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Detection of antigen of *Mycobactrium avium* and *Salmonella species* from caged pet birds

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Abstract

Pet birds are a not-so-well known veterinarian's client ship fraction. These birds are potential carriers and/or transmitters of zoonotic diseases. This study summarized the status of some selected zoonotic bacteria in some pet shops such as *Mycobacterium avium subsp. avium* (*MAA*) and *Salmonella* infection. A total of 230 fecal samples were collected and examined by traditional and molecular techniques. The prevalence was 11(4.7%) and 16(7%) by culture in *MAA* and *Salmonella*, respectively. Higher prevalence of *MAA* was detected in older birds 8/11 and female birds 7/11. Moreover, fecal samples were examined for all different types of atypical *Mycobacteria* and revealed total prevalence of (8.2%). Studying the antibiogram and detection of some virulence genes (*inv*A, *avr*A and *sop*B) were carried out on the isolated *Salmonella spp*. All isolated *Salmonella species* were serotyped as *Salmonella Typhimurium*. The public health and environmental significance of the isolated strains was discussed.

Keywords: *Pet birds, Mycobactrium avium, Salmonella species.*

Introduction

The term "Pet bird" designates birds housed and bred for an exclusively ornamental use (**Tully, 2009**). Pet birds associated bacterial zoonoses represent a relatively neglected area compared with food borne zoonoses. The close contact between household pets and people offers favourable conditions for transmission by direct contact (e.g. petting, licking or physical injuries) or indirectly through contamination of food and domestic environments (**Song *et. al.*, 2013**). The overcrowding also induces intense stress to the birds and cause quick debilitation of the weakest individuals and higher sensitivity to infections (**Boseret *et al.* 2006**). *MAA* is the etiologic agent of avian tuberculosis, a chronic contagious disease described in a wide variety of domestic and wild bird species (**OIE, 2018**). It is primarily described an intestinal disease, passing between susceptible hosts by fecal oral rout of transmission (**Pollock, 2006**). The infection leads to a slowly progressive disseminated disease with a protected preclinical phase at which large number of bacilli may be shed in the feces where they can survive in the soil for several years (**Cromie *et al.,* 1993**). In diagnosis of *MAA*, Microscopical examination, culturing and/or molecular methods remains the most definitive means of diagnosing mycobacterial infection in birds (**Yang and Fang, 2010**). *Salmonella species* are one of the major causes of food poisoning diseases of human and birds. It is the most responsible cause of transmissions to human causing many outbreaks in last years (**Gast, 2013**). It is mainly diagnosed by isolation on selective media and the suspected colonies confirmed biochemically and serologically **(Fakher *et al.* 2006).** The aim of this study was to declare the prevalence of *MAA*, other atypical *Mycobacteria spp.*, *Salmonella* infection, their antibiogram and detection of virulence genes in the isolates in pet birds in some selected pet shops

to protect human and other contacting birds or animals and environment.

Materials and Methods

Sampling:

A total 230 of fecal samples were collected from pet birds (either individually or on sterilized filter paper) at popular markets, shops and clinics in different area such as (Zebra finch, Cockatiel, Yellow faced amazon, Palm cockatoo, Indian ringnecked parakeet, Lesser sulphur-crested cockatoo and Zebra).

Each sample was divided into two parts; one for detection of *MAA* and the other was submitted to Reference Lab. for Veterinary Quality Control on Poultry production for examination of the presence of *Salmonella species*.

Isolation and Identification of *Mycobacterium avium subsp. avium*

Sample processing and decontamination:

Samples were preprocessed with hexadecylpyridinium (HPC) in order to eliminate rapidly growing microorganisms according to **(OIE, 2018)**.

Identification of the agent by Zehil-Nelseen staining:

Loopfull from the decontaminated sediment was stained by Zeihl-Nelseen stain and inspected under oil immersion lens (**Shitaye *et al*., 2008).**

Isolation of the agent by culture:

The decontaminated sediment was cultured on Lowenstein–Jensen, Middlebrook 7H10 and 7H11 media and inspected daily in the first week, then weekly until 8 weeks (**OIE, 2018**).

Biochemical identification of the AFB isolates Biochemical identification of the AFB isolates was based on multiple tests, which included the p-nitrobenzoic acid (PNB) growth assay, niacin production, Tween-80 hydrolysis, heat stable catalase activity, and nitrate reductase activity (**Yang and Fang, 2010**) and (**Sharam *et al*., 2013**).

Polymerase Chain Reaction (PCR) analysis:

Extraction of the DNA and sampling during extraction:

(According to thermo scientific, GeneJET Genomic purification kit #K0721, #0722)

Detection of M. tuberculosis complex.

Real time PCR was performed according to the kit obtained from biovision® Real-time PCR according to (**Cousins *et al.,* 2011**).

The reaction was run in Applied Biosystem StepOne Real Time PCR System and FAM fluorogenic signal was collected and the cycle threshold of the reactions was detected by StepOne ™ software version 2.2.2 (Life Technology).

Table (1). Comparative analysis of different techniques used for diagnosis of avian tuberculosis **(Radomski *et al*. 2010).**

Type of test	Performed in	Time for reporting results	Diagnosis					
			Rapid	Easy	Specific	Sensitive	Definitive	Differential
Acid fast staining	Live bird	1 hr	+	+	-	-	-	-
Culture		1-2 month	-	-	+	+	-	-
PCR		4 days	-	-	++	++	-	-
Real time PCR		4-6 days	-	-	++	++	-	-

Isolation and identification of *Salmonella* spp.

Isolation, identification and Antibiotic sensitivity test

All steps were done according to **(ISO 6579, 2002)**, Serological identification of *Salmonella* species was done according to (**Grimont and Weill, 2007**) by using diagnostic antisera (Sifin).

Antibiotic sensitivity test

The antibiogram of *Salmonella* isolates was done by disc-diffusion test against 9 antimicribial agents ampicillin, chloramphenicol, ciprofloxacin, gentamicin, levofloxacin, nalidixic acid, norfloxacin, trimethoprim-sulfamethoxazole, according to the Clinical and Laboratory Standards Institute/ Formerly National Committee for Clinical Laboratory Standard **(CLSI/NCCLS, 2009**).

Briefly, 3-5 pure bacterial colonies were selected and put on 2 mL Muller Hinton broth in test tube. The test tubes were incubated at 37°C aerobically for slight turbidity compared against 0.5 McFarland tube. Muller Hinton agar plate was inoculated with previously prepared culture using sterile bacterial cotton swab in three different directions. Then the antibiotic disks were distributed on the inoculated plate. The plate was incubated at 37°C aerobically for 24 hrs as previously described. Inhibition zones were measured to detect the resistant and sensitive strains.

Polymerase chain reaction (PCR) for detection of virulence genes of *Salmonella Typhimurium* isolates:

Conventional PCR technique was used for detection of virulence determinants by detection of 3 virulence genes (*Inv*A, *avr*A, *sop*B,) in the 16 *Salmonella Typhimurium* strains by conventional PCR technique .

DNA extraction was performed using QIAamp DNA mini kit (Qiagen, Germany, GmbH Catalogue no.51304).

Oligonucleotide primer: primers were used supplied from metabion (Germany) and PCR conditions were mentioned as in Table (2). All isolates were confirmed by using Conventional PCR technique for detection of *inv*A gene.

PCR amplification: a volume of 25 µL PCR reaction containing 12.5 µL of Emerald Amp Max PCR Master Mix (Emerald, Japan), 1 µL of each primer of 20 pmol concentrations, 4.5 µL of Depic water and 6 µL of template was used in a Biometra thermal cycler. The reference strains provided by the External Quality Assurance Services (EQAS) were used as positive controls of *S. Typhimurium*. DNA of the negative control (*E. coli* NCIMB 50034).

Analysis of the PCR products: The PCR products were separated by electrophoresis on 1% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V\cm. for gel analysis 5 µL of the products was loaded in each gel slot. Gene ruler 100-1000 bp ladder. Thermo scientific was used to determine the fragment size. The gel was photographed using a gel documentation system (applied).

Table (2). Virulence genes targets and primers, including nucleotide sequences, PCR conditions, and references

Gene Designation	Location on SP1/gene function	Oligonucleotide sequences (5\–3\)	PCR conditions [a]			Product Size (bp)	References
			Denaturing	Annealing	Extensio n		
*inv*A	Type III secretion system apparatus SPI-1/invasion of macrophages	**F:** GTG AAA TTA TCG CCA CGT TCG GGCAA **R:**TCA TCG CAC CGT CAA AGG AACG	94ºC for 60 seconds	64ºC for 30 seconds	72ºC for 30 seconds[b]	284	**Salehi *et al.*, `2005**
*sop*B	SPI-5/inositol polyphosphate, phosphatase that promotes macropinocytosis, regulates SCV localization, and promotes fluid secretion	**F**: TCA GAA GRC GTC TAA CCA CTC **R**:TAC CGT CCT CAT GCA CAC TC	95ºC for 30 seconds	64ºC for 30 seconds	72ºC for 30 s seconds[b]	517	**Huehn *et al.*, 2010**
*avr*A	SPI-1/controls *Salmonella*-induced inflammation	**F:**CCT GTA TTG TTG AGC GTC TGG **R:**AGA AGA GCT TCG TTG AAT GTCC	95ºC for 30 seconds	64ºC for 30 seconds	72ºC for 30 seconds[b]	422	

a PCR was done for 35 cycles.
b After 30 cycles, final extension step of 4 minutes at 72ºC was performe

Results

Table (3). Infection rate of pet birds with *Mycobacterium avium subspecies avium* and *Salmonella spp.* in the examined samples based on culture technique

No. of examined samples	M. avium subspecies avium		*Salmonella spp.*	
	No.	%	No.	%
230	11	4.7	16	7

% calculated according to the no. of examined samples.

Table (4). Detection of Atypical *Mycobacteria* spp. isolated from fecal samples of pet birds according to biochemical tests.

No. of examined samples	Isolated Mycobacteria					Total isolated *Mycobacterium spp.*
	M. avium subsp. Avium	*M. fortutum*	*M. chelonae*	*M. diernhoferi*	*M. peregrinum*	
230	11	4	2	1	1	19/230 (8.2%)

Table (5). Comparison between different diagnostic techniques used in detection of *Mycobacterium avium.*

No. of examined samples	Used techniques					
	Microscopical examination		Culture		Real time PCR	
	No.	%	No.	%	No.	%
230	5	2.2	11	4.7	18	7.8

Table (6). Analysis of some risk factors associated with the prevalence of avian tuberculosis in pet birds.

Type of risk	Total no. of positive samples	Rate of infection
Age	11	
More than 5 years		8/11
Less than 5 years		3/11
Sex		
Female		7/11
Male		4/11

Table (7). Serotyping of *Salmonella Typhimurium*

Serovar	"O" antigens	Phase 1"H" antigens	Phase 2"H" antigens
Salmonella Typhimurium	1, 4,[5],12	I	1,2

Table (8). Results of antibiotic sensitivity test of *Salmonella* isolates

antimicrobial Discs	**Potency** (n=16)			
	Resistant		**Sensitive**	
	No.	**%**	**No.**	**%**
Ampicillin	11	69%	5	**31%**
Chloramphenicol	6	37. 5%	10	**62. 5%**
Ciprofloxacin	0	0%	16	**100%**
Gentamicin	0	0%	16	**100%**
Levofloxacin	0	0%	16	**100%**
Nalidixic acid	16	100%	0	**0%**
Norfloxacin	6	37. 5%	10	**62. 5%**
Trimethoprim-sulfamethoxazole	0	0%	16	**100%**
Tetracycline	**0**	**0%**	**16**	**100%**

% calculated according to the no. of tested *Salmonella Typhimrium* isolates (16).

Results of Real time PCR used for detection of *M. avium subsp. Avium:*

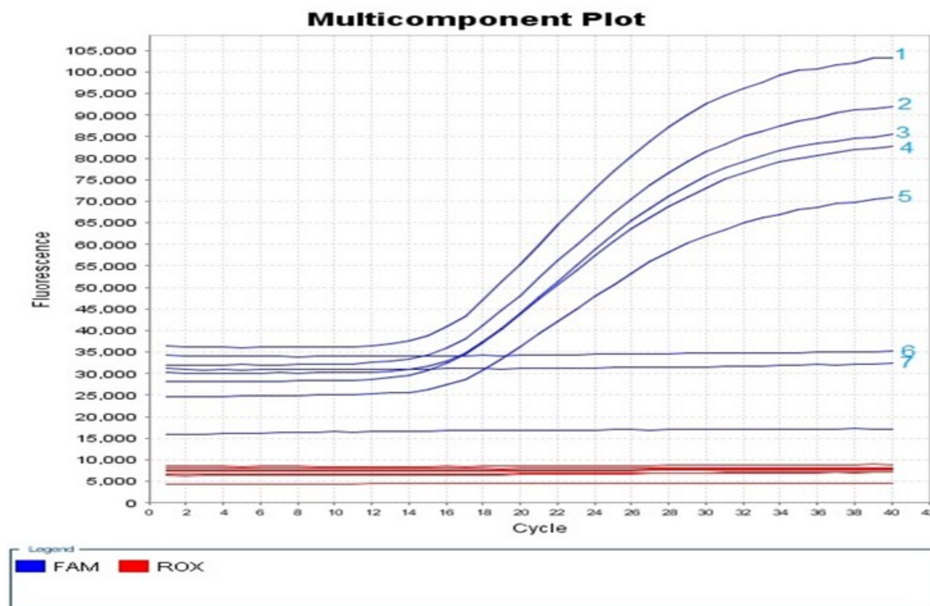

Fig. (1): Showing some results of real time PCR.
1 showing positive control of *Mycobacterium avium subsp. avium*
From 2 to 5 showing positive samples “ their Ct ranges from 14 to 20”
6 showing negative control and samples.
7 showing negative samples
All curves their Ct ranges from 14 to 20 indicating positive samples

Polymerase chain reaction (PCR) for detection of resistant genes in isolated strains of *Salmonella Typhimurium*:

All *Salmonella Typhimurium* were examined by for presence of 3 virulence genes *inv*A, *avr*A and *sop*B genes by using conventional PCR and the results were *inv*A gene were found in all tested isolates, the *avr*A gene were found in 33.3% of samples and *Sop*B genes were found in 66.7% of Samples.

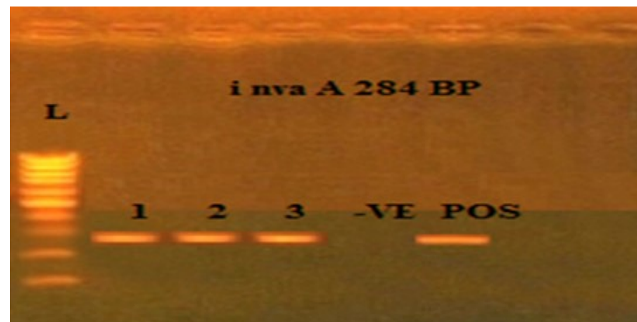

Photo. (1): Agarose gel electrophoresis showing *Salmonella* specific PCR of *Salmonella Typhimurium* using primer set for the *inv*A (284 bp) gene

Lanes 1 - 3: positive samples of *Salmonella* Typhimurium
Lane -VE: Negative control (*E. coli* NCIMB 50034)
Lane POS: Positive control (*Salmonella Typhimurium* EQAS)
Lane L: DNA ladder.

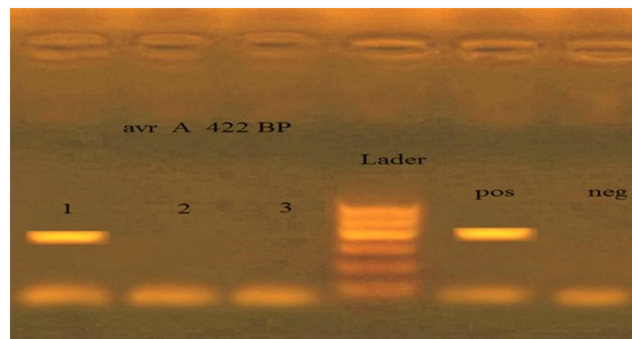

Photo. (2): Agarose gel electrophoresis showing *Salmonella* specific PCR of *Salmonella Typhimurium* using primer set for the *avr*A (422 bp) gene

Lane Ladder: DNA ladder
Lanes 1: positive *Salmonella Typhimurium* samples for *avr*A
Lane neg: Negative control (*E. coli* NCIMB 50034)
Lane pos: Positive control (*Salmonella Typhimurium* EQAS) .

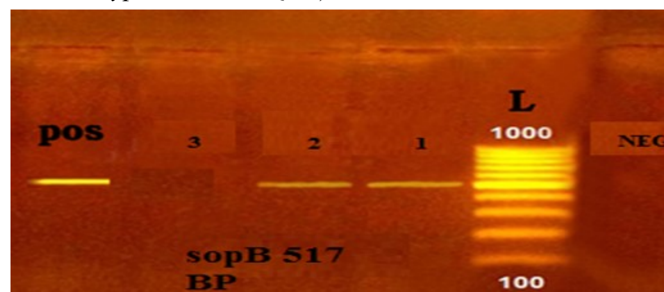

Photo. (3): Agarose gel electrophoresis showing *Salmonella* specific PCR of *Salmonella Typhimurium* using primer set for the *sop*B (517 bp) gene

Lane L: DNA ladder
Lanes 1-2: positive *Salmonella Typhimurium* samples for *sop*B
Lane neg: Negative control (*E. coli* NCIMB 50034)
Lane pos: Positive control (*Salmonella Typhimurium* EQAS)

Discussion

Pet-birds are potential carriers and/or transmitters of zoonotic diseases. Some of them could have an important impact on human health. This study summarized the status of two of the most important bacterial zoonoses in some pet shops such as *Mycobacterium avium subsp. avium* (*MAA*) and *Salmonella* infection.

In most birds, avian mycobacteriosis primarily involves the alimentary tract. The mycobacteria are usually ingested and initially colonize the intestinal wall. Tubercles that form in the intestinal wall allow for fecal shedding of *M. avium* (**Cromie *et al.*, 1993**) where they can survive in the soil for several years (**Doneley *et al.*, 1999**).

In this study we examined 230 fecal samples of pet birds for presence of *MAA*, our results showed that, the infection rate of *MAA* is (4.7%) from the examined birds drooping's. Higher results were recorded by (**Dvorska *et al.*, 2007**) who detect the infection rate with *MAA* (11.1%). Moreover, (**Shitaye *et al.*, 2008**) found higher isolation rate 42.9% (9/21). Therefore, the true prevalence remains unknown because of the long incubation and preclinical periods following exposure, which can vary widely, may have masked any short-term trends in incidence. Moreover, quarantine prevents group-to-group spread but does not curb spread within a quarantined enclosure (**Pollock, 2006**).

The isolation of *MAA* infection determined in this study from fecal samples suggests that the birds become infected through the alimentary tract as described in other studies as the most common rout of infection **(Tell *et al.*, 2001).**

In comparing different diagnostic techniques for isolation of *MAA*; microscopical examination revealed 5(2.2%) A fecal acid fast stain was performed but, in this case, was not diagnostic because few acid-fast bacteria were observed. The fecal acid-fast stain is relatively insensitive and requires the presence of approximately 10,000 bacteria per gram of feces to be detected. **(Gerlach, 1994).** Also, it is difficult to differentiate between pathogenic and nonpathogenic strains of *Mycobacteria*, both of which may be present in the feces **(Ellingson *et al.*, 1998).**

In many cases, smear microscopy fails to stain the bacteria by standard methods. This is the major limitation of this technique. Moreover, this technique depends on the microbial load. If the microbial load is low in the sample then it cannot be used successfully to observe the bacteria in the sample.

Fecal culture which is the golden standard technique as indicated by (**OIE, 2018**) revealed 11(4.7%) isolation rate, but its drawbacks are; time consumption, especial care in handling clinical specimens and probability of growth of other rapidly growing *Mycobacteria* which contaminate the media producing false negative results and decreasing the sensitivity and specificity **(Mayahi *et al.,* 2013).** Lower fecal culture results was indicated by (**Shitaye *et al.,* 2008**) who detected only one positive sample in some area and no positive samples in other area.

Molecular technique's results indicated 18 (7.8%). It is rapid and accurate method for diagnosis of avian tuberculosis. Nowadays, quantative real time PCR is considered to be the best method. The only drawback, its high costs (**Manarolla *et al.,* 2009**).

Higher PCR results was indicated by (**Lena *et al.,* 2018**) as 38(18%) in central pacific area while (**Hernandez-Drivers *et al.,* 2008)** detected within average results as (2/45, 4%).

In analysis of some risk factors associated with prevalence of avian tuberculosis in pet birds, our study revealed that, the prevalence was higher in elder ages than younger ages 8/11 and 3/11, respectively. That is due to longer exposure period providing more time for expression of the slowly progressive disease. Similar results was indicated by (**Kindu and Getaneh, 2016**) who detected higher prevalence in older ages than younger (68.4%) and (31.6%), respectively on the other hand, different results were obtained by (**Witte *et al.,* 2010**) who detected higher prevalence of avian Mycobacteriosis in younger birds than adult one.

Concerning sex as a risk factor, higher prevalence was indicated in female birds than males as 7(63.6%) and 4(36.4%), respectively. Different results were estimated by (**witte *et al.,* 2010**) who detected higher prevalence in male birds (50.2%) than female birds (45.8%). While similar results were indicated by (**Kindu**

and Getaneh, 2016) who detected higher results in female birds than male birds as (93.6%) and (6.4%), respectively.
Salmonella species are one of the major causes of food poisoning diseases of human and birds. In this study 230 fecal samples were examined for the presence of *Salmonella species* and we detected *Salmonella species* in 16(7%) out of 230.
This results was disagreed with (**Marin *et al.,* 2014**) who isolated *Salmonella species* in high precent from wild bird (52.6%), however, in agreement with (**Seepersadsingh and Adesiyun, 2003**) who isolated the *Salmonella species* in low percent in (0.9 %) from examined pet bird.
All *Salmonella species* isolated in this study was serotyped *Salmonella Typhimurium* this result was compatible with what (**Madadgar *et al.,* 2009**) who reported that all *Salmonella* isolates from pet birds were also *Salmonella Typhimurium* and not compatible with (**Seeper -sadsingh and Adesiyun 2003**) who did not found *Salmonella Ttyphimurium* in the *Salmonella* isolates which isolated from the examined pet birds.
All *Salmonella* isolates were examined for antimicrobial susceptibility against 9 different antimicrobial agents. All isolates were highly sensitive to Ciprofloxacin, Gentamicin, Levofloxacin, Trimethoprim sulfamethoxazole, Tetracycline by 100% and moderately sensitive to Norfloxacin, Chloramphenicol by 62.5% and less sensitive to Ampicillin by 31% while all isolates were resistant to Nalidixic acidour.
Our results was in agreement with (**Matias *et al.,* 2016**) who examined *Salmonella Typhimurium* isolate against most of antibiotics which we used like ampicillin, chloramphenicol, gentamicin; nalidixic acid; ciprofloxacin, tetracycline, and trimethoprim/sulfamethoxazole. His result was agree with our results with gentamicin, nalidixic acid; ciprofloxacin, tetracycline, and trimethoprim/sulfamethoxazole, while disagree with results of ampicillin, and chloramphenicol.
All *Salmonella Typhimurium* isolates were examined by PCR for presence of 3 virulence genes *inv*A, *avr*A and *sop*B genes. *inv*A was found in all isolates and *avr*A gene was found in 33.3% of isolates, this result was agreed with (**Elkenany *et al.,* 2019**) who found *inv*A gene in all examined isolates and *arv*A gene in 38.9% of examined and disagree with (**Osman *et al.,* 2014**) who did not found *avr*A gene in examined *Salmonella Typhimurium* isolates. While *sop*B gene was detected in 66.7 %of isolates and this result was agreed with (**Osman *et al*., 2014**) and (**Krawiec *et al.,* 2015**) who detected *sop*B gene in 100% and 94.7% respectively.

Recommendations

Concerning Avian tuberculosis, treatment of birds is not recommended due to the long incubation period of the disease and the appearance of signs in some birds only and commonly in late stage of the disease with intermittent shedding of the organism in the environment which can survive in the soil for several years, it become a must to perform a regular survey in pet birds due to their life longevity which increase the risk of carrying and spreading infection to other birds, animals and human.
Bird acquisition and breeding are a popular and a commercial hobby. This bird can transmit some of the zoonotic diseases like Avian tuberculosis and Salmonellosis so we must disseminate health awareness to preserve of different bird species we must do some steps:
In pet birds shops and markets we must disinfect the birdhouses and provide a good and clean feed and water to birds
Culling of the affected bird flock.
Follow strict biosecurity procedures along with good hygienic practices.
Pet birds in house must be handled in care with special clothes, gloves and regular disinfection of cages.
Regular monitoring of pet bird with tuberculin test.

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ination of Salmonella

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