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Determination of Antibacterial Drug Residues in Commercial Eggs Distributed in Urmia, Iran

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Abstract

Background: The presence of antibacterial drug residues in food caused many concerns for consumers. This study was designed to determine of antibacterial drug residues in commercial eggs distributed in Urmia, Iran.

Methods: Disc diffusion microbial inhibition test was performed using three media seeded with *Bacillus subtilis* at different pH values (6, 7.2 or 8) and a fourth medium seeded with *Micrococcus luteus*. Two hundred commercial eggs were collected randomly and examined for antibacterial drug residues. Standard solutions of antibiotics were used as control.

Results: The results of this study revealed that twenty five samples (12.5%) of the prepared eggs were positive for antibacterial substances and all of them were related to macrolides group.

Conclusion: The presence of these residues in eggs could cause health hazards like hypersensitivity reaction, development of resistant organisms to these antibacterial agents, destruction of gastrointestinal natural flora, etc. In conclusion, the antibiotic residues in commercial eggs have to be monitored as routine test due to their side effects on human health.

Introduction

As an important protein sources, birds are reared for meat and egg production. Drugs as an essential part of poultry production are used to prevent and control diseases, reduce stress due to environmental changes, vaccination and other management practices (Kabir et al., 2004). Drugs given to birds orally or parentally may be found in tissues (Donoghue and Hairston, 2000; Kan and Petz, 2000). Their presence in tissues of birds is more likely in instances where the drug is fed continuously over a long period of time and when used beyond recommendations (Kabir et al., 2004). Antibacterial agents are among the drugs which are commonly used in poultry production (Kilinc and Cakli, 2008). The accumulation of antibacterial agents in the body tissues and other products such as egg are due to using drugs and are known as drug residues (Ezenduka et al., 2011).

Worldwide national and international public health agencies have a deep concern about the presence of antibiotic residues in meat, egg and edible viscera of food-producing animal (Ašperger et al., 2009; Mahmoudi et al., 2014). Undesirable changes in bowel micro flora and immunological reaction in susceptible persons may be caused after consumption of a large amount of various animal products containing antibiotic residues (Mahmoudi et al., 2014; Mottier et al., 2003).

The screening methods are initial steps in determining

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the presence of drug residues in studying samples. These screening methods must have the following characteristics 1) inexpensive, 2) possible to be performed in large scales, 3) able to detect multiple types of antibiotic residues and 4) high sensitivity and specificity and making accurate results (Pikkemaat et al., 2008). Various microbiological and chemical assays have already been developed and validated to detect and quantitate antibiotic residues. The microbiological assay is used for screening antibiotic residues in food products at macro level (Tajik et al., 2010).

Nowadays, four plate test (FPT) is commonly used for antibiotic residues screening (Kilinc and Cakli, 2008; Okerman et al., 1997). This method is a four plate agar diffusion test in which two different microorganisms *Bacillus subtilis* and *Micrococcus luteus* are used as indicator organisms, with three different pH levels of the media (Bogaerts and Wolf, 1980). Diffusion of an antibacterial substance is shown by the formation of inhibition zones of one or both microorganisms (Kilinc and Cakli, 2008; Okerman et al., 1997). Using different pH levels in the test media are essential to increase the detection limits of antibiotics (Bogaerts and Wolf, 1980). FPT is used to assay multiple antibiotic substances as listed in Table 1 (Chang et al., 2000).

The aim of this study was to determine of antibacterial drug residues in commercial eggs distributed in Urmia, Iran.

Materials and methods

Sample preparation

Commercial egg samples (200 in total) were collected every 7 days from distributors in various part of Urmia city from May to September in 2012. For sampling, crates of eggs, available at the selected sources, were numbered from the first to the last crates. The egg at the top left corner in the first crate was assigned No. 1 until the last egg in the bottom right corner. Numbering was continued with the egg at the top left corner of the second crate in that order to the last egg. A total of 10 eggs were then randomly selected from the total number of eggs using simple random sampling format. The egg samples were then taken to the laboratory immediately for analysis (Kabir et al., 2004).

Preparation of culture medium

Nutrient broth and mueller hinton agar (MHA; Merck, Darmstadt, Germany) media were prepared according to the manufacturers recommendations. Blank paper discs 12 mm in diameter were sterilized and used for inoculating samples and controls onto plates (Kabir et al., 2004). MHA agar pH 6, MHA agar pH 7.2 and MHA agar pH 8 were prepared using pH meter (pH 510 Eutech; CyberScan, Singapore) by adding hydrochloric acid and sodium hydroxide and autoclaved. After cooling to 45-50 ˚C and adding the supplement (trimethoprim) of MHA agar pH 7.2, the media were poured into the plates (Kilinc and Cakli, 2008).

The bacteria used in FPT were *B. Subtilis* (PTTC 1365) and *M. luteus* (PTTC 1169) that have been prepared from Department of Microbiology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran. An overnight culture of the test organisms in 10 ml of nutrient broth was used to inoculate plates. Broth suspension of each test organism was adjusted with a sterile normal saline solution to a concentration approximately equal to McFarland standard turbidity (equivalent to 3×10^8 cell/ml; Kabir et al., 2004).

MHA media were inoculated by sterile cotton swab sticks which were already dipped into the suspension of the test organisms. The plates were thoroughly swabbed uniformly, one quadrant at a time to achieve a confluent growth. Inoculated plates were allowed to dry for about 5-10 min (Kabir et al., 2004).

Four different inoculated media were used for antibiotic detection including 1) MHA agar pH 6 seeded with *B. subtilis*, 2) MHA agar pH 7.2 with trimethoprim (Sigma chemical Co. St. Louis, Mo., USA) added to a final concentration of 50 µg/l medium and seeded with *B. subtilis*, 3) MHA agar pH 8 seeded with *B. subtilis*, 4) MHA agar pH 8 seeded with *M. luteus* (Kilinc and Cakli, 2008).

Testing of sample eggs

The surface of each egg sample was cleaned using a sterile hand towel soaked in 70% (v/v) alcohol. A small opening was made at the tip of the egg using a sterile forceps.

The albumen was then drained out carefully through the pore and the yolk transferred into a sterile falcon tubes. Ten ml of phosphate buffer (pH 7) was added to the yolk and homogenized. Using a sterile forceps, paper discs were dipped into the yolk, allowed to soak and excess yolk were drained from the discs before placing them on the surface of seeded MHA plates (Kabir et al., 2004).

Antibiotic standards were penicillin G sodium salt, sulfadimidin, and streptomycin tylosin. Stock solutions were prepared as 0.0062 g/l0 µl for penicillin, and 0.5 g/10 µl for sulfadimidin, streptomycin and tylosin. Two fold dilutions were prepared from these stock solutions. Control discs were prepared by adding 0.01 ml control solutions and placed on agar surface. Blank paper disk without any sample was placed on agar surface as negative control as well (Kilinc and Cakli, 2008).

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After application of the test and control discs, plates were incubated at 37 ºC for 18-20 h and then investigated for the presence of inhibition zones of test organism around the test and control discs (Kabir et al., 2004).

Results

A total of 200 commercial eggs were tested for the presence of antibacterial drug residues out of which 25 (12.5%) were found to be positive. The inhibition zones of positive samples are shown in Table 2. A positive result was indicated by the complete inhibition of growth on the surface of the agar around discs on one or more test plates, in an inhibition zone not less than 2 mm wide (Kilinc and Cakli, 2008).

All positive samples in present study were detected from medium 4 (MHA agar pH 8 seeded with *M. luteus*) that indicate the presence of penicillin type and macrolide type antibiotic residues in egg samples.

Inhibition zones were measured and correlated to antibiotic concentrations. Detection limits for different antibiotics tested were as follows: penicillin G (sodium salt) on medium 1, 0.4 ng; sulfadimidin on medium 2, 62.5 ng; streptomycin on medium 3, 31.25 ng; tylosin on medium 4, 4 ng.

Table1: Detection of multiple antibiotic substances by FPT assay

pH of agar medium	Test bacteria	Inferred antibiotics
6	*B. subtilis*	Penicillin-type Tetracycline-type
7.2	*B. subtilis*	Sulfa drugs
8	*B. subtilis*	Aminoglycoside-type
8	*M. luteus*	Penicillin-type Marcolides-type

Table 2: Results of microbial inhibition test of commercial eggs of Urmia, Iran

Day	Positive	Negative	Total of tested eggs	Projected number of eggs with residues	Size of inhibition zone (mm)
1	0	10	10	-	-
8	1	9	10	11	13
15	1	9	10	23	13
22	0	10	10	-	-
29	0	10	10	-	-
36	1	9	10	51	13
43	1	9	10	65	13
50	2	8	10	70, 72	11, 13
57	2	8	10	81, 85	11, 10
64	1	9	10	99	13
71	1	9	10	110	13
78	1	9	10	115	13
85	1	9	10	123	12
92	2	8	10	133, 136	11, 10
99	2	8	10	141, 149	11, 13
106	2	8	10	152, 154	10, 10
113	1	9	10	163, 165	11
120	2	8	10	170, 173	13, 13
127	2	8	10	188, 189	13, 12
134	2	8	10	194	12
Total	25	175	200	-	-

Discussion

Drug residues in food are considered as an important food safety concern due to being public health hazards. In subjects of hypersensitivity to sulfonamides, skin allergies could occur following consumption of some foods like eggs containing high concentrations of sulfonamide residues. Consumer's anaphylactic reaction has been previously reported due to consumption of chickens containing penicillin residues (Teh and Rigg, 1992).

The results obtained in this survey indicated a high incidence of veterinary drug residues in the eggs in Urmia. Quite consistent with the results of current study, other researchers diagnosed macrolides group as the major contaminant antibiotics in the egg yolk by FPT method (Hakimzadegan et al., 2014; Kabir et al., 2004). Tiamulin, tylosin and erythromycin of macrolides group are commonly used against poultry diseases. Erythromycin is used for treatment of arthritis caused by *staphylococcus aureus* and tylosin and tiamulin are frequently used to treat *Mycoplasma* infections in poultry, especially in laying hens (Smith et al., 2007). In addition to macrolides as major contaminants in the tested eggs, aminoglycosides and tetracycline were diagnosed as minor

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contaminants which were not detected in present study (Hakimzadegan et al., 2014). However, similar to present study, macrolides were the only antibiotic contaminants in egg in Nigeria (Kabir et al., 2004).

In commercial eggs, the handicap of using yolk directly makes the detection underestimated, given the fact that it is viscous and diffuses poorly on agar (Kabir et al., 2004). An initial deproteination procedure may enhance detection of drugs (Arnold and Somogyi, 1984). Screening of drug residues in egg yolk by microbiological methods is more appropriate than egg white, because of its lower content of lysozymes and more deposition and longer persistence of drugs in egg yolk (Alm El Dein and Elhearon, 2010; Kan and Petz, 2000).

Sensitivity and specificity of microbiological methods are generally low for residue testing. Nevertheless, their simplicity makes them suitable for screening purposes (Tajik et al., 2010). A number of microbiological screening tests have been described alone or in conjunction with other physico-chemical and immunological methods. These tests are more suitable for regulatory purposes and provide a greater accuracy in the assessment of contamination level of poultry products with veterinary drug residues (Kabir et al., 2004).

M. luteus used by FPT has a broader sensitivity range to antibacterial drugs particularly penicillins, sulfonamides and aminoglycosides (Kabir et al., 2004; Read et al., 1971; Tsai and Kondo, 2001). According to recent knowledge, a combination of *B. subtilis* (pH 7.2) as a test-microorganism and the addition of trimethoprim showed the highest sensitivity to the presence of sulfonamide residues in food (Kilinc and Cakli, 2008; Kilinc et al., 2007). Trimethoprim is a chemical substance used as a therapeutic agent because of its inhibitory effect against bacterial enzymes (Marcinčák et al., 2006). MFP test should be able to detect the presence of antibiotic residues at the level of MRL. It is possible to state that FPT without any modification is not able to detect sulfonamide residues at the level of MRL. Valid food legislation approved the application of FPT method as a screening test for drug residues (Marcinčák et al., 2006).

It is believed that bird's treatment is a more important source of antibacterial residue than the use of medicated feeds which appears to have contributed to contamination of the table eggs in which antibacterial residues were detected (Ezenduka et al., 2011). Drug residues would be stored by hens days to weeks after dosing period even without any additional drug transfer. This is of concern, because eggs are continuously supplied to many households by backyard poultry rearing while no official program monitors drug residues and consumers response to the drug residue hazards is sustainable and passive (Alm El Dein and Elhearon, 2010; Donoghue et al., 1996).

Conclusion

Many troubles such as teratogenic and carcinogenic effects, allergic reactions and antibiotic resistance can threaten human health due to presence of residues in food originated from animals. Although, producing food originated from animals free from chemicals or drug residues is impossible, but deep consideration is needed towards proper and controlled drug use, and safer animal food products. First step is the monitoring of withdrawal time of drugs followed by systematic education of farmers and regular veterinary supervision. In order to diminish the frequent usage of antibacterial drugs in poultry, occurrence of residues in food and the bacterial resistance to drugs, veterinarians should develop alternative management options like vaccinations. We provide the first insight into the extent of contamination of poultry products intended for human consumption with veterinary drug residues in Iran. Obviously, there is a need for a national residue avoidance and control program in Iran in accordance with international regulations.

Conflicts of interest

The authors declared no conflict of interest in this research.

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