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Bacteriological and molecular studies on *Salmonella* species isolated from poultry farms

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A B S T R A C T

This study comprise the examination of 100 samples of feed(40), water (36) and litter (24) collected randomly from different chicken shops, houses and farms in Kafr El-Sheikh Governorate. Results of this survey revealed isolation of 14/100(14%) *S*almonella species (3)(12.5%) isolates from litter, (5)(13.8%) isolates from water and (6)(25%) isolates from farms . The prevalence rate to Salmonella was (14%) Sensitivity test was made and isolates exhibit resistance against ciprofloxacin and cefotaxime with (89%) and highly sensitivity to amikacin with (100%) so amikacin is the first drug choice for treatment of Salmonella infection. A PCR based assay was developed to developed to detect the prevalence of Salmonella in samples and to evaluate plasmid and chromosome-borne virulence genes (*stn*-*inv*A) which considered as a target genes for the detection of Salmonella The results of detection of resistance genes (*qnr* S-*blaTEM*) as a resistance genes in all isolates of Salmonella.

Key words: *Antibiogram, Feed, Resistance and virulence genes, Salmonella*

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1. INTRODUCTION

Salmonella is a Gram-negative, non-spore forming rod which can ferment glucose belonging to the family Enterobacteriaceae and can reduce nitrate to nitrite. (Grimont et al., 2000)

Members of the genus Salmonella are facultative anaerobes; motile or non-motile by flagellae, and most heterogenous bacteria. Eller Mier and Slauch, (2006)

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 (Gantios et al, 2009).

*S*almonella species spread from poultry to human, and also from human to human, often through foods as eggs and meat. *S*almonella can cause an intestinal infection of human knows as salmonellosis or enteric fever (Chowdhuri et al., 2011).

*S*almonella Enterica is one of the major borne diseases due to its endemic nature, high morbidity and characterized by its zoonotic

importance on high public concern (Moussa et al., 2012).

Most of *S*almonella serotypes are pathogenic to the human. The infection with *S*almonella in the human called *S*almonellosis which has common symptoms as abdominal pain, diarrhea, muscle pain, fever, Drowsiness, nausea and vomiting (Andino and Hanning, 2015).

Eggs and poultry products are the main reservoirs of salmonellae *S*almonella can pass from them through the food chain and transmitted to the human (Howard et al., 2012).

Pathogens in poultry products are vary depending on the country, the system of poultry production and the control measures in the place of the production poultry may infect with and carry the *S*almonella serovars with no specific clinical picture of the disease (Herikstad et al., 2002).

The widely used antimicrobial agents for poultry growth promotion or increasing production and also in the treatment aims can raise the concern with regard to the antimicrobial resistance, which are observed widely in many Salmonella serovars (Duong et al., 2006).

The majority of antimicrobial resistant phenotypes of Salmonellae and other pathogens are obtained from the plasmids or extra chromosomal genes. Plasmids carry DNA mobile elements, as transposons or integrons which are very important in the occurrence of multi-drug resistance among the gram-negative bacteria or Salmonellae (Harbottle et al., 2006).

The objective of this study is the bacteriological and molecular studies of Salmonella species isolated from poultry feed. Salmonella is considered as one of the major economic problems facing poultry industry all over the world because of its significant losses which are mainly due to poor feed conversion and carcass condemnation at processing.

2. MATERIALS AND METHODS

2.1. Sampling:

A total of 100 chicken samples were taken from poultry houses ,chicken shops and broiler farms (24 litter, 40 feed and 36 water). at different intervals throughout February 2017 and February 2018 located in Kafr-Elsheikh. Samples were collected as aseptically as possible to prevent cross contamination.

2.2. Isolation and Identification of Salmonella (ISO 6579, 2002)

All samples cultured in peptone water (Oxoid) were incubated at 37°C for 24h after which they were inoculated in Rappaport-Vassiliadis broth (Oxoid) at the ratio of 1:10 and incubated at (42±1°C) °C For 24 ±2h Per iso 6579:2002 and then streaked on XLD agar (Oxoid) which was further incubated for 24h at 37°C.The suspected colonies (pink with black center) and typical colony on SS agar (Oxoid) (colorless with or without black center) were picked. then stored on semi-solid agar for further investigation. All the suspected pure colonies of suspected *S*almonellae were furtherly subjected to biochemical reactions (Methyl red , Voges – Proskauer , Indole , Oxidase testes according to Cruickshank et al., (1975) and Urease test (Edwards and Ewing 1972).

2.3. Serological typing of Salmonella organisms

Salmonella isolates that were preliminary identified biochemically, were subjected to serological identification according to Kauffmann-White scheme (Kauffmann, 1974).

2.4 Antimicrobial susceptibility tests:

Media used antibiogram assay according to (ISO 6579 2002) .The Mueller- Hinton agar medium (Nccls) (Oxoid) National Committee for Clinical Laboratory Standards

"NCCLS" (2001) *S*almonella isolates were examined in vitro for their susceptibility to the following antimicrobial disc as cefotaxime (CTX), 30 µg, doxycycline (DO), 20 µg, cephradine (CE), 20 U, Erythromycin (E) 20 µg, amikacin (AK). 30 µg, flumequine (FL), 30 µg, ceftazidime (CAZ), 30 µg lincomycin (L-MY), 25 µg, Clindamycin (DA), 25 µg spectinomycin (SPT-SH), 20µg Ciprofloxacin (CIP), 5 µg Neomycin (N), 30 µg

2.5. Identification of isolates of Salmonella by PCR

According to The QIAamp DNA mini kit instructions (Sambrook et al., 1989) The QIAamp mini spin column was carefully opened and 500 ml buffer AW2 were added without wetting the rim. The cap was closed, and centrifugated at full speed for 3 min. The QIAamp mini spin column was placed in a new 2 ml collection tube and the old collection tube was discarded with the filtrate. Centrifugation at full speed for 1 min was done. The QIAamp mini spin column was placed in a clean 1.5 ml microcentrifuge tube, and the collection tube containing the filtrate was discarded. The QIAamp mini spin column was carefully opened and 100 µl buffer AE were added. The QIAamp mini spin column was incubated at room temperature (15-25ºC) for 1 min, and then centrifugated at 8000 rpm for 1 min. Oligonucleotide primers used in PCR(Table 1, 2, 3) for detection of resistance genes (*bla*TEM, *qnr*S) and virulence genes (*Stn*, invA) of salmonella:

2.6 Detection of Salmonella resistance gene: according to (Colom k.,2003)

In this study, a total of four Salmonella serotypes were S. Kentucky, S. Blegdam, S. Ferruch and S. Yovokome were screened for presence of two resistance genes including *qnr*S and *bla*TEM

2.7 Detection of Salmonella Virulence associated genes: according to Murugkar, H.V (2003)

A total of 9 Salmonella isolates were three S. Kentucky, three S. Blegdam, two S. Ferruch and one S. Yovokome. Four different types of Salmonella and were screened by multiplex PCR to detect two virulence genes *inv* A, *stn*. Cycling conditions of the primers during cPCR Temperature and time conditions of the two primers during PCR are shown in (Table 4)

Table (1) Primers for detection of resistance gene of Salmonella

Primer	Sequence 5- 3-	Amplified product	Reference
*bla*TEM	F : ATCAGCAATAAACCAGC R : CCCCGAAGAACGTTTTC	516 bp	Colom et al. (2003)
*qnr*S	F : ACGACATTCGTTCAACTGCAA R :TAAATTGGCACCCTGTAGGC	417 bp	Robicsek et a. (2006)

Table (2) primers used in PCR. For detection of virulence genes of *S*almonella:

Primer	Sequence 5- 3-	Amplified product	Reference
stn	F : TIG TIG CGC TAT CAC TGG CAA CC R : ATT CGT AAC CCG CTC TCG TCC	617 bp	Murugkar et al. (2003)
*inv*A	F:GTGAAATTATCGCCACGTTCGGGCAA R : TCATCGCACCGTCAAAGGAACC	284bp	Oliveira et al. (2003)

Table (3) Preparation of PCR Master Mix according to Emerald Amp GT PCR master mix (Takara) code No. RR310A kit as shown in

Component	Volume/reaction
Emerald Amp GT PCR mastermix (2x premix)	12.5 µl
PCR grade water	4.5 µl
Forward primer (20 pmol)	1 µl
Reverse primer (20 pmol)	1 µl
Template DNA	6 µl
Total	25 µl

Table (4) Cycling conditions of the different of primers during PCR:

*Gen*e	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
stn	94°C 5 min	94°C 30 sec	59°C 40 sec	72°C 45 sec	35	72°C 10 min
*inv*A	94°C 5 min	94°C 30 sec	55°C 40 sec	72°C 30 sec	35	72°C 7 min
*bla*TEM	94°C 5 min	94°C 30 sec	55°C 40 sec	72°C 45 sec	35	72°C 10 min
qnrS	94°C 5 min	94°C 30 sec	55°C 40 sec	72°C 45 sec	35	72°C 10 min

3. RESULTS

3.1. prevalence of Salmonella:

The Prevalence rate of Salmonella was 14%. Which revealed 14 Salmonella isolates from poultry samples (6 isolates from feed , 5 isolates from water and 3 isolates from litter).

3.2. Serotyping of Isolated Salmonella:

The isolates were three S. Kentucky,(3/9) three S. Blegdam (3/9), one S. Yovokome (1/9) and two S. Ferruch (2/9) in table (5).

Table (5) Serotyping of isolated Salmonella from chicken in feed, litter and water samples

Salmonella isolates	No. of isolates	serotyping
S. Kentucky	3	(8, 20 :I : Z_6)
S. Blegdam	3	(9, 12: g,m,q.)
S. Yovokome	1	(8, 20: d: 1.5)
S. Ferruch	2	(8, e, h:1.5)
Total isolates	9	

3.3. Sensitivity of Salmonella strains in different antibiotics

The nine isolated *S*almonella serovars were tested for antibiotic sensitivity for (cefotaxime, ciprofloxacin, cephradine, clindamycin, erythromycin, flumequine, neomycin, lincomycin, ceftazidine, doxycycline, Spectinomycin and amikacin).We were found to be (89%) resistant to cefotaxime and ciprofloxacin, while all of isolates *S*almonella serovars were found to be (100%) sensitive to amikacin.

Table (6) Numbers and Percentages of Salmonella Serotypes exhibiting resistance.

Antimicrobial agent	Resistant isolates		Sensitive isolates		Inter mediate isolates	
	No	%	No	%	No	%
Cefotaxime	8	89	1	11	0	0
Ciprofloxacin	8	89	1	11	0	0
Cephradine	5	55.5	4	44.5	0	0
Clindamycin	5	55.5	4	44.5	0	0
Erythromycin	6	66.6	4	33.4	0	0
Flumequine	4	44.5	5	55.5	0	0
Neomycin	4	44.5	5	55.5	0	0
Lincomycin	4	44.5	5	55.5	0	0
Ceftazidine	2	22.2	7	77.8	0	0
Doxycycline	3	33.3	5	55.5	1	11
Spectinomycin	3	33.3	5	55.5	1	11
Amikacin	0	0	9	100	0	0

3.4. Detection of Salmonella resistance gene:

It was found that all four *S*almonella isolates. The *blaTEM* amplicon (516 bp) was obtained in all *S*almonella isolates and *qnr*S amplicon (417 bp) was obtained in all nine Salmonella isolates (Fig. 1). A total of 9 *S*almonella isolates were three S. Kentucky, three S. Blegdam, two S. Ferruch and one S. Yovokome. Four different types of *S*almonella and were screened by multiplex PCR to detect two virulence genes *inv* A, *stn* (284pb, 617pb, respectively (Fig. 2). The obtained results revealed that *invA* was the most prevalent in all Salmonella isolates thus, all the Salmonella isolates were found highly invasive to cells. Also, Salmonella enterotoxin encoding gene (*stn*) was found in of the isolates.

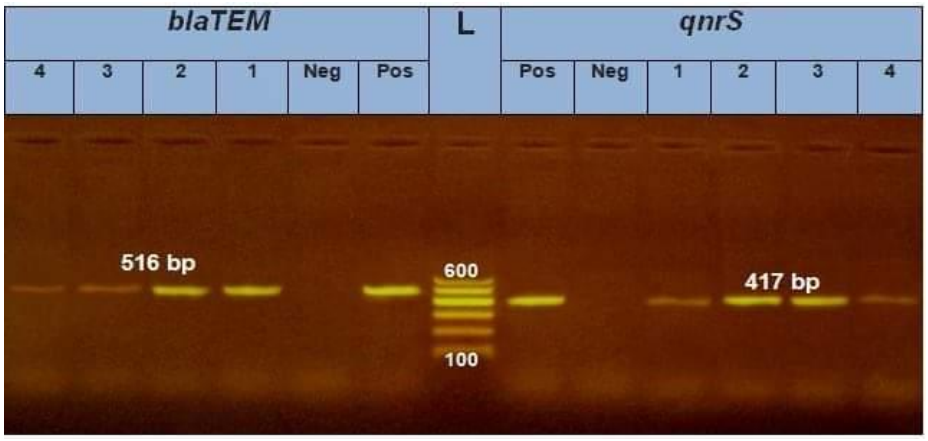

Fig (1) Agarose gel electrophoresis showing specific PCR of Salmonella serotypes using primer set for qnrs gene (417bp)-L=ladder & Lane (P)= positive control& Lane (N)= negative control and Lanes (1-2-3-4) were positive for this gene and primer set for bla TEM gene (516 bp)- L= Ladder & Lane(P)= Positive control & Lane (n)= negative control and lanes (1- 2- 3-4) were positive for this gene.*3.5 Detection of Salmonella Virulence associated genes*

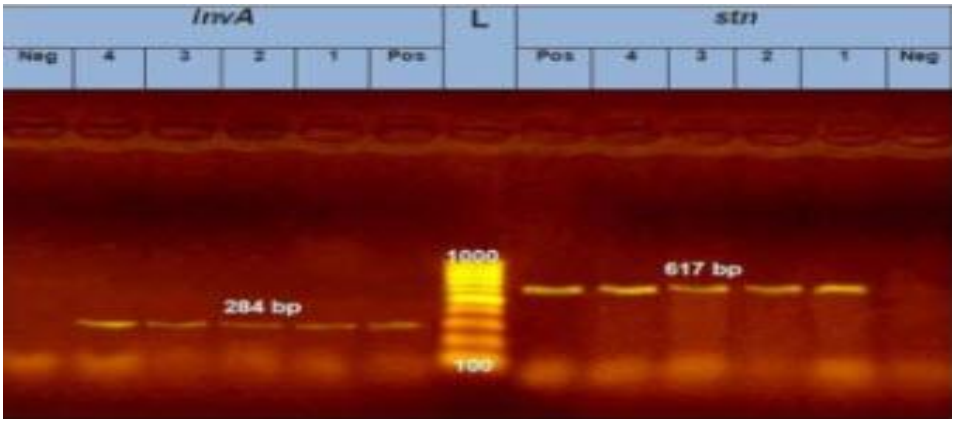

Fig (2) Agarose gel electrophoresis showing specific PCR of Salmonella serotypes using primer set for invA gene (284 pb)- L= Ladder & Lane (P)= positive control & Lane(N)= negative control and Lanes (1- 2- 3- 4) were positive for this gene and primer set for Stn gene (617bp)- L= Ladder & Lane (P)= positive control & Lane (N)= negative control and lanes (1- 2- 3- 4) were positive for this gene.

4. DISCUSION

Salmonella in poultry became a big problem due to its economic importance in all stages of poultry industry from the production line to the marketing. The extensive usage of antibiotics in humans and veterinary medicine raises the possibility of multidrug resistant Salmonellae (Cruchaga et al., 2001). In this study, (100) Samples were collected from litter, water and feed from various broiler farms, houses and poultry shops in Kafr El- Sheikh. These samples were examined for the presence of Salmonella and showed that (14%) of samples were positive for Salmonella. The positive samples were (6) from feeders, (3) from litter and (5) from water.

The current results were partially agreed with Schluter et al, (1994) where they reported an isolation rate of (13.3%) from flocks of lying chicken and to some extent with Murugkar, (2005) who examined (231) cloacal swabs from diarrheic poultry and found (34) isolates of Salmonella with (14.7%) prevalence rate. In addition to Dahal (2007) when detected Salmonella in (13%) of totally (400) examined samples and finally also agreed with the results of Yhiler and Bassey (2015), where they reported Salmonella from (170) samples of feeders, drinkers and litter with (12.9%) prevalence

rate in water samples, (14.1%) in litter and (10%) in feeders.

The results of this study may increase slightly over the results of Ammar et al, (2010) Who recorded a prevalence rate of (12%) of S. Typhimurium which was the most common isolated Salmonella serovar from samples of broilers and laying breeding reproducers in Algeria. Also, the results of Reham (2004) with (12%) prevalence rate for isolations of Salmonella Species detected from samples of broilers.

On the other hand, the results of this study are less than the results reported Osman (2014) who isolated (45) Salmonella species from collected (150) samples from broiler farms with prevalence rate (30%), Harison et al. (2001) when found (29%) prevalence rate of Salmonella in (300) raw chicken samples collected from chains of supermarket and shops of butchers, Cardinal et al, (2003) when recorded (32%) prevalence rate after examining (300) carcasses of chicken from shops of retail in Dakar, Senegal, Bada-Alambedji et al. (2006), who recorded (62.5%) prevalence rate of Salmonella in (120) samples of chicken carcasses from flocks, super markets and poultry slaughter houses, Soomro et al, (2010) when recorded (38%) prevalence rate.

In the present study, serotyping results of isolated Salmonella from chicken were 3/9 (33.3%) of S. Kentucky, 3/9 (33.3%) S. Blegdam, 2/9 (22.2%) S. Ferruch and 1/9 (11.1%) S. Yovokome. This results agreed with the results of Bada-Alambedji et al, (2006) who found (30%) of the isolated Salmonella was S. Kentucky as the most frequent serotype. Also, found different serotypes as S. Muenster (13.3%), S. Brancaster (8.8%), S. Hadar and S. Enteritidis (6.6%). Also, The results were disagreed with the results of Abd El- Tawab (2015) when found S. Kentucky in isolations from chicken samples , Also found other serotypes as S. Typhimurium, S. Daula, S. Colindale, S. Bargny, S..Newport, S. Apeyeme, Molade, S. Tamale, S. Labadi, S. Lexington, S. Enteritidis, S. Magherafelt, S. Santiago, S. Rechovot, S. Takoradi and S. Shubra., Hassan et al, (2016) who found S. Kentucky in (25.45%) of the isolations and also found Salmonella Infantis (56.36%), S. Enteritidis (5.45%), S. Ferruch and S. Virchow , Ammar et al, (2016) Was disagreed with the current findings where S. Kentucky in (12.5%) of isolates of Salmonella beside other serotypes S. Enteritidis (56.25%) and S. Typhimurium (18.75%).

In The present work, Salmonella showed highly resistance (89%) to ciprofloxacin and cefotaxime and high sensitivity (100%) to amikacin which Was agreed with Hassan et al., (2016) who found S. Kentucky isolates resistance against the majority of antibiotics where 100% resistant to ciprofloxacin, 85% showed resistance against both of cefotaxime and ceftazidime. Also, our results agreed with results of Rania (2017) who detected 100% sensitive to amikacin and 100% resistance to ciprofloxacin. Also. Partially was agreed with Marwa., (2017) who detected 100% resistance of Salmonella to cefotaxime.

The antibiogram was results disagreed with Yah and Eghafone (2007) who reported that Salmonella isolates from chicken were high resistant to tetracycline, sulphamethaxazole, trimethoprim and lower resistant to ciprofloxacin and cefotaxime, Akter et al. (2007) who reported that Salmonella serovars isolates were sensitive to ciprofloxacin (80%),

In this 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 was agreed with Yojiaro et al, (2010) who showed *bla*TEM-1 and *bla*TEM-104 from gram negative bacteria isolated from farms in Egypt .Also 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 bla cmy-2 in isolates of S. Enterica serovar Enteritidis. Also, Chen et al. (2004) identified the *bla*TEM-1 in Salmonella Enterica serovars in United States and China.

The for mentioned results was higher than result of Amira et al, (2014) who identified B-lactamase encoding genes, *bla*TEM (40%)- also, Ahmed et al, (2009) who Identified (50%) of *bla*TEM in 47 S. Typhimurium out of ninety-four S. Enterica isolates from animal in Japan.

In the Salmonella isolates showed more resistance to fluoroquinolones in antimicrobial susceptibility tests which were examined by PCR for *qnr*S (Plasmid–mediated quinolones resistance genes) detected 100% were positive for *qnr*S. This result was higher than wafaa et al. (2015) who detected (18 %) 4/22 positive for qnrs while Ahmed et al, (2009)who reported that *qnr*S and *qnr*B were detected in two isolates of S. Enterica serovar Enteritidis and one isolate of S. Enterica serovar Typhimurium, respectively. *qnr*S was identified previously in S. Enterica serovar Typhimurium isolated from animal in Japan.

This finding was consistent with many previous reports Abd Eltawwab et al., (2013), Singh et al., (2013), Osman et al.,(2014), Mohamed et al., (2014), Rowlands et al., (2014), Amira et al., (2015), Abdeltawab et al., (2015), and Ammar et al.,(2016) that established the presence of i*nv*A gene in nearly all Salmonella irrespective of serovar or source. While, Lofstrom et al., (2004), Kumarss et al., (2010), Barman et al., (2013), Sallam et al., (2014) and Olobatoke et al., (2015) reported a lower percentage of *inv*A in 10%,11.4%, 64%, 24.4% and 87.5% respectively.

In the present results, *stn* gene was found in 100% of the isolates. These results were the same as reported by Das et al., (2012) Barman et al, (2013), Sallam et al., (2014), Naik et al., (2015) and Amira (2015) who reported 100% were positive for *stn* gene.

While lower results are reported by Rahman et al., (2011), , Shi et al.,(2013), Ammar et al., (2016) who reported *stn* gene 38.23%, 95.7%, and 41.7% respectively.

This result is similar exactly to Das et al .,(2012) who reported that *inv*A and *stn* were detected in (100%) and 100% isolates respectively in total (134) samples of poultry feed, Zou et al ., (2012) who detected *stn* and *inv*A in all (425)Clinical S. Enteritidis isolates of human origin,

All of serovars produced 284 pb *inv*A gene and 617 pb amplicon for enterotoxin *stn* gene. that they are the specific targets of Salmonella identification and are capable of producing gastroenteric illness to human.

5. It was concluded that :

Salmonellae were isolated with incidence rate (14%).*S*almonellae isolation from chicken feed were (15%) 6/40, from litter (12.5%) 3/24 and from water (13.8%) 5/36.Serotyping of isolated Salmonella was (3) S. Kentucky (8,20: I: Z6), (3) S. Yovokome (8,20: d:1.5) and (2) S. Ferruch (8,e,h:1.5).

The most isolated Salmonella were found to be resistant to ciprofloxacin (fluoroquinolones) and cefotaxime (cephalosporin) with 89% and 100% sensitive to amikacin so amikacin is the first choice for treatment of Salmonella infection.

Molecular characterization of beta lactamase resistance genes in Salmonella serovars showed presence of *bla*TEM gene in all four isolates of Salmonella (100%) and plasmid-mediated quinolone resistance gene showed presence of *qnr*S gene in all four isolates of Salmonella

PCR technique is used to detection of most common virulent genes to detect two virulence gene *inv*A gene with (100%) (highly invasive to cells) and *stn* gene with (100%) Salmonella enterotoxin gene in all isolates.

6. CONCULOSIONS

The Present study suggested that *inv*A and *stn* (virulence genes) are much conserved in Salmonella isolated feed, litter and water. It could be used independently as a gene marker for the rapid detection of the virulent strains of Salmonella.

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