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EVALUATION OF THE TOLERANCE OF BIOFILM-FORMING *SALMONELLA* ISOLATED FROM DEAD IN SHELL EMBRYOS TO SOME DISINFECTANTS

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ABSTRACT

Salmonella is a hazardous bacterium that can lead to dangerous human infections, as well as catastrophic losses in chicken production. Disinfectants are frequently used in chicken houses to prevent the spread of zoonotic infections such as *Salmonella* strains. The emergence of bacteria strains resistant to various disinfectants is a serious problem when using disinfectants. The resistance of certain *Salmonella* serotypes to quaternary ammonium compounds is a feature of these phenomena and some *Salmonella spp.* may carry the *qacED1* and *qacA/B* genes, which are responsible for this resistance. So the purpose of this study was to identify *Salmonella* serotypes and determine the most important virulence genes of the serotypes obtained from samples taken from dead chicken embryos. Evaluation of the resistance of *Salmonella* strains against various disinfectants (Quaternary ammonium compounds QACs, iodine, and virkon S) and determined the minimum inhibitory concentration (MIC) to investigate the antibacterial potential of plant essential oil components, such as thymol, cinnamaldehyde, and zingiberene, against *Salmonella* serotypes. A total of 115 samples were collected from dead chicken embryos, after isolation *Salmonella* isolates were reported to be 16/115 (13.9%). The most prevalent serotypes were *Salmonella typhimurium, Salmonella kentucky, Salmonella anatum* and *Salmonella poona*. Through the treatment of the bacteria at various concentrations, the effectiveness of the disinfectant was determined. Research has shown that the type and concentration of the disinfectant affect its biocidal activity. PCR was also used to detect the presence of *qacED1* and *qacA/B* genes.

Keywords: *Salmonella* serotype; QACs; *qacED1;* thymol; cinnamaldehyde.

INTRODUCTION

In the past several years, the poultry industry has faced numerous difficulties, and *Salmonella* infections being one of the main among them. (Ruvalcaba-Gómez *et al.,* 2022). Within the *Enterobacteriaceae* family, the *Salmonella* genus is classified as an enteric Gram-negative, facultative anaerobe, non-spore-forming bacilli. (Bhunia, 2008; Barrow *et al.,* 2012).

There are over 2600 serovars in the *Salmonella* species (Mohanapriya *et al.,* 2023). *Salmonella* serovars can spread horizontally between flocks including the

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vertical transmission of *Salmonella* bacteria to eggs, which results in the death of the embryos or the death of recently hatched chicks, causing significant morbidity and mortality (Vo *et al.,* 2006; Yang *et al.,* 2019). The primary cause of *Salmonella*, with a 48.5% incidence, is hatcheries. (Wang *et al.,* 2023) and *Salmonella* infection is frequently linked to both mortality of recently hatched chicks and dead-in-shell embryos. (Lister and Barrow, 2008).

The membrane proteins from bacteria that invade host intestinal cells are encoded by the invA gene (Yulian *et al.,* 2020). The invasion of host epithelial cells by *Salmonella* isolates is significantly facilitated by invA. For the majority of *Salmonella* species, this gene is extremely specific (Pardo *et al.,* 2019).

Many genes that encode virulence are the main factor that determines how severe *Salmonella* infection occurs. Most of the *Salmonella* virulence indicators found on *Salmonella* pathogenicity islands (SPIs) which are either chromosomal or plasmid-based include adhesion, invasion, intracellular proliferation, and toxin genes. (Miller *et al.,* 2010). There are two primary SPI areas: the first harbors invasion genes, while the second is critical for intracellular pathogenicity and plays a major part in *Salmonella* infections that spread throughout the entire organism.

The poultry industry's extensive use of antibiotics has led to the emergence of *Salmonella* strains that are resistant to numerous antimicrobials, including sulfonamides, ampicillin, quinolones and tetracyclines (De Mesquita *et al.,* 2022).

A key component of the pathogenicity of many bacterial species, including *Salmonella* spp., biofilm is one of the principal causes of chronic infections and environmental persistence (Seixas *et al.,* 2014). One method that *Salmonella* is known to use to survive and proliferate in chicken farms is the creation of biofilms. (Merino *et al.,* 2019). These biofilms may include antibiotic-resistant bacteria, as well as other elements that contribute to their environmental persistence. (Dhanani *et al.,* 2015 & Guillén *et al.,* 2020). Features of virulence associated with resistance to antibiotics, and *Salmonella's* ability to form biofilms are thought to be an increasing risk to public health in both human and poultry production. (Karabasanavar *et al.,* 2020).

Also, Abd El-basit *et al.* (2019) reported that all *Salmonella* strains tested by PCR were found to have the *adrA, csgD,* and *gcpA* genes. Additionally, each isolate of *Salmonella* formed biofilms, which increased their resistance to antibiotics and disinfectants and made the disease harder to cure. This caused many issues for the food industry, because it became a constant source of contamination.

The primary component of biosecurity programs is disinfectants (Dvorak, 2005). Using disinfectants is essential in poultry production to reduce the risk of infection and contamination with *Salmonella* (Galis *et al.*, 2013). Thus, the increasing lack of responsiveness to disinfectants is considered a serious hazard (Mc Carlie *et al.,* 2020). It is believed that *Salmonella* is a major cause of the spread of QAC resistance (Long *et al.,* 2016). The development of antimicrobial resistance has been related to the widespread use of disinfectants (Chapman, 2003).

MATERIAL AND METHODS

1. Sampling:

The samples were taken from dead chicken embryos collected from various broiler hatcheries. A total number of 115 samples from the intestines, liver, kidney, yolk sac, and spleen. After egg shells were cleaned with 70% ethyl alcohol and cut open with clean scissors, the dead embryos were examined for post-mortem and organ samples were taken out for additional bacteriological analysis.

2. Bacteriological examination

2.1. Isolation of bacterial agents

5 grams of all samples were pre-enriched in 45 milliliters of tryptic soy broth for 16–20 hours at 37°C after being aseptically cut into small pieces. A loopful from the pre-enrichment culture was streaked onto the xylose lysine deoxycholate agar (XLD; Oxoid), which was then incubated for 24 hours at 37°C. On XLD medium, the probable *Salmonella* colonies were purified. The isolates were biochemically identified according to **Zhang *et al.* (2013)**. Using "O" and "H" antisera from (Difco), the isolates were serotyped using a slide agglutination test following the Kauffmann-White system.

2.2. Morphological examination: It was carried out following Cruichshank *et al.* (1975).

2.3. Biochemical identification

Using biochemical tests, pure colonies of isolates were recognized, according to Quinn *et al.* (2002) and Zhang *et al.* (2013).

3. Serological identification of *Salmonella*

The organisms' serotypes were determined using **Kauffmann and Das-Kauffmann (2001)**.

4. Congo red dye agar test (CR Test):

The test was conducted using a strategy of Berkhoff and Vinal (1986). After being streaked on Congo red agar, the colonies were cultured at 37°C for 24 hours. Red colonies showing up within 24 hours were noted as a positive response. Negative colonies were deemed negative after they failed to bind the dye and continued to be white or gray even after 24 hours.

5. Antimicrobial susceptibility testing:

The following antimicrobials were found to be susceptible to the serotyped *Salmonella*: colistin (10µg), streptomycin (10µg), tetracycline (30µg), co-trimoxazole (25µg), neomycin (30µg) and amoxycillin (10µg), cephradine (30µg), cefotaxime (30µg) and enrofloxacin (5µg), using the disc diffusion method according to CLSI, (2013). Antimicrobial discs and the installed medium were provided by (Oxoid). To evaluate resistance or susceptibility, inhibition zones were evaluated. The acquired results are presented in the table (3).

6. Evaluation of the biocidal activity of the disinfectants:

Dilutions of disinfectants were made from the stock solutions to provide the following ratios: for iodine 1: 25, 1:50, 1: 100 and 1: 200 v/v of distilled water (DW), for QAC at rate 1: 25, 1:50, 1: 100 and 1: 150 v/v of DW, Virkon S at rate 1: 100, 1:200, 1: 500 and 1: 2000 v/v of DW. Two milliliters of previously made bacterial serial dilutions (10^7 CFU/mL) were combined with eight milliliters of diluted disinfectant solutions to test the disinfectants' biocidal activity. The mixture was homogeneously mixed according to the steps provided by Aksoy *et al.* (2020). The concoctions approved 24 hrs standing period. After distributing 0.1 ml of the mixture on nutrient agar, it was incubated at 37 °C for 24 hours. The disinfectants' dilution was evaluated according to the evaluation criteria for the antibacterial activity of the disinfectants.

7. Molecular identification of virulence genes, biofilm-formed genes and resistance genes of disinfectant:

DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 20 µl of proteinase K and 200 µl of lysis buffer at 56°C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged, following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

7.1. Oligonucleotide Primer. Primers used were supplied from Metabion (Germany) are listed in table (1).

7.2. PCR amplification.

PCR Primers were utilized in a 25- µl reaction containing 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentrations, 5.5 µl of water, and 5 µl of DNA template. The reaction was performed in an applied biosystem 2720 thermal cycler.

7.3. Analysis of the PCR Products.

The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the PCR products were loaded in each gel slot. A gene ruler 100 bp ladder (Fermentas, thermo, Germany) and Genedirex 100-3000 bp DNA ladder H3 RTU (Genedirex, Taiwan) were used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

8. Minimum inhibitory concentration (MIC) determinations of essential oils:

Initially, sterile 96-well polystyrene plates were filled with various concentrations (2048, 1024, 512, 256, 128, 64, 32, 16, 8, 4, 2, and 1 µg/mL, respectively) of antimicrobial compounds (such as thymol, Zingiberene, and cinnamondehyde). Antimicrobial agents (10 µL) were applied to wells 1 through 13 whereas well 14 served as a growth control without any antimicrobial agent addition. The direct bacterial suspension method was then used to create bacterial suspensions with turbidity equal to 0.5 McFarland standards. Following the addition of 100 µL (10^7 CFU/mL) suspensions of *Salmonella* isolates to each well, the wells were sealed, and for checking the findings, each plate was incubated for 24 hours at 37°C. At 37°C for 24 hours, the lowest concentration at which no discernible bacterial growth occurs is known as the minimum inhibitory concentration (MIC) as described by Tang *et al.* (2022).

Table 1: Primers sequences, target genes, amplicon sizes and cycling conditions.

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
adr A	ATGTTCCCAAAAATAATGAA TCATGCCGCCACTTCGGTGC	1113 bp	94˚C 5 min.	94˚C 30 sec.	50˚C 1 min.	72˚C 1.2 min.	72˚C 12 min.	**Bhowmick *et al*., 2011**
gcp A	CTATTTCTTTTCCCGCTCCT GTGCCGCACGAAACACTGTT	1713 bp	94˚5 min.	94˚C 30 sec.	57˚C 1 min.	72˚C 2 min.	72˚C 12 min.	
hilA	CATGGCTGGTCAGTTGGAG CGTAATTCATCGCCTAAACG	150 bp	94˚C 5 min.	94˚C 30 sec.	60˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	**Yang *et al*., 2014**
Qac ED1	TAA GCC CTA CACAAA TTG GGA GAT AT GCC TCC GCA GCG ACT TCCACG	362 bp	94˚C 5 min.	94˚C 30 sec.	58˚C 40 sec	72˚C 40 sec	72˚C 10 min.	**Chuanchuen *et al*., 2007**
invA	GTGAAATTATCGCCACGTTC GGGCAA TCATCGCACCGTCAAAGGA ACC	284 bp	94˚C 5 min.	94˚C 30 sec.	55˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	**Oliveira *et al*., 2003**
avr A	CCT GTA TTG TTG AGC GTC TGG AGA AGA GCT TCG TTG AAT GTC C	422 bp	94˚C 5 min.	94˚C 30 sec.	58˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	**Huehn *et al*. 2010**
Qac A/B	GCAGAAAGTGCAGAGTTCG CCAGTCCAATCATGCCTG	361 bp	94˚C 5 min.	94˚C 30 sec.	53˚C 40 sec.	72˚C 40 sec.	72˚C 10 min.	**Noguchi *et al*., 2005**
omp A	AGTCGAGCTCATGAAAAAG ACAGCTATCGC AGTCAAGCTTTTAAGCCTGC GGCTGAGTTA	1052 bp	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 1 min.	72˚C 10 min.	**Kataria *et al*., 2013**

RESULTS

1. Bacteriological examination

Salmonella strain isolation and identification have been confirmed serologically using "O" and "H" antisera, and the obtained serotypes were recorded in (**Table 2**). *Salmonella*-positive sample percentages were 16/115 (13.9%), for dead embryos of chickens. *S. Typhimurium* (n=3), *S. Kentucky* (n=7), *S. anatum* (n=5), and *S. poona* (n=1) were isolated from dead embryos collected from broiler hatcheries.

2. Congo red agar test

Out of the 16 isolates examined in this study (13.9%), a positive result for the CR test. After being cultured in CRA at 37 °C, every isolate of *Salmonella* serotypes formed a rdar colony morphotype. **(Fig. 1).**

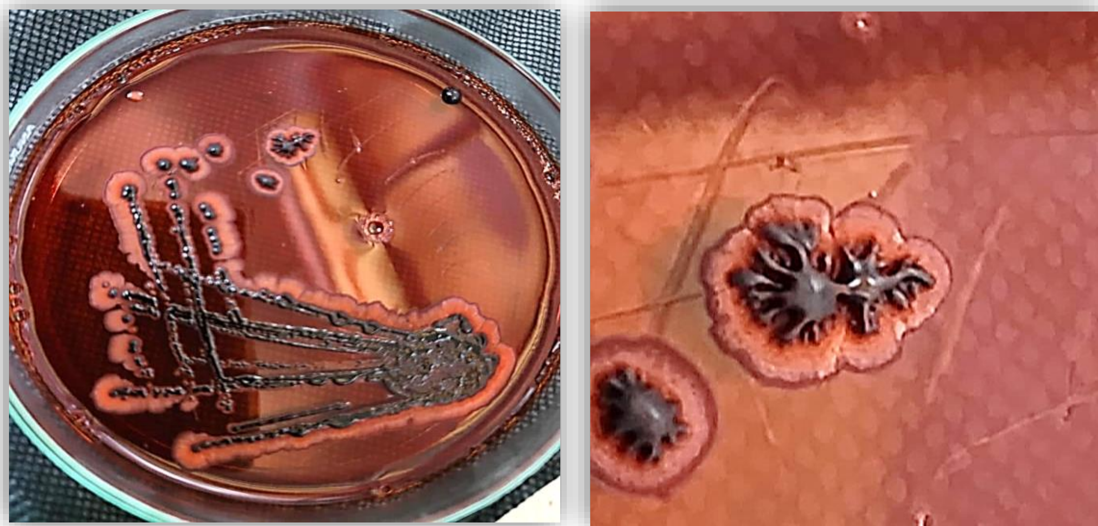

Figure (1): Colony morphotypes on Congo red agar: rdar morphotype of *Salmonella* isolates

Table 2: Rates of *Salmonella* serotypes, Congo red activity and antigenic structures identified from dead-shell embryos.

Type of samples	*Salmonella*-positive			Congo red activity		Antigen			Total (%)
	Serotype	Freq	Total (%)	positive	negative	O	H Phase 1	H Phase 2	
Yolk sac	*S. Kentucky*	4	4/115 (3.4%)	4	0	8, 20	I	Z6	4/115 (3.4%)
Liver	*S. Typhimurium*	3	3/115 (2.6%)	3	0	1,4,{5},12	I	1,2	3/115 (2.6%)
Intestine	*S. poona*	1	1/115 (0.8%)	1	0	1,13,22	z	1,6	1/115 (0.8%)
Kidney	*S. Anatum*	5	5/115 (4.3%)	5	0	3, {10}{15}{15,34}	e, h	1,6	5/115 (4.3%)
Spleen	*S. Kentucky*	3	3/115 (2.6%)	3	0	8, 20	I	Z6	3/115 (2.6%)
Total	**-**	**16**	**16/115 (13.9%)**	**16**	**0**	**-**	**-**	**-**	**16/115** (13.9%)

3. Antimicrobial susceptibility testing

The isolated *Salmonella* serotypes were sensitive to colistin, cefotaxime, amoxycillin and enrofloxacin at rates of 81.25, 62.5, 68.75 and 56.25% respectively. Conversely, however, isolates showed antibiotic resistance to neomycin, streptomycin, co-trimoxazole, cephradine and tetracycline at rates of 93.75, 87.5, 75, 68.75 and 56.25% respectively **(Table 3**).

Table 3: Antibiogram phenotypic patterns of isolated *Salmonella*.

Antibiotics		Antibiogram phenotypic pattern					
		susceptible		Intermediate		Resistant	
		No.	%	No.	%	No.	%
Colistin	CL10	13	81.25	-	-	3	18.75
Streptomycin	S10	2	12.5	-	-	14	87.5
Tetracycline	TE30	4	25	3	18.75	9	56.25
Co-Trimoxazole	COT25	3	18.75	1	6.25	12	75
Neomycin	N30	1	6.25	-	-	15	93.75
Amoxycillin	AMX10	11	68.75	2	12.5	3	18.75
Cephradine	CE30	2	12.5	3	18.75	11	68.75
Cefotaxime	CTX30	10	62.5	4	25	2	12.5
Enrofloxacin	ENR5	9	56.25	2	12.5	5	31.25

4. Molecular identification of virulence genes and biofilm resistance genes:

The most prevalent virulence genes identified in the isolated *Salmonella* serotypes were *ompA, hilA, avrA,* and *invA*. *Salmonella* serotypes were successfully amplified for the two most common biofilm resistance genes, *gcpA* and *adrA*. (See in **Figures 2 & 3)**

5. Genotyping results of quaternary ammonium compounds (QAC) resistance tests:

After extracting the DNA from *S. typhimurium, S. kentucky, S. anatum, and S. poona*, the *qacED1* and *qacA/B* genes were detected by PCR. The QAC-treated bacteria were subjected to genotyping testing. The findings demonstrated that every isolate of *Salmonella* had the genes *qacED1* and *qacA/B*, which are treated with a disinfectant (**Figure 4**).

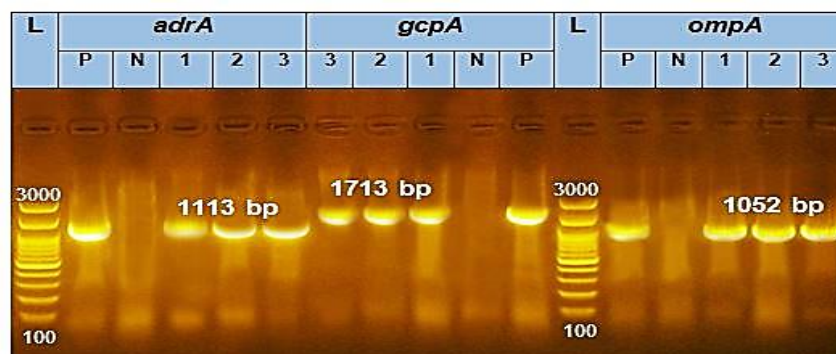

Figure (2): PCR results for *Salmonella* isolates are separated on an agarose gel electrophoresis to identify the *adrA, gcpA,* and *ompA* genes in the genomic DNA.

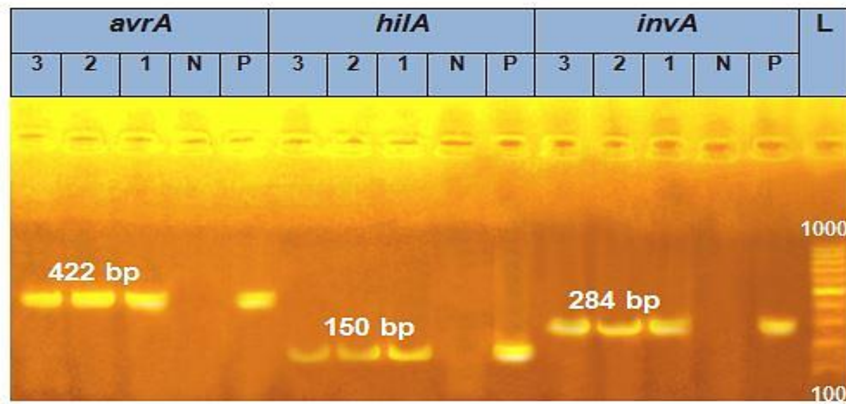

Figure (3): Agarose gel electrophoresis of PCR products for *Salmonella* isolates to detect virulence genes *avrA, hilA,* and *invA* gene in genomic DNA.

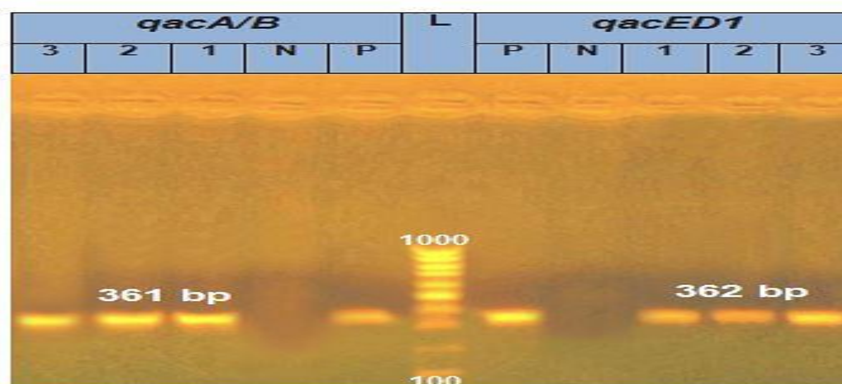

Figure (4): Detecting the *qacED*1 and *qacA/B* genes in genomic DNA of *Salmonella* isolates using agarose gel electrophoresis.

6. Evaluation of the resistance to disinfectants:

6.1. Effect of iodine:

When using (1:25 and 1:50) dilutions of iodine as a disinfectant, the growth of the four microorganisms under examination was totally prevented. While *S Kentucky, S. Typhimurium,* and *S. Anatum* were completely inhibited from growing by the 1:100 diluted iodine, while *S. poona* was not affected. There was no noticeable inhibitory effect from using more diluted iodine (1:200) on *Salmonella* serotypes (**Table 4**).

Table 4: The effect of iodine

Dilution	Bacteria			
	S. Kentucky (CFU)	S. Typhimurium (CFU)	S. poona (CFU)	S. Anatum (CFU)
1/25	-ve	-ve	-ve	-ve
1/50	-ve	-ve	-ve	-ve
1/100	-ve	-ve	+ve	-ve
1/200	+ve	+ve	+ve	+ve

6.2. Effect of QAC:

All bacterial serotypes under examination observed total growth inhibition when exposed to QAC at dilutions of 1:25, 1:50, and 1:100. The diluted QAC (1:150) was unable to prevent the growth of *Salmonella* isolates. (**Table 5 and Figure 5A**).

Table 5: The effect of QAC.

Dilution	Bacteria			
	S. Kentucky (CFU)	**S. Typhimurium (CFU)**	**S. poona (CFU)**	**S. Anatum (CFU)**
1/25	-ve	-ve	-ve	-ve
1/50	-ve	-ve	-ve	-ve
1/100	-ve	-ve	-ve	-ve
1/150	+ve	+ve	+ve	+ve

6.3. Effect of Virkon S:

Following treatment with (1:100 and 1:200) dilutions of Virkon S for all treatments, none of the studied bacteria (*S. Kentucky, S. Typhimurium, S. Anatum,* and *S. poona*) displayed any discernible growth. While Virkon S (1:500 and 1:2000) did not exhibit any discernible inhibitory impact over *Salmonella* serotypes when used at higher dilutions. (**Table 6 and Figure 5B**).

Table 6: The Effect of Virkon S.

Dilution	Bacteria			
	S. Kentucky (CFU)	**S. Typhimurium (CFU)**	**S. poona (CFU)**	**S. Anatum (CFU)**
1/100	-ve	-ve	-ve	-ve
1/200	-ve	-ve	-ve	-ve
1/500	+ve	+ve	+ve	+ve
1/2000	+ve	+ve	+ve	+ve

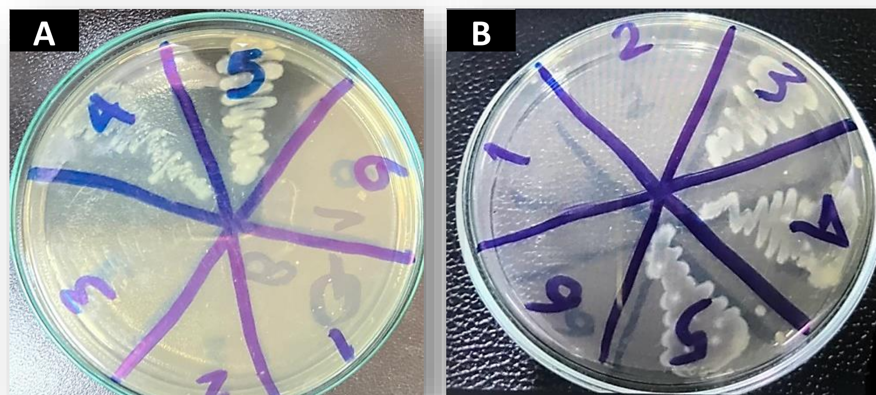

Figure (5): Antibacterial activity (clear areas) of various disinfectants to bacteria isolates (**A**): QAC (**B**): Virkon S.

7. MIC of essential oils:

The three essential oils' minimum inhibitory concentrations (MICs) were determined in this study. The products, namely thymol, cinnamaldehyde and Zingiberene, showed some degree of serotype-inhibitory action on *Salmonella*. **Figure (6)** shows the MIC values of three components found in essential oils against isolates, with thymol, cinnamaldehyde and zingiberene. Thymol against *S. typhimurium* , *S. kentucky, S. poona* and *S. anatum* indicating MIC values of 32, 32, 64 and 128 µg/mL, respectively, signifying the components having the lowest

MIC values. Generally, a greater inhibiting of bacterial growth is indicated by a decreased MIC value. The MIC values of zingiberene against *S. typhimurium* , *S. Kentucky, S. poona* and *S. anatum* show MIC values of 64, 512, 256 and 256 µg/mL, respectively. The MIC values of cinnamaldehyde against *S. typhimurium* , *S. Kentucky, S. poona* and *S. Anatum* indicating MIC values of 64, 128, 128 and 128 µg/mL, respectively.

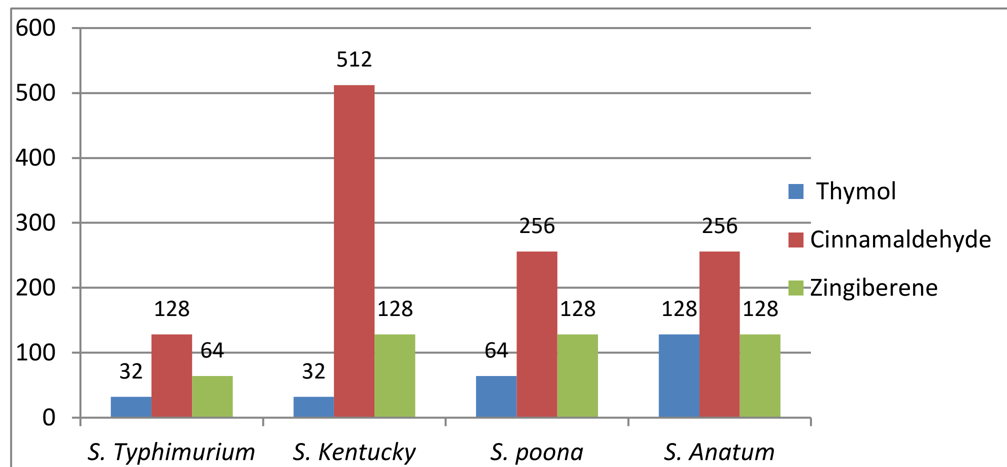

Figure (6): Minimum inhibitory concentrations (MIC) of essential oils.

DISCUSSION

Salmonella is a major foodborne pathogen responsible for many infectious diseases in animals and humans worldwide (Raguenaud *et al.,* 2012: Manoj *et al.,* 2015). In the present study, sixteen isolates of *Salmonella* were found in dead embryos taken from broiler hatcheries. *Salmonella* isolation rates were 13.9% overall Table (2). The findings of the microbiological examination of the organs confirmed with Shehata *et al.* (2019), who used the intestine, kidney, spleen, liver, and yolk sacs in order to isolate *Salmonella* spp. from chicken dead embryos; with an isolation rate of 12.5%.

The findings confirmed that *S. Kentucky, S. typhimurium, S. anatum,* and *S. poona* were isolated from dead embryos of chickens which agrees with Lacroix-Lamande *et al.* (2023) that isolated *S. Typhimurium* from embryos of dead chickens but disagreed with Shehata *et al.* (2019) which reported that *S.Enteritidis, S.Amsterdam* and *S.Atakpame* were isolated from dead chicken embryos obtained from hatcheries for broilers. During hatching, *Salmonella* can enter embryos either vertically or horizontally (Bailey *et al.,* 1994: Hameed *et al.,* 2014).

Our findings verified the presence of the virulence genes *invA, hila, avrA* and *ompA* in all *Salmonella* strains using specific invA primer (Figures 2 and 3). The findings confirmed with Kelly *et al.* (2023) and Freshindy *et al.* (2021), which found that the results of the virulence gene *invA* were found in each of the seven (100%) among the MDR *Salmonella* strains,and agreed with Mashayekh *et al.* (2022) who reported that *invA* and *agfA* virulence genes were found in 30 isolates (100%) according to PCR results. Shehata *et al.* (2019) showed that 95% of the isolated *Salmonellae* had identified *invA*, demonstrating the importance of *invA* as a vital indicator in the molecular detection of *Salmonella* in poultry (Dong et al., 2011). Numerous virulence factors are carried by *Salmonella* spp., which is one of the reasons why salmonellosis is so common in both humans and animals. When antibiotics are used extensively to treat salmonellosis, resistant bacteria usually develop. PCR represents one of the greatest tests for determining virulence genes (Ansharieta *et al.,* 2021).

Salmonella species diversity can be observed in the variability in biofilm production; strains of *S. Typhimurium* are known to be effective producers of biofilms in a variety of environmental settings (Beshiru *et al.,* 2018). So, our studies revealed that the *adrA* and *gcpA* genes were found with a 100% incidence rate (Figure 1&2) and that all strains of *Salmonella* (*S. Kentucky, S. Typhimurium, S. anatum,* and *S. poona*) had biofilm genes. This finding was generally agreed with Dorgham *et al.* (2019) who showed that *adrA, gcpA* and *csgD* genes were shown to have an incidence of 88.8%, 100% and 100% respectively, also fully agreed with (Seixas *et al*., 2014) who reported that, 129 isolates (97.0%) tested positive for *gcpA* out of the 133 *Salmonella* isolates that were obtained from environment and animal sources. All isolates (100%) had the *adrA* and *csgD* genes. On the other side, our results didn't agree with (Hawash *et al.,* 2017) found that in Egypt's poultry farms, surveillance on *Salmonella* bacteria revealed that all samples had positive *csgD* gene PCR results and negative *adrA* and *gcpA* gene results.

QACs are widely used cationic surface-active detergents in poultry farms because of their strong antimicrobial qualities and low relative toxicity moderately efficient in detecting the presence of organic materials, low toxicity, not corrosive and not irritating. As a result, it is the preferred disinfectant for tools like hatching trays and incubators (Haynes and Smith*,* 2003). In the present study, the *qacE*D*1* and *qacA/B* genes were 100% of the *Salmonella* isolates (Figure 3). These results were nearly in accordance with Amira (2016) who found that 93.1% of *Salmonella* isolates had the *qacED1* gene. Also, our results agreed nearly with Rungtip *et al.* (2007) who found that more isolates of Salmonella tested positive for *qacEΔ1* but negative for intI1. The *qacEΔ1* gene may be incorporated into a chromosome or carried on additional elements. These non-integron-associated *qacEΔ1* genes play a role in the development of decreased *Salmonella* susceptibility to BKC. It could help to explain why, despite cleaning and disinfection efforts following depopulation, this pathogen continues to persist in laying and grill flocks over multiple flock cycles. Conversely, however Nabil and Younis (2019) reported that the *qacA/B* gene was negative, which might have happened as a result of different *Salmonella* serotypes being linked to particular genes. Additionally, it could be explained by the fact that *Salmonella* and other Gram-negative bacteria have a higher frequency of the *qacEΔ1* gene than the *qacA/B* gene.

Corresponding to the findings from sensitivity to antimicrobial tests, *Salmonella* isolates were shown to be resistant to tetracycline, neomycin, streptomycin, co-trimoxazole, cephradine, and neomycin, but sensitive to colistin, cefotaxime, amoxycillin, and enrofloxacin, as shown in table (3). These results varied from Nabil and Younis (2019) who explained that resistance to streptomycin and tetracycline was higher, with percentages of 70.6% and 94.1%, respectively, Zishiri *et al.* (2016) who found that resistance to ampicillin (47%), sulfamethoxazole-trimethoprim (84%), tetracycline (93%), and streptomycin (12%) was seen in the isolated *Salmonella*; Zdragas et al., (2012) who stated that they were resistant to tetracycline (2%) and streptomycin (5%); Cardoso et al., (2006) who recognized that a strain of *Salmonella* obtained from broiler chickens exhibited a 100% tetracycline resistance.

This study examined the effects of several widely used disinfectants on *S. typhimurium, S. anatum, S. kentucky, and S. poona* depending on the concentration of the disinfectant as shown in **Table (4, 5 & 6).** The result illustrated that QAC proved to be the strongest disinfectant followed by iodine and *Virkon S*. Results from treatments with QAC, iodine, and Virkon S showed that the concentration of each disinfectant affecting its ability to kill bacteria, with more diluted disinfectants encouraging the growth of pathogens.

Overall, our findings in Table (5) and Figure (5A) agreed with the findings of Aksoy *et al.* (2020) who stated that after treatment with (1:100) diluted QAC for all treatments, none of the studied bacteria (*S. enitiridis, S. infantis, and S. typhimorium*) displayed any visible growth. Tomi *et al.* (2024) demonstrated that QACs are a category of antibacterial that targets the surface of bacteria, disrupting and leaking cellular contents and exhibiting decreased growth strains of *Salmonella*.

Findings of the iodine disinfection table (4) agreed with the findings given by Aksoy *et al.* (2020) and Ramesh *et al.* (2002) but Aksoy *et al.* (2020) Examining the resistance of *Salmonella* serotypes against widely used disinfectants (iodine) revealed that the killing activity of the disinfectant was dependent on concentration and increasing this factor resulted in increasing the killing activity of the disinfectant. This showed that using iodine as a disinfection agent was completely preventive for the growth of the three investigated bacteria when using (1:100 and 1:200) dilutions.

Additionally, Virkon S was shown to be efficient against *Salmonella* Table (6) and Figure (5B), which is in agreement with earlier findings of Møretrø *et al.* (2008) who showed that Virkon S needs certain conditions and concentrations to be effective against Salmonella, and Dunowska *et al.* (2005) pointed out that routinely disinfecting surfaces that have been previously cleansed could benefit from using Virkon S.

In our research, *Salmonella* serotypes are inhibited by thymol, cinnamonaldehyde, and zingiberene, according to the results of the physicochemical analysis, as Figure (6) illustrates. Inhibiting the growth of *Salmonella* isolates, thymol and gingiberene are more effective than cinnamondehyde. These results were nearly in accordance with Wu *et al.* (2023) who reported that thymol (128 µg/mL) natural antibacterial agent, was found to have the best inhibitory impact on *Salmonella* isolates through the evaluation of MIC. Chi *et al.* (2023) showed that zingiberene, which may inhibit bacterial development by targeting the cell wall and membrane, acts as the primary antibacterial active component of ginger essential oil (GEO). Mesomo *et al.* (2013) determined that oxygenated monoterpenes, such as zingiberene, were the main component of GEO. Zingiberene and curcumin have been shown to possess potent antibacterial properties. Thongson *et al.* (2005) found that as compared to ginger extracts, GEO had greater antibacterial activity against isolates of *Salmonella typhimurium*.

It is critical to remember that essential oils with a high concentration of aldehydes and phenols, including thymol, zingiberene, and cinnamondehyde, have a stronger antibacterial effect than those with terpenoid alcohols (Dhifi *et al.,* 2016). It has been discovered that thymol and zingiberene cause the bacterial biofilm to break down, allowing internal contents to drain out and ultimately leading to cell death (Kachur and Suntres 2020).

The groups of aldehydes found in cinnamonaldehyde can quickly break down the structure of polysaccharides and enter cells. Moreover, the aldehyde groups can function as sterilizing agents by acting on protein transporters (Ding *et al.,* 2023; Zhang *et al.,* 2021).

CONCLUSIONS

Salmonellae have been isolated from dead embryos in chicken hatcheries, which supports the importance of protecting hatchery and farm biosecurity regulations. In the current study, analyzing the resistance of four *Salmonella* serotypes (*S. typhimurium, S. anatum, S. kentucky, and S. poona*) against three frequently used disinfectants (QAC, iodine and virkon S) showed that the killing activity of the disinfectant was dependent on concentration. Physicochemical investigation revealed that

zingiberene, cinnamonaldehyde, and thymol inhibited *Salmonella* serotypes. The presence of the *qacED1* and *qacA/B* genes in the bacterial genomes of *Salmonella* serotypes demonstrated a link between the genotype and the resistance of the bacteria against QAC.

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تقييم مقاومة السالمونيلا المعزولة من أجنة بيض كابس والمكونة للبيوفيلم لبعض المطهرات

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السالمونيلا هي بكتيريا خطيرة يمكن أن تؤدي بالإضافة إلى خسائر كارثية في إنتاج الدجاج إلى إصابات بشرية. كثيرا ما تستخدم المطهرات في مزارع الدجاج لمنع انتشار العدوى مثل سلالات السالمونيلا. وبعد تكوين سلالات بكتيرية مقاومة للمطهرات مشكلة خطيرة عند استخدام انواع مختلفة من المطهرات. ووجد أن بعض أنماط السالمونيلا مقاومة لمركبات الأمونيوم الرباعية وقد تحمل جينات *qacED1* و *qacA/B* المسؤولة عن هذه المقاومة. لذا كان الهدف من هذه الدراسة هو التعرف على سلالات السالمونيلا وتحديد أهم الجينات الضارة. وخلصت الدراسة إلي أن الزيوت النباتية الأساسية مثل الثيمول والسيناملدهيد والزنجبيرين كان لها تأثير مثبط علي الأنماط المختلفة من السالمونيلا المعزولة وتحديد الحد الأدنى للتركيز المثبط (MIC) وتقييم مقاومة السالمونيلا ضد المطهرات المختلفة (مركبات الأمونيوم الرباعية QACs، واليود، وvirkon S). فتم تجميع ١١٥ عينة من أجنة الدجاج الميت، بعد أن تم عزل سلالات السالمونيلا و كانت ١١٥/١٦ (١٣,٩٪)، وكانت السلالات الأكثر انتشارا هي السالمونيلا تيفيموريوم، وسالمونيلا كنتاكي، وسالمونيلا أناتوم، وسالمونيلا بونا. ومن خلال معالجة البكتيريا بتركيزات مختلفة تم تحديد فعالية المطهرات. فأظهرت الأبحاث أن نوع المطهر وتركيزه يؤثران على نشاطه كمبيد بيولوجي. كما تم استخدام تفاعل البوليميراز المتسلسل (PCR) للكشف عن وجود جينات qacED1 وqacA/B في سلالات السالمونيلا المعزولة حيث ثبت وجودها.