

RESEARCH ARTICLE



Temporal variations in patterns of *Escherichia coli* strain diversity and antimicrobial resistance in the migrant Egyptian vulture

Pradeep Sharma ^a, Sunil Maherchandani^a, B. N. Shringi^a, Sudhir Kumar Kashyap^a and K. S. Gopi Sundar ^{b,c}

^aDepartment of Veterinary Microbiology and Biotechnology, College of Veterinary and Animal Science, Rajasthan University of Veterinary and Animal Sciences, Bikaner, India; ^bProgram SarusScape, International Crane Foundation, Baraboo, WI, USA; ^cCranes and Wetlands Programme, Nature Conservation Foundation, Mysuru, India

ABSTRACT

Aims: Multiple antimicrobial resistance in *Escherichia coli* of wild vertebrates is a global concern with scarce assessments on the subject from developing countries that have high human-wild species interactions. We studied the ecology of *E. coli* in a wintering population of Egyptian Vultures in India to understand temporal changes in both *E. coli* strains and patterns of antimicrobial resistance.

Methods and Results: We ribotyped *E. coli* strains and assessed antimicrobial resistance from wintering vultures at a highly synanthropic carcass dump in north-west India. Both *E. coli* occurrence (90.32%) and resistance to multiple antimicrobials (71.43%) were very high. Clear temporal patterns were apparent. Diversity of strains changed and homogenized at the end of the Vultures' wintering period, while the resistance pattern showed significantly difference inter-annually, as well as between arrival and departing individuals within a wintering cycle.

Significance of study: The carcass dump environment altered both *E. coli* strains and multiple antimicrobial resistance in migratory Egyptian Vultures within a season. Long-distance migratory species could therefore disseminate resistant *E. coli* strains across broad geographical scales rendering regional mitigation strategies to control multiple antimicrobial resistance in bacteria ineffective.

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Introduction

Escherichia coli occurs widely in the intestines and faeces of all vertebrates, and its prevalence and diversity in wild species varies with host taxonomy, geography, diet, and environmental conditions particularly the degree of synanthropy of the hosts' surroundings [1–4]. The recognition of wild vertebrate species carrying *E. coli* is not recent, but the number of wild species and the range of environmental conditions that have been studied to date is biased to a handful of locations globally, with notable paucity of studies from densely-populated, developing countries [5].


Wide-ranging wild species hosting *E. coli* are subjected to widely varying environments including exposure to human-dominated environments, in a relatively short period of time facilitating development of multiple antimicrobial resistance in *E. coli* [6]. The prevalence of multiple antimicrobial resistance in commensal *E. coli* is a strong indicator of selective pressure to use of antimicrobials [7,8]. The level of resistance in bacteria of wild animals correlates well with the degree of association with human

activity [5,9]. Also, more synanthropic bird species show increased multidrug resistance *E. coli* [10,11]. Wild vertebrate species, especially those that use human-dominated landscapes as part of their life history, are therefore likely to be 'reservoirs and melting pots of bacterial resistance' with increasing potential of resistant *E. coli* colonizing intestine and resulting in antimicrobial resistant infections if they end up in urinary tract or the bloodstream adding concern to human health [4,12].

Studies using wild animals have revealed microbial traits of *E. coli* that indicate its suitability as an indicator organism of multiple ecological aspects. *E. coli* showed a much greater geographical effect in non-domesticated, resident wild species relative to isolates in humans suggesting that differential movement patterns influence *E. coli* strains that predominate in a species [3]. Similarly, in wild species, *E. coli* exhibit very strong geographic effects, which is likely due to a combination of host taxonomy, diet and climate [2,13,14,15]. Phylogeny of *E. coli* of wild birds varied concomitant to exposure to humans and human-associated vertebrates such as domestic fowl underscoring the influence of anthropogenic

CONTACT Pradeep Sharma  pradeep@gmx.it  Department of Veterinary Microbiology and Biotechnology, College of Veterinary and Animal Science, Rajasthan University of Veterinary and Animal Sciences, Bikaner, Rajasthan, India

^{*}Present address: Department of Animal Husbandry, Government of Rajasthan, India.

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components [5,10]. The near-absence of studies of the of *E. coli* in wild vertebrates in densely-populated countries is worrisome especially given the increased risk of wild species interacting with humans and domestic animals, and are urgently required [4]. In particular, documentation of the temporal and spatial patterns of antimicrobial drug resistance in different habitats and species in developing countries is essential to developing a cogent understanding of wildlife as a source of antimicrobial resistance [16,17]

Two consistent patterns of *E. coli* ecology are emerging from studies in wild vertebrates: (1) Population structure of *E. coli* strains strongly reflect the host's environment and feeding habits, and (2) multiple antimicrobial resistant strains increase in more synanthropic settings. In this study, we tested if these two patterns hold in a unique environmental setting using an explicit temporal study design, and a migrant scavenger species, the Egyptian vulture (*Neophron n. percnopterus*) in its wintering grounds in north-western India.

The Egyptian vulture is ideal to test both patterns due to its natural history and habits, but microbial studies using this host species are absent to date [18]. The species breeds across a wide geographic range in Europe nesting on cliffs, displaying dietary plasticity that includes both wild and domestic vertebrates [18–20]. The migratory route of the Egyptian vulture can differ annually, but the species travels non-stop and rapidly to wintering grounds, displaying very strong inter-annual site fidelity, with movements in wintering sites restricted to a small area if food is abundant [20]. The population of Egyptian vultures wintering in north-western India stay for several months (October to March), some of them at a highly unusual and synanthropic site, a communal dump for livestock carcasses in Jorbeer, Rajasthan. Livestock carcasses from different clinical settings as well as dairies in Bikaner city are collected, dumped and skinned by resident workers. Such carcasses can be hubs for interactions between different host species that potentially harbour infectious agents [21]. Scavenging raptors' diet in communal dumps are largely carcasses of farm animals which contain high rates of antimicrobial resistance [22]. The carcass dump, therefore, can facilitate intense interaction of diverse micro-biota including *E. coli* of human, livestock and multiple congregating, wintering wild bird species, in addition to exposure to unknown quantities and types of veterinary drugs in the carcasses. Conditions were therefore primed to expose Egyptian vultures to different *E. coli* assemblages relative to their breeding grounds, and also to develop multiple antimicrobial resistance.

The migrating Egyptian Vultures at Jorbeer are therefore exposed to widely varying environmental and synanthropic conditions in their breeding and wintering grounds. In line with the migratory

behaviour of the vultures and conditions at Jorbeer, we asked three broad questions in our study: (1) does the population structure of commensal *E. coli* of the migrating vultures respond to spatial and temporal changes?; (2) could *E. coli* of the vultures attending communal carcass dumps show multiple antimicrobial resistance and if so; (3) would patterns of such resistance show clear spatial and temporal changes to match with the vultures' wintering environment? Related to these questions and widely observed patterns of *E. coli* ecology in wild animals, we framed and tested two specific hypothesis that are conceptualized in the Supplemental Material (Fig. S1): (a) *the environmental hypothesis* – the diversity of *E. coli* strains in the vulture population recently arrived at Jorbeer (representing conditions and exposure at breeding grounds) will change as the vulture population spends more time in the wintering grounds, and that there will be increasing homogeneity in commensal *E. coli* strains just prior to departure reflecting exposure to similar environmental conditions at wintering grounds; and (b) *the resistance hypothesis* – the highly synanthropic nature of the carcass dump will result in very high incidence of antimicrobial resistance, with patterns of resistance changing progressively over the wintering season reflecting varying exposure to differing drugs and changing diet at the carcass dump. For both aspects, we also hypothesized strong inter-annual variations due to migratory behaviour of the species.

Materials and methods

Sample collection

Samples were collected throughout the wintering season of the vultures at a large carcass dump (approximate 0.25 Sq. Kms area) known as Jorbeer (N 27°57.958' E 73°22.598') in Rajasthan state, north-western India. Focal individuals of Egyptian Vultures were watched, and were approached immediately as they voided faecal lumps. One sample was taken from each individual vulture, samples were collected during mornings for 90 minutes in a single day for a given month of sampling, and were processed the same day within one hour. We minimized the probability of sampling the same individual vulture by moving focus rapidly to a different individual vulture after each sample collection. Since there was always over 500 vultures in the dump, we could not be certain of the landing location of previously sampled individuals. Samples were collected once during the pre-departure period (February 2011), and then sequentially in the subsequent year to reflect the subsequent full wintering period, which included arrival time (October 2011), resident period (November 2011), and departure time (March 2012) (Table 1). The sample therefore had a complete cross-section of one complete wintering period

Table 1. Sampling periods and frequency of isolation of the *E. coli*.

Month and year of sampling	No. of samples	Frequency of isolation of <i>E. coli</i>	Frequency of isolation (in percent)
February, 2011	13	12	92.31
October, 2011	13	12	92.31
November, 2011	21	19	90.48
March, 2012	15	13	86.67
Total	62	56	90.32

(2011–2012), and representation of the inter-annual variation represented by the bout of sampling in February 2011.

Isolating *E. coli*

In the laboratory, the swabs were inoculated in nutrient broth (Himedia, Mumbai) and incubated for 24 hours at 37°C. and processed further with standard biochemical tests for phenotypic characterization [23].

Isolating *E. coli*. In the laboratory, the swabs were inoculated in nutrient broth and incubated for 24 hours at 37°C. In short: A loop full of culture was streaked onto MacConkey agar (Himedia, Mumbai) and incubated aerobically at 37°C for 24 hours, pink colonies typical of *E. coli* were processed further streaked on EMB agar (Himedia, Mumbai) and incubated at 37°C for 24 hours. Colonies showing metallic sheen were randomly picked and streaked on nutrient agar and incubated at 37°C for 24 hours. Gram negative rods from catalase positive and oxidase negative colonies were processed further with standard biochemical tests for phenotypic characterization (23).

Ribotyping

As the design of this experiment focused on study of temporal changes in *E. coli* strains and not source-tracking, 16S–23S rRNA intergenic spacer region (16S-23S rRNA ISR) polymorphism (Ribotyping) was regarded as a suitable technique for studying intra species phylogeny in bacteria as this is least affected by horizontal gene transfers [24], while the flanking rRNA genes are highly conserved. Also, ribotyping is suitable in epidemiological studies for determining relatedness and discrimination of strains [25,26].

DNA from confirmed *E. coli* isolates was subsequently processed for ribotyping using 16S-23S r-RNA Intergenic Spacer Region (ISR) polymorphism by PCR. The Microbial Type Culture Collection (MTCC) 443 strain of *E. coli* was used as a positive control to validate the results. In short the ribotyping involved the following sequential steps:

- (1) DNA was extracted, checked for purity, integrity and ribotyped by published protocols [27–29].
- (2) Subsequently, the polyacrylamide gel electrophoresis (PAGE) images were analysed by PyElph software [30]. Bands across the lanes were matched based on position and number of bands, and a binary matrix, designating presence of band as ‘1’ and absence as ‘0’, was made using the software.
- (3) To confirm validity and reproducibility of the ribotyping process 14 isolates of the *E. coli*, one each from 14 random faecal samples, picking a different colony other than one used in previous ribotyping, were ribotyped again by 16S-23S r-RNA ISR polymorphism PCR as used in the previous ribotyping, which showed no changes compared to the previous ribotyping.

Antimicrobial susceptibility testing

The antimicrobial resistance pattern of the isolates was determined by disc diffusion method [31] using CLSI standards (2010). All of the pure *E. coli* cultures were subjected to resistotyping to 13 antimicrobials of different classes. Antimicrobials that were commonly used in veterinary as well as human medicine were included. Eleven classes of commonly used veterinary antimicrobials including combination of ampicillin with sulbactam (a beta-lactamase inhibitor) were chosen to study if the resistance patterns are influenced by the usage. In addition, we selected imipenem specifically as a stand-alone antimicrobial not used in veterinary practices to see if the *E. coli* could still develop resistance to this antimicrobial as well. This way, a total of 13 antimicrobials were used for antimicrobial susceptibility testing. All agar media and antimicrobial discs were sourced from HiMedia, Mumbai, India.

Control strain MTCC 443 was used as control. Multiple antimicrobial resistance (MAR) was defined as resistance to three or more than three antimicrobials of different chemical classes. A binary matrix (resistant = 1; sensitive = 0) was constructed based on response of the isolates to antimicrobials. The ‘intermediate’ responses were converted either into ‘resistant’ or ‘sensitive’ using lower and higher diameter of zone of inhibition, relative to reference values (of ‘intermediate’ response), respectively for statistical analyses.

Statistical analyses

All analyses were carried out on the R platform [32]. We wanted to estimate relatedness of *E. coli* strains sampled in each month to ascertain the validity of the environmental hypothesis. For this we used two methods: discriminant analysis of principal components (DAPC), and analysis of molecular variance

(AMOVA), using packages *ade4* and *poppr* respectively [33,34].

DAPC is a model-free method that does not assume populations are panmictic, and explores structures of populations by locating individuals into clusters after partitioning the variance of the sample into within- and between- group components. DAPC is sensitive to the number of principal components, so we used the function *xvalDapc* in package *ade4* to select the correct number of components. We used 90% of the data as a training set with 1,000 replicates, and derived the minimum number of principal components as that having the lowest root mean squared value and the highest degree of successful reassignment of the samples. AMOVA was used to determine the degree of differentiation of variations between designated partitions (months and year in this case) using molecular markers. We used a hierarchy of year and month to understand if the *E. coli* strains varied not just between months of one full wintering period, but also if strains varied between years. Using the package *ade4* and 1,000 permutations, we tested significant deviations of the data from a random population structure. In addition to computing significance levels of variations between months and years using AMOVA, we plotted the clustering of the populations using DAPC to visually assess the degree of partitioning of populations.

We tested the resistance hypothesis, or if patterns of antimicrobial resistance varied between months and years, using three different methods, each for a different resolution: non-metric multidimensional scaling (NMDS), dissimilarity between months using Jaccard's index, and Sørensen's β -diversity. These were implemented in R packages *vegan*, *simba* and *betapart* respectively [35–37]. Using package *vegan*, we implemented NMDS to assess if patterns of antimicrobial resistance exhibited by *E. coli* varied across months. NMDS is a gradient analyses that uses a rank-based approach to represent as closely as possible the dissimilarity between objects in a reduced number of dimensions. Original distance data is substituted by ranks, which does not require to meet assumptions regarding distributions of data sets. NMDS uses an iterative process, and produces stress values that can be used to assess the fit of the algorithm, with stress values < 0.2 indicating a reasonable to good fit, and values > 0.3 indicating that the ordination is arbitrary. We produced an NMDS plot to visually assess dissimilarities of microbial resistance of *E. coli* in different months. We carried out a statistical test using presence/absence matrices of the resistance pattern obtained each month using the multi-response permutation procedure (MRPP) also using package *vegan*. The MRPP statistic δ evaluates if there is a difference between two or more groups of sampling units, and represents the weighted-mean of

within-group means of pairwise dissimilarities in the sampling units. A significant value would indicate considerable differences in antimicrobial resistance patterns between months. We then assessed pairwise monthly differences with Jaccard's indices using the function *com.sim* in package *simba*. To overcome sample size limitations, we ran 1,000 permutations of the data and derived statistical significance values to assess if variation was significant between months. This additional analysis was required since month-wise variation using only NMDS plots can be difficult to interpret directly owing to the arbitrary scales of the axis, and the reduction of dimensions.

The overall diversity of antimicrobial resistance of the *E. coli* occurring in wintering vultures at Jorbeer could be due to two different processes. The first is 'turnover', indicating acquirement of new resistance by *E. coli* incrementally as the wintering period progressed. Turnover would show sequential and consistent changes in resistance patterns over a period of time. The second is 'nestedness', where a large part or all of the resistance pattern is acquired in bursts over relatively short periods. This would suggest very rapid change in resistance patterns of the *E. coli* corresponding to a particular sampling period, with few to no additional new resistance being added as time lapsed. We assessed which pattern contributes to the overall β -diversity (or the Sørensen's dissimilarity equivalent, β_{SOR}), that can be parsed into two additive components [37]. The first is the level of turn-over (β_{SIM} ; or Simpson's dissimilarity, which is independent of the number of antimicrobials), and the second was the level of nestedness (β_{NSE}). These three measures, $\beta_{SOR} = \beta_{SIM} + \beta_{NSE}$, would therefore provide not only the diversity of antimicrobial resistance experienced by vultures at Jorbeer, but also indicate the process that contributed to the observed resistance pattern. We used the function *beta.sample* to resample the data set 1,000 times to avoid systematic biases that can arise due to sample sizes, and obtained means and standard deviations of all three measures.

Finally, we assessed if resistance to an individual antimicrobial occurred more than by random chance in one or more months using the 'indicator species analysis' with package *indicspecies* [38]. We computed a single-antimicrobial single-month analyses to check how many of the antimicrobials showed significant variation in resistance patterns much more than by random chance in a single month. We also assessed if resistance of individual antimicrobials varied in more than one month significantly more than by random chance [39]. In short, while β -diversity provided metrics useful to understand the overall pattern and processes, the indicator species analysis identified

individual antimicrobials whose non-random variation contributed to the overall patterns.

Results

Environmental hypothesis

The occurrence of *E. coli* was high with very low variation throughout the sampling period (90.32 ± 2.66 (SD) % of samples tested positive; range = 86.67 – 92.31%). All the *E. coli* isolates ($N = 56$) showed variable patterns consisting of bands ranging from 800 to 3000 bp in the ribotyping visually displaying 16S-23S rRNA ISR polymorphism. The most parsimonious number of principal components for DAPC was seven (Supporting Material Figure S2), and the scatterplot confirmed our hypothesis of *E. coli* populations changing over the winter, and homogenizing in the later part of the wintering season (Figure 1). The *E. coli* community in October was distinctly different from the other months, and were most similar in the months of November and March (Figure 1). AMOVA confirmed significant difference within and between samples ($P = 0.001$ in each case; Supporting Material Figure S3). However,

E. coli communities were not significantly different between years ($P = 0.50$) despite a considerable portion of the spread for February non-overlapping with other months as visible on the scatterplot (Figure 1).

Resistance hypothesis

Results confirmed that *E. coli* in vultures that feed in carcass dumps had high incidence of MAR (72%) (Table 2). No resistance to imipenem and gentamicin was observed. Distinct variation in resistance patterns of cephalexin and cefepime between February 2011 and October 2011 was apparent (Table 2). None of the *E. coli* was sensitive to cefepime in February 2011, but all isolates from March 2012 were sensitive to cefepime. The resistance patterns of chloramphenicol in February 2011 and March 2012 were identical (100% sensitivity). Overall, the *E. coli* showed temporally variable resistance patterns to 11 out of 13 antimicrobials used (Table 2).

Results of NMDS had an overall stress of 0.137 and a nearly linear stress plot ($R^2 = 0.98$; Supporting Material, Figure S4). The NMDS graph showed clear separation between years (as depicted by the non-overlapping polygon for February), and also revealed

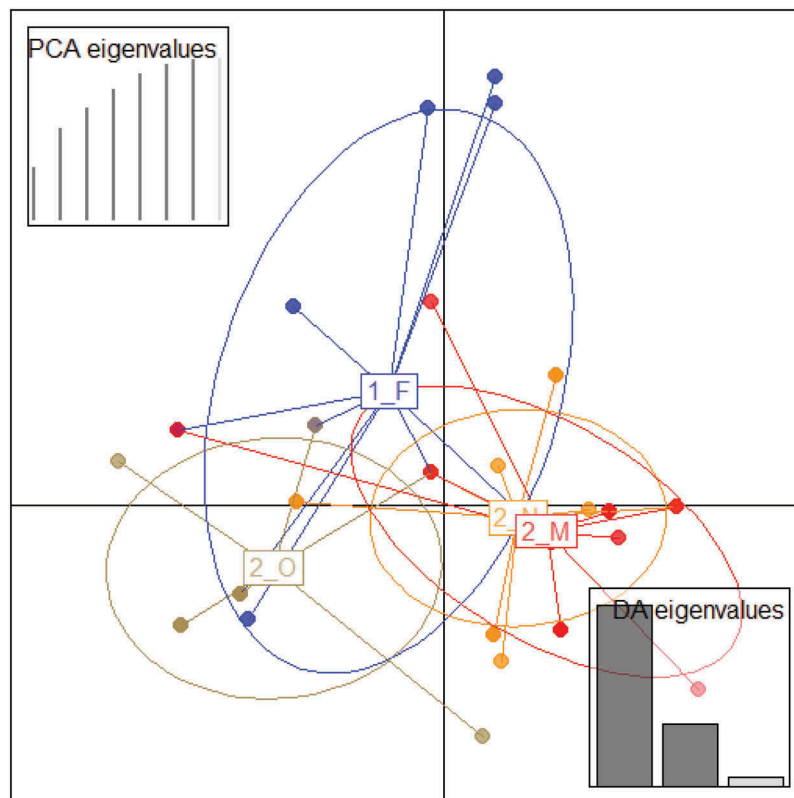


Figure 1. Scatterplot from discriminant analysis of principal components (DAPC) of the first two principal components discriminating *E. coli* communities colour-coded by month (F – February; O – October; N – November; M – March) across two wintering periods (1 – 2010-2011; 2 – 2011-2012). Each point represents actual data, and lines represent each population's membership. Ellipses represent 67% confidence intervals based on a bivariate normal distribution. Number of principal components at which the maximal reassignment of samples occurred are depicted as black lines the PCA graph on the top-left corner, with subsequent components in grey line.

Table 2. Antimicrobial resistance pattern of the *E. coli* in four sampling efforts.

Sr No.	Name of Antimicrobial	Response of the <i>E. coli</i>	February, 2011 (N = 12)	October, 2011 (N = 12)	November, 2011 (N = 19)	March, 2012 (N = 13)	Overall (N = 56)
1	Ampicillin	Sensitive	0.00	0.00	0.00	7.69	1.79
		Intermediate	33.33	0.00	0.00	0.00	7.14
		Resistance	66.67	100.00	100.00	92.31	91.07
2	Gentamicin	Sensitive	100.00	100.00	100.00	100.00	100.00
		Intermediate	0.00	0.00	0.00	0.00	0.00
		Resistance	0.00	0.00	0.00	0.00	0.00
3	Imipenem	Sensitive	100.00	100.00	100.00	100.00	100.00
		Intermediate	0.00	0.00	0.00	0.00	0.00
		Resistance	0.00	0.00	0.00	0.00	0.00
4	Ampicillin+ Sulbactam	Sensitive	100.00	83.33	68.42	53.85	75.00
		Intermediate	0.00	0.00	21.05	46.15	17.86
		Resistance	0.00	16.67	10.53	0.00	7.14
5	Cephotaxime	Sensitive	0.00	91.67	42.11	53.85	46.43
		Intermediate	0.00	0.00	52.63	0.00	17.86
		Resistance	100.00	8.33	5.26	46.15	35.71
6	Cefepime	Sensitive	0.00	91.67	78.95	84.62	66.07
		Intermediate	25.00	0.00	5.26	15.38	10.71
		Resistance	75.00	8.33	15.79	0.00	23.21
7	Ofloxacin	Sensitive	75.00	91.67	47.37	69.23	67.86
		Intermediate	0.00	0.00	21.05	7.69	8.93
		Resistance	25.00	8.33	31.58	23.08	23.21
8	Erythromycin	Sensitive	0.00	0.00	47.37	0.00	16.07
		Intermediate	0.00	50.00	0.00	53.85	23.21
		Resistance	100.00	50.00	52.63	46.15	60.71
9	Co-trimoxazole	Sensitive	58.33	75.00	73.68	53.85	66.07
		Intermediate	0.00	0.00	0.00	0.00	0.00
		Resistance	41.67	25.00	26.32	46.15	33.93
10	Rifampicin	Sensitive	91.67	58.33	73.68	53.85	69.64
		Intermediate	0.00	0.00	0.00	0.00	0.00
		Resistance	8.33	41.67	26.32	46.15	30.36
11	Chloramphenicol	Sensitive	100.00	83.33	78.95	100.00	89.29
		Intermediate	0.00	0.00	5.26	0.00	1.79
		Resistance	0.00	16.67	15.79	0.00	8.93
12	Ciprofloxacin	Sensitive	66.67	83.33	52.63	46.15	60.71
		Intermediate	8.33	8.33	10.53	38.46	16.07
		Resistance	25.00	8.33	36.84	15.38	23.21
13	Tetracyclin	Sensitive	41.67	16.67	10.53	7.69	17.86
		Intermediate	16.67	41.67	31.58	7.69	25.00
		Resistance	41.67	41.67	57.89	84.62	57.14

varying levels of overlaps within the wintering period of 2011–2012 (October and November 2011, and March 2012; see Figure 2). MRPP confirmed an unambiguous differentiation of antimicrobial resistance patterns between months ($\delta = 0.001$, chance corrected within-group agreement $A = 0.172$, 1,000 permutations). Pair-wise comparison of mean dissimilarity using Jaccard's index revealed that the *E. coli* from most months had significantly different resistance patterns (Table 3). The Jaccard's index and NMDS clearly suggest that the most diverse resistance patterns of *E. coli* was in November. Resistance pattern in October was most similar to other months. The Sørensen's β -diversity ($\beta_{\text{SOR}} = 0.554 \pm 0.05$) was almost equally partitioned between turnover ($\beta_{\text{SIM}} = 0.328 \pm 0.09$) and nestedness ($\beta_{\text{NSE}} = 0.226 \pm 0.07$). Indicator-species analysis revealed that rifampicin and tetracyclin displayed temporal variations in resistance patterns occurring more than expected by random chance in February 2011 (Table 4). Cefotaxime and cefepime also showed this variation in October 2011 (Table 4(a)), and within the wintering cycle (October, November 2011 and March, 2012; Table 4(b)).

Discussion

Prevalence

Information on prevalence of *E. coli* is not known in Egyptian vultures from other locations, but similarly high levels of prevalence was observed in the scavenging Eurasian griffon (90%) and other captive birds of prey (89%) [40,41]. High prevalence, however, was not common across all vultures (36–38% in cinereous vultures) or all raptors (15% in common buzzards) suggesting that species-level investigations are necessary to evaluate *E. coli* prevalence in this group of birds [42]. Contrary to our observations, effect of spatial differences were insignificant on the prevalence of *E. coli* in red kites, also a highly synanthropic species [43]. High occurrence of *E. coli* strains matches well with the strongly synanthropic habit of the Egyptian vultures in their wintering grounds. This in turn matches past observations of higher prevalence in more synanthropic host species [11,44].

Temporal variation in *E. coli*

DAPC was very useful to reveal temporal variations in the *E. coli* community found in Egyptian vultures. The

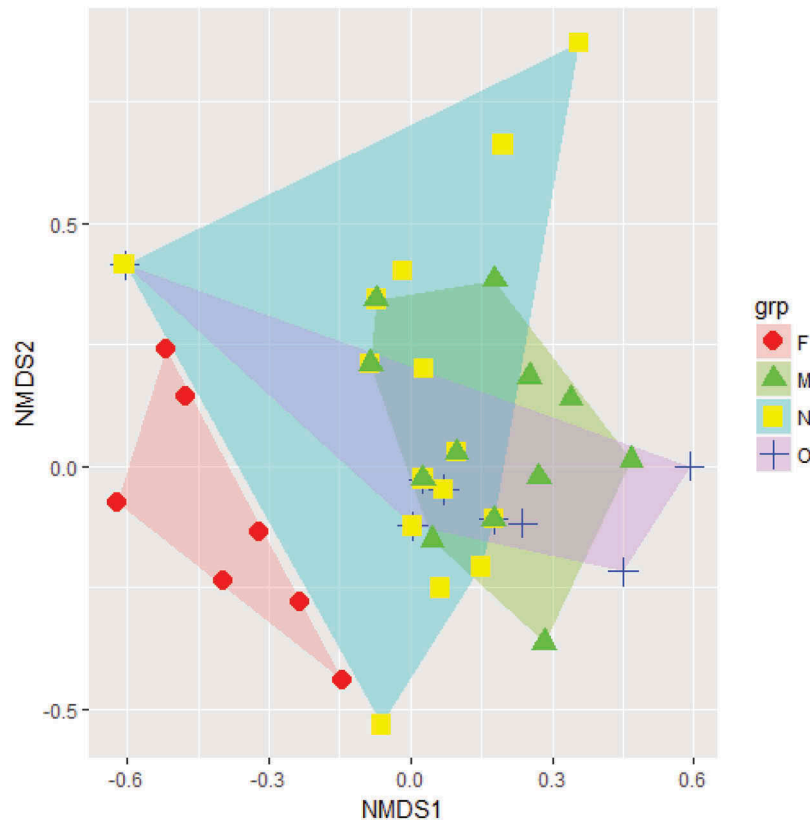


Figure 2. Non-metric multidimensional scaling (NMDS) plot representing resistance to varied antimicrobials by *E. coli* in Egyptian vultures across different months (F – February; O – October; N – November; M – March) in two-dimensional space.

Table 3. Pair-wise comparisons of dissimilarity in *E. coli* ribotypes in a wintering Egyptian Vulture population in Jorbeer using Jaccard's index. (* – $P < 0.05$; n.s. – not significant).

	October 2011	November 2011	March 2012
February 2011	n.s.	*	*
October 2011		*	n.s.
November 2011			*

strong overlapping of November and March (see Figure 1) suggests that homogenization of *E. coli* communities occur relatively early in the wintering season. Our results show that despite a statistical non-significance, inter-annual variations in *E. coli* communities were visible. However, it will be necessary to obtain data that completely cover full wintering periods for multiple years to understand if inter-annual variations are significant, and if they occur

across multiple years. It would be useful to conduct similar studies with a temporal design in both migratory and non-migratory species to understand if patterns obtained in our study are shared across different environmental conditions and species, or if these patterns occur only in highly synanthropic species.

Patterns of MAR

Results confirmed our hypotheses that *E. coli* in Egyptian vultures feeding in carcass dumps will have a very high incidence of MAR (72%). Raptors have been known to harbour higher incidence of MAR relative to other bird species [45]. High MAR incidence during November to February reflects the unique conditions at the carcass dump, but high incidence in October reflects conditions in vultures'

Table 4. Results of the indicator species analyses showing antimicrobials with temporal variation more than by random chance in (a) single months, and (b) multiple months.

A. Single-antimicrobial, single-month (Indicator values, with P -value in parenthesis)					
Antimicrobial	February, 2011	October, 2011	November, 2011	March, 2012	
Cephotaxime		0.602 (0.015)			
Cefepime		0.596 (0.015)			
Rifampicin	0.568 (0.015)				
Tetracyclin	0.527 (0.01)				
B. Single-antimicrobial, multiple-months.					
Antimicrobial	Indicator Value (P)	February, 2011	October, 2011	November, 2011	March, 2012
Cephotaxime	0.953 (0.005)		+	+	+
Cefepime	0.965 (0.005)		+	+	+

breeding areas. This is suggestive of Egyptian Vultures being exposed to treated carcasses throughout their life cycle. This level of occurrence reflects the unique conditions present at the carcass dump – exceedingly high levels of human activity, and the presence of mostly pre-treated animal carcasses for vultures to feed on. Studies on wild birds have unfortunately not always noted environmental conditions, including an objective measurement of synanthropy, making it difficult to draw ecological conclusions using merely observed incidence values of MAR in past studies [5].

Gentamicin, an aminoglycoside, was the only commonly used antimicrobial that all samples of *E. coli* showed sensitivity to (see Table 2). This is significant in view of more than 50 aminoglycoside-modifying enzymes conferring widespread resistance with *E. coli* harbouring ‘key-resistance’ to aminoglycosides [46,47].

Determinants of resistance to tetracyclines, specifically plasmid borne gram-negative efflux genes are widespread [48]. Inter-species transfer of these genes can take place between different species of bacteria as well.

Grouping the resistance to ampicillin, cephalexin and cefepime, the highest resistance was observed in the *E. coli* sampled in February, 2011 (67, 100, and 75% respectively). However, no resistance to combination of ampicillin and sulbactam was observed in this period suggesting relatively high proportion of extended spectrum β -lactamases (ESBL) producing strains. Cephalexin and cefepime displayed most conspicuous and statistically significant changes in pattern of resistance both at the end of the 2010–2011 wintering season in February 2011 as well as across the 2011–2012 wintering period from October 2011 to March 2012. (Table 4(a,b)). This translates into resistance to cephalexin and cefepime more likely to be temporally structured and that the change of habitat could have affected the associated resistance determinants. This variation could possibly be attributed to more than 400 widespread horizontally transferred genes coding β -lactamases in *E. coli* and permissibility of genome of *E. coli* to such genes, though chromosomal genes providing intrinsic resistance are not uncommon [49]. While many studies report ESBL in *E. coli* of wild species, the response of migration on cephalosporin resistance determinants is poorly known [17].

NMDS analysis indicated that most antimicrobials showed inter-annual variation in resistance patterns, except the ones the *E. coli* were completely resistant or susceptible to throughout the study. This variability was high for cephalexin, cefepime, erythromycin rifampicin, very low for cotrimoxazole, ofloxacin and absent in chloramphenicol (Table 2). The highest diversity of the resistotypes observed in November 2011 (Figure. 2) is suggestive of vultures arriving from different breeding grounds (See Supporting

Material Figure S1), potentially leading to intense and increased interactions of *E. coli* and associated resistance determinants. This is also, therefore, the time period that also likely most closely reflects resistance profiles relating to their breeding grounds. Conducting studies simultaneously in the breeding and wintering grounds over multiple years can provide undisputable insights related to variation in resistance profile patterns, and to ascertain if some antimicrobials show consistent inter-annual variations more than others. Such an investigation is critical in management strategies to identify antimicrobial classes more prone to dissemination through wild migratory species.

We are not aware of other attempts to understand the processes driving overall diversity (Sørensen’s β -diversity in our case) of resistance profiles of *E. coli*. A nearly-equal partitioning of overall diversity to turnover and nestedness shows that our resistance hypothesis was substantiated, but also that there were episodic increases in resistance to novel antimicrobials. The strong dissimilarity of resistance patterns using Jaccard’s index, and the identification of two individual antimicrobials (cephalexin and cefepime) with marked variations in resistance profile in a few months than could be expected by chance, provide additional evidence of multiple active processes by which *E. coli* in Egyptian vultures are gaining MAR.

The observed distinctive temporal patterns (Figure. 2) of these strains suggest that, apart from imparting resistance determinants, the dump did act as a ‘melting pot’ of antimicrobial resistance with the potential to disseminate resistant *E. coli* to multiple geographical locations (breeding grounds of the vultures) corroborating the contention of [12].

It seems that resistotypes are more sensitive to the effect of temporal and geographical variations compared to the *E. coli* isolates in this study. This points to the involvement of extra-nuclear genetic interactions governing resistance factors (resistance plasmids and integrons), which could additionally provide variation in resistance patterns along with chromosomal DNA compared to exclusive chromosomal DNA based changes accounted for clonal differences in *E. coli*. Antimicrobial agents contribute to selection pressure in commensals *E. coli* [50]. While some phylogenetic groups (A and some D group strains) of *E. coli* are more permissive to develop resistance to third generation cephalosporins whereas group B2 strains are less likely to develop resistance compared to the other groups [51–53]. This variability influences the strain structures of *E. coli* in hosts frequently exposed to antimicrobials [4]. Clearly, MAR *E. coli* strains are surviving, persisting and propagating in the gut of the vultures. Combining the observed patterns of changes in population

structure (ribotypes) and MAR patterns provide further insights into *E. coli* ecology. For example, selection pressure may have contributed to the homogenization of the *E. coli* strains observed at the end of the migratory period 2011–2012. The recurrence of some strains in multiple years (see Figure. 1) suggests that selection pressures working over multiple years could favour persistence of MAR *E. coli* strains over relatively susceptible ones.

These findings are significant from an epidemiological point of view in that, while places like the Jorbeer carcass dump are important food sources to a number of raptor species of global conservation concern, they are also likely locations where diet (livestock carcasses with antimicrobials) and selection pressure can produce dangerously high levels of MAR *E. coli* in migratory bird species. Apart from MAR *E. coli*, Egyptian vultures could carry other potentially pathogenic and virulent MAR bacteria like *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* [54,55] as well suggesting the spectrum of MAR bacteria of public health significance associated with vultures (and other migratory birds) is bigger than presently known.

MAR bacterial strains could be dispersed and colonized at new locations due to migratory bird species [56,57]. Environmental conditions and presence of high levels of antimicrobials as found in Jorbeer are likely common in many developing countries that have high human and livestock densities. Studies on antimicrobial resistance in bacteria of wild species are very rare in such countries [5]. Coverage of varied conditions and wild vertebrate species using robust spatial and temporal designs are entirely absent. This lacuna of even basic information requires to be urgently rectified given the unregulated and injudicious usage of antimicrobials, intensive livestock presence, high human densities, combined with very high degree of synanthropy shown by wild bird species in south Asia. We advocate multi-disciplinary approaches involving ecological, veterinary and microbiological components. Vultures render immense ecological benefit by disposing livestock carcasses without human resources investment, and the conservation of these endangered species is exceedingly important. However, the role of such migratory species in future remedial strategies to control spread of antimicrobial resistance should also be carefully looked into.

This study shows that Egyptian vultures feeding in a livestock carcass dump in north-west India: (1) had high occurrence (90.32%) of *E. coli*, with strains showing host-species restriction and limited diversity with clear temporal structuring, and (2) *E. coli* showed 71.43% MAR, with the resistance patterns showing strong spatial and temporal structuring. *E. coli* in migrating Egyptian vultures, therefore, displayed excellent ability to incorporate and reflect resistance determinants prevailing both at the dump and the period of sampling.

This strongly suggests that solely local or regional measures to spread of antimicrobial resistance is unlikely to be adequate, and we echo previous calls to execute more comprehensive and interdisciplinary mitigation methods concomitant with the ecology of migratory species such as Egyptian vultures [5].

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Disclosure statement

No potential conflict of interest was reported by the authors.

Notes on contributors

Dr. Pradeep Sharma is Veterinary Microbiologist working with Government of Rajasthan, India. His research interests are dynamics of bacterial strains and antimicrobial resistance in wild avian species in various synanthropic ecological systems. He is also interested in epidemiology and dynamics of zoonotic transmission of bacteria between domesticated and wild species in human-dominated landscapes in India.

Dr. Sunil Maherchandani is Professor in Department of Veterinary Microbiology and Biotechnology at Rajasthan University of Veterinary and Animal Sciences, Bikaner, Rajasthan. His fields of interest are in studying adaptive antibiotic resistance mechanisms with special reference to multiple antibiotic resistant bacteria and virus host interactions.

Dr. B. N. Shringi is Professor in Department of Veterinary Microbiology and Biotechnology at Rajasthan University of Veterinary and Animal Sciences, Bikaner, Rajasthan. His field of interests are immunology of Brucella, Blue-tongue virus and other infectious agents of veterinary importance.

Dr. Sudhir Kumar Kashyap is Professor and Head in Department of Veterinary Microbiology and Biotechnology at Rajasthan University of Veterinary and Animal Sciences, Bikaner, Rajasthan. His fields of interest are in studying molecular epidemiology of microbes and molecular diagnostic methods.

Dr. K.S. Gopi Sundar is a conservation biologist and ornithologist who manages Program SarusScape, a collaborative venture of the Nature Conservation Foundation in India and the International Crane Foundation in the USA. Gopi is primarily interested in the ecology and conservation of birds that live amid human-dominated landscapes. He is also the global Co-Chair of the IUCN Stork, Ibis and Spoonbill Specialist Group. He has collaborative projects currently ongoing in Nepal, India and Australia.

ORCID

Pradeep Sharma  <http://orcid.org/0000-0002-7000-9495>
K. S. Gopi Sundar  <http://orcid.org/0000-0003-3477-5691>

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