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Bacteriological studies on *Salmonella* isolated from chickens and eggs in Taif city with special reference to antibiotics resistance pattern

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Abstract

In this study 100 samples of chicken eggs and 100 samples of chicken liver were collected for an average study of *Salmonella*, which is one of the main causes of intestinal infection. Bacteria were isolated 30 isolates: 21 isolates from eggs and 9 from liver. After their defined, the most prevalent serogroupe was *Salmonella* Enteritidis 66.6% of the isolates and *Salmonella* Typhi was 33.3%. The sensitivity of the strains was tested against 16 antibiotics. Around 86.6 % of *Salmonella* isolates showed multiple antimicrobial resistance, about 6.6% and 3.3% were resistant to different antibiotics respectively. A large proportion of the *Salmonella* isolates were resistant of 93.3% nalidexic acid, 90% ampicillin, 86.6% trimethoprim + sulfamethoxazole and tetracycline. 12 out of 30 isolates were Extended spectrum β-Lactamases (ESβL) which detected by Cefinase phenotypically and the rate reached 43.3 %. PCR technique was conducting for detection of the invA gene in 30 isolates and all isolates were positive for this gene.

Keywords: *Salmonella*, Chicken, Antibiotic, Resistance, Serology.

INTRODUCTION

Salmonella species one of the pathogens which cause chronic and acute diseases in poultry. *Salmonella* Pullorum and *Salmonella* Gallinarum poultry-specific serovars which develop avian salmonellosis causing severe health problem to birds as well as serotypes as *Salmonella* Enteritidis and *Salmonella* Typhimurium and others causing paratyphoid infections [40], [41]. Bacterial resistance to different antimicrobials is a critical topic to humans and animals as it harms both mortality and morbidity caused by resistant bacteria with high economic losses due to the tendency of bacterial strains spreading from animals to another and for humans and vice versa [49]. This is the cause of the emergence and re-emergence of cross-resistance in different bacteria [31]. 1990, research revealed that many *Salmonella* strains acquired multidrug resistance (MDR) pattern due to the miss-use of antimicrobials, which have a significant impact on public health [51]. *Salmonella* categorized among Food-borne diseases which occur mainly in the developing countries and industrialized communities causing global public health problem [27], [50].

Molla demonstrated that the miss application of antibiotics leads to the selection environment due to the pressure on *Salmonella* to develop resistance strategy to evade from the antimicrobial and able to survive [33]. The resistance pattern of *Salmonella* to antimicrobial is increasing due to the miss-use of antibiotics globally. Gram-negative bacteria as *Salmonella* producing enzymes have the resistance pattern against beta-lactam antibiotics like Carbapenems, third and 4th generation of Cephalosporins, and Monobactams, which also named as EsβLs [14].

There is a comprehensive observation that the resistance to Cephalosporins in many serotypes of *Salmonella* enterica development rapidly and is due to plasmid-mediated production of β-lactamases [15]. The detection process for different

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resistance phenotypes is a critical challenge and complicated process in order to control the ESβL-producing *Salmonella* spreading, which causes the failure of treatment efforts [44].

The significant danger for humans and livestock is the presence of the ESβL-producing microorganisms in the poultry houses, which considered a potential source of spreading the antimicrobial-resistant bacteria causing severe infections among the living community [8], [52]. InvA gene has been detected in most of *Salmonella* strains samples and it was not possible to be detected the same gene in other pathogens. This confirms the potential diagnostic importance of the InvA gene as a polymerase chain reaction (PCR) specific target for *Salmonella* [38]. There is a considerable risk for veterinarians and workers in contact with colonized or infected animals and their products with antimicrobial-resistant bacteria [4].

This study aims to perform and accomplish the isolation, identification, serotyping, antibiotic sensitivity testing to detect the multidrug-resistant patterns, phenotypic detection of ESβLs, and genotypic detection of the invA gene for *Salmonella* microorganisms.

MATERIALS AND METHODS

200 specimens have been assembled from liver chickens and eggs. These were 100 liver and 100 egg yolk. The specimens were collected from private farms and markets in Taif city and examined for the presence of *Salmonella* microorganisms. All the specimens were labeled and transported to the laboratory in the icebox with minimum delay.

Isolation and identification (Microscopical and Biochemical) of *Salmonellae* were performed according to [37]. Diagnostic liquid stable poly and monovalent antisera for the determination of O, H and Vi antigens for the serological identification of *Salmonellae*. In Central Laboratory for Quality Control on Poultry Production (CLQP) in Egypt, the samples were serotyped according to [25].

Antibacterial sensitivity testing, the disk diffusion test was performed according to Clinical and Laboratory Standards Institute [12] and antibacterial agents [36] used were Ciprofloxacin: CIP (5µ), Aztreonam: ATM (30µ), Chloramphenicol: C (30 µ), Cefotaxime sodium: CTX (30µ), Ampicillin: AMP (10µ), Ceftazidime: CAZ (30µ), Amikacin: AK (30µ), Cephalothin: KF (30µ), Cefepime: FEP (30µ), Ceftriaxone: CRO (30µ), Gentamicin: CN (10µ), Sulphamethoxazole/Trimethoprim: SXT (25µ), Imipenem: IPM (10µ), Ampicillin/sulbactam: SAM (20µ), Nalidixic acid: NA (30µ), Tetracycline: TE (5µ).

Detection of ESβL was achieved through BD BBL Cefinase was achieved by BD BBL Cefinase -Lactamase Detection Discs (ESβL Screening Test). The BD BB™ Cefinase™ paper discs (BD Biosciences, Sparks, Md.) is impregnated with Chromogenic cephalosporin (nitrocefin) disc method is the recommended method by [12] for ESBL detection. Confirmatory testing uses both Combined Disc Diffusion Test (CDD): CAZ/CLA: Ceftazidime/Clavulanic acid (30/10) and CTX/CLA: Cefotaxime/Clavulanic acid (30/10) BD BBL Sensi-Disk ESBL Confirmatory Test disks (BD Biosciences, Sparks, Md.) according to [12].

For polymerase chain reaction (PCR) testing, DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Primers for invA gene have unique sequences and amplify a unique PCR product [35] were supplied from Metabion, Germany are listed in the Table (1). The reaction was conducted in Applied biosystem 2720 thermal cycler. Agarose gel electrophoreses [42] were conducted for the PCR products and the documentation system took the photo of the gel and the data was interpreted.

Table 1: InvA gene oligonucleotide primers sequences

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
invA	GTGAAATTATCGCCACGTTCGGGCAA	284	94˚C 5 min.	94˚C 30 sec.	55˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	[35]
	TCATCGCACCGTCAAAGGAACC							

RESULTS

Bacteriological isolation and identification

Bacteriological examination for *Salmonella* isolation from 200 samples of eggs and chicken liver revealed that a total of 30 *Salmonella* isolates 21 isolates from the egg and 9 isolates from the liver with an incidence of 15%. On XLD agar medium [36] *Salmonella* colonies showed a black center with a light transparent zone Photo. (1). *Salmonella* colonies on Hektoen enteric (HK) agar plate [36] appeared blue-green colonies with or without black centers Photo. (2). On MacConkey (MAC) agar medium [36], *Salmonella* colonies reveal white colonies Photo. (3). On Brilliant Green (BG) agar medium [36] *Salmonella* colonies were red to pink, white colonies surrounded by a red zone Photo. (4).

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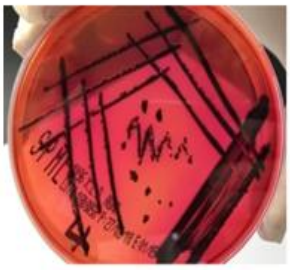

Photo. (1): *Salmonella* on XLD agar medium colonies appear pink with black center.

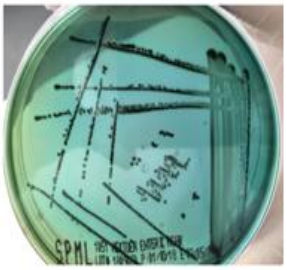

Photo. (2): *Salmonella* on HK agar medium colonies appear with black center.

Photo. (3): *Salmonella* on Mac agar medium colonies reveal white.

Photo. (4): *Salmonella* BG Agar medium appears as red to pink-white colonies surrounded by a red zone.

Biochemical identification

Lysine iron (LI) (+ve), methyl red (+ve), triple sugar iron agar test (TSI) Red slant and blackbutt, with gas production, urea test (–ve), indole (–ve), citrate utilization (+ve) and Voges Proskauer (–ve) tests were used for biochemical confirmation of *Salmonella* spp. Photo. (5).

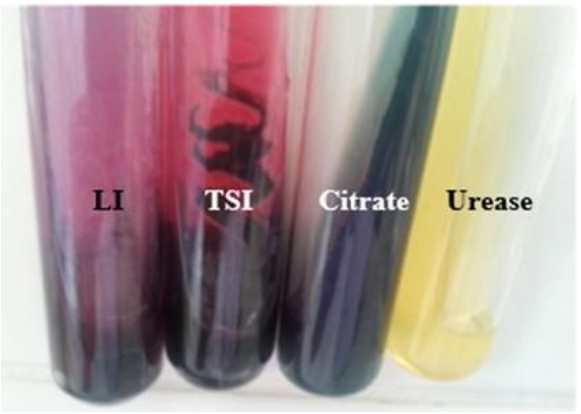

Photo. (5): LI, TSI, Citrate and urease tests for *Salmonella* spp.
LI: Purple in color. TSI: Red slant and blackbutt, with gas production. Citrate: blue in color. Urease: Yellow in color.

Serological results

The incidence of the 30 isolated *Salmonellae* from 200 samples according to their serotypes indicated that 20 *Salmonella* Enteritidis in an incidence of 66.6%, followed by 10 *Salmonella* Typhi, was 33.3% in Table (2).

Table 2: Incidence and percentage of different *Salmonella* serovars isolated from different samples

Samples	*Serotype*	No.	% to positive samples (30)	*%* to total samples (200)
Liver and eggs	*Salmonella* Enteritidis	20	66.6%	10%
Eggs	*Salmonella* Typhi	10	33.3%	5%

***Salmonella* serovars isolated from samples susceptibility to anti-microbials:**

Distribution of antimicrobial resistance

There was two *Salmonella* isolates pan-susceptible and one *Salmonella* isolates susceptible to one antimicrobial drug; three isolates were susceptible to two antibiotics. The rest isolates were resistant to Nalidixic acid (93.3%), Ampicillin (90%), Tetracyclines (86.6%), and Sulphamethoxasole + Trimethoprim (86.6%).

Multiple resistance patterns and distribution

Around 86% of the *Salmonella* isolates showed multiple resistance for three antimicrobials, 6.6% and 3.3% isolates showed resistance for four antimicrobial drugs, respectively (Table 3), Photo. (6). Resistance to 1/2 antimicrobial types was seen in 3.3% of the isolates. 4 *Salmonella* isolates showed resistance to ten antimicrobial drugs.

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Table 3: Collective resistance pattern of isolates to the antimicrobial used.

Antimicrobial agents		Isolates			% Resistant
		R	I	S	
Sulphamethaxole +Trimethoprim	SXT	26	-	4	86.6%
Amikacin 30 µg	AK-30	-	-	30	0%
Imepenem 10 µg	IPM-10	-	3	27	0%
Tetracyclines 30 µg	TE-30	26	-	4	86.6%
Ampicillin 10 µg	AMP-10	27	2	1	90%
Nalidixic acid 30 µg	NA-30	28	-	2	93.3%
Chloramphenicol 30 µg	C-30	12	-	18	40 %
Gentamicin 10 µg	CN-10	-	2	28	0%
Ciprofloxacin 5 µg	CIP-5	23	7	-	76.6%
Azetreonam 30 µg	ATM-30	7	2	21	23.3%
Ampicillin +Sulbactam	SAM-20	7	3	20	23.3%
Cefepem	FEP-30	8	2	20	26.6%
Ceftriaxone 30 µg	CRO-30	7	3	20	23.3%
Cephalothin 30 µg	KF-30	14	6	10	46.6%
Cefotaxime 30 µg	CTX-30	2	-	28	6.6%
Ceftazidem 30 µg	CAZ-30	2	-	28	6.6%

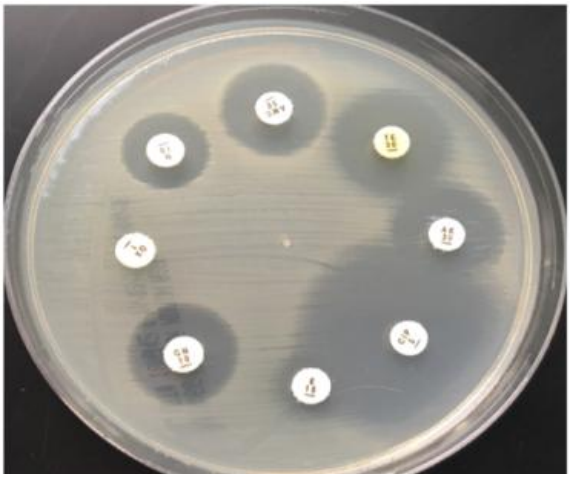

Photo 6: Effect of antimicrobial on *Salmonella.*

Phenotypic detection of ESβL-producing *Salmonellae*

BD BBL Cefinase -Lactamase Detection Discs (ESβL Screening Test)

12 isolates from total of 30 *Salmonella* isolates were ESβL phenotypically detected through using the Cefinase test (43.3 %).

BD BBL Sensi-Disk ESBL Confirmatory Test disks

Out of 30 *Salmonella* isolates 12 were ESβL when confirmed phenotypically by combined disc diffusion test with a rate of 43.3%.

PCR for amplification of invA gene from the DNA of *Salmonella*

The results observed in Photo (7) revealed that the invA gene was detected in 30 isolates with incidence rate 100%.

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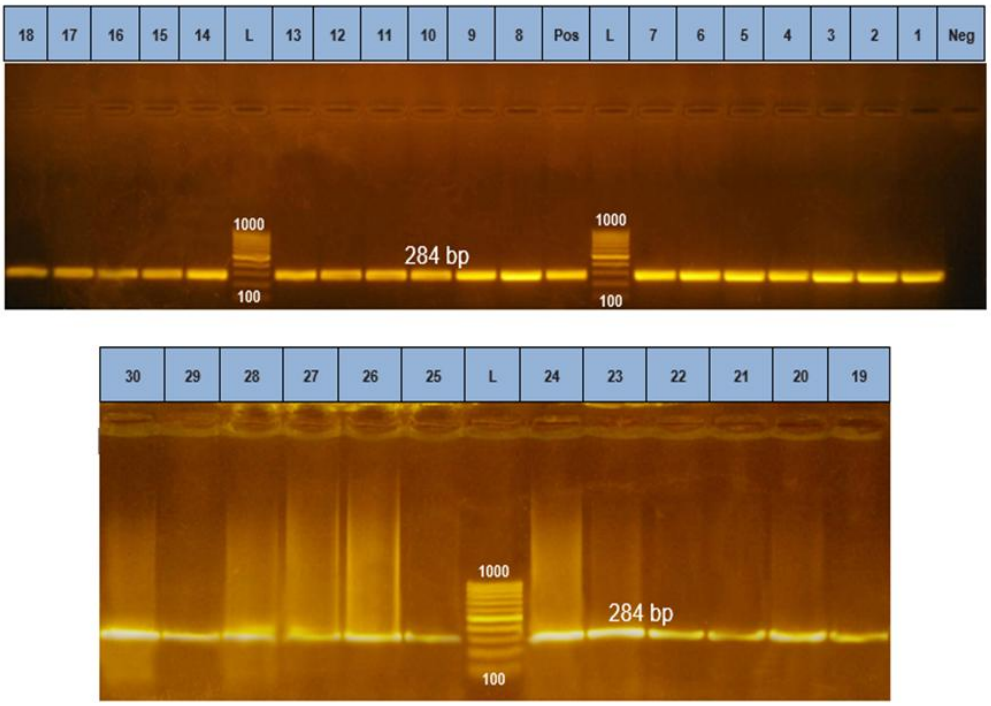

Photo 7: Agarose gel electrophoresis showing *Salmonella* specific PCR of *Salmonella* isolates using primer set for the *invA* (284 bp) gene.

DISCUSSION AND CONCLUSION

In our study, results showed that *Salmonella* spp. were isolated with an incidence of 15%, that results agreed with [1], [3], [5], [39], [46] who recovered *Salmonella* with incidence of 25%, 10%,12%, 9.17%, 9.8%, 18.8%, 12% and 18.8%.
Lower results were obtained by [24], [29], [32], [43], [48] and who recovered *Salmonella* with incidence of 3.6 %, 4.4%, 6.3%, 5% and 4%.and [7] and [26] who recovered *Salmonella* with incidence of 1.6%, 0.8%, 0.74% and 1.6% respectively.
Salmonella isolates were serotyped using O and H polyvalent and monovalent antisera. The results of [30], using the same procedure we adopted in the present investigation the collected samples were analyzed according to ISO 6579:2002 and positive isolates serotyped according to Kauffman White Le Minor technique showed that all collected samples were *Salmonella* contaminated ranged among (1.5%) and (38.6%).

The most prevalent in this study were in decreasing order, *Salmonella* Enteritidis (66.6%) and *Salmonella* Typhi (33.3%). These results were agreed with [2] who isolated *S.* Typhi with a percentage of 20% from chickens and also agreed with [47] who said that some serovars of *Salmonella* such as *S.* Typhi its host adaptation seem to have developed with loss of the intestinal lifestyle and the accretion of the ability to cause systemic infection.

To control *Salmonella*, antibiotic therapy may aid in overcoming an outbreak [45]. And antibiotic therapy should be based on the results of susceptibility testing [37]. The emergence of antimicrobial drug resistance is a matter of concern. Persons with infections caused by antimicrobial drug-resistant *Salmonella* spp., particularly Nalidixic acid-resistant *Salmonella* spp., are more likely to die, are more likely to be hospitalized, and are hospitalized for longer periods than patients with infections caused by susceptible strains [20], [21], [28].

The report in these study of the antibiotics resistant pattern against 16 types of antibacterial discs of the (30) *Salmonella* isolates from as analyzed in this study were as follows; Ampicillin, (90%), Nalidixic acid (93.3%), Tetracycline and Sulphamethoxazole/Trimethoprim (86.6%), Ciprofloxacin (76.6%), Chloramphenicol (40%), Cephalothin(46.6%) Aztreonam, Ampicillin/Sulbactam and Ceftriaxone (23.3%), Cefepime (26.6%), Cefotaxime and Ceftazidime (6.6%) Amikacin Imepenem and Gentamicin (0%).

In these study, results were correlated to that reported by [16] who found a high percentage of resistant *Salmonella* strains to Amoxicillin/Clavulanic acid (16.1%), Kanamycin (17.7%), Spectinomycin (32.3%), Trimethoprim-Sulfamethoxazole (24.2%), Trimethoprim (30.6%), Ampicillin (33.8%), Gentamicin (21%), Tetracycline (79%), Ciprofloxacin (33.9%), Nalidixic acid (37.1%) and Streptomycin (72.5%).
ESβLs carrying *Salmonella* isolates have emerged globally in the last decade. There is a significant concern since the drug of choice is Cephalosporins for children's salmonellosis treatment. Many blaCMY, blaCTX, blaTEM, and blaSHV, genes were detected to encode ESβLs resistance in *Salmonella* [34], [52].

The phenotypic expression analysis revealed that the subsequently β-lactamase were 43.3% from which 43.3% were positive by screening test and a confirmatory test. Goyal described the ESβL detected by the disk potentiation technique was 64.5% [17].

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However, the Phenotypic confirmatory test couldn't reveal the ESβL in the bacterial isolates, which also produce other classes of AmpCs, TEMs and beta-lactamases. Many causes for false-negative reactions, among them TEM type ESβLs with single amino acid replacements, consist only low-level oxyimino beta-lactam activity [23]. Many additional ESβL enzymes have superior oxyimino beta-lactam activity but deficient in intrinsic extended-spectrum enzyme efficacy [9].

All *Salmonella* isolates were positive for invA, as reported by different studies globally [6], [13], [10]. It was anticipated that these genes would be distinguished in all of the isolates as their impact on cell invasion. PCR test is a rapid tool for the detection of invA gene, the target gene for the detection of *Salmonella* spp. And the sopB gene, which relates to enteritis in birds [22], [38], [53].

Most of the pathogenicity elements and acquired antibiotic resistance genes by *Salmonella* are located on virulence-associated plasmids (extrachromosomal genetic elements) or intrachromosomal segments created from different genomes [11], [18], [19]. Some studies reported the simultaneous presence of virulence-associated plasmids and antimicrobial resistance genes in *Salmonella* [11].

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AUTHOR CONTRIBUTIONS

Abeer, Nashwa and Ahmed designed the project, performed experiments, analysed data, and prepared the manuscript; Abeer, Mai, Nashwa and Ahmed aided in the analysis of data, provided valuable comments and ideas, and technical assistance during the development of the project; Abeer, Nashwa and Ahmed designed and supervised the work. Abeer. And Ahmed wrote the manuscript.

CONFLICT OF INTEREST

All authors have not any conflicts designed the project, performed experiments, analyzed data and prepared the manuscript.

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