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Differentiation of Human and Migratory Water Fowl by Multiplex *Escherichia coli* Differential Amplification Technique (MECDAT) in South Punjab, Pakistan

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Abstract

Escherichia coli, a gram negative, facultative anaerobic, non-sporulating rod, bacteria are commonly found in lower intestine as a part of the normal flora of gut in all warm blooded organisms. Most *E. coli* strains provide many beneficial functions including protection from other pathogenic bacteria. When *E. coli* strains acquire genetic material from others, they can become pathogenic. *E. coli* strains can be categorized into 5 principal pathogenicity groups; Enteropathogenic *E. coli* (EPEC), Enteroaggregative *E. coli* (EAEC), Enteroinvasive *E. coli* (EIEC), Enterotoxogenic *E. coli* (ETEC) and Enterohaemorrhagic *E. coli* (EHEC). All these strains can cause diarrhea, gastrointestinal infections, urinary tract infections, neonatal meningitis and other illnesses in humans as well as in animals. *EHEC O157* is identified readily in the clinical laboratory with standard culturing techniques. All other strain need molecular methods for their presence in any infected materials.

In this study we isolated a total of 40 cultures and detected different strains of *E. coli* from humans and birds (water fowls). In all isolates antibiotic sensitivity by disc diffusion method with genetic characterization within the *E. coli*. The result shows that all human *E. coli* were resistant to 3 antibiotics (Ampicillin, Co-Trimoxazole, and Cefuroxime), while birds *E. coli* strains are sensitive to these antibiotics indicates different genomic lineage. In human isolates EHEC has highest share, however it was not significantly placed. In correlation studies (Pearson's Correlation) indicates that its significance to the use of only chloramphenicol (p=0.044). ANOVA as well as Pearson's and Spearman's coefficients show that there is no association with any of the drugs that could be useful for their treatment. In could be concluded from the antibiotic profile, distribution pattern that the two groups are epidemiologically are different. Furthermore, it appears that birds don't contribute to the spread of disease in humans/animals.

Keywords *E. coli*; Pathogenicity; Human infection; Antibiotic resistance

Introduction

Intestinal *E. coli* is well characterized as an opportunistic pathogenic species that causes infection in human and animals [1]. Most of these studies are based on serological identification, virulence factors [2], and the mechanism of pathogenicity [3]. Recently, *E. coli* 3 strains associated with diarrheal disease is placed into five pathogenicity groups; Enteroinvasive *E. coli* (EIEC), Enterotoxogenic *E. coli* (ETEC), Enteroaggregative *E. coli* (EAEC), Enteropathogenic *E. coli* (EPEC), and Enterohaemorrhagic *E. coli* (EHEC). Vero cytotoxin-producing *E. coli* (VTEC)/Shiga toxin-producing *E. coli* (STEC) are two other common names for later *E. coli* strains [1,4-7]. Infection with EIEC strains result in watery diarrhea and occasionally dysentery syndrome along with blood, mucus and leukocytes in the stool, tenesmus and fever [7,8]. The primary host for these EIEC human infections appears to be similar to the disease caused by Shigella species. [3,9]. Until now they don't have any reported animal reservoirs [10,11]. The ETEC outbreaks have been connected to the consumption of contaminated food or water [12]. Strains of ETEC are best known to cause traveler's diarrhea [12,13] and infection to infants in developing countries [3,7]. These subspecies of *E. coli* don't invade tissues neither they leave the intestine of the host [14,15].

The EAEC on the other hand is most recently been connected to a category of infectious diarrheagenic *E. coli* that adhere to tissue culture cells *in vitro*. They tend to produce aggregation, adherence and association to produce persistent, watery diarrhea in young children. Till now, no mode of pathogenicity has been elucidated for them in literature [16]. The forth type of *E. coli* (EPEC) induce a profuse watery and bloody diarrhea [17]. It is the leading cause of infantile diarrhea in developing world [3,18]. Transmission has been associated to consumption of contaminated drinking water and meat products, [15]. Molecular pathogenesis of EPEC has proven to involve plasmid mediated protein referred to as EPEC Adherence Factor (EAF) [17,19].

The Verotoxin producing *E. coli* (VTEC) strain infects humans and cause severe complications of Hemolytic Uremic Syndrome (HUS) [20,21]. These are sometime also referred as Enterohaemorrhagic *E. coli* (EHEC) or Shiga toxin-producing *E. coli* (STEC) [11,22]. All these strains produce Shiga toxin (also known as Verotoxin), a major cause of food borne illness [23,24]. The Vero cytotoxin has shown to cause damage to 4 renal and endothelial cells [25].

The best known strains in these organisms have been *O157:H7* that have produced devastations in developed countries [26-28]. Most EHEC strains carry a plasmid which encodes a haemolysin gene and chromosomally located Locus of Enterocyte Effacement (LEE) factor [11,26]. The avian pathogenic *E. coli* (APEC) strains also cause intestinal as well as extra intestinal diseases via respiratory tract infection in birds [29-31]. These species share virulence factors that include type 1 and P fimbriae, coliaerobactin, lipopolysaccharide (LPS), K1 capsular antigen, and Temperature Sensitive Hemagglutinin (TSH) [1]. All of these are linked to uncharacterized specific chromosomal location [1,32].

The APEC strains can cause most common form of colibacillosis that initiates with respiratory infection symptoms (air sacculitis) which later spreads and cause generalized infections (perihepatitis, pericarditis, and septicemia) [33]. Data from some studies show that positive relation exists between APEC and human Extraintestinal Pathogenic *E. coli* (ExPEC). Numbers of examples are listed in literature like uropathogenic *E. coli* (UPEC) causing uremic syndrome [33-35] and newborn meningitiscausing *E. coli* (NMEC). This also suggests that some APEC strains could be considered potential zoonotic agents [1,3,33].

In our case, a study was initiated to isolate and genotypically classify *E. coli* pathogens (groups) from humans and migratory water fowls and find their commonality. This would provide deeper insight about the two reservoirs. Antibiotic patterns within the two groups will also give some evidence about their linkage between human and migratory birds both at genetic as well as phenotypic nature of *E. coli* species.

Materials and Methods

Pure cultures of 20 *E. coli* human were included in the study after collection from Combined Military Hospital (CMH) Multan Cantonment and other private laboratories of 5 Multan, Pakistan. Further, another 20 were isolated and collected from Water Fowls cloacal/buccal swabs.

Preservation of samples

The isolated and identified cultures were preserved in MicroBank vials (ProLab, Canada) and kept in freezer at -80°C (Sanyo, Japan).

Study area

The present study was conducted at Micro/Molecular Biology Laboratory (MMBL), Institute of Pure and Applied Biology (IPAB), Bahauddin Zakariya University Multan.

Media preparation

All solutions and media were sterilized by autoclaving (Hirayama, Japan) at 121°C at 15 lbs/sq. inch for 20 minutes. Each plate (Germany) was made by adding approximately 20 mL of the selective/differential media after it cools down to 47°C. After solidification of the media, they were placed in refrigerator until needed for assay.

Antibiotic sensitivity test

Each of antibiotic sensitivity tests were performed on Antibiotic Sensitivity Sulfonamide (ASS) Agar (Merck, Germany). All stock culture *E. coli* were grown overnight in Nutrient broth (Oxide, UK).

Optical density was adjusted to 0.5 OD McFarland's tube and cultures were spread on ASS plate with the help of sterile cultural swabs (China). To each plate an Octadisc™ (HiMedia Laboratories, India) with selected antibiotics were placed in the middle of plate with sterilized forceps with gentle press.

The selected Octadiscs™ were impregnated with antibiotics Tetracycline (TE 30 µg), Gentamicin (GEN 10 µg), Co-Trimoxazole (COT 25 µg), Ceftriaxone (CTR 30 µg), Cefuroxime (CXM 30 µg), Chloramphenicol (C 30 µg), Ampicillin (AMP 10 µg), and Ciprofloxacin (5 µg). Plates were incubator overnight. Antibiotic sensitivity was read by millimeter scale next day. The results were recorded as given in Tables 1 and 2.

DNA extraction and PCR

The DNA was extraction from each isolate with both alkaline lysis method and CTAB methods [36,37]. Multiplex PCR technique was used for detection of different *E. coli* subspecies. The amplifications were done with 8 primers: bfpa, eae, eae-y, lpfa 1.1, lpfa 1.3, lpfa 2.2, stx1, stx2. List of primer is provided in Table 1.

No.	Primers	Primer Sequences (5´ ® 3´)	Amplicon	Primer (mM in Rx)	Tm °C
1	STX2-F	ATC CTA TTC CCG GGA GTT TAC G	587	1	62
	STX2-R	GCG TCA TCG TAT ACA CAG GAG C		1	64
2	EAE-y-F	CAG GTT GGG GTA ACG GAC TTT AC	472	1	65
	EAE-y-R	TTG CTT GCG TTT GAG ACT TAC CGT TG		1	66
3	LPFA1.1-F	GTG CTG GAT TCA CCA CTA TTC ATC GC	389	0.4	68
	LPFA1.1-R	GCC TTG TCT GCA CTG GCA TTA ACT TC		0.4	68
4	STX1-F	CAG TTA ATG TGG TKG CGA AGG	348	1	60
	STX1-R	CAC CAG ACA ATG TAA CCG CTG		1	61

5	BFPA-F	AAT GGT GCT TGG GCT TGC TGC	326	0.4	63
	BFPA-R	GCC GCT TTA TCC AAC CTG GTA		0.4	61
6	IPFA2.2-F	CTA CAG GCG GCT GAT GGA ACA	297	0.4	63
	IPFA2.2-R	GCT AAT ACC AGC GGC AGC ATC GT		0.4	66
7	IPFA1.3-F	GGT TGG TGA CAA ATC CCC G	244	0.4	59
	IPFA1.3-R	CGT CTG GCC TTT ACT CAG A		0.4	57
8	EAE-F	CTT TGA CGG TAG TTC ACT GGA CTT C	166	0.4	66
	EAE-R	GAA GAC GTT ATA GCC CAA CAT ATT TTC AGG		0.4	68

Table 1: Antibiotic sensitivity result.

All PCR reactions were done on conventional Thermocycler (Eppendorf, Germany) in 20 µL reaction volume (Invitrogen, USA). Amplifications were run in two step modules with 55 cycles as indicated in Tables 2a and 2b. Amplicons were analyzed on 1% Agarose (US Biological, USA) in 1X TBE buffer gel electrophoresis in mini gel box (Hoefer, USA) with power supply (Pharmacia, Sweden) at 80 volts. The amplified DNAs were stained in 0.5 µg/mL Ethidium bromide (US Biological, USA) (stock 10 mg/mL) for 10 minutes. All DNA bands were visualized under UV light transilluminator (UVtec, USA) and photographed.

Reaction mixture	Volume requried
Template (Sample)	1 µl
Master Mix (5X) (REDTaq)	4 µl
Primer concentration	10 µl
PCR grade water	5 µl
Total	20 µl

Table 2a: Reaction mixture for PCR.

Reaction mixture	Volume required
Template (Sample)	2 µl
Master Mix (5X) (REDTaq)	4 µl
Primer conc.	10 µl
PCR grade water	4 µl
Total	20 µl

Table 2b: Reaction mixture for PCR.

Results

Antibiotic sensitivity

Comparison of antibiotic sensitivity within the two groups indicates that they belong to two separate clades with some overlapping (Table 3).

Groups		Human Sig.	Birds Sig.
TE	Between Groups	0.189	0.28
	Within Groups		
GEN	Between Groups	0.353	0.171
	Within Groups		
COT	Between Groups	-	0.437
	Within Groups		
CTR	Between Groups	0.67	0.904
	Within Groups		
CXM	Between Groups	-	0.883
	Within Groups		
CIP	Between Groups	0.394	0.189
	Within Groups		
C	Between Groups	0.044	0.855
	Within Groups		
AMP	Between Groups	-	0.303
	Within Groups		

Table 3: ANOVA (N=40) (2 tailed analysis with *E. coli* genetic species).

The human isolates were sensitive to Chloramphenicol (p=0.044 with CL 95%) out of 8 antibiotics tested (Table 1). While on the contrary, in bird *E. coli* isolates does not show any significant association any of the drugs tested (Table 2).

Correlation data indicates that all genetic isolates are negatively related to resistance to antibiotic gentamycin with no significance. In birds, Chloramphenicol is highly negative significant to Cefuroxime. However, Tetracycline, Ceftriaxone show significant association with the type of isolates (Table 4).

Interestingly gentamycin is only drug which is in negative non-significant to all isolates. This allows us to infer that resistance in human isolates to 2nd and 3rd generation antibiotics and in linkage to waterfowls.

Sr. No.	*E. coli* Strain	Humans (%)	Birds (%)
1	Atypical EPEC	3 (15.0)	3 (15.0)
2	Typical EPEC	4 (20.0)	6 (30.0)
3	EHEC	6 (30.0)	4 (20.0)
4	STEC	3 (15.0)	2 (10.0)
5	LEE-STEC	1 (5.0)	0
6	Others	0	1 (5.0)
7	Negative	3 (15.0)	4 (20.0)

Table 4: Distribution of different isolates according to genetic *E. coli* groups.

Multiplex *E. coli* differential amplification technique (MECDAT)

In this study, the isolated organisms are genetically grouped and resistances to antibiotics are used to address the issue of spread of human strains in birds or vice versa. Results show that Application of Multiplex *E. coli* Differential Amplification Technique (MECDAT) is successfully applied in surveillance of *E. coli* in humans / birds. Data on the group's antibiotic sensitograms indicates that the two groups are with some genetically overlapping differences. Genetic groups show that atypical and typical EPEC share 35% of human total isolates. While in birds they have about 45% strains positive. On the other hand, 50% of human isolates produce shiga toxin as against 30% in water migratory bird isolates (Table 2).

Discussion

We isolated total of 40 *E. coli* cultures from humans and migratory birds during migratory bird season. Of these, collected cultures 20 each from human and other 20 from water fowls were included in the study. The human isolates show resistance to 3 antibiotics Ampicillin (AMP), Co-Trimoxazole, and Ceftriaxone (CXM) (Table 1). Each resistance pattern evolves itself through genetic selection/genetic transfer mechanisms [38,39]. In literature, it was observed that diarrheal *E. coli* isolates were 86.4% resistant to AMP, and 29.6% to CXM, [40]. On the contrary, in birds *E. coli* was sensitive to all these antibiotics (Table 1). In Jamaica, study highlights the prevalence of multiple drug resistant *E. coli* among healthy broiler chickens in Jamaica, West Indies, possibly associated with expression of tetracycline resistance [41]. The data show that it didn't appear to be a common source in multiple drug resistance strains of avian or human origin, the genes encoding resistance are similar [41,42]. These results suggest that genes are disseminated in the environment and more investigative certification of the possibility for avian sources acting as reservoirs for tetracycline resistance [41] (Table 5).

Correlations											
Pearson's Correlation		Genetic species	Type	TE	GEN	COT	CTR	CXM	CIP	C	AMP
Genetic species	P. Correlation	1									
	Sig. (2-tailed)										
Type	P. Correlation	0.013	1								
	Sig. (2-tailed)	0.938									
Tetracycline	P. Correlation	-0.086	0.799*	1							
	Sig. (2-tailed)	0.598	0								
Gentamycin	P. Correlation	-0.076	-0.162	-0.051	1						
	Sig. (2-tailed)	0.639	0.319	0.755							
Co-Trimoxazole	P. Correlation	-0.056	0.839*	0.632*	-0.112	1					
	Sig. (2-tailed)	0.733	0	0	0.493						
Ceftriaxone	P. Correlation	-0.084	0.813*	0.712*	-0.05	0.796*	1				
	Sig. (2-tailed)	0.607	0	0	0.759	0					
Cefuroxime	P. Correlation	0.139	0.673*	0.572*	-0.016	0.653*	0.547*	1			
	Sig. (2-tailed)	0.392	0	0	0.921	0	0				
Ciprofloxacin	P. Correlation	0.105	0.712*	0.416*	-0.256	0.682*	0.523*	0.384***	1		
	Sig. (2-tailed)	0.518	0	0.008	0.111	0	0.001	0.014			
Chloramphenicol	P. Correlation	0.148	-0.343****	-0.380****	-0.009	-0.271	-0.319****	-0.436**	0.102	1	
	Sig. (2-tailed)	0.363	0.03	0.016	0.955	0.091	0.045	0.005	0.532		

Ampicillin	P. Correlation	0.006	0.874*	0.673*	-0.179	0.761*	0.770*	0.559*	0.649*	-0.278	1
	Sig. (2-tailed)	0.97	0	0	0.268	0	0	0	0	0.082	

Note: *Correlation is significant at the 0.01 level (2-tailed). **Negative Correlation is significant at the 0.01 level (2-tailed). ***Correlation is significant at the 0.05 level (2-tailed). ****Negative Correlation is significant at the 0.05 level (2-tailed)

Table 5: Correlation of different factors and Antibiotics with E. coli genetic strains.

In our hand, 17 human samples provide amplifications with 3 with no results (Table 2). EHEC was found to be higher in these isolates with no significance to variants. In birds, a similar pattern, 16 samples showed amplification with 4 being negative (Table 4). Studies by Sjolund and coworkers on wild birds and humans concluded that *E. coli* isolates were similar [42]. Use of Multiplex *E. coli* Differential Amplification Technique (MECDAT), as used in this study, will provide a long way to identify subspecies with some modifications. Similar results have been cited elsewhere in the literature. ANOVA results as well as correlation coefficients indicate that there is no association with any of the antibiotic that could be useful for their treatment. The Pearson's correlation coefficient on antibiotics showed that Chloramphenicol is significantly placed (p=0.044) at 0.05 confidence limit. Interestingly in birds, genetic grouping showed overlapping differences to humans. In bird *E. coli* a typical EPEC class dominates. The EHEC microbe has rarely been reported in wildlife with exception to deer and sporadically in domestic animals and birds [11,17]. The presence of EHEC, though in small numbers is important in spread of strain to the farthest flinched area with ease. This also indicates some sort of special association of these *E. coli* in the digestive tract of human versus birds. In literature special type of pili has been assigned to these *E. coli* for its colonization [43]. This also has been illustrated from the studies on the gut microbiota of different migratory birds. These findings are unique, and reported first time in these migratory waterfowls from Pakistan. The presence of STEC in humans and birds show minor differences (15.0% vs. 10.0%) respectively (Table 4). Importance of *E. coli O157* type infection is partly concludes from this study. But like in other studies, presence of 10% of STEC appears too much higher than recent investigators identification by Neher et al. from India [44]. Though it is also suggested that presence of seed *E. coli* strain in these waterfowls may be important in the spread of new infections, probably not EHEC, centers from where they can enhance colonization and 9 thereby spread in the community. Such spread has been documented elsewhere in the literature [45,46]. In one report, microbiological culturing along with genomics has indicated that an outbreak could occur by indirect contact with wild birds' faeces of VTEC infection in humans [27]. In a two-year study (2007-2009) in Canada, an important conclusion was gathered which confirms the isolation of *E. coli* O157 from faecal pats. Presence of these organisms suggest that it is inducted in by contact chain between calves and cattle by ventilation, manure systems and importantly linked to bird number per milking cow [47]. Wildlife is normally not exposed to clinically use of antimicrobial agents, but can acquire antimicrobial resistance through contact with humans, domesticated animals, environment or water polluted with feces new putative infection cycles between wildlife, domesticated animals and humans [48]. Literature has been accumulated which spans for number of years, indicates that strains initially show small antimicrobial resistance to Ampicillin, Gentamicin, Streptomycin, Ciprofloxacin, Chloramphenicol and Tetracycline that later increased to high levels [49]. In a recent study carried out on 101 *E. coli* from broilers and layer hens in Bangladesh with colibacillosis illustrates this similar phenomenon [50]. Another misnomer about avian *E. coli* isolates is there no pathogenicity. The data on avian pathogenic *E. coli* (APEC) is not yet clear on the significance of infective ExPECs [50-52]. Recent epidemiological studies have shown similitude in isolates of human and avian Extraintestinal Pathogenic *Escherichia coli* (ExPEC) for their virulence genes [43]. This suggests a probable common pathogenic mechanism in poultry and mankind. Molecular typing on animal faecal and from food samples revealed that they cluster into the same molecular type [51,53]. This also pinpoints that animal/bird faeces might play a significant reservoir role for *EHEC O157* spread. The survival studies in bird's intestinal tract of pathogenic bacteria show that they can survive and can be transferred to humans from food, animals through faecal contact [54]. Data clearly suggest that migratory birds can carry *E. coli O157:H7* and 10 disseminates to a large area and distances in short time [54,55]. This all suggests strict monitoring of all possible food contamination to be reinforced [53,56,57]. In our data, this plausible issue couldn't be explained, elaborative nor suggested otherwise. However, numerous similar studies provide evidence that APEC ST95 O1 strains cluster with human ExPEC strains. This again clearly demonstrates its zoonotic potential [58,59] which we did not explore further in our work. Study on the APEC strains in a rat model, reveals that APEC's ability to cause meningitis in mammals, including humans their possible potential [60]. This imparts good support to our hypothesis that APEC strains have the potential of zoonosis [30]. Chandran et al. [61], on the contrary, provides evidence to our conclusion that STEC and EPEC isolates were genetically distinct from nonpathogenic *E. coli* and clustered independently. Some researchers conclude that the presence of virulence genes alone cannot be used to determine the pathogenicity of strains. Results show that potentially pathogenic STEC and EPEC strains can be found in some of the avian hosts studied and may contaminate surface water and potentially impact human health [62]. Our study concludes and depicts that strict, continuous monitoring with molecular tools must be a part in epidemiologically important pathogens for predictive suggestions. These notable differences in either human or animals should be made public for better preventive measure as proposed by Ewers and coworkers and others later [45,63]. We at this stage also recommend the following:

- Continuous monitoring of 8 commonly used antibiotic resistances in human and from other animals, birds be done.
- More funds for surveillance schemes are allocated globally, especially, in the developing world.
- Pulse Field Gel Electrophoresis (PFGE) profiles and Sequence analysis facilities/centers will enable us to get close to the phylogenic evolution of each strain that is seen in our daily life.
- Contribution of genetic transfer/modifications by extra-chromosomal elements is assessed with genetic exchange methods.
- Yearly monitoring in these migratory birds should be done with molecular techniques.

Conclusion

In our study, however, waterfowl birds, reported first time, do show less similar patterns within the two groups. Moreover, besides these similarities, differences in the isolates provide some evidence for the lineage that may have common origin as well as addition of any isolated source (Table 4). Recent data on ExPEC microbes show that they produce "newer β-Lactamases" that are encoded on plasmids that are classified as class C Cephalos porinases (Amp C) β-Lactamases, Extended Spectrum β-Lactamases (ESBLs) and Carbapenemases (e.g., Imipenem, Verona Integrons-encoded Metallo-β-Lactamase (VIM), New Delhi Metallo-β-Lactamase (NDM types), that are now reported widespread [1,48]. Presence of resistance in human isolates, especially with these β-Lactamases, needs further investigation. Recently *Escherichia Albertii* is identified as a newly emerging enteric pathogen [64,65] that has been identified in both animals and humans with subclinical association to some birds [66]. Ooka et al. [65] has rightfully identified *E. Albertii* identified different *eae* gene that might be misidentified as EHEC or EPEC. In our work also some of the isolates, both in human and birds, were not identified by this subspecies identification system and need further improvements that should also include *E. Albertii* like organisms. Additionally, we showed that a great number of avian ExPEC are probable-Lactamase producing strains, sinceresistance to amoxicillin/clavulanic acid were recorded as 70.4% in the isolates [50]. Identification of Enterohaemorrhagic strains in birds as well in human quae us to make strict surveillance a mandatory option in food borne pathogens. Thus in future, our focus and concentration would 12 be towards the phylogenic mechanistic evolution in birds and animals using molecular techniques.

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