ISSN Print: 2394-7500
ISSN Online: 2394-5869
Impact Factor: 5.2
IJAR 2019; 5(2): 19-24
www.allresearchjournal.com
Received: 11-12-2018
Accepted: 15-01-2019

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Diversity of bacterial flora and their EXO enzyme and antibiotic profiling from bird droppings/fecal matter from Western Ghats, Kerala, India

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Abstract
Diversity which is one of the most widely studied ecologic properties, is fundamental to the field of ecology itself. It is widely reported and often promoted as an indicator of ecosystem state due to its relationships with productivity, functioning, and stability. Very little is known about the normal gastrointestinal flora of wild birds, or how it might affect or reflect the host's life-history traits. The aim of this study was to survey the species richness of bacteria in the feces of a wild population of birds of Western Ghats, Kerala. Bacterial diversity of birds was analyzed and out of the sixteen isolates, six were found to be potent and capable of producing more than two enzymes qualitatively and thus efficient on their exoenzyme production. Molecular characterization revealed their identities to be *Burkholderiasp, Bacillus pumilus, Lysinibacillus sp., Paenalcaligenes suwonensis, Kurthiagibsonii*, and *Pseudomonas aeruginosa*. Antibiotic sensitivity tests by disc diffusion assay showed that some of them were resistant to multiple drugs.

Keywords: Bacterial diversity, bird fecal matter, EXO enzyme, antibiotic sensitivity testing

Introduction
Avian feces are most common and can be in form of semi-solid/ water and its color varies among the species of birds. Some may be whitish, or in the form of ashes and dark brown to black in colour. There are several millions of micro flora present in bird droppings including pathogenic and non-pathogenic species and the opportunistic ones (Adegunloye, 2006) [16]. The nutrient composition of avian faeces varies with the feed ration, type of bird, the proportion of litter droppings, the manure handling system, and also with the type of litter. They can also vary in their physical and chemical compositions and those factors affecting the composition can include the types and number of birds per unit area, density of feed nutrients, environmental factors and several other management factors (Vest *et al.*, 2009) [20]. The chemical composition of feces includes water, nitrogen, phosphorus and potassium with some other minerals and this supports the growth of millons of microorganisms. Bird feces contain diverse important elements that make it to be used as manure. The organic matter content makes it suitable to be used as soil conditioner. It is moist and because of its nutrient and organic matter content, the manure is a suitable breeding ground for pestiferous flies like houseflies, flesh flies, black garbage flies and biting stable flies. The manure is in turn a source of odor, caused by the production of different fatty acids such as butyric, valeric, capronic and caprylic acids (Adegunloye, 2006) [16].
Bird droppings do pose a public health risk and cause illness. Humans become infected by inhaling dust containing dried faeces, urine, or respiratory secretions of infected birds (Chang *et al.*, 2004) [7]. But the high nutrient content of bird muck provides an excellent sanctuary for potentially useful microbial enzymes. They have variety of uses and have potent applications in the industry. This is an unexplored area where researches are infrequent. In addition, the microbial enzymes have been paid more consideration due to their active and stable nature than enzymes of plant and animal origin (Anbu *et al.*, 2015). Most of the microorganisms are unable to grow and produce enzyme under harsh environments which cause toxicity to microorganisms. Though some microorganisms have undergone various adaptations, enabling them to grow and produce enzymes under harsh conditions (Sardessai & Bhosle, 2004) [17].

Recently several outlines of study have been initiated to isolate new microbial strains from harsh environments for the production of different enzymes having the properties to yield higher (Gopinath *et al.,* 2005) [10].

In the present study, the bird fecal samples and were collected from forest regions in and around Kochi and the biodiversity of micro flora was analyzed. The exo enzyme profiling was done to identify the potent Antibiotic sensitivity tests were conducted for checking multiple drug resistance.

Materials and Methods

1. Collection of samples and isolation of bacterial flora

A total of 5 faecal samples, which, were collected from Common myna, Jungle fowl, spotted dove, Malabar grey hornbill and Red whiskered bulbul residing near Western Ghats, Kochi, Kerala. Each sample was aseptically transported to the bacteriology laboratory of Merit Bio labs, Kochi. The samples were immediately inoculated to nutrient broths and were incubated at 37°C for 24 hours and after 24 hours, the incubated broths were streaked into nutrient agar (Hi Media, India) to obtain pure culture of the bacterial isolates.

2. Exoenzyme profiling of potent bacterial isolates

Qualitative analysis for different enzyme activities by potent bacterial isolates were assessed by growing them on xylan agar, casein agar, starch agar media, pectin agar and carboxy methyl cellulose agar. The isolates were inoculated on respective media and incubated at 28 °C for 24 hours; the appearance of clear zone surrounding the colony was measured after adding specific reagent and used as indicator for extracellular enzymatic activities.

Xylanase activity

Xylanase activity was performed on xylan agar medium. After 24 hours of incubation, plates were flooded with iodine solution. The appearance of clear zone surrounding the colony was considered positive for xylanase enzyme.

Pectinase activity

Pectinase activity was performed on pectin agar medium. After 24 hours of incubation, plates were flooded with iodine solution. The appearance of clear zone surrounding the colony was considered positive for pectinase enzyme.

Protease activity

Protease activity was performed on casein agar medium. After 24 hours of incubation, plates were checked for the appearance of clear zone surrounding the colony, which was considered positive for protease enzyme.

Amylase activity

Amylase enzyme activity was assessed by growing the organisms on starch agar medium containing 1% soluble starch. After 24 hours' incubation, the plates with fungal colony were flooded with 1% iodine in 2% potassium iodide. The appearance of clear zone surrounding the colony was considered positive for amylase enzyme.

Cellulase activity

Cellulase activity was performed on carboxy methyl cellulase (CMC) agar medium. After 24 hours of incubation, plates were flooded with Congo red for 15 minutes and washed using 0.1N NaCl solution. The appearance of clear zone surrounding the colony was considered positive for cellulase enzyme.

3. Preservation of stock culture

Twenty percent sterile buffered glycerin and NA slant with liquid paraffin was used to preserve the isolated bacteria for further study (Buxton and Fraser, 1977).

4. Molecular identification of potent isolates

Genomic DNA was isolated and purified (Ausubel *et al.,* 1987). Spin 2 mL of culture at 12000 rpm for 10 minutes (4 ^{0}C). Discard the supernatant and repeat the same step. Discard the supernatant and add 875 µL TE and vortex re-suspend. Add 5 µL proteinase K and 100 µL SDS (10%). Mix gently Add 1 ml Phenol: Chloroform mixture (1:1), mix gently. Spin at 12000rpm for 10 minutes (4 ^{0}C). Transfer the supernatant to a fresh tube (Pipette it out till it reaches the interface) and repeat the above 2 steps with Chloroform twice. Transfer the supernatant and add 0.1 volume sodium acetate (5 molar, pH – 5.2) (If 1 ml 100 µL and double the volume of isopropanol stored at -20 ^{0}C). Keep at -20 ^{0} C overnight / 1-2 hours. Centrifuge at 8000 rpm for 10 minutes. Wash with ethanol – 500 µL then spin at 8000 rpm for 2 minutes. Pour of the ethanol and air dry. Dissolve DNA in TE (50 µL). Stored at 4 ^{0} C.

Agarose gel electrophoresis for DNA quality and quantity check

The quality of the DNA isolated was checked using agarose gel electrophoresis. 1µl of 6X gel-loading buffer (0.25% bromophenol blue, 30% sucrose in TE buffer pH-8.0) was added to 5µl of DNA. The samples were loaded to 0.8% agarose gel prepared in 0.5X TBE (Tris-Borate-EDTA) buffer containing 0.5 µg/ml ethidium bromide. Electrophoresis was performed with 0.5X TBE as electrophoresis buffer at 75 V until bromophenol dye front has migrated to the bottom of the gel. The gels were visualized in a UV Tran illuminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad)

PCR Analysis

PCR amplification reactions were carried out in a 20 µl reaction volume which contained 1X PCR buffer (100mM Tris HCl, pH-8.3; 500mM KCl), 0.2mM each dNTPs (dATP, dGTP, dCTP and dTTP), 2.5mM MgCl2, 1 unit of AmpliTaq Gold DNA polymerase enzyme, 0.1 mg/ml BSA, 4% DMSO, 5pM of forward and reverse primers and template DNA.

Table 1: Primers used

Target	Primer pair	Direction	Sequence (5' → 3')
16SrRNA	16S-RS-F	Forward	CAGGCCTAACACATGCAAGTC
	16S-RS-R	Reverse	GGGCGGWGTGTACAAGGC

Table 2: The conditions used for PCR amplification are listed

Sl. no	Step	Temperature	Time
1	Initial Denaturation	94^{0}C	1.5 min
3	Annealing	56^{0}C	30 s
4	Extension	72^{0}C	2 min
5	Final Extension	72^{0}C	10 min

*Steps 2, 3& 4 are repeated in 30 cycles

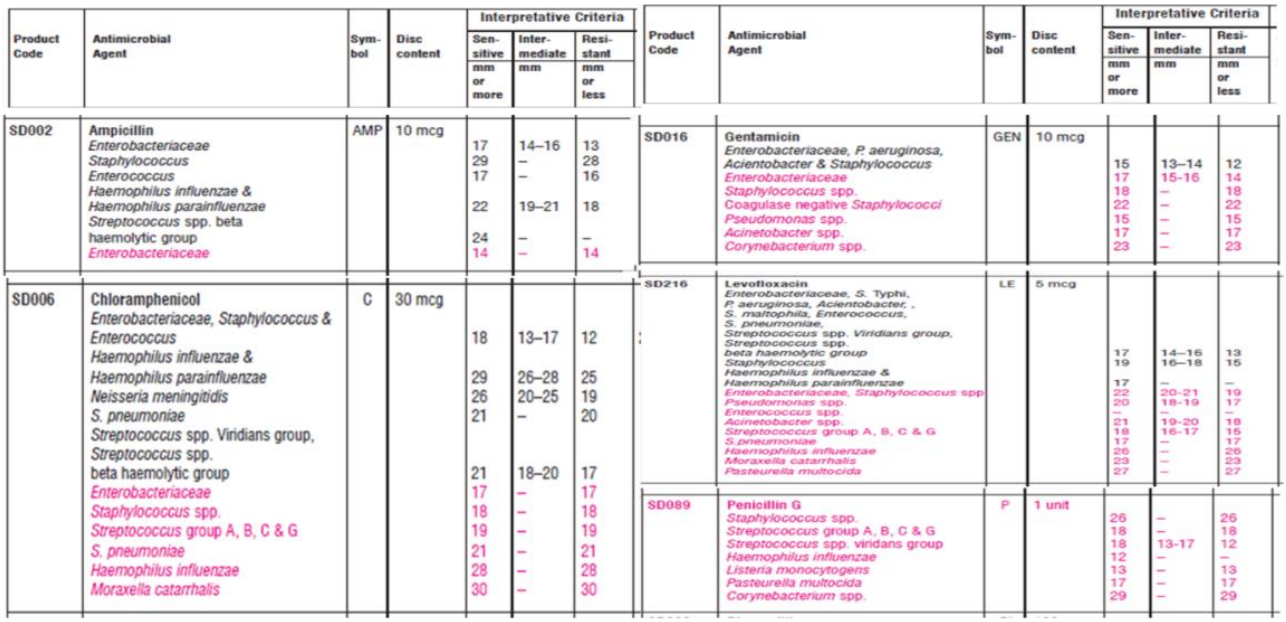
The PCR amplification was carried out in a PCR thermal cycler (Gene Amp PCR System 9700, Applied Biosystems).

Sequence Analysis

The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.6 (Drummond *et al.*, 2012) [8].

5. Antibiotic sensitivity tests

To determine the drug sensitivity pattern of the isolated bacteria, commercially available antimicrobial discs (Hi Media, Mumbai) were used for the test. *In vitro* antibiotic sensitivity tests were done using disc diffusion test following the method described by Kirby-Bauer (Bauer *et al.*, 1966) [3]. 1-2 mL of freshly growing bacterial cultures were poured on to nutrient agar and spread uniformly. Antibiotic discs were placed apart on the surface of the inoculated plates aseptically with the help of a sterile forceps and incubated at 37^0C for 24 hrs. After incubation, the media plates were examined and the diameters of the zone of inhibition were measured. Depending on the area of the zone diameters for individual antibiotic, the sensitivity was recorded as highly sensitive, moderately sensitive, less sensitive and resistant as per manufacturer's instructions.



Product Code	Antimicrobial Agent	Sym-bol	Disc content	Interpretative Criteria: Sen-sitive mm or more	Inter-mediate mm	Resi-stant mm or less
SD002	Ampicillin	AMP	10 mcg			
	Enterobacteriaceae			17	14–16	13
	Staphylococcus			29	–	28
	Enterococcus			17	–	16
	Haemophilus influenzae & *Haemophilus parainfluenzae*			22	19–21	18
	Streptococcus spp. beta haemolytic group			24	–	–
	Enterobacteriaceae			14	–	14
SD006	Chloramphenicol	C	30 mcg			
	Enterobacteriaceae, Staphylococcus & *Enterococcus*			18	13–17	12
	Haemophilus influenzae & *Haemophilus parainfluenzae*			29	26–28	25
	Neisseria meningitidis			26	20–25	19
	S. pneumoniae			21	–	20
	Streptococcus spp. Viridians group, *Streptococcus* spp. beta haemolytic group			21	18–20	17
	Enterobacteriaceae			17	–	17
	Staphylococcus spp.			18	–	18
	Streptococcus group A, B, C & G			19	–	19
	S. pneumoniae			21	–	21
	Haemophilus influenzae			28	–	28
	Moraxella catarrhalis			30	–	30

Product Code	Antimicrobial Agent	Sym-bol	Disc content	Interpretative Criteria: Sen-sitive mm or more	Inter-mediate mm	Resi-stant mm or less
SD016	Gentamicin	GEN	10 mcg			
	Enterobacteriaceae, P. aeruginosa, Acientobacter & Staphylococcus			15	13–14	12
	Enterobacteriaceae			17	15-16	14
	Staphylococcus spp.			18	–	18
	Coagulase negative *Staphylococci*			22	–	22
	Pseudomonas spp.			15	–	15
	Acinetobacter spp.			17	–	17
	Corynebacterium spp.			23	–	23
SD216	Levofloxacin	LE	5 mcg			
	Enterobacteriaceae, S. Typhi, P. aeruginosa, Acientobacter, , S. maltophila, Enterococcus, S. pneumoniae, Streptococcus spp. *Viridians group, Streptococcus* spp. *beta haemolytic group Staphylococcus*			17	14–16	13
				19	16–18	15
	Haemophilus influenzae & *Haemophilus parainfluenzae*			17	–	–
	Enterobacteriaceae, Staphylococcus spp			22	20-21	19
	Pseudomonas spp.			20	18-19	17
	Enterococcus spp.			–	–	–
	Acinetobacter spp.			21	19-20	18
	Streptococcus group A, B, C & G			18	16-17	15
	S.pneumoniae			17	–	17
	Haemophilus influenzae			26	–	26
	Moraxella catarrhalis			23	–	23
	Pasteurella multocida			27	–	27
SD089	Penicillin G	P	1 unit			
	Staphylococcus spp.			26	–	26
	Streptococcus group A, B, C & G			18	–	18
	Streptococcus spp. viridans group			18	13-17	12
	Haemophilus influenzae			12	–	–
	Listeria monocytogens			13	–	13
	Pasteurella multocida			17	–	17
	Corynebacterium spp.			29	–	29

References: 1. Bauer, Kirby, Sherris and Turck, 1966, Am. J. Clin. Path., 45 : 493 2. Performance Standards for Antimicrobial Disk Susceptibility Tests, M100-S25, CLSI Vol. 35 No. 3, Jan. 2015. For more details refer to this volume. 3. EUCAST, Breakpoint tables for interpretation of MICs & zone diameters, version 5.0, valid from 01.01.2015.

Fig 1: Antibiotic susceptibility tests. Zone Size Interpretative Chart for antibiotics from HiMedia as per CLSI

Results

1. Collection of samples and isolation of bacterial flora

Five different droppings were collected from Common myna (1E), Jungle fowl (E2), Spotted dove (E3), Malabar grey hornbill (4E) and Redwhiskered bulbul (5E) residing near Western Ghats, Kochi, Kerala and sixteen morphologically different isolates were obtained.

2. Exoenzyme profiling of potent bacterial isolates

Different enzyme activities were tested for all the bacterial isolates obtained. The results are listed in table 3 and figure 2.

2.1. Xylanase activity

Only isolates showed positive reaction for xylanase activity

2.2. Pectinase activity

Out of sixteen, only five isolates showed positive results for pectinase enzyme production.

2.3. Protease activity

Four bacterial isolates shows zone on casein agar plates indicating their protease activity.

2.4. Amylase activity

Two out of sixteen isolates showed amylase activity on starch agar.

2.5. Cellulase activity

Three of the isolates showed zone of inhibition on CMC agar showing their capability for cellulase activity.

Table 3: Showing exoenzyme profiling of bacterial isolates

Sl. no	Enzyme	Bacterial isolates															
		$1E_1$	**$1E_2$**	**$1E_3$**	**$1E_4$**	**$1E_5$**	**$E2_1$**	**$E2_2$**	**$E2_3$**	**$E3_1$**	**$E3_2$**	**4E1**	**4E2**	**5E1**	**5E2**	**5E3**	**5E4**
1	Xylanase	-	-	-	-	-	+	+	-	+	-	-	+	+	-	-	+
2	Pectinase	-	-	-	-	-	+	+	-	+	-	-	-	+	-	-	+
3	Protease	-	-	-	-	-	+	-	-	+	-	-	+	+	-	-	-
4	Amylase	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-
5	Cellulase	-	-	-	-	-	-	+	-	+	-	-	-	+	-	-	-

*+ → Presence of enzyme, - → Absence of enzyme

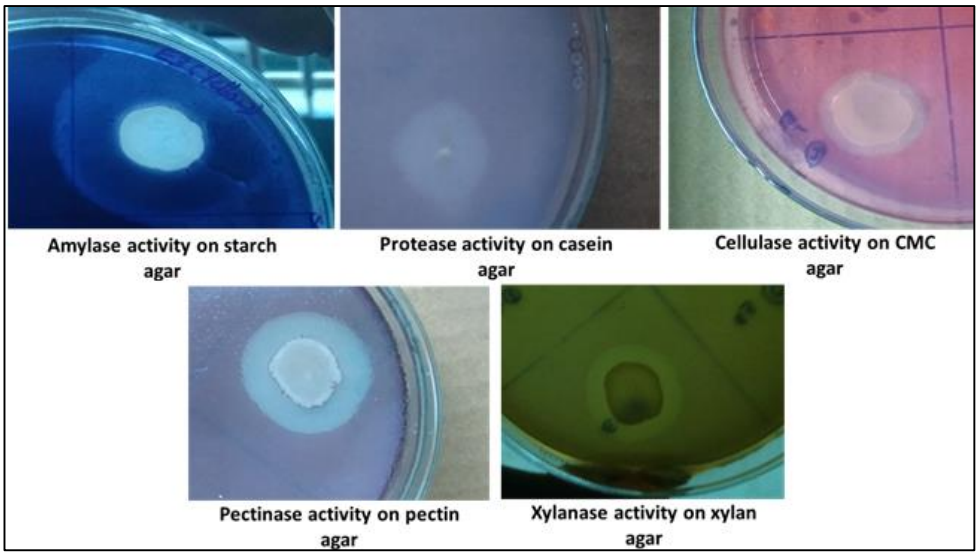


Fig 2: showing qualitative enzyme assays using special media

Based on the qualitative enzyme assays, sixisolates ($E2_1$, $E2_2$, $E3_1$, $4E_2$, $5E_1$ and $5E_4$) out of the sixteen were considered to be more potent due to the presence of different enzymes and thus selected for further studies.

3. Molecular identification of potent isolates

Genomic DNA was isolated (Ausubel *et al*., 1987) (Figure 7). A portion of the 16S rDNA was amplified using a primer pair for16S rDNA (Shivaji *et al.,* 2000). The identity of the sequences was determined by comparing the 16S rDNA sequence with the sequences available in the NCBI nucleotide databases using BLAST (Basic Local Alignment Search Tool) algorithm (Altschul *et al*., 1990) (Figure 3 and Table 4).

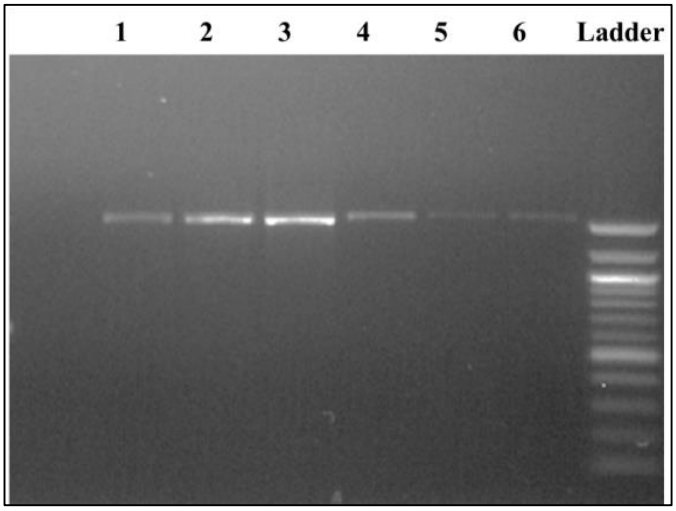


Fig 3: Agarose gel showing the bands of Ladder- Lambda DNA / EcoR1/Hind III/ Double digest 1, Isolates 6- $5E_4$, 5- $5E_1$, 4-$4E_2$, 3-$E3_1$, 2-$E2_2$, 1-$E2_1$.

Table 4: Molecular identity of isolated Bacterial isolates

Sl. no	Isolate	Identity
1	$E2_1$	*Burkholderiasp*
2	$E2_2$	*Bacillus pumilus*
3	$E3_1$	*Lysinibacillus sp.*
4	$4E_2$	*Paenalcaligenes suwonensis*
5	$5E_1$	*Kurthiagibsonii*
6	$5E_4$	*Pseudomonas aeruginosa*

4. Antibiotic sensitivity tests

The six potent isolates were tested for antibiotic sensitivity, with 5 antibiotics (HiMedia, Mumbai) belonging to different classes, namely Ampicillin (10 µg/mL), Chloramphenicol (30 µg/mL), Gentamycin (10 µg/mL), Levofloxacin (5 µg/mL) and Penicillin (1 unit). The results were interpreted as per the manufacturers' instructions and listed in table 5 and figure 4.

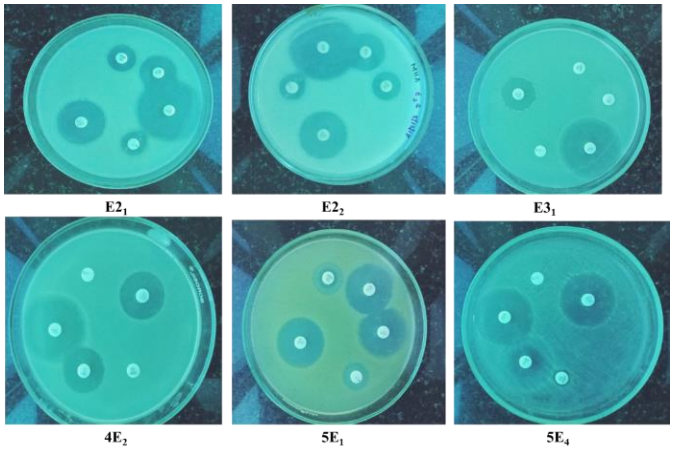


Fig 4: showing the antibiotic sensitivity test by disc diffusion assay

Table 5: Results of Antibiotic sensitivity test by disc diffusion assay

Name of Antibiotics	Isolates					
	$E2_1$	$E2_2$	$E3_1$	$4E_2$	$5E_1$	$5E_4$
Ampicillin (10 µg/mL)	S	R	R	R	R	R
Chloramphenicol (30 µg/mL)	S	I	R	I	S	R
Gentamycin (10 µg/mL)	S	S	S	S	S	S
Levofloxacin (5 µg/mL).	S	S	S	S	S	S
Penicillin (1 unit)	I	R	R	R	R	R

*R-Resistant, I-Intermediate, S-Sensitive

Most of the isolates were found to be resistant or intermediately resistant to the common antibiotics used.

Discussion

Experiential studies documenting the intestinal bacterial flora of wild birds are sparse and have concentrated unreasonably on either those species considered most likely

to transmit bacteria to humans or acquire bacteria from human sources, as reviewed by Benskin *et al.*, 2009 [4]; or on surveys of dead birds which were recovered following disease outbreaks, particularly those in visible locations such as urban parks or gardens (Faddoul *et al.*, 1966; Kirkwood *et al.*, 1995) [9, 14]. Progressively, it is acknowledged that the vertebrate gastrointestinal microbiome influences, and is influenced by, a varied range of factors, including ecological variables such as diet and habitat (Janiga *et al.*, 2007; Literak *et al.*, 2012) [12, 15]; micro-environmental factors connected with the anatomical structure of the digestive tract (Berg 1996; Stevens and Hume 1998) [6, 18]; and, in birds, by bacteria from the reproductive tract (Stewart and Rambo 2000; Hupton *et al.*, 2003) [19, 11]. Changes in the bacterial community structure have been documented to have important consequences for host health in humans (Kau *et al.*, 2011; Wu *et al.*, 2011) [13, 21] and may have an equally important impact on host fitness in birds. Thus, recording the normal micro flora of the avian gut by identifying bacterial presence and richness, as well as factors affecting their distribution in a natural population of birds, is of fundamental importance if we are to fully understand the complexities of bacterial interactions within birds, and escalate whether and how communities of gut bacteria influence the host life history.

There are scarce data concerning the factors that influence the composition and dynamics of the gastrointestinal microbial communities in birds, although there is indication of similarities of micro flora within family groups, and consistency within individuals over time. Currently, the fitness consequences of this variation in gut bacterial species richness are largely unknown (Benskin *et al.*, 2010) [5]. Thus in the present study, the bacterial diversity in bird fecal matter from parts of Western Ghats, Kerala, India via molecular level identification and the exoenzyme and antibiotic profiling of the potent isolates were analyzed.

Conclusion

Five different types of bird droppings were collected from and sixteen morphologically different isolates were obtained. Based on the qualitative enzyme assays, six isolates ($E2_1$, $E2_2$, $E3_1$, $4E_2$, $5E_1$ and $5E_4$) out of the sixteen were considered to be more potent due to the presence of different enzymes and thus selected for further studies. Moleular identification revealed their identity as *Burkholderia sp., Bacillus pumilus, Lysinibacillus sp., Paenalcaligene sinensis, Kurthiagi bsonii,* and *Pseudomonas aeruginosa*. The six potent isolates were tested for antibiotic sensitivity, with 5 antibiotics (Hi Media, Mumbai) belonging to different classes, namely Ampicillin (10 µg/mL), Chloramphenicol (30 µg/mL), Gentamycin (10 µg/mL), Levofloxacin (5 µg/mL) and Penicillin (1 unit) and found that most of them were multiple drug resistant. Further investigations have to be carried out to characterize their pathogenic nature.

Acknowledgement

The first author acknowledges faculties and staff of Merit Biolabs, Vennala, Kochi, and Kerala for supporting the work with necessary facilities.

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