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A study on isolation, identification and molecular characterization of bacterial pathogens in faecal matter of captive exotic birds in Bhubaneswar smart city

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ABSTRACT

A total of 8 (cloacal and faecal) samples were aseptically collected from different cages of captive exotic birds (Budgerigars, Java sparrow and love birds) from Janpath and Soubhagya nagar area of Bhubaneswar for the isolation, identification and molecular characterization of bacterial flora. All the collected samples were streaked in different selective media for the study of the type of bacteria. Gram staining and biochemical tests were performed for morpho-physiological characterization of the bacteria. This test confirmed the prevalence of *E. coli* among the isolated bacteria. The antibiogram of the isolated bacteria was performed in which isolated bacteria were found to be highly resistant to cloxacillin, tetracycline and ampicillin and highly sensitive to gentamicin and amikacin. Antibacterial properties of extracts from pulp of wood apple and bark of arjun were evaluated in *invitro* condition and are found to be ineffective on the isolated bacteria. The presence of virulence gene *stx1* in the *E. coli* was detected using multiplex PCR. Since, many samples were found to contain *E. Coli,* the handlers must take optimal care during the handling to avoid transmission.

1. Introduction

Now a days, the content of adorning and exotic birds in the urban environment is very common. Birds and their diversity have been considered as good indicators of ecosystem health and in the UK, bird diversity is used as one of the 15 quality of life indicators (Gregory *et al.,* 2003).

The importance of wild birds as probable vectors of disease has resumed empirical interest, especially regarding human health. There are many types of bacterial infections that birds do suffer. Understanding the spread of bacterial pathogens in wild birds may serve as a useful model for examining the spread of other disease organisms, both amongst birds, and from birds to other taxa. Information relating to the normal bacterial flora in gastrointestinal region is limited for the majority of wild bird species, with only few well-studied examples concentrating on bacteria that are zoonotic and relate to avian species of commercial interest (Benskin *et al.,* 2009). Through direct or indirect contact of the diseased or carrier birds many zoonotic diseases are transferred from cageor pet birds to human. Bacteria are one of the most common causes of zoonotic diseases. Hence, proper isolation, identification and characterization of the bacteria collected from infected/carrier birds are essential to control zoonotic diseases.

Wildlife animals that are kept in captivity are very defenceless against opportunistic diseases and they may act as pool of pathogenic bacteria (Ahmed *et al.,* 2007). In most cases, the birds are probably susceptible to these infections due to underlying problems that have allowed for a large bacterial population to overwhelm their normal immunity, or the birds themselves are already weakened due to stress, poor nutrition, or poor husbandry. Most common bacterial pathogens that are noticed in birds includes *Escherichia coli*, *Pasteurella* spp., *Pseudomonas*

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spp., *Salmonella* spp., *Shigella* spp., *Klebsiella* spp. and *Vibrio* spp.

Out of all the bacterial pathogens, *Escherichia coli* can be considered as the most ubiquitous effective enterobacteria in captive animals and is associated with systemic disease in birds (Mattes *et al.,* 2005). The pathogenesis of enteritis by *E. coli* in birds is still not clear, but the presence of diarrheagenic strains may show a public health risk. Although innocuous *E. coli* predominate among the normal flora of the vertebrate intestine, pathogenic forms exist that causedisease of varying severity in humans and otheranimals. Pathogenic strains of *E. coli* are determined by specific virulence factors and their effect in susceptible species. The *Escherichia coli* diarrheagenic (diarrheagenic E. coli - DEC) are an important cause of endemic and epidemic diarrhoea in the world. With the application of polymerase chain reaction (PCR), one can detect genes involved in the pathogenicity of several bacterial isolates, allowing simple identification. In view of this, the small piece of research is focused on isolation, identification and characterization of pathogenic bacteria from faecal samples of captive exotic birds.

2. Material and Methods

2.1. Collection of samples

A total of 8 cloacal swab and faecal samples were collected from 8 different cages of captive exotic birds (Budgerigars, java sparrow and love birds) from Janpath & Soubhagyanagar area of Bhubaneswar.

Each sample was transported to the Mycobacterium Culture Laboratory of Department of Epidemiology & Preventive Medicine, College of Veterinary Science and Animal Husbandry, Orissa University of Agriculture and Technology, Bhubaneswar-03.

2.2. Isolation of bacterial pathogens

Samples were inoculated in the Nutrient Broth and incubated at 37^0C for 24 hrs. After 24 hours cultureswere streaked on different Enriched media such as EMB, SS, TCBS, KSA and MacC (Himedia, India).

2.3. Preservation of Stock Culture

Bacterial cultures was streaked on the NA slants and preserved at 40 for further use.

2.4. Morphological and Biochemical characterization

The morphology of bacterial isolates was performed with Gram's staining. For biochemical characterization, each isolated bacteria was identified with different tests like oxidase, catalase, urease, citrate, indole, mannitol motility and Triple sugar iron test (Himedia, India). After incubation at 37^0C for 48 hours, the tubes were examined for any change in the slant or butt (Cheesbrough, 1984).

2.5. Haemolytic activity

To determine the haemolytic property of isolated bacteria, the colonies of bacteria were inoculated on Blood Agar media (BA) and incubated at 37^0C for 24 hours. The haemolytic pattern of the bacteria was categorized according to the types of haemolysis produced on BA and this was made as per recommendation of Carter (1986).

2.6. Antibiotic Sensitivity test (ABST)

The drug sensitivity pattern of the isolated bacteria was determined using commercially available antimicrobial discs. In vitro antibiotic sensitivity tests was done using disc diffusion test. Antibiotic discs were placed aseptically on the surface of the inoculated plates with the help of sterile forceps and incubated at 37^0C for 24 hours. After incubation the plates were examined and the diameters of the zone of inhibition were measured using high antibiotic zone scale (Himedia). Depending on the area of the zone diameters for individual antibiotic was recorded as sensitive, intermediate and resistant.

2.7. Anti-bacterial activity of Wood apple and Arjun extract

The following plant materials were collected for the *in vitro* treatment of the bacteria.

Particulars of plant and plant extract collected:

Sl.no.	Local Name	Botanical Name	Source
1	Wood-Apple	*Limonia acidissima*	Ladies Hostel Complex, OUAT
2	Arjuna	*Terminalia arjuna*	Laboratory of the Department of Epidemiology and Preventive Medicine

2.8. Extraction of anti-bacterial compound from Wood apple (Limonia acidissima) and Arjun (Terminalia arjuna):

The fruits (*L. acidissima*) were taken and thoroughly washed under tap water. All the clean samples were separated mechanically into fruit pulp. It was dried in a hot air oven at 60^0 for one week and coarsely powdered using a mixer grinder and stored in an air tight container for further use.

20 gm of dried *L. acidissima* pulp powder was added to 200 ml of methanol and kept in the water bath at 80^0 for 2 hours. The extract was then evaporated using Rotary Vacuum evaporator and then the pure extract was collected in a jar. It was then poured in a glass petri plate and left for drying inside the laminar airflow. Then it was kept in hot air oven at 80^0 till it was dried completely. Then the dried powder was stored in the refrigerator for further use. The powder was mixed with PBS solution at 10mg/ml concentration and mixed thoroughly. The solution was poured into the holes of the cultured plates. Same procedure was repeated for water instead of methanol.

Same procedure of extraction was followed for bark of the Arjun plant.

2.9. Extraction of bacterial genomic DNA

300µL of overnight bacterial culture was taken in 1.5ml tube. 200µL of resuspension buffer was added to it followed by 200µL of lysis buffer. The mixture was incubated at room temperature for 2mins. 20µL of Proteinase K was added to it and was incubated at 65^0C for 20mins with intermediate shake. After incubation, 200µL of precipitation buffer was added to it and then the mixture was transferred to spin column. It was then centrifuged at 10,000rpm for 2mins. 500µL of wash buffer was added to spin column and was centrifuged at 10,000rpm for 2mins. The wash step was repeated for 2 times. Then it was centrifuged at 10,000rpm for 2mins to drag the spin cup. 100µL of elution buffer was added to spin column and was then centrifuged at 10,000rpm for 5mins. It was kept at -20^0C for future use.

2.10. Detection of virulence gene in E. coli

A total of four pairs of primers were used for the detection of virulence associated gene of *E. coli* like *eae* (454bp), *bfpA* (550bp), *stx1* (349bp) and *stx2* (110bp) genes, according to the method described by Costa *et al*., (2010). The reaction mixture for PCR of *E.coli* was prepared by taking ampliqon master mix with forward primer, reverse primer, sample DNA template and nuclease free water and then the amplification was carried out using multiplex PCR. Amplification conditions comprised initial denaturation at 94^0C for 5 min, 35 cycles of 1.5 min at 94^0C, 1.5 min at 50^0 /56^0C and 1.5 min at 72^0C and final extension for 10 min at 72^0C.

2.11. Primers for development of multiplex PCR to detect the pathogenic strains of bacteria

2.12. Agarose gel electrophoresis of PCR product

The PCR products were analysed for positive amplification by agarose gel electrophoresis on 1.5% agarose w/v gels by loading 10µL of PCR product into wells and 100bp DNA ladder was used as a marker. A current of 100V was applied and the PCR products were visualized by UV illumination (BioImaging system).

3. Results and Discussion

Information regarding the normal gastrointestinal bacterial flora is limited for the majority of wild bird species, with the few well-studied examples concentrating on bacteria that are zoonotic or relate to avian species of commercial interest (Benskin *et al*., 2009). However, spreading of bacterial pathogens from one species of birds to another species and from birds to other animals including human beings is considered to be important for study. Hence, the present study tried to focus on presence of pathogenic bacteria in the faecal matter.

Sarker *et al.* (2012) studied the faecal sample of 72 water birds and isolated *E. Coli* (54.16%), *Salmonella spp*

Sl.No.	Genes	Initiators	Sequence (5/- 3/)	Product (bp)
1	*eae*	EAE-1	AAACAGGTGAAACTGTTGCC	
		EAE-2	CTCTGCAGATTAACCTCTGC	454
2	*bfpA*	EP-1	CAATGGTGCTTGCGCTTGCT	
		EP-2	GCCGCTTTATCCAACCTGGT	550
3	*stx1*	STX-1A	CAACACTGGATGATCTAG	
		STX-1B	CCCCCTCAACTGCTAATA	349
4	*stx2*	STX-2A	ATCAGTCGTCACTCACTGGT	
		STX-2B	CTGCTGTCACAGTGACAAA	110

(31.94%), *Staphylococcus spp* (27.78%), *Bacillus spp* (26.38%), and *Proteus* spp. (8.33%) through bacteriological media, biochemical tests and antibiogram profiling. However, literature related to identification of bacterial flora present in the faecal matter of the captive exotic birds is scanty. In the present study, selective culture media like EMB, KSA, SS, TCBS and MacC were used for the identification of bacterial flora. It was found that all the cultures were positive for EMB whereas, with SS four samples were positive and four were negative. Similarly, with KSA four were positive and four were negative. With TCBS all samples were negative. Similarly, five samples were tested positive for lactose fermentation and three samples were negative for lactose fermentation. Through the study, it was noticed that most of the samples were positive for EMB and hence the dominating bacteria was *E. coli.* Subsequently, morpho-physiological characterization was done through biochemical tests and Gram staining from which the domination of *E. coli* in the faecal samples was confirmed. Since the study conducted by Sarker *et al.* (2012) was on water birds, who were more exposed to environmental influence, bacterial flora diversification was more. The present work was on captive/caged exotic birds which restricted the bacterial variety to one or two.

However, no bacteria were found to be involved in rupturing RBCs as confirmed through haemolytic test.

A study conducted by Miranda *et al.* (2008) on the resistivity in poultry intestinal *E. coli* found that the resistance rates of intestinal *E. coli* to all the antimicrobials significantly increased during the course of therapeutic treatment. In the present study antibiotic sensitivity of the isolates were conducted using 16 different antibiotic disks. Out of these, Cloxacillin showed 100% resistance for the isolates, Tetracycline and Ampicillin were found to be resistant for 66.67% of isolates, Amikacin showed sensitivity for 44.45% of the isolates and Cefalexin, Cefotaxime and Gentamicin showed very high sensitivity of 99.99%. It is interesting to note here that another contemporary study conducted in the same laboratory on faecal samples of commercial poultry birds indicated resistance of isolates to all varieties of above mentioned antibiotics. It is likely due to frequent use of antibiotics and consequent developed resistance in poultry birds in the commercial farms.

A report on antimicrobial activity of the extracts of leaves of *Limonia acidissima* against four Gram negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus vulgaris*) and five Gram-positive bacteria (*Bacillus subtilis*, *Enterococcus faecalis*, *Micrococcus luteus, Staphylococcus aureus*, *Streptococcus pneumoniae*) was published by Naidu *et al*. (2014). Similar results through bark extract of *Terminalis arjuna* on Gram negative bacteria was reported by Mandal *et al*. (2013). But in the present study, no antibacterial activity of different concentrations of *L. acidissima* and *T. arjuna* extracts was noticed against the *in vitro* cultured isolates. A study conducted by Seeley *et al*. (2014) on a captive flock of budgerigars identified *E. coli* with the virulence gene (*eae*) that contributed to mortality of the birds. Present study detected virulence gene stx1 in *E. coli* at 349 bp using four primers for the genes like *eae, bfpA, stx1* and *stx2* with the help of multiplex PCR.

Table 1

Isolation of bacteria on different selective media

Sl. No.	Sample	EMB (A)	SS (B)	KSA (C)	TCBS (D)	MacC (E)
1	Cage-1	+	-	+	-	LF
2	Cage-2	+	+	-	-	LF
3	Cage-3	+	+	-	-	NLF
4	Cage-4	+	-	+	-	LF
5	Cage-5	+	+	+	-	NLF
6	Cage-6	+	-	-	-	LF
7	Cage-7	+	+	+	-	NLF
8	Cage-8	+	-	-	-	LF

Table 2

Biochemical characterization of final isolates

Sl.	Isolates	Urease	Catalase	Oxidase	Citrate	Indole	Mannitol	Motility	Glucose	Lactose	Sucrose	H_2S	Gas
1	CA1	-	+	-	+	+	+	+	+	+	+	-	+
2	CA3	-	+	-	+	+	+	+	+	+	+	-	+
3	CA4	-	+	-	+	+	+	-	+	+	+	-	+
4	CA5	-	+	-	+	+	+	+	+	+	+	-	+
5	CA6	-	+	-	+	+	+	-	+	+	+	-	+
6	CA7	-	+	+	+	+	+	+	+	+	+	-	+
7	CA8	-	+	-	+	+	+	+	+	+	+	-	+
8	CC1	+	+	-	+	-	+	+	+	-	-	+	+
9	CC2	-	+	-	+	+	+	-	+	+	+	-	+
10	CC4	-	+	-	+	+	+	-	+	+	+	-	+
11	CC5	-	+	-	+	+	+	+	+	+	+	+	+
12	CC7	+	+	-	+	-	+	+	+	-	-	-	+
13	CD5	-	+	-	+	+	+	+	+	+	+	+	+

Table 3

Morphology of isolated bacteria through Gram Staining

Sl. no.	Isolates	Results	Shape of the Bacteria
1	CA1	Gram negative	Small rod
2	CA3	Gram negative	Rod
3	CA4	Gram negative	Rod
4	CA5	Gram negative	Small rod
5	CA6	Gram negative	Small rod
6	CA7	Gram negative	Rod
7	CA8	Gram negative	Rod
8	CC1	Gram negative	Rod
9	CC2	Gram negative	Rod
10	CC4	Gram negative	Rod
11	CC5	Gram negative	Small rod
12	CC7	Gram negative	Small rod
13	CD5	Gram negative	Rod

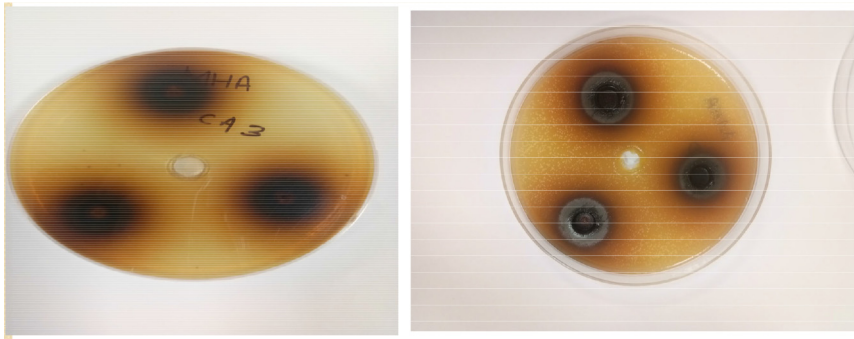
Table 4

Antibiotics sensitivity of the isolated bacteria

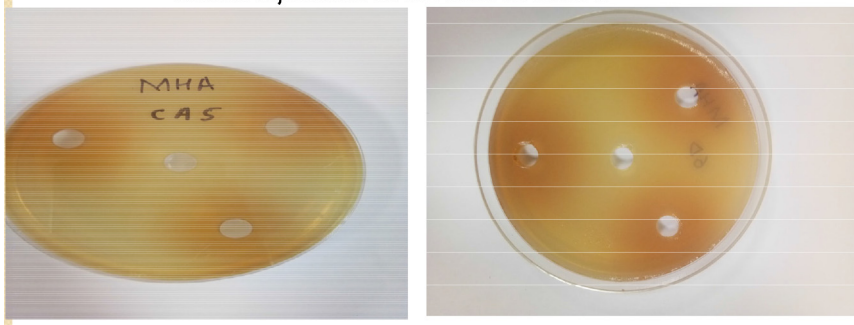
Sl.no.	samples	S=18 R=14 CN	S=22 R=14 CTX	S=20 R=19 AMC	S=15 R=12 GEN	S=21 R=17 CIP	S=11 R=10 CL	S=18 R=13 AZM	S=19 R=14 TE	S=20 R=14 CXM	S=17 R=14 AK	S=19 R=13 AMX	S=17 R=13 AMP	S=18 R=14 CAZ	S=21 R=15 CPZ	S=12 R=11 PB	S=40 R=30 COX
1	CA3	19S	29S	18R	17S	23S	11S	20S	10R	25S	17S	R	13S	11S	19I	11S	R
2	CA6	20S	30S	19R	17S	25S	11S	19S	10R	24S	17S	R	10R	R	21S	13S	R
3	CA8	22S	30S	26S	18S	20I	10R	24S	10R	25S	18S	33S	27S	10R	31S	11R	R
4	CC1	22S	30S	22S	16S	26S	11S	14I	22S	R	16I	R	13R	R	23S	12S	R
5	CC2	22S	31S	10R	18S	16R	11S	25S	R	25S	18S	R	12R	14R	20I	13S	R
6	CC4	22S	27S	22S	17S	26S	12S	18S	10R	24S	16I	R	15I	10R	17I	11R	R
7	CC5	23S	30S	22S	17S	28S	11S	19S	10R	24S	16I	R	13R	R	22S	13S	R
8	CC7	24S	28S	28S	17S	36S	12S	21S	19S	21S	16I	12S	19S	11R	25S	12S	R
9	CD5	21S	28S	21S	17S	28S	11S	18S	10R	24S	17S	R	14I	R	21S	14S	R

R-Resistant, I-Intermediate, S-Sensitive

CN-Cefalexin, CTX-Cefotaxime, AMC-Amoxyclav, GEN-Gentamicin, CIP-Ciprofloxacin, CL-Colistin, AZM- Azithromycin, TE-Tetracycline, CXM-Cefuroxime, AK-Amikacin, AMX-Amoxicillin, AMP-Ampicillin, CAZ-Ceftazidime, CPZ- Cefoperazone, PB-Polymyxin-B, COX-Cloxacillin



Effect of arjuna bark on the cultured bacteria



Effect of wood apple on the cultured bacteria 17

A total of 3 samples were subjected for identification pathogenic strains of *E. coli* through thermal-cycler. The four genes, *eae, bfpA, stx1* and *stx2* were used to identify pathogenic *E.coli,* out of which stx1 gene showed positive result for pathogenic *E. coli* at 349 bp.

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