Diagnostic Investigation of Salmonella in Captive Budgerigars Using Standard Microbiological Techniques and invA Gene Specific PCR

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Abstract

A total of 384 faecal samples were collected from apparently healthy budgerigars and processed to investigate the status of *Salmonella* in captive budgerigars by Standard Microbiological Techniques (SMT) and PCR with *inv*A gene. (10.42%) were *Salmonella* positive by SMT while (18.49%) were positive by PCR. The incidence by SMT was (11.31%, 12.50% and 6.25%) while, by PCR (25%, 16.67% and 9.38%) in zoos, pet shops and household groups, respectively. The sensitivity and specificity of the PCR were (100% and 91%) respectively. Individual samples that examined by SMT and PCR were compared with Pooling method which considered as initial screening method that eliminates all *Salmonella*-negative samples. Enriched faecal broth and PCR with *inv*A gene can be used as rapid method for direct detection of *Salmonella* in the faecal samples of carrier captive birds. *Salmonella* isolation rate was (87.5%, 7.5%, 5% and 0%) in Spring, Summer, Winter and Autumn respectively.

Introduction

A budgerigar *(Melopsittacus undulatus)* is one of psittacine birds (which is a common term for members of order Psittaciformes and family Psittacidae which including parrots and parakeets). Parrots are popular as pets due to their sociable nature, intelligence, bright colors, and ability to imitate human voices, in addition to their longevity. Economically, parrots can be beneficial to communities as sources of income from the pet trade. Depending on locality, parrots may be either wild caught or be captive bred, though in most areas without native parrots, pet parrots are captive bred ***(Akhter et al, 2010).***

A variety of *Salmonella* serotypes, including those frequently isolated from humans, have been isolated from parrots and parakeets, with clinical signs ranging from

asymptomatic to per-acute death. Contacts with wild or captive birds have a possible threat to human health ***(Hoelzer et al, 2011)***.

Diagnosis of salmonellosis can be achieved by culture of faeces, blood, spleen, liver, and intestinal contents. The bacterial culture has traditionally been the ''gold standard'' for identification of *Salmonella* spp. from faecal specimens **(*Arnold et al, 2004)***. These methods are laborious, require substantial manpower and last 4–7 days to complete ***(Malorny and Hoorfar, 2005)***.

More recently, molecular techniques like Polymerase Chain Reaction (PCR) was developed to accelerate the identification of serotypes and used as diagnostic tool to detect *Salmonella* in different clinical materials ***(Hong et al, 2008)***. This study aimed to investigate *Salmonella* status in captive budgerigars and qualify its possible zoonotic importance to human with special reference to effect of seasonal variation.

Material and methods

This study was carried out on (384) apparently healthy budgerigars from different sources and classified into 3 groups: zoos (168 birds), pet shops (120 birds) and household (96 birds) during different seasons.

1- Samples: A sterilized waxed paper were placed on the floor of the cages to minimize possible contamination ***(Bangert et al, 1988)***. A total of 384 freshly voided faecal dropping were swabbed immediately with a sterile cotton swab then was inoculated in test tube contained peptone water according to ***(ISO, 2002)***.

2- Standard Microbiological Techniques (SMT): The samples were cultured according to ***(ISO, 2002)***. The microscopical examination and biochemical identification were carried according to ***(Finegold and Martin, 1982).***

3- Serotyping of *Salmonella* isolates: By the Kauffman-White scheme as described by ***(Edwards and Edwing, 1972)*** at Animal Health Research Institute, Dokki, Giza.

4- PCR for detection of *Salmonella*: It was carried out at Central Laboratory Unit. Faculty of Veterinary Medicine. Suez Canal University.

4-1- Preparation of faecal samples for PCR assay, according to ***(Oliveira, 2003)***.

4-2- Pooling of enriched faecal samples according to ***(Singer, 2006)***.

4-3- DNA extraction from pooled enriched faecal samples using Bacterial DNA Extraction Kit (Spin-column):

4-3-1- Preparation of pooled samples for DNA extraction according to ***(Gamal-Eldein et al, 2008).***

4-3-2- Procedures of Bacterial DNA Extraction Kit (Spin-column), ***BioTeke Corporation, China***.

4-4- PCR amplification using *inv*A gene according to **(*Oladapo et al, 2013)*** with some modifications during work: Initial denaturation at 94°C/60 sec. 35 cycles of amplification at 94°C/60 sec. Annealing at 62°C/30 sec. Extension at 72°C/30 sec. and final extension at 72°C /7min.

4-5- PCR machine: A DNA thermal cycler (model Mastercycler Gradient, Eppendorf, Germany), was used for amplification of DNA.

4-6- Electrophoresis and Photo documentation equipment.

- Horizontal gel electrophoresis apparatus. (MS) MajorScience, Mini-300.
- Photographic apparatus-UVP- Biospectrum, multispectral imaging system, An Analytik Jena Company, Cambridge, UK.

5- Statistical analysis: The sensitivity and specificity was calculated with McNemar's test of MedCalc program, according to ***(Soria et al, 2012).***

Table 1: *Oligonucleotides primer of invA gene (eurofins (mwg/operon) company, Germany).*

Primer	Primer Sequence.	Melting point	Amplicon lenght (bp)
***inv*A forward**	5' GTG AAA TTA TCG CCA CGT TCG GGC AA-3'	64.8	284
***inv*A reverse**	5'-TCA TCG CAC CGT CAA AGG AAC C-3'	62.1	

Results

The examined budgerigars were subjected to clinical and bacteriological examinations. They were behaviorally normal and clinically were apparently healthy showing no clinical signs for any disease. Table (2) showed that, by SMT (10.42%) of the faecal samples were positive for *Salmonella*. The incidence was (11.31%, 12.50% and 6.25%) in zoos, pet shops and household groups, respectively. While this positivity increased by PCR to (18.49%), and the incidence became (25%, 16.67% and 9.38%) in zoos, pet shops and household groups respectively.

Among each budgerigars group (30, 30 and 20 pools) were obtained from zoos, pet shops and household groups respectively, with *Salmonella* incidence of (53.33%, 33.33% and 30%) respectively. Statistically, non-significant relation was found between positivity or negativity and the source of pooled samples either from zoos or pet shops or household groups as (P-value)= 0.076, non-significant at ($P > 0.05$).

The pooled samples were screened for the presence of *Salmonella* by detecting the invA gene which expressed with bands at 284 bp in the electrophoretic gel as shown in photo (1).

Forty *Salmonella* isolates were recovered from 384 apparently healthy budgerigars that belonged to 4 different serovars. The most common isolated serovar was *S.* Paratyphi A 19 out of 40 (47.5%), followed by *S.* Typhimurium 14 out of 40 (35%). Also, *S.* Chester 3 out of 40 (7.5%), 2 out of 40 (5%) for *S.* Infantis and 2 (5%) untypable *Salmonella* as seen in Table (3).

Table 2: *Incidence of Salmonella in captive budgerigars individually by SMT and PCR compared to pooling method.*

Sample source	No. of examined birds	+ve SMT No.	%	+ve PCR No.	%	No. of pooled samples	+ve pooled samples No.	%
Zoos group	168	19	11.31	42	25.00	30	16	53.33
Pet shops group	120	15	12.50	20	16.67	30	10	33.33
Household group	96	6	6.25	9	9.38	20	6	30.00
Total	384	40	10.42	71	18.49	80	32	40.00

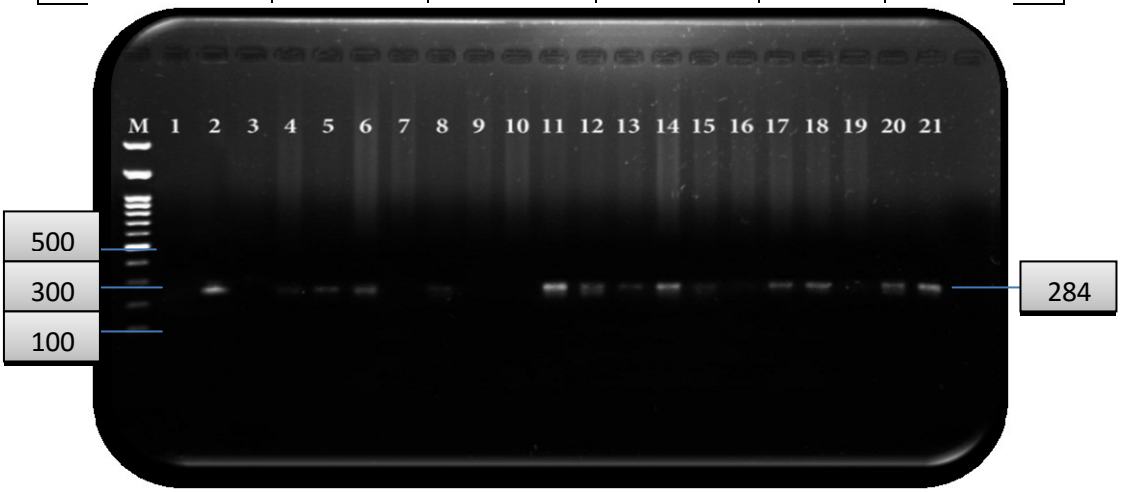

Figure 1: Electrophoretic gel showing positive bands at 284 bp of invA gene. M: 100 bp DNA ladder; lane 1: Control –ve; lane 2: Control +ve ; lanes: 4, 5, 6, 8, 11, 12, 13, 14, 15, 17, 18, 20, 21 are positive for invA gene; lanes 3, 7, 9, 10, 16, 19 are negative for invA gene.

Table (4) concerning *Salmonella* isolation with special reference to the seasonal variation showed that, *Salmonella* incidence was (87.5%, 7.5%, 5% and 0%) in Spring, Summer, Winter and Autumn respectively.

Table 3: *Serotyping and antigenic formula of 40 Salmonella isolates.*

***Salmonella* isolates**	**No. (40)**	**%**	**Somatic antigen "O"**	**Flagellar antigen "H"**	
				Phase I "H1"	**Phase II "H2"**
***S.* Paratyphi A**	19	47.50	2, 12	a	[1, 5]
***S.* Typhimurium**	14	35.00	1, 4, [5], 12	i	1, 2

***S.* Chester**	3	7.50	4	e, h	enx
***S.* Infantis**	2	5.00	6, 7	r	1, 5
Untypable strains	2	5.00	-	-	-

Table 4: *Salmonella isolation by SMT confirmed with serotyping with special reference to seasonal variations*

Season	+ve SMT Samples No.	%	*Salmonella* strains				
			S. Paraty-phi A	S. Typhim-urium	S. Chester	S. Infantis	S. Untypable
Autumn	0	00.0	0	0	0	0	0
Winter	2	5.0	2	0	0	0	0
Spring	35	87.5	17	12	3	2	1
Summer	3	7.5	0	2	0	0	1
All dates	40	100.	19	14	3	2	2

Discussion

The budgerigar is one of the earliest known captive psittacines, it is the third most popular pet in the world after the domesticated dog and cat ***(Perrins, 2003)***. Salmonellosis is a common bacterial zoonotic disease and can be a serious disease of psittacine birds. Asymptomatic *Salmonella* carriage in wild birds is high, they acquire the organisms and become carriers without any visible signs and considered as apparently healthy birds **(*Tizard, 2004)***. *Salmonella* detection by SMT are generally time-consuming, tedious, costly and require well-trained technicians ***(Nori and Thong, 2010)***. In-vitro amplification of DNA by the PCR method is a powerful tool in microbiological diagnostics ***(Malorny et al, 2003).***

There is no doubt that, the incidence rate of *Salmonella* obtained by PCR is more accurate, reliable and true. However, both results (10.42% and 18.49%) were relatively lower than that of ***(Deem et al, 2005)*** who recorded (67.3%) positive *Salmonella* in blue fronted Amazon parrot, and ***(Akhter et al, 2010)*** who isolated *Salmonella* with percentage of (46.67%) from faeces of apparently healthy caged parrots. The results were nearly similar to ***(Rigby et al, 1981)***. Also, they were higher than that approved by ***(Hidasi et al, 2013)*** who detected only one *Salmonella* spp. (0.20% of isolates and 0.33% of individuals) from faecal samples of 300 parrots. This may be attributed to different geographical range, food type and sampling techniques.

According to ***(Oliveira et al, 2003)*** Rappaport-Vassiliadis Broth (RVB) was sensitive for the detection and identification of *Salmonella* by PCR. Moreover, when the incidence of *Salmonella* detection by PCR was compared to the total number of individual enriched samples, it was found that, there was great increase in the total and in each

group individually by PCR as shown in Table (2). This could attributed to the advantage of PCR with selective enrichment to detect more positive samples than the SMT, because the selective enrichment dilutes PCR inhibitory substances and inhibits competitive microflora, which allows the target microorganism to grow thus increasing the quantity of target DNA. In addition, the failure to detect some *Salmonella* positive samples by SMT was possible related to the fact that isolates in these samples produced colonies lacking the characteristics of *Salmonella* colonies leading to false-negative results. It could be also related to the amount of *Salmonella* present on the sample.

A technique that can further increases the efficiency of processing large numbers of samples is the incorporation of sample pooling ***(Singer et al, 2006)***. As a field trial, 384 enriched (RVB) faecal samples were pooled into 80 pools by using 96-well numbering plate. *inv*A gene was recognized as a unique gene and an international standard for the detection of *Salmonella* species. Therefore, it was used in PCR amplification of pooled samples for *Salmonella* detection. As seen in Table (2) and Photo (1), the percentage of positive pooled samples generated 284 bp DNA fragments was (40%). This result much higher than ***(Jafari et al, 2007)*** who reported that, 5 out of 85 (5.8%) of pooled faecal samples from chickens were positive for *Salmonella*. This may be attributed to higher incidence of *Salmonella* in budgerigars.

These results revealed that, pooling was considered as initial screening method that ideally eliminates all *Salmonella*-negative samples from further analysis and results in the isolation of individual colonies from all *Salmonella*-positive samples.

The sensitivity of the PCR was determined to be (100%) when it was compared with SMT. This value agreed with ***(Gamal-Eldein et al, 2008 and Sareyyüpoğlu et al, 2008)***. In the contrary, it was higher than ***(Weeks et al, 2002)*** who reported (80%) sensitivity, while, the specificity was (91%), this result was lower than that of ***(Gamal-Eldein et al, 2008 and Sareyyüpoğlu et al, 2008)*** who recorded (95% and 99%) respectively. No amplification could be observed with bacterial strain other than *Salmonella* strains. This indicated the higher sensitivity and specificity of the PCR method. The accuracy of the PCR were determined respectively to be (92%).

The results showed in Table (3) approved that, 4 different serovars of *Salmonella* were recovered from apparently healthy budgerigars including human-specific serotypes such as *S.* Paratyphi A which cause enteric fever in humans and *S.* Typhimurium which is the common etiologic agent of salmonellosis in

humans ***(Lin et al, 2007)***. *S.* Paratyphi A was the most common isolated serovars and this disagreed with ***(Sanchez et al, 2002 and Styles, 2005)*** who approved that, *S.* Typhimurium was the most isolated serotype from budgerigars and other psittacine birds. 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 *Salmonella*.

The effect of different seasons on *Salmonella* incidence in budgerigars was reported in Table (4). The highest isolation rate (87.5%) was in Spring compared to total isolates in different seasons, followed by (7.5%) in Summer, (5%) in Winter and completely absent in Autumn. It could be attributed to the activation of *Salmonella* growth with temperature variation in Spring, overcrowding, bad hygiene and spread of insects or rodents. This disagreed with ***(Mahmud et al, 2011)*** who cleared that, *Salmonella* infection was higher (23.6%) in Summer than in Winter (12.9%) season. This may be due to low number of collected samples in Summer in relation to Spring, or due to different localities and geographical areas.

In conclusion, RVB-PCR was able to detect the *Salmonella* from apparently healthy birds that present at even low level or could detect the non-cultural or the nonviable *Salmonella* from faeces. Furthermore, it has the ability to detect and allow analysis of minute amounts of microbial DNA sequences. Moreover, the *inv*A gene sequence of *Salmonella* can be used as rapid, sensitive and accurate method for direct detection of *Salmonella* in the faecal samples of carrier captive birds.

It is recommended to make periodical examination for captive budgerigars in different rearing systems by pooling method as it is initial screening for the case study. When showed positive; individual detection must be done by PCR technique.

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الفحص التشخيصي للسالمونيلا في طيور الدر الاسترالية الأسيرة باستخدام الطرق التقليدية وتفاعل البلمرة المتسلسل مع الرجوع إلى تغيرات فصول السنة

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الملخص العربي

تم تجميع ومعالجة ٣٨٤ عينة براز مِنْ طيور الدر الاسترالية السليمة ظاهرياً لفحص حالة السالمونيلا باستعمال التقنيات الميكروبيولوجية القياسية وتفاعل البلمرة المتسلسل و*inv*A gene . كَانتْ نسبة السالمونيلا بالزرع البكتريولوجي (١٠,٤٢%) بينما كانت (١٨,٤٩%) باستعمال تفاعل البلمرة المتسلسل. نسبة انتشار السالمونيلا بتقنياتِ الميكروبيولوجي القياسية كَانتْ (١١,٣١%، ١٢,٥٠% و٦,٢٥%) وبواسطة تفاعل البلمرة المتسلسل كانت (٢٥ %، ١٦,٦٧% و٩,٣٨ %) في حدائق الحيوانات، محلات طيور الزينة والمجموعات المنزلية على التوالي. نسبة الحسّاسية والخصوصية لتفاعل البلمرة المتسلسل كانتا (١٠٠ % و٩١ %)على التوالي. يعتبر تجمع واشتراك العينات طريقة فحص أوليةِ والتي تستبعد كليا كُلّ عينات السالمونيلا السلبيةِ. تفاعل البلمرة المتسلسل للبراز المُغنى مَع *inv*A gene يُمْكِنُ أَنْ يُستَعملَ كطريقة سريعة وحسّاسة للكشفِ المباشرِ عن السالمونيلا في براز الطيورِ البرّيةِ الأسيرة الحاملة للمرض. نسبة عزل السالمونيلا تتأثر بتغير فصول السنة: وكانت النسبة (٨٧,٥%، ٧,٥%، ٥%) في فصل الربيع و الصيف و الشتاء على التوالي ولم يتم عزلها في فصل الخريف.