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MICROBIAL SCREENING OF EGGS FOR DETECTION OF ANTIMICROBIAL DRUG RESIDUES - A SIMPLE AND COST-EFFECTIVE METHOD

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ABSTRACT
This study was carried out to determine the antimicrobial drug residues in commercial chicken eggs obtained from various outlets of layer farms in Tamil Nadu. Microbial tube test method was performed using the bacterial strain *Micrococcus luteus.* Sixty commercial eggs were collected randomly and examined for antimicrobial drug residues. The results of this study revealed 20 (33.3%) egg samples were positive for antimicrobial residues related to pleuromutilin group (Tiamulin). However, residues detected in 33.3% of eggs were all below the MRL set by European Union (EU). The presence of these residues in eggs could cause health hazards includes toxicological, carcinogenic and genotoxic impacts, effect on normal gut flora, hypersensitivity reactions and bacterial resistance. Thus, the antibiotic residues in commercial eggs must be monitored as routine test in order to guarantee food safety due to their side effects on human health.

KEYWORDS: antimicrobial drug, chicken eggs, Tiamulin, EU.

INTRODUCTION

Poultry industry is growing rapidly in India and the consumption of eggs has also been increasing resulting in more demand. The increasing demand for poultry eggs has necessitated the large scale production of poultry and subsequent use of veterinary drugs, especially antimicrobials. However, the use of these antimicrobial agents has recently become a serious public health issue (Jafari, 2007). These antimicrobials are used for therapeutic purposes and are added in feed and water in sub-therapeutic doses for prophylaxis and growth promotion and its residues can be detected in poultry eggs, if proper withdrawal protocols are not followed (Ezenduka *et al*., 2014; Diaz-Sanchez *et al*., 2015). The consumption of eggs containing residues could result in public health hazards including: development of antimicrobial resistance, alteration of intestinal microflora (Salehzadeh *et al*., 2006) and hypersensitive reactions (Samanidou *et al*., 2008). To ensure consumer safety, worldwide regulatory authorities have set the Maximum Residual Limits (MRL) for several veterinary drugs including antimicrobials (European Union, 2010; Codex Alimentarius Commission, 2012) and it is necessary to abide by these set limits to make sure that these residues are not present at levels in animal products intended for human consumption.

For antibiotic residue detection, numerous methods are available but the easy, fast and cheap method is the microbiological assay which is the first step in screening samples to prove the existence or absence of antibiotic residues (Hakimzadegan *et al*., 2014). Microbiological methods are the most common and practical method to determine antibiotic residues in food, because, these methods are compared with other methods in terms of cost and time savings are more advantageous (Pikkemaat, 2009). As a screening method, the microbiological method minimizes the number of test samples that would eventually be tested with a more sensitive test for confirmation. Although not always specific, these methods are recommended for screening as they can detect many classes of drug residues in animal products in a single test (Fagbamila *et al*., 2012). This study therefore was designed to provide information on antimicrobial drug residues in commercial eggs collected from Tamil Nadu.

MATERIALS & METHODS

Sample collection

A total number of 60 egg samples were collected from various commercial layer farm outlets in Tamil Nadu, India. Then the egg samples were kept in the refrigerator until further use.

Sample preparation

The surface of each egg sample was cleaned using 70% alcohol. A small opening was made at the tip of the egg using sterile forceps and the eggs were separated into 3 parts–Egg yolks, Egg albumen and whole egg was drained out carefully, extracted and analysed for antibiotic residues.

Extraction of Egg Samples

The chemicals and solvents used for the extraction of antibiotic residues from egg samples were of analytical and purchased from Merck and Sigma-Aldrich. Extraction of antibiotic residues from eggs samples were done by using the method described by Godfred Darko *et al*., 2015 with some modifications.

Preparation of culture medium

The following culture media were used: Nutrient agar and Nutrient broth obtained from HIMEDIA. Nutrient broth (1.3 g) was weighed into a conical flask and 100 ml of distilled water added, as per the manufacturer's

instructions. It was then sterilized in an autoclave at a pressure of 15 mm Hg and a temperature of 121°C for 15 minutes, after which it was cooled to about 50°C. ThenBromo Cresol Purple indicator (0.002 g/l) was added in the autoclaved nutrient broth medium. (Shelef *et al*., 1997, Jambalang, A.R., 2012)

Preparation of bacterial strain

The bacterial strain used was *Micrococcus luteus* (MTCC 7590), obtained from Microbiological Type Culture Collection (MTCC). The freeze dried bacterial culture was activated according to the instructions given by MTCC. Single colony obtained from petriplate was inoculated into 5ml nutrient broth and incubated at 37ºC for 18 -24hrs. Broth suspension of test organism was adjusted with a sterile physiological saline to a concentration approximately equal to 0.5 McFarland standard equivalents to 1.5×10^8CFU/ml. All the procedures were done aseptically under biosafety cabinet (Kilinc *et al*., 2008).

Experimental Design

Egg samples collected	Egg samples separated into		
Commercial chicken eggs 60	Whole egg 60	Egg yolk 60	Egg albumen 60

Analysis of Egg Samples by Microbial Screening

Screening of antimicrobial drug residues were conducted by using microbiological tube test method with a suitable indicator organism for the antibiotic studied (Ref: Kirbis, 2006)

Antibiotictested	Indicator bacteria
Tiamulin	*Micrococcus luteus*

Microbiological tube test method

Procedure

1800µl of nutrient broth with pH indicator bromocresol purple was taken in a test tube and then 100µl of egg sample and 100µl of test bacterium was pipetted into the test tube and mixed thoroughly. The test tubes with positive growth controls containing culture organism in the broth culture and a negative control containing broth also kept. All the test tubes were incubated at 37ºC for 18-24hrs depending on the culture organism and their growth. The test tubes that remained purple after the incubation were recorded as positive for antimicrobial residues, and those that turned yellow were recorded as negative for antimicrobial residues (Jambalang, A.R., 2012)

RESULTS

Out of 60 egg samples analyzed, 20(33.3%)were found to be positive for antibiotic drug residues against the test organism *Micrococcus luteus*. A positive result was indicated by color change *i.e*., test tubes that remained purple after the incubation were recorded as positive and those that turned yellow were recorded as negative for antimicrobial residues. In this study, antibiotic residues were analyzed for albumen and yolk separately because some antimicrobials would occur in the yolk and some in the albumen and testing of only the egg yolk or egg albumen could lead to false negative results (Jambalang, A.R., 2012). Tiamulin residues were detected in both egg yolk and egg white of all the positive samples. Physicochemical properties of the drugs, the physiology of the hen and egg formation will determine how much drug will be deposited (Woodward KN., 1991). The LOD for the antibiotic tiamulin was found to be 100 µg/kg which is below the accepted maximum residue level (MRL) for the tested antibiotic (1000µg/kg) set by European Union.

Number and percentage of positive samples			
Total sample	Egg yolk	Egg albumen	Whole egg
60	60	60	60
20 (33.3%)	20(33.3%)	20(33.3%)	20(33.3%)

Antibiotic	Bacterial strain	LOD st.s. (µg/ kg)	LOD egg (µg/kg)	MRL egg (µg/kg)
Tiamulin	*Micrococcus luteus*	100	100	1000

LOD st.s -Limit of detection in standard solution
LOD egg - Limit of detection in egg
MRL egg - Maximum residue level in egg

FIGURE 1: *Micrococcus luteus*

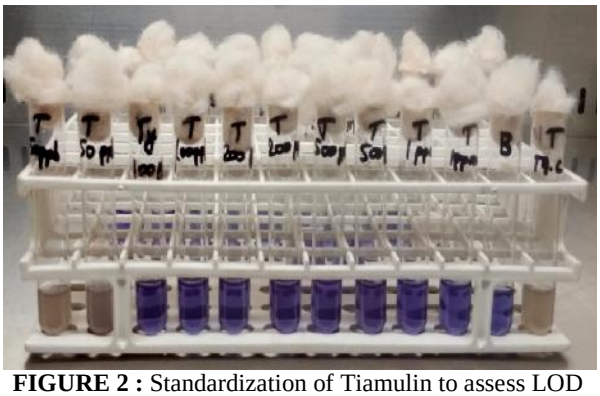

FIGURE 2 : Standardization of Tiamulin to assess LOD

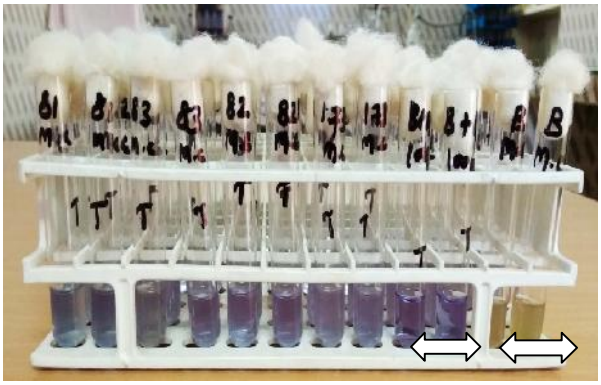

FIGURE 3: Antimicrobial assay of Antibiotic Tiamulin in Egg samples

DISCUSSION

Veterinary drugs are essential in poultry production to prevent and control infectious and non-infectious diseases, assist in reducing stress due to environmental changes, vaccination, debeaking and other management practices (Kabir *et al*., 2004). The increased demand for eggs as an alternative and cheapest source of animal protein places a lot of pressure on the growing poultry industry and compel the indiscriminate use of veterinary drugs particularly the antimicrobials to ensure disease free growth promotion in broilers and egg production in layers, with farmers failing to observe the recommended withdrawal periods (Fagbamila *et al*., 2010). Nevertheless, the use of antibiotics in food producing animals can leave their traces in food stuffs such as meat, milk and eggs.

The results obtained in this study indicated the presence of antimicrobial residue tiamulin in egg samples tested. Tiamulin is mainly used for swine and poultry and it is indicated for the prophylaxis and treatment of dysentery, pneumonia, and mycoplasmal infections (Wang *et al*., 2011). Tiamulin is commonly used to treat Mycoplasma infection in laying hens (Smith *et al*., 2006) even though the residue level of the positive samples were not above the MRL level but still the development of antibiotic resistance in humans is unavoidable. Tiamulin resistance is rare but emerging and cross-resistance to tylosin and erythromycin occurs (Krasucka *et al*., 2010). Since the mechanism of development of resistance to antibiotic is due to the usage of antibiotic at sub-therapeutic doses, so there may be possibility of development of resistance among the consumers (human) due to continuous ingestion of milk, meat and eggs with antibiotic residues even below the MRL (Singh *et al*., 2014).

CONCLUSION

The detection of antimicrobial residues in animal products (milk, meat and eggs) is of immense importance because of their direct impact on public health. Microbial screening methods are essential to know about the existence or absence of drug residues in foods of animal origin and have been recommended as official and conventional methods because of their simplicity. Also, it is not feasible to employ sophisticated instruments when large number of samples needs to be analysed, as they are very expensive and consume lot of time. Currently, there are very few reports available describing the screening methods for the detection of antimicrobial residues in eggs and extension of this method to identify various classes of antimicrobial drugs is need of the hour.

Conflicts of interest

The authors declared no conflict of interest in this research.

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REFERENCES

Kilinc, B. and Cakli, S. (2008) Screening for antibiotic residues in the trout by the Four Plate test, Premi test and ELISA test. *Eur. Food Res. Technol*. 226(4), pp.795-799

Codex Alimentarius Commission (2012) Maximum residue limits for veterinary drugs in foods. Updated as at the 35th session of the Codex Alimentarius Commission. CAC/MRL 2: 1-40.

Darko, G., Mensah, J.K., Dapaah, S.S. and Odei, J. (2015) Estimated dietary exposure to veterinary residues in chicken and eggs. *Int. j. food contam.*, *2*(1), p.16. https: // doi. org /10.1186/s40550-015-0022-2

Diaz-Sanchez, S., Moscoso, S., delos Santos, F.S., Andino, A. and Hanning, I. (2015) Antibiotic use in poultry: A driving force for organic poultry production. *Food Protection Trends*, **35,** pp.440-447.

European Union (2010) Commission regulation (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. *Official Journal of the European Union*.

Ezenduka, E.V., Ike, O.S. and Anaelom, N.J. (2014) Rapid detection of antimicrobial residues in poultry: A consequence of non-prudent use of antimicrobials. *Health*, 6(02), p.149.https://doi.org. 10.4236/health. 2014. 62023

Idowu, F., Junaid, K., Paul, A., Gabriel, O., Paul, A., Sati, N., Maryam, M. and Jarlath, U. (2010) Antimicrobial screening of commercial eggs and determination of tetracycline residue using two microbiological methods. *International Journal of Poultry Science*, *9*(10), pp.959-962.

Fagbamila, I.O., Ngulukun, S.S., Ardzard, S.S., Sati, N., Ajayi, O.T., Ankeli, P.I., Akalusi, Y. (2012) "Validation of commercial test kit for microbiological screening of antimicrobials in chicken eggs." *Research Journal of Veterinary Sciences* 5: 75-80.https://doi.org. 10.3923/ rjvs. 2012.75.80

Hakimzadegan, M., Khalilzadeh Khosroshahi, M. and Hasseini Nasab, S. (2014) Monitoring of Antibiotic Residue in chicken eggs in Tabriz city by FPT. *Int J Adv BiolBiomRes*, ***2*(1),** pp.132-140.

Jafari, M.T., Khayamian, T., Shaer, V. and Zarei, N., (2007) Determination of veterinary drug residues in chicken meat using corona discharge ion mobility spectrometry. *Analytica chimica acta*, *581*(1):147-153.

Jambalang, A.R. (2012) *The development and validation of a bacteriological screening test for antimicrobial residues in eggs* (Doctoral dissertation, University of Pretoria).

Kabir, J., Umoh, V.J., Audu-Okoh, E., Umoh, J.U. and Kwaga, J.K.P. (2004) Veterinary drug use in poultry farms and determination of antimicrobial drug residues in commercial eggs and slaughtered chicken in Kaduna State, Nigeria. *Food Control*, ***15*(2)**, pp.99-105.

Kirbiš, A. (2006) Microbilogical 5-plate screening method for detection of tetracyclines, aminoglycosides, cephalo sporines and macrolides in milk. *Slov Vet Res*, ***43*(4),** pp.161-168.

Krasucka, D., Mitura, A., Cybulski, W., Kos, K. and Pietroń, W. (2010) Tiamulin Hydrogen Fumarate-Veterinary Uses and HPLC Method of Determination in Premixes and Medicated Feeding Stuffs. *Acta Poloniae Pharmaceutica–Drug Res*, ***67,*** pp.682-685.

Pikkemaat, M.G. (2009) Microbial screening methods for detection of antibiotic residues in slaughter animals. *Analytical and bioanalytical chemistry*, *395*(4), pp.893-905.

Salehzadeh, F., Madani, R., Salehzadeh, A., Rokni, N. and Golchinefar, F. (2006**)** Oxytetracycline residue in chicken tissues from Tehran slaughterhouses in Iran. *Pakistan Journal of Nutrition*, **5(4)**, pp.377-381.

Samanidou, V. and Nisyriou, S. (2008) Multi- residue methods for confirmatory determination of antibiotics in milk. *Journal of separation science*, **31(11)**, pp.2068-2090.

Shelef, L.A. & Firstenberg- Eden, R. (1997) Novel selective and non- selective optical detection of micro-organisms. *Letters in applied microbiology*, *25*(3), pp. 202-206.

Singh, S., Shukla, S., Tandia, N., Kumar, N. and Paliwal, R., (2014) Antibiotic Residues: A Global Challenge. *Pharma Science Monitor*, ***5*(3)**.

Smith J., Drum D., Dai Y., Kim J., Sanchez S., Maurer J., Hofacre C., Lee M. (2007) Impact of antimicrobial usage on antimicrobial resistance in commensal Escherichia coli strains colonizing broiler chickens. *Applied and Environmental Microbiology*. **73**: 1404-1414.

Wang, J., Mac Neil, J.D. and Kay, J.F. eds. (2011) *Chemical analysis of antibiotic residues in food* (Vol. 38) John Wiley & Sons.