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Isolation and molecular identification of enteropathogen *E. coli* in captive pheasants of Arignar Anna zoological park, Vandalur Chennai, Tamil Nadu

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Abstract
Pheasants are susceptible to a wide range of diseases, in which enteropathogens are one of the major health problems, which might be transmitted from one group of birds to other. Seventy-six fresh dropping samples were collected aseptically using readymade transport medium swabs and transported immediately to the laboratory. All the samples were processed immediately for isolation and identification of important bacterial enteropathogen *E. coli* using standard conventional and molecular procedures. The isolation of *E. coli* began with collected cultural swabs were inoculated in EMB agar. KB010 is a comprehensive test system that can be used for identification biochemical characterization of *E. coli*. Molecular identification of *E. coli by* DNA extraction and polymerase chain reaction. Out of 76 samples, twenty three samples were positive (30.26% Per cent) and growth of *E. coli* organisms in EMB agar appeared as blackish centers with a green metallic sheen. Molecular Identification of *E. coli* by polymerase chain reaction the amplicon size was 477 bp.

Keywords: Captive pheasants enteropathogen *E. coli* isolation& identification–molecular method

Introduction
Pheasants belong to the family 'Phasianidae' and Order 'Galliformes'. Pheasants are brightly coloured, large bodied and ground dwelling birds. Pheasants are susceptible to a wide range of diseases, in which enteropathogens are one of the major health problems, which might be transmitted from one group of birds to other. This study helps in identification of enteropathogens providing accurate therapeutic and management measures to prevent the infection in captive pheasants of India as well as other countries and surely bring to lime light on various bacterial as well as parasitic agents related to diseases among captive pheasants. Further, the findings will help to place emphasis on effective prevention and control of diseases of zoonotic potential related to rearing of captive pheasants as well as better management of declining pheasants.

Materials and Methods
The fresh dropping samples were collected aseptically using readymade Cary-Blair medium transport swabs (Himedia, Mumbai, India), in suitable aseptic containers. For direct DNA extraction and PCR detection 2g of dropping samples were collected in suitable plastic packets with ice. All the samples were transported immediately to the laboratory under chilled conditions. All the samples were processed immediately for isolation and identification of important bacterial enteropathogen *E. coli* using standard conventional and molecular procedures.

Biochemical characterization of *E. coli*
KB010 is a comprehensive test system that can be used for identification and differentiation of *Escherichia coli*. *Escherichia coli* are gram negative, lactose fermenting cocco-bacillary rods which are frequently isolated from food, feces, water and other relevant clinical samples. Throughout the course of the study the culture samples were used for screening each KB010 kit is a standardized colorimetric identification system utilizing eight conventional biochemical tests and four carbohydrate utilization tests. The tests were based on the principle of pH change and substrate utilization. On incubation *E. coli* exhibited metabolic changes indicated by a colour change in the media which was either visible spontaneously or after addition of a reagent.

The organism to be identified was isolated on a common medium like Nutrient Agar. Each well was inoculated with 50 µl of the above inoculum by surface inoculation method. Temperature of incubation was 35° ± 2 °C and duration of incubation was 18-24 hours.

Molecular identification of *E. coli*

DNA extraction from the dropping samples was done by using QIAamp DNA Stool mini Kit (50) as per the protocol given from Qiagen, Germany.

Primers for *E. coli*

Primer set designed by Traute Janben (2001) [4] was used for detection of virulence associated gene of APEC (Avian Pathogenic *E. coli*) such as TypeI Fimbriae (*fimC)* was purchased from Ocimum Biosciences. The details of primers were given below:

Table 1: Shows target gene sequence amplicon size and references

Target gene	Sequence (5´- 3´)	Amplicon size (bp)	Reference
FimC	F: GGG TAG AAA ATG CCG ATG GTG R: CGT CAT TTT GGG GGT AAG TGC	215-235 671-691	(*Traute Janben et al.,* 2001) [4]

Polymerase Chain Reaction for *E. coli*

The PCR amplification will be carried out in Eppendorf Mastercycler (Eppendorf, Germany) with following thermal programme. Initial denaturation at 94 °C for 2 min followed by 25 cycles of denaturation at 94 °C for 1 minute, annealing at 59 °C for 1 minute and primer extension at 72 °C for 90 seconds followed by final extension at 72 °C for 7 minutes.

Agarose gel electrophoresis of PCR product

The PCR products were tested for positive amplification by agarose gel electrophoresis on 1.5% agarose w/v gels by loading 10 micro liter of PCR product into wells and 100bp DNA (GeNei) ladder was used as a marker. A current of 120 V was applied to each gel and PCR products were visualized by UV illumination.

Result

In Arignar Anna Zoological Park, out of 76 samples 30.26% of the samples (n=23) were positive for *E. coli*

Isolation of *E. coli* bacteria by culture

During the observation for the growth of *E. coli* organisms in the samples streaked over Eosin-Methylene Blue (EMB) agar medium followed by incubation at 37 °C for 24hrs, the colonies of *E. coli* organism were found to appear as blackish centers with a green metallic sheen as show in (Plate 1).

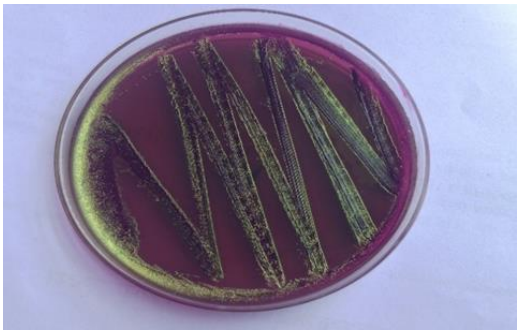
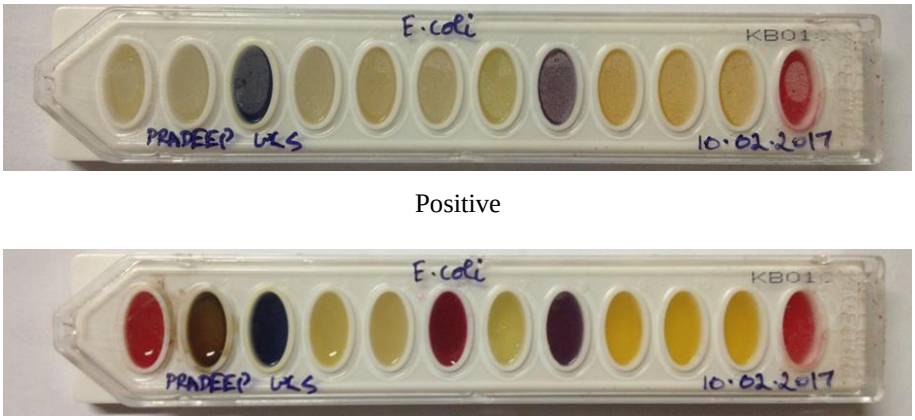


Plate 1: *E.coli* (Colonies with metallic sheen) on EMB agar

Biochemical characterization of *E. coli*

Colonies revealing characteristic metallic sheen on EMB agar were subjected to biochemical tests for identification of *E. coli* by using a KB010 HilMViC kit. Results are presented in (Plate 2 and Table 1).



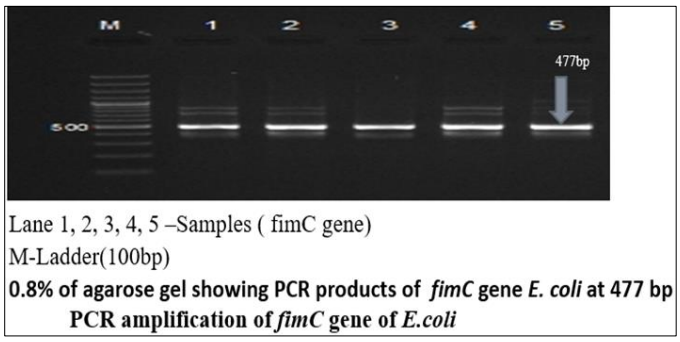
Positive

Plate 2: KB010 HilMViC kit for identification *E. coli*

Table 1: Identification index for Escherichia coli by using KB010-Hi MViC test kit

T	Test	Reagents	Principle	Positive reaction	Result
1.	Methyl red	1-2 drops of methyl red reagent	Detects acid production	Red	+
2.	Voges Proskauer's	1-2 drops of baritt reagent A and 1-2 drops of baritt reagent B	Detects acetoin production	Pinkish red	-
3.	Citrate utilization	-	Detects capability of organisms to utilize citrate as a sole carbon source	Blue	-
4.	Indole	1-2 drops of kovac's reagent	Detects deamination of tryptophan	Reddish pink	+
5.	Glucuronidase	-	Detects glucuronidase activity	Bluish green	+
6.	Nitrate reduction	1-2 drops of sulphanilic acid and 1-2 drops of N, N dimethyl-1-Napthylamine	Detects nitrate reduction	Pinkish red	+
7.	ONPG	-	Detects beta galactosidase activity	Yellow	+
8.	Lysine utilization	-	Detects lysine decarboxylation	Purple/light purple	+
9.	Lactose	-	Lactose utilization	Yellow	+
10.	Glucose	-	Glucose utilization	Yellow	+
11.	Sucrose	-	Sucrose utilization	Yellow	+
12.	Sorbitol	-	Sorbitol utilization	Yellow	-

Molecular Identification: *E. coli* organisms by polymerase chain reaction, the amplicon size was 477bp (Plate 3).



Lane 1, 2, 3, 4, 5 –Samples (fimC gene)
M-Ladder(100bp)
0.8% of agarose gel showing PCR products of *fimC* gene *E. coli* at 477 bp
PCR amplification of *fimC* gene of *E.coli*

Plate 3: PCR confirmation of genus *E. coli*

Discussion

The encountering of *E. coli* organisms in captive pheasants of this study was in agreement with the findings reported by Gopee *et al*. (2000) [2] who also stated that the frequency of *E. coli* isolation was found to be significantly higher in mammals when compared with birds and reptiles.

The primers sequences used in this study with pheasants under captive condition for amplification of DNA for the detection of *E. coli* were in agreement with the findings given by Janben *et al.* (2001) [4] who noted that *fim C* gene was detected with highest prevalence in avian pathogenic *E. coli* (APEC).

Diagnosing the *E. coli* infections in the faecal samples from captive birds, as done in this study was in accordance with the reports furnished by Graham and Graham (1978) [3].

The identification of sick birds, in the enclosures needed to be based on the identification of various signs like isolated resting activity in a group, reduced activities, ruffled feathers and watery droppings in this study. However, the loose motion was the particularly noticed sign in the sick birds, under study. This was in agreement with the findings reported by Price (1992) who quoted on the incidences of diarrhea in both *E. coli* infections, as well as in Salmonellosis. Further, Altman *et al.* (1997) [1] also quoted about watery stools in avifauna affected by various bacterial infections like *E. coli* infections, Salmonellosis etc. Though clinical signs needed to be given major significance, it is noteworthy to mention that the members of the order *Galliformes* were prone to many infectious and husbandry related conditions, and like many birds, they frequently masked any signs of illness, until very late in the course of disease as a survival strategy to prevent the predators attack of a weak or sickly individual.

Isolation of birds showing signs of sickness and further treatment accordingly, thereby reduce the risk of infection among the pheasants.

The bacterial pathogens encountered in this study are in one or the other way zoonotic pathogens. Gloves must be worn, in addition to wearing of foot wears and coveralls, during the engagement with the Pheasants in order to avoid the zoonosis related problems among the animal keepers working in these captive pheasants enclosure. Since pheasants under study were found to be harbour *E. coli* which had significant zoonotic potential with them. Further, suitable awareness programmes might be held, with regard to prevention of zoonosis from the pheasants. Usage of blow gun over the substrate in addition to change of substrate might be of much useful features to tackle zoonosis related infections in pheasants.

Animal keepers associated with husbandry-practices in the enclosures of captive Pheasants needed to clean their hands well with more water and soap many times, especially before taking food or water and these measures might be helpful in reducing the incidences of *E. coli* infections in handlers of pheasants to a great extent. Similar precautions were recommended for field veterinarians those are working in a zoo or zoological park.

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