



SHORT COMMUNICATION

## Characterization of *exo-s*, *exo-u*, and *alg* virulence factors and antimicrobial resistance in *Pseudomonas aeruginosa* isolated from migratory Egyptian vultures from India

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This study of *Pseudomonas aeruginosa* in fecal droppings of migratory Egyptian vultures (*Neophron p. percnopterus*) revealed eight positive samples ( $n=25$ ) by a 16S rRNA gene-based PCR in two consecutive winter seasons. Disk diffusion sensitivity testing revealed three multiple antimicrobial resistant (MAR) isolates. Genotypic characterization showed mutually exclusive *exo-s* and *exo-u* virulence genes in five and three isolates, respectively, while the *alg* gene was present in all of the isolates. MAR isolates with virulence genes were detected in both seasons. The Egyptian vultures could potentially be vectors of pathogenic and MAR *P. aeruginosa*, thereby affecting regional control and preventive measures.

Keywords: *Pseudomonas aeruginosa*; virulence; vultures; antimicrobial resistance; carcass dump

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Emerging resistance to multiple antimicrobial compounds and the spread of nosocomial infections of *Pseudomonas aeruginosa*, which is able to thrive in a variety of ecological niches (1), are areas of alarming concerns. Birds can be infected by bacteria originating from human and animal sources and could also be potential reservoirs and disseminators of pathogens (2), including *P. aeruginosa*. In parts of Asia, livestock carcasses are a major food source for scavenging raptors and could potentially be a source for infectious agents and antimicrobial resistant bacteria to these birds. Avian mobility and migration could affect the spatial distribution of pathogens and should be regarded as a potential crucial epidemiological factor for certain pathogens (3). *P. aeruginosa* can cause pathogenicity in raptors (4), thereby constituting a potential threat to populations of endangered raptors. With this in mind, this study was planned to investigate the occurrence, multiple antimicrobial resistance, and characterization of three virulence

markers of *P. aeruginosa* in the fecal matter of migratory Egyptian vultures (*Neophron p. percnopterus*) wintering at a carcass dump.

### Material and methods

Hundreds of endangered Egyptian vultures (5) (henceforth 'the vultures') overwinter and feed at a large livestock carcass dumping site known as *Jorbeer* in the desert of Thar in northwest India (N 27°57.958' E 73°22.598'). The vultures breed in the higher ranges of Pakistan, Afghanistan, few central Asian countries, and parts of Europe (5). Approximately 20 carcasses of stray and dairy cattle, camels, dogs, equines, and poultry offal were being dumped daily at the site during the study period. Most carcasses originated from dairy farms, veterinary clinics, and research centers and could possibly contain residual veterinary antimicrobials. The dump also hosted many other avian species, migratory as well as resident vultures and eagles, and hundreds of resident stray dogs.

Fresh fecal samples were collected from roosting sites of the vultures by non-invasive means. A total of 25 samples were collected in two consecutive winter seasons, that is, February 2011 ( $n = 11$ ) and March 2012 ( $n = 14$ ). All the sampled birds were apparently healthy and did not show any clinical symptoms of disease. The birds always existed in excess of 500 individuals, as estimated by line-transect method; thus, the probability of duplicate sampling from a single individual was statistically insignificant ( $P < 0.05$ ). *P. aeruginosa* was phenotypically confirmed and characterized by standard methods. In short, all samples were enriched in nutrient broth (HiMedia, Mumbai) at 37°C for 12 hours, streaked on cetrinide agar (HiMedia, Mumbai), and incubated aerobically at 37°C for 12 hours. The resulting colonies were streaked on nutrient agar plates (HiMedia, Mumbai) and incubated at 37°C for 24 hours to obtain pure cultures. Gram-negative rods from oxidase- and catalase-positive colonies were processed further with standard biochemical tests. DNA isolation was performed following a published protocol (6), with some minor modifications. Species-specific primers (F-5' GGGGAT CTTCGGACCTCA 3' and R-5' TCCTTAGAGTGCC ACCCG 3', product 956 bp) were employed in a 16S rRNA gene-based PCR identification (7). Genotypic characterization of the virulence markers *exo-s*, *exo-u*, and *alg* was conducted using earlier described methods (8–10) with the following primer sequences: *exo-s* (product 504 bp) - (F) 5' CTT GAA GGG ACT CGA CAA GG 3', (R) 5' TAA GTG ATG CGC CTG GAC TT 3'; *exo-u* (product 1,500 bp) - (F) 5' GCA GCC TAT CGT GCA AG 3', (R) 5' GCG TGC AGT GAT TGC GA 3'; *alg* (product 313 bp) - (F) 5' CGT CTG CCG CGA GAT CGG CT 3', (R) 5' GAC CTC GAC GGT CTT GCG GA 3'.

PCR amplification of the 16S rRNA gene was carried out in a 50 µl reaction volume using the Promega gene amplification kit (Madison, Wisconsin, USA) by mixing 10 µl 5 × assay buffer, 1 µl F-primer (10 pM/µl), 1 µl R-primer (10 pM/µl), 1-µl dNTP (10 mM), 3 µl MgCl<sub>2</sub> (1.5 mM/µl), 0.25 µl Taq DNA polymerase (5 U/µl), 30.75 µl deionised water, and 3 µl template DNA (25 ng/µl). The PCR was performed in Eppendorf Mastercycler Gradient (Eppendorf AG, Hamburg, Germany). The PCR for 16S rRNA amplification involved initial denaturation at 96°C for 1 min, followed by 40 cycles of 94°C for 30 sec, 58°C for 30 sec, 72°C for 1 min, and final extension of 72°C for 10 min. Reaction volumes and concentrations of mastermix were similar for *exo-s*, *exo-u*, and *alg* PCRs, but the annealing temperature was 55°C for 1 min. PCR reaction products were analyzed by electrophoretic separation on 1.2% agarose gels stained with ethidium bromide.

Subsequently, *P. aeruginosa* isolates were tested for *in vitro* antimicrobial sensitivity to 13 routinely used antimicrobials of different chemical classes by disc diffusion (11), employing a Clinical Laboratory Standard Institute

(CLSI) protocol using the *P. aeruginosa* ATCC 27853 strain to validate the results.

## Results and discussion

The frequency of isolation was 32.0% ( $n = 25$ ) as eight samples from the vultures were positive for *P. aeruginosa*. Mutually exclusive *exo-s* and *exo-u* virulence factors were confirmed in five and three samples, respectively, while *alg* was present in all the isolates. Three isolates, from both seasons, showed MAR. All the isolates were resistant to ampicillin and susceptible to amikacin, ciprofloxacin, gentamicin, and polymyxin B. The isolates showed reduced susceptibilities to bacitracin, carbenicillin, cephalothin, colistin, kanamycin, cephalexin, nalidixic acid, and nitrofurantoin. Complete susceptibility to ciprofloxacin is notable, as resistance to fluoroquinolones is encoded exclusively by chromosomal genes, while resistance to beta-lactam antibiotics and aminoglycosides could be plasmid mediated (12), indicative of the role of conjugative plasmids for acquiring resistance genes. These findings are significant as the vultures were without any prior therapeutic exposure to antimicrobials, and also information on MAR *P. aeruginosa* from avian wildlife is very rare, especially from Asia. The presence of *exo-s*, *exo-u*, and *alg* genes, which are important determinants of the virulence of *P. aeruginosa* (1, 13, 14), is probably a novel finding. All bacteria, isolated from the vultures, internally or externally, can potentially cause horizontal disease transmission both in wildlife and humans (15). The observed persistence of the potentially virulent and MAR isolates in both wintering seasons, the migratory movements of the vultures, and their interaction with other species of migratory raptors at *Jorbeer* were substantial reasons underlining the spatial and ecological efficiency of the vultures as a potential epidemiological vector of *P. aeruginosa* and associated resistance and virulence genes.

These findings highlight a risk of impairing regional measures to control emerging MAR and pathogenic strains of *P. aeruginosa*. Moreover, because of the pathogenicity of *P. aeruginosa* in other raptorial species (4) and its remarkable host adaptabilities, further studies are advocated to investigate potential detrimental effects (if any) to these endangered vultures along with dedicated molecular studies to determine the mechanisms responsible for the acquirement of the antimicrobial resistance by the *P. aeruginosa*.

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## Conflict of interest and funding

No conflict of interest to declare.

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