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Molecular characterization of Salmonella serovars isolated from Chicken

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ABSTRACT

The aims of the current study were bacteriological and molecular studies on *Salmonella* species isolated from poultry farms A total of one hundred diseased chicken (100 samples of each of liver, gall bladder and Intestine content) suffering from diarrhea were collected from different broiler private farms in Giza governorate, Egypt. Samples were collected in a sterile container in an ice bag and then transferred aseptically to the laboratory for conventional bacteriological analysis. Bacteriological examination revealed that *Salmonella* from liver were (2.66%), gall bladder (3%) and intestine (1.66%). The prevalence rate to *Salmonella* was (7.66%). Antibiogram pattern was applied, and isolates exhibit resistance against rifampicin, amoxycillin and doxycycline and highly sensitivity to streptomycin with (100%) variable results were recorded with the remaining test antibiotic molecular characterizations of β-lactamase resistance genes in salmonella serovars gave variable results. A PCR based assay was developed to detect the prevalence of *Salmonella* in samples and to and chromosome-borne virulence genes (bla*TEM*, blaSHV and bla*CTX*).

1. INTRODUCTION

Salmonellae are gram-negative facultative anaerobes. Due to their position as zoonotic and food borne pathogens, they have significant losses in animal production concerns. (Moussa et al., 2012). Salmonella serotypes determined by surface antigens, are divided into (enteritidis and bongori). The Infection of warm-blooded animals is caused by several serotypes of Salmonella enterica (Brenner et al, 2000). Certain serotypes of salmonella as Enteritidis Can penetrate (invade) poultry reproductive system causing contamination of egg contents which has been a major cause of human illness for many years (decades) (Gantios et al, 2009)

Farm of poultry play a significant role in the transmission of Salmonella to the commercial life. When chicken comes in contact with infected birds through their home environment, food, litter, rodents and insects, it is infected with salmonella (LiljebJelke.et al., 2005). Many serotypes of Salmonella are humanly pathogenic. Salmonellosis, which has widespread symptoms such as stomach pain, diarrhea, muscle pain, fever, sleepiness, nausea, and vomiting, is a human infection of Salmonella. (Andino and Hanning, 2015)

Eggs and poultry products are the main reservoirs of salmonellae. Salmonella can pass to food chain and transmitted to the human (Howard et al, 2012). The aims of the current study were bacteriological and molecular studies on Salmonella species isolated from poultry farms

2. MATERIAL AND METHODS

2.1. Samples collection

A total of one hundred diseased chickens (100 samples of each of Liver, Gall bladder, and Intestine content) in Giza governorate. Samples were collected in a sterile container in an ice bag and then transferred aseptically to the laboratory for conventional bacteriological analysis

2.2. Bacteriological examination

One gram of each sample was inoculated in 10 ml buffered peptone water then incubated at 37℃ for 24 h. One ml of preincubated buffered peptone were inoculated in (MKTTn broth) and incubated at 37℃ for 24 hrs. All samples were streaked onto XLD and SS agar and incubated overnight at 37℃. Suspected colonies were picked up on nutrient agar and incubated at 37℃ for further morphological, biochemical identification according to Quinne et al (2002) Iso 6579-2017

2.3. Serological identification of salmonella

Isolates which biochemically proved to be salmonella were subjected to serological identification according to white Kauffman scheme 2007 by slide agglutination technique using omnivalent and polyvalent antisera for O and H antigen antisera obtained from DENKA. SEIKEN CO. LTD. Japan

2.3. Antibiogram pattern

Discs used for Antibiogram (oxidl). Salmonella serovars were tested for their susceptibility to 12 antimicrobial discs (Table 1) using disk diffusion assay according to instruction described by CLSI (2011

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Table 1: Discs used for Antibiogram (oxidl)

Antibiotics (Abbreviation), Concentration	
Cefotaxime (CTX), 30 µg	Doxycycline (DO), 20 µg
Cephradine (CE), 20 µg	Erythromycin (E), 20 µg
Amikacin (AK), 30 µg	Flumequine (FL), 30 µg
Ceftazidime (CAZ), 30 µg	Lincomycin (L-MY), 25µg
Clindamycin (DA), 25µg	Spectinomycin (SPT-SH), 20 µg
Ciprofloxacin (CIP), 5 µg	Neomycin (N), 30 µg

Table 2: Primers sequences and amplification cycles

Target Gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
blaTEM	ATCAGCAATAAAC-CAGC CCCCGAAGAAC-GTTTTC	516 bp	94°C 5 min	94 °C 30 min	54 °C 40 sec	72 °C 45 sec	72 °C 10 min	colom et al., (2003)
blaSHV	AG-GATTGACTGCCTTTT TG ATTTGCTGAT-TTCGCTCG	392 bp	94°C 5 min	94 °C 30 min	54 °C 40 sec	72 °C 45 sec	72 °C 10 min	
blaCTX	ATG TGC AGY ACC AGT AAR GTK ATG GC TGG GTR AAR TAR GTS ACC AGA AYC AGC GG	593 bp	94°C 5 min	94 °C 30 min	54 °C 40 sec	72 °C 45 sec	72 °C 10min	Areham-bault et al., (2006)

2.4. PCR Technique

DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modification from the manufacturer's recommendations. Briefly, 200 µl of the Sample suspension was incubated with 10 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.

2.4.1. Oligonucleotide primer

Primers used are listed in Table (2), supplied from Metabion (Germany)

2.4.2. PCR amplification

PCR mixture was conducted in a 25µl reaction containing 12.5µl of Emerald Amp Max PCR Master mix (Takara, Japan) 1 µl of each primer of 20 pM of concentration, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an applied bios system 2720 thermal cycler.

2.4.3. Analysis of PCR Products

The products of PCR were separated by electrophoresis on 1.5% agars gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5 V/cm. For gel analysis, 20 µl of the PCR products were loaded in each gel slot. Gene ruler 100 bp ladder (Fermentas, Thermo Scientific, Germany) was 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 (Monstein et al.2007). Primers have specific sequence and amplify a specific product as shown in Table 2

3. RESULTS

3.1. Incidence of Salmonella

The incidence of Salmonella SPP in different chicken Liver, Gall bladder and Intestine was shown in Table 3

3.2. Serotyping of Isolated Salmonella

The results of serotyping of isolated *salmonella* were illustrated in Table 4.

Table 3: Prevalence of *Salmonella* in collected samples from diseased chicken

Type of samples	Number of Samples	Results			
		Positive samples number	%*	Negative samples number	%*
Liver	100	8	2.66%	92	97.34%
Gall bladder	100	9	3%	91	97%
Intestine	100	6	20%	94	98%

N.B The percentage was calculated according to total number of examined samples (300)

N.B The percentage was calculated according to total number of examined samples (300)

Table 4: The Incidence rates if salmonella serovars from diseased chicken.

Type of the isolated strains	Number and percentage	Antigenic Structure
S. Typhimurium	5 (68.2%)	1,4,[5],12:i:1,2
S. Kentucky	4 (54.5%)	8,20:i:z 6
S. Lumberherst	1 (13.64%)	3,10:e,h:e,n,z 15
S.Enteritidis	3 (40.92%)	1,9,12:g,m:-
S. Ferruch	2 (27.28%)	8:e,h:1,5
S. kedougou	1 (13.64%)	1,13,23:i:l,w
S. Virchow	3 (40.92%)	6,7,14:r:1,2
S. Paratyphi A	1 (13.64%)	1_,1,12:a:[1,5]
S. Gallinarum	1 (13.64%)	1,9,12:-:-
S. Nitra	1 (13.64%)	2,12:g,m:-
S. Euston	1 (13.64%)	11:r,i:e,n,x,z15

3.3. Serotyping of Isolated Salmonella

The isolated Salmonella serovars were tested for antibiotic sensitivity for (sulphamethoxane methoprime, amikacin, gentamicin, cefoperazone, norfloxacin, rifampicin, cefotaxime, erythromycin, nalidixic acid, levofloxacin, streptomycin, amoxycillin and doxycycline). All salmonellae were sensitive to sulphamethoxane metheprim, nalidixic acid, and streptomycin while with Norfloxacin all tested salmonella were sensitive except S. Lumerhust were resistant. All salmonellae were resistant to erythromycin, Amikacin, Rifampicin, amoxycillin and doxycycline. All examined salmonellae were resistant to Levofloxacin except S. Typhimurium 2, S. Ferruch and S. Euston. All salmonellae were resistant to cefoperazone except S. Gallinarum and S. Nitra, S. Typhimurium 1, S. Ferruch, S. Virchow and S. Euston were intermediate to cefatoxim and other salmonellae were resistant to enrofloxacin.

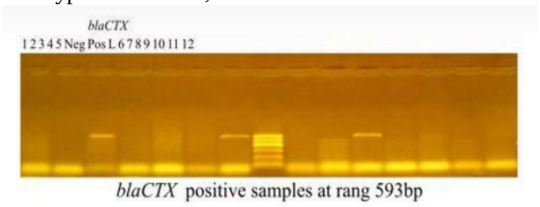

Figure (1) Agarose gel electrophoresis showing specific PCR of *Salmonella* serotypes using primer set for bla *CTX* gene (593 pb)- L= Ladder and Lane (P)= positive control and Lane(N)= negative control and Lanes (3- 8) were positive for this gene. The Agarose gel electrophoresis showing specific PCR of *Salmonella* serotypes using primer set for bla *TEM* gene (516 bp)

3.4. Detection of Salmonella resistance genes and virulence genes.

The agarose gel electrophoresis showing specific PCR product of Salmonella serotypes using primer set for bla CTX gene (593 pb) was illustrated in figure 1, for bla TEM gene (516 pb) was illustrated in figure 2, and for bla SHV gene (392 pb) was illustrated in figure 3.

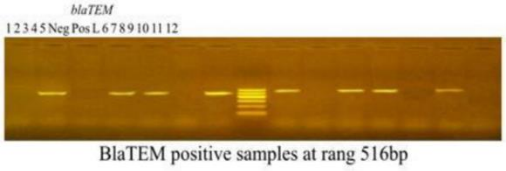

Figure (2) Agarose gel electrophoresis showing specific PCR of *Salmonella* serotypes using primer set for bla *TEM* gene (516 bp)- L= Ladder and Lane(P)= Positive control and Lane (n)= negative control and lanes (2-4-5-6-8-9-11) were positive for this gene. The agarose gel electrophoresis showing specific PCR of Salmonella serotypes using primer set for bla *SHV* gene (392 bp).

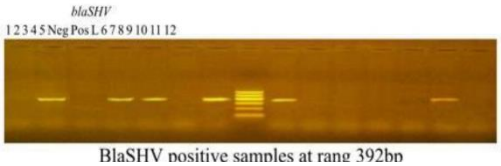

Figure (3) Agarose gel electrophoresis showing specific PCR of *Salmonella* serotypes using primer set for bla *SHV* gene (392 bp)- L= Ladder and Lane(P)= Positive control and Lane (n)= negative control and lanes (2-4-5-6-11) were positive for this gene

4. DISCUSSION

Salmonella in poultry has become a major problem of economic importance Extensive use of antibiotics in humans and veterinary medicine raises the possibility of multidrug-resistant Salmonella (Cruchaga et al, 2001) In the present study, 100 samples from the liver, gall bladder and intestine were obtained from diseased chickens. These samples were tested for the presence of Salmonella and revealed that (7.66%) of the samples were positive. This result nearly agreed with the results of Murugkar, (2005) who examined (231) cloacal swabs from diarrheic poultry and found (34) isolates of Salmonella with (14.7%) prevalence rate. and at final higher than results of Balala et al, (2006) when recorded the rate of Salmonella (4.9%) in 325 samples Selvaraj et al, (2010) who found the prevalence rate of Salmonella was (4.9%) and Abd El – Ghany et al, (2012) when recorded a prevalence rate range between (3.84%) and (5.18%) after examining 4 flocks of broiler chicken in Kaliobeya, Egypt. Rabie et al,.(2012) who recorded (2.4%) prevalence rate of Salmonella in raw meat of chicken, when found (6) Salmonella isolates in Processing Stages with (3.75%) prevalence rate, Agada et al, (2013) reported that the prevalence rate was (10.9%) as, the highest rate in litter at (31.1%), feed (1.1%) and (0%) in hand swabs, also Dahshan et al, (2015) who recorded a prevalence rate of (3.1%) when examined (225) samples from litter, water and bird droppings, collected from broiler poultry farms in Sharkia, Egypt, Abd El–Tawab (2013) with (10.9%) rate of prevalence after examining of (579) poultry samples was collected from different Governorates in Egypt Also this study results are higher than results of Brizio and prentice (2015) when found (2.2%) prevalence rate of Salmonella in examined (452) samples. Nossair (2016) who recorded (2.13%), Ameh et al, (2016) mentioned that from chickens in slaughterhouses and farms He found that the prevalence rate of Salmonella was (8%). El_Sharkawy et al, (2017) isolated salmonella from broiler chicken in Kafr-Elshiekh at percentage of (10.89%) Hassan et al (2016) and waghamore et al (2017) isolated salmonella from poultry in percentage of 73.3% ,66.66% 88.46% and 78.3% respectively. Five S.Typhimurium, four S. Kentucky,one S. Lumberhurst , threeS.Enteritidis, two S. Ferruck, one S. Kedougou, three S.Virchowand one S.Paratyphi A , one S. Gallinarum , oneS. Nitra and one S. Euston were isolated from internal organs of chickens with a percentage of (68.2%), (54.5%), (13.6%), (40.9%), (27.28%), (13.6%), (40.9%), (13.6%), (13.6%), (13.64%) and (13.6%) as showed in table (4) respectively.

These results coincided with results obtained Dutil (2010) when found S. Kentucky (43%), S. Enteritidis (27%), S. Typhimurium (10%) and S. Hiedeberg (4.4%) as most common (4) serotypes of Salmonella isolated from chickens and results of Abd El-Tawab (2013) when found S. Kentucky in isolations from samples of chicken. On country, El_ Sharkawy (2017) who found Salmonella Typhimurium in (86.6%) of the isolates, also reported (9%) S. Enteritidis and non- typable in (4.5%), The Bacterial resistance occurs due to unnecessary usage of antibiotic as feed additives in poultry farms (Hur et al., 2011). In our study, Amoxycillin and Doxycycline and highly sensitivity to Streptomycin with (100 %) variable results were recorded is match with Hassan et al., (2016) who found S. Kentucky isolates resistance against antibiotics majority where 100% resistant to ciprofloxacin, 85% showed resistance against both of cefotaxime and ceftazidime, also agreed with Marwa., (2016) who detected 100% resistance to cefotaxime. Also Soomro et al.,(2010) who observed resistance to ampicillin and sensitivity to Streptomycin, cefotaxime, gentamicin and Ciprofloxacin, Donado Godoy et al., (2012) reported resistance to the isolated Salmonella from chicken (15%) to ciprofloxacin, tetracycline, trimethoprim, streptomycin and nalidixic acid, Fallah et al., (2013) who observed the sensitivity to cefotaxime and 100% were resistant to nalidixic acid, tetracycline and streptomycin, Lin et al., (2015) also This result disagreed with Ibrahim (2016) who found highest numbers of S. Kentucky isolates showed (83.3%) resistance against chloramphenicol and 66.7% ciprofloxacin, 50% resistance against gentamicin and streptomycin, ampicillin, cephalothin (16.7%), S. Kentucky isolates from the humans were 100% sensitivity to ampicillin and streptomycin and 50% resistance against ciprofloxacin, gentamycin and chloramphenicol, Also were different with Dalia et al., 2018 who record 75% sensitive to ciprofloxacin and norfloxacin. In our results, 89% of Salmonella isolates were multidrug resistant to at least 2 classes of the antibiotics. This highly rate of multi drug resistance Salmonella in chicken were also recorded in other study in Egypt 92.8% by Abd-Elghany et al., (2014). Also, our results are moderately like the previously recorded in many studies from different countries all over the world as in Spain (100%) (Carra minana et al., 2004), Brazil (90.5%) (De Oliveira etal., 2005) Turkey (100%) (Yildirim et al., 2011), Korea (87.2%) (Kim et al.,2012), Romania (83.2%) (Mihaiu et al., 2014) and (87%) (Marwa., 2016). The Derivatives (B. lactams) are the broad-spectrum antibacterial agents widely used in human and veterinary medicine. Resistance to cefotaxime in the Gram-negative bacteria is primarily mediated by B-. Lactamases. many different B-. Lactamases have been described but TEM type B-. Lactamases are most predominant in the bacteria which is Gram negative (Brad ford, 2001) In the current study, the blaTEM gene was detected 100% of Salmonella isolates that discovered resistant to cefotaxime which was (B – lactams) are broad spectrum antibiotic agents widely used. This result agreed with Ahmad and Shimamoto (2012) analyzed the mechanisms of multidrug-resistance in 21 isolates of S. Enterica serovar Enteritidis and four isolates of S. Enterica serovar Typhimurium also, they identified blacmy-2 in isolates of S. Enterica serovar Enteritidis. Our result was lower than result of Amira et al, (2014) who identified B – lactamase encoding genes, blaTEM (40%)- also, Ahmed (2009) who Identified (50%) of bla TEM in 47 S. Typhimurium out of ninety-four S. Enterica isolates from animal in Japan. Also, Ahmed and Shimamoto, (2012) could identify the B – Lactamase encoding gene bla TEM-1 from twenty-one S. Enterica isolates from diseased broiler in Egypt Hamidian et al, (2009) found that 2 of 129 S. Enterica isolates from patients with diarrhea in Tehran, carried blaTEM gene.

5. CONCLUSION

Salmonellae were isolated with incidence rate (7.66%). Antibiogram pattern was applied, and isolates exhibit resistance against Rifampicin, Amoxycillin and Doxycycline and highly sensitivity to Streptomycin with (100%). Molecular characterization of Beta lactamase resistance genes in Salmonella serovars showed presence of (blaTEM, blaSHV and blaCTX

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