



## Evaluation of Apparent Microflora and Study of Antibiotic Resistance of Coliforms Isolated from the Shells of Poultry Eggs in Moscow-Russia

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### Authors' contributions

*This research was carried out in collaboration among all authors. Author MMJA was involved with data collection and processing, statistical analysis, manuscript writing and editing. Authors PIV and SLA designed the study, performed literature review, manuscript writing and was also involved in data collection and analysis. All authors read and approved the final manuscript.*

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### ABSTRACT

**Background:** Antibiotic resistance is increasingly an issue in both human health and veterinary medicine. It is important to regularly assess the resistance of strains which have high potential to transmit this resistance. Numerous researches have shown so far that coli forms are part of these bacteria and coliforms from breeding environments (in particular poultry farming) are the most concerned because of their permanent exposure to antibiotics and adaptation that could result.

**Aim:** This work was carried out to investigate the sensitivity to antibiotics of coliforms isolated from the shell of poultry eggs.

**Methods:** A total of 191 egg samples (149 chicken eggs and 42 quail eggs) were collected in supermarket of the city of Moscow, coliforms bacteria were isolated the shells of these eggs and their sensitivity to antibiotics was achieved by employing modified Kirby-Bauer's disc method. The results have been analysed and interpreted using the Guidelines of Ministry of health of Russian Federation.

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**Results and Discussion:** It turns out that, among the 191 samples studied only 16.2% (31) of the chicken eggs contained coli forms, 7.3% (14) contained mold and 76.4% (146) were sterile while 81% (34) of quail eggs were sterile, 19% (8) contained mold and none contained coliforms. Of the 60 bacteria isolated, 39 (65%) were resistant to at least one antibiotic and the highest MAR Index observed was 0.58 while the lowest was 0.08. Of the 720 anti biograms performed, we observed 557 (77.4%) cases of sensitivity, 72 (10.0%) intermediate cases and 91 (12.6%) cases of resistance. The highest resistances were observed on tetracycline (50%), amoxycillin (40%) and ampicillin (30%) while the weakest resistances were observed on trimethoprim (11.7%), cefazolin (10%), ciprofloxacin (5%) and 1.7% for ceftriazone, fosfomycin and nitrofurantoin. A comparison between the sensitivity of the isolates to Cefazolin and their sensitivity to Cefazolin + Clavulanic acid showed that the difference between these two antibiotics was highly significant (P = 0.00).

**Conclusion:** Antibiotic resistance of coliform bacteria isolated was high, with the highest MAR Index observed equal to 0.58. The bio security relating to the use of antibiotics must be reinforced, better use of current known antibiotics is necessary to prolong their effectiveness over time.

**Keywords:** Apparent microflora; antibiotic; susceptibility; coli forms; multi-drug resistance.

## 1. INTRODUCTION

The presence of microorganisms on eggs is a major problem in poultry farming and all related industrial sectors since it can potentially result in the occurrence of a public health problem. It has been shown that contamination of eggs most often occurs shortly after laying due to contact with dirty surfaces [1]. Several studies have shown that the penetration of microorganisms inside the eggs is possible when the quantity of microorganisms on the shell becomes very large [2]. It has been reported that the penetration of bacteria like *Staphylococcus aureus* into eggs can be facilitated by *Escherichia coli* O157: H7 [3]. The bacteria most involved in egg contamination are *Salmonella* spp., *E. coli*, *Campylobacter jejuni* and *Listeria monocytogens* [4,5]. These microorganisms can be pathogenic and the symptoms of the diseases they induce can be mild or more serious, endangering the life of the consumer [6]. Whether they are pathogenic or not, the real problem with these bacteria is the multidrug resistance which they increasingly present to different antibiotics [7]. For more than half a century, antibiotics have been used in poultry farming as a prophylactic and / or curative agent against infections. They are also used to promote the proliferation of certain microorganisms beneficial to poultry while eliminating or reducing those which are pathogenic [8]. In some countries, the use of antibiotics as growth promoters has been banned, in particular, due to the presence of antibiotic residues in foods containing eggs and the development of resistance traits which could be transmitted to bacteria pathogenic for human [7]. However, although the danger is clearly identified, many breeders continue to use antibiotics while others try to turn to alternative

methods. Thus, this study was conducted to evaluate the apparent microflora of the shell of poultry eggs with the aim of highlighting and quantifying the resistance of coliforms isolated from shells of eggs produced in Moscow markets and supermarkets.

## 2. MATERIALS AND METHODS

This study was conducted in the bacteriology laboratory of the Department of Microbiology and Virology in the Faculty of Medicine at Peoples' Friendship University of Russia from December 2019 to March 2020.

### 2.1 Collection and Treatment of Samples

The eggs used in this study were purchased from supermarkets (Auchan, Diksi, Fix Price and Perekrestok) in Moscow. A total of 191 eggs were collected (149 chicken eggs and 42 quail eggs) and brought back to the laboratory in the cells which contained them. In the laboratory, the eggs were put in a sterile polythene bag and the study was only looked at their surface for isolation of coliform bacteria.

### 2.2 Isolation and Preservation of Bacteria from Egg Shell Surface

After placing each egg in a sterile polythene bag, the shell was washed with 5 mL of sterile physiological water. Subsequently, 200 µL of the washing solution was taken using a micropipette and seeded in a petri dish containing sterile ENDO medium(from HIMEDIA,Ref M029-500G), then we incubated at 37°C for 24 h. The isolated bacteria were stored at 4°C in CRYOINSTANT® (tubes produced by Deltalab containing porous

beads, suitable for the conservation of microbiological cultures)

### 2.3 Antibiotic Sensitivity Tests

The modified Kirby-Bauer's disc method described by Mahfuzul et al., [9] was used to study the antibiotic sensitivity of isolated coliforms. Initially, the isolated coliforms stored at 4°C were brought to room temperature and 50 µL of each preservation solution containing the bacteria was introduced into tubes containing 5 ml sterile BHI (Brain Heart Infusion Broth) medium (from HIMEDIA®, Ref 173-500G). After 18 to 24 hours of incubation, 1.5 ml of the broth was introduced into 1.5 microcentrifuge tubes and then centrifuged for 15 minutes at 3000 RCF. The centrifugate was recovered and washed then resuspended in 250 µL of sterile physiological water, the McFarland 0.5 standards concentration was used to obtain the final dilution (in 5 ml of sterile physiological water) to be used (50 µL) for antibiogram [9]. Then, using a dispenser, the antibiotic discs were aseptically placed on the surface of the previously inoculated Muller Hinton (MH) medium (from HIMEDIA®, Ref 173-500G) and the diameters of the clear inhibition zones were measured. The following 12 antibiotics were used: amoxicillin (AMC), 30 µg/disc; ampicillin (AMP), 25 µg/disc; cefazolin (CZ), 30 µg/disc; cefazolin/ clavulanic acid (CAC), 30/10 per disc; ceftazidime (CAZ), 30 µg/disc; ceftriaxone (CTR), 30 µg/disc; ciprofloxacin (CIP), 30 µg/disc; fosfomycin (FO), 200 µg/disc; imipenem (IMP), 10 µg/disc; nitrofurantoin (NIT), 200 µg/disc; tetracyclin (TE), 30 µg/disc and trimethoprim (TR), 30 µg/disc.

### 2.4 Interpretation of Results and Data Analysis

The inhibition diameters measured were interpreted referred to the Clinical & Laboratory Standards Institute [10]. Resistance R, Intermediate I and Sensitive S interpretations were obtained automatically using algorithms written in Excel software with the parameters described in Table 1 and all the graphs were drawn using Sigmaplot.

## 3. RESULTS AND DISCUSSION

### 3.1 Prevalence of Coliforms in Chicken and Quail Egg Shells and Apparent Microflora

Among the 191 samples studied (149 chicken eggs and 42 quail eggs), the study was observed

that only 16.2% (31) of eggs contained coliforms, 7.3% (14) contained mold and 76.4% (146) were sterile. Exclusively for chicken eggs, we observed that 24.8% (37) of chicken eggs contained microorganisms with 20.8% (31) containing coliforms and 4.0% (6) contained mold. Furthermore, 81% (34) of quail eggs were sterile while 19% (8) contained mold and none contained coliforms (Table 2). This result is different from those obtained by Neamatallah et al., [11]. and Amer [12]. Only 60 coliforms were isolated. The absence of coliforms on the shells of quail eggs and most of the chicken eggs used in this study could be explained by the fact that these eggs were washed with disinfectant solutions. In fact, some farmers specializing in poultry farming and egg production clean and disinfect the eggs before they are sent to markets and supermarkets. The disinfection of eggs is generally done by various chemical substances such as hydrogen peroxide spray in combination with UV, chlorine spray, peracetic acid spray alone or in combination with ultraviolet [13]. However, this disinfection operation is not always systematic and sometimes although carried out, it is not always possible to eliminate all the microorganisms present on the eggs. Beyond the washing, it is important to note that the findings of this study correspond with USDA [14] statement that, micro-organisms can be found on egg shell. The presence of coliforms and molds on certain eggs could be explained by contamination of the eggs by the different environments through which they passed from their conception in the hen's body to the storage place. Indeed, the probability of contamination of eggs when they are laid is high because the egg emerges from the body of the hen in the same way that faeces are excreted and the flora found in these faeces (from the intestinal tract) is, therefore, likely to infect eggs, especially, if the egg is soiled with chicken droppings, even the shell is not cracked the bacteria can enter through natural warm shell pores [15]. The same observation was made by Ashish and Rajesh [16] and Adesiyun et al., [17]. It has been shown that other factors are involved in egg contamination. These include improper handling of eggs by retailers, poor storage in the retail shops or markets where these eggs are sold in unhygienic conditions such as substandard packaging, presence of litter material, etc. [18].

### 3.2 Sensitivity to Antibiotics

The inhibition diameters (in mm) for different antibiotics ranged from 6 to 20 for Amoxicillin

(AMC), 6 to 29 for Ampicillin (AMP), 6 to 25 for Cefazolin (CZ), 19 to 29 for Cefazolin/clavulanic acid (CAC), 17 to 27 for Ceftazidime (CAZ), 10 to 32 for Ceftriaxone (CTR), 14 to 36 for Ciprofloxacin (CIP), 10 to 34 for Fosfomycin (FO), 14 to 30 for Imipenem (IMP), 10 to 27 for Nitrofurantoin (NIT), 10 to 36 for Trimethoprim (TR) and 6 to 35 for Tetracyclin (TE). The table shows that a total of 12 antibiograms were performed on each of the sixty bacteria, making a total of 720 trials. Of these 720 tests, the study was observed 557 (77.4%) cases of antibiotic sensitivity, 72 (10.0%) intermediate cases and 91 (12.6%) cases of resistance to the antibiotics disc. The low antibiotic resistance rate of bacteria in this study differs from the results obtained by Sheikh et al., [19] in a similar study in Canada. Of the 60 bacteria isolated, 39 were resistant to at least one antibiotic (65%). This result is in agreement with that observed by Mahfuzul et al., [9] who obtained an overall resistance percentage of 67.14% on bacteria isolated on the egg shells in Dhaka city in Bangladesh. Furthermore, graphical representations of the specific sensitivity of the 60 isolates to the 12 antibiotics and the proportions of the resistances observed were made respectively in Fig. 1. This Figure shows that the highest resistances were observed on tetracycline (50%), amoxicillin (40%) and ampicillin (30%) while the weakest resistances were observed on trimethoprim (11.7%), cefazolin (10%), ciprofloxacin (5%) and

1.7% for ceftriazone, fosfomycin, and nitrofurantoin. From table eggs of commercial layers, Mahfuzul et al., [9] obtained a similar classification for the 3 antibiotics with high resistance but the percentage resistance was higher with notably 100% for tetracycline, 88.7% for amoxicillin and 77.8% for ampicillin. In addition, no resistance was observed on ceftazidime (0%), imipenem (0%) and cefazolin + clavulanic acid (0%). The highest sensitivities were observed on cefazolin + clavulanic acid (98.3%), ceftazidime (95%), fosfomycin (96.7%) Imipenem (93.3%), nitrofurantoin (93.3%), Trimethoprim (88.3%) and ciprofloxacin (85%). A high percentage of intermediate sensitivity was observed for amoxicillin (41.7%). So, if all these bacteria with an intermediate sensitivity to amoxicillin were to become resistant, the percentage of bacteria in this study resistant to amoxicillin would increase to 81.7%, which would be close to the results obtained by Ashish and Rajesh [16]. The high resistance rates to tetracycline, ampicillin and amoxicillin were closely in agreement with the results obtained by Papadopoulou [20] who highlighted the resistance of certain coliforms such as *E. coli* (to tetracyclin, ampicillin), *Enterococcus faecalis* (to ampicillin and tetracyclin), *Enterobacter cloacae* (to ampicillin, amoxicillin + clavulanic acid, and tetracycline). However, concerning the other antimicrobial agents such as ciprofloxacin and fosfomycin, the results of our study were different

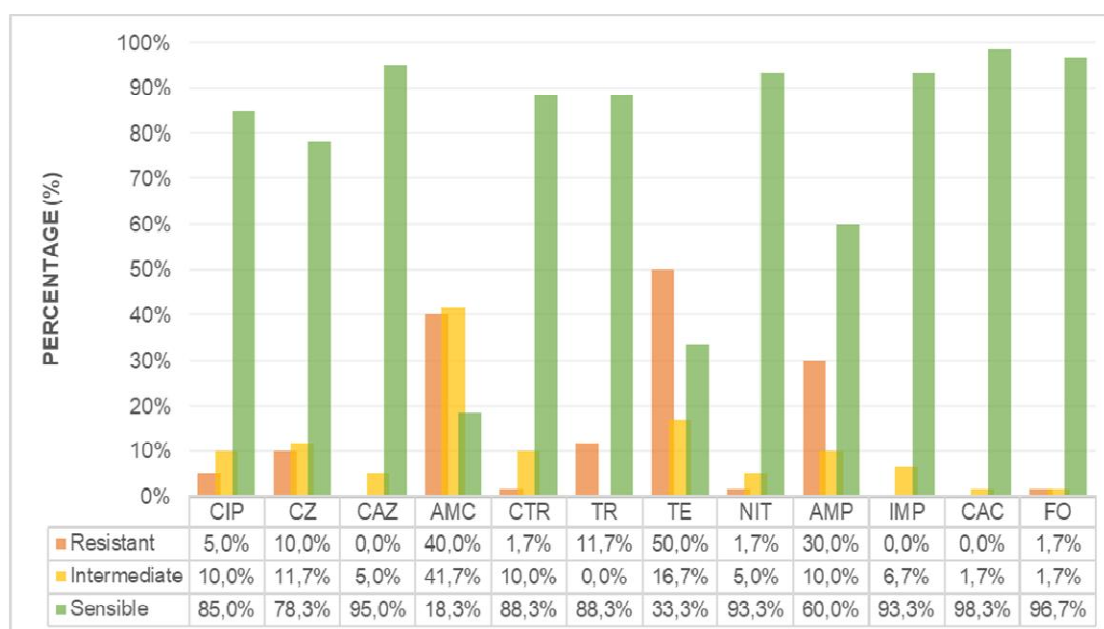


Fig. 1. Sensibility to antibiotics of coilforms isolated

**Table 1. Interpretation criteria for antibiotic sensitivity of enterobacteria [10]**

Interpretation	Antibiotics/limits of inhibition diameters (d)											
	CIP	CZ	CAZ	AMC	CTR	TR	TE	NIT	AMP	IMP	CAC	FO
<b>R</b>	d≤15	d≤14	d≤14	d≤13	d≤13	d≤13	d≤14	d≤13	d≤13	d≤13	d≤14	d≤12
<b>I</b>	16-20	15-17	15-17	14-17	14-20	14-15	15-18	14-17	14-16	14-15	15-17	13-15
<b>S</b>	d≥21	d≥18	d≥18	d≥18	d≥21	d≥16	d≥19	d≥18	d≥17	d≥16	d≥18	d≥17

*R=Resistant, I=Intermediate, S=sensible, AMC=amoxycillin, AMP=ampicillin, CZ=cefazolin, CAC=cefazolin/clavulanic acid, CAZ=ceftazidime, CTR= ceftriaxone, CIP= ciprofloxacin, FO= fosfomycin, IMP=imipenem, NIT=nitrofurantoin, TE= tetracyclinand TR= trimethoprim*

**Table 2. Prevalence of coliforms in chicken and quail egg shell and apparent microflora**

	Number & percentage			Total
	Without microorganism	Presence of coliforms	Presence of mold	
<b>Chicken egg</b>	112	31	6	149
Percentage	75,2%	20,8%	4,0%	
<b>Quail egg</b>	34	0	8	42
Percentage	81,0%	0,0%	19,0%	
<b>Total</b>	146	31	14	191
Percentage	76,4%	16,2%	7,3%	

**Table 3. MAR index and resistance pattern of the isolates**

Number of isolates	Resistance profile	MAR index
12	TE	0.08
2	AMC	0.08
1	AMP	0.08
2	AMP, CZ	0,17
6	AMC, TE	0,17
1	AMC, CZ	0,17
1	AMC, TR, NIT	0,25
5	AMC, TE, AMP	0,25
4	AMC, TE, TR, AMP	0,33
1	AMC, CZ, FO, AMP	0,33
2	AMC, TE, CIP, AMP	0,33
1	AMC, TE, TR, CZ, AMP	0,42
1	AMC, TE, CZ, CIP, CTR, TR, AMP	0,58

from those obtained by Papadopoulou [20], Ashish and Rajesh [16] and Eid [21] who all observed that bacteria of the same type with those isolated in our study had a very high resistance rate to these substances. In the case of amoxicillin (40%), the results were different from those obtained by Ashish and Rajesh [16] who recorded resistance at a higher level of 80%. On the other hand, the results for this antibiotic were also different from those of Sheikh et al., [19] who recorded resistance of 92.86% whereas Motayo et al., [22] found 16.8%. The results on resistance to Ceftriaxone (1.7%) were in agreement with those obtained by Mahfuzul et al., [9]. A comparison between the sensitivity of the isolates to Cefazolin and their sensitivity to Cefazolin + Clavulanic acid showed that the difference between these two antibiotics was highly significant ( $P = 0.00$ , Fischer Test). It is important to remember that no resistance to Cefazolin + Clavulanic acid has been observed. This lack of resistance could be explained by the fact that Cefazolin + Clavulanic acid is not usually used in poultry breeding in Russia. Indeed, Clavulanic acid has been used since the 1980s as a first pharmacological strategy for

combating resistance based on the inhibition of betalactamases [23]. Moreover, the study observed that, whenever resistance to cefazolin was encountered, the bacteria concerned were sensitive to cefazolin + clavulanic acid. The high resistance observed for tetracycline, amoxicillin and ampicillin would be due to the excessive use of these antibiotics in poultry breeding.

### 3.3 Mar Index and Resistance Pattern of the Isolates

Table 3 shows the summary of the multidrug resistance levels of the isolated bacteria. The percentage of each resistance level was calculated taking into account only the 39 bacteria that had at least one resistance to one of the antibiotics used in the study. The highest level of multidrug resistance corresponds to resistance against 7 antibiotics and only one bacterium (2.6%) has such resistance level and this bacterium has the MAR Index of 0.58 and was resistant to amoxycillin, ampicillin, tetracyclines, cefazolin, ciprofloxacin, ceftriazone, trimethoprim. It was also observed that a single bacterium (2.6%) had multidrug

resistance to 5 antibiotics. In addition, 9 bacteria (23.1%) were involved in a multidrug resistance to 2 antibiotics while 6 bacteria (15.4%) were involved in a multidrug resistance to 3 antibiotics. The resistance profile of each resistant strain was established and the multi-resistance index of the strains was calculated (Table 3). The MAR Index ranged from 0.08 to 0.58. The highest number of resistant strains (15; 38.5%) was observed for resistance to one antibiotic and the table of MAR Index and resistance profiles showed that the antibiotics involved in this category were tetracycline (12), amoxycillin (2) and ampicillin (1). All bacteria in this first category had an AMR Index of 0.8. It was observed that 24 isolates (61.5%) were resistant to at least two antibiotics while 15 bacteria (38.5%) were resistant to at least 3 antibiotics. This result is not very different from those of Adesiyun [17], who reported that 46.6% of coliform strains were resistant to at least five antimicrobial agents. It is however different from that of Eid [21] who reported that 94% of the strains were resistant to at least 5 antibiotics. For resistance to three antibiotics, our results differ from those of Ashish and Rajesh [16] who observed that 73.73% of isolates were found to be resistant to at least 3 antibiotics while our study revealed 38.5%. The bacterium with the second highest AMR index (0.42) was resistant to amoxycillin, tetracycline, trimethoprim, cefazolin and ampicillin. An AMR index of 0.17 was recorded for 8 bacteria and 6 of them were resistant to tetracycline and amoxycillin while the other 2 were resistant to cefazolin and ampicillin. As noted in the previous paragraphs, it emerges from this resistance pattern that amoxycillin, ampicillin and tetracycline are involved in all levels of resistance. This resistance to these 3 antibiotics could be an indicator of the frequency of exposure of the bacteria tested to these antibiotics and therefore of their frequency of use in poultry farming in Moscow.

#### 4. CONCLUSION

Multidrug resistance of bacteria to antibiotics is a phenomenon which is increasingly becoming frequent and could result from the prolonged exposure of multiple bacterial strains to various antibiotics during the prophylactic or curative treatment of animals and sometimes from harsh prescription of antibiotics. To reduce this exposure, Biosecurity must be strictly enforced and antimicrobials should be exclusively used or prescribed by professionals and a study of the sensitivity to antibiotics should be made in

advance for establishment of the most suitable curative treatment and an emphasis should be put on the application of alternative measures (such as use of probiotics) as a prophylactic measure. Finally, cleaning and disinfection must be done systematically using a chemical agent to destroy the nucleic acids thus avoiding the interbacterial transmission of resistance and multidrug resistance.

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#### COMPETING INTERESTS

Authors have declared that no competing interests exist.

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