



Comparative Screening of Chloramphenicol Residue in Chicken Tissues Using Four Plate Test and Premi® Test Methods

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ABSTRACT

Background: The safety of food with animal origin means that the food consumed is considered as safe when synthetic chemical agents are absent or present at very low concentrations. The aims of the present study were to validate the Premi® Test and four plate test (FPT) methods as well as screen and estimate the occurrence of chloramphenicol (CAP) residue in collected chicken tissues including liver, kidney and thigh muscle from Kermanshah, west of Iran.

Methods: A total of 150 chicken samples were purchased from different poultry slaughterhouses in Kermanshah province, west of Iran and subjected to the FPT and Premi® Test.

Results: The Premi® Test could not detect CAP residue at concentrations below 3 and 6 ppm in aqueous solution and kidney fluid, respectively. The highest sensitivity of FPT in the detection of CAP residue was optimally found in the agar medium inoculated with *Bacillus subtilis* at pH 7.2. The Premi® Test was more sensitive than FPT in the kidney fluid and aqueous solution. Regarding FPT results, CAP residue was found in 20% (n=30), 8.66% (n=28) and 11.33% (n=17) of liver, kidney and muscle samples, respectively. In the case of Premi® Test, the most contaminated samples were liver (24%), followed by kidney (22.66%) and muscle (19.33%).

Conclusion: It can be concluded that illegal use of CAP in Iranian poultry industries should be taken into account seriously.

Introduction

The safety of food with animal origin means that the consumed food is considered as safe when synthetic chemical agents such as antibiotic drugs, pesticides, insecticides and herbicides are absent or present at very low concentrations.¹ The development of scientific standards and approaches that reduced the potential risk of undesirable effects of chemical residues in humans is of important consequence for food industries and consumers.² One of the much-debated chemical agents in the animal food production chain is chloramphenicol (CAP). CAP is an efficient antibiotic which has been banned for treatment of farm animals in the European Union (EU) and in many other countries due to its serious side effects especially aplastic anemia.^{3,4} Monitoring animal facilities for controlling and prevention of illegal CAP usage is necessary because it is unlikely that farmers respecting the guidelines established by the European Commission.^{5,6} Previous studies has been reported the presence of CAP residue in seafood, milk, meat and poultry products.^{1,4,7-10}

During the last decades, various sensitive screening methods including microbiological tests and immunoassays have been evaluated for monitoring and

determination of non-allowed substance residues in food products.⁶ Microbial screening methods such as four plate test (FPT) are commonly used for large-scale screening of an antibiotic or a group of antibiotic residues in animal food products. The FPT is based on the growth inhibition of three *Bacillus subtilis* plates and one *Staphylococcus aureus* plate containing three different pH.^{11,12} FPT is a qualitative method and its most important disadvantages are lack of specificity and the long required incubation time.¹¹ Although, FPT is not recognized as a sensitive method to monitor and determine the zero-tolerance level of some veterinary drug residues, it is frequently used in the reference laboratories of Iranian Veterinary Organization.^{4,13,14} Recently, Premi® Test has been introduced as an alternative, suitable, fast, easy to use and inexpensive test for detecting several antimicrobial compounds in meat and meat products, seafood and egg.^{11,15,16} The method is also based on microbiological detection of antibiotic residues by growth inhibition of *Bacillus stearothermophilus*.¹⁶

Although our knowledge on CAP in the microbiological tests such as FPT and Premi® Test has been improved in the recent year, when the various animal tissues examined for CAP residues, the proposed method must be validated.

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Hence, the aims of the present study were to (i) validate the Premi®Test and FPT methods according to the Commission Decision 2002/657/EC (2002), (ii) screen and estimate the occurrence of CAP residue in collected chicken tissues including liver, kidney and muscle from different poultry slaughterhouses in Kermanshah province, west of Iran, and (iii) introduce the best validated microbial screening method for estimating the occurrence of CAP residue in chicken tissues.

Materials and Methods

Chemicals and reagents

The CAP standard was obtained from Sigma-Aldrich (Germany). Premi®Test kit (Cat. No. R3925) with detection limit of 2.5-3 ppm was purchased from R-Biopharm (Germany). CAP and blank discs were supplied from Himedia Ltd. (India) and Padtan Teb Corporation (Iran), respectively. All media and reagents were obtained from Merck (Germany). *Bacillus subtilis* (ATCC 6633) and *Staphylococcus aureus* (ATCC 6538) were purchased from the culture collection of the Iranian Research Organization for Science and Technology, Tehran, Iran.

Chicken sampling

In 2014, a total of 150 chicken samples were purchased from different poultry slaughterhouses in Kermanshah province, west of Iran and subjected to the FPT and Premi®Test. After slaughter, chicken samples were transferred to the laboratory at refrigerated temperature ($4\pm 1^\circ\text{C}$). Then, kidney, liver and thigh muscle were obtained and stored at frozen condition ($-20\pm 2^\circ\text{C}$) until use.

Premi®Test

Preparation of the samples was done according to the instruction of Premi®Test kit. In summary, frozen chicken samples were thawed at refrigerated temperature ($4\pm 1^\circ\text{C}$). An aliquot of 2 cm² of each tissue samples was cut into pieces and pressed using Premi®Test Multipress in order to extract about 250 μl of tissue juices. 100 μl of each tissue juice was slowly added onto the agar in the ampoule. The ampoules containing liver and thigh muscle juices were stand at room temperature for 20 min for a pre-diffusion. In the case of kidney tissue, the ampoule was covered with foil and incubated at 80°C for 20 min to inactivate lysozyme present in the kidney fluid. After this step, the tissue juices were washed twice with double-distilled water. The discrimination of end point growth in tubes was conducted based on changing color of negative control sample during incubation in the block heater at 64°C . According to the instruction of the Premi®Test kit, this was occurred after 3 h. Therefore, the ampoules were covered with foil and incubated in the block heater at 64°C for 3 h. After changing color of negative control sample, all test ampoules were withdrawn from the block heater. In the Premi®Test, agar ampoule consists of spores of the *Bacillus stearothermophilus*. The bacterial spores germinate when the test ampoule heated and produce carbonic acid. This acid leads the bromocresol purple

indicator in the ampoule to change from purple to yellow. The presence of antimicrobial drug residues inhibit the bacterial growth and the test ampoule remains purple.¹¹

Four Plate Test

Preparation of test microorganisms

Preparation of bacterial inoculum doses of *B. subtilis* (1.5×10^5 CFU/ml) and *S. aureus* (1.5×10^8 CFU/ml) were conducted according the previously reported method by Shahbazi et al.⁶

Preparation of culture media

For the FPT, the Mueller Hinton Agar (MHA) with three different pH including 6, 7.2 and 8 were prepared and autoclaved. The agars were cooled to between $45\text{--}50^\circ\text{C}$ and then the bacterial suspensions (*B. subtilis*: 1.5×10^5 CFU/ml and *S. aureus*: 3.6×10^6 CFU/ml) were transferred and cast into petri dishes with a diameter of 90 mm. In the present study, four different media were studied as follow: 1) MHA with pH 6.0; 2) MHA with pH 7.2; and 3) MHA with pH 8.0, inoculated with *B. subtilis*; and 4): MHA with pH 8.0, seeded with *S. aureus*.^{4,6}

Preparation of Sample

The frozen chicken samples were thawed at refrigerated temperature, chopped and homogenized using meat homogenizer. 5 g of homogenized samples were centrifuged at $10000\times g$ for 10 min in a refrigerated centrifuge. The supernatant was placed into a water bath at 54°C to promote inactivation of the complement system and other natural inhibitory antimicrobial agents. The paper disc impregnated with 10 μl of the supernatant was placed onto the surface of the earlier prepared MHAs. Positive (CAP disc) control also was considered in the present test. The MHAs inoculated with *B. subtilis* and *S. aureus* were incubated in an upright position at 37°C and 30°C for 24 h, respectively. The radius of the inhibition zones of one or both microorganisms was measured. The zone of inhibition equal to or greater than 2 mm was indicated a positive result. The area of the inhibition zone was calculated as πr^2 .⁶

Determination of Premi®Test and FPT sensitivities in aqueous solution and kidney fluid

In order to evaluate sensitivities of the described methods in aqueous solution, the concentration ranges from above the MRLs to below the minimum detectable limit claimed by the Premi®Test kit were constructed. Then, each prepared aqueous solution was examined in the Premi®Test and FPT (pH 6, 7.2 and 8). In the case of kidney fluid, the blank kidney samples were spiked with the concentration ranges from above the MRLs to below the minimum detectable limit claimed by the Premi®Test kit. Then, the preparation sample was conducted according to the outlined method as described above.

Determination of detection capability (CC β) and specificity

These two parameters were determined through the

analysis of twenty blank chicken samples spiked with different concentrations of CAP using Premi®Test and FPT.

Determination of CAP stability in aqueous solution and kidney fluid

The stability of CAP in solution and kidney fluid was evaluated using three different storage conditions (-20 °C, 5 °C and room temperature). For this purpose, three individual CAP stock solutions were prepared and kept at different temperature for eight weeks. The stability examination of samples using Premi®Test was repeated at 7 intervals (0, 7, 21, 28, 35, 42 and 49 days) and 5 intervals (0, 7, 14, 21 and 28 days) for aqueous solution and kidney fluid, respectively.

Statistical analysis

The analysis was performed using SPSS 16.0 for Windows (SPSS, Chicago, IL, USA) software package. Significance level was considered as $p < 0.05$ in all experimental data.

Results and Discussion

Determination of CAP residue in aqueous solution and kidney fluid using Premi®Test and FPT

The initial work was based on the previously published method^{14,15} with some modifications, where the sensitivities of Premi®Test and FPT methods were compared in the aqueous solution and kidney fluid. In general, various animal tissues such as fat, skin, kidney, liver and muscle may be used as a matrix for screening and detecting veterinary drugs and contaminants. Nevertheless, the kidney tissue is routinely screened for residues of antimicrobial drugs and contaminants because most of residues tend to accumulate in this matrix.^{17,18} The compared findings of CAP determination in aqueous solution and kidney fluid using Premi®Test and FPT are presented in Table 1. As can be seen, the Premi®Test could not detect CAP residue at concentrations below 3 and 6 ppm in aqueous solution and kidney fluid, respectively. The kidney fluid significantly decreased the sensitivity of Premi®Test ($P < 0.05$), compared with the obtained sensitivity in aqueous solution. Kilinc et al., and Cantwell and O'keeffe investigated the effect of trout muscle and kidney tissues on detection limits of CAP with Premi®Test, respectively. According to their results, tissue components such as proteins, saccharides and fat influence the determination of antibiotic residue present in trout muscle and kidney tissues.^{12,14} A previous study reported that the sensitivity of Premi®Test was significantly increased when pre-treatment involving mechanical denaturing and chemical extraction of the

tissue with acetonitrile/acetone (70:30 v/v) was conducted.¹⁵ Hence, further studies are required to optimize the extraction condition of chicken tissue samples.

As can be seen in Table 1, the highest sensitivity of the FPT method in the detection of CAP residue was optimally found in the agar medium inoculated with *B. subtilis* at pH 7.2. The sensitivity of the FPT for CAP residue was found to be 8 ppm in both aqueous solution and kidney fluid (Table 1). The detection limits of the FPT method were remarkably higher than the maximum residual level (MRL) recommended by EU.¹⁹ Hence, it is not sufficiently sensitive to detect the CAP residue in chicken tissue samples. In general, the sensitivity of FPT method may different depends on the diffusion of the compounds into the agar medium, pH value of the used medium and also matrix effect.^{4,6} In a previous work, Shahbazi et al., reported that pH value of the agar medium had significant effect on increasing the sensitivity of FPT method and subsequently decreasing its detection limit for tetracycline residues in chicken tissue samples.⁶ Several studies demonstrated that the different pH of agars had remarkable effect on the inhibition zone of FPT assay,²⁰⁻²² which their results are in good agreement with our findings.

Based on the results of the present study, the Premi®Test was more sensitive than FPT in both matrices. This finding is in good agreement with previous studies.^{11,12,14,23} Pikkemaat et al., compared the performance of three microbiological methods including FPT, Nouws Antibiotic Test (NAT) and Premi®Test as the primary screening tests for detecting of several antibiotic residues in slaughter animals. They found that antibiotic residues can be detected by the Premi®Test significantly better than the FPT and NAT.¹¹ Gaudin et al., showed that the detection capability of Premi®Test for sulfamethazine and sulfadiazine residues in egg was lower than other commercial tube tests such as Explorer® Test.²³

Stability of CAP in aqueous solution and kidney fluid

According to the Commission Decision 2002/657/EC (2002), the stability of antimicrobial drug residues should be examined in aqueous solution and matrix tissues. In the current study, stability of the CAP in aqueous solution and kidney fluid was determined at three different storage conditions (-20 °C, 5 °C and room temperature). As can be seen in Table 2, CAP was stable when stored at -20 °C, 5 °C and room temperature as follow: in aqueous solution: 49, 49 and 1 days and in kidney fluid: 28, 7 and 1 days, respectively. It was well known that antibiotic drugs are sensitive to different form of breakdown in aqueous solution and tissue matrices.

Table 1. Sensitivity of the Premi®Test and FPT for aqueous solution and kidney fluid of chloramphenicol.

Chloramphenicol	Determined sensitivity, ppm Premi®Test	Determined sensitivity, ppm FPT				Manufacturer's claimed limit for Premi®Test, ppm	MRL (kidney), ppm
		pH6	pH7.2	pH8	pH8s*		
Aqueous solution	3	10	8	12.5	14	2.5-3	-
Kidney fluid	6	12	8	14	18	2.5-3	-

*pH8s: medium seeded with *S. aureus* (pH 8.0).

Several researchers investigated stabilities of numerous antibiotics such as CAP, macrolides, lincosamides, beta-lactams, tetracyclines and quinolones in solutions and matrices.^{8,24,25} They demonstrated that the antibiotic stability depends on several factors such as the type of buffer solution and matrix as well as the temperature. According to the results of Leston et al., CAP was stabilized -20 °C and 4 °C approximately for 7 and 6 weeks, respectively,⁸ which are in accordance with our results.

Table 2. Stability of chloramphenicol in aqueous solution and kidney fluid, stored at -20°C, 5°C and room temperature.

Chloramphenicol	Stability, days		
	-20°C	5°C	Room Temperature
Aqueous solution	49	49	1
Kidney fluid	28	7	1

Specificity of Premi®Test and FPT in kidney fluid

Based on the Commission Decision 2002/657/EC (2002), an analytical method should be able discriminate between the analyte and closely related substances such as isomers, metabolites, degradation products and endogenous substances to prevent false positive results. However, since screening assays such as Premi®Test and FPT were used for the detection of all antimicrobial substances in animal products, this parameter is not associated to these tests. The second consideration is that matrix substances may have effect on the specificity of the analytical method. Therefore, twenty blank kidney fluid samples were spiked with the CAP standard solution to determine the potential of interfering matrix. According to our findings, no false positive results were found; this means that the presence of the matrix itself did not change the results of Premi®Test and FPT.

Detection capability of Premi®Test and FPT

According to the guidelines on the implementation of 2002/657/EC,²⁶ value for detection capabilities ($CC\beta$) of a screening test should be calculated. The $CC\beta$ is defined as “the lowest concentration at which a method can detect truly in a contaminated sample with a statistical certainty of 1- β .” As shown in Table 3, in the Premi®Test, $CC\beta$ ($\beta=5\%$) value was 6, 6 and 10 ppm for kidney, liver and muscle tissues, respectively. In the FPT, this value was found to be 8, 8 and 12 ppm for kidney, liver and muscle tissues, respectively. The results of the present study indicated that Premi®Test was more sensitive than the FPT. However, this value was very higher than recommended Minimum Required Performance Limit (MRPL) of CAP (0.3 $\mu\text{g/g}$) in foods with animal origin.²⁶ It was found that the $CC\beta$ of CAP was equal to limit of sensitivity of the analyte in the kidney fluid. In both methods, all the blank and spiked samples were showed to give negative and clear positive responses,

respectively. In the case of kidney juice sample, the significantly higher $CC\beta$ or lower false negative result of Premi®Test in compare with FPT is due to this fact that the kidney juice sample should be pre-incubated at 80 °C for 10 min before applying to the ampule according to instruction of the test. This can lead to inactivation of natural growth inhibiting compounds such as lysozymes present in kidney fluid and subsequently decreasing of false negative results.^{11,16}

Commission Decision 2002/657/EC indicates that during validation, an analytical method should be evaluated for applicability. Based on the results of the present study, in the case of muscle tissue, a negative response was found at concentrations equivalent to the $CC\beta$ of the method. When comparison was conducted among spiked kidney, liver and muscle, a considerable matrix effect was found. It can be concluded that kidney is more suitable matrix than muscle for detection of CAP. Previous studies reported that since drug releasing level in muscle tissue is generally lower than parenchymal tissues such as liver and kidney as well as most of antimicrobial drugs rapidly eliminated from muscle tissue, the possibility of detecting a positive meat sample is rather low.^{27,28}

Screening of CAP residue using FPT and Premi®Test

With regards to the results of FPT method (Table S1 in Supplementary Materials), the inhibition zone equal to or greater than 2 mm was indicated as a positive result. Therefore, 5.33% (n=8), 4% (n=6) and 1.33% (n=2) of liver, kidney and muscle samples respectively were found contaminated with CAP residue on the pH 6 plate inoculated with *B. subtilis*. CAP residue was found in 20% (n=30), 8.66% (n=28) and 11.33% (n=17) of liver, kidney and muscle samples, respectively on the pH 7.2 plate. Indeed, the most contaminated samples were observed in the liver tissue on pH 7.2 plates. The frequency of CAP residue was obtained 15.3% including 10 (6.66%) of liver, 8 (5.33%) of kidney and 5 (3.33%) of muscle samples on the pH 8 plate, whereas only 1 (0.66%) of liver sample was found contaminated with CAP residue on the pH 8 plate seeded with *S. aureus*. The inhibition zones of positive (CAP disc) control for MHA with pH 7.2, MHA with pH 8.0 and MHA with pH 6.0 inoculated with *B. subtilis* as well as MHA with pH 8.0 seeded with *S. aureus* were found to be 25.12 ± 0.01 , 12.56 ± 0.02 , 11.33 ± 0.03 and 9.42 ± 0.03 , respectively. The statistical analysis of the data showed a significant difference between the content of CAP residue in chicken kidney, liver and muscle on the different pH plates ($p < 0.05$). It was noteworthy that all tissue samples which were positive at pHs 6 and 8 were also positive at pH 7.2. Based on the results of the FPT, the most positive samples were liver (32.66%), followed by kidney (28%) and muscle (16%) (Table S1 in Supplementary Materials).

Table 3. Sensitivity of the Premi®Test and FPT in the chicken samples.

Chloramphenicol	Response at $CC\beta$			Limit of Sensitivity		
	Kidney	Liver	Muscle	Kidney	Liver	Muscle
Premi®Test	+	+	-	6	6	10
FPT	+	+	-	8	8	12

The most important reason of the high contamination of liver samples is related to the fact that this organ is considered as an excretory organ.^{6,27} No significant ($p > 0.05$) difference was found between the percentage of CAP residue of liver and kidney samples, whereas a statistically significant difference ($p < 0.05$) between liver and kidney with muscle tissue was observed. In the case of Premi[®]Test, the most contaminated samples were liver (24%), followed by kidney (22.66%) and muscle (19.33%).

Conclusion

The results of the present study indicated that Premi[®]Test was more sensitive than the FPT. Based on our findings, illegal uses of CAP in Iranian poultry industries should be taken into account seriously.⁴ However, Premi[®]Test and FPT methods cannot be used for the estimation of CAP residue in different chicken tissue samples at maximum residue levels (MRLs) of this illegal veterinary drug. Hence, further sensitive and selective methods such as HPLC and ELISA is required to determine the CAP residue in chicken samples.

Conflict of interests

The authors claim that there is no conflict of interest.

Supplementary Materials

Supplementary file contains Table S1 is available on the journal's web site along with the published article.

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