In-House Validation of a Commercial Enzyme-Linked Immunosorbent Assay kit for Screening of Furazolidone Residue in Chicken and Egg

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Abstract

Furazolidone, a nitrofuran antibiotic which was once widely used in the livestock industry and aquaculture, is now prohibited in several countries. This drug was prohibited by Commission Decision 2003/181/EC because of its potential carcinogenic and mutagenic effects on human beings. Detection of furazolidone is difficult because it is readily metabolized within animal tissues but its tissue bound metabolite, 3-amino-2-oxazolidinone (AOZ) can be detected using different techniques. Here, we describe the in-house validation of a commercial enzyme-linked immunosorbent assay (ELISA) kit from Europroxima, The Nederlands, for screening AOZ in chicken and egg. According to European Commission Guideline CRL/2010, the kit was validated by evaluating two performance characteristics- specificity and detection capability (CCβ). Tissue-bound AOZ was separated/isolated by acid hydrolysis followed by derivatization with 2-nitrobenzadehyde before detection with ELISA. Detection capability (CCβ) of the kit for chicken samples was 0.2µg/kg where it was 0.3µg/kg for the egg. False compliant rates were 0% for both cases. The limit of detection (LoD) was 0.03µg/kg for both types of matrices. Cut-off level *Fm* for chicken samples was 0.789 OD where it was o.686 OD for the egg. Our results show that the ELISA kit used in this study is suitable for regulatory purposes.

Keywords: AOZ (3-amino-2-oxolidinone), ELISA, validation, Furazolidone

1. Introduction

Chicken and eggs produced from the poultry farms are a very good source of protein in Bangladesh, and they supply about 22-27% of the total animal protein consumed in our country [1]. Due to income growth, increased population, urbanization, and dietary changes of the people, demand for meat and egg has increased dramatically. Therefore, to meet the demand of these protein sources, many poultry farms were established in the last few years. Use of antibiotics plays a great role to reduce the mortality rate of chicken. Nitrofuran group of antibiotics is frequently used in the poultry farms in many countries for their robust antibacterial, pharmacokinetics, and growth promoting activities [2].

Furazolidone, a member of nitrofuran group of antibiotics and its marker metabolite 3-amino-2-oxazolidinone (AOZ) were found to be genotoxic, mutagenic, and hepatotoxic in mammals [3]. The European Union (EU) banned the use of nitrofuran drugs in food producing animals due to concerns about the carcinogenicity of the drug residues and their potential harmful effects on human health [4-5]. The use of nitrofurans in livestock has also been prohibited in countries such as Australia, the USA, the Philippines, Thailand, and Brazil [6]. In response to the recommendation of Directorate F-Food and Veterinary Office (FVO), European Commission; Ministry of Fisheries and Livestock, Government of Bangladesh, issued a notification in 2007 (Vide memo No. MOFL/F-5/(FVO)/23/2007/ 698 dated 12-11-2007) not to use nitrofuran in any animal feed and medicine in any form [7].

Furazolidone, nitrofurazone, furaltadone, and nitrofurantoin are the members of the nitrofuran group of antibiotics. Following ingestion, these nitrofuran parent drugs are rapidly metabolized to their corresponding tissue bound metabolites 3-amino-2-oxazolidinone (AOZ), semicarbazide (SEM), 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ) and 1-aminohydantoïne (AHD) respectively [8]. These metabolites have been reported to remain in the tissues for many weeks after administration [9]. About 67-90% of AOZ was found to be present in the incurred muscle and liver of pig samples, even after cooking, frying, grilling, roasting, and microwaving. It was also reported that, eight months of storage did not have a significant effect on the residual concentration of nitrofurans in incurred samples [10]. AOZ was found to be deposited in egg yolk, eggshell, and in the white portion of the egg and was stable up to 12 months during storage at 4°C [11-12]. Because of their metabolic stability, metabolites of nitrofuran derivatives are used as a marker/indicator of drug residues in analytical methods [13]. There is no maximum residue limit (MRL) for nitrofuran antibiotics in chicken and egg but the EU set minimum required performance limit (MRPL) at 1 µg/kg [14].

As far as we know, there is no laboratory in our country that provides antibiotic residue analysis services for foods of animal origin regularly. However, fish inspection and quality control (FIQC) laboratories established with the help of the European Union are providing analytical services only for shrimp. Our laboratory has been working on developing and validating analytical methods for screening and confirmatory analysis of veterinary drug residues in animal foods origin to facilitate international trade and monitoring local markets. The receptor laboratory must investigate the performance of a screening method or commercial kit obtained from the originator laboratory whether it is already validated by them [15]. In the present study, in-house validation (abridged validation) of a commercial ELISA kit from Europroxima, Netherlands, was carried out for screening AOZ in chicken and egg matrices following the EU Guideline CRL/2010 [15].

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2. Materials and Methods

2.1 Chemicals and standard solution

AOZ ELISA kit was bought from Europroxima, The Netherlands; lot nr: SN 6959 (expiry date 02/2019). Test kit contained antibody-coated microtiter plate, sample dilution buffer, AOZ stock solution, NPAOZ standard solution, AOZ-conjugate, anti-AOZ-antibody, red chromogen stop solution and washing buffer. Nitrofuran metabolites AOZ, AMOZ, AHD, SEM and their Nitrophenyl derivatives NPAOZ, NPAMOZ, NPAHD, NPSEM and other chemicals were bought from Sigma Aldrich (Germany).

2.2 Blank matrix

Chicken breast muscle and hen egg were used throughout the study as blank matrices. From the perspective of our country, it was difficult to standard reference blank matrix maintaining at appropriate storage conditions. Therefore, fed chicken (Local variety) and their egg collected from the specific rural areas (Dighapatia, Natore) of the country.

2.3 Principle of AOZ ELISA

Microtiter plate based AOZ consists of well pre-coated with the antibody against AOZ. This ELISA abides by the principle of competitive enzyme immunoassay and is used for qualitative and semi-quantitative detection of AOZ. Horseradish peroxidase (HRP) labeled AOZ and standard solution or samples to be analyzed, are added to the wells. Free AOZ released from the samples or standards and AOZ-HRP conjugate competes for the specific antibody binding sites. The content of the wells is then allowed for incubation for 30 minutes in the dark followed by a washing step that removes non-bound reagents. Substrate/ chromogen solution (tetramethylbenzidine, TMB) is added to the wells. Bound AOZ-HRP conjugate is visualized when the colorless substrate/ chromogen is transformed into colored product, Enzyme-substrate reaction is stopped by the addition of sulfuric acid. The color intensity developed after the addition of stop solution is measured photometrically at 450 nm. The optical density is inversely proportional to the AOZ concentration in the sample.

2.4 Calibration curve preparation

A standard dose-response curve was prepared earlier in this laboratory [16]. Preparation of the graph was aimed to verify the sensitivity of the kit in terms of limit of detection (LoD) claimed by the manufacturer. Briefly, the graph was prepared using the stock NPAOZ (nitrophenyl derivative of AOZ) solutions supplied by the manufacturer at different concentrations- 0.01, 0.026, 0.064, 0.16, 0.40, and 1.00µg/kg in duplicates. The ELISA graph for AOZ in assay buffer is logarithmic and the responses of the concentrations are expressed as B/ Bo ratio in %, where B is the absorbance at a given concentration of the analyte and Bo the absorbance of the zero standard. A semi-logarithmic graph was created by plotting %B/Bo values in the Y axis and AOZ equivalent concentration in the X axis which is shown in Fig. 1. In this type of graph, the concentration of AOZ equivalent is inversely proportional to %B/Bo value.

2.5 Spiking blank sample

Target concentrations of analyte for spiking blank samples should be lower or at the MRPL as recommended by the EU [15, 17]. The minimum required performance limit (MRPL) of AOZ in edible food set by the EU is 1µg/kg. In this study, the blank samples of chicken were spiked with AOZ standard at 0.2 and 0.3µg/kg where the egg samples were spiked only at 0.3µg/kg.

2.6 Sample extraction and derivatisation procedure

Extraction of tissue bound AOZ (3-amino-2-oxazolidinone) from fortified samples and their derivatization was carried out according to the kit manufacturer's instruction performed earlier by Rana et al [16]. Briefly, 1gm of the homogenized sample was taken in a 15 ml centrifuge tube, and 4 ml double distilled water, 0.5 ml 1 M HCl and 150 µl derivative agent (10 mM 2-nitrobenzaldehyde in dimethyl sulfoxide) was added to it. After a head over head mixing, 2.5 ml 0.25M K_2HPO_4, 0.4 ml 1 M NaOH, and 5 ml ethyl acetate were added to the tube. All the components were mixed again for 1 minute followed by centrifugation for 10 minutes at 2000 x g. A 2.5 ml ethyl acetate layer was transferred into a 4 ml glass tube and subjected to evaporation at 50ºC under nitrogen blow. The residue was dissolved in 1ml sample dilution buffer and extracted with l ml hexane. The hexane layer was removed by centrifugation at 2000xg for 10 minutes. Finally, 50 µl of the aqueous phase was used for ELISA.

2.7 Validation protocol

To adopt the ELISA kit in our laboratory, we have performed abridged validation as recommended by the European Guideline for the validation of screening methods [15]. The performance characteristics to be determined for abridged or in-house validation were- specificity and detection capability (CCβ). By definition abridged validation is less intensive than full validation. Its purpose is solely to indicate that the transferred method will work reliably in the receptor laboratory.

2.7.1 Specificity and detection capability (CCβ)

During abridged validation, a reduced number of samples is required compared to full validation. In this case, 20 samples for one matrix are enough whichever is the screening target concentration [15]. The MRPL for AOZ in foods of animal origin is 1 µg/kg set by the EU. If the assay kit shows 5% or less false positive rate, then the selected target concentration will be the detection capability of the method CCβ (smallest content of analyte that may be detected in a sample with an error probability of β*)* and it must be at or lower the MRPL [15]. To determine the CCβ and specificity of the method, we took into consideration the MRPL level, and the CCβ of the same kit for screening AOZ in shrimp (0.3µg/kg) determined earlier in our laboratory (data yet to publish). Samples from both types of matrices were spiked at 0.2µg/kg. If the target concentration (blank sample spiking concentration) failed to meet the requirements of validation, then it was increased to 0.3µg/kg.

Two days were scheduled for this study. Each day, 10 blank samples and the same samples spiked with AOZ at the target concentration (the estimated CCβ) were analyzed by two operators. Detection capability and specificity were determined both from classical and statistical approaches explained in the guideline of Community Reference Laboratories residues (CRL) 20/1/2010 for the validation of screening methods [15-16].

2.7.2 Classical approach

Twenty blank samples of both egg and chicken were spiked with AOZ at 0.2 and 0.3 µg/kg respectively. Responses of blank and spiked samples of both types of matrices were recorded. The lowest response of the blank and the highest response of the spiked samples were noted. If none of the responses of spiked samples of a type of matrix overlaps with the range of responses of its blank samples, then spiking concentration (i.e. screening target concentration) will be the CCβ of the method for that type of matrix with 0% false compliant rate, and the highest response of the spiked samples will be the cut-off factor for screening AOZ in that matrix. If there are overlaps between two sample populations which is greater than 5% (two overlaps among responses of blank and spiked samples) then, CCβ of the method will be higher than the target concentration, and the assay must be repeated with higher screening target concentration.

2.7.3 Statistical approach

In the statistical approach, the positivity threshold *T* and the cut-off factor *Fm* were calculated. 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 if *T* was greater than *Fm* and smaller than *B*. Moreover, the number of spiked samples with OD below the cut-off level was identified. If more than 5% of the spiked samples at the screening target concentration (estimated CCβ) gave an OD greater than the cut-off level, the concentration chosen for the spiking/ fortification was considered too low for validation. Detection capability CCβ is validated when the cut-off factor $Fm<B$, and false-positive rate is at or below 5%.

3. Results and Discussion

3.1 Kit sensitivity

United States directives use the limit of detection (LoD) as a performance characteristic of a qualitative analytical method for validation purposes. It is defined as the lowest concentration level of an analyte in a sample that can be determined statistically different from a blank at a specified level of confidence. It does not account for false positive or false negative [18]. In European directives, detection capability (CCβ) is used instead of LoD. It is defined as the smallest content of analyte that may be detected in a sample with an error probability of β [15]. The manufacturer of the kit expressed the sensitivity of the kit in terms of limit of detection (LoD) and specificity of the kit towards AOZ (100%). Therefore, LoD declared by the manufacturer was verified using the same formula they used. In this study, LoD was calculated from the average response of 20 blank samples and the standard deviation (SD) using the formula, LoD= mean response of blank B+ 3SD. The resulted value was converted to %B/Bo and read from the calibration curve (Fig. 1). We found LoD of the kit 0.03µg/kg for both egg and chicken samples, while the value for both matrices claimed by the manufacturer was 0.05µg/kg. In our previous study, LoD was found to be 0.05 for the shrimp matrix [16]. Jester et al [19], evaluated the AOZ kit from r-BIOPHARM, Germany, and found LoD 0.05 µg/kg for shrimp matrix. However, LoD should not be used as cut-off level for screening purposes to avoid false positive results.

3.2 Determination of detection capability CCβ and cut-off factor Fm

Analytical responses of blank and spiked samples for both egg and chicken were summarized in Table 1 and illustrated in Figs. 2-4. Detection capability CCβ and specificity were calculated using both classical and statistical approaches described in Annex I and II of EU guideline CRL/2010 [15].

3.2.1 Classical approach

At first, both chicken and egg samples were spiked with AOZ at 0.2µg/kg and their analytical responses were represented in Figs. 2-4. One response of the spiked chicken samples overlaps with the five responses of its blank samples (Fig. 2). So, a clear cut-off level was not established for screening the samples with AOZ positive or negative. From these data, it was inferred that detection capability CCβ might be greater than 0.2µg/kg, and the screening target concentration of 0.2µg/kg could not be truly detected using this method. We again fortified 20 blank samples of chicken at 0.3µg/kg, and the responses were illustrated in Fig. 3. This time, there were no overlaps among the responses of blank and spiked samples of the chicken matrix. Therefore, the highest response of the spiked samples (0.778) was the cut-off level, and 0.3µg/kg was the CCβ of the method with a 0% false compliant rate.

If we analyze the range of responses of egg samples (Fig. 4), the cut-off level will be 0.731 (highest response of fortified samples that did not overlap any responses of blank), and CCβ of the method for this matrix was 0.2µg/kg with 0% false compliant rate.

Theoretically, if 19 of the spiked samples were declared non-compliant, then CCβ is equal to the level of fortification. Additionally, if all of the fortified samples are declared non-compliant, then CCβ is lower than the level of fortification. In practice, the levels of fortification for each matrix were chosen to ensure that all of the fortified samples were declared non-compliant to avoid the problem

of false negatives. However, we should keep in mind that the level of fortification must be as low as possible.

3.2.2 statistical approach

Figs. 3-4 represent the distribution of ODs of chicken and egg samples respectively. There is a clear difference of OD between blank and spiked samples for both types of matrices, and therefore it allowed a good detection at 0.3µg/kg for chicken (Fig. 3) and 0.2 µg/kg for egg (Fig. 4). So, the detection capability CCβ of the kit for chicken and egg would be 0.3 and 0.2µg/kg respectively.

From the data, the mean response of blank samples *B*, mean response of spiked samples *M*, positivity threshold *T*, and cut-off factor *Fm* were calculated and presented in Table 1. For the egg, the calculated threshold value *T* and cut-off factor *Fm* were equal to 0.975 and 0.686 OD respectably. For chicken, calculated threshold value *T* and cut-off factor *Fm* were equal to 0.844 and 0.789 OD respectably. According to EU guidelines, the kit/method is valid for screening AOZ in chicken and egg because *of Fm<T* for both matrices (Table 1). When the OD of one sample is greater than *Fm*, we can assume that the sample is screen negative.

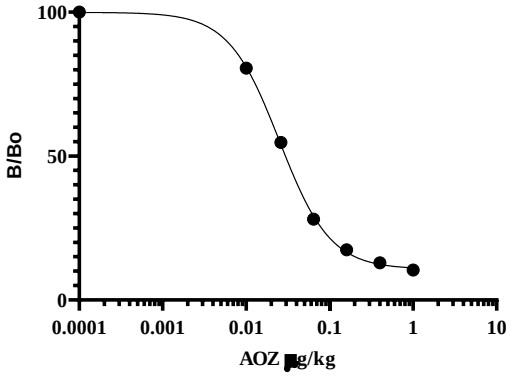

Fig. 1: Standard curve. The X-axis is in logarithmic scale. Each point represents the mean of two replicates. R squared value = 0.9996 and IC_{50} value = 0.025 µg/kg. This Fig. was taken from the previous work of Rana et al [16] for the calculation of LoD

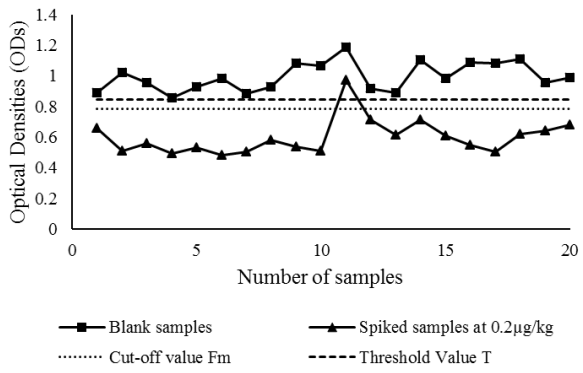

Fig. 2: Graphical illustration of the distribution of optical densities (ODs) of blank chicken samples and the same samples spiked with AOZ at 0.2 µg/kg. The number of samples for each population is 20

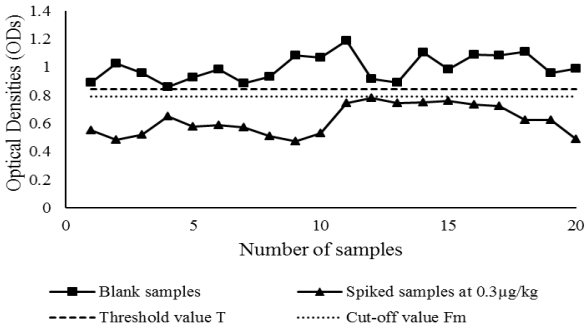

Fig. 3: Graphical illustration of the distribution of optical densities (ODs) of blank chicken samples and the same samples spiked with AOZ at 0.3 µg/kg. The number of samples for each population is 20

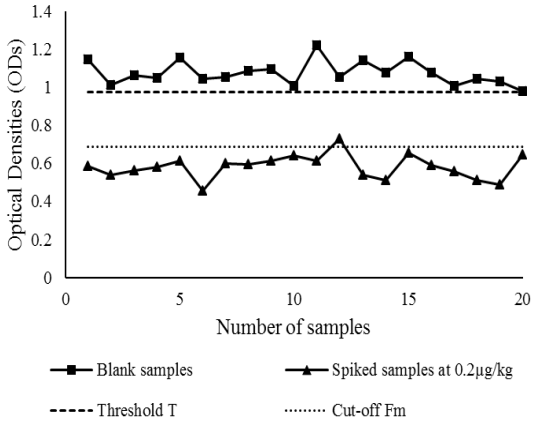

Fig. 4: Graphical illustration of the distribution of optical densities (ODs) of blank egg samples and the same samples spiked with AOZ at 0.2 µg/kg. The number of samples for each population is 20

Table 1: Determination of threshold value *T*, cut-off factor *Fm* and detection capability CCβ for AOZ ELISA kit.

Matrix	N_b	B±SD_b (uOD)	Ms±SD_s (uOD)	˙(uOD)	N_s	Fm (uOD)	Nfp	CCβ (µg/kg)
Chicken	20	0.996±0.093	0.620±0.106	0.844	20	0.789	0/20	0.3
Egg	20	1.076±0.062	0.581±0.064	0.975	20	0.686	0/20	0.2

ELISA, enzyme linked immunosorbent assay; B, mean of blank samples; M_s, 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; Nfp, number of false positive; T, threshold value; Fm, cut-off factor; uOD, unit of optical density.Detection capability CCβ is validated when *Fm < B*, and the false-positive rate is at or below 5%; when *T<Fm<B*, the false positive rate is greater than 5% [15]. In our result, *Fm<B* for both types of matrices and false positive results were 0% (Table 1, Figs. 3-4). Therefore, CCβ for both types of matrices was validated.

Some authors evaluated the performance characteristics of commercial ELISA for screening marker metabolite AOZ of furazolidone antibiotic in different matrices. Krongpong et al [20] reported that Ridascreen Nitrofuran ELISA kit, R-

Biopharm, Darmstadt, Germany was capable of detecting AOZ at 1.0 µg/kg in eel samples. Elizabeta et al [21] performed in-house validation of ELISA kit from r-BIOPHARM, Germany in hen egg, and found the CCβ 0.5µg/kg with 0% false positive rate. Cooper et al [22] developed first ELISA for the detection of AOZ in prawn with CCβ 0.4µg/kg having a 5% false positive rate. On the contrary, the CCβ of the ELISA kit from Europroxima, The Nederlands, used throughout this study was 0.2µg/kg for egg and 0.3µg/kg for chicken with 0% false compliant rate for both matrices. The detection capability of an ELISA kit for the same matrix may vary due to the operator's skill, the sensitivity of the ELISA kit, and finally, the selection of target concentration for the fortification of blank samples.

4. Conclusion

A practical example of in-house validation of a commercial immunochemical method (ELISA kit) for screening AOZ, metabolite of the nitrofuran drug furazolidone, in chicken muscle and hen egg has been reported. According to the EU guideline, if the originating laboratory performed complete validation of the kit, there is no need for the receptor laboratory to perform full validation for a specific matrix again. But the receptor laboratory must perform abridged/in-house validation of the kit to verify two performance characteristics - specificity and detection capability. Some countries including the USA validate the kit considering LoD as a parameter for determining the sensitivity. But LoD does not show the false compliant result, where detection capability (CCβ) shows. Here, we also determined the LoD. The value of LoD (0.03µg/kg) of the kit was lower than the manufacturer declared value (0.05µg/kg) though it was found the same in the case of the shrimp matrix (found in our previous study). In this study, validation results of specificity (false compliant rate is 0%) and detection capability (0.2µg/kg for egg and 0.3µg/kg for chicken) are compatible with the EU requirements for qualitative assay. Detection capabilities of the kit for both matrices being tested were sufficiently lower than the MRPL (1 µg/kg). There is no validation report on the performance characteristics of the AOZ ELISA kit from Europroxima, The Nederlands for screening furazolidone antibiotic residues in foods of animal origin. Therefore, this validation is very important to adopt the kit for performing AOZ residue screening in foods from the perspective of Bangladesh to facilitate international trade and to monitor the local market as well. The screening or qualitative immunochemical method gives a result/response which is considered as above or below a cutoff level. For routine analysis, a daily threshold *T* and a daily cut-off factor *Fm* should be calculated based on the analyses of negative and positive quality controls (QCs). These values must be updated or, verified when a new batch or a lot of the kit arrives. The assay is valid only if the threshold value *T* is greater than *Fm* value daily.

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