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RESEARCH ARTICLE

Isolation and Antimicrobial Characterization of Aerobic Pathogenic Bacteria from Egg Shell and Egg Contents in Table Eggs in Erbil

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Abstract

Contaminated eggs are the major reservoirs for many foodborne pathogens and their ability to produce toxins causing diseases or even death in human. One of the essential quality characteristics of eggs is hygienic quality, lack of good hygienic practices, which may lead to loss the quality and deterioration of eggs. This study was focused on fresh chicken eggs to determine the prevalence and level of contamination in process as well as risk of antibacterial resistance of isolated bacteria. The level of contamination of table eggs with pathogenic bacteria and the antimicrobial resistance (AMR) were examined. Total numbers of 72 fresh chicken eggs were collected from eight flocks including conveyer belt and storage eggs from two large scale poultry layer farms in Erbil. The total bacteria on its eggshell was counted, bacterial loads were ranged between 5.174-5.854 log cfu/ eggshell. Bacteria isolated colonies subculture on Macconkey agar and gram negative baccilli were inoculated on VITEK® 2 ID-GNB (identification-Gram-negative bacilli) and antimicrobial susceptibility testing (AST)-GN69 cards. The bacterial pathogenic contamination ratio was 22.2% Staphylococcus and 22.2% enterobacteriaceae mostly (*E-coli* 11%). Whereas no bacteria were found in polls egg contents. The highest resistance rate was detected against Cefazolin, Amoicillin, Trimethoprim/ Sulfamethoxzol, Nitrofurantoin, Ciprofloxacin, Levofloxacin. The high sensitivity rate was recorded against Piperacillin/Tazobactam, Imipenem, Ertapenem, Gentamycin, Tobramycin, Cefipime, Cefitraxone. The results of this study conclude that the bacterial load on egg shells and contents were at the accepted level while the isolation of pathogenic bacteria due to lack of hygiene practice should be considered, also we suggest that the farmer must fumigate the fresh eggs with a good hygienic practice before marketing.

Introduction

Chicken table eggs are a healthy food for human particularly for infants and elderly, at the same time; chicken eggs are rich source of proteins, minerals, fat and vitamins [1, 2]. However, table eggs have been described as the most critical food carrier of pathogenic bacteria mostly in the etiology of food borne disease in human [3].Consuming treated eggs could cause food borne diseases like salmonellosis [2].In the developed countries microbial load of table eggs is routinely evaluated before selling, the safety of this product is important which is often linked to food poisoning outbreaks [4].The total count of aerobic bacteria in the air of the experimental poultry houses has been found to be positively correlated with the initial bacterial eggshell contamination in the hen house [5, 6].Food-borne diseases are distributed widely in most developing countries due to the egg contamination. Bacteria spread in the hen house environment is the common cause of egg contamination, pathogenic bacteria have the ability to penetrate the surface of the egg, through the cuticle, into the egg shell pores. However, eggs are susceptible to bacterial growth once the shell membranes are broken [7]. In Erbil governorate, few data are published about the bacterial contamination on the shell of consumption eggs by *Enterobacteriaceae*. The *Enterobacteriaceae* family includes many genera of pathogenic bacteria, one of which is *Salmonella* [8].The level of bacteria in the eggshell and internal contents varies greatly due to many factors

such as season, stress, stocking density, flock size, individual flock management, farm management, infection from pests and hygiene, which play a major role in the level of bacteria present in eggs [9]. Numerous studies focused on egg shell quality indicated a higher quality of eggs from cages [10, 11]. The eggshell thickness was lower and the eggshell hardness was higher in eggs that were produced in cages, [12]. In North of Iraq little is known about the level of the bacterial contamination in table eggs. The present study focused on fresh chicken eggs particularly eggshell, egg content to determine the prevalence and level of contamination in process as well as the risk of antibacterial resistance of isolated bacteria.

Material Method

Selection in Study Area

The study was conducted during the period from July to September 2018 at different areas in Erbil district. The fresh chicken egg was collected from two large scale poultry layer farms followed by Erbil Veterinary directorate, Kawi Qarachux poultry layer farm (ISA Brown in five flocks), and Hayat Altaaba poultry layer farm (1.Luhmann, 2.Tetra, 3.Hy-Line) (Table 1). All hens at all farms received the standard vaccines recommended; the flocks were housed in conventional cages. Samples of 172 eggs were randomly collected, which 15-20 eggs from the egg belts and hen nests of each cage in every hall. In total, 15-20 eggs per-flocks, and 20 eggs from storage or in site of grading and packaging of eggs were collected. 3-5 surface swabs were taken from different cages in the hall.

Eggs were manually sampled (using gloves) directly from conveyor belts, then placed in open carton filler flats, and transported by a car, in ambient conditions, to the laboratory where they were kept for a maximum of 24 h in ambient conditions before analyzing [13].The farms were visited and sampled when the chickens were between 35-43weeks of age.

Table 1: Overview of the visited flocks of laying hens farm, system, age of the birds and the hybrid used with Counts of aerobic bacteria on the eggshell for the different flocks

Farm	System	District	Flock	Capacity	Age (Wk)	Hybird
Kawi Qarachux	Cage	Qushtapa	H1	120000	45	ISA Brown
Kawi Qarachux	Cage	Qushtapa	H2	62500	43	ISA Brown
Kawi Qarachux	Cage	Qushtapa	H3	62500	43	ISA Brown
Kawi Qarachux	Cage	Qushtapa	H4	62500	43	ISA Brown
Kawi Qarachux	Cage	Qushtapa	H5	62500	45	ISA Brown
Hayat Altaaba	Cage	Shamamk	H1	54000	43	Lohmann
Hayat Altaaba	Cage	Shamamk	H2	54000	42	Hy-Line
Hayat Altaaba	Cage	Shamamk	H3	54000	38	Tetra

Eggshell Contamination

The intact egg was placed in a whirl plastic bag with 10 mL of wash solution (0.1% buffered peptone water (Lab M, 104).Each egg was rubbed through the bag for 1 min as described by [13].The eggs were removed from the bags and 100uL of the wash solution was plated on nutrient agar. The plates were incubated overnight at 37°C and colonies were counted and recorded for the determination of the total count of aerobic bacteria.

The colonies selected and subjected to the further microbiological examinations on different media such as MacConkey agar for enterobactereaca, EMA agar for *E coli* and XLD agar for *Salmonella serovars and* Manitol salt agar for *staphylococcus uraus* identification. The shell thickness was determined with a micrometer on three places. The mean value was used for calculations

Internal Egg Contents Contamination

Three eggs were disinfectant and broken of egg shell. The contents were Pooled and homogenized egg contents were mixed thoroughly by hand for 10 minute. 5 ml of homogenate egg content was aseptically was mixed to 45mL of (0.1%) Buffered Peptone Water in a zip-lock bag as described by Samiullah et al 2014 [14].100uL Plated on each nutrient agar, MacConkey agar and Manitol salt agar, incubated as mentioned above and the bacteria colonies counted and recorded if present.

Environmental Swab

Sterile swabs were taken from cages directly. The swabs then were transferred into 10uL the 0.1% buffered peptone water and

incubated at 37 °C for 24 hours. Then cultured on Nutrient agar media and incubated at 37 °C for 24 hours. After 24 hours, colony of suspected bacteria were found, the colonies were taken from the nutrient agar media and sub cultured on specific media for specific organism. The prepared dilutions were subjected to the following microbiological examinations on different agar media; MacConkey agar for Enterobactereaca, EM**B** agar for E coli and XLD agar for *Salmonella serovars and Manitol salt agar (MSA) for staph uraus* identification.

Bacterial Characterization

The primary culture on agar media that showed significant growth was examined and the morphological character of a single well isolated colony was removed using sterile wire loop and re-plated on specific media and incubated again for 18 to 24 h at 37°C just before testing. Tested Colonies were stained by Gram's stain. Bacterial identification was using VITEK 2 compact system (BioMérieux, Marcy I'Etoile, France), which is a semi-automated bacterial identification and susceptibility testing system. Suspensions of the cultures were made in 0.45% saline solution, adjusted to the turbidity of a 0.6 McFarland Standard, and used to loaded into the appropriate VITEK 2 ID and AST cards, the procedures were strictly followed. By manufacturer.

Statistical Analysis of Data

Statistical analysis of the data was performed using GLM procedure of SAS version 9.1 (SAS 2005).The relationships between both eggshell thickness and hardness with log eggshell contamination.

Results and Discussion

The study was conducted to evaluate the microbial quality of chicken eggs before selling is a imperative for egg hygienic. The total bacterial on its eggshell was counted, bacterial loads in Kawi Qarachux flocks and Hayat Altaaba flocks were ranged between (5.296-6.114) and (5.078 -5.174) log cfu/ eggshell in hen house respectively and 5.224-5.710 log cfu/ eggshell on Conveyer Belt and Egg storage respectively Figure (1, 2).The results showed that bacterial contamination of fresh egg in hen house various depending to the farm management which not agreed with the result of previous studies which reported that sterility of fresh eggs at lay concluded that 90% to100% of hens eggs are microbiologically sterile at lay [7,15].

Whereas in agreement with the result of Board and Tranter (1995) founded that the level of contamination on egg shells have a wide range of variation from 2-7 log cfu/egg shell, as well as results of Bruce and Drysdale, (1994) founded that the eggs laid in dirty environment was enclosed more bacteria than eggs laid in clean environment. In this study the samples from cage farm were found predominantly contaminated with *Staphylococcus spp* 22, 22% and aerobacteriaceae 22, 22% which E. *coli* composed 11.11% (Table 2, 3).Similarly, studies have found that the highest grade of eggshell contamination with *Staphylococcus spp* which can tolerate dry and extreme conditions was present in dust, soil and feces, which is the major reason of its Contamination of eggshells [18, 19].

As well as similar results of [20] showed that the surrounding environment and storage conditions including temperature and storage period can affect the level of bacterial contamination [20].In the second hand Enterobacteriaceae and *E coli* populations can be used as a measure of food quality and sanitary processing conditions [21].The presence of these bacteria in large numbers in eggs isolated from different sites in the present study indicates the poor sanitary conditions.

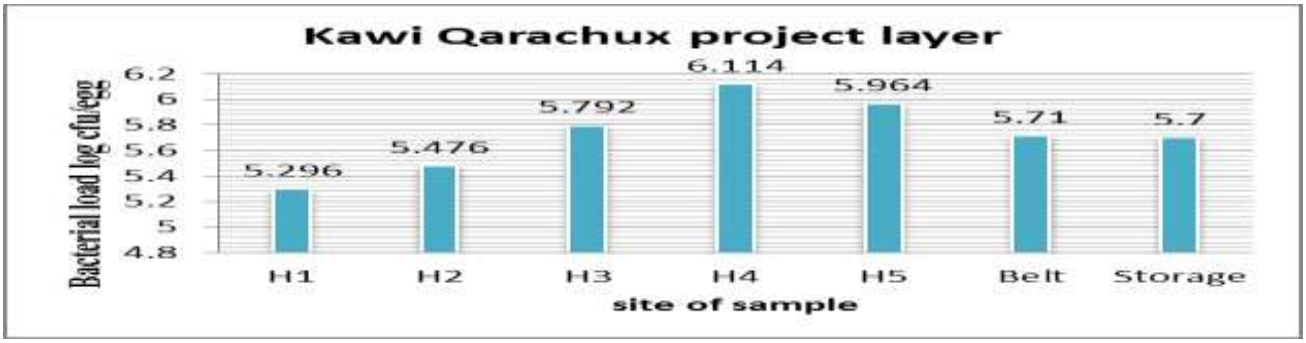

Figure 1: Bacterial load on eggshell log/M in deferent flocks of Kawi Qarachux project layer

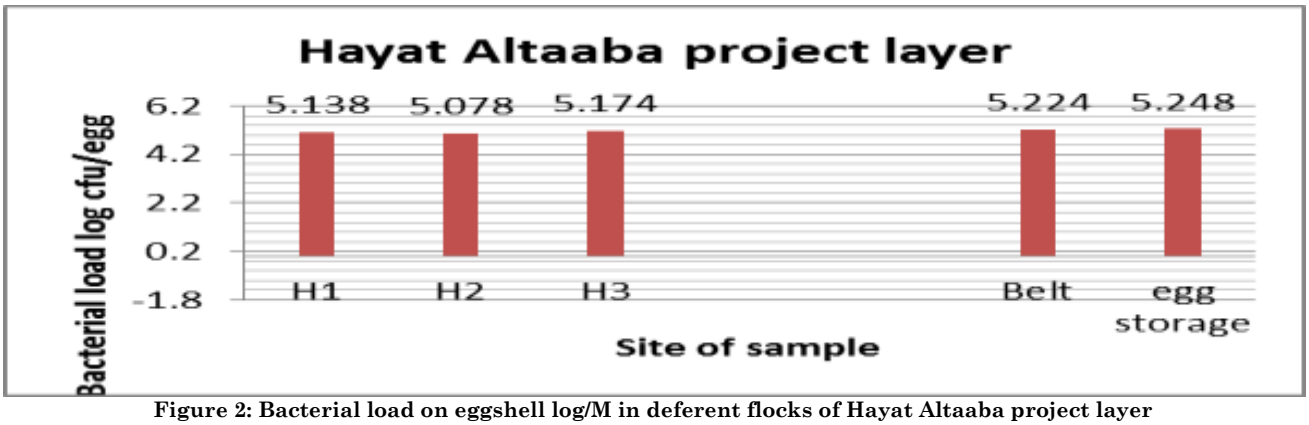

Figure 2: Bacterial load on eggshell log/M in deferent flocks of Hayat Altaaba project layer

Table 2: Represented bacterial isolates, differential culture media, staining and Vitek2

Total bacteria	Media	Biochemical test	test Stain
Staphylococcus	**Blood agar, MSA**	Coagulase –ve	**Gram +**
Enterobactereaca	MacConkey agar	**Vitek 2 system**	**Gram -**
E-coli	**EMB agar**	**Vitek 2 system**	**Gram -**
Others gram negative	MacConkey agar	**Vitek 2 system**	**Gram -**
Salmonella	**XLD agar**	**Vitek 2 system**	**Gram -**

The results of the present study was showed that the bacterial load on egg shell and no bacteria isolated from egg contents were measured at the allowed level, while isolation of pathogenic bacteria from eggshell due to lack of hygiene practice should be considered. De Reu et al., 2006 reported that a higher initial eggshell contamination with total count of aerobic bacteria was found at eggs from both non-cage systems and conventional cage systems. The differences in farm construction or management play an important role in the bacterial eggshell contamination De Reu et al., (2009).Presence of *Staphylococcus spp* and *E. coli* from cage swab in the surface of the hen house of poultry farm was found to be positively correlated with the bacterial eggshell contamination (Table 3). This showed the result agreement by researchers [6, 13].Arathy Sabarinath1 et al (2009) found that the high number of bacterial isolations from the eggshell collected from poor hygienic conditions at the layer farms. The study indicates the need for optimum hygienic conditions at the farm level to decrease the bacterial load. The quality of the egg depends on reasons before the laying phase and after oviposition. Hen´s health, feed safety, environmental conditions, grading and pack systems, processing, handling and transportation were essential factors that influence on the shelf-life and internal quality of the eggs [33].

Table 3: Determination of overall prevalence of bacterial pathogens

Name of samples	Total number of positive isolates					Total no of pathogens isolated from different samples with Percentage
	E. coli	***Citrobacter freundii***	***Staphylococcus* spp.**	***Enterobacter aerogenes***	***Enterobacter cloacae***	
Egg shell (50) 69.4%	6	1	13	5	2	27 (54%)
Egg content (10) 13.9%	0	0	0	0	0	0 (00.0%)
Cage swab (12) 16.7	2	0	3	0	0	5 (41.66%)
Total (72) 100%	**8(11.11%)**	**1 (1.38 %)**	**16 (22.22 %)**	**5 (6.94 %)**	**2 (2.76 %)**	**32 (44.44%)**

Regarding prevalence of bacterial isolated during this study, a total of 32 pathogenic aerobic bacterial including gram positive and gram negative were from all the examined samples presented in (Table 2, 3). The results showe that *staphylococci spp* represented the highest percentage of isolated bacterial (22.22%), *E.coli* 11.11%, Citrobacter *freundii* (1.38%), *Enterobacter aerogenes* (6.94 %), *Enterobacter cloacae* (2.76%).In agreement with the previous results which reported that the most common food borne pathogens associated with food of animal origin are *Salmonella, , Staphylococcus aureus* and *Escherichia coli*[25].In addition, a study was revealed that *S. aureus* was demonstrated to be a common and wide spread food poisoning organism, and found that natural eggshell

contamination of table eggs was conquered by gram positive *Staphylococcus spp*, which represented the highest percentage of bacterial isolates De Reu et al.(2005, 2006, 2007). *Staphylococcus* also found to be the most dominating species in the air of the poultry houses [18], While Escherichia *coli* represented 11.11% of the total isolates. Previous study reported that the human disease syndromes caused by ingestion of enteropathogenic *E. coli* [16, 32].

The results of egg content contamination for enterobacteriacea were revealed that no samples of pooled egg contents were positive for enterobacteriacea and salmonella, this may be due to a good practice of control program of salmonella via vaccination and antimicrobial feed additives. However Hincke *et al.* (2000) found lysozyme and shell gland specific protein ovocalyxin present in the shell, are also involved in the bacterial defense and prevent bacterial penetration into egg. Similar finding examined egg contents contaminations were observed in Bablyon, Iraq and Shahrekord, Iran were reported no positive results for egg contents [27, 15]. Regarding the egg thickness eggshell hardness were showed there is a significant different between eggshell in conveyer (1.544) with Luhman eggshell (0.936), while result in eggshell thickness there is significant different between sample eggshell with eggshell luhman only and there is no significant different between them. As well as result in eggshell contaminations are significant different between luhman with eggshell storage, eggshell conveyer and eggshell ISA Brown, this study revealed that luhman was the lowest value.

The result of regression were showed in (Table 6) between bacterial load and eggshell hardness there is a weak relation R2= (0.1) which is undependable but thickness there is a strong relation R2= (0.41) which could be dependable correlated with bacterial load while correlation both with bacterial load are correlated to thickness only (R2= 0.41) (Table 5) were as several workers to correlate eggshell porosity with bacterial penetration with varying results. Fromm, D., Monroe, R.J. (1960) supported a correlation, while Messens *et al.* (2005a) refuted these earlier findings and found no relationship between shell thickness and the likelihood of *Salmonella* Enteritidis to penetrate the eggshell.

Table 4 Determination of overall prevalence of bacterial pathogens Description of VITEK2 MIC (mg/L)/ category of susceptible of AST-GN69 results of isolated gram negative bacteria

		AMP	AMX/CLA	AMP/SUL	PIP/TAZ	CFZ	CAZ	CRO	CEF	ETM	IMP	GEN	TOB	CIP	LEV
E coli	6	(≥32) R	(8) S	(≥32) R	(≤ 8) S	(≤ 64) R	(≤ 1) S	(≤ 1) S	(≤ 1) S	(≤ 0.5) S	(≤ 0.25) S	(≤ 1) S	(≤ 1) S	(≥4) R	(≥8) R
Enterobacter aerogennes	5	(16) R	-	(≤ 4) S	(≤ 64) R	(≤ 1) S	(≤ 1) S	(≤ 1) S	(≤ 0.5) S	(0.5) S	(≤ 1) S	(≤ 1) S	(1) S	(1) S	(64) R
Enterobacter Cloacae	2	(≥32) R	-	(8) S	(≤ 64) R	(8) S	(≤ 1) S	(≤ 1) S	(≤ 0.5) S	(1) S	(≤ 1) S	(≤ 1) S	(≤ 0.25) S	(2) S	(64) R
Citrobacter freundii	1	(≤ 2) S	(≤ 2) S	(≤ 4) S	(≤ 4) S	(≤ 1) S	(≤ 1) S	(≤ 1) S	(≤ 0.5) S	(≤ 0.25) S	(≤ 1) S	(≤ 1) S	(≥4) R	(≥8) R	(64) R

AMP: ampicillin; AMX/CLA: amoxicillin/clavulanic acid; PIP/TAZ: piperacillin/tazobactam; CFZ Cefazolin, AMP/SUL: ampicillin/sulbactam, IMP: Imipenem; CAZ: Ceftazidime; CEF: Cefepime; CRO: Ceftriaxone,; TOB: Tobramycin; GEN: Gentamicin; CIP: Ciprofloxacin; LEV: Levofloxacin, ETM Ertapenem

Table 5: Determination of overall prevalence of bacterial pathogens Mean ± SE of Hardness, thickness of the eggshell with different of bacterial load Cfu/ml log for the different flocks

Egg sample	Hardness Mean ± SE kg/cm	Thickness Mean ± SE mm	bacterial load Cfu/ml log Mean ± SE
Lohmann	**0.936 ±0.217 b**	**0.316 ± 0.069 b**	**4.404 ± 0.734 b**
Hy-Line	**1.235 ±0.098 ab**	**0.392 ± 0.010 ab**	**5.078 ± 0.103 ab**
Tetra brown	**1.441 ±0.165 ab**	**0.393±0.008 ab**	**5.174± 0.071 ab**
Egg storage Hayat altaaba	**1.202 ±0.115 ab**	**0.452 ±0.036 a**	**5.248 ± 0.012 ab**
ISA Brown	**1.304 ±0.103 ab**	**0.388 ± 0.005 ab**	**5.728 ± 0.064 a**
Conveyer Belt Kawi Qarachux	**1.544 ± 0.156 a**	**0.420 ± 0.024 a**	**5.710 ± 0.038 a**
Egg storage Kawi Qarachux	**1.255 ±0.125 ab**	**0.463 ±0.042 a**	**5.700± 0.045 a**

A= significant B= non significant

Table 6: Determination of regression between Hardness, thickness of the eggshell with different of bacterial load Cfu/ml log for the different flocks

	bacterial load **Cfu/ml log Mean ± SE**	**R^2**
Hardness	Y=a+b1xh	0.10
Thickness	Y= a+b2xth	0.41
Hardness+Thickness	Y= a+b1xth +b2xth	0.43

Y= l

The Vitek2 antibiotic susceptibly of the gram negative isolates against different antibiotics is shown in (Table 4) among all the antibiotics tested all the bacterial isolates were highly sensitive to Gentamicin, Imipenem, Ceftazidimem Cefepimem Ceftriaxonem. Tobramycin, Ertapenem with maximum MIC dilution ranged (≤ 0.25 to ≤ 1,) whereas resistance to Cefazolin MIC ranged from ≤64 µg/ml, Ciprofloxacin with MIC ≥4µg/ml, Levofloxacin with MIC ≥8µg/ml and it appeared to have the resistance to *E.coli* and *Enterobacter spp*.

In between tested antibiotics. This may be contributed to Cefazolin, Ciprofloxacin and Levofloxacin being a misused in Kurdistan-Iraq also isolates were found to be resistant to Ampicillin/sulbactam with MIC ≥32µg/ml and Trimethoprim/Sulfamethoxzole with MIC ≥320µg/ml. The study results revealed the same as observed by Mascaretti (2003) reported the result combinations with ß-lactamase inhibiting drugs such as amoxicillin-clavulanate and ampicillin-sulbactam have been used as alternatives in treating severe infections involving resistant *E. coli* strains. All the isolates from this study were susceptible against gentamycin which is showing similarity with the results of [30]. The reliability of direct identification and antimicrobial susceptibility testing with any automated method should be tested by individual laboratories before the method is considered for routine use [31].Attention to lack of information prescription makes quantification of risk factors of antibiotic resistance, the acquisition of these resistant strains can result in human infections and this may eventually lead to treatment failures [36].

These treatment failures can bring serious financial burden on nations in the treatment of resistant bacteria associated with human infections. We were concluded that the present study provide the recent dataset of the prevalence of *Sataphyloccocus* and *E. coli* in fresh chicken egg at hen houses in Kurdistan-Iraq. In addition, bacterial load on egg shell and contents were at the accepted level while isolation of pathogenic bacteria due to lack of hygiene practice were attention. We suggest that the farmer must fumigate the fresh eggs with a good hygienic practice before marketing.

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