**Original Paper**

**Eggshell contamination with special reference to different methods of disinfection on the isolated bacteria**

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**ABSTRACT**

The study was conducted on 198 table eggs that were collected from markets, and manual and automatic farms in EL-Fayoum governorate, to assess the existence of foodborne pathogens (Escherichia coli and Staphylococcus aureus), either on the outer eggshell or inner egg contents and to evaluate the reduction capacity of three antimicrobial agents (citric acid 3%, Sodium bicarbonate 3% and hydrogen peroxide (H2O2) 3%) against eggshells bacterial load. The obtained results found that Staph. aureus has recorded the highest prevalence (90%) in table eggs, Almost, E. coli and Staph. aureus isolates exhibited high resistance to the tested antibiotics, Also, the E. coli isolates were positive to virulence genes (tet A gene and dfr A gene). Finally, the effect of tested antimicrobial agents on the experimentally contaminated eggs showed that H2O2 (3%) was more effective in reducing the load of E. coli on eggshells than the other antimicrobial agents tested, while bicarbonate 3% expressed a significant ability to reduce Staph. aureus.

1. **INTRODUCTION**

The modern trends of poultry industry have become directed towards production of healthy and safe food in the developing countries. Eggs can provide an excellent source of high-quality protein, affordable, cheap, multivitamin, good fats, and trace elements, while offering a moderate substance with strong bactericidal properties (Carrillo et al., 2012).

Moreover, the high nutritive value of eggs makes them vulnerable to microbial contamination that threatens the eggs’ shelf life (Gole et al., 2014). The microbial quality of table eggs also adversely affects consumers through transmission of pathogenic microorganisms causing intoxication and public health hazards (Al Momanii et al., 2018). Egg contents could be contaminated by a vertical transmission during laying (Gantois et al., 2009) or horizontally by soil, faeces, nesting particles, hands of workers, during processing, during transportation, and during storage, as the bacteria can be colonized on the shell and penetrate the egg through its pores (Messens et al., 2007).

The most foodborne pathogens often contaminate eggshells are E. coli and Staph. aureus (Maktabi et al., 2018). Most of E. coli strains are harmless bacteria and commonly inhabit the mammals and birds intestine which easily spread through water, litter, faecal matter, dust and food (Gupta and Sharma, 2015). The strain, O157:H7 has been reported to be pathogenic to humans, causing bloody diarrhea, haemolytic uremic syndrome, urinary tracts infections, pneumonia meningitis and peritonitis in humans (Musgrove et al., 2008). The thermostable enterotoxins from Staph. aureus causing nausea, vomiting, abdominal pain, cramps and diarrhea within 6 hours of ingestion of contaminated egg (EFSA, 2014).

Food poisoning outbreaks due to consumption of eggs contaminated with pathogenic microorganisms may be higher in developing countries where little or no control measures are applied. Keeping the eggs clean and sterile is extremely important for public health. Chemical disinfection has more appropriate action against bacterial load with the rise of microbial antibiotic resistance (Michael et al., 2014), while choosing the perfect disinfectant that’s effective, safe, and keeps egg quality without change in color or odour could be a great challenge.

Citric acid is one of the most effective choices, especially under standard doses, because it improves the flavor of foodstuffs without causing any harmful effects (Park et al., 2009). Hydrogen peroxide is another inexpensive chemical substance with strong bactericidal properties (Ukuku et al., 2005).

2. **MATERIAL AND METHODS**

2.1. Sample collection

A total number of 198 table egg samples, 150 eggs were used for microbial isolation, identification, and antibiotics...
sensitivity, while 48 eggs were used for antimicrobial efficacy assessment. The eggs were randomly collected from poultry farms, markets, supermarkets, and groceries in EL-Fayoum governorate from February to April 2020. Each sample was placed in a sterile plastic bag and carried to AHRI laboratory (Animal Health Research Institute) without delay in preparation and microbiological examination.

2.2. Microbiological examination

2.2.1. Preparation of samples

Each sample was washed in a sterile plastic bag with 100 ml sterile saline. Eggshells were tested by the rinse method as described by Moats (1980). The eggs were prepared for the evacuation of their contents according to (APHA, 2004), and the contents were tested after egg evacuation according to Speck (1976).

2.2.2. Microbial Isolation and identification

Isolation and identification of E. coli from egg contents were carried out according to (Cheesbrough, 2006) While the isolation and identification of coagulase-positive Staphylococcus aureus were done according to (AM, 2003, ISO, 2003b). The isolates were confirmed by PCR. Oligonucleotide sequences, target gene, amplicon size, and cycling condition were showed in table (1).

<table>
<thead>
<tr>
<th>Genetic target</th>
<th>Primer</th>
<th>Primer sequences</th>
<th>Primary denaturation</th>
<th>Secondary denaturation</th>
<th>annealing temperature</th>
<th>Extension</th>
<th>Final extension</th>
<th>Amplicon size</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA Gene</td>
<td>Forward</td>
<td>TCTTCACTCGCAAGTCGAAGG</td>
<td>94 °C 5 min.</td>
<td>30 sec.</td>
<td>58 °C 45 sec.</td>
<td>30 sec.</td>
<td>72 °C 10 min.</td>
<td>729 bp</td>
<td>(Van et al., 2008)</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TCTTTGAGTTTCAACCTTGCGGTCG</td>
<td>94 °C 45 sec.</td>
<td>30 sec.</td>
<td>94 °C 3 min.</td>
<td>5 min.</td>
<td>72 °C 75 °C</td>
<td>729 bp</td>
<td>(Van et al., 2008)</td>
</tr>
</tbody>
</table>

2.3. Antibiotics sensitivity test

The Antibiogram of E. coli and Staph. aureus isolates from the collected eggs were performed using the agar disc diffusion technique as described by (Bauer, 1966) using seventeen antibiotic discs (Himedia, India). The results were interpreted according to the standards of Clinical Laboratory Standards Institute (CLSI, 2016).

The presence of genes associated with resistance to streptomycin [aadA1 of 447 bp, its sequence are (F) TATCCAGCTAAGCGCGAACT, (R) ATTTGCCGACTACTCTCTG] and for tetracycline [tet(A) at 577 bp, its sequence are (F) GGAGTGCCAAAGGTGAACAGC, (R) GAGTACAGGTAGACTTCTG] were determined by PCR. The presence of genes associated with resistance to trimethoprim [dfrA1 of 367 bp, its sequence are (F) TTTTGAGTTTCAACCTTGCGGTCG, (R) CAGTACAGGTAGACTTCTG] and for streptomycin [aadA1 of 447 bp, its sequence are (F) CTCTATTTGCTAACAGCTG, (R) TATCCAGCTAAGCGCGAACT] were determined by PCR.

2.4. Efficacy of antimicrobial agents on eggshells

2.4.1. Preparation of bacterial suspension

Tubes containing 5 ml trypticase soy broth (TSB) were inoculated with the colonies previously isolated, then incubated at 37°C for 20 h on a shaker at 10 RPM. About 1.5 ml was moved to a different tube with 60 ml TSB and incubated as before, then centrifuged at 4000 RPM for 7 min. The supernatant was discarded, and the pellets were re-suspended in 10 ml phosphate-buffered saline (PBS). Eggs were inoculated by the previously prepared concentrated suspension as described below.

2.4.2. Eggs inoculation

In each series of tests, six medium-sized fresh eggs (a total of 48 eggs) were prepared according to (Rodriguez-Romo and Yousef, 2005). Aliquot 500 ml of sterile PBS was added to dilute the concentrated bacterium suspension to achieve the concentrations of 5.76 and 5.84 Log CFU/ml for E. coli and Staph. aureus respectively. Eggs were placed within every bacterial suspension for 30 min. and dried under a laminar flow for 1 h at ambient temperature.

2.4.3. Disinfection of eggs with the antimicrobial agents

Sterile stomacher plastic bags containing 200 ml from each citric acid, H2O2 and sodium bicarbonate was prepared individually at a concentration of 3% for egg treatment (Park et al., 2009). The final bacterial concentrations in eggs treated with various antimicrobial agents were compared to the bacterial concentrations in eggs immersed once in controls (BPS without disinfection) (Upadhyaya et al., 2013).

2.5. Statistical analysis

One way analysis of variance was used (ANOVA) and significance was done used Tukey test using SPSS (version 16; SPSS Inc., Chicago, USA).

3. RESULTS

3.1. Prevalence of E. coli and Staph. aureus in eggshell and inner contents

The microbial prevalence of eggs is summarized in Table (2). The highest prevalence rate from collected eggs was recorded by Staph. aureus (90%) followed by E. coli (70%). Only 20% of eggshells were contaminated with E. coli and Staph. aureus and the contamination did not reach to the inner content of eggs. From both eggshell and egg contents, staphylococcus aureus was the highest bacteria isolated (70%) followed by E. coli (50%).

<table>
<thead>
<tr>
<th>Samples</th>
<th>No of egg</th>
<th>Prevalence (%)</th>
<th>Staph. Aureus Prevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive eggshells only</td>
<td>10</td>
<td>20%</td>
<td>10</td>
</tr>
<tr>
<td>Positive egg contents only</td>
<td>0</td>
<td>0%</td>
<td>0</td>
</tr>
<tr>
<td>Positive both eggshells &amp; contents</td>
<td>75</td>
<td>50%</td>
<td>105</td>
</tr>
<tr>
<td>Contamination free</td>
<td>45</td>
<td>30%</td>
<td>15</td>
</tr>
<tr>
<td>Total contaminated eggs</td>
<td>105</td>
<td>70%</td>
<td>135</td>
</tr>
</tbody>
</table>

3.2. Effect of source of collection on microbial prevalence in table eggs.

The results showed that all eggs collected from local markets and manual farms were contaminated with Staphylococcus aureus against 66.6% from automatic ones. Similarly, 95% of the egg from local markets were against 66,6% from automatic ones.
3.3. Molecular detection of isolated bacteria:
From all positive isolates, 20 samples (randomly selected) of E. coli and Staph. aureus were used for PCR amplification targeting the tuf and 16s rRNA, respectively. All the samples (100%) were positive as shown in fig. 1.

![Image](323x91 to 539x247)

![Image](323x514 to 544x699)

Fig. 1 Agarose gel electrophoresis 1.5% showing PCR products Lane (L): molecular weight maker (100 bp DNA ladder. Thermo) a: tuf gene of E.coli. Lanes (1) to (20) were positive DNA samples showed a band at 258 bp. Lane (POS) was positive control. Lane (N) was negative control.

b: 16s rRNA gene of Staph. Aureus. Lanes (1) to (20) were positive DNA samples showed a band at 791 bp. Lane (POS) was positive control. Lane (N) was negative control.

3.4. Antibiotic sensitivity test against E. coli and Staph. aureus:
The antibiotic susceptibility of E. coli isolates against different antibiotics were shown in Table (4) and histogram (1). Among all the antibiotics tested, E. coli was highly resistant (100%) to Ofloxacin, Ciprofloxacin, Cefuroxim, Amoxicillin, Ampcillin Sulbictam, Cefepem, Rifampin and Oxytetracyclim. Only 40% of the isolated E. coli was sensitive to Amikacin, followed by Kanamycin (15%) then Apramycin (10%), Staph. aureus was sensitive to Apramycin (40%), Erythromycin (35%), Vancomycin (30%), and Amikacin (25%), and sensitivity decreased gradually to Penicillin (15%), Rifampin (10%), Ciprofloxacin (10%), and Streptomycin (5%), while showed the highest resistance (100%) to Tetracycline and Sulfamethoxaseth.

The results proved that all of the E. coli isolates were positive to the tet A gene (577 bp) and dfr A gene (367 bp). It was shown that out of 20 E.coli isolates, 19 isolates (95%) were positive for aadA1 gene at 447 bp and only 13 isolates (65%) were positive for ere A gene at 419bp (Fig. 2).

3.5. Effect of antimicrobial agents on E. coli and Staph. aureus:
Changes in E. coli count after treatments with citric acid, H2O2, and bicarbonate at concentration of 3% were displayed in Fig. 3. There was a reduction in the bacterial count by 0.36, 1.16, and 0.80 Log CFU/ml, respectively. Various disinfection treatments showed significant reductions (P<0.05) in bacterial concentrations compared to PBS alone (control). There was a numerical reduced bacterial concentration in E. coli between citric acid 3%, H2O2 3%, and bicarbonate 3%, but the difference wasn't significant. H2O2 (3%) was superior to other treatments in bacterial reduction. Similarly, there was a reduction in Staph. aureus count after treatment with citric acid 3%, H2O2 (3%), and bicarbonate 3% by 0.90, 0.83, and 1.14 Log CFU/ml, respectively. Staph. aureus appeared more sensitive to bicarbonate 3% than other antimicrobial agents tested.
4. DISCUSSION

Eggs sold in local Egyptian markets are commonly coming from different sources as deep litter farms, battery farms, automatic farms, manual farms and from backyard houses, which vary in egg handling, cleaning, disinfection, and storage. This gives different management levels for eggs ranging from very high technology in automatic farm to unwashed behind the backyard houses, rather than cross-contamination and bad storage in markets.

The recorded prevalence rate of *E. coli* were in accordance with (Islam et al., 2018) who reported that the major egg contaminants were *E. coli* (34.6%).

Staphylococci spp. is one of the foodborne pathogens that are important for human and veterinary medicine. Our prevalence rates were nearly similar to (Islam et al., 2018) who reported that of *Staphylococci* isolated from egg content and shell were 41.2% and 58.8%, respectively. The lower prevalence was reported by (Eid et al., 2015).

Freshly laid eggs from disease-free hens generally devoid from pathogenic organism while poor storage, poor cleaning, delay in collection, and other environmental factors such as humidity, temperature, dust, litter and dirty nests causes primarily heavy contamination that could migrate to the inner contents (Evêncio-Luz et al., 2012).

The recorded microbial prevalence rate in the manual farms which depends on floor system and nest boxes, lacking technology and poor hygienic practice of farm workers was higher than automatic ones. While the microbial prevalence rate in markets was the highest that may be due to bad storage and collection from backyards owners as eggs have no cleaning or washing practices. Those results came in accordance with (Messens et al., 2007) who found that the aerobic bacterial contamination of eggs and eggshells, was higher for nest eggs collected from non-cage systems compared to nest eggs collected from furnished cages or eggs from conventional cages.

In layers farms reared on deep litter system, the presence of hen manure may pose a significant environmental load on feed, air, and water that reflected on microbial quality of the egg. Environmentally, non-caged production isn't the best choice for sustainable egg production (Gunnarsson et al., 2020).

Eggs in markets even supermarkets stored and offered at room temperature, not at the refrigerator, this multiply the contamination came from the farm, this may explain the highest prevalence in markets egg samples (Al Momani et al., 2018).

The antibiotic-resistant bacteria which isolated from table eggs can be transmitted to human by even egg handling and the ingestion of raw eggs or egg dependent products. In the present study, *E. coli* was completely resistant to ciprofloxacin, this agreed with the results of (Saleiman et al., 2013). The results obtained indicated that *E. coli* was completely resistant to Rifampin, Amoxicillin, and Oxytetracyclines. These results were disagreed with those observed by (Okorie-Kanu et al., 2016).

About 94.09% of *E. coli* isolates were resistant to all tested antimicrobial drugs that were agreed with (Eid et al., 2015).

From the obtained results, about 35%, 30%, 10%, and 5% of *Staph. aureus* isolates exhibited sensitivity against Erythromycin, vancomycin, Ciprofloxacin, and Streptomycin respectively. The higher sensitivity was reported by (Nwankwo and Nasiri, 2011) who found that the sensitivity pattern of *Staph. aureus* to Erythromycin, vancomycin, Ciprofloxacin and Streptomycin were 52.4%, 100%, 78.9%, and 44.2% respectively.

The resistance rate of both *E. coli* and *Staph. aureus* against Oxytetracycline was (100%), a result that was nearly similar to the result of (Eid et al., 2015) reported approximately 92.9%.

In the present study, Amikacin, Kanamycin and Apramycin showed higher efficacy than other antibiotics used against *E. coli*, while Apramycin, Erythromycin and Vancomycin showed higher efficacy against *Staph. aureus*.

Eggshells contamination can reduce eggs’ safety and shelf-life. Therefore, decontamination of egg surfaces should begin as soon as possible, using efficient antimicrobial agents as much as possible, this will help to achieve health goals.

Immersion of eggs in H2O2 solution resulted in the largest reductions in *E. coli* on eggshells compared to other tested antimicrobial agents. Those results agreed with (Maktabi et al., 2018) who reported that H2O2 and citric acid were similar in reducing *E. coli* contamination in eggs and different food types.

In our studies, *Staph. aureus* appeared more sensitive to bicarbonate (3%) compared with H2O2 (3%) and citric acid (3%). These results were nearly similar to (Sander and Wilson, 1999) who observed that H2O2 (3%) caused significant reductions in *Staph. aureus* counts in contaminated eggs. H2O2 vapor decontamination, effectively reduced *Staph. aureus* from rooms and equipment (French et al., 2004).

Lemon juice (citric acid) and baking soda (bicarbonate) are readily available, naturally environmentally friendly, and low in toxicity (Fong et al., 2014). Citric acid has higher antimicrobial efficacy than sodium bicarbonate in the treatment of hard surfaces (Bjornsdottir et al., 2007).

*E. coli* as gram-negative bacteria are more susceptible to organic acids (citric acid) than gram-positive bacteria (*Staph. aureus*) that impair the diffusion of the organic acids into the cell, preventing antimicrobial action (Rutala et al., 2000). Control programs and measures should apply in home-produced eggs for personal consumption or local sale, to avoid the risk of infection from their consumption.

5. CONCLUSION

Table eggs collected from different sources may harbor some natural pathogens such as *E.coli* and *Staph. aureus* infections, Table eggs may be also a vital reservoir of resistance genes with a high incidence of antibiotics resistance. This demands the limitation of antibiotic use in poultry farms, more attention, and rigorous hygienic measures in layer farms. Disinfection of table egg is beneficial to decrease microbial load on eggshell, invasion.
to content and avoid potential transfer of risk to the consumer. Continuous efforts are required, strict hygienic and effective sanitary measures and proper management practices need to be adopted during egg production in poultry farms.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest for current data.

6. REFERENCES


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