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COMPARISON OF PHYLOGENIC TREE AND GENE SEQUENCE OF SALMONELLA ENTERITIDIS ISOLATED FROM DIFFERENT BIRDS

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ABSTRACT: Salmonella is bacterium causing high morbidity and mortality rates in the birds'. The present study was performed on a total of 100 samples from diseased and apparently healthy quail came from governorates (Giza, Cairo, Damietta and kafrelsheik). Collected samples included different organs (liver, heart, lung, and bone marrow). And a comparison between the genetic tree and gene sequences of Salmonella isolated from {chicken-ducks and rabbit}. The bacteriological examination revealed that out of 100 collected samples, 22 samples (22 %) were positive for Salmonella isolation. the serotyping of Salmonella species isolates showed a major variety of serotypes which included Salmonella Enteritidis (27.3%); Salmonella Typhimurium and Salmonella Senftenberg (22.8% for- each); Salmonella Agona, (18.1%) and Salmonella Magherafelt (9%) . Results of the sensitivity testing of *salmonella* isolated from quail. Showed that (72.8%, 68.2%) of strains were resistant to Nalidixic acid and Streptomycin, but sensitive to Gentamicin, Trimethoprim-sulfamethoxazole, (68.2%) of strains. Also, all Salmonella isolates showed multidrug resistance. We selected three strains to be sequenced with accession number MT267777 to MT267779 and it resembled Salmonella enterica subsp. enterica serovar Enteritidis strain from China, Korea, and UK with 100% identitiy percent.

Key words: Salmonella enteritides, antibiotic sensitivity, sequence gene.

INTRODUCTION

One of the main important pathogen is salmonella as it is food born disease that causes severe economic losses and down grade food product Centers for Disease Control, (2015)

Salmonella is bacteria of Gram- negative, facultatively anaerobic, usually motile it is one from the family Entero bacteriaceae Douglas et al., (2015). Salmonella present in the alimentary tract but not one of the normal flora of poultry if it was infected. It causes decrease villi number, irritation of the intestinal wall that lead to lower absorption Pelicano *et al.*, (2005) also it secretes toxins like ammonia or amines, which affect the hosts liver. isit present in poultry and some of its species has zoonotic effect like (Salmonella enterica) so humane could infected through ingestion of contaminated food especially poultry product Saba *et al*., (2013).

Poultry plays an important role in the transmission of Salmonella to human as there are several serovars were isolated from poultry and human EFSA, (2019).

Quail birds are small migratory birds so it faces many parasites, infectious, and noninfectious diseases, and as it is a bird it takes the same diseases as poultry especially salmonella and may cause zoonotic diseases Yee *et al*., (2009), Ngulukun *et al*., (2010).

Biochemical and serological tests are the most used commonly used methods for detection and identification of Salmonella spp. But methods are delay in diagnosis, treatment and control of infections. Ranjbar *et al.,* (2013). Salmonellosis is still a major food borne disease in human and the significance of Salmonella species as causes of human and animal disease has increased in the recent years. Saba *et al.,* (2013).

Antibiotic resistance is widespread and resistance has been elevated by world health organizations as one of the top health challenges. The escalating cases of antibiotic resistance have raised concerns that we are entering a "post antibiotic era" meaning we might enter an era where there won't be effective antibiotics to treat many life threatening infections Douglas et *al.,* (2015).

Various virulence genes are important for Salmonella pathogenesis, such genes are placed on different genome elements as plasmids, chromosome, Salmonella genomic islands (SGIs), and integrated bacteriophage DNA and Salmonella pathogenicity islands (SPIs) (Card *et al.*, (2016), Riyaz-Ul-Hassan *et al.,* (2004); and Jamshidi *et al.,* (2010) . The stn gene is in attendance in all Salmonella serotypes and contained a unique sequence that considered as suitable PCR target for detection of Salmonella strains in field samples Ammar *et al.,* (2019).

So, it is work aims to isolate and identity attempting to isolate the salmonella microbe from quail with its characterization, which helps in ease and speed of diagnosis and studying the resistance pattern of isolates to antibiotics used in quail. And a comparison between the genetic tree and gene sequences of Salmonella isolated from different birds.

MATERIALS AND METHODS

Collected Samples: A total of 100 samples from diseased and healthy quail came from governorates (Giza, 30-Cairo, 20-Damietta, 20- and Kafrelsheikh, 30). Collected samples included different organs (liver, heart, lung, and bone marrow). The samples were collected under aseptic conditions and safety precautions to prevent- cross - contamination according to Middleton *et al., (*2005). A comparison between the

genetic tree and gene sequences of Salmonella isolated from {chicken-ducks and rabbit}.

Isolation and Identification of Salmonella according to ISO 6579-1: 2017. Briefly, Samples were weighed and suspended in buffered peptone water (as 1:10 dilution) then incubated at 37ºC ±1ºC for 16-20 hours aerobically. The pre- enrichment broth after incubation was mixed and 0.1 ml of the broth was transferred into a tube containing 10 ml of Rappaport-Vassiliadis medium with soya (RVS broth). Another 1 ml of the pre enrichment broth was transferred into a tube containing 10 ml of Muller-Kauffmann tetra thionate novobiocin broth (MKTTn broth). The inoculated RVS broth was incubated at 41.5 ^{o}C±1^{o}C for 24 ± 3 hours and the inoculated MKTTn broth at 37^{o}C±1^{o}C for 24 ± 3 hours. Then a loop-full of material from the RVS broth and MKTTn was transferred and streaked separately onto the surface of Xylose Lysine Deoxycholate Agar (XLD agar), Hektoen Enteric (HE agar) and MacConkey's Agar separately. The plates were incubated in an inverted position at 37^{o}C±1^{o}C for 24 ± 3hours aerobically then checked for growth of typical Salmonella colonies .The typical and selected colonies were identified by biochemical tests (Urea agar, Triple sugar iron, and Lysine iron).

Serotyping of isolated *Salmonella* species according to ISO 6579-3: 2014 and reading of *Salmonella* species by Kauffman – White scheme Grimont and Weill, (2007) using Salmonella antiserum (Sifin Co., Japan®).

antiobiotic sensitivity test:

The antibiogram of isolates was done by disc diffusion test according to Koneman *et al.,* (1997) against 10 antibiotic discs purchased from Oxoid (Amoxicillin +Clavulanic acid, Chloramphenicol, Ciprofloxacin, Gentamicin, Nalidixic acid, Nitrofurantoin, Norfloxacin, Streptomycin, Trimethoprim-sulfamethoxazole and Tetracycline). The interpretation according to the Clinical and Laboratory Standards Institute/ Formerly National Committee for Clinical Laboratory Standard according to CLSI/NCCLS, (2017).

dna amplification and sequencing:

The (22) positive samples for isolation were confirmed by PCR. The DNA was extracted from samples using a QIAmp viral DNA mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The salmonella enteritidis genes were amplified using gene- specific primers and Phusion® high fidelity DNA polymerase (Thermo Fisher Scientific, MA, USA), according to the manufacturers protocol. We selected represented samples from positive flocks from different governorates to be sequenced. Purification was carried out using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Sequencing was performed using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, California, USA) with gene-specific primers, and the nucleotide sequence was obtained from an ABI 3500 Genetic Analyzer (Life Technologies, California, USA).

RESULTS AND DISCUSSIONS

Poultry can become infected with different Salmonella serotypes with high morbidity and mortality during the first three weeks of their life, and may also become carriers bacteria spread horizontally or vertically leading to embryo mortality or rapid death of newly hatched birds. The prevalence of Salmonella infection in Chinese poultry

farms has been widely described Yang, et *al.,* (2019). Salmonella enteric serovar Typhimurium and S. enterica serovar Enteritidis are the most frequently encountered species from foods like poultry, pork and beef products Vose, *et al.,* (2013).

In the present study, 100 quail samples (included 50 from life disease and 50 from freshly dead one) were collected from (Giza, Cairo, Damietta and kafrelsheik governorates) were examined bacteriologically for the presence of Salmonella. Postmortem examination of the dead quails showed septicemia, fibroins pericarditis, perihepatitis, peritonitis, airsaculitis and some cases showed abs cessation of the viscera. The bacteriological examination revealed that 22 out of 100 collected samples, (22 %) were positive for Salmonella isolation (Table2, 3). The obtained results were in contrast to those recorded by Dipineto *et al.,* (2014) in which no Salmonella was isolated from quail flocks examined. Similar results were obtained by Palanisamy and Bamaiyi (2015).

(Table, 4.) Salmonella enter-itidis was recovered only from dead bird's liver, heart and bone marrow and likely associated with systemic infection . S. Enteritidis contaminated eggs leading to public health concerns. The diseased birds may show lesions of pericarditis, per hepatitis and septicaemia. Islam *et al.,* (2016).

In this study, the serotyping of Salmonella species isolates showed a major variety of serotypes which included Salmonella Enteritidis (27.3%); Salmonella Typhimurium and Salmonella Senftenberg (22.8% for each), Salmonella Agona, (18.1%) and Salmonella Magherafelt (9%) (Table, 5). Harsha *et al.,* (2011) and Bacci *et al.,* (2012) recorded that the most frequently isolated serotypes in the quail samples S. Enteritidis (17.1%). Also *S.* Enteritidis, *S.* Typhimurium, *S.* Sinstorf and *S.*Vejle were isolated from chicks while, *S.* Enteritidis, *S.* Muenster and *S.* Cuckmere were isolated from turkey poults. Similarly, Jodas and Hafez (2002). Isolated different types of Salmonella spp. identified S. enterica subspecies Enterica; S. Corvalis, S. Give, S. Lexington, S. Minnesota, S. Schwarzengrund, S. Rissen, and S. Typhimurium from meconium samples. Freitas *et al., (*2013) and Udhayavel *et al.,* (2016).

(Table, 6). The excessive and massive usage of antibiotics on in-tensive food especially poultry represent the cornerstone for the emer-gence, persistence and spread of the resistant bacteria repre-sent a major threat to human health globally WHO, (2014). The resistance bacteria in food animals can transmit to humans directly contact with the animal or indirectly from environment that receives these bacteria from infected animals and fecal mate-rials FAO, (2011) and WHO, (2011). The sensitivity testing of *salmonella* isolated from quail. Showed that (72.8%, 68.2%) of strains were resistant to Nalidixic acid and Streptomycin, but sensitive to Gentamicin and Trimethoprim-sulfamethoxazole, (68.2%) of strains. Also, all Salmonella isolates showed multidrug resistance. In a study by Jahan *et al.,* (2018). The lowest percentage was 20% to Nalidixic acid, Rahman, *et al.,* (2011).

Reported that in vitro amplification of DNA by PCR method is a powerful tool in microbiological diagnostics and, showed that the PCR-based assays were more sensitive than the culture method

for detection of Salmonella. In addition to that obtained by Freitas *et al.,* (2010) who concluded that the mPCR was able to detect the presence of these bacteria in a short period and enabled the identification of serotype Enteritidis in one of the samples found positive for Salmonella species. Moreover, Akiba *et al., (*2011). We make PCR amplification of Hypthetical protein of salmonella Entraitidis and the result was positive for 22 samples from 100 at 304pb.

We selected three strains to be sequenced with accession number MT267777 to MT267779 and it was resemble to Salmonella enterica subsp. enterica serovar Enteritidis strain from china, korea, Uk with 100% identitiy percent. This result has resembled to Akiba *et al.,* (2011).

CONCLUSION

Salmonella enteritis' is counted as one of the major bacteria causing severe problems in poultry farms. So, to minimize the economic losses in the poultry production firm hygienic measures should be applied. Further investigations should continue to characterize the antibiotic resistance genes and the epidemiology link between poultry and human. Biosecurity on the poultry farms should be the first line of defense against infectious diseases.

Table (1): Sequences of primer and the size of amplified products.

Bacterial strains	**Target gene**	**The Sequence of the primers 5'-3'**	**PCR Size (bp)**	**refrence**
S. Enteritidis	SEN1383 hypothetical protein	F:TGTGTTTTATCTGATGCAAGAGG' R: -TGAACTACGTTCGTTCTTCTGG'	304	Ranjbar, et al , 2014

Table (2): Incidence of salmonella recovered from examined quail samples in different governorates

Locality	**Salmonella recovered from examined quail**	
	No examined	**No. positive %**
Cairo	30	6(20%)
Giza	20	3(15%)
Damietta	20	5(25%)
Kafrelsheik	30	8(26%)
Total	100	22(22%)

*Percentage according to total number of the examined samples in each governorates.

Table (3): Numbers and Percent of the positive and negative examined samples.

Birds status	No. of samples	positive number	positive %	Negative number	Negative %
Scarified birds	50	5	10	45	90
Freshly dead	50	17	34	33	66
total	100	22	22	78	78

*Percentage according to total number of the examined samples.

Table (4): Incidence of Salmonella in different organs of quails.

Bird status	No. Examined of positive	Organ examined							
		Liver	%*	lung	%*	Heart	%*	Bone marrow	%*
Scarified birds	5	2	40	0	0	1	20	2	40
Freshly dead	17	8	47	2	11.7	2	11.7	5	29.6
Total	22	11	50	2	9	3	13.6	6	27.2

*Percentage according to total number of the examined samples.

Table (5): Serotyping results of Salmonella species isolated from quails.

Serotype	No. of isolates	%
Salmonella Enteritidis	6	27.3
Salmonella Typhimurium	5	22.8
Salmonella Senftenberg	5	22.8
Salmonella Agona	4	18.1
Salmonella Magherafelt	2	9
Total	22	100

*Percentage according to total number of the examined samples.

Table (6): Results of antimicrobial sensitivity of salmonella isolates recovered from examined quail (total number of samples = 22)

antimicrobial agents	Resistance		Intermediate		Sensitive	
	No	%*	No	%*	No	%*
Amoxicillin + Clavulinic acid	5	22.7	15	68.2	2	9.1
Chloramphenico	12	54.6	5	22.7	5	22.7
Ciprofloxacin	6	27.2	4	18.2	12	54.6
Gentamicin	3	13.6	4	18.2	15	68.2
Nalidixic acid	16	72.8	3	13.6	3	13.6
Nitrofurantoin	9	40.9	10	45.5	3	13.6
Norfloxacin	8	36.4	5	22.7	9	40.9
Streptomycin	15	68.2	4	18.2	3	13.6
Trimethoprim-sulfamethoxazole	5	22.7	2	9.1	15	68.2
Tetracycline	5	22.7	9	40.9	8	36.4

*Percentage calculated according to total number of the examined samples.

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الملخص العربي

مقارنة بين الشجرة الوراثية وتسلسل الجينات من السالمونيلا انترتيدس المعزولة من الطيور المختلفة

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السالمونيلا هي بكتيريا سالبة الجرام مسؤولة عن مجموعة متنوعة من الأمراض المعدية: حمى التيفود والتهاب المعدة والأمعاء والتسمم الغذائي وتسمم الدم. أجريت الدراسة الحالية على ما مجموعه ١٠٠ عينة من السمان المريضة والتي تبدو سليمة جاءت من محافظات (الجيزة ، القاهرة ، دمياط وكفر الشيخ). وشملت العينات التي تم جمعها أعضاء مختلفة (الكبد والقلب والرئة ونخاع العظام). ومقارنة بين الشجرة الجينية والتسلسلات الجينية للسالمونيلا المعزولة من {الدجاج والبط والأرانب}. أظهر الفحص البكتريولوجي أنه من أصل ١٠٠ عينة تم جمعها ، كانت ٢٢ عينة (٢٢٪) موجبة لعزل السالمونيلا. أظهر التنميط المصلي لعزلات السالمونيلا تنوعًا كبيرًا من الأنماط المصلية التي شملت سالمونيلا انترتيديس (٢٧.٣٪). السالمونيلا تيفيموريوم والسالمونيلا سينفتنبرج (٢٢.٨٪ لكل منهما) ؛ السالمونيلا اجونا (١٨.١٪) والسالمونيلا ماجرافيلت (٩٪). أظهر اختبار حساسية المضادات الحيوية في المختبر لسلالات السالمونيلا المعزولة من السمان أن (٧٢.٨٪ ، ٦٨.٢٪) من السلالات كانت مقاومة لحمض الناليديكسيك والستربتومايسين ، لكنها كانت حساسة للجنتاميسين وتريميثوبريم-سلفاميثوكسازول ، (٦٨.٢٪) من السلالات. كما أظهرت جميع عزلات السالمونيلا مقاومة للأدوية المتعددة. اخترنا ثلاث سلالات ليتم ترتيب تسلسلها برقم الإدخال MT267777 إلى MT267779 وكانت تشبه Salmonella enterica subsp. enterica serovar Enteritidis سلالة من الصين وكوريا بنسبة ١٠٠٪.