

DETECTION OF OXYTETRACYCLINE IN BROILER CHICKEN MEAT MARKETED IN SEVERAL CITIES IN JAVA ISLAND USING ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) METHOD

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ABSTRAK

Oksitetrasiklin (OTC) adalah salah satu jenis antibiotika golongan tetrasiklin berpektrum luas yang banyak digunakan pada industri ternak ayam. Namun penyalahgunaan dengan dosis berlebihan berpotensi terhadap terbentuknya residu pada produk ternak yang dapat membahayakan konsumen berupa reaksi alergi maupun resistensi. Penelitian ini bertujuan untuk mendeteksi residu OTC dalam daging ayam pedaging asal pasar tradisional dan swalayan di Depok-Bekasi, Bandung, Cilegon, Surakarta dan Yogyakarta secara *indirect competitive enzyme-linked immunosorbent assay* (icELISA). Hasil analisis pada 67 daging ayam pedaging menunjukkan hanya ada 1 (1,5%) sampel yang terdeteksi adanya residu OTC sebesar 86,1 ng/g dan masih di bawah batas maksimum residu OTC yang diijinkan (100 ng/g). Meskipun demikian, penerapan regulasi yang ketat terhadap penggunaan OTC ini di bidang peternakan ayam serta monitoring keberadaan residunya pada produk ternak sebelum dipasarkan sangat diperlukan untuk mencegah pengaruh buruk dari keberadaan residu tersebut pada produk pangan asal ternak.

Kata kunci : oksitetrasiklin (OTC) residu, icELISA, daging ayam pedaging

ABSTRACT

Oxytetracycline (OTC) is one of the tetracycline (TCs) broad-spectrum antibiotics widely used in the chicken industry. However, improper use of OTC with excessive doses potentially leads to residue formation in animal products that can be harmful to consumers in the form of allergic reaction or resistance. This study aimed to detect OTC residues in broiler chicken meat, marketed in traditional markets and supermarkets in Depok, Bekasi, Bandung, Cilegon, Surakarta and Yogyakarta using indirect competitive enzyme-linked immunosorbent assay (icELISA) method. The analyses of 67 broiler meat samples showed only 1 (1.5%) sample was positive for the OTC residue at 86.1 ng/g which meant below the maximum residue limits of permissible OTC (100 ng/g). Nevertheless, a stricter regulation for the use of OTC in the poultry industry and the monitoring of its residue in chicken products prior to marketing is still necessary to avoid the adverse effects of the residue present in animal products.

Keywords: oxytetracycline (OTC) residue, icELISA, broiler chicken meat

INTRODUCTION

Oxytetracycline (OTC) is a broad spectrum antibiotic in the tetracycline class which is widely used for the prevention and control of diseases in poultry industry (Berendsen and van Rhijin, 2006)

because of its availability, relatively cheaper price, and more easily use by oral administration through drinking water or feed. OTC is a broad-spectrum antibiotic that is bacteriostatic, that is difficult to be metabolized and partly excreted in the form of parent compounds due to its high

solubility in water (Slana and Dolenc, 2013).

However, overuse and insufficient lengths of withdrawal of OTC antibiotic in animal husbandry may result in the presence of residues in animal products (Centikaya *et al.*, 2012) that may endanger human health and even lead to its presence in the environment (Aga *et al.*, 2003). For the purpose of monitoring and ensuring food safety of consumers to the dangers of residues in food products, CODEX (CAC, 2012) sets the maximum residue limits (MRL) for tetracycline (CTC, TC and OTC) as the sum of parent compounds and its metabolites in poultry products which are 200 ng/g for meat, 600 ng/g for liver and 400 ng/g for eggs. Similarly, Indonesia also sets the MRL for OTC of 100 ng/g for meat and 50 ng/g for eggs through SNI No. 01-6366 2000 (DSN, 2000).

Antibiotics as feed additives are still widely used in Indonesia. Bahri *et al.* (2005) reported that the use of tetracycline and sulphonamide antibiotics as feed additives in chicken by 74.43% (5 out of 7) feed factories in Bogor, Cianjur, Tangerang, Bekasi and Sukabumi Regencies. Therefore, since 2009 the Indonesian Government has prohibited the use of antibiotics including tetracycline as a feed additive through the Law Number 18 of 2009 regarding Livestock Animal Health.

The prevention and control of TCs residues through monitoring are necessary, and can be conducted using several detection methods that have been developed either using a high performance liquid chromatography (HPLC) (Shalaby *et al.*, 2011) or a liquid chromatography mass spectrophotometry (LCMS) (Pena *et al.*, 2007). However, these instruments are expensive and the preparation and clean up procedures are time consuming.

Enzyme-linked immunosorbent assay (ELISA) is a detection method that offers some advantages as it is simple, rapid, inexpensive, can detect samples in large quantities and has been applied to detect OTC residues in different food sample types such as shrimp (Wongtangprasert *et al.*, 2014), milk (Gaurav *et al.*, 2014) and honey (Mahmoudi *et al.*, 2014). The purpose of this study was to conduct a method validation of an indirect competitive ELISA (icELISA) for OTC residue detection in broiler chicken meat using locally generated polyclonal OTC-BSA antibodies (Widiastuti *et al.*, 2103) and to determine the OTC residue level in broiler chicken meat samples marketed in traditional markets and

supermarkets from several cities in Java by using the validated method.

MATERIALS AND METHODS

ELISA development

The polyclonal OTC-BSA antibodies were generated locally and produced in New Zealand White rabbits based on the method developed by Zhang *et al.* (2007). Isolation and purification of the polyclonal antibodies were conducted on a Hi-Trap Protein A column (Pharmacia) (Widiastuti *et al.*, 2013). A competitive indirect ELISA (icELISA) in this study was developed with some modifications. Briefly, a microplate (Maxisorb, manufactured from Nunc, Roskilde, Denmark) (microplate A) with 96 wells was coated with 10 µg/mL OTC-BSA (100 µL/well) in bicarbonate buffer solution (0.05 M, pH 9.6) and incubated overnight at room temperature. The microplate was washed with distilled water, and each well was blocked with 200 µL/well of blocking solution (100 mL PBS containing 3 g skim milk, and added with 200 µL of 10% sodium azide), followed by incubating for 1 hour at room temperature, then the plate was washed 3 times with distilled water. After the blocking solution was removed, the plate was washed 3 times and dried. Separately, in another microplate (microplate B), 125 µL of each sample, control (the OTC antibody which is mixed with the sample matrix), and OTC standard solutions (at 5 different concentration ranges: 25, 50, 100, 200 and 400 ng/g) were added by 125 µL PBS-methanol and a blank solution of 250 µL, a mixture of PBS-methanol (1:1), were incubated for 1 hour at room temperature. From microplate B, 100 µL of blank solution, samples and control were taken and placed into the microplate A and incubated for 1 hour at room temperature. The microplate A was washed 3 times with water and dried and each well was added with a substrate of IgG-HRP (1/20,000, 100 µL/well) and incubated for 45 min at room temperature. The microplate A was washed again 3 times, and dried. After that, each well was added with a substrate of 3,3', 5,5'-tetramethylbenzidine (TMB) and incubated for 30 min at room temperature. The reaction was stopped by the addition of 50 µL of 1.25 M H₂SO₄ and spectrophotometrically read with an automatic microplate at a wavelength of 450 nm using an ELISA reader (Labsystems Multiskan 354-00832, manufactured by Fisher Scientific Pittsburgh, PA). Absorbances were corrected by

blank reading. The result of the optical density (OD) was expressed in inhibition concentration (% IC) as follows :

$$\% \text{ IC} = 1 - \left(\frac{\text{Standard absorbance} - \text{Blank absorbance}}{\text{Control absorbance} - \text{Blank absorbance}} \right) \times 100\%$$

Method Validation

Several parameters of method validation namely the linearity, recovery, detection and confirmation using an HPLC were conducted in this study.

HPLC Analysis

The analysis using HPLC was adopted from the method developed by Cinquina *et al.* (2003) and had been validated by Widiastuti *et al.* (2010).

Sample Collection

Sixty seven chicken meat samples weighing approximately 200 gram were collected and analyzed for the presence of OTC residue. Samples were bought from traditional markets and supermarkets from several cities, namely Depok, Jakarta, Bandung, Cilegon, Surakarta and Yogyakarta, and carried in a cold condition (with ice cubes) and then stored in a refrigerator at -20^o C until further analyses were conducted.

Sample Preparation

Samples to be analyzed were thawed at room temperature and finely chopped. Each sample containing 5 gram minced broiler chicken meat was added with 10 mL of PBS-methanol (1:1), then shaken for 30 min, and centrifuged at 3000 rpm for 30 min. The upper layer solution was separated, and the remained samples were recentrifuged at 3000 rpm for 15 min. Finally, the sample solutions were analyzed for the determination of OTC residues using the ELISA procedure as described above.

RESULTS AND DISCUSSIONS

Evaluation of OTC Residue Detection using icELISA

Linearity was obtained between the OTC-BSA (1/1000) and IgG-HRP (1/20,000) for the OTC standards at concentrations of 25, 50, 100, 200 and 400 ng/mL in PBS-methanol (1:1) and in sample matrix, and the % IC were then constructed to make a calibration curve and the correlations (r^2) obtained were 0.9756 in PBS-methanol and 0.9878 in sample matrix, which

resembled one another and meant no difference for dissolving OTC in PBS-methanol or in matrix sample.

Recoveries were carried by fortification of the standards of OTC on blank minced fresh chicken meat samples at final concentrations of 50, 100 and 200 ng/g and preceded for analyzing by the ELISA method as described above. The recovery ranges were 71.8 to 115.8% and the average was 100.5%. Those results were close to Kumar *et al.* (2010) findings at 72.0 to 92.0% who used the monoclonal antibodies for detecting OTC in chicken meat samples.

The detection limit was estimated at IC₁₀ and it was at 24.4 ng/g (ppb). This finding was not as sensitive as the observation by Le *et al.* (2012) who found the detection limits were at 2.01 ppb for OTC and 0.13 ppb for its epimer by the use of monoclonal antibodies. Xu *et al.* (2013) obtained the detection limit of 1.43 ppb also by using the monoclonal antibodies. Kumar *et al.* (2010) found the sensitivity of 1 ppb using immunochromatography of monoclonal antibody-based ELISA. Those findings meant the use of monoclonal antibodies is more sensitive and specific in detecting OTC compared to polyclonal antibodies.

The difference lies on the production of antibodies. Monoclonal antibodies are those secreted by a single clone of B lymphocytes, while the polyclonal antibodies are produced by a mixture of various B lymphocyte clones (Leenaars and Hendriksen, 2005), showing that monoclonal antibodies are more superior than the polyclonal antibodies. This can be explained because the polyclonal antibodies will produce large amounts of non-specific antibodies which can sometimes give background signals in some applications.

In this study, cross reaction to other TCs or other classes of antibiotics could not be performed because the IC₅₀ (50% inhibitory concentration) could not be achieved. There are several reasons of its failure. It might due to inadequate antibody used, so the concentration of the antibody should be increased or it was probably due to insufficient amount of antigen coated to the microplate, so more antigen for coating was needed. However, in spite of its failure, the method is sufficient as a qualitative detection for a screening method to control the abuse of OTC residue in poultry industry as its detection limit obtained (24.4 ng/g) is still below the regulatory maximum residue limit (MRL) of 100 ng/g. Therefore, a further

Table 1. Evaluation and Comparison of ELISA and HPLC Methods for OTC Residue Detection in Broiler Chicken Meat

| Parameters | ELISA | HPLC* |
|---|--------|--------|
| Linearity test (r^2) | 0.9878 | 0.9997 |
| % of Recoveries test (fortified by OTC standards at 50, 100 and 200 ng/g) | 100.52 | 114.56 |
| Detection limit (ng/g) | 24.4 | 5.3 |

*Data reproduced from Widiastuti *et al.* (2010)

improvement to achieve a better sensitivity is still needed.

Comparison of ELISA and HPLC Detection Methods

The performance evaluation between ELISA and HPLC methods are displayed in Table 1. This comparison was also applied on 11 chicken meat samples analyzed and shown in Table 2.

The detection using ELISA showed 5 samples were positives at the concentration range of 50.7 to 112.2 ng/g, whereas the detection using HPLC showed 8 were positives at the concentration range of 6.9 to 114.9 ng/g. The difference is caused by their differences in the detection limits of those two methods, namely 24.4 ng/g for ELISA method and 5.3 ng/g for HPLC method. The calibration curve was constructed from those 5 positive samples using ELISA and HPLC methods and the resulting correlation (r^2) of 0.90 was not as good as Kumar *et al.* (2010) who obtained r^2 of 0.99 for analyzing 25 chicken meat samples.

The results for OTC residue using ELISA detection tends to give higher values than those using HPLC due to the presence of impurities (interference) which has a chemical structure similar to the target compounds that could potentially give false positive results (Toldra and Reig, 2006). This might be caused by the absence of the purification process in the ELISA method, which is different to the HPLC method preceded by a chemical extraction process and purification before identified using HPLC.

OTC Residue in Marketed Chicken Meat Samples

Monitoring of 67 broiler chicken meat samples was a complementary to demonstrate the

Table 2. Comparison of ELISA and HPLC Determination of OTC Residue in Broiler Chicken Meat Samples

| Sample Codes | OTC Concentration (ng/g = ppb)* | |
|--------------|---------------------------------|-------|
| | ELISA | HPLC |
| 1 | ND | 6.9 |
| 2 | ND | ND |
| 3 | ND | ND |
| 4 | ND | ND |
| 5 | ND | 15.3 |
| 6 | ND | 22.4 |
| 7 | 86.1 | 68.7 |
| 8 | 57.8 | 56.5 |
| 9 | 50.7 | 57.2 |
| 10 | 112.2 | 114.9 |
| 11 | 91.8 | 109.3 |

ND : no residue detected at ≤ 24.4 ng/g (ELISA) or ≤ 5.3 ng/g (HPLC)

ability of the ELISA method in detecting large quantities of samples. Actually the minimum number of samples of poultry must be one per 200 tones of annual production (Council Directive 96/23/EC in 2010) or equal to approximately of 300 chicken meat samples (from Java island) for 1 month sample collection time out of 1.750.000 tones for the overall national production in 2010.

The analyzed results of OTC residue detection on 67 chicken meat samples are summarized in Table 3. The samples collected in

Table 3. Analysis of OTC in Marketed Broiler Chicken Meat Samples using ELISA Method

| Sampling Locations | Number of Samples | | OTC Concentration (ng/g) |
|--------------------|-------------------|--------------------------|--------------------------|
| | Samples Analyzed | Sample Positives for OTC | |
| Depok and Bekasi | 16 | 1 | 86.1 |
| Bandung | 14 | - | ND |
| Cilegon | 10 | - | ND |
| Surakarta | 13 | - | ND |
| Yogyakarta | 14 | - | ND |
| Total | 67 | 1 | 86.1 |

ND : no residue detected below 24.4 ng/g

2009 from traditional markets and supermarkets in Depok, Bekasi, Yogyakarta, Surakarta, Bandung and Cilegon. The results showed only 1 (one) or 1.5% of the samples was positive for OTC residue at the concentration of 86.1 ng/g, because samples contained below 24.4 ng/g OTC residue with ELISA method would be stated as not detected. However, that result did not mean no other samples were positive for the OTC residue if they were analyzed with the HPLC (for examples 6.9, 15.3 and 22.4 ng/g as shown in Table 2).

The data above showed that marketed chicken meat samples collected from several cities in Java island is not at risk because the OTC residue mostly were absence. The reason of very low of OTC contamination findings might be caused by the fact that OTC antibiotic was no longer used and the farmers were careful of the withdrawal time. This finding is not different to the previous study that detected the OTC using the HPLC (Widiastuti *et al.*, 2010).

Salehzadeh *et al.* (2006) revealed that 25 (27.77%) of 90 chicken meat samples in Iran, which were detected by HPLC contained OTC concentrations that exceeding the MRL, whereas Shahid *et al.* (2007) found that 4 out of 7 samples of chicken meat in Pakistan also positive for OTC residue in a concentration range of 30 to 85 ppb. Differently, the research done by Cetinkaya *et al.* (2012) in Turkey with the use of LCMSMS, did not find any residue on 60 samples analyzed.

However, the number of samples included in this survey was relatively small compared to the total number sold in the markets and slaughterhouses. The government needs to set up

strict rules and procedures to monitor the residues in the chicken meat products as antibiotics are frequently administered by unqualified farmers or para veterinary field staffs. Therefore, awareness campaigns and educational programs targeted at them on the accurate and proper use of veterinary drugs will help avoid many of the unwanted consequences.

ELISA provides an inexpensive and fast technique to monitor the presence of OTC residue in meat samples, and to avoid false positive results, a confirmatory test is needed using HPLC or sophisticated detection methods of mass spectrometry such as LCMSMS (Impens *et al.*, 2003, Kim *et al.*, 2013) as a complementary method. ELISA is sufficient to be used for monitoring a large number of samples, meaning that in one microplate of 96 wells can accommodate about 41 (forty one) samples in a duplication analyses. Additionally, ELISA method also offers faster analysis time and cheaper cost compared to chromatography methods that need sample preparation and detection in such chromatography instrumentation.

CONCLUSION

This present icELISA method for detecting OTC residue in chicken meat is sufficient to be used for monitoring a large number of samples or as a supplement for the HPLC method. This method still has to be improved further to achieve a better detection limit and to be capable of providing cross reaction to other TCs antibiotics. The analyses on 67 broiler chicken meat marketed in several cities in Java Island showed only 1 (1.5

%) sample was positive for the OTC residue at 86.1 ng/g and most samples were safe for human consumption.

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