deviation	94.6	96.2
RSD	0.07	0.07

able 9: Robustness testing using amoxicillin at 50 µg/kg for selected fish samples

		Spiked at 50 µg/kg		Non Spiked	
	Run	Pangasiuss	Dorade	Blank Reading	Fish species
Results at 0 hrs	1	1019	1069	2433	Pangasius
	2	1024	959	2398	Pangasius
	3	1017	1019	2399	Pangasius
	4	1201	1069	2064	Dorade
	5	1155	1033	2200	Dorade
	6	1020	1067	2109	Dorade
Results after 14 hrs	7	1120	1120	2399	Pangasius
	8	1059	1080	2064	Pangasius
	9	1260	1195	2399	Pangasius
	10	1011	1113	2068	Dorade
	11	1089	1089	2210	Dorade
	12	1099	1092	2205	Dorade
Average		1089.5	1075.4	2245.7	
SD		81.6	57.9	150.8	
RSD		0.07	0.05	0.07	

able 10: Specificity and cross reactivity tests using the Macrolides kit

Blanks cpm	Salmon -150 µg/kg cpm	Salmon -300 µg/kg cpm	Pangassius-500 µg/kg cpm	Catfish-150 µg/kg cpm	Catfish-300 µg/kg cpm	Trout-500 µg/kg cpm
5377	5097	5239	5239	5536	5511	4694
5538	4860	4931	4931	5224	5393	5262
5538	5106	4800	5034	5223	5803	5412
5076	4703	4900	5351	4966	5558	5686
5571	5039	4950	5121	5236	5807	5728
Macrolide calculated control point cpm= 2118						

able 11: Specificity and cross reactivity tests using mixed standards of different ntimicrobial on

Aminoglycosides kit

Antibiotics used in the mix and their respective MRL	Mixed standard for spiking blank fish samples and respective spiking level, µg/kg	Fish Species	CPM Values for the spiked fish samples
Tetracycline (MRL 100 µg/kg)	Tetracycline spiked at 1000 µg/kg	Cat fish	5742
Penicilin G (MRL 50 µg/kg)	Penicilin G spiked at 500 µg/kg	Cat fish	6286
		Cat fish	5780
Sulfamethazine (MRL 100 µg/kg)	Sulfamethazine spiked at 1000 µg/kg	Cat fish	5776
Tilmicosin (50MRL µg/kg)	Tilmicosin spiked at 500 µg/kg	salmon	5418
		salmon	5700
		salmon	5584
		Trout	5776
		Trout	5584
		Trout	6155
		Trout	5962
		Trout	5777
		Trout	5800
		Trout	5671
		Trout	6010
		Trout	5699
		Trout	5700
		Trout	5800
		Trout	5810
		Trout	5156
Aminoglycosides calculated control point cpm= 3346			

Figures

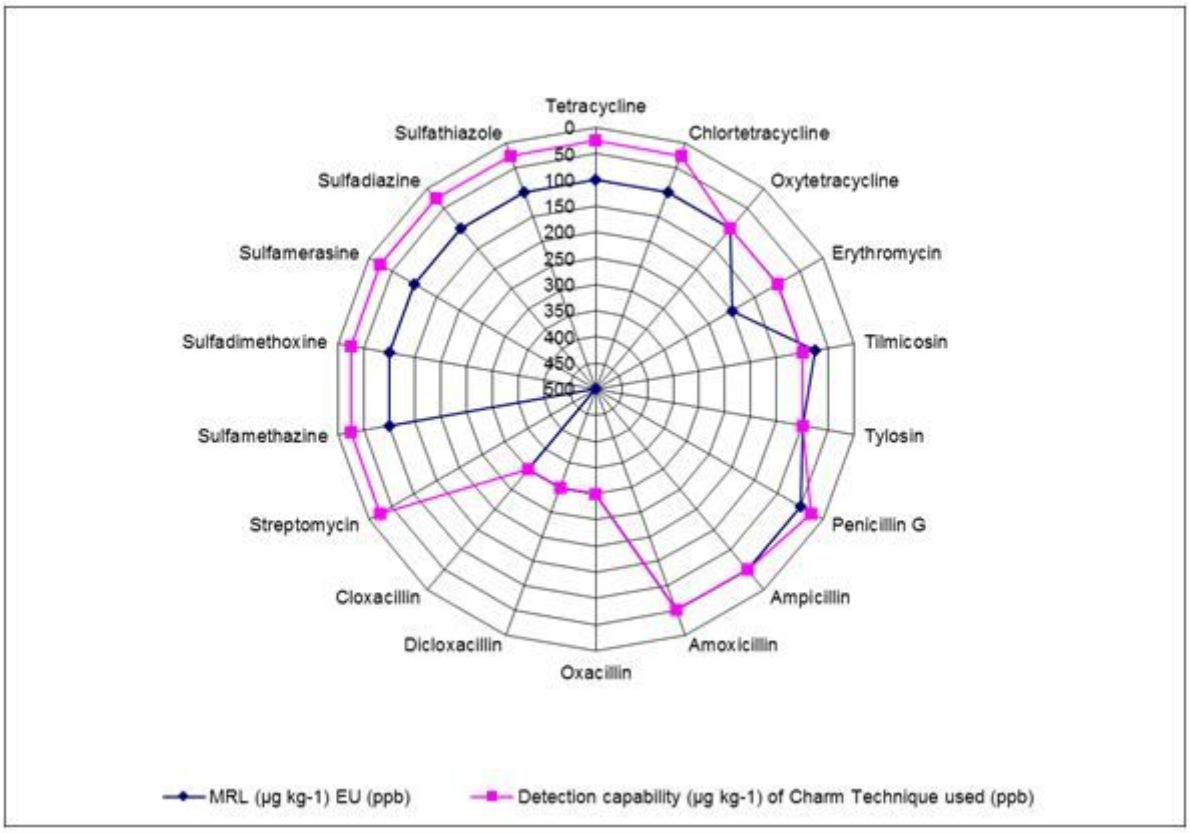

Figure 1

The detection capabilities and maximum residue limits for the different antimicrobials

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- CCJOD1900279RevisedSupportingInformationMarch2020.docx