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Isolation and Characterization of Bacteria Contaminating Eggshell from Imported and Locally Produced Chicken

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Abstract | Eggs are regarded as nutritionally complete foods and are a good source of protein. Contamination of eggs with bacteria that may compromise egg quality and transmit diseases or intoxication to humans, posing public health problems This study was undertaken to assess the potential risk of bacterial contamination in table eggs available in the market, as well as the probability of transmission of pathogenic bacteria from the eggs to the community. For this purpose, a total of 60 samples comprised of local and imported table chicken eggs (30each) were randomly collected from the local markets of Baghdad province and samples were taken by cotton swabs from their shells. All the samples were subjected to isolation and identification of bacterial isolates. The presumptive isolates of local eggs were *E. coli*, while imported eggs, where *S. aureus*, Additionally, these positive samples were further confirmed by morphological characteristics of culturing media using nutrient agar for both as a general growth medium, eosin methylene blue for *E. coli* and mannitol salt agar for *S. aureus*, by microscopic examination using Gram's stain, and by *16sRNA* gene. Two isolates (1 from each group) were subjected by PCR followed by gene sequencing, genetic analysis and comparison to the reference strain of *E. coli* and *S. aureus*. The result of the present study highlights the need for applied food safety measures across the table egg production chain to prevent the development of some pathogenic microorganism by revealing the critical role of storage temperature on egg quality parameters, as well as the great influence of the housing environment on the microbial profile of produced eggs. Regular monitoring and corrective control measures should be set to maintain egg quality and safety at acceptable levels.

Keywords | Egg contamination, *E. coli*, *Staphylococcus aureus*, Food-borne

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INTRODUCTION

Food-borne microorganisms are key pathogens influencing food safety and cause human sickness globally because of food consumption. Many of these microbes have zoonotic importance, which has a considerable impact on both public health and the economy. Bacteria cause two-thirds of all human food-borne diseases worldwide, with impoverished countries bearing a disproportionately high burden (Awny *et al.*, 2018). Many food-borne zoonotic bacterial diseases are primarily found in food animals, with animal products serving as the primary mode of transmission. People are most likely to become infected with zoonotic bacteria through meat, dairy products, or eggs. Pathogenesis of these bacteria is caused by the production of toxins and

structural pathogenic factors (Abebe *et al*., 2020).

Typical symptoms of foodborne infections include abdominal discomfort, diarrhea, vomiting, nausea, fever, respiratory problems, and, in severe cases, death. These symptoms are caused by ingested pathogens, as in the case of foodborne diseases (Gourama, 2020) Eggs are a wonderful, nutrient-dense, healthy food that is widely consumed around the world. It supplies all important amino acids, minerals (calcium, iron, potassium, and phosphorus), and vitamins (vitamins A, B2, B9, B6, B12, and choline) to humans (Abbas *et al*., 2024). Because of their low cost, eggs and egg products are essential components of human diets. However, eggs that are incorrectly handled may pose a public health threat. The current study aimed to isolate and identify the egg-borne bacteria from egg storing trays and different parts of table eggs such as eggshell and yolk. Eggs are exposed to contamination due to faulty sanitary conditions during production to storage, high temperature, dust, hand touching, and all other surrounding pollution states (Islam, 2018).

Eggs are regarded as nutritionally complete foods and are a good source of protein. Egg contamination with bacteria can reduce egg quality and convey diseases or intoxication to consumers, posing a public health risk. The egg contents have been microbiologically analyzed for total bacterial count including *Staphylococcus aureus*. Isolated coliforms were recognized into *Citrobacter* spp., *Enterobacter* spp., *Escherichia coli*, and *Klebsiella* spp., while the isolated molds species were *Alternaria* spp., *Aspergillus* spp., and molds count as well as the presence of *Salmonella* (Awny *et al*., 2018). Microbial contamination is a major concern, limiting the use of table eggs. As a result, table eggs should be properly decontaminated before use as food items. The regulatory rules seek to limit exogenous and endogenous contamination of table eggs. Egg contamination is caused by a variety of bacteria. Contamination of eggs and egg products with bacteria raises the risk of many infectious illnesses in humans (Eddin *et al*., 2019).

Eggshells can be contaminated by a variety of causes, including fallopian tube infection or external contamination that can reach the eggs. Environmental or human contact with eggs can also introduce foodborne germs to the eggshells. Eggs can become contaminated during their manufacture, storage, distribution, processing, or food preparation. Increasing the microbial load on the eggshell increases the risk of germs passing through the shell and into the interior egg content (Hsu *et al*., 2023).

While most bacteria do not affect healthy humans, others flourish and reproduce in the body, producing illnesses. Food-borne diseases (including food-borne intoxications and food-borne infections) are illnesses that occur because of consuming contaminated food and are also known as food poisoning. Food-borne diseases are a substantial health burden globally, causing high morbidity and mortality (Almaary, 2023). According to estimates of the global burden of food-borne diseases, nearly one in every ten people are affected by contaminated food each year, with 4,20,000 dying as a result. Children under the age of five are believed to be at significant risk, with 1,25,000 children dying each year from food-borne infections (Kirk *et al*., 2017; Chowdhury *et al*., 2023).

The current study aims to investigate the risk potential of table eggs for harbouring microbial pathogens which could pose risk for onward speared not only in birds but also amongst people.

MATERIALS AND METHODS

Samples collection

A total of 60 random chicken table eggs (30 local eggs, 30 imported eggs) were randomly collected from markets in Baghdad city.

Isolation and identification of Bacterial spp.

Samples are isolated and identification by taking a swab from the surface of whole eggs shell, and streak on surface of nutrient agar, then on eosin methylene blue agar for Escherichia coli and on mannitol salt agar for *Staphylococcus aureus*. Macroscopic appearance was determined by examination of the (shape, color, edges of colonies, consistency) and other apparent characteristic features of colonies of bacteria. Microscopic appearance considered a preliminary diagnosis of bacterial infection, that detection of bacterial spp. by taken a thin smear of each culture was made, on slide then heated to fixed and flooded with crystal violet for 1 minute, washed with distal water, flooded with gram iodine for 30 seconds, washed with distal water, decolonization with 95% ethyl alcohol, washed with water immediately and flooded with safranin for 30 seconds and again washed with water, and observed under oil immersion objective.

Molecular detection

Molecular detection was performed by genomic DNA extraction from isolated egg culture samples of *E. coli* and *Staphylococcus aureus*. The DNA was extracted according to manufacturer instructions using Favor Prep Bacterial/ Cultured Cells Genomic DNA Extraction Mini Kit (Intron/ Korea) for detection of *E. coli* and *Staphylococcus aureus* as shown in Table 1 and Figure 1. The extraction mixture consisted of RBC Lysis Buffer, FATG Buffer, FABG Buffer, W 1 Buffer, Wash Buffer * (concentrate), Elution Buffer, FABG mini column collection tube, user manual and ethanol was added to preparation of wash

buffer (96-100)%.

Table 1: DNA extraction mini kit from cultured cells.

Cat. No:	FABGK 100 (100 preps)
RBC Lysis Buffer	135 ml
FATG Buffer	30 ml
FABG Buffer	40 ml
W 1 Buffer	45 ml
Wash Buffer * (concentrate)	25 ml
Elution Buffer	30 ml
FABG Mini Column	100 PCS
Collection Tube	200 PCS
User Manual	1

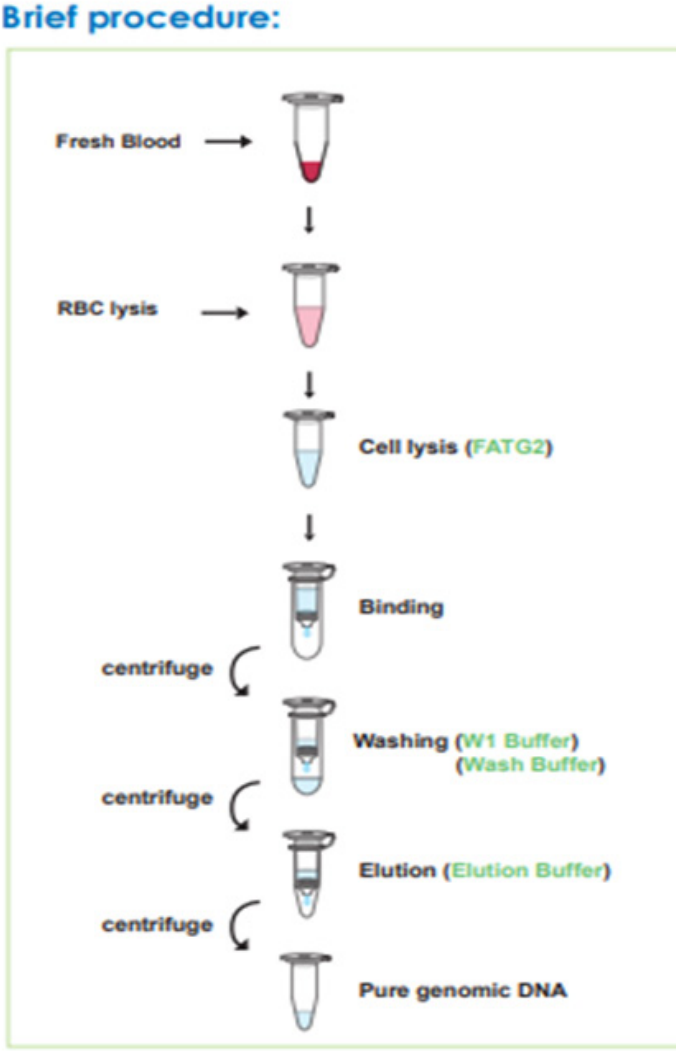

Figure 1: Brief procedure of DNA extraction from suspected samples.

Amplification of DNA genome

The amplification of DNA was performed using specific primers for detection of *E. coli* and *Staphylococcus aureus* genome (16srRNA) as shown in Table 2. The extracted DNA was identified by using the Maxime PCR Premix kit (I-Taq) (Intron, Korea), which consists of 5ul I-Taq TM DNA Polymerase, 2.5 mM for each dNTPs, 1x Reaction Buffer(10x) and 1x Gel Loading buffer. The reaction components of PCR composed of 5ul Taq PCR Premix, 1ul Forward primer, 1ul Reverse primer, 1.5ul DNA and 16.5ul Distill water thermal-cycling was performed as follows and then amplification of DNA following the cycling parameters as shown in Table 3 This experiment was performed in Bacteriology Laboratory of Zoonoses research unit in College of Veterinary Medicine University of Baghdad.

Identification using a PCR assay targeting the 16srRNA gene

Conventional PCR was applied to amplify a 1250 bp of *E. coli* and *Staphylococcus aureus* genome (16srRNA) using specific primers. The primers were lyophilized and were dissolved in the free ddH2O to give a final concentration of 100 pmol/µl as stock solution and kept a stock at -20 to prepare 10 pmol/µl concentration as work primer suspended. A total of 10 µl of the stock solution in 90 µl of the free ddH2O water was prepared to reach a final volume 100 µl. PCR amplicon were subjected to electrophoresis on a 1% agarose gel with 3 microliters of red stain solution (10 mg/ml). Gels were visualized using a UV transilluminator and captured via a gel documentation method. A 100-bp DNA ladder (Bioneer, Korea) served as a size marker for DNA molecules.

Sequencing of 16srRNA

After using the polymerase chain reaction and successfully detecting diagnostic probes for bacteria in the diagnostic gene 16srRNA by specific primers these products were sent for Sanger sequencing using AB13730XL, a computerized DNA sequencer (Macrogen Corporation, South Korea). The software program was used for sequence analysis and detection of phylogenetic tree by Neighbor-joining method (NJ) comparing to the sequences data from NCBI by using nucleotide BLAST for measuring percentage identity.

Table 2: Primers used in this study to detect the 16srRNA.

Primer	Sequence	Primer sequence	Tm (°C)	GC%	Product size (bp)
16s RNA	27F	5'- AGAGTTTGATCCTGGCTCAG- 3'	56.92	50.00	1250bp
	1492R	5'- GGTTACCTTGTTACGACTT- 3'	52.20	42.11	

Table 3: The optimum condition of *16srRNA* gene detection.

No.	Phase	Tm (°C)	Time	No. of cycle
1-	Initial denaturation	95°C	5 min	1 cycle
2-	Denaturation	95°C	sec45	35 cycles
3-	Annealing	58°C	sec45	
4-	Extension	72°C	1min	
5-	Extension 2	72°C	5 min.	1 cycle

RESULTS AND DISCUSSION

A swap of sixty samples taken from eggshells grown on nutritional agar, EMB agar, and Mannitol salt agar revealed the presence of bacterial contamination in both local and imported eggshells. The most abundant bacteria spp. in local eggs belong to Enterobacteriaceae (mostly *E. coli*) while the imported egg contained (*Staphylococcus aureus*) (Pesavento *et al*., 2017; Ganesh, 2024) counted the bacterial load of eggshell. It was discovered that *E. coli* was present in significant amounts compared to other microbes. Similarly, *S. aureus* contamination of the shell was discovered, conclusion that it could occur during passage through the cloaca. *E. coli* and *Staphylococcus aureus* were described based on morphological characteristic of culture medium, in both macroscopic and microscopic appearance, and molecular detection. *E. coli* was cultivated on nutrient agar showing Circular, smooth, colorless colonies as shown in Figure 2. Streaking on EMB agar was used for isolation and identification and was thought to be a quick and accurate way for distinguishing *E. coli* from other Gram-negative bacteria. The analysis of the isolates showed a metallic sheen green colony, which precipitates green metal pigments identified as a round slippery colony, Figure 3 as reported earlier (Saad, 2025).

Figure 2: Colonies of *E. coli* on nutrient agar, show smooth colorless colonies.

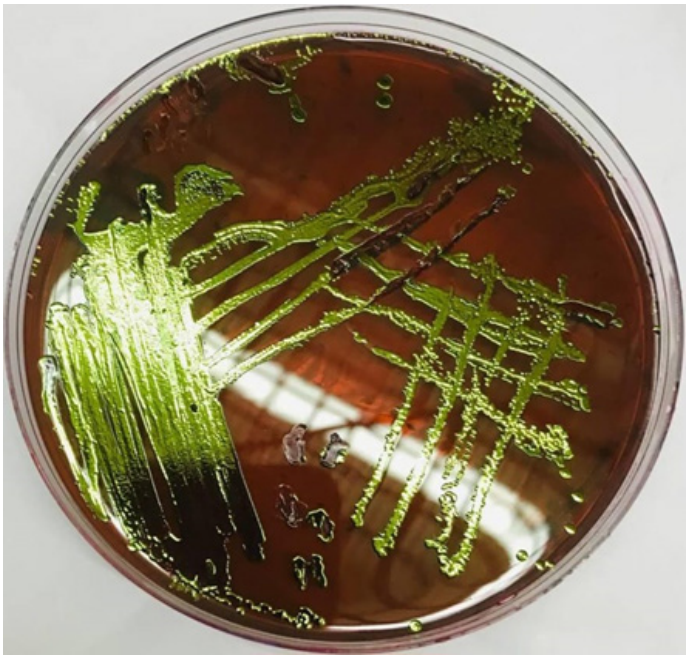

Figure 3: Colonies of *E. coli* on EMB agar appeared as green metallic sheen color.

Staphylococcus aureus, firstly cultured on nutrient agar demonstrated round, smooth, and convex colonies with soft shiny surface, consistency that are characteristic with golden-yellow pigment (Figure 4). Samples were streaked on on mannitol salt agar which is considered a selective and differential medium used to isolate and identify *Staphylococcus aureus* because it inhibits the growth of most other bacteria, while S. aureus can grow and ferment mannitol, producing yellow colonies and yellow zone around the colonies due to acid production Figure 5 (Al-Dmour *et al*., 2023).

Figure 4: Colonies of *staphylococcus aureus* on nutrient agar, show round smooth golden yellow colonies.

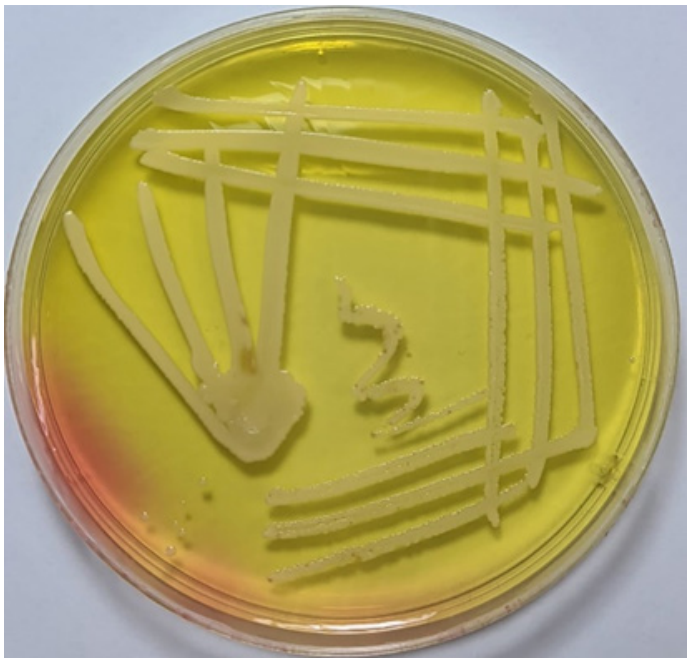

Figure 5: Colonies of *Staphylococcus aureus* on MSA agar, show fermentation of mannitol with yellow colonies and yellow zone around it.

Gram staining of suspected colonies revealed Gram-negative, short rod shape, no spore formation, single cell and in pairs for *E. coli* under light microscopy (Dimri *et al*., 2020) as in Figure 6. *Staphylococcus aureus* colonies appeared as Gram positive, purple, spherical (cocci) cells, commonly in clusters simulating a group of grapes (Figure 7) (Mohammed *et al*., 2024).

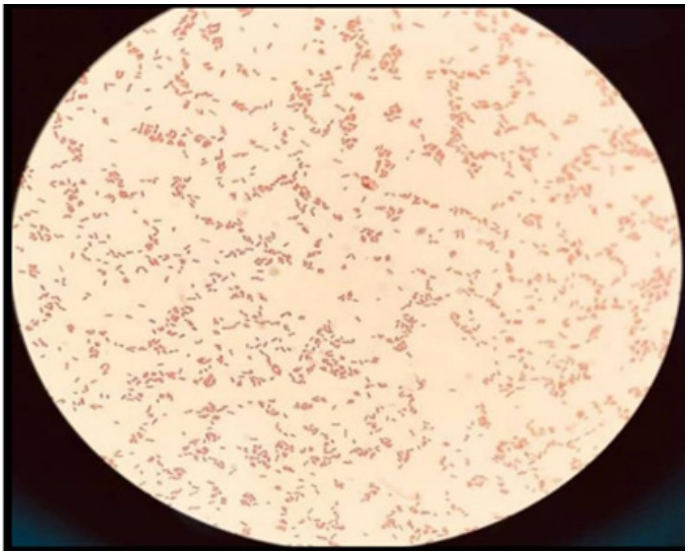

Figure 6: Microscopic section of *E. coli* under light microscope appears as short rod, red color, single cells (100x).

Identification of *Staphylococcus aureus* and *E. coli* was confirmed using conventional PCR method was revealed in the band size of 1250bp. The products were separated on electrophoresis on 1.5% agarose at 5 volt/cm^2. Lane 1 was used for utilizing *Staphylococcus aureus* and lane 2 was used for *E. coli* isolated from cultured media of eggshell samples. *S. aureus* is one of the commonest food poisoning bacteria. *S. aureus* food poisoning is due to its ability to produce in the food a broad range of exotoxins and enterotoxins. Ingestion the foods containing one or more performed exotoxins or enterotoxins cause the staphylococcal food poisoning (Balaban and Roosly, 2000). Conventional methods used for identification and confirmation of *S. aureus* and *E. coli* are time consuming. Molecular techniques, such as PCR have been used extensively for several years for identification and characterization of foodborne pathogens in food samples, these procedures aligned with earlier reports (El-Hadedy and El-Nour, 2012).

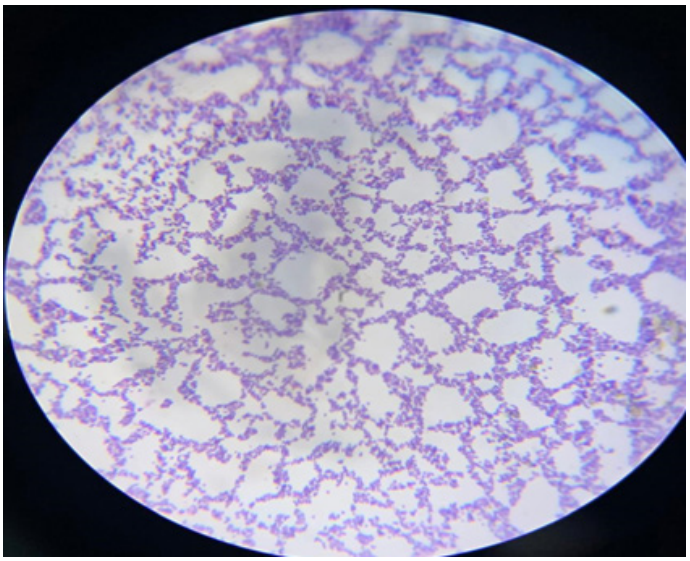

Figure 7: Microscopic section of *Staphylococcus aureus* under light microscope appears as spherical cocci, purple color, grape clusters (100x).

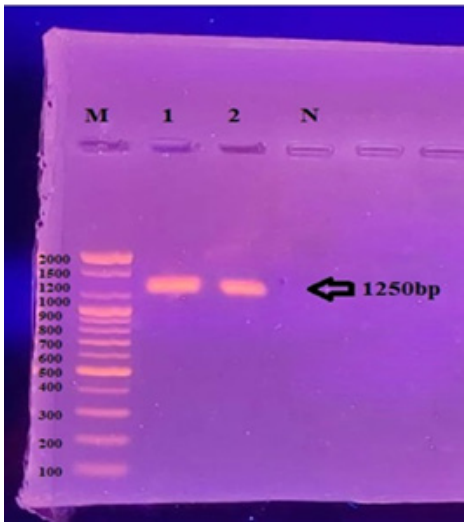

Figure 8: PCR product size of 1250bp of *Staphylococcus aureus* and *E. coli*. 1.5% Agarose Gel PCR amplification products of 16sRNA Gene that is obtained from eggshell samples. M: represent marker. Sample No. (1, 2) represent positive sample.

Table 4: Sample submission and sequence analysis.

Identities	Sequence ID with submission	Sequence ID with compare	Source	Nucleotide	Location	Type of substitution	No. Of sample
99%	ID: PV300528.1	ID: OR196041.1	*Escherichia coli*	C\A	780	Transvertion	1
				T\G	854	Transvertion	
99%	ID: PV300529.1	ID: OP959872.1	*Staphylococcus aureus*	G\T	381	Transvertion	2
				A\G	942	Transition	
				G\A	1007	Transition	
				C\A	1026	Transvertion	

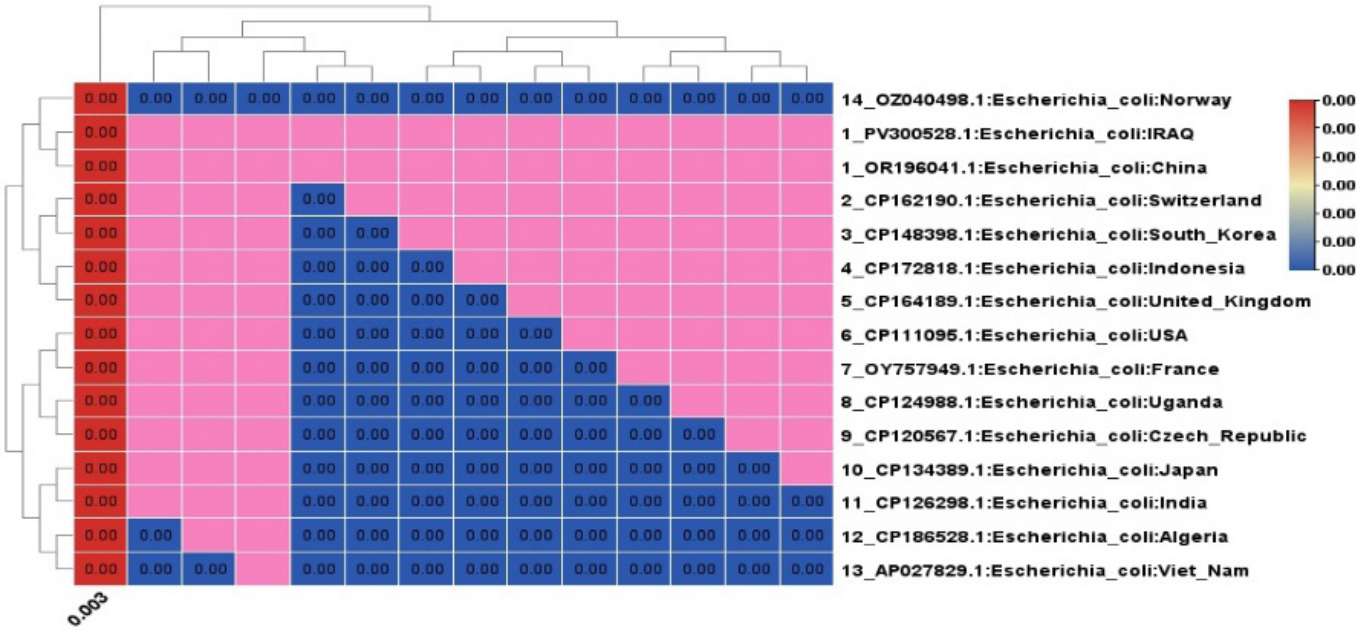

Figure 9: Phylogenetic tree relationship, based on 16sRNA gene for *E. coli* bacteria isolates and the closely related isolates deposited on the GenBank/NCBI website.

A total of two isolates after amplification with conventional PCR method were sent for sequence to clarify the gene detection and for comparison with other isolates. *E. coli* detected with a transversion type of substitution and with compare sequence ID: OR196041.1 although the *Staphylococcus aureus* was sent with compare sequence ID: OP959872.1 and transversion substitution. Both isolates were 99% identical with sequences available in the public domains NCBI (Saeed *et al*., 2022) as shown in Table 4.

Phylogenetic analysis is an advanced scientific approach used to shed light on the complex evolutionary past and convoluted interactions within a broad array of organisms. Traditionally, phylogenetic analysis was primarily conducted through comparative studies of dozens of morphological traits detectable in fossil samples. The information derived from fossils, however, was highly limited due to the extent of preservation and a relatively small number of finds. Currently, molecular phylogenetic analysis is in high demand due to the abundance of molecular information available, including DNA sequences and protein structures, and it is instrumental in the processes of evolutionary investigation (Horiike, 2016). In this present study of the occurrence of these 2 isolates the *E. coli* phylogenetic tree was related to isolates of Norway, Iraq, Switzerland, South Korea and China as shown in Figure 9.

The second isolate of *Staphylococcus aureus* was detected and confirmed by the sequence and the Phylogenetic tree clarified the results were related to the isolates of China, USA, Netherlands, Japan and Italy as shown in Figure 10.

If the species in question are distantly related phylogenetically, the chosen molecules should have a low evolutionary rate due to increased sequences. Since nucleotide or amino acid substitution on genes or proteins increases sequences with varying evolutionary rate among species that are distantly related, they will reach a saturating state if it is high, thus unsuitable. However, a nucleotide sequence on a gene is known to reach its saturation point faster compared to when an amino acid sequence on a protein it encodes. In such a case, therefore, it is appropriate to use housekeeping genes, naturally with

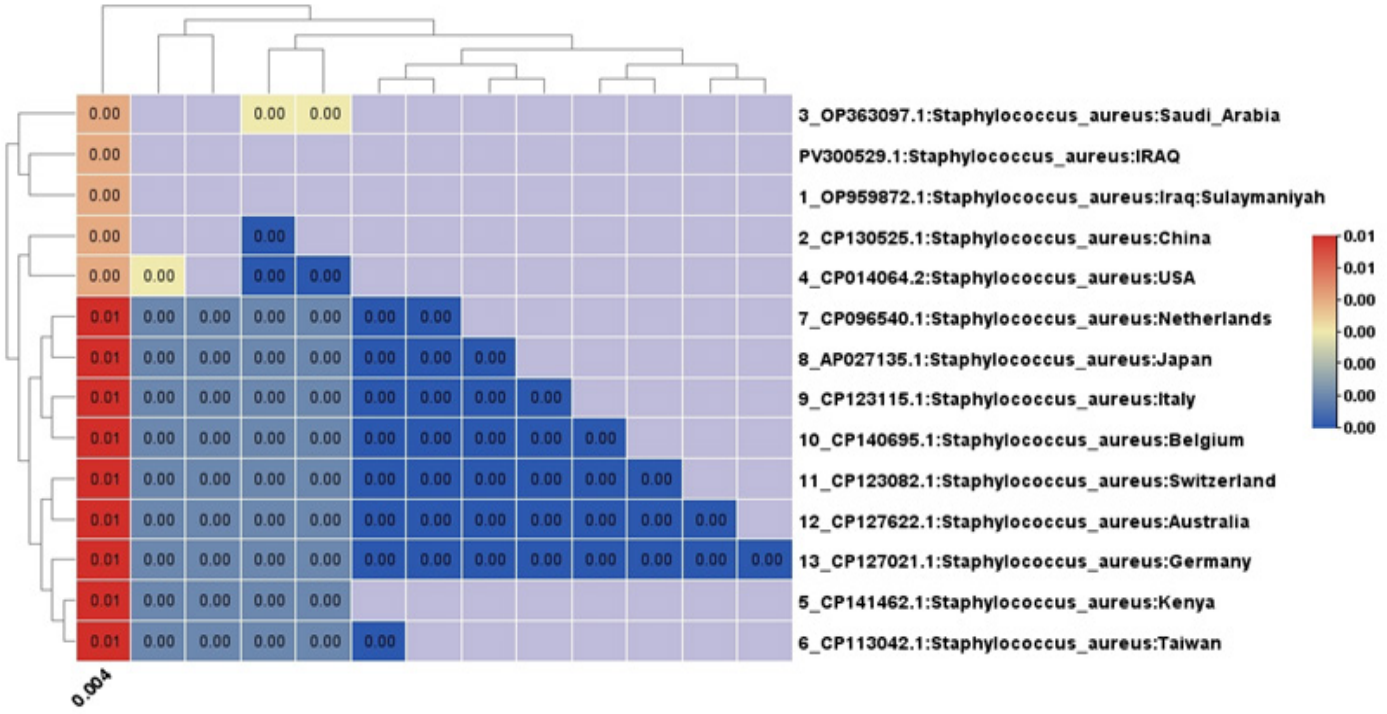

Figure 10: Phylogenetic tree relationship, based on *16sRNA* gene for *Staphylococcus aureus* bacteria isolates and the closely related isolates deposited on the GenBank/NCBI website.

a low evolutionary rate. For example, 16S ribosomal RNA and gyrB are commonly used for prokaryotic organisms while 18s ribosomal RNA and Histone 3 are appropriate for eukaryotic organisms and readily available. If the two species are closely related phylogenetically, on the other hand Molecules with strong evolutionary rates should be picked. Low evolutionary rates lead to fewer substitution occurrences, resulting in less precision. In this situation, tissue-specific genes that have a high evolutionary rate are suited (Smith, 2021).

The findings indicated that the local eggs were contaminated with *E. coli* and that eggs might operate as a vector for diseases in the food system because *E. coli* is a normal intestinal flora of birds and humans, and some *E. coli* strains have acquired virulent genes, allowing them to cause diarrhea and other related disorders. The imported eggs were contaminated with *Staphylococcus aureus*, which could be a possible source of disease and food poisoning in people (Fahim *et al*., 2021) because *E. coli* and *Staphylococcus aureus* strains containing virulent genes with public health implications could infect the external or internal contents of eggs, causing disease in consumers. Bacterial contamination of eggs can occur via vertical and horizontal pathways (Authority, 2005). Hens have a common cloacal entry for digestive, urinary, and reproductive processes, which could contribute to eggshell contamination when the egg passes along this channel (De Reu *et al*., 2006).

CONCLUSION AND RECOMMENDATIONS

In conclusion, the environment in which table eggs are produced, handled, and stored has an impact on their quality and safety. As a result, the eggs should be stored in a clean, well-ventilated area with consistent temperature and relative humidity. Furthermore, continuous monitoring of the quality of stored eggs is recommended. Additionally, cold storage is recommended for improved egg quality.

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NOVELTY STATEMENT

After numerous diseases spread in Baghdad city because of food poisoning in eggs, this manuscript was chosen to understand the most common causes of foodborne illnesses aiming to clarify the causative agent and determine the source of infections.

AUTHOR'S CONTRIBUTION

SSHAA-B: Proposed the idea, provided the bacterial isolation work, helped with the lab work, and part of the

literature collection and reviewing. MDMS: Contributed with the Molecular detection and genetic analysis of the bacteria and the lab work as well as the writing of the results in relation to isolation and lab results. SMK: Added further support by collecting samples from random local markets.

Generative AI and AI-assisted technology statement

The authors declare that no Genrative AI was used in the creation of this manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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