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# Validation of a Commercial Enzyme-Linked Immunosorbent Assay for Screening Tetracycline Residues in Foods of Animal Origin from the Perspective of Bangladesh

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***Authors' contributions***

*This work was carried out in collaboration between all authors. Author AYKMMR designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author SUT performed analyses of the study. Authors MMN, MHI and MJI managed the literature searches. Author ASMS supervised the whole work. All authors read and approved the final manuscript.*

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## ABSTRACT

Enzyme-linked Immunosorbent Assay (ELISA) is one of the most important screening methods of antibiotic residue analysis in different food matrices. This study was carried out to validate the ELISA kit of R-Biopharm (RIDASCREEN) for screening tetracycline antibiotic residues in the muscle of chicken, beef, and shrimp in accordance with the European Commission (EC) decision 2002/657/EC. The kit was validated in terms of different characteristic performances, e.g., detection capability, specificity, applicability, ruggedness, and stability. Detection capability CCβ of the method was 100 µg/kg for three types of matrix with a false compliant rate of 5% for beef and

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chicken, and 0% for shrimp samples. The threshold value T was equal to 1.85, 1.558, and 1.532 OD for beef, chicken, and shrimp samples, respectively, whereas the cut-off value Fm was equal to 0.621, 0.519, and 0.424 OD, respectively. The kit was valid because Fm<T for all the cases. Sensitivity of the kit decreased after six months of first experiment, but still can be used successfully because the Fm values clearly lowered the T values, and the reading ODs did not overlap any ODs of spiked samples. The ELISA kit for tetracycline was robust and cost effective because there was no need to use solid phase extraction. In case of monitoring tetracycline antibiotic residues, the kit is applicable for the muscle of chicken, beef, and also shrimp with the same detection capability value.

*Keywords: RIDASCREEN; validation; tetracycline; ELISA; detection capability.*

## 1. INTRODUCTION

Antibiotics are widely used for preventative and curative treatment of livestock and aquacultures and also as growth promoting agent for farming animals [1,2]. Tetracyclines are broad-spectrum antibiotics which are commonly used in veterinary medicine to prevent diseases and as an additive in foods of animal origin to promote growth [3]. Growth promotion and nutrient efficacy are considered to be accomplished with relatively small doses of antibiotics mixed with the feed by the manufacturers or farmers [4-6]. Various antibiotics take different time periods to be excreted from the body. Therefore, the antibiotic residues could be found in food of animal origin if proper withdrawl time for corresponding antibiotic was not maintained and became potential hazard to human health [3]. Residues of antibiotic could also be found in environment because of their entry into the food chain through feces and urine. Withdrawal period of oxytetracycline, chlortetracycline and tetracycline in animals are 10, 5 and 5 days respectively [7]. Indiscriminate use of antibiotics left noticeable residues in meat, milk, cheese, butter and other livestock products which may cause allergies and microbiological resistance [4,8]. For these reasons, different international communities have set maximum residue limits (MRLs) for the antibiotics to be used in livestock as veterinary medicines. To ensure human food safety, MRL for the presence of safe level of TC residues in animal product has been set by Codex Alimentarius Commission and United State Food and Drug Administration (FDA) as 200 µg/kg in muscle, 600 µg/kg in liver and 1200 µg/kg in fat and kidney [9]. European Commission has established MRL for tetracycline in milk, meat and other foods by European regulation 2377/90 [10] and subsequent modifications [11]. The MRL for milk and muscle were set at 100 µg/kg for all species, and the levels set for liver and kidney were 300 and 600 µgkg/kg, respectively [12].

Screening is the first step in monitoring the presence of antibiotic residues which conclude whether a sample contains antibiotic residues at or above the MRL. In case of positive screening, it is necessary to use physiochemical methods for the confirmation of the identity and the quantification of the substance [13]. Enzyme linked immunosorbent assay (ELISA) is a very sensitive and reproducible method which is approved by the EU for screening tetracycline family antibiotic residues in foods at MRL level [14,15]. Before use, the method must be validated in terms of some performance characteristics- detection capability CCβ, specificity, applicability, ruggedness and stability [13,14].

Bangladesh is a developing country. Number of poultry and animal farms are increasing with the increase of population. The country exports frozen shrimp to international markets especially in European Countries. Considering the threat to public health and to international trades, Bangladesh Atomic Energy Commission (BAEC) has established a veterinary drug residue analysis laboratory with the help of International Atomic Energy Commission (IAEA) at the Institute of Food and Radiation Biology, BAEC for monitoring antibiotic residues in foods. The aim of this study was to validate an ELISA kit-from r-biopharm (RIADASCREEN) for screening the presence of tetracycline group antibiotic residues in foods of animal origin.

## 2. MATERIAL AND METHODS

### 2.1 Chemicals and Standard Solutions

RIDASCREEN tetracycline kit from r-biopharm, Art. No. R3505 (lot no. 11295), was used

throughout the validation process. Test kit contained microplate, tetracycline standard solution, tetracycline-conjugate, anti-tetracycline-antibody, red chromogen stop solution, buffer and washing buffer. According to European Commission guidelines, less sensitive/specific analyte among the class should be used for validation [15]. Oxytetracycline (OTC) bought from Sigma Aldrich was used as spiking reference standard. All chemicals and reagents were used as analytical grade.

## 2.2 Matrices

Three types of matrix- muscle of beef, chicken and shrimp were used throughout the study. Negative control (Blank) samples of different origin were supplied by International Atomic Energy Agency (IAEA) under a Technical Co-operation Project BGD 5031. A total of 60 samples (20 samples each of Beef, chicken and shrimp) were analyzed for validation purposes.

## 2.3 Preparation of Standards

Oxytetracycline standard was prepared at 20mgml-1 in methanol and stored at -20℃. 1mgml-1 working standard solution was prepared from stock solution in water and stored at 4℃.

## 2.4 Principal of ELISA Kits

RIDASCREEN tetracycline kit is a competitive enzyme immune assay for the qualitative and quantitative analysis of tetracycline group antibiotics (oxytetraycline, chlortetracycline, doxycycline, tetracycline and their epimers). The microtiter wells are coated with tetracycline-protein-conjugate. Tetracycline standards or sample solutions and anti-tetracycline antibodies are added. Free tetracycline and immobilized tetracycline compete for the tetracycline antibody binding sites. Any unbound antibody is then removed in washing step and enzyme labeled secondary antibody, which is directed against the anti-tetracycline antibody, is added. The unbound conjugate is removed by a washing step. Subsequently, a substrate/chromagen mixture is added to the wells and incubated at a fixed temperature. The bound conjugate converts the chromagen into a blue product. Adding stop solution leads to a color change from blue to yellow. The measurement is made photometrically at 450 nm. The absorption is inversely proportional to the concentration of antibiotic in the sample.

## 2.5 Spiking Blank Sample

Blank samples were spiked with oxytetracycline (OTC). Ideally, the chosen concentration of analyte for spiking blank samples should be lower or at the MRL [14,16]. We tried at half MRL (50 µg/kg) but some optical density values of spiked samples were very close to blank samples. Therefore, to minimize the false negativity and to use the kit for monitoring program of antibiotic residues in foods of animal origin, we choose to spike the blank sample at MRL (100 µg/kg).

## 2.6 Sample Extraction Procedure

Extraction of tetracyclines from spiked and unknown samples was carried out according to Kit manufacturer's instruction. Briefly, after homogenizing the samples (spiked and blank), 1 gm of each was taken into 50 ml tube, and 9 ml of 20mm PBS buffer (pH 7.4) was added. After mixing, the tubes were shaken for 10 minutes and centrifuged at 4000g at room temperature (RT) for 10 minutes. 1 ml supernatant was transferred from each of the tube to a new vial and 2 ml hexane was added. Samples were vortexed and centrifuged at 4000g at RT for 10 minutes. Fifty microliter of aqueous phase was collected and used for the ELISA assay. In case of shrimp, extraction with hexane was omitted. ELISA assay was carried out according to manufacturer's procedures.

## 2.7 Validation

The kit was validated according to the European Guideline for the validation of screening methods [14], which is based on the criteria of European Decision No. 2002/657/EC [13]. The performance characteristics to be determined for validation of the kit were: applicability specificity, detection capability CCβ, ruggedness and stability. The analyses were blindly performed by two operators.

### 2.7.1 Specificity and Detection capability (CCβ)

According to European Guidelines of Community Reference Laboratories residues 20/1/2010 for the validation of screening methods [14], the number of samples required for validation of screening method depends on degree of statistical confidence required for the result and relationship between the target concentration

and the regulatory limit (MRL). Lower the target concentration compared to regulatory concentration, fewer replicates are needed for the same degree of confidence. For example-

(i) If the Screening Target Concentration is set at half MRL or lower, 20 "Screen Positive" Control Samples (with the occurrence of one or no false-compliant results) is sufficient to demonstrate that CCβ (smallest content of the analyte that may be detected in a sample with an error probability of β) is less than the MRL.
(ii) If the Screening Target Concentration is set between 50% and 90% of the MRL, at least 40 "Screen Positive" Control Samples with no more than 2 false-compliant results will be sufficient to demonstrate that CCβ is less than the MRL.
(iii) If the sensitivity of the screening test is such that the Screening Target Concentration approaches the MRL, more "Screen Positive" Control Samples may be required. A maximum of 60 replicates with no more than 3 false-compliant results is needed to demonstrate that CCβ is fit for the purpose.

If the screening method is applicable to one matrix but to different animal species, 60 different samples could be taken from the different species (e.g. 20 porcine muscles, 20 bovine muscles, and 20 poultry muscles). According to the guideline, these larger studies can be undertaken in sequential stages i.e. the first twenty blank samples and the same samples fortified with antibiotic tested, and if more than one spiked sample falls below the cut-off level, the validation can be abandoned at this point, the Screening Target Concentration has to be increased and the validation exercise repeated.

According to manufacturer's instruction, the kit can be used for determination of tetracycline in meat, milk, fish, shrimp and honey samples. In this study, we took 20 beef, 20 chicken and 20 shrimp samples considering the three type of muscle as one matrix because all the procedure for extraction and detection of TCs from these matrices are almost similar with the exception for shrimp. In this case addition of hexane is not required.

Twenty blank samples from each of three type matrices supplied by IAEA were used to study specificity. Samples were spiked at MRL (100 µg/kg) and chosen as target concentration. Ten samples from each were analyzed by one operator and rest tens were carried out by another operator. Six days were scheduled for the validation of the kit. Each day, 10 samples were analyzed (10 blank samples and the same samples spiked with the antibiotic).

After the analysis of 60 spiked (or incurred) samples at MRL where less than or equal to 5% of false-compliant results may present, the spiking level would be the detection capability CCβ of the method.

Statistical approach that took into account the β error (i.e. false-compliant rate) of 5% in selecting detection capability was chosen as recommended in the guideline of Community Reference Laboratories Residues 20/1/2010 for the validation of screening methods explained in Annex II [15]. The positivity threshold T and the cut-off value Fm were calculated from the same Annex. Cut-off factor/level is the response in a screening test which indicates that a sample contains an analyte at or above the screening target concentration. The positivity threshold T and the cut-off factor Fm are matrix specific.

$$T = B - 1.64 \times SDb \quad (1)$$

where B is the mean and SDb is the standard deviation of the optical densities (ODs) of the blank samples, and

$$Fm = M + 1.64 \times SDs \quad (2)$$

where M is the mean and SDs is the standard deviation of the ODs of the spiked samples.

The assay was considered valid only when Fm<T and CCβ was validated when Fm<B. The number of spiked samples with mean OD below the cut-off level (i.e. screen positive) was identified.Moreover, the false-positive rate was determined. If T<Fm<B, the false-positive rate is greater than 5%. In the case of Fm<T, the false-positive rate is below 5%. If more than 5% of the spiked samples at the screening target concentration gave an OD greater than the cut-off level (i.e. false-negative), this concentration chosen for the spiking study was considered too low for validation.

### 2.7.2 Applicability

Applicability of tetracycline ELISA kits to three types of matrix- beef, chicken and shrimp was tested. This study was performed together with specificity and detection capability. Twenty

spiked samples from each type of matrices were used separately to determine detection capability. It was also checked whether the same CCβ could be used for the three types of muscle tissues from beef chicken and shrimp.

### 2.7.3 Ruggedness

Ruggedness study is usually performed by changing minor changes in the method to observe the effect of the changes on different performance parameters. This parameter was not performed because of the simplicity of the method. Earlier kit Art No. R 3503 of same manufacturer used solid phase extraction procedure but the Art No. R 3505 did not need solid phase extraction step. Otherwise there were shortage of kit during the study and it was not so easy to get the kit instantly in the perspective of Bangladesh. However, we have checked matrix effect and the effect of operators on CCβ that could also be included in ruggedness study.

### 2.7.4 Stability

According to Commission Guideline, if the stability of the analyte is known, there is no need to determine the stability again. We did not determine the stability of tetracycline since its stability has already been determined by other researchers [17]. However, we studied the stability of the kit which was performed after six month of the first experiment.

## 3. RESULTS AND DISCUSSION

### 3.1 Specificity and Detection Capability

The specificity and detection capability CCβ of the ELISA kit for three types of matrix were determined separately. Twenty pair (blank and spiked) samples were analyzed for each type of matrix. The distributions of ODs of blank and spiked samples at100 µg/kg are illustrated in Figs. 1, 2 and 3. Difference of ODs between blank and fortified samples of beef, chicken and shrimp were so high that it allowed a good detection at100 µg/kg. For blank beef samples, highest and lowest ODs were 2.343 and 1.816 respectively, and for spiked samples they were 0.761 and 0.27 respectively (Fig. 1). In case of blank chicken samples, highest and lowest ODs were 2.191 and 1.608 respectively whereas they were 0.647 and 0.294 for spiked samples respectively (Fig. 2). For blank shrimp samples, highest and lowest ODs were 2.121 and 1.579 respectively whereas they were 0.412 and 0.221 for spiked samples respectively (Fig. 3). For all the cases none of the ODs of the spiked samples overlaps with the range of ODs of blank samples.

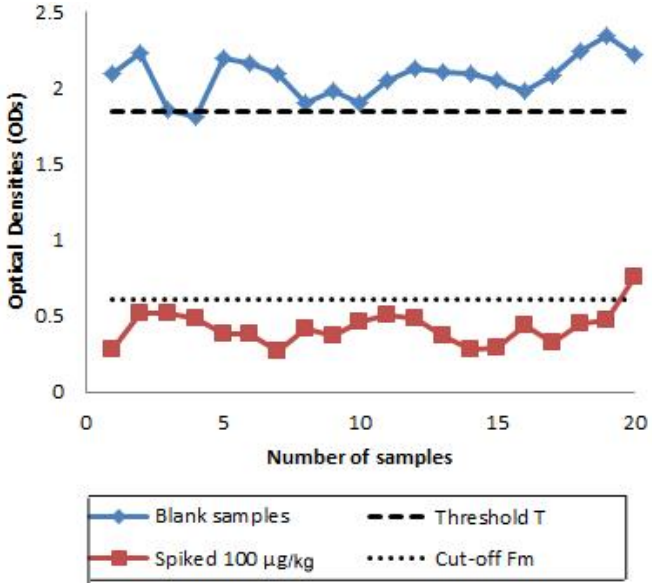


**Fig. 1. Graphical illustration of the distribution of optical densities (ODs) of blank samples (beef muscle) and blank samples spiked at 100 µg/kg with oxytetracycline.**

*This figure represents the distribution of the ODs after the analyses of 20 blank samples of beef muscle of different origin supplied by IAEA and the analyses of the same 20 samples fortified at 100 µg/ kg with oxytetracycline.*

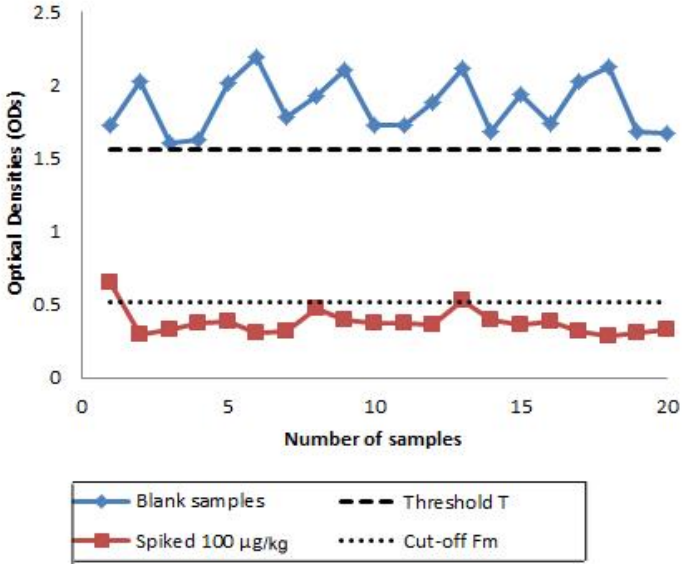


**Fig. 2. Graphical illustration of the distribution of optical densities (ODs) of blank samples (chicken muscle) and blank samples spiked at 100 µg/kg with oxytetracycline.**

*This figure represents the distribution of the ODs after the analyses of 20 blank samples of chicken muscle of different origin supplied by IAEA and the analyses of the same 20 samples fortified at 100 µg/kg with oxytetracycline.*

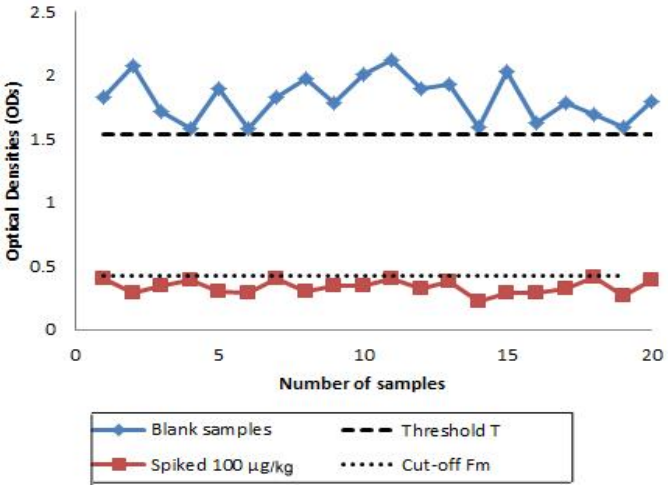


**Fig. 3. Graphical illustration of the distribution of optical densities (ODs) of blank samples (shrimp muscle) and blank samples spiked at 100 µg/kg with oxytetracycline.**

*This figure represents the distribution of the ODs after the analyses of 20 blank samples of chicken muscle of different origin supplied by IAEA and the analyses of the same 20 samples fortified at 100 µg/kg with oxytetracycline.*

The mean ODs for blank and spiked samples are presented in Table 1. From these data, the positivity threshold T, cut-off factor Fm and mean response of the blank B were calculated. In competitive immunoassays, OD of blank sample is greater than the OD of spiked sample and the concentration of the antibiotic is inversely proportional to the OD. According to the Commission Decision 2002/657/EC [13] and the European Guideline for the validation of screening methods [14], the detection capability CCβ is validated when the cut-off factor Fm < B (for competitive ELISA) and the kit is valid when Fm<T.

For beef, chicken and shrimp, threshold values T were equal to 1.85, 1.558 and 1.532 OD respectively whereas, cut-off values Fm were equal to 0.612, 0.519 and 0.424 OD respectively. Mean response B of blank samples for beef, chicken and shrimp were 2.077±0.138, 1.866±0.189 and 1.816±0.174 OD respectively. Therefore, the detection capability CCβ (100µg/kg) of the method is valid because Fm<B for all three type of matrices and the assay is also valid because Fm<T for all mentioned matrices.

According to EU guideline, the laboratory has to determine the false positive rate also. If T < Fm < B, false-positive rate is greater than 5%. In the case of Fm < T, the false-positive rate is below 5%. If T is chosen as the decision limit to determine the positivity of samples, the false positive rate for beef, chicken and shrimp samples are equal to 5, 0 and 0% respectively (Table 1 and Figs. 1, 2 and 3). If Fm is chosen as decision limit to determine negativity of samples, the false negative rate for beef, chicken and shrimp samples are equal to 5, 5 and 0% respectively (Table 1 and Figs. 1, 2 and 3).

The detection capability CCβ of the ELISA kit for three types of muscle matrix was fixed at 100 µg/kg because mean Fm values of the three type matrices could be applicable to determine screen positive sample within the false negative rate at or below 5%. In this regard, Okerman et. al 1998 showed that the matrix does not influence the detection of tetracyclines substantially, contrary to other antibiotics [18]. Finally, when the OD of one sample is less than or equal to Fm, we can assume that the concentration of tetracycline equivalent (as described by the kit's manufacturer) is lower than or equal to 100µg/kg. Tetracycline equivalent means oxytetracycline, tetracycline, chlortetracycline and doxycycline. During routine analyses, the recommendation is to calculate a daily threshold T and a daily cut-off factor Fm, which would be based on the analyses of negative and positive quality controls (QCs) on each day. The assay is valid only if T is greater than Fm daily.

## 3.2 Applicability

Applicability of ELISA kit for screening tetracycline residues in muscle matrices been proved during specificity study and determination of detection capability CCβ. It would be concluded that the ELISA kit is applicable for the screening of tetracycline group antibiotic residues in the muscle of beef, chicken and shrimp with the same CCβ 100µg/kg having <5% false negative rate.

## 3.3 Ruggedness

Ruggedness studies use the deliberate introduction of minor reasonable variations by the laboratory and the observation of their consequences on the results [14]. Matrices and operators could be included in ruggedness study. In this case, applicability and ruggedness could be performed combined. We could not perform the ruggedness study properly due to lack of ELISA kit and its cost. However, we did not find significant variations between the performances of two operators. Effect of matrices is shown in Table 1. The method is rapid, simple and applicable in same degree to the three types of muscle tissues.

## 3.4 Stability of Analyte

Stability of tetracycline antibiotic was conducted by Berendsen et al. [17] in solution and in muscle matrix. At -18℃, tetracycline in solution was storable for maximum 3 months and there were no severe degradation observed in matrix. They observed that especially oxytetracycline and tetracycline were instable in methanolic solutions which were also demonstrated by Okerman et al. [19]. According to their findings, tetracycline and oxytetracycline stock solutions should be prepared fresh on a weekly basis if they are stored at 4°C and every two months (tetracycline) or every three months (other tetracyclines) if stored at -18°C. The difference in stability of oxytetracycline and tetracycline compared to chlortetracycline and doxycycline is confirmed by Okerman et al. [19]. They reported no loss of activity for chlortetracycline and doxycycline during 6 months storage at -20°C whereas oxytetracycline and tetracycline loose approximately 25% of their activity in the same period.

## 3.5 Stability of the Kit

The shelf life of the kit is one year when stored at 2-8℃ as mentioned by manufacturer. After six month of first experiment, we performed the sensitivity of the kits using seven blank and spiked samples of beef, chicken and shrimp and the result summarized in Table 2. The sensitivity of the kit became lower with the time but still can be used successfully because the Fm value clearly lower the T value and the reading ODs did not overlap any one of ODs of spiked samples.

**Table 1. Determination of the threshold value T and cut-off value Fm and detection capability CCβ**

| Matrix | $N_b$ | $B \pm SD_b$ (uOD) | $M \pm SD_s$ (uOD) | T (uOD) | $N_s$ | Fm (uOD) | No. of false positive | No. of false negative | CCβ (µg/kg) |
|---|---|---|---|---|---|---|---|---|---|
| Beef | 20 | 2.077±0.138 | 0.424±0.114 | 1.85 | 20 | 0.612 | 1/20 | 1/20 | 100 |
| Chicken | 20 | 1.866±0.189 | 0.372±0.086 | 1.558 | 20 | 0.519 | 0/20 | 1/20 | 100 |
| Shrimp | 20 | 1.816±0.174 | 0.336±0.054 | 1.532 | 20 | 0.424 | 0/20 | 0/20 | 100 |

*B, mean of blank samples; M, mean of spiked samples; $SD_b$, standard deviation of blank samples; $SD_s$, standard deviation of spiked samples; $N_b$, number of blank samples; $N_s$, number of spiked samples; uOD, unit of optical density*

**Table 2. Performance of ELISA kit after six months of first experiment in terms of threshold value T, cut-off value Fm and detection capability CCβ**

| Matrix | $N_b$ | $B \pm SD_b$ (uOD) | $M \pm SD_s$ (uOD) | T (uOD) | $N_s$ | Fm (uOD) | Number of false positive | CCβ (µg/kg) |
|---|---|---|---|---|---|---|---|---|
| Beef | 7 | 1.113±0.114 | 0.373±0.048 | 0.927 | 7 | 0.451 | 0/7 | 100 |
| Chicken | 7 | 0.952±0.132 | 0.442±0.110 | 0.735 | 7 | 0.621 | 0/7 | 100 |
| Shrimp | 7 | 1.744±0.203 | 0.322±0.067 | 1.412 | 7 | 0.43 | 0/7 | 100 |

*B, mean of blank samples; M, mean of spiked samples; $SD_b$, standard deviation of blank samples; $SD_s$, standard deviation of spiked samples; $N_b$, number of blank samples; $N_s$, number of spiked samples; uOD, unit of optical density.*

## 4. CONCLUSION

To our knowledge, this study represents the first example of the validation of a commercial ELISA kit for screening tetracycline residues in muscle of beef, chicken and shrimp in perspective of Bangladesh. The test kit was validated considering EU Commission regulations. Regarding the Commission's decision 2002/657/EC [13], a screening method should have false-negative rate lower than 5% at the level of interest. False-negative rate showed by the kit for beef, chicken and shrimp samples were 5, 5 and 0% respectively with same detection capability100µg/kg. The technical advantage of this kit is that the extraction procedure does not need solid phase extraction cartridges (SPE). The methods are easy, specific and robust. Therefore, the kit fulfills the EU criteria and applicable to the screening of TC residues in muscle of beef, chicken and shrimp. Mean of the Fm values for three types of matrix could be used as single cut-off factor but it is better to use corresponding Fm values since there is little matrix effect on ODs in case of beef. We also suggest that, every laboratory should update/adjust the cut-off value before analysis of sample using control positive and control negative samples for antibiotic residue monitoring program.

## CONSENT

It is not applicable.

## ETHICAL APPROVAL

As per international standard or university standard, written approval of Ethics committee has been collected and preserved by the author(s).

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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