Phenotypic and Genotypic Characterization of *Klebsiella Pneumoniae* Obtained from Egyptian Vultures and Steppes Eagles from India

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ABSTRACT

Wild migrating Egyptian vultures (*Neophron percnopterus percnopterus*) and Steppes eagles (*Aquila nipalensis*) can potentially harbor and spread pathogenic microorganisms and may associated with antibiotic resistance genes over long distances. In the present study, we reported the prevalence of *Klebsiella pneumoniae* from feces of wild Egyptian vultures and Steppes eagles, which were sampled for two consecutive winter seasons, at a carcass dump in the state of Rajasthan India, and to the best knowledge of the authors as a first record from the Indian subcontinent. After phenotypic characterization and genotypic confirmation based on 16S-23S rDNA internal transcribed spacer (ITS) region showing a prevalence of 11% (n=27), and these isolates displayed resistance to multiple antibiotics including beta-lactams, glycopeptides, macrolides, polypeptides and sulfonamides groups. All isolates were screened for virulence associated serotypes K1, K2 and K5 with serotype specific primers and the potentially pathogenic K5 serotype was confirmed in one isolate obtained from an Egyptian vulture. Occurrence of multiple antibiotic resistance and K5 serotype of *K. pneumoniae* in endangered Egyptian vultures, and steppe eagles requires further investigations in the context of epidemiology and pathogenicity of *K. pneumoniae* including concerning effects on population health and conservation context of these scavenging raptors.

Keywords: Steppe Eagle, Egyptian Vulture, *Klebsiella pneumoniae*, K5 serotype.

INTRODUCTION

Emerging antibiotic resistance of infectious diseases among domestic animals, wildlife and human being draw attraction toward increased interface between humans, domestic animals and free-ranging wildlife. The role of wild animals including bird of prey as reservoirs and disseminators of pathogens of potential zoonotic importance has major implications for public health (1, 2). Since the migratory scavengers feed at refuse dumps, abattoirs and other sources of domestic refuse and offal from various and different places of world they may act as disseminators of bacterial pathogens associated with those food sources (3). Raptors feeding on carcass dumps are exposed to a variety of pathogenic organisms. Hundreds of endangered Egyptian vultures *N. p. percnopterus* and Steppe eagles (*Aquila nipalensis*), henceforth referred to as 'the raptors', overwinter at Jorbeer carcass dump (4) situated 10 km from Bikaner city, in state of Rajasthan.

Klebsiella pneumoniae is a gram negative opportunistic pathogen associated with many enteric infections of animals, mastitis in cattle (5), bacteremia in calves (6), metritis in mares (7), pneumonia and urinary tract infections in dogs, and pneumonia, septicemia in foals (8). Epidemic and endemic nosocomial infections caused by *Klebsiella pneumoniae* are also the leading cause of morbidity and mortality in human beings (9).

At present, strains expressing capsule serotypes K1 and

K2 of *K. pneumoniae* are considered especially likely to be virulent, although only a few of the 77 different K antigens have been systematically studied in this regard (10). Also serotypes K1, K2 and K5 are often associated with severe infections in humans, animals and are highly virulent in experimental infection in mice (11, 12).

Extensive survey of the literature including peer-reviewed journals revealed a dearth of information on occurrence of *Klebsiella* spp. from Egyptian vultures, and no report of the organism from Steppe eagles. No information is available on the role of scavenging raptors in epidemiology of the potentially pathogenic serotypes including K1, K2 and K5 of *K. pneumoniae*. Information on multiple antibiotic resistance in fecal bacteria of scavenging raptors is generally lacking and no information could be accessed on *Klebsiella* specifically from the Indian subcontinent.

The objectives of this investigation were to (i) study prevalence of *Klebsiella pneumoniae* in fecal matter of the migratory scavenging raptors (ii) employ phenotypic characterization of the *K. pneumoniae*, (iv) genotypic confirmation of the isolates from the scavenging raptors employing 16S-23S rDNA internal transcribed spacer (ITS) region specific primers (v) confirmation of virulent capsular serotype K1, K2 and K5 with serotype specific primers; and (vi) study the occurrence of multiple antibiotic resistance in these isolates.

MATERIAL AND METHODS

Study site

Jorbeer carcass dump, with coordinates: N 27˚57.958' E 73˚22.598' (± 3m), is a dumping site for livestock carcasses near Bikaner in Rajasthan state of India. The site is in the Thar Desert at 235 m above sea level, with a landscape dominated by arid and sparse desert vegetation composed of scattered trees and shrubs. Carcasses were mainly of cattle but included camels, dogs, equines and poultry waste. Approximately 15–20 carcasses were being dumped each day at the site providing a regular and ample source of food to the scavenging raptors. The probability of carcasses containing different type of veterianry drugs considered high as some of these originate from veterinary university clinics, and other veterinary clinical setting.

The carcass dump hosts five other species of vultures (White-rumped *Gyps bengalensis*, Long-billed *G. indicus*, Eurasian Griffon *G. fulvus*, Cinereous *G. monachus*, and Red-headed vultures *Sarcogyps calvus*), Black kite (*Milvus milvus*), and few other breeding as well as migrating eagles belonging to the genus *Aquila* (4). Many avian species, migratory as well as resident, also inundate the dump site along with hundreds of resident stray dogs.

Sample collection, isolation and identification

Fresh fecal samples were collected from roosting sites of the raptors by noninvasive means through sterile cotton swabs (Himedia Laboratories Pvt. Ltd., Mumbai, India). The birds were identified and monitored using binoculars (Nikon Monarch 10x50, Nikon Inc., USA) and fecal samples collected aseptically from inside of lump of drooping by HiCulture™ Transport Swabs (Himedia Laboratories Pvt. Ltd., Mumbai, India) as soon as the raptors defecated. 27 fresh fecal samples of the raptors in two consecutive winter seasons (includes 25 Egyptian vultures and two steppe eagles) were examined for presence of *Klebsiella pneumoniae*. The raptors always existed in excess of 500 in numbers with in the sampling period and thus probability of duplicate sampling was statistically insignificant ($P<0.05$). The pure

Table 1: Phenotypic characterization of *K. pneumoniae* isolated from Steppe eagles and Egyptian vultures.

S. No.	Biochemical Test	Reaction
1	Catalase test	Positive
2	Oxidase test	Negative
3	Capsule staining	Capsulated
4	Oxidation- fermentation test	Fermentative
5	IMViC Pattern	-ve-ve+ve+ve
6	Colony Muco-viscosity	Positive
7	Growth on 10°C	Negative
8	Growth on 44.5°C	Positive
9	Growth on MacConkey agar	Mucoid pink colonies
10	Growth on SCAI Agar	Yellow mucoid dome shaped colonies
11	Growth on EMB Agar	Dark mucoid non-metallic colonies
12	Growth on BCP Agar	Yellow mucoid colonies
13	Growth on TSI Agar	Acid/Acid/No H_2S
14	Gelatin Liquefaction	Negative
15	Aesculin Hydrolysis	Positive
16	Nitrate Reduction	Positive
17	Malonate Utilization	Positive
18	Arginine Hydrolysis	Negative
19	Lysine Decarboxylation	Positive
20	Phenylalanine Deamination	Negative
21	ONPG (o-Nitrophenyl-β-d Galactopyranoside)	Positive

colonies of organisms were phenotypically confirmed and characterized by standard biochemical tests and colony characters according to the techniques described by Edwards and Ewing (13) (Table 1). All of the confirmed *K. pneumoniae* isolates were also examined for sugar fermentation reaction of 15 sugars (Table 2).

The genotypic confirmation was based on 16S-23S rDNA internal transcribed spacer (ITS) region as method described by Liu *et al.,* (14). PCR reactions were carried out in 50 µl volumes with final concentrations of 3 µl of extracted template DNA, as template DNA obtained through technique described by Chen and Kuo (15), 10 µl 5x PCR assay buffer (with 1.5 mM MgCl2), 1 µl dNTP (10 mM), 1 µl each forward and reverse primer set, 0.25 µl Taq DNA polymerase (5 U/ µl) and 33.75 µl MiliQ water. The cycling conditions were 10 min at 94 ˚C followed by 35 cycles of 30 s at 94 ˚C, 20 s at 57 ˚C, and 20 s at 72 ˚C then 10 minutes hold at 72 ˚C. PCR products (130 bp) were separated by 8% native PAGE (Hoefer SE 600 Hoefer Inc., Massachusetts, USA).

Detection of serotype (K1, K2 and K5) and antibiotic susceptibility testing

Genotypically confirmed *K. pneumoniae* were evaluated for presence of serotype K1, K2 and K5 using serotype specific primers with genomic DNA as template and PCR conditions as described by Turton *et al.,* (16) and the PCR products were separated by 8% native PAGE (17).

Each confirmed *K. pneumoniae* isolate was examined for susceptibility to 24 antibiotics of different chemical classes (Table 3) and resistance pattern was determined by disc diffusion method of Bauer *et al.,* (18) on Mueller Hinton agar (Himedia Laboratories Pvt. Ltd., Mumbai, India).

Table 2: Differentiation of *K. pneumoniae* isolates based on sugar fermentation and gas production

	Isolate ID		
Name of Sugar	EV1	SE1	EV2
Xylose	+G	+G	+G
Dulcitol	+G	--	--
Arabinose	+G	--	+G
Lactose	+-	+G	--
Mannose	+G	+G	+G
Inositol	+G	+G	+G
Raffinose	+G	+G	+G
Rhamnose	+G	+G	+G
Maltose	+G	+G	+G
Sucrose	+G	+G	+G
Sorbitol	+G	+G	+G
Dextrose	+G	+G	+G
Fructose	+G	+G	+-
Mannitol	+G	+G	+G
Trehalose	+G	+G	+G

+ Positive for Fermentation; G Positive for gas production; - Negative for fermentation or gas production

RESULTS AND DISCUSSION

In the present study, *Klebsiella pneumoniae* showed a prevalence of 11.11% (n=27) as phenotypic (Table. 1) and genotypic characterization confirmed three *K. pneumoniae* isolates (Figure 1); two from Egyptian vultures and one from a steppe eagle. Furthermore out of three isolates, one isolate from the Egyptian vulture was detected as K5 serotype (280bp) and the K1 and K2 serotypes were not found in any of the isolates

Table 3: Multiple Antibiotic resistance pattern of *K. pneumoniae* isolates from Egyptian vultures (EV) and Steppe eagles (SE)

	Isolate ID		
Antibiotics	EV1	SE1	EV2
Ampicillin	R	R	R
Bacitracin	R	R	R
Ciprofloxacin	S	S	S
Ceftazidime	S	S	S
Cephlothin	R	I	I
Norfloxacin	S	S	S
Oxacillin	R	R	R
Erythromycin	R	R	R
Imipenem	S	S	S
Vancomycin	R	R	R
Gentamicin	S	S	S
Tetracyclin	I	I	I
Rifampicin	R	R	R
Trimethoprim	S	S	S
Kanamycin	I	S	S
Amoxycillin+Clavulanic Acid	R	R	R
Ampicillin + Sulbactam	S	S	S
Amoxycillin	R	R	R
Cefepime	S	S	S
Cefotaxime	S	S	S
Clinadmycin	R	R	R
Faropenem	R	I	R
Polymyxin	R	R	R
Sulfadiazine	R	R	R

R: Resistant; S: Sensitive; I: Intermediate

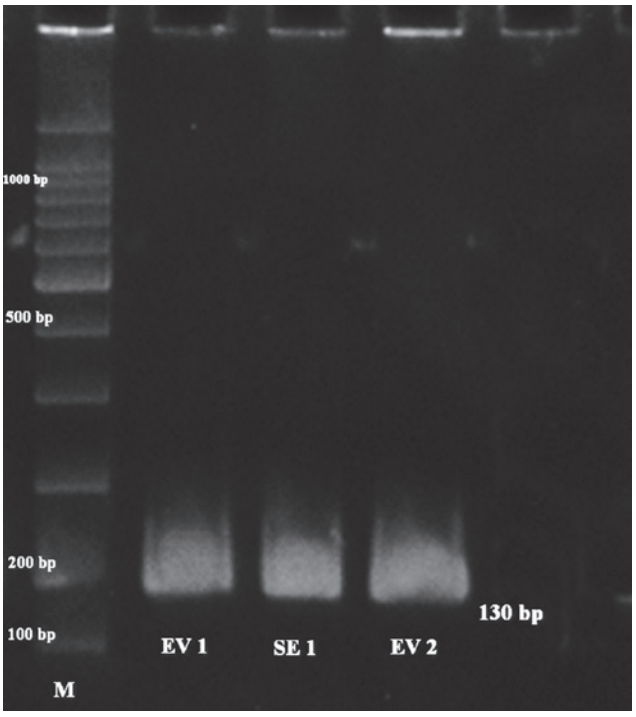

Figure 1: Genotypic confirmation of *K. pneumoniae* based on 16S-23S rDNA internal transcribed spacer (ITS) region.

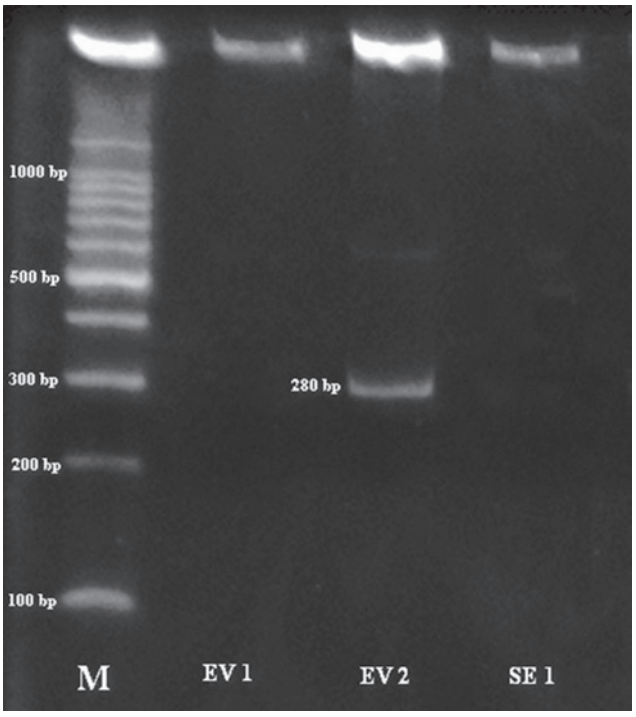

Figure 2: Genotypic detection of serotype K5 of *K. pneumoniae* using serotype specific primers.

(Figure 2). Except for three sugars dulcitol (33.3%), arabinose (66.6%) and lactose (66.6%), all sugars were fermented by all the isolates (Table 2). All isolates showed multiple antibiotic resistance and all of the isolates were resistant to 11 antibiotics used in the study and remaining antibiotics showed variable patterns of efficacy against the isolates (Table. 3).

The availability of literature on *Klebsiella pneumoniae* isolated from Egyptian vultures and steppes eagles is very scarce but *Klebsiella* spp. have been reported from different avian genera such as red kites (*Milvus milvus*), Egyptian vultures, and Antarctic skua (*Catharacta* spp.) showing a prevalence of 7.96% (n=113) (19), 8.82% (n=68) (20) and 4.54 % (n=22) (21) respectively. Prevalence of fecal *Klebsiella* spp. from Red-billed choughs (*Pyrrhocorax pyrrhocorax*) was 15.0%, 12.8% and 15.6% for three different locations in Spain (22). *K. pneumoniae* was isolated from wild turkey vultures (*Cathartes aura*) (23) and peregrine falcon (*Falco peregrinus*) (24) in USA showed prevalence of 5% (n=20) and 42.85% (n=14) respectively. In the context of phenotypic characterization and sugar fermentation patterns of *K. pneumoniae*, similar result were obtained in present study as described in previous literature in which *K. pneumoniae* was obtained from various animals and humans (25, 26 and 27).

To the best knowledge of the authors, the presence of K5 serotype of *K. pneumoniae* from feces of the wild Egyptian vulture is a first report although particular serotypes of *K. pneumoniae* have been incriminated in epidemics in animals (28), community-acquired liver abscesses, pneumonia, endo-ophthalmitis, and as an opportunistic nosocomial pathogen in humans worldwide, especially Asia and the USA (10, 29). Serotypes K2, K5 and K7 were found to be the most pathogenic in equines (30), whereas serotypes K1, K2, K5 caused metritis epidemics in mares (31). K5 strain caused pneumonia in humans (32) and outbreak of enteritis and septicemia in canines (33). Apart from isolated case reports describing *K. pneumoniae* as a cause of pathogenic lesions in the infra-trochlear area and brainstem in Barn owl (*Tyto alba*) (34) the ability of the bacteria to cause disease at different spatial scale in raptorial birds including endangered Egyptian vultures, largely remains unknown requiring further studies in reference to the population health of the species.

The presence of *Klebsiella* spp. from substantially different spatial and biological sources suggests a widespread distribution, adaptation and pathogenicity in diverse host species. This study firstly documents Egyptian vultures, which can migrate thousands of kilometers (35), as host and carrier of

serotype K5. This finding is epidemiologically important as bacteria from vultures can potentially spread to other wildlife or human populations passively, possibly causing diseases (3, 1 and 36). The raptors studied were apparently healthy as no visible clinical symptoms could be observed suggesting *K. pneumoniae* can exist as commensals in their gastro-intestinal tract. Reported prevalence of 7.29% (n=96) of the K5 serotype, associated with pneumonia in dromedary camels (*Camelus dromedarius*) in and around Bikaner city (37), proposes additional novel host species, which can contribute to this microbial ecological niche of carcass dumps receiving such pneumonic camel carcasses.

Strains of *Klebsiella* spp. are naturally resistant to aminopenicillins (ampicillin and amoxicillin) and other penicillins due to chromosomal class-A β-lactamase production, but susceptible to most other β-lactam antibiotics (38). The observed bacterial resistance to antibiotics belonging to macrolide, cyclic polypeptides, β-lactams (including penem subgroup), rifamycin, sulfonamide, and specially combinations of β-lactam/β-lactamase inhibitor classes is significant since the sampled migratory raptors were wild, and in all probability, were without any prophylactic history concerning antibiotics. The probability of ingestion of residual antimicrobials by the raptors, in carcasses of livestock, administered prior to their death was high as many carcasses originated from veterinary clinics. Practically it is possible that such regular and long exposure of residual antibiotics to the raptors could be attributed to the development of resistance in the *K. pneumoniae* isolates. A second alternative however important explanation is that the transfer of resistance genes from resistant bacteria to susceptible ones was possible as many of the bacterial resistance genes are plasmid borne. *Escherichia coli* strains are potentially harbored by wild birds of diverse species and spread conjugative resistance plasmids (R plasmids) (39, 40). However *Klebsiella* spp. are poorly studied in this context even if the bacteria harbor widely distributed R plasmids, which can be transferred to other bacterial strains and species (41) conferring resistance to quinolones and β-lactams antibiotics (42). Such transmission of R plasmids to distant geographical locations may possibly be facilitated by different dynamically interacting populations of migrating scavenging raptors, which although breed in different geographic locations still winter in similar ecological niches of carcass dumps such as Jorbeer. The third reason for the observed resistance to antimicrobials could be the ingestion of already resistant *K. pneumoniae* originating from the carcasses of livestock species including camels, which are susceptible hosts of *K. pneumoniae*, as food sources are known to affect the gastro-intestinal micro-flora. It is also possible that all the three proposed mechanisms responsible for the observed antimicrobial resistance, as mentioned above, were not mutually exclusive and probably worked simultaneously. Further expansions of this study to investigate these mechanisms may illuminate exactly which processes govern the occurrence of the observed resistance in these wild raptors in this study. The ability of *K. pneumoniae* to spread intercontinentally (43), the presence of New Delhi metallo-β-lactamase-1 (NDM-1) enzymes in *K. pneumoniae* strains isolated from India conferring resistance to carbapenem antibiotics (44), and widespread distribution of such strains (45) are rational reasons to study the role of these migratory raptors in epidemiological perspectives of *K. pneumoniae* and its serotypes.

The populations of birds under study, which are declining, thrive in similar ecological niches of carcass dumps sharing almost the same food sources, which may facilitate potential spread of pathogenic bacteria such as *K. pneumoniae* among the birds' populations. Also since the species under study are wild and migratory any potential disease or outbreak caused by the virulent strains of K5 and resistance to multiple antibiotics may probably go unnoticed and spread to distant geographic locations. Thus focused molecular and clinical studies are needed to ascertain pathogenicity, adaptability and virulence of the K5 serotypes to ascertain if these avian genera are biological hosts or mere mechanical vectors. More exhaustive epidemiological studies are needed to study the prevalence and pathogenicity of other potentially pathogenic serotypes of *Klebsiella* among these raptors.

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