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Salmonella infection in laying hens and table eggs with a trail to improve egg quality using acidic and alkaline electrolyzed water

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ABSTRACT

Salmonella is a serious organism in the commercial poultry industry. Eggs contaminated with *Salmonella* have been internationally significant sources of human illness for several decades. Most egg-associated illness has been attributed to *Salmonella enteritidis*, but a few other serovars (notably *S. heidelberg* and *S. typhimurium*) are also sometimes implicated. This study for isolation and identification of *Salmonella* from 5 Layers farms in El-Menofia Governorate by cultural and Molecular methods. The overall prevalence of *Salmonella* spp. was 15% (n=15/100). 6 *Salmonella* isolates were found from egg samples (4 isolates from eggshell, 2 isolates from egg contents). 9 isolates from liver, egg follicle, intestine and cloacal swaps were detected. The percentage of *S. kentucky*, *S. typhimurium*, *S. infantis* and *S. enteritidis* were 33.3%, 26.7%, 20% & 20%, respectively. The highest serotype isolated was *S. kentucky* (33.3%). Aerobic plate count was performed for the 60 eggshells (before the treatment as control), then 20 eggshells were treated with slightly acidic electrolyzed water, 20 eggshells were treated with slightly alkaline electrolyzed water and 20 eggshells treated with slightly acidic electrolyzed water then slightly alkaline water, respectively. Decontamination of eggs with slightly acidic electrolyzed water (SAEW) showed higher bactericidal effect compared to slightly alkaline (SAIEW). The highly effect obtained when using the acidic and alkaline water together. Therefore, decontamination of the egg surface would be a critical step in improving the microbiological safety and to extend the shelf life of the eggs used for human consumption.

INTRODUCTION:

Salmonellosis is categorized as an important zoonotic disease in public health. Infection can be acquired by direct contact and indirect contact with poultry. Indirect transmission can occur through contact with contaminated objects around poultry farms.

The presence of pathogenic bacteria as *Salmonella* in food may pose a serious health problem (food poisoning and foodborne infection), considering the fact that many food containing eggs and egg products unergo non ther-

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mal treatment, or insufficient thermal treatment to neutralize these pathogens (**Baumann and Sadkowska 2012)**.

Numerous outbreaks of diseases in humans have been caused by *Salmonella* and other food poisons bacteria from the consumption of contaminated eggs or their products. In 2015, approximately 95,000 food-poisoning outbreak cases and by *Salmonella* were registered in the European Union, and *Salmonella* in eggs accounted for 10% of all strong-evidence outbreaks, which were associated with the highest number of reported food outbreaks (**De Reu et al. 2008**).

Salmonella contamination of laying hen flocks and eggshells is associated with bad management and unhygienic environmental conditions. Poultry can become infected with many different types of *Salmonella*. The most important species are *Salmonella typhimurium*, *Salmonella enteritidis*, *Salmonella hadar*, *Salmonella livingstone* and *Salmonella senftenberg*. *Salmonella* and other pathogenic bacteria can rapidly penetrate the shell and contaminate internal contents of the egg **(De Reu et al. 2008).**

Salmonella enteritidis was able to penetrate the shell of an egg most frequently after approximately 5 days. This penetration may result in the deterioration of egg quality during storage and cause a major economic loss to the poultry industry **(Holck et al. 2018**).

Therefore, decontaminating eggshells is required to improve the microbiological safety or to extend the shelf life of table eggs for human consumption. Eggs can be contaminated with bacteria, and the egg content is an ideal growth medium for pathogenic bacteria, such as *Salmonella*, *Escherichia coli* or *Enterobacter*, which are hazardous to humans (**Chousalkar et al. 2010**).

Currently, most egg processors utilize chemical sanitization systems to decontaminate the surfaces of eggshells prior to packaging. Various disinfectants were used to reduce the microorganisms on the surface of hatching eggs, experimentally and in practice. Formaldehyde is widely used as a conventional chemical disinfectant for eggs, which can result in toxic residue and endanger hatchability, low in chick quality, and pullet growth performance (**Oliveira et al. 2020**).

The efficacy of acidic electrolyzed water (AEW) for the microbial safety and quality of eggs did not significantly affect albumin height or eggshell strength **(Bialka et al. 2004).** Slightly acidic electrolyzed water (SAEW) with a pH value of 5.0 to 6.5 contains a high concentration of hypochlorous acid (HOCl), and its application is widely accepted as an environmental friendly sanitizer method (**Zang et al. 2015**).

In recent years, the use of SAEW as an egg surface decontamination method has been met with increasing interest. Some studies have demonstrated that SAEW could be used as a disinfectant in egg processing (**Cao et al. 2009; Ni, et al. 2014**).

MATERIALS and METHOS:

Sampling:

Different samples were collected from 5 local layer farms in El- El-Menofia Governorate during years (2023-2024) (60 fresh table eggs, 50 live hens,50 dead hens). cloacal swabs were taken from the 50 live hens (pooling of each 5) and (livers, intestines and egg follicles) were collected from the 50 dead hens (pooling of each 5) and subjected to postmortem examination and transported to the laboratory as quick as possible under a septic condition for bacteriological examination as showed in table (A). Fresh table eggs were purchased and transported to the laboratory within 5 hrs, weighing 55 to 59 g per egg. Eggs were visually inspected and cracked eggs were removed.

Table A. Types and numbers of samples and age of layer chicken.

	Type of sample	No. of samples	Chicken age (wks)
50	Pooled livers	10	
Dead hens	Pooled intestines	10	
	Pooled egg follicles	10	10-15
50	Pooled cloacal swabs	10	
Live hens			
60	Eggshell	60	Before mar-
Table eggs	and content		keting
Total		100	

Clinical, Postmortum findings:

The diseased birds were examined clinically for recording the clinical signs and the freshly dead birds were subjected to postmortem (PM) examination.

Bacteriological examination:

Preparation of sample homogenate (ISO, 6887-1/ 2017).

Twenty-five grams of the examined samples were aseptically transferred to a sterile stomacher bag and homogenized with 225 ml sterile buffered peptone water (0.1%) for 30-60 seconds to give an initial dilution of 1/10. Transfer by means of pipette 1 ml of the initial suspension into a tube containing 9 ml of sterile diluent. Mix thoroughly by using vortex for 5-10 seconds to obtain 1:100 dilutions. Repeat this operation to obtain dilutions 1:1000, 1: 10000 and etc. dilutions.

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Slightly alkaline electrolyzed **water** (**PH 8.5) and slightly acidic electrolyzed water (PH 6) preparation:**

Preparation of electrolyzed water (EW) according to **Al-Haq et al. (2005), Hricova et al. (2008), Athayde et al. (2018), and Tolba et al. (2023).**

Electrolyzed water (EW) of both SAlEW (PH 8.5) and SAEW (PH 6) was prepared through electrolysis of tap water with sodium chloride (NaCl) 0.2% (2 g/liter) of tap water. A current of 9-10 volt and 8-10 amber was passed through electrolysis chamber with two poles, anode (+) and cathode (-) for 10 min. The exchange of ions occurred between two separate sides through a bridge. At the anode side, SAEW was formed due to the generation of hypochlorous acid (HOCl), hypochlorite ions (OCl-) and chlorine gas (Cl2). While, at the cathode side, SAlEW was formed as a result of generation of sodium hydroxide (NaOH). The PH of EW was estimated using a digital meter (FSSAI, 2015)

Anode: **2 NaCl→Cl2 (g) + 2 e— + 2 Na+, 2 H2O (l)→4 H + (aq) + O2 (g) + 4 e—, Cl2+ H2O (l) →HCl + HOCl**

Cathode: **2 H2O (l) + 2 e—— →2 OH—— (aq) + H2 (g), 2 NaCl + 2OH—— →2NaOH + Cl**

Isolation of ***Salmonella*:**

Salmonella was isolated according to standard methods **(ISO, 6579-1/2017)** (Microbiology of feed stuffs - horizontal method for detection of *Salmonella* species).

All collected samples (intestine, liver, egg follicle and egg) were treated as 25 g sample + 225g buffered peptone water and incubated at 37º C±1ºC for 18 hrs ± 2 hrs. Then 0.1 ml culture was inoculated in selective enrichment broth [Rappaport-Vassaliadis soya broth (RVS broth) (MERCK), Muller-Kauffmn Tetrathionate Novobiocin broth (MKTTn) (Oxoid)] and incubated at 41.5 ± 1ºC, 37 ± 1ºC for 24 ± 3 hrs respectively. A loopful from each broth culture was inoculated onto selective plating medium Xylose Lysine desoxycholate agar (XLD) (Oxoid) and Brilliant Green agar media (HiMedia) and incubated at 37ºc±1ºc for 24 hrs±3hrs. then the pure culture was examined morphologically (Films from pure cultures were stained with Gram stain which showed pink to red gram negative bacteria.), then biochemically.

Serological identification:

Salmonella isolates were subjected to serological identification in animal health research institute in Dokki by slide agglutination test according to Kauffman-White Scheme (**Kauffman 1974**) for determination of somatic (O) and flagellar (H) antigens (**Cruickshank et al. 1975**) using *Salmonella* antiserum (DENKA SEIKEN Co., Japan).

Molecular identification:

The *Salmonella*-specific primer sets for comparison and the corresponding thermocycler annealing temperatures that were used are presented in Table B. The primer sets have been published previously and are commonly used in many studies for detection of *Salmonella*. PCRs were carried out in a GenAmp PCR System 9700 thermocycler (Applied Biosystems, Weiterstadt, Germany). A typical 25-µl PCR mixture contained 0.4 µM concentrations of each primer, 200 µM concentrations of each dNTP (Roche Diagnostics, Mannheim, Germany), 1× PCR buffer (20 mM Tris-HCl [pH 8.4], 50 mM KCl), 1.5 mM MgCl2, 0.75 U of Platinum Taq polymerase (Invitrogen, Karlsruhe, Germany), and 5 µl of sample DNA (approximately 10^6 CFU per reaction tube). The incubation conditions were 95°C for 1 min, followed by 35 or 38 cycles of 95°C for 30 s, 55 to 64°C depending on the primer set used (Table B) for 30 s, and 72°C for 30 s. A final extension of 72°C for 4 min was employed. For selectivity tests, *Salmonella* DNA was cycled 35 times and non-Salmonella DNA was cycled 38 times in order to detect possible no target PCR fragments.

A 10-µl aliquot of a PCR product was loaded on a 1.8% agarose gel containing 0.5 µg of ethidium bromide/ml and electrophoresed at 6 V/cm for 90 min. Marker X (Roche Diagnostics) was used in the electrophoresis as the molecular weight standard. The gel was documented with a video camera. A positive response was defined as the presence of a visible band at the expected size, while a negative response was defined as the lack of any band at the expected size.

Table B. Primers sequences, target genes, amplicon sizes and cycling conditions.

Target	Primers sequences	Amplified segment (bp)	Primary Denaturation	Amplification (35 cycles) Secondary denaturation	Annealing	Extension	Final extension	Reference
S. typhimurium STM4495	GGT GGC AAG GGA ATG AA CGC AGC GTA AAG CAA CT	915	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec.	72˚C 50 sec.	72˚C 10 min.	**Liu et al. 2012**
S. enteritidis sefA	GCAGCGGTTACTA TTGCAGC TGTGACAGGGAC ATTTAGCG	310	94˚C 5 min.	94˚C 30 sec.	52˚C 30 sec.	72˚C 30 sec.	72˚C 10 min.	**Akbarmehr et al. 2010**
S. infantis ISR2-ISR3	GACGC-TATCAATTCAAGC AGAC ATACGATACTA-CAATACCCGACG	268	94˚C 5 min.	94˚C 30 sec.	59˚C 30 sec.	72˚C 30 sec.	72˚C 10 min.	**Chiang et al. 2018**
S. Kentucky ORF (putative membrane protein)	TTCCAATT-GAAACGAGTGCG G ACTAACCGCTT-GGGTTGTTGCTGT	170	94˚C 5 min.	94˚C 30 sec.	51˚C 30 sec.	72˚C 30 sec.	72˚C 10 min.	**Zhu et al. 2015**

Aerobic plate count determination for 60 egg shells before and after their treatment with electrolyzed water

According to **(ISO 4833-1:2013 part 2)**

The surface of whole egg was swabbed aseptically with sterile cotton swab and then diluted with normal saline. The samples were further serially diluted and 1 ml of respective dilution was poured on the surface of nutrient agar (NA) (Himedia, India) and incubated at 30°C for 72hrs. Then aerobic plate counts were recorded for the 60 eggshells.

Treatment of egg shells with electrolyzed water:
(according to Yuan X et al. 2022)

The 60 eggs were classified into three groups: group A (SAEW group), group B: (SALEW group) and group c (SAEW followed by SALEW). The eggshells were sprayed for 28 seconds with electrolyzed water using a hand operated manually sprayer. After treatment, the 60 eggshells were allowed to dry for 30 min and then subjected to aerobic plate count.

RESULTS

Clinical signs and Postmortem findings:

The observed clinical signs included sudden drop in feed consumption, ruffled feather, pale combs and diarrhea. in chronic carriers: drop in egg production

Necropsy examination as showed in table (1)

Table 1. Gross lesions of Suspected affected birds during necropsy examination.

Lesions	Infected farm no					
	F-1	F-2	F-3	F-4	F-5	Positive samples for *Salmonella*
Friable, bronze discoloration liver with white focal necrosis	-	3	1	1	-	2
Congested and enlarged liver	1	-	2	4	1	2
Congested haemorrhagic, and discolored egg follicles with stalk formation	3	2	4	2	4	1
Mild congested and haemorrhagic egg	1	1	2	-	2	1
Sever enteritis	4	3	-	2	3	2
X2			4.40			
p-value			0.353			

X2: The chi-square the result is not significant at $p < 0.05$"-" absent and F=

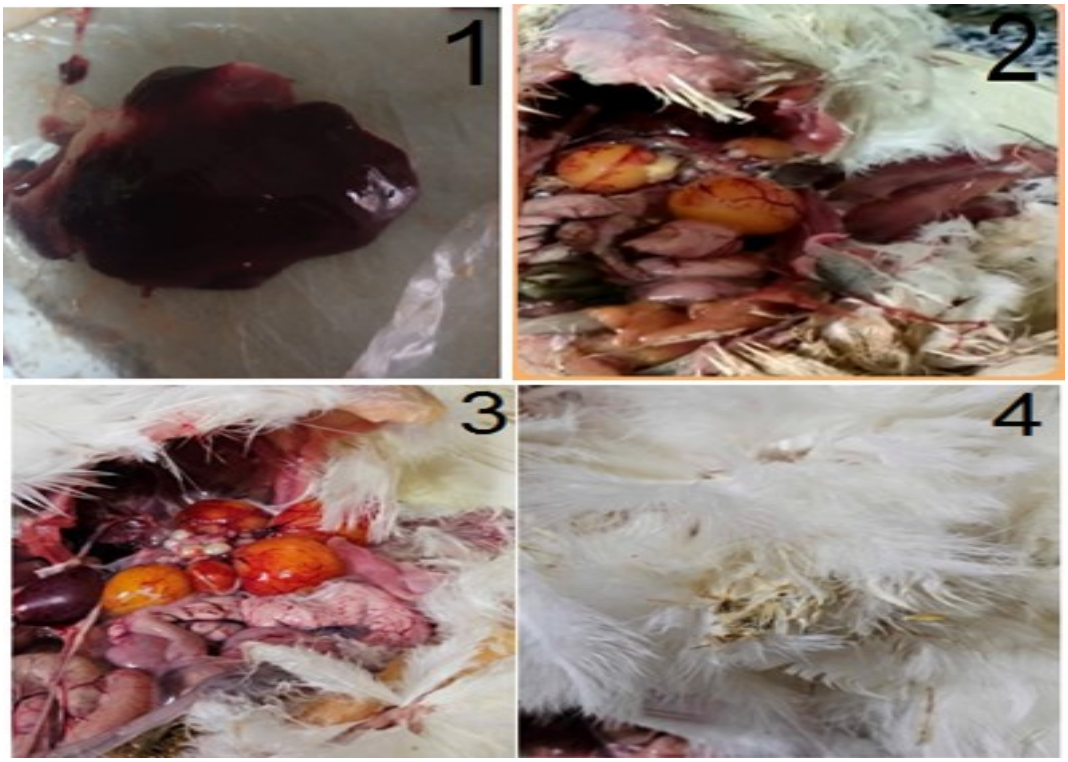

Fig (1): Congested and enlarged liver in *Salmonella* affected layer chicken.
Fig (2): *Salmonella* affected egg follicles shows haemorrhagic, congested and discolored with stalk formation.
Fig (3): Layer flock 82 days with congested, haemorrhagic eggs and sever entritis.
Fig (4): Layer flock 82 days suffer from diarrhea with mortality 10 daily/10000.

Serotyping of the isolates:

All 15 isolates using slide agglutination test revealed different antigenic structure as illustrated in (Table 2).

Among all *Salmonella*, 15 serotypes were identified from the examined samples: *S. kentucky* 5 (33.3%), *S. typhimurium* 4 (26.7%), *S. enteritidis* 3 (20%) and *S. infantis* 3 (20%), respectively

Table 2. Antigenic structure of all (15) isolates using slide agglutination test:

Isolated strain			Antigenic structure	
NO	%	Identified isolates	O	H
5	33.3	*S.kentucky*	8,20	i:z6
3	20	*S.infantis*	6,7,14	r :1,5
4	26.7	*S.typhimurium*	1,4,[5],12	i:1,2
3	20	*S.enteritidis*	,9,12	g,m: 1,7

Prevalence of *Salmonella* spp. among different examined layer hens:

A total of 100 different samples of layer hens were examined for the presence of *Salmonella*. The present study showed the overall prevalence of *Salmonella* spp. as 15 % (n=15/100). The higher isolation rate was from liver (40%) followed by intestine and egg follicle (20% for each), cloacal swabs 1 (10%) and 6.7% from eggshell while egg content (3.3) as showed in table (3).

For liver samples: 4 isolates were found to be *Salmonella* from 10 liver samples and serotyped as *S. enteritidis* (1). *S. infantis* (1), *S. kentucky* (2).

For intestine samples: 2 isolates were found to be *Salmonella* from 10 intestine samples and serotyped as *S. typhimurium* (2).

For cloacal swabs: 1 isolate was found to be *Salmonella* and serotyped as *S. typhimurium*

For egg follicles samples: 2 isolates were found to be *Salmonella* and serotyped as *S. enteritidis* (1). *S. infantis* (1)

Table 3. Types and frequency distribution of *Salmonella* serovars isolated from the examined samples (n=100)

Source of samples	No of examined samples	No	Positive Prevalence of *Salmonella* spp %	X2	p-value	Types of isolated *Salmonella* serovars	No. of positive Samples
Liver	10	4	40	7.215	.124	*S.kentucky*	2
						S.infantis	1
						S.enteritidis	1
Intestine	10	2	20			*S.typimurium*	2
Egg follicle	10	2	20			*S.infantis*	1
						S.enteritidis	1
Cloacal swabs	10	1	10			*S.typimurium*	1
Egg shell		4	6.7			*S.kentucky*	3
						S.typimurium	1
	60						
Egg content		2	3.3			*S.infantis*	1
						S.enteritidis	1
Total	100	15					

X2: The chi-square the result is not significant at $p < 0.05$

Table 4. Aerobic plate count (APC) (log CFU/egg) of control and treated eggs with EW (n=60)

Statistical analysis	**Control**	**EW treated egg**		
		SAEW	SAIEW	SAEW and SAIEW
Min	5.00	2.48	2.30	1.12
Max	6.56	4.03	4.10	2.60
Mean± SE	5.93±.101[a]	3.47±.090[b]	3.54±.111[b]	1.34±.101[a]

According to results showed in Table (4) The Aerobic plate count control (non treated) eggshell ranged from (5 .0 to 6.56 log CFU/egg) with a mean value of 5.93±0.1[a].

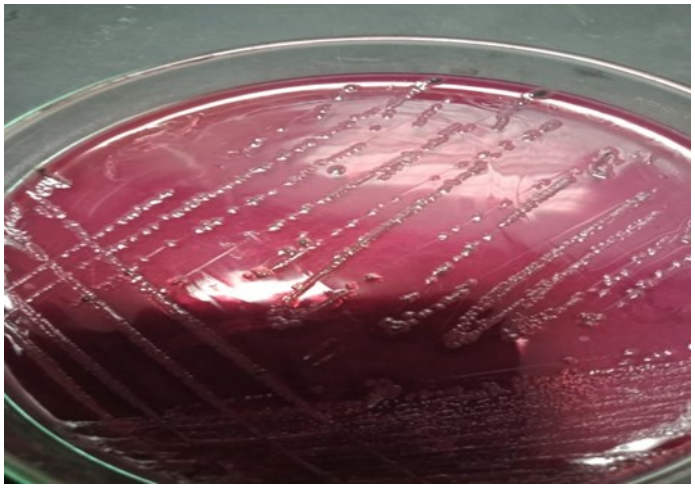

Fig (5): The *Salmonella* isolates on XLD

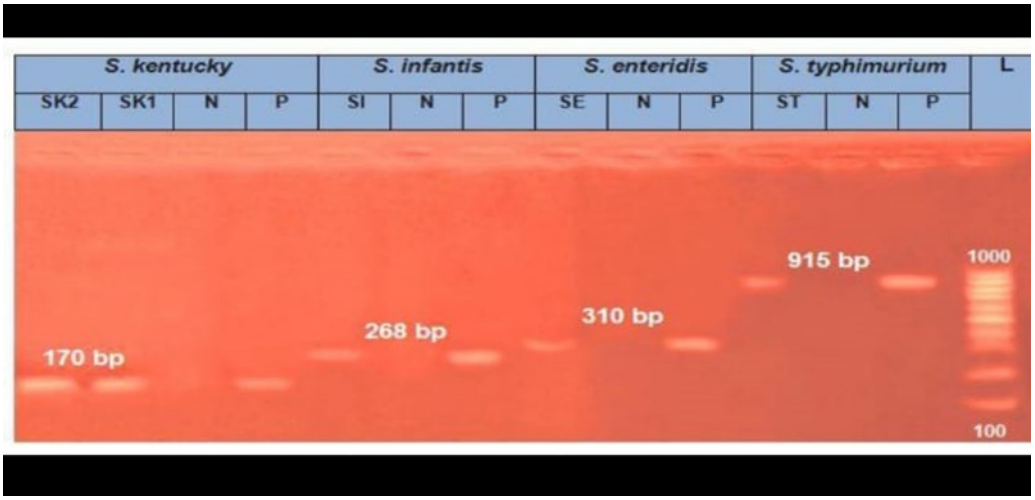

Fig (6): PCR for identification of *Salmonella* isolates.

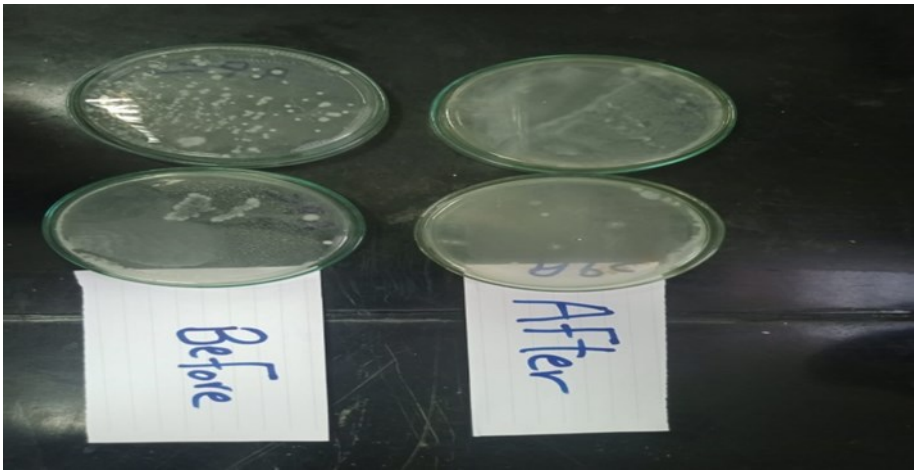

Fig (7): Effect of alkaline and acidic SAEW on total bacterial count

DISCUSSION:

In poultry, avian salmonellosis considered an important disease causing serious problem threatened the development of poultry industry especially in developing countries of Asia and Africa and remains a persistent threat of both human and animal health (**El-Sharkawy et al. 2017**).

Salmonella enterica is probably the best known food poisoning organism and can be found in a wide variety of foods. Cases and incidence of salmonellosis have reduced but it is still one of the major causes of outbreaks of food poisoning. In the present study showed the overall prevalence of *Salmonella* spp. as 15 % (n=15/100). This result agreed with **Akhtaruzzaman et al. (2020)** who recorded that the overall prevalence of *Salmonella* spp. from layer birds and from inner content of laid eggs of different poultry farms were 15.6% (n=14/90).

Salmonella isolated from liver 4 (40%), intestine 2 (20%), egg follicle 2 (20%), egg 6 (10%) and cloacal swabs 1 (10%). This result in agreement with (**Gole et al. 2014 a**) reported that A field survey investigating the prevalence of Salmonella shedding on commercial layer farms found significant variability in *Salmonella* prevalence at various stages of lay on farm. Also in the present study *S.typhimurium* isolated from intestine and swaps Shedding from the known positive laying hens can be intermittent and remain undetected for several weeks (**Gole et al. 2014 b**). On the other hand, contamination of egg internal contents with *S. typhimurium* has been documented after experimental infection of hens at the onset of lay via oral and aerosol routes (**Okamura et al. 2010)**.

The prevalence of *Salmonella* in eggshell in the present study was 6.6% while in egg content was 3.3%. This agrees with **Hoque et al. (2019) and Akhtaruzzaman et al. (2020),** who reported that Prevalence of *Salmonella* spp. was significantly higher in eggshell compared to egg contents and may be associated with human illnesses during consumption of contaminated poultry eggs.

Two *Salmonella* were isolated from 60 laid egg samples. The isolation rate was 3.3% reporting transovarian transmission in poultry salmonellosis.

Serotyping has been used for identification and epidemiological investigation, each serovars is identified by combination of lipopolysaccharide moieties on the cell surface (O antigens) and different flagellar protein (H antigen)
(Prendergast et al. 2013).

Serological identification of 15 *Salmonella* isolates were confirmed by slide agglutination test (Table 2) according to white Kauffman le minor scheme. This results showed that the most frequently reported serovars are *S. kentucky* 5(33%), *S. typhimurium* 4 (26.7%), *S. enteritidis* and *S.infantis* 3(20%) respectively. These results agreed with (**Diker et al. 2020)** who recorded that *S. kentucky* was the most common serotype in samples taken from laying hens covering 9 different provinces of Türkiye but failed to isolate *S. typhimurium* in any of the samples. The results not agreed with **Hodagari et al. (2020)** who reported that the most common serotype in laying hens is *S.* typhimurium and others' (**Snow et al. 2007; Huneau-salaün et al. 2009; Hulaj et al. 2016; Velasquez et al. 2018)**, which reported *S. enteritidis* as the most prevalent serovar. There are significant differences in *Salmonella* serovars between countries, even in different regions of the same country (**Carlı et al. 2001**).

During the recent years, S. *kentucky* has emerged as a global zoonotic pathogen as it is frequently isolated from both poultry and humans (**Xiong et al. 2020**). so, it cannot be ignored that it is among the most common *Salmonella* serotypes associated with poultry worldwide and its increasing trend in layer hens in recent years (**Alessiani et al.**

2022).

Salmonella. typhimurium transmitted in between chicken due to the contact of live birds or through consumption of contaminated food (e.g. chicken meat) directly by the micro-organisms or through their enterotoxins (**Niyonzima et al. 2016).**

In the present study, the isolates also identified by PCR. In recent years many researches try to establish methods which can reduce the time for the detection and identification of *Salmonella*. Detection of bacteria by conventional methods is time consuming and doesn't allow the detection of viable but non culturable one. PCR has emerged as an approach to overcome these problems. The exploration of gene targets for evaluation of absence and presence of bacteria is still a matter of importance. Several genes *inv*A, *fim*A and *ace*K were used for identification of genus *Salmonella*. The long persistence of DNA even after bacterial death may lead to positive result, there for the detection of RNA much less stable in the environment and detect the viable bacteria is considered more attractive (**O'Regan et al. 2008).**

In the present study, the obtained mean values of APC for samples were 5.93 ± 101^{a} in eggshells, this agrees with **Chaemsanit et al. (2015)** who reported that The APC range of bacteria on eggshells were (2.9 to 6.2 log CFU/ egg) in market layer.

Bacterial inactivation is a crucial aspect of sanitation and hygiene. Slightly acidic electrolyzed water (SAEW) has been proved as an effective antimicrobial agent for inactivating *E.coli*, *S.aureus* and *Salmonella* spp in vitro (**Issa-Zacharia et al. 2010**).

Conventional chemical disinfectants used for egg disinfection could result in toxic residue and endanger hatchability, chick quality, and pullet growth performance. Slightly acidic electrolyzed water (SAEW) is known as a novel disinfectant for egg sterilization due to its high efficiency and no residue. In this study, a comprehensive assessment of slightly acidic electrolyzed water and alkaline electrolyzed water used in the disinfection channel was conducted to assess the microbial count, eggshell quality. The total colony count means 5.93 ± 0.1^{a}; the alkaline electrolyzed water was $3.54 \pm 111^{b,}$ the acidic electrolyzed water 3.47 ± 111^{b} and the acidic and alkaline water together $1.34\pm.101^{a}$. This result agreed with Zhang et al. (2021), show that the sterilization efficiency of acidic water is high. Spray disinfection of SAEW for 3 and 4 min caused a complete inactivation of *S. enteritidis* and *E. coli* on the eggshell.

In this study spraying technique of disinfectant were applied, this technique applied also by **Liu et al. (2022**).

Slightly acidic electrolyzed water shows the potential to be used for sanitization of eggshells as an environmentally friendly disinfection agent.

Efforts including critical control point programs in food manufacture are needed to reduce the incidence of *Salmonella* in food. Consumers-awareness efforts would protect public health from foodborne pathogen.

CONCLUSION

Slightly acidic electrolyzed water (SAEW) showed the potential to be used for sanitization of egg shells with higher bactericidal effect compared to alkaline electrolyzed water (SAlEW). The highly effect performed by using the SAEW and SAlEW water respectively together. Therefore, decontamination of the egg surface would be a critical step in improving the microbiological safety and to extend the shelf life of the eggs used for human consumption.

Competinginterests:
The authors declare no competing interests.

Author´s Contributions:
All authors contributed equally to this work.

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