GSC Advanced Research and Reviews

eISSN: 2582-4597 CODEN (USA): GARRC2

Cross Ref DOI: 10.30574/gscarr

Journal homepage: https://gsconlinepress.com/journals/gscarr/

(RESEARCH ARTICLE)

Thin layer chromatographic detection of colistin sulfate antibiotic residues in poultry tissues

Md. Nazmul Hasan, Md. Shafiqul Islam *, Md. Rakibul Hasan and Kazi Rafiqul Islam

Department of Pharmacology, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh.

GSC Advanced Research and Reviews, 2021, 07(01), 001–008

Publication history: Received on 18 February 2021; revised on 25 March 2021; accepted on 27 March 2021

Article DOI: https://doi.org/10.30574/gscarr.2021.7.1.0063

Abstract

Residues of antibiotic drugs in food and food products have been received much attention in recent years because of growing food safety concerns for public health. There are serious effects of antibiotic residues in meat for human consumption (e.g., increasing antimicrobial resistance, carcinogenicity, mutagenicity, and hypersensitivity). In this study, we used broiler chicks as a laboratory animal to detect the colistin sulfate antibiotic residue for human health concerns. The day-old broiler chicks were collected and reared for up to 31 days. The treatment was started from the day 16th until sacrifice. The chicks were randomly divided into three groups namely control group (Group A), discriminate antibiotic group (Group B) and indiscriminate antibiotic group (Group C) on the 14th day. The discriminate group was treated with an antibiotic, colistin sulfate maintaining the withdrawal period of one week. In case of indiscriminate group the withdrawal period was not maintained and antibiotic treatment was continued till the day of sacrifice. The body weights of the birds were recorded daily. The mean body weight was highest in indiscriminate group (1261.15±16.37gm) followed by discriminate group (1156.15±18.23gm) and the lowest was in control group (1008.49±18.11gm). The differences among mean weight gain were statistically significant ($P<0.05$) in antibiotic treated group compared with control group. The Thin Layer Chromatography revealed that all the samples were positive in indiscriminate group and in case of Liver, Kidney and Spleen, it was 100%. In case of discriminate antibiotic group all the samples were positive except thigh and breast muscle and the percentage was highest in Liver sample (66.67%). There was no positive sample in control group. The results were statistically significant ($P<0.05$). From the above findings, this research could be considered a need based research in Bangladesh to ascertain the influential effect of antibiotic abuse in poultry industry.

Keywords: Colistin sulfate; Antibiotic residue; TLC; Broiler

1. Introduction

Antibiotics are low to medium molecular weight compounds exhibiting a variety of chemical and biological properties. This is mainly employed for chemotherapeutic and prophylactic purposes and also used as feed additives to promote growth and improve feed efficiency [1]. However, the antibiotic residues from milk, meat and egg may persist for longer period after treatment, when administered without maintaining withdrawal period properly. Various antibiotics take different time periods to be excreted from the body. It becomes a potential hazard to human health. In poultry, Antibiotics are widely used as therapeutic, prophylactic, growth promoting agents and nutritive purposes in poultry production [2, 3]. This wide spread use of antibiotics in poultry industry resulted in the presence of residuals in foodstuffs leading to a potential health hazards for consumers which include; carcinogenicity, mutagenicity, bone

* Corresponding author: Md. Shafiqul Islam
Department of Pharmacology, Faculty of Veterinary Science.
Bangladesh Agricultural University, Mymensingh-2202, Bangladesh.

marrow toxicity and allergy [4] as well as appearance of a resistant strains of pathogenic bacteria [5]. Now a days colistin sulfate is one of the most widely used antibiotic in poultry sector.

Colistin has been used for decades in veterinary medicine, especially in swine and veal calves.. Most of the colistin applications in animals are for oral group treatments. It is highly effective against strains of Escherichia coli, Pseudomonas aeruginosa, Salmonella spp., Haemophilus spp., Shigella spp., Pasteurella spp., Brucella spp., Aerobacter aerogenes and Bordetella bronchiseptica. The mechanism of action of colistin sulfate is to alter the permeability of the cell membrane of bacteria. Colistin resistance has been emerging rapidly following its reintroduction as shown in different reports with an associated increased mortality [6, 7, 8]. The use of colistin in combination is also more frequently considered and clinical studies are on-going. From the above facts, it may be mentioned these antibiotic residues might be potential hazards for human as well as animal health and a great obstacle to export poultry meat and products. In this context, these research work was undertaken to detect the presence of residues of colistin sulfate in broilers tissues by thin layer chromatography (TLC) test. The main purpose of this test through indoor trial research was to detect the residue of colistin sulfate in edible poultry tissues.

2. Material and methods

2.1. Experimental design

18 apparently healthy day-old "Cobb-500" broiler chicks were purchased from CP Hatchery Ltd, Valuka, Mymensingh. . On the 16th days of age chicks were randomly divided into three groups (Group A, B & C). Each group contains 6 birds. The birds of Group-A, B and C were kept in different cages. Group A was kept as untreated control & received non-medicated water. Group B & C were administered with colistin sulfate @2gm/1L drinking water. After 7 days, at the age of day 23; antibiotic supply was stopped in the group-B and withdrawal period was maintained. In group-C the antibiotic supply was continued until the day of scarifice. Birds received their freshly prepared daily medication in the morning hour of each day. The concentration of colistin sulfate in the water to give the required dose per kilogram of body weight was calculated by determining the water consumption and body weight of each bird on the day of medication.

2.2. Sample Collection

At the end of the experiment six birds from each group were sacrificed ethically. Liver, kidney, breast muscle, thigh muscle, fat and spleen samples were collected. Immediately after collection of sample, these were washed individually several times in physiological saline to remove clotted blood and debris. All samples were marked separately and preserved at -20º C in polythene zipper bags for their extraction and analysis.

2.3. Sample Preparation

The samples were stored in the deep freeze at −20°C until further advanced procedures were performed. Samples were grinded with a mortar & pastel properly. These samples were taken into properly cleaned and sterilized petridishes with proper care. From this 4g of sample was taken into beaker with the help of electric balance and spatula. The homogenization was done with the addition of 10 ml phosphate buffer (pH-7.2). After proper mixing, protein contents of these samples were precipitated with the addition of 2 ml trichloroacetic acid (30%) maintaining sufficient care and attention. Then these samples were taken into properly cleaned and sterilized centrifuge tubes for centrifugation. The centrifugation was performed @60000 rpm for 20 min with the help of automatically time regulated centrifuge machine. Then the supernatant was collected in a new tube. The supernatant was extracted with an equal volume of diethyl ether to perform de-fatation. Then mixture was kept for 10 min to become into a separate layer. Then these mixtures were separated from each other, and upper oily layer was discarded but only the bottom layer was collected. This extraction of supernatant was repeated twice with diethyl ether. Then, the extracts were collected into screw cap vial with proper care and kept into refrigerator for further advanced analysis. Total procedure was performed as the reference cited by Poppelka et al. [9].

2.4. Thin layer chromatography (TLC)

2.4.1. TLC apparatus

TLC plate (MN-Germany), TLC tank and UV detection box (UV light: F18W-Germany) were used. TLC was performed according to Tajick and Shohreh, Islam et al, Ali et al, Das et al. [9, 10, 11, 12] with some required adjustments. TLC plate was cut into appropriate size (4x5 cm) from 20x20 cm. A straight line was drawn across the plate approximately 2 cm from the bottom by a pencil. Another straight line was drawn across the plate below 1 cm from the upper edge of the plate. Desired spots marking were marked on the bottom line where analytes were dropped. Spots were applied to the

plate using thin capillary glass pipettes. A volume of 50 µl was used for spotting. Plate was placed in TLC tank (contained mobile phase; Butanol: distilled water: acetic acid = 60:20:20) and covered by lid and it was left until the mobile phase reached the upper line. Spots were visualized in UV detection box at 256 nm. Spots marking were done by pencil for calculation of retention factor (Rf).

2.5. Calculation of Rf values

These measurements are the distance travelled by the solvent, and the distance travelled by individual sample spots. Same Rf value of standard and sample considered similar compound.

2.6. Data analysis

Experimental data were introduced and stored in Microsoft Excel-2010 and results were analyzed.

3. Results

Table 1 Efficacy of colistin sulfate on body weight gain.

Sl No.	Control Group	Discriminate group	Indiscriminate group
1	959.63	1184.63	1276.63
2	982.88	1102.80	1217.80
3	1035.06	1173.06	1291.06
4	992.13	1114.13	1213.13
5	1083.94	1219.94	1312.94
6	997.31	1142.31	1255.31
Mean±SEM	1008.49±18.11	1156.15±18.23	1261.15±16.37

The Table 1 represents that the average body weight gain was the highest in indiscriminate antibiotic group which was 1261.15±16.37 whereas the lowest was 1008.49±18.11 in control group. Discriminate antibiotic group showed moderate weight gain that was 1156.15±18.23. The differences among means of three groups were statistically significant (P<0.05). The multiple comparisons during one way ANOVA revealed that there was no significant difference in means between group B and C. On the contrary, between group A & C, and group A & B, significant differences were observed.

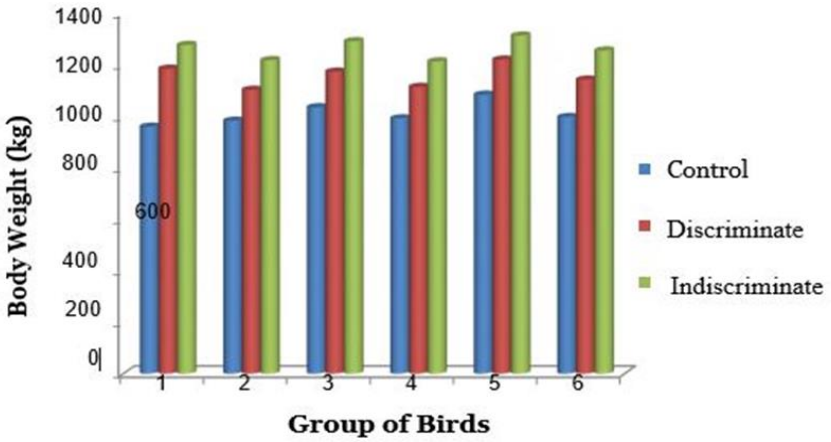

Figure 1 Body weight gain of three individual groups in 14 day period (16th -30th)

Figure 1 represents body weight gain of broilers of control, discriminate and indiscriminate antibiotic groups in 14 day period (16th -30th). It also shows standard error mean of three individual groups.

Table 2 Percentage (%) of colistin sulfate positive and negative samples (Discriminate group).

Sample frequency	Positive (%) frequency	Negative(%) frequency
Liver (6)	66.67 (4)	33.33 (2)
Kidney (6)	50.00 (3)	50.00 (3)
Spleen (6)	33.33 (2)	66.67 (4)
Thigh muscle (6)	0.00 (0)	100.00 (6)
Breast muscle (6)	0.00 (0)	100.00 (6)
Fat tissue (6)	16.67 (1)	83.33(5)

In the discriminate group liver, kidney, spleen and fat tissue showed positive frequency and the percentage were 66.67%, 50.00%, 33.33% and 16.67% respectively. The other samples (Thigh and Breast muscle) were negative in the TLC analysis.

Table 3 Percentage (%) of colistin sulfate positive and negative samples (Indiscriminate group).

Sample frequency	Positive (%) frequency	Negative (%) frequency
Liver (6)	100.00 (6)	0.00 (0)
Kidney (6)	100.00 (6)	0.00 (0)
Spleen (6)	100.00 (6)	0.00 (0)
Thigh muscle (6)	83.33 (5)	16.67 (1)
Breast muscle (6)	66.67 (4)	33.33 (2)
Fat tissue (6)	83.33 (5)	16.67 (1)

In case of indiscriminate group all the samples showed positive frequency in various percentages. In the control group all the samples showed negative antibiotic residual frequency in TLC analysis as there was no use of antibiotic in control group.

Table 4 Overall percentage (%) of positive samples in three different groups

The overall percentage of colistin sulfate residues of the three different groups are given below showing their standard deviation with the standard error mean.

Sample	Antibiotic group (Discriminate)	Antibiotic group (Indiscriminate)	Control group
Liver	66.67	100.00	0.00
Kidney	50.00	100.00	0.00
Spleen	33.33	100.00	0.00
Thigh muscle	0.00	83.33	0.00
Breast muscle	0.00	66.67	0.00
Fat	16.67	83.33	0.00
Mean±SEM	27.77±11.12	88.88±5.56	0.00

The highest average was in indiscriminate antibiotic group which was 88.88±5.56 and the lowest was in control group (0). In the discriminate antibiotic group, the average percentage was 27.77±11.12.The differences among means of three

individual groups were statistically significant (P<0.05). The multiple pairwise comparison revealed that the difference of means between discriminate and control group was not significant. On the other hand, the other two pairs showed statistically significant difference among means.

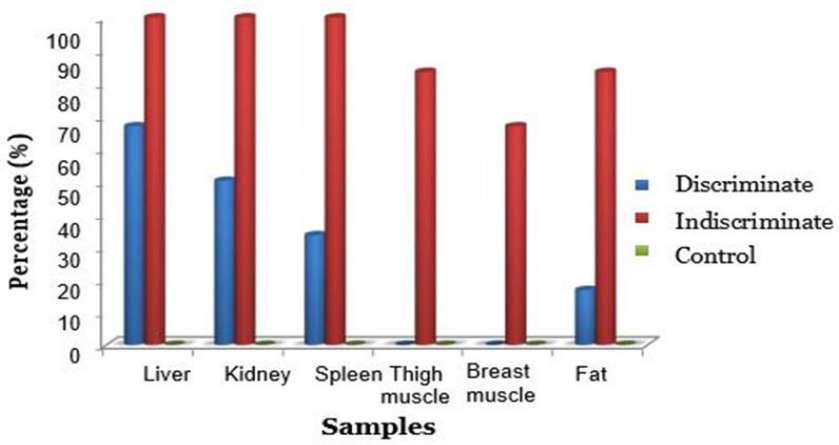

Figure 2 Percentage of colistin sulfate residue samples in three group

4. Discussion

Antimicrobial residues in food of animal origin have received much attention in developed countries to ensure food safety. Many countries have monitoring programs to avoid antimicrobial residue in food of animal origin [13]. In Bangladesh, there are regulations regarding the use of antimicrobials or the maximum allowable antimicrobial concentrations in food. Additionally, there are no systems to monitor the presence of antimicrobial residue in animal products in Bangladesh. Therefore, screening of food products from animal origin intended for human consumption for the presence of antimicrobial residue is essential to ensure food safety. In this study, antimicrobial residue in poultry meat was evaluated. The most commonly sold antimicrobial classes in the major livestock especially in poultry production in 15 countries from Europe, Asia and Australia were penicillins, tetracyclines, macrolides and aminoglycosides, especially since each of these classes has been in use for more than 50 years [14]. These antimicrobials are administered to broilers by injections (intramuscularly or subcutaneously) and orally in food or water [15]. Screening techniques are the first step in determination the presence of antimicrobials in food of animal origins; these techniques may use biological methods, biochemical methods and physicochemical methods [16]. Microbiological inhibition tests are cheap and permit to analyze a large number of samples in a short time. Microbiological screening relies on a common property of all antibacterials; they inhibit growth of microorganisms [17].

Chicken meat has high quality proteins and low fat, along with important vitamins and minerals [18]. Also chicken production is quicker and cheaper than other meat sources .By forbidding of some animal meats consumption, like pork, due to religious rules in Islamic countries, and also higher price of red meats like beef and lambs etc., chicken meat gains more attention in these countries and plays an important role in public nutrition. An increased demand for chicken meat, forces the poultry breeding industry to produce more amount of meat, which then leads to a further increase in the use of drugs such as antibiotics or hormones for growth stimulation and weight promotion [19]. Chicken liver contained the highest of proportion of antibiotic residues than the rest of three samples. This finding has similarities with the report of Naeem et al.[20] that chicken liver contained the highest level of enrofloxacin, ciprofloxacin and other antibiotic residues than kidney and muscles. Due to the high risk of veterinary drug residues in foods of animal origin, the maximum residues limit (MRL) regulation for use of each pharmacologically active substance has been developed by European Union (EU).

The live body weight gain throughout treatment period (last 14 days) was highest in indiscriminate antibiotic used group but indiscriminate group showed lower gain. On the other hand, control group revealed the lowest weight gain of all. Antibiotics alter various functions in poultry causing increased feed efficiency. The use of antibiotic induces significant changes in microbial population. Use of antibiotics growth promoters in poultry has been a popular practice from the past decades. But injudicious usage not only lessens the cost but also make the meat safe for consumption. So, it is evident that if proper withdrawal period is maintained, the body weight is gained more profitably. As use of antibiotics is expensive and there is no apparent health benefit of using antibiotics until sacrifice, rather potential harmful effects might prevail, there is no use of using antibiotics up to last day of rearing. The body of broilers is adapted

to gain weight at a certain extent where it might be incapable of gaining body weight even if more feeding and growth promoter is provided [21]. The lower weight gain in control group was expected but it is not recommended by the study that antibiotics are must be used to promote growth of live broilers. Rather proper management and scientific feeding practices can attain such goal more profitably than feeding of antibiotics. Furthermore, it will be consumer and environment friendly. Now a day, alternatives to antibiotic growth promoters is an interesting topic. Various researches showed that use of natural feed additive as alternative to antimicrobial growth promoters is highly efficient [22]. Recently medicinal plant extracts are being used to promote growth and performance [23]. The most beneficial effects of using plant extracts are no side effect, antibiotic resistance doesn't develop, cost-effectiveness and eco-friendliness [22]. So, we should also consider using medicinal plants as growth promoters in broilers.

The TLC method was used to separate and identify colistin sulfate from poultry samples. Thin layer chromatography is a simple non expensive and exact technique which can execute easily in most laboratories. Among chromatographic techniques HPLC have high accuracy but have some limitations [24]. For direct investigation of residues on poultry farms TLC have low costs, it is fast and can analyze at least 10 samples at the same time. A similar type of study was conducted by [25] to separate and identified the ciprofloxacin and enrofloxacin residues from chicken liver, kidney and muscles using TLC. From the TLC analysis of colistin sulfate residue on poultry we find more or less same consequences. In the discriminate group liver, kidney, spleen and fat tissues showed positive frequency and the percentage were 66.67%, 50.00%, 33.33% and 16.67% respectively. The other samples (Thigh and Breast muscle) were negative in the TLC analysis. And in case of indiscriminate group all the samples showed positive frequency in various percentages. In the control group all the samples showed negative antibiotic residual frequency in TLC analysis as there was no use of antibiotic in control group. Finally, the highest average was in indiscriminate antibiotic group which was 88.88±5.56 and the lowest was in control group (0). In the discriminate antibiotic group, the average percentage was 27.77±11.12.The differences among means of three individual groups were statistically significant ($P<0.05$). The multiple pairwise comparison revealed that the difference of means between discriminate and control group was not significant. On the other hand, the other two pairs showed statistically significant difference among means.

It is evident that the indiscriminate use of colistin sulfate doesn't bear any fruitful effect on broiler. Rather there is potential harmful effects of antibiotics residue which might enter the human food chain and produce deleterious impact on human health. Moreover, the higher cost is a drawback to the profitability of the farmers. So, they must realize that use of antibiotics at large can't increase their profitability rather decrease the profits. The lower blood parameters are the clear indication of decreased immunity of the live birds. So, if the antibiotics are used prudently and proper withdrawal period is maintained, there is lower risk of antibiotics resistance and other residue related problems and there is increased chance of profitability of broiler farmers. So from the above discussion it is clear that, antibiotic residue already exist in our food chain, especially in broiler. However, a comprehensive study require in Bangladesh to detect and estimate all the antibiotics used in broiler and layer chicken to take potential steps to protect the mankind and environment from antibiotics residue hazards.

5. Conclusion

The indoor trial study was conducted to assess the presence of colistin sulfate antibiotic residue in the edible tissue of broiler chicken. As it was an indoor investigation, so the main goal of this research was to detect the antibiotic in the muscle tissue even after maintaining a successful withdrawal period. Body weights of two groups of broiler showed highly significant changes during the use of antibiotic compared with the control group. Among the blood parameters the hemoglobin and the total erythrocyte showed significant difference whereas the packed cell volume did not show any significant changes. Percentage for positive samples, data was collected through the detection by TLC screening. The data obtained from the analyzed samples showed that, among the total of 72 samples, 42 (58.33%) samples were positive and 30 samples (41. 67%) found negative. A large numbers of samples were found positive and in liver, kidney and spleen the percentage were 66.67%, 50.00% and 33.33% in case of discriminate antibiotic group. For the indiscriminate antibiotic group, the percentage in liver, kidney and spleen were 100%. In both discriminate and indiscriminate group the samples like thigh muscle, breast muscle and fat also showed positive frequency of antibiotic residue in various percentages. But the percentages in these samples were less than the liver, kidney and spleen samples. The results confirmed the presence of antibiotic residues of colistin sulfate in broiler chicken and ready to eat chicken luncheon samples which pose a potential hazard to consumers. As the great number of samples was found with antimicrobial residues, it was stated that probably in some cases chicken meat producers do not respect the regulation about withdrawal periods of the centenary products. The use and sometimes misuse of antimicrobials in food animal production have resulted in the emergence and dissemination of resistant pathogens and resistance genes. Antimicrobial resistant bacteria in food animals can affect not only animal health, but also public health when they enter the food chain. Evidence suggests that more judicious use of antimicrobials in food animals will reduce the selection of resistant bacteria and help to preserve these valuable drugs for both human and veterinary medicine. Veterinary

authorities should control the use of antibiotics in poultry farms and banned their use as growth promoter. Rules should be taken to ensure the proper withdrawal periods before slaughtering and marketing. Besides, a monitoring policy should be implemented to ensure the conformity of poultry meat with international standards. National authorities should adopt a proactive approach that promotes programs aimed at reducing the need for antimicrobials in food animals and ensuring their prudent use. The method described in this study is a simple, easy inexpensive which can be readily adopted by any laboratory for the detection antibiotic residues in tissues of food-producing animals. Further investigation is required for the quantitative determination of antibiotic residues in food products.

Compliance with ethical standards

Acknowledgments

This work was supported by the Ministry of Education, Government of the People's Republic of Bangladesh by a grant in research. (Project No. 37.20.0000.004.033.020.2016.1053; LS2019925).

Disclosure of conflict of interest

The authors state no conflict of interest.

Statement of ethical approval

The experimental broilers were used ethically and at the end of the experiment sacrificed humanely following the ethical and welfare guidelines set by the Animal Welfare and Experimental Ethics Committee of Bangladesh Agricultural University [approval 5 number: AWEEC/ BAU/2021(09)].

References

[1] Swatantra S, Shukla, Tandia N, Nitesh K, Paliwal R.: Antibiotic Residues: A global challenge. Pharma Science Monitor. 2014; 5(3): 184-197.

[2] Donoghue DJ. Antibiotic residues in poultry tissues and eggs: Human health concerns? Poultry Sci. 2003; 82(4): 618-621.

[3] Jinap S, Cheong CK, Hajeb P, Ismail-Fitry MR. Sulfonamides determination in chicken meat products from Malaysia. International Food Research Journal. 2010; 17(4): 885-892.

[4] Nisha AR. Antibiotic Residues-A Global Health Hazard. Veterinary World. 2008; 1(12): 375–377.

[5] Hussein MA, Khalil S. Screening of some antibiotics and anabolic steroids residues in broiler breast marketed in El-Sharkia governorate. Life Sci. 2013: 10(1).

[6] Capone A, Giannella M, Fortini D, Giordano A, Meledandri M et al. High rate of colistin resistance among patients with carbapenem-resistant Klebsiella pneumoniae infection accounts for an excess of mortality. Clinical microbiology and infection. The official publication of the European Society of Clinical Microbiology and Infectious Diseases. 2013; 19(1): 23-30.

[7] Kontopoulou K, Protonotariou E, Vasilakos K, Kriti M, Koteli A, Antoniadou E, Sofianou D. Hospital outbreak caused by Klebsiella pneumoniae producing KPC-2 beta-lactamase resistant to colistin. The Journal of hospital infection. 2010; 76(1): 70-3.

[8] Zarkotou O, Pournaras S, Voulgari E, Chrysos G, Prekates A, Voutsinas D, Themeli-Digalaki K, Tsakris A. Risk factors and outcomes associated with acquisition of colistin-resistant KPC-producing Klebsiella pneumoniae: A matched case-control study. Journal of clinical microbiology. 2010; 48(6): 2271-4.

[9] Tajik MA, Shohreh B. Detection of antibiotics residues in chicken meat using TLC. International Journal of Poultry Science. 2006; 5(7): 611-612.

[10] Islam MS, Hasan MR, Islam MS. Thin layer chromatographic investigation of antibiotics residues in edible poultry tissues in Bangladesh. World Journal of Biology Pharmacy and Health Sciences. 2021; 05(03): 024–032.

[11] Ali MR, Sikder MMH, Islam MS, Islam MS. Investigation of discriminate and indiscriminate use of doxycycline in broiler: an indoor research on antibiotic doxycycline residue study in edible poultry tissue. Asian Journal of Medical and Biological Research. 2020; 6 (1): 1-7.

[12] Das D, Islam MS, Sikder MMH, Alom F, Sharmin MA, Ashraf MZF. Presence of antibiotic residue and residual effect of tylosin tartrate in broiler. International Journal of Natural and Social Sciences. 2020; 7(2): 29-35.

[13] Ellis RL. Food Additives and Contaminants. Development of veterinary drug residue controls by the Codex Alimentary Commission, a review. 2008; 25(12): 1432-1438.

[14] Page SW, Gautier P. Use of antimicrobial agents in livestock. Rev Sci et Tech. 2012; 31(1): 145-188.

[15] Kirbis A. Microbiological screening method for detection of aminoglycosides, β-lactames, macrolides, tetracyclines and quinolones in meat samples. Slovenian Veterinary Research. 2007; 44(1/2): 11-18.

[16] Chafer-Pericas C, Maquieira A, Puchades R.Fast screening methods to detect antibiotic residues in food samples. TrAC Trends in Analytical Chemistry. 2010; 29(9): 1038-1049.

[17] Wasch DK, Okerman L, Croubels S, Brabander HD, Hoof JV, Backer PD. 1998: Detection of residues of tetracycline antibiotics in pork and chicken meat: correlation between results of screening and confirmatory test. Analyst. 1998; 123: 2737-2741.

[18] Givens DI. The role of animal nutrition in improving the nutritive value of animal derived foods in relation to chronic disease. Proceedings of the Nutrition Society. 2005; 64: 395-402.

[19] Paryad A, Mahmoudin M. Effect of different levels of supplemental yeast (Saccharomyces cerevisiae) on performance, blood constituents and carcass characteristics of broiler chicks. African Journal of Agricultural Research. 2008; 3(12): 835-842.

[20] Naeem M, Khan K, Rafiq S. Determination of residues of quinolones in poultry products by high pressure liquid chromatography. Appl. Sci. 2006; 6(2): 373-379.

[21] Marcio CC, Jose AB, Amauri AA, Alexandre O Different antibiotic growth promoters induce specific changes in the cecal microbiota membership of broiler chicken. PLOS ONE. 2017; 12: 1-14.

[22] Demir E, Sarica S, Ozcan MA, Suicmez M. The use of natural feed additives alternative to antibiotic growth promoters in broiler diets. European Poultry Science. 2005; 69: 110-116.

[23] Barreto MSR, Menter JFM, Pereira PWZ, Rizzo PV. Plant extracts used as growth promoters in broilers. Brazilian Journal of Avian Research. 2008, 12: 1-13.

[24] Choma MI. TLC separation of flouroquinolones: searching for better selectivity. Journal of Liquid Chromatography & Related Technologies. 2003; 26(16): 2673-2685.

[25] Amjad H, Iqbal J, Naeem M. Analysis of some residual antibiotics in muscle, kidney and liver samples of broiler chicken by various methods. Proc. Pak. Acad. Sci. 2005; 42(4): 223-231.