Serological and Molecular Studies on Multi-drug Resistant *Salmonella* Isolated From Captive Budgerigars (Melopsittacus undulatus)

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Abstract

The present work was carried out for serological and molecular screening of virulence genes associated with *Salmonella* in captive budgerigars. A total of 805 apparently healthy birds were collected from different sources, and subjected to clinical and bacteriological examination. *Salmonella* species were isolated at rate of (4.97%) with recognition of 4 different serovars in which *Salmonella* Paratyphi A was the most common isolated serotype. All isolates were highly sensitive to Ciprofloxacin, Enrofloxacin and Norfloxacin. Multiplex-PCR using (*inv*A, *spv*C and *stn*) was devised to confirm the isolates and predict their virulence. (*inv*A;284bp) was detected in all *Salmonella* isolates with (100%), while (*stn*; 617bp) and (*spv*C; 392bp) were detected in some *Salmonella* isolates.

Introduction

A budgerigar *(Melopsittacus undulatus)* is one of most popular psittacine birds. Nowadays, this popularity is worldwide reaching to an international trade of the living birds. Naturally, budgerigars are found in Australia, however, they are not found in Egyptian wildlife, therefore all birds present in houses are derived from pet stores or shop fairs. Many zoonotic diseases are transferred from cage or pet birds to human through direct or indirect contact of the diseased or carrier birds ***(Akhter et al, 2010)***. Salmonellosis is a common bacterial zoonotic disease and can be a serious disease of psittacine birds. Asymptomatic *Salmonella* carriage in wild birds is thought to be high, as many species acquire the organisms and become carriers without any visible signs and considered as apparently healthy birds ***(Tizard, 2004)***. In-vitro amplification of DNA by the PCR method is a powerful tool in microbiological diagnostics. Several genes have been used to detect *Salmonella* in faecal samples. Invasion gene "*inv*A" is a target gene of *Salmonella* responsible for adhesion and invasion in the host system.

Salmonella enterotoxin "*stn*" associated with the actual manifestation of pathogenic processes ***(Murugkar et al, 2003)*** and "*spv*C" which is present in plasmid and associated with virulence ***(Oladapo et al, 2013)***. So, this work undertaken to isolate, identify and compare the incidence of *Salmonella* as a zoonotic microorganism in captive budgerigars collected from zoos, pet shops and households. Also, to screen the virulence genes of isolated *Salmonella* using Multiplex-PCR.

Material and methods

1- Examined birds: A total of 805 apparently healthy budgerigars were collected from 3 different sources; zoos (500 birds), pet shops (187 birds) and household (118 birds).

2- Sampling: A sterilized waxed paper were placed on the floor of the cages to minimize possible contamination ***(Bangert et al, 1988)***. A total of 805 freshly voided faecal dropping were swabbed immediately with a sterile cotton swab and placed in 5ml peptone water **(**Himedia**)** as a pre-enrichment media according to ***(ISO, 2002)***.

3- Isolation of *Salmonella* species: Collected samples were cultured according to ***(ISO, 2002)***. The microscopical examination and biochemical identification were carried according to ***(Finegold and Martin, 1982).*** Serological identification of isolates was carried out in serological unit, Animal Health Research Institute, Dokki, Giza". According to Kauffmann-White Scheme as described by ***(Edwards and Ewing, 1972).*** Antimicrobial sensitivity test was carried on all isolates according to the procedures given by ***(NCCLS, 2002)*** using 16 commercial antibiotic discs **(**Oxoid**)** at Animal Health Research Institute, Ismailia.

4- Molecular typing of isolated *Salmonella* species: It was carried out at Central Laboratory Unit (CBU). Faculty of Veterinary Medicine. Suez Canal University.

4-1- Extraction of DNA from *Salmonella* isolates by boiling *(Croci et al, 2004).*

4-2- Multiplex-PCR using *inv*A, *stn* and *spv*C genes: Two pairs of oligonucleotides primers specific for each *Salmonella* gene (*inv*A, *stn* and *spv*C genes) were used for multiplex-PCR as shown in Table (1). Multiplex-PCR was carried out in 25µl reaction volume in a 0.2 ml PCR tube contained 12.5µl 2X PCR Master Mix, 1µl of each primer, 2µl template DNA and 4.5µl nuclease free water. Then Placed in an Eppendorf Mastercycler Gradient and subjected to the following protocol: Initial denaturation at 94°C/90 sec. 35 cycles of amplification at 94°C/60 sec. Annealing at 58°C/45 sec. Extension at 72°C/90 sec. and

final extension at 72°C/7min. *S.* Typhimurium was used as positive control and negative control PCR reaction with no DNA template also were included in this assay. Then 10µl of the final PCR product was separated by electrophoresis on 2% agarose gel with 100bp DNA ladder **(GeneDirex)** at 100volts for 30min and visualized by staining with ethidium bromide under UV light.

5- Statistical analysis: It was carried out using the Chi square test by M stat program.

Table 1: *Oligonucleotides primers used for detection of Salmonella by PCR (eurofins (mwg/operon) company, Germany)*

Primer	Primer Sequence.	Amplicon lenght (bp)	Reference
***inv*A forward**	5' GTG AAA TTA TCG CCA CGT TCG GGC AA-3'	284	**Oladapo et al., 2013**
***inv*A reverse**	5'-TCA TCG CAC CGT CAA AGG AAC C-3'		
***stn* forward**	5 - TTG TGT CGC TAT CAC TGG CAACC – 3	617	**Murugkar et al., 2003**
***stn* reverse**	5 - ATT CGT AAC CCG CTC TCG TCC – 3		
***spv*C forward**	5'-GGGGCGGAAATACCATCTACA 3'	392	**Alessiani et al., 2014**
***spv*C reverse**	5'-GCGCCCAbGGCTAACACG -3'		

Results and discussion

This work sheds light upon *Salmonella* spp. affecting captive budgerigars kept in zoological gardens, pet shops and houses. The examined birds subjected to clinical and bacteriological examinations.

Clinically, all birds were apparently healthy.

Bacteriological investigation showed that, 40 (4.97 %) of faecal samples were positive for *Salmonella* (Table 2). Similar results were revealed the presence of *Salmonella* in healthy

budgerigars by ***(Enas, 2008)***, in other healthy psittacines by ***(Akhter et al, 2010)*** and in other healthy wild birds by ***(Samah and Azhar, 2013)***. On the other side, ***(Ortiz–Catedral et al, 2009)*** failed to isolate *salmonella* from healthy psittacine birds. The incidence of *Salmonella* was 3.8 % in zoos, 8.02 % in pet shops and 5.1% in household groups. The decreased incidence of *Salmonella* isolation in this work was disagreed with ***(Akhter et al, 2010)***. Otherwise, these results were higher than that recorded by ***(Bezerra et al, 2013)***. This could be attributed to various types and size of samples or using different methods for *Salmonella* detection, or its geographic location and types of food consumed ***(Padungtod and Kaneene, 2006)***. The incidence of *Salmonella* isolation in zoo birds was lower than that recorded by ***(Jang et al, 2008)*** and higher than that recorded by **(Enas, 2008)**. Nevertheless, the relatively close confines of captivity mean an increased pathogen load in the environment in which companion and aviary parrots live which may leads to greater exposure of these birds to bacteria and parasites ***(Doneley, 2009)***. Statistically, there was no relationship between the source of samples and the number of positive cases.

As shown in Table (3), 4 different *Salmonella* serovars were isolated from budgerigars. (47.5%) *S.* Paratyphi A, (35%) *S.* Typhimurium, (7.5%) *S.* Chester, (5%) for both *S.* Infantis and untypable *Salmonella*. *S.* Paratyphi A was the most common isolated serovar in households group with percentage of (66.67%) followed by (60% and 31.58%) in pet shops and zoos groups respectively. This may be due to direct or indirect contact with the bird fanciers, owners, zoo visitors and zoo keepers which might be diseased or carrier for the *Salmonella*. These findings were disagreed with ***(Styles, 2005)*** who approved that, *S.* Typhimurium was the most isolated serotype from budgerigars and other psittacine birds. Isolation of *S.* Paratyphi A from budgerigars reflect its zoonotic importance as a restricted human pathogen and causes only systemic disease ***(McClelland et al, 2004)***. On the other hand, *Salmonella* can be a normal inhabitant of humans and many animals with no evidence of clinical signs. Many people don't develop disease however, others may develop diarrhea, abdominal cramps and fever within 12-72 hr. of exposure ***(Souza, 2009)***.

The isolation of *S.* Typhimurium with the highest rate of (42.10%) was in zoos group followed by (40%) in pet shops group and failed to be isolated from household group. This results could be accepted due to presence of a lot of free-ranging wild birds in and around the zoos, such as crows, cattle egrets, house

sparrows, doves and pigeon that could carry the disease and transmitted to the zoo birds. Nearly similar results were recorded in Giza zoo by ***(Oraby, 1993)***, in zoo of Pakistan by ***(Javed et al, 1994)***, and in Tehran by ***(Rahmani et al, 2011)*** who approved that, *Salmonella* Typhimurium was the most prevalent serotype in parks and pet shops. Statistically, there was a highly significant relationship between the different *Salmonella* serotypes and the various sources of budgerigars and their faecal samples.

In our study, all *Salmonella* isolates were highly sensitive to Ciprofloxacin, Enrofloxacin and Norfloxacin, while there was great resistance to Amoxicillin, Erythromycin, Tetracycline, Gentamycin, Streptomycin. These results agreed with ***(Akhter et al, 2010 and Samah and Azhar, 2013)*** and disagreed with ***(Vigo et al, 2009)*** who reported that, all *Salmonella* strains isolated from blue and gold Macaw were susceptible to Gentamicin, Streptomycin and Tetracycline, and also with ***(Enas, 2008)*** who revealed that, Gentamycin and Tetracycline were the most effective drugs against the isolated *Salmonella* isolate from budgerigars. All *Salmonella* strains showed multiple drug resistance (MDR) at least to 5 antibiotics. *S.* Paratyphi A showed resistance to 11 antibiotics, while it was sensitive to 5 drugs. Both *S.* Typhimurium and *S.* Chester were resistant to 10 antibiotics and sensitive to 6. *S.* Infantis was resistant to 9 and sensitive to 6 antibiotics. Finally, the untypable strains were resistant to 5 and sensitive to 8 drugs. This may be attributed to the uncontrolled use of antibiotics in animals. In addition, the unregulated use of antibiotics by humans.

A multiplex-PCR containing three sets of PCR primers (*inv*A, *stn* and *spv*C genes) was created to confirm the *Salmonella* isolates and predict its virulence. As shown in Photo (1), the amplification of *inv*A, *stn* and *spv*C genes revealed that, *inv*A gene bands of 284 bp were found in all tested *Salmonella* isolates, which suggested that, it is conserved gene among *Salmonella* serovars and is the predominant necessary one to express virulence in the host, causing infection. This results in agreement with ***(Shanmugasamy et al, 2011)*** who reported presence of *inv*A gene in all *Salmonella* they tested. Moreover, this finding at variance with ***(Oladapo et al, 2013)*** who confirmed the absence of *inv*A gene in 3 isolates out of 8, and ***(Bacci et al, 2006)*** who detected *inv*A gene in 62 out of the 63 strains of *Salmonella* screened. This implied that, the isolate that doesn't carry the gene may not be virulent and unable to invade epithelial cells.

The presence of *stn* gene were detected by the presence of 617 bp PCR product in some *Salmonella* isolates which agreed with ***(Muthu et al, 2014)***, but disagreed with ***(Murugkar et al, 2003)*** who carried out PCR assay for the detection of the *stn* gene in 95 *Salmonella* isolates from 5 different serovars and 4 different sources and revealed its presence in all the isolates. Also ***(Ziemer and Steadham, 2003 and Samah and Azhar, 2013)*** revealed 100% positive results for *stn* gene in all isolates. *S.* Paratyphi A and *S.* Typhimurium were positive for *stn* gene this finding was similar to that reported by ***(Murugkar et al, 2003 and Samah and Azhar, 2013)***, while it was absent in *Salmonella* Chester, *Salmonella* Infantis and in the untypable strains, which came in variance with ***(Ziemer and Steadham, 2003)*** who stated that, *Salmonella* Infantis was positive for *stn* gene.

Presence of *spv*C gene in the present study was confirmed by amplification of 392 bp PCR product in some *Salmonella* isolates such as *S.* Typhimurium and *S.* Infantis. While it was absent in *S.* Paratyphi A, *S.* Chester and untypable strains. This finding was consistent with reports of ***(Alessiani et al, 2014)*** who approved the presence of *spv*C gene in *S.* Typhimurium. In addition, ***(Lin et al, 2007)*** who reported the absence of *spv*C gene in *S.* Paratyphi as well as *S.* Typhi which are a human-specific pathogens, the etiologic agents of enteric fever, carry Vi antigen which is not carried by the great majority of the other *Salmonella*. *spv* gene is responsible for the systemic infection and multi-drug resistance in both human and animals **(Gebreyes *et al*., 2009)**. In nature some plasmids can be transferred from one bacterium to the next through conjugation. This ability contributes to the spread of drug resistance in bacterial species ***(Rychlik et al, 2005)***.

In conclusion, Multiplex-PCR approved that, strains of *S.* Paratyphi A were positive for *inv*A and *stn* genes, and negative for *spv*C gene, while *S.* Typhimurium strains were positive for *inv*A, *stn* and *spv*C genes. *S.* Infantis strains were positive for *inv*A and *spv*C genes, and negative for *stn* gene. Finally, both *S.* Chester and the untypable strains were positive for *inv*A gene only and negative to the others. These results approved that, with more investigations, the Multiplex-PCR could help in the determination of bacterial serovars in absence of their serogroup data. This was similar to ***(Peterson et al, 2010)*** who identified 135 out of 142 *Salmonella* isolates by multiplex-PCR in the absence of traditional antibody-based serotyping. So, it can be a rapid, sensitive and specific means to identify, serotype *Salmonella* with

offering quick data for antibiotic sensitivity.

This work approved that, apparently healthy budgerigars could be carriers for different serovars of *Salmonella*. It is very important to apply good hygienic steps to obtain healthy birds free from salmonellosis. Furthermore, the pet shops should be under the supervision of the General Authority for Veterinary Services and wildlife authority before and after license issue.

Table 2: *Number and Percentage of positive faecal sample for isolation of Salmonella species in relation to number of examined budgerigars.*

Source of faecal samples	No. of examined birds	No. of positive samples	Percentage %
Zoos group	500	19	3.8
Pet shops group	187	15	8.02
Household group	118	6	5.1
Total	805	40	4.97

Chi square (χ^2) = 5.14, Degree of freedom (df)= 2, (P- value)= 0.076,non-significant at (P > 0.05)

Table 3: *Percentage of different Salmonella serotypes in zoos, pet shops and household groups.*

***Salmonella* serotype**	**Zoos**		**Pet shops**		**Household**		**Total**	
	No.	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**
***S.* Paratyphi A**	6	31.58	9	60.00	4	66.67	19	47.50
***S.* Typhimurium**	8	42.10	6	40.00	0	0	14	35.00
***S.* Chester**	3	15.79	0	00.00	0	00.00	3	7.50
***S.* Infantis**	2	10.53	0	00.00	0	00.00	2	5.00
Untypable strains	0	00.00	0	00.00	2	33.33	2	5.00
Total	19	100	15	100	6	100	40	100

(χ^2) = 21.31, (df)= 8, (P- value)= 0.0064, highly significant at ($P \leq 0.01$)

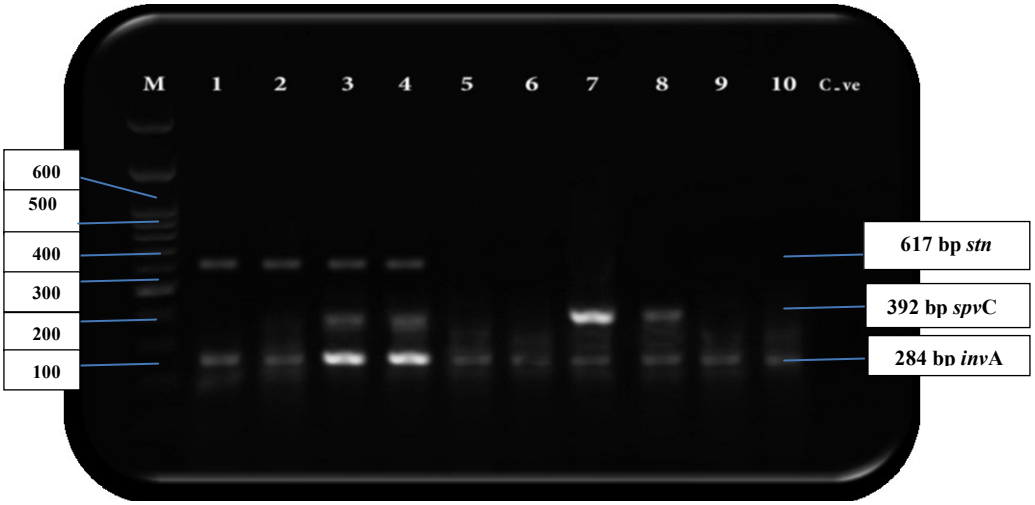

Figure 1: *Agarose gel electrophoresis of multiplex-PCR of isolated Salmonella strains. M: 100 bp DNA ladder; Lane 1,2 S. Paratyphi A; Lane 3,4 S. Typhimurium; lane 5,6 S. Chester; lane 7,8 S. Infantis and Lane 9,10 untypable Salmonella. C-ve control negative.*

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دراسات سيرولوجية وجزيئية على السالمونيلا متعددة المقاومة والمعزولة من طيور الدر الاسترالية الاسيرة

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الملخص العربي

هذا العمل قد تم لمسح الخصائص السيرولوجية والجزيئية لميكروب السالمونيلا وجينات الضراوة المرتبطة معها في طيور الدر الاسترالية التي تعيش في الأسر. العدد الكلي ٨٠٥ طائر سليم ظاهريا تم تجميعهم من أماكن مختلفة (حدائق حيوانات، محلات طيور الزينة و المنازل الخاصة) وتم فحصهم سريريا وبكتريولوجيا. عزلت السالمونيلا بنسبة (٤,٩٧%) مع وجود اربع عترات مختلفة من السالمونيلا وكانت سالمونيلا باراتيفي A أكثر العترات عزلاً. كل العترات المعزولة كانت أكثر حساسية للسيبروفلوكساسين، الإنروفلوكساسين والنورفلوكساسين. كما قمنا بإجراء تفاعل البلمرة المتسلسل المتعدد باستخدام ثلاث بادئات جينية هي *inv*A, *stn*, *spv*C وذلك للتأكد من عترات السالمونيلا المعزولة بالإضافة إلى التنبؤ بمدى ضراوتها. تم اكتشاف (*inv*A;284bp) في كل السالمونيلا المعزولة بنسبة (١٠٠%). بينما (*stn*; 617bp) , (*spv*C; 392bp) تم اكتشافهم في بعض العترات المعزولة فقط.