

Isolation of Bacteria From Dead - In - Shell Chick Embryos of Psittacines

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ABSTRACT

The objective of the present study is to explore the bacteria associated with the dead-in-shell eggs of psittacines. A total of 132 dead-in-shell eggs of different psittacine breeds (Macaws, Lories, Conures, Amazons, Eclectus, Cockatoos, Grey parrots and Jardine parrots) were obtained from the incubation unit of Cavin estate exotic bird farm, Chennai, Tamil Nadu and subjected to microbiological analysis. The overall prevalence of bacteria isolated from the dead-in-shell was 80.30% (106/132). Among these, Gram-negative bacteria were predominant (49.24%, 65/132) was observed followed by Gram-positive organisms (28.78%, 38/132) and Gram-positive filamentous organisms (2.27%, 3/132) were also isolated from dead in shell embryos. Among the Gram-positive bacteria, *Staphylococcus* sp. (21.12%) was found to be more followed by *Bacillus* sp. (3.78%), *Corynebacterium* sp. (2.2%) and *Enterococcus* sp. (1.51%). Similarly, among the Gram-negative bacteria, *Escherichia* sp. (25%) was identified more followed by *Pseudomonas* sp. (6.60%), *Serratia* sp. (6.60%), *Acinetobacter* sp. (4.5%), *Enterobacter* sp. (3.78%), *Yersinia* sp. (3.77%), *Citrobacter* sp. (2.27%), *Buttiauxella* sp. (1.52%), *Klebsiella* sp. (1.51%), *Cedeaceae* sp. (1.51%) and *Erwinia* sp. (0.75%). Among the psittacine species, more numbers of the dead in shell cases were found to be in conures (37%) and lories (14.5%). Commonly found bacterial isolates were subjected to antibiotic sensitivity test. All the isolates were found to be sensitive to enrofloxacin and cefotaxime whereas, most of the bacteria showed resistance to trimethoprim + sulphamethoxazole and kanamycin. Resistance to other antibiotics were variable. Multiple resistance was not observed for any of the isolate.

Key words: Dead in shell, Embryos, Psittacine, Lories, bacterial isolates

INTRODUCTION

Psittacines or parrots and parakeets, which belong to the order Psittaciformes

are popular display animals and pets due to their colourful behaviours and widely recognised intelligence. Psittacine birds are characterised by having a

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strong, curved bill, an upright stance, strong legs, and clawed zygodactyl feet (Reavill and Dorrestein, 2018). They are distributed mostly in the tropical and subtropical continents and regions including Australia, south east Asia, central and South America, Africa, Caribbean and Pacific islands. Many parrots are brightly coloured, and some are multi-coloured. Despite these unique and attractive characters, psittacines lack in parenting which may affect the production of live young birds. Further, natural incubation of captive psittacines depends on many factors including parent's breeding behaviour, health, diet, species, origin (wild caught or hand raised), experience, environment, egg integrity, and nest box design (Martin and Romagnano, 2006).

Artificial incubation is now being practiced which has tremendous insight in the field of psittacine breeding. This is more beneficial when compared to the natural incubation as it prevents the cross contamination of disease from the parents to off-springs and therefore the probability of developing healthy chicks increased to a higher extent.

A dead-in-shell embryo is of the major emerging problem with the artificial incubation. Bacteria represent primary or secondary agent of dead-in-shell embryos. A number of general bacteria especially in the family *Enterobacteriaceae* can be isolated from dead embryos, dead-in-shell, weak chicks or infected yolk sacs of young birds (Barrow, 1994). Trans-shell transmission, translocation from the digestive tract and vertical transmission are the ways in which bacteria gain access to the developing

embryo (Bruce and Drysdale, 1994). Therefore, microbiological examination is required to study the reason behind the failure in hatching. Literature available on the etiological agent responsible for dead-in-shell embryos in artificial incubation of psittacines is limited. The objective of the study is to identify and explore the plethora of bacterial pathogens that are associated with the dead in shell mortalities.

MATERIALS AND METHODS

Sample collection

Cavin Estates Exotic birds farm, Chennai is located on the south-eastern coast of India in the north-eastern part of Tamil Nadu on a flat coastal plain known as the Eastern Coastal Plains. A wide variety of exotic birds *viz.* Amazons, Macaws, Lories, Lorikeets, Cockatoos, Conures and Eclectus are available at Cavin estates. The birds are maintained in aviaries with nest box facilities to enhance breeding. A separate incubation unit is functioning in cavin estates for artificial incubation of eggs collected from those captive psittacines. The incubators are maintained at a temperature of 37.3°C and humidity of 40 to 60%. The eggs are subjected to 10 -12 rotations per day

A total of one hundred and thirty two dead-in-shell eggs were collected from the incubation unit of cavin estates farm from June to December 2018. Eggs collected were preserved in refrigerator. Each egg was carefully opened under sterile conditions and swabs from the unabsorbed yolk, egg white and visceral organs of the embryos were aseptically collected and stored for bacteriological examination.

Bacteriological screening

The yolk swabs were inoculated in nutrient broth and incubated at 37°C for 18 hrs. The broth cultures were then streaked on nutrient agar and incubated at 37°C for 24 hrs. After incubation, the agar plates were examined for cultural characteristics by determining the size, shape, elevation, edges, surface, and colour of the colonies. Well isolated colonies were subcultured on to selective media to obtain pure colonies. The pure colonies were subjected to Gram's staining and examined under the oil immersion (100x) to classify them as Gram positive or Gram negative (Merchant and Packer, 1967 and Cheesbrough, 1991)

Gram positive bacteria were identified by their morphology (rods, cocci, spore forming), mannitol fermentation, haemolysis on blood agar, catalase test and bile aesculin hydrolysis. Gram negative bacterial colonies were inoculated on MacConkey agar to differentiate lactose fermenters from non lactose fermenters and then subjected to a set of standard biochemical tests *viz.* indole, methyl red, Voges-Proskauer, citrate utilisation, triple sugar iron agar test, lysine decarboxylase, bile aesculin hydrolysis, urease test and oxidase test. *Salmonella* sp. was confirmed by production of black centered colonies on XLD agar and *E. coli* was confirmed by growth of colonies with green metallic sheen on EMB agar. The results were analysed as per the guidelines of Bergey's manual of systematic bacteriology (Bergey et al., 1994) and ABIS online analytical tool in the following website address.

(http://www.tgw1916.net/bacteria_abis.html).

All media used in this study were prepared following the manufacturer's (Himedia) recommendations. Antibiotic sensitivity test for the bacterial isolates was carried out using the standard disk diffusion method (Bauer et al., 1966) using Mueller Hinton agar plates. A total of six antibiotic discs were placed at equal distance and plates were incubated at 37 °C for 18 to 24 hrs. The sensitivity and resistant patterns of the isolates were derived by measuring the diameter of inhibition zone around the antibiotic discs following the manufacturer's (Himedia) instructions. Antimicrobial agents used in the study were cefataxaime 30 mcg (CTX), enrofloxacin 5 mcg (ENR), tetracycline 30 mcg (T30), kanamycin 30 mcg (K), gentamicin 10mcg (GEN) and trimethoprim + sulphamethoxazole 1.25/23.75 mcg (COT).

RESULTS AND DISCUSSION

The overall prevalence of bacteria isolated from the dead-in-shell chick embryos of psittacines was 80.30% (106/132). Among these, high level of Gram-negative organisms (49.24%, 65/132) was observed followed by Gram-positive organisms (28.78%, 38/132) and Gram-positive filamentous bacteria (2.27%, 3/132) were also isolated from the dead in shell chick embryos (Table 1; Figure 1). Among the Gram-positive bacteria, *Staphylococcus* sp. (21.12%) was found to be more followed by *Bacillus* sp. (3.78%), *Corynebacterium* sp. (2.2%), and *Enterococcus* sp. (1.51%). Similarly, among the Gram-negative bacteria *Escherichia* sp. (25%) was identified more followed by *Pseudomonas* sp. (6.60%), *Serratia* sp. (6.60%), *Acinetobacter* sp.

(4.5%), *Enterobacter* sp. (3.78%), *Yersinia* sp. (3.77%), *Citrobacter* sp. (2.27%), *Buttiauxella* sp. (1.52%), *Klebsiella* sp. (1.51%), *Cedeacae* sp. (1.51%) and *Erwinia* sp. (0.75%) (Table 2). Among the psittacine species, more numbers of the dead in shell cases were found in conures (37%) and lorries (14.5%) depicted in figure.

2. Commonly found bacterial isolates were subjected to antibiotic sensitivity test. All the isolates were found to be sensitive to enrofloxacin and cefotaxime whereas, most of the bacteria showed resistance to trimethoprim + sulphamethoxazole and kanamycin. Resistance to other antibiotics were variable. Multiple resistance was not seen among the isolates.

Table 1 Bacterial isolates obtained from shell chick embryos of psittacines

Bacterial isolates obtained	Number of sample positive
Gram negative bacteria	65
Gram positive bacteria	38
Gram positive with filamentous type	3
Total	106

Fig. 1 Bacterial isolates obtained from shell chick embryos of psittacines

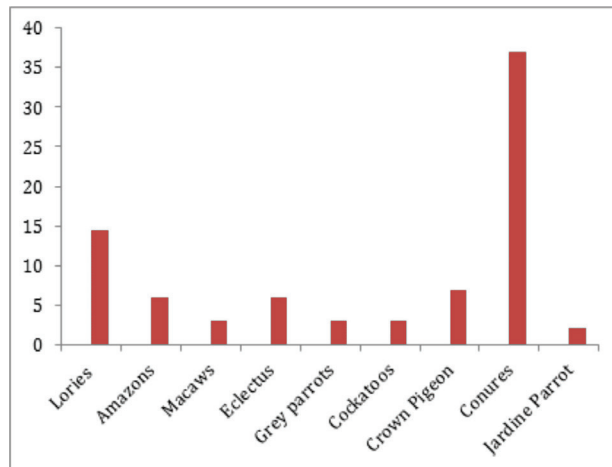


Table 2 List of Gram positive and gram negative bacteria isolated from different Psittacine species

Gram negative bacteria	Lories	Gray parrot	Amazon	Conures	Cockatoos	eclectus	Crown pigeon	Macaws	Total
<i>Acinetobacter</i> sp.		2		3		1			6
<i>Butiauxella</i> sp.			1						1
<i>Citrobacter</i> sp.	2		1						3
<i>Escherichia coli</i>	4	2		26	1				33
<i>Yersinia</i> sp.	1	1	1	2			1		6
<i>Enterobacter</i> sp.	3							1	4
<i>Pseudomonas</i> sp.						1			1
<i>Serratia</i> sp.	2							3	5
<i>Erwinia</i> sp.							1		1
<i>Cedeace</i> sp.							2		2
<i>Klebsiella</i> sp.		1		1					2
Gram positive bacteria									
<i>Bacillus</i> sp.		1	1	1		2			5
<i>Staphylococcus</i> sp.	6	1		14	1		6		28
<i>Enterococci</i> sp.				2					2
<i>Corynebacterium</i> sp.	1	2							3
Gram positive with filamentous type			1	2					3

Dead-in-shell in artificial incubation due to bacterial contamination is a critical issue and should be considered important as the exposure of eggs to environment is minimal when compared to that of natural incubation. The possible reasons for dead-in-shell in artificial incubation are faecal contamination of the egg shell, design and ventilation of hatchery, high stocking density, poor sanitation of hatchery room and their equipment resulting in poor hatchability. Out of the 132 samples screened, 106 samples showed bacterial contamination due to both Gram positive and

Gram negative bacteria. A total of 41 Gram positive bacteria and 65 Gram negative bacteria were isolated. The percentage of Gram negative bacteria isolated was higher than that of the Gram positive genera. This is correlated well with the results of Osman et al. (2013). *Escherichia* sp. was isolated most commonly among the Gram negative (25%) organism which is in accordance with the findings of Cortes *et al.* (2004) and Amer et al. (2017). The higher rate of *Escherichia coli* isolation in the dead-in-shell may be due to the presence of *E. coli* in faecal contaminated water which may

contaminate the egg shells (Cortes et al., 2004).

The other Gram negative bacteria isolated in higher percentage were *Acinetobacter* sp. (4.5%) and *Yersinia* sp. (3.75%). This result goes in parallel with the work of Osman et al. (2013) and Casadio et al. (2014). *Pseudomonas* sp., reported as a potential pathogen causing embryonic mortalities (Walker et al., 2002) was isolated in a higher percentage (6.60%) when compared to the other Gram negative genera next to *Escherichia* sp. In this study,

the incidence of *Salmonella* sp. is zero and this was in agreement with the results of Jahantigh. (2010). However Amer et al. (2017) and Osman et al. (2013) reported low percentage of *Salmonella* sp. in their studies. The other Gram negative bacteria isolated were *Enterobacter* sp. (3.78%), *Citrobacter* sp. (2.27%), *Buttiauxella* sp. (1.52%), *Klebsiella* sp. (1.51%), *Cedeaceae* sp. (1.51%) and *Erwinia* sp. (0.75%). The number of Gram positive and negative bacteria isolated and their percentage are listed in table 2.

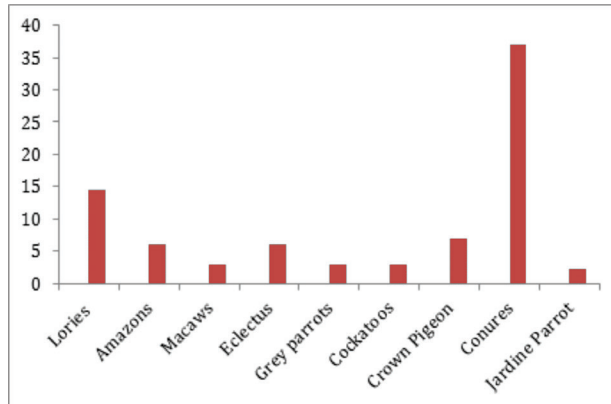
Table 3 Dead in shell mortality due to bacterial growth

Species	Number of samples examined	Number of showed bacterial growth	Positive percentage
Lories	24	19	79
Amazons	10	5	50
Macaws	09	4	44
Eclectus	12	4	33
Grey parrots	10	10	100
Cockatoos	04	2	50
Crown Pigeon	11	11	100
Conures	52	51	98
Total	132	106	

Among the Gram positive genera, *Staphylococcus* sp. was found to be in higher percentage (21.12%). This is similar to the results of Cortes et al. (2004). The other Gram positive bacteria isolated were *Bacillus* sp., *Enterococcus* sp. and *Corynebacterium* sp. as reported by many authors (Osman et al., 2013, Razmyar and Zamani, 2016 and Amer et al., 2017).

Most of the dead in shell cases were reported in Conures, followed by lories (Fig .2). No bacterial contamination was detected in 26 dead-in-shell cases. This may be due to malposition of the egg and irregular humidity levels in the incubation room as reported by other authors (Sanctuary et al., 1925) or fluctuating humidity levels (Jordan, 2001).

Fig 2 Overall dead in shell mortality percentage in different species of psittacine



In this study, it was observed that the frequency of isolation of pathogenic bacteria viz. *Pseudomonas* sp. and *Salmonella* sp. were lower or nil when compared to the *E. coli* and *Staphylococcus* sp. The results suggest that the egg contamination in artificial incubation may be due to improper handling of egg without sanitisation during shifting of eggs from the cage to incubator and moving eggs with in the incubator. The surface contaminants may penetrate the egg shell and enter the yolk sac which may probably results in contamination. The ability of bacteria viz. *Acinetobacter* sp., *Staphylococcus* sp. and *Serratia* sp. to penetrate ostrich egg shell has been studied (Knobl et al., 2012).

The antibiotic sensitivity profile of the most frequently isolated bacteria and bacteria of pathogenic importance was studied. Variable sensitivity/resistant patterns were observed among the isolates. Except *Staphylococcus* sp. and *Acinetobacter* sp., which showed sensitivity to all the antibiotics tested, the

others showed resistance to two or more antibiotics. The highest rate of resistance was against kanamycin and trimethoprim + sulphamethoxazole followed by gentamicin and tetracycline. All the isolates were found to be sensitive for cefotaxime and enrofloxacin. Most of the isolates showed resistance to kanamycin and trimethoprim + sulphamethoxazole. None of the isolates showed multidrug resistance. However, 100% resistance to three antibiotics were observed in *Klebsiella* sp. (gentamicin, kanamycin, trimethoprim + sulphamethoxazole) and *E.coli* (kanamycin, tetracycline and trimethoprim + sulphamethoxazole.).

It can be concluded that bacterial contamination was found to be the major challenge in artificial incubation in psittacine species. Thus, the study recommends the use of clean room facilities for artificial incubation and establishment of hygienic practices to avoid bacterial contamination thereby enhancing hatchability of eggs.

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