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

Enas, M. S.¹, Kamel, A. M.¹, Khafagy, A. R.², and Gamal-Eldein, M. A. M.¹

¹Wildlife Management and Zoo Medicine Dept. and Bacteriology,  
²Immunology and Mycology Dept. Faculty of Veterinary Medicine. Suez Canal University.

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 (*invA, spvC and stn*) was devised to confirm the isolates and predict their virulence. (*invA; 284bp*) was detected in all *Salmonella* isolates with (100%), while (*stn; 617bp*) and (*spvC; 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 "*invA*" 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 "spvC" 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 invA, stn and spvC genes: Two pairs of oligonucleotides primers specific for each Salmonella gene (invA, stn and spvC 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)

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer Sequence.</th>
<th>Amplicon length (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>invA</em> forward</td>
<td>5’ GTG AAA TTA TCG CCA CGT TCG GGC AA-3’</td>
<td>284</td>
<td>Oladapo et al., 2013</td>
</tr>
<tr>
<td><em>invA</em> reverse</td>
<td>5’-TCA TCG CAC CGT CAA AGG AAC C-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>stn</em> forward</td>
<td>5’-TTG TGT CGC TAT CAC TGG CAACC –3</td>
<td>617</td>
<td>Murugkar et al., 2003</td>
</tr>
<tr>
<td><em>stn</em> reverse</td>
<td>5’-ATT CGT AAC CCG CTC TCG TCC –3</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>spvC</em> forward</td>
<td>5’-GGGGCGGAAATACCATCAGCA 3’</td>
<td>392</td>
<td>Alessiani et al., 2014</td>
</tr>
<tr>
<td><em>spvC</em> reverse</td>
<td>5’-GCGCCCABGGCTAACACACG -3’</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**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 avairy 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 (invA, stn and spvC genes) was created to confirm the Salmonella isolates and predict its virulence. As shown in Photo (1), the amplification of invA, stn and spvC genes revealed that, invA 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 invA gene in all Salmonella they tested. Moreover, this finding at variance with (Oladapo et al., 2013) who confirmed the absence of invA gene in 3 isolates out of 8, and (Bacci et al., 2006) who detected invA 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 spvC 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 spvC gene in S. Typhimurium. In addition, (Lin et al, 2007) who reported the absence of spvC 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 invA and stn genes, and negative for spvC gene, while S. Typhimurium strains were positive for invA, stn and spvC genes. S. Infantis strains were positive for invA and spvC genes, and negative for stn gene. Finally, both S. Chester and the untypable strains were positive for invA 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.

<table>
<thead>
<tr>
<th>Source of faecal samples</th>
<th>No. of examined birds</th>
<th>No. of positive samples</th>
<th>Percentage %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zoos group</td>
<td>500</td>
<td>19</td>
<td>3.8</td>
</tr>
<tr>
<td>Pet shops group</td>
<td>187</td>
<td>15</td>
<td>8.02</td>
</tr>
<tr>
<td>Household group</td>
<td>118</td>
<td>6</td>
<td>5.1</td>
</tr>
<tr>
<td>Total</td>
<td>805</td>
<td>40</td>
<td>4.97</td>
</tr>
</tbody>
</table>

*Chi square (χ²) = 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.

<table>
<thead>
<tr>
<th><em>Salmonella</em> serotype</th>
<th>Zoos</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td><em>S.</em> Paratyphi A</td>
<td>6</td>
<td>31.58</td>
<td>9</td>
<td>60.00</td>
<td>4</td>
<td>66.67</td>
<td>19</td>
<td>47.50</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S.</em> Typhimurium</td>
<td>8</td>
<td>42.10</td>
<td>6</td>
<td>40.00</td>
<td>0</td>
<td>0</td>
<td>14</td>
<td>35.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S.</em> Chester</td>
<td>3</td>
<td>15.79</td>
<td>0</td>
<td>00.00</td>
<td>0</td>
<td>00.00</td>
<td>3</td>
<td>7.50</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S.</em> Infantis</td>
<td>2</td>
<td>10.53</td>
<td>0</td>
<td>00.00</td>
<td>0</td>
<td>00.00</td>
<td>2</td>
<td>5.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untypable strains</td>
<td>0</td>
<td>00.00</td>
<td>0</td>
<td>00.00</td>
<td>2</td>
<td>33.33</td>
<td>2</td>
<td>5.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>19</td>
<td>100</td>
<td>15</td>
<td>100</td>
<td>6</td>
<td>100</td>
<td>40</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*(χ²) = 21.31, (df) = 8, (P-value) = 0.0064, highly significant at (P ≤ 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.

References
Veterinariae. Volume (41): Article No. 1157.


NCCLS (National Committee for Clinical Laboratory Standards). (2002): Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals approved standards. 22(1).


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

إيناس محمد سعد1، عاطف محمد كامل أحمد1، أحمد أحمد رفعت خفاجي2، محمد عبدالحليم مصطفى جمال الدين1

قسم الحياة البرية وحدائق الحيوان و2قسم البيكترولوجي والمناعة والفطريات- كلية الطب البيطري- جامعة قناة السويس

الملخص العربي

هذا العمل قد تم لمسح الخصائص السيرولوجية والجزئية لميكروب السالمونيلا وجينات الضراوة المرتبطة معها في طيور الدر الاسترالية التي تعيش في الأسر. العدد الكلي 508 طائر سليم ظاهرا تم تجميعهم من أماكن مختلفة (حدائق حيوانات، محلات طيور الزينة والمنازل الخاصة) ومن فحصهم سريري وبكتريولوجي. عزلت السالمونيلا بنسبة (79.4%) مع وجود اربع عترات مختلفة 

من السالمونيلا وكانت سالمونيلا بارا تيفي A أكثر العترات عزلًا. كل العترات المعزولة كانت أكثر حساسية للسيبروفلوكساسين، الإنروفلوكساسين والنورفلوكساسين. كما قمنا بإجراء تفاعل البلمرة المتسلسل باستخدام ثلاث نباتات جينية هي invA, stn, spvC المشتقة باستخدام ثلاث بادئة جينية هي (invA; 284bp) في كل السالمونيلا المعزولة بالإضافة إلى التنبو بعدي ضراوتها. تم اكتشاف (spvC; 392bp) و (stn; 617bp) في بعض العترات المعزولة فقط.