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# Diclofenac residues as the cause of vulture population decline in Pakistan

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**The Oriental white-backed vulture (OWBV; *Gyps bengalensis*) was once one of the most common raptors in the Indian subcontinent[1]. A population decline of >95%, starting in the 1990s, was first noted at Keoladeo National Park, India[2]. Since then, catastrophic declines, also involving *Gyps indicus* and *Gyps tenuirostris*, have continued to be reported across the subcontinent[3]. Consequently these vultures are now listed as critically endangered by BirdLife International[4]. In 2000, the Peregrine Fund initiated its Asian Vulture Crisis Project with the Ornithological Society of Pakistan, establishing study sites at 16 OWBV colonies in the Kasur, Khanewal and Muzaffargarh–Layyah Districts of Pakistan to measure mortality at over 2,400 active nest sites[5]. Between 2000 and 2003, high annual adult and subadult mortality (5–86%) and resulting population declines (34–95%) (ref. 5 and M.G., manuscript in preparation) were associated with renal failure and visceral gout. Here, we provide results that directly correlate residues of the anti-inflammatory drug diclofenac with renal failure. Diclofenac residues and renal disease were reproduced experimentally in OWBVs by direct oral exposure and through feeding vultures diclofenac-treated livestock. We propose that residues of veterinary diclofenac are responsible for the OWBV decline.**

Between 2000 and 2002 we performed gross post-mortem examinations on 259 adult and subadult OWBVs, of which 219 (85%) had grossly apparent urate deposits, characteristic of visceral gout, on the surface of internal organs (Fig. 1). Visceral gout in birds is most commonly the result of renal failure leading to hyperuricaemia and the deposition of uric acid on and within the internal organs, and can be caused by degenerative, metabolic, infectious, or toxic diseases[6]. To verify renal disease, and to determine the cause, detailed necropsies and diagnostic testing were performed on a subset of 42 OWBVs (33 adults and 9 juveniles; 28 with visceral gout and 14 without visceral gout) that were found within approximately 24 h of death. The remaining OWBVs were significantly decomposed and thus were unsuitable for diagnostic evaluation, although the grossly apparent and characteristic lesions of visceral gout allowed the presence or absence of the disease to be determined. Of the 14 OWBVs without visceral gout, we determined the cause of death in 8 (57%), which included trauma, intestinal foreign bodies, lead poisoning, organophosphate poisoning and gun-shot (Supplementary Information). We were unable to determine the cause of death in the remaining six vultures. Among the 28 OWBVs with visceral gout, only one (4%) had an identifiable disease (infection with *Mycobacterium avium*) in addition to visceral gout. All but two of the visceral gout cases were in good physical condition based on the subjective assessment of normal pectoral muscle mass and adequate body fat. In all of the OWBVs with visceral gout, the only significant histopathological lesion was severe, acute renal

Table 1 **Diclofenac residue testing results in kidney samples from wild OWBVs with and without renal failure**

| Case no. | Date | Site | Age | Gout | Diagnosis | Diclofenac residues ($\mu g\,g^{-1}$) |
|---|---|---|---|---|---|---|
| 33 | 2001 | KS | Juv | Yes | None | 0.051 |
| 74 | 2002 | KH | Ad | Yes | None | 0.054 |
| 16 | 2001 | KS | Ad | Yes | None | 0.060 |
| 53 | 2002 | KH | Ad | Yes | None | 0.064 |
| 60 | 2002 | ML | Ad | Yes | None | 0.064 (0.076) |
| 39 | 2001 | KH | Ad | Yes | None | 0.077 |
| 69 | 2002 | ML | Ad | Yes | None | 0.079 |
| 57 | 2002 | KH | Ad | Yes | None | 0.080 |
| 40 | 2002 | ML | Ad | Yes | None | 0.091 |
| 20 | 2001 | KS | Ad | Yes | None | 0.097 |
| 15 | 2001 | KS | Ad | Yes | None | 0.099 |
| 35 | 2001 | KS | Ad | Yes | None | 0.106 (0.077) |
| 41 | 2002 | KH | Ad | Yes | None | 0.109 |
| 75 | 2002 | KH | Ad | Yes | None | 0.114 |
| 55 | 2002 | ML | Ad | Yes | None | 0.124 |
| 54 | 2002 | ML | Ad | Yes | None | 0.177 |
| 71 | 2002 | KH | Ad | Yes | None | 0.179 |
| 61 | 2002 | ML | Ad | Yes | None | 0.186 |
| 42 | 2002 | KH | Ad | Yes | None | 0.199 |
| 44 | 2002 | KH | Ad | Yes | None | 0.233 |
| 4 | 2000 | KS | Ad | Yes | *M. avium* infection | 0.450 |
| 38 | 2001 | ML | Ad | Yes | None | 0.451 |
| 59 | 2002 | ML | Ad | Yes | None | 0.504 |
| 45 | 2002 | ML | Ad | Yes | None | 0.642 (0.197) |
| 56 | 2002 | KH | Ad | Yes | None | 0.643 |
| 2 | 2000 | LH | Ad | No | Wire collision | BDL |
| 3 | 2000 | CW | Juv | No | Hit by car | BDL |
| 12 | 2001 | ML | Ad | No | None | BDL |
| 14 | 2001 | KS | Ad | No | Lead poisoning | BDL |
| 28 | 2001 | KS | Ad | No | Normal (trapped) | BDL (BDL) |
| 31 | 2001 | KH | Juv | No | Fell from nest | BDL |
| 46 | 2002 | KH | Juv | No | Fractured tibia | BDL |
| 47 | 2002 | KH | Juv | No | Intestinal foreign body | BDL |
| 49 | 2002 | KF | Ad | No | None | BDL |
| 50 | 2002 | ML | Juv | No | None | BDL |
| 51 | 2002 | KH | Juv | No | None | BDL |
| 52 | 2002 | ML | Juv | No | None | BDL |
| 58 | 2002 | ML | Ad | No | Organophosphate | BDL (BDL) |

BDL indicates 'below detection limit' of diclofenac assay (0.005–0.01 $\mu g\,g^{-1}$). Results in parentheses are from the Toxicology Laboratory at the University of Pennsylvania New Bolton Center, which were performed as independent verification. This additional testing also did not detect acetaminophen (0.05), flunixin (0.05), ibuprofen (0.50), phenylbutazone (0.10), oxyphenylbutazone (0.05), indomethacin (0.05), ketoprofen (0.25), mefenamic acid (0.50), salicylic acid (1.0), tolmetin (0.05), or naproxen (1.0) (detection limits, in parentheses, are in $\mu g\,g^{-1}$). Ad, adult; CW, Chichawatni; Juv, juvenile; KF, Katora Forest; KH, Khanewal district study site; KS, Kasur district study site; LH, City of Lahore; ML, Muzaffargarh–Layyah district study site.

tubular necrosis and uric acid crystal formation in the kidneys and other tissues (detailed histopathological description is provided in Supplementary Information). Fibrosis or other changes indicating chronic renal disease were not present. Inflammatory lesions indicating an infectious disease were not consistently present in any of the visceral gout cases.

These findings were most compatible with acute renal failure due to a toxic cause. Additional testing eliminated known causes of avian renal disease, including toxic concentrations of cadmium, lead (39 vultures) and mercury (37 vultures)[7] (Supplementary Tables 1 and 2), as well as infection with the avian influenza[8], infectious bronchitis[9] (21 vultures) and West Nile viruses[10] (13 vultures) (Supplementary Table 3). Testing was also negative for toxic concentrations of arsenic, and for toxic or deficient concentrations of copper, iron, manganese, molybdenum and zinc (39 vultures) (Supplementary Tables 1 and 2). Carbamate and organophosphate pesticides (34 vultures), organochlorine pesticides and polychlorinated biphenyls (13 vultures) were either not detected or were detected at below toxic concentrations (Supplementary Tables 4, 5 and 6). We failed to isolate any viruses from the kidney, spleen, lung or intestine using at least four passages on chicken embryo fibroblast, duck embryo fibroblast, peregrine falcon (*Falco peregrinus*) embryo fibroblast and Vero cell cultures for 13 vultures (Supplementary Table 7).

The primary food source for OWBVs in Pakistan is dead domestic livestock. Therefore, we hypothesized that ingested veterinary pharmaceuticals might be responsible for renal disease in the scavenging birds. We conducted a survey of 74 veterinarians and veterinary pharmaceutical retailers in the region to identify drugs that were known to be both toxic to kidneys and absorbed orally. The only drug identified in the survey that met these criteria was diclofenac, a non-steroidal anti-inflammatory drug (NSAID) used as an analgesic, anti-inflammatory and antipyretic[11]. Liquid chromatography and mass spectroscopy detected diclofenac residue concentrations of 0.051–0.643 $\mu g\,g^{-1}$ in the kidneys of 25 out of 25 (100%) vultures that died of renal failure, and in 0 out of 13 (0%) vultures that died from other causes (Table 1). The association between renal failure and diclofenac residues was very highly significant ($\chi^2 = 38$, 1 degree of freedom, $P = 0.0$). Independent testing on three positive and two negative samples verified these results, and did not detect the presence of any other NSAID (Table 1). Diclofenac residues were found in vultures collected between 2000 and 2002, and at study sites separated by up to 350 km.

Although there are no reports of the use of diclofenac in birds, other NSAIDs such as indomethacin[12], flunixin meglumide[13] and ketoprofen (M. Busch & B.A.R., personal communication) may cause renal disease in chickens, cranes and quail, and in African white-backed vultures (*Gyps africanus*), respectively. NSAIDs, including diclofenac, are also recognized as causing renal disease in mammals[11,14]. To verify the renal toxicity of diclofenac in OWBVs, we administered single oral doses of veterinary diclofenac to four captive, non-releasable juvenile OWBVs. Two were given the mammalian label dose of 2.5 $mg\,kg^{-1}$ (high dose) and two were given 0.25 $mg\,kg^{-1}$ (low dose). Both of the high-dose and one of the low-dose vultures died as a result of renal failure and visceral gout within 36–58 h after administration, and all had the same microscopic renal lesions as the field cases. Plasma samples collected 1 h after administration for one high-dose and one low-dose vulture indicated normal uric acid levels[15] (53 and 33 $mg\,l^{-1}$, respectively). By 24 h after administration, both vultures had developed hyperuricaemia (775 $mg\,l^{-1}$ for both). Diclofenac residue concentrations of 0.29, 1.1 and 0.16 $\mu g\,g^{-1}$ were present in the kidneys of the two high-dose vultures and the one low-dose vulture that died as a result of renal failure. Although the other low-dose vulture developed hyperuricaemia (138 $mg\,l^{-1}$ at 1 h and 654 $mg\,l^{-1}$ at 24 h after administration), it remained clinically normal 4 weeks after administration, and did not have microscopic renal lesions or detectable diclofenac residues at necropsy. Two untreated control birds that were fed the same diet also did not have renal lesions or detectable diclofenac residues at necropsy.

The most probable source of diclofenac exposure is the consumption of treated livestock. In Pakistan, veterinary diclofenac is widely marketed over the counter by multiple companies for treating all types of hoofed livestock. A second survey of 84 drug retailers and veterinarians in nine districts in the Punjab province (Dera Ghazi Khan, Kasur, Khanewal, Layyah, Multan, Muzaffargarh, Pakpattan, Rajanpur, Sahiwal) and one district in the Sindh province (Hyderabad) found that all 84 sold diclofenac; 77 sold it daily, and 71 reported that it had become available within the last 5 years. Furthermore, livestock that die of disease or injury are typically left for scavengers to remove.

**Figure 1** Necropsy photograph of the abdominal cavity of an OWBV with visceral gout, showing uric acid precipitates on the serosal surfaces of the liver.

To verify that carcasses of treated livestock contain sufficient diclofenac concentrations to cause renal failure and death in the vultures that scavenge upon them, ten juvenile OWBVs were fed meat from a buffalo or goat that was injected intramuscularly with 2.5 mg kg$^{-1}$ veterinary diclofenac once daily for 3 days (per label instructions) and that were slaughtered 4 h hours after the last injection. Resulting diclofenac residue concentrations in the buffalo kidney, liver and muscle were 5.7, 1.5 and 0.76 μg g$^{-1}$, respectively, and in the goat kidney, liver and muscle they were 0.94, 0.22 and 0.19 μg g$^{-1}$, respectively. Ten more OWBVs were fed buffalo meat that contained 6.4 μg g$^{-1}$ of diclofenac. On the basis of the product of diclofenac concentration in the meat and the amount of food consumed, eight OWBVs received doses of 0.005–0.3 mg kg$^{-1}$. Two of these vultures died from renal failure at 4 and 6 days after exposure, and at necropsy they had diclofenac residue concentrations of 0.07 and 0.38 μg g$^{-1}$ in the kidney. A necropsy was carried out on one surviving, clinically normal vulture at 8 days after exposure, at which time it did not have renal lesions or detectable diclofenac residues. The other five surviving vultures remain clinically normal at approximately 6 months after exposure. Two OWBVs received doses of 0.5–0.6 mg kg$^{-1}$. One of these died from renal failure 1 day after exposure, and at necropsy it had a diclofenac residue concentration of 0.25 μg g$^{-1}$ in the kidney. The other surviving, clinically normal vulture had a necropsy performed on it at 8 days after exposure, at which time it did not have renal lesions or detectable diclofenac residues. Ten OWBVs received doses of 0.8–1.0 mg kg$^{-1}$. All ten of these vultures died from renal failure. Kidney diclofenac residue concentrations of 0.120–0.906 μg g$^{-1}$ were present in six out of six of these vultures tested. These ten vultures were negative for other recognized avian nephrotoxins (Supplementary Information). All 13 vultures that died from renal failure after consuming meat from diclofenac-treated animals had the same histopathological renal lesions as the vultures that were exposed directly with diclofenac, and as the field cases with visceral gout. Six control vultures that were fed untreated goat (meat that did not contain detectable diclofenac residues) remained clinically normal 8 and 25 days into the experiment. Two were necropsied at day eight, and they did not have renal lesions or detectable diclofenac residues. These results are summarized in Table 2. In addition, all of the experimental OWBVs were held in captivity, and were clinically normal for 30–75 days before diclofenac exposure, indicating that visceral gout was not incidentally related to captivity. The combined mortality rate of 13 out of 20 (65%) in the exposed vultures and 0 out of 6 (0%) in the control vultures indicates a statistically highly significant relationship between renal failure and exposure to diclofenac ($\chi^2 = 7.8$, 1 degree of freedom, $P = 0.005$). When the direct and food-borne exposure levels were combined, a statistically highly significant ($\chi^2 = 12.3$, 3 degrees of freedom, $P = 0.0064$) dose-dependence for diclofenac toxicity can also be demonstrated.

Alternatively, wild OWBVs might be exposed to diclofenac through contaminated water sources[16]; however, because NSAID-associated renal failure in birds appears to be acute and dose-dependent[12,13], the very low water concentrations (on the scale of ng l$^{-1}$)[16] would be unlikely to cause toxicity. Also, the rapid and complete elimination of most NSAIDs in mammals[14] and birds[17] makes bioaccumulation in either the treated animals or in the scavengers that feed on them unlikely. The lack of detectable diclofenac residues in the kidneys of any unaffected wild OWBV and in the kidneys of the surviving experimental vultures also suggest that bioaccumulation does not occur.

The potential ecologically toxic effects of human and veterinary pharmaceuticals are a growing concern[16,18]. Sporadic poisonings of scavenging birds by organophosphate pesticides and barbiturates used in livestock have been documented previously[19,20]. Anthelmintics may be shed in livestock faeces and may kill ecologically important invertebrates[21]. However, in stark contrast to the current situation with the OWBV, pharmaceutical residues have not been implicated previously as a cause of major ecological damage. The identification of diclofenac as the cause of the OWBV decline in Pakistan provides an opportunity for conservation intervention. The high rate of visceral-gout-associated vulture mortality in India[3,22,23] as well as the widespread use of veterinary diclofenac in India (R. Risebrough, personal communication) suggests strongly that diclofenac may also be responsible for vulture declines in the rest of the Indian subcontinent wherever diclofenac is used for the treatment of livestock. □

Table 2 **Results of experimentally feeding meat from diclofenac-treated animals to OWBVs**

| Vulture no | Exposure (mg kg$^{-1}$) | Result | Diclofenac residues (μg g$^{-1}$) |
|---|---|---|---|
| 11 | 0.007 | Gout | 0.38 |
| 12 | 0.025 | Survived | ND |
| 8 | 0.027 | Survived | ND |
| 10 | 0.028 | Survived | ND |
| 7 | 0.029 | Survived | ND |
| 9 | 0.029 | Survived | ND |
| D | 0.140 | Gout | 0.07 |
| F | 0.240 | Survived | BDL (8 days) |
| H | 0.550 | Gout | 0.25 |
| B | 0.600 | Survived | BDL (8 days) |
| 64 | 0.820 | Gout | 0.54 |
| 65 | 0.820 | Gout | ND |
| 62 | 0.840 | Gout | 0.22 |
| 63 | 0.840 | Gout | 0.12 |
| 70 | 0.860 | Gout | 0.74 |
| 67 | 0.860 | Gout | ND |
| 68 | 0.860 | Gout | ND |
| 66 | 0.880 | Gout | 0.26 |
| 73 | 0.910 | Gout | 0.91 |
| 72 | 0.940 | Gout | ND |
| 1 | None (control) | Survived | ND |
| 2 | None (control) | Survived | ND |
| 3 | None (control) | Survived | ND |
| 4 | None (control) | Survived | ND |
| cc1 | None (control) | Survived | BDL (8 days) |
| cc2 | None (control) | Survived | BDL (8 days) |

Values in parentheses in the last column indicate time after exposure. OWBVs that died and displayed gross characteristics of visceral gout on necropsy are labelled as 'Gout' in the third column. BDL, below detection limit of diclofenac assay (0.005–0.01 μg g$^{-1}$); ND, analysis not done.

## Methods

### Histopathology

Tissue samples were fixed in 10% neutral-buffered formalin, and 5-μm sections were cut and stained routinely with haematoxylin and eosin.

### Toxicology

Testing for arsenic, cadmium, copper, iron, lead, manganese, mercury, molybdenum and zinc was performed by the inductively coupled plasma method. Organophosphate and carbamate testing included measuring brain acetylcholinesterase levels[24] as an indirect indicator of exposure[25], and direct detection by liquid chromatography and mass spectroscopy on liver, brain or stomach contents. Organochlorine pesticide and polychlorinated biphenyl testing was performed by liquid chromatography and mass spectroscopy. Reported concentrations are based on the wet weight of the samples.

### Diclofenac analysis

Kidney samples (0.5 g) were homogenized in 4 ml of acetonitrile and centrifuged to pellet debris. Three millilitres of the supernatants were then passed through solid-phase extraction cartridges (Waters Sep-Pak Plus t-C18) at 1 ml min$^{-1}$. The cartridges were then washed with an additional 3 ml of acetonitrile, the eluates pooled, and concentrated to final volumes of 1 ml. For samples that weighed less than 0.5 g, the protocol was adjusted proportionally for the smaller weights.

Diclofenac was detected and quantified by high-performance liquid chromatography and mass spectroscopy (Agilent 1100 series equipped with a Water's Xterra MS C18 (3.9 mm × 150 mm, 5 μm) and guard column (3.9 mm × 20 mm, 5 μm)). Diclofenac standard (Sigma, D6899) was dissolved into 1:1 (v/v) acetonitrile:water at 1,040 μg ml$^{-1}$, diluted to a final concentration of 10.4 μg ml$^{-1}$, and this stock was used to prepare calibration standards of 0.104, 0.052, 0.026, 0.0104 and 0.0052 μg ml$^{-1}$ in acetonitrile, which generated a linear calibration curve with $R^2$ values equal to 0.999. Samples and standards (20 μl) were subjected to a binary gradient elution profile, which consisted of 0.1% acetic acid in water (solution A) and 100% acetonitrile (solution B) as follows: starting conditions 75% A/25% B for 0.1 min, a 15-min linear gradient from 75% A/25% B to 5% A/95% B, followed by a 5-min column-wash step in 5% A/95% B, and a 10-min

re-equilibration step with 75% A/25% B before the next injection. Flow rate was 0.7 ml $min^{-1}$ and the column temperature was 40 °C. The mass spectrometer (Agilent 1946D) was equipped with an electrospray ionization inlet and mass spectra were acquired in the negative ion mode. The mass spectrometer was set to selectively monitor for mass ions 294 and 296 *m*/*z* of which mass 294 *m*/*z*, the deprotonated molecular mass, was used to quantify the diclofenac residues. The mean recovery from spiked avian kidney tissue was approximately 70%, and this value was used to calculate the final sample concentration of diclofenac.

### Virus isolation

Virus isolation in cell culture was performed in primary avian cell cultures prepared by the method of ref. 26 from chicken, duck and peregrine falcon embryos, and in Vero cells. Tissue samples were prepared by homogenization in Eagle's minimal essential medium with penicillin, streptomycin and amphotericin B, centrifuged to remove debris, filtered through 0.45-μm filters, and added to the cells. Cell cultures were observed daily for cytopathology. At the end of each passage (5–8 days), the cells and media were aspirated, stored at −80 °C, rapidly thawed, and added to new cells for the next passage.

### Polymerase chain reaction assays for infectious agents

RNA and DNA were extracted from 0.1–0.3 g of tissue with a commercial RNA extraction kit (Trizol Reagent; BRL Life Technologies) or a commercial DNA extraction kit (Puregene; Gentra Systems) as per the manufacturer's instructions. In addition to positive and negative control RNA or DNA for each agent, intact sample RNA and DNA were verified by demonstrating the presence of avian cellular β-actin gene RNA or DNA, and the absence of nonspecific inhibitors was verified by spiking samples with positive control RNA or DNA.

The polymerase chain reaction with the reverse transcription (RT–PCR) procedure for infectious bronchitis virus was as published in ref. 27—the positive control for this assay was the Arkansas strain. The RT–PCR procedure for avian influenza was as published in ref. 28—the positive control for this assay was viral RNA obtained from the USDA National Veterinary Services Laboratory, Ames, Iowa. The RT–PCR procedure for West Nile virus was as published in ref. 29—the positive control for this assay was viral RNA obtained from the USDA National Veterinary Services Laboratory, Ames, Iowa.

### Identification of *M. avium*

Acid-fast bacilli were detected with Ziehl–Neelsen stains of tissue impression smears. *Mycobacterium avium* was identified by PCR amplification and restriction enzyme polymorphisms in the heat-shock protein gene[30].

### Uric acid measurement

Uric acid levels were measured in heparinized plasma with a colorimetric assay for the production of hydrogen peroxide by uricase.

### Animal subjects

Animals were maintained and experiments performed in accordance with the Animal Health and Welfare Regulations of Bahauddin Zakariya University, Pakistan.

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# An optimal bronchial tree may be dangerous

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**The geometry and dimensions of branched structures such as blood vessels or airways are important factors in determining the efficiency of physiological processes. It has been shown that fractal trees can be space filling[1] and can ensure minimal dissipation[2–4]. The bronchial tree of most mammalian lungs is a good example of an efficient distribution system with an approximate fractal structure[5,6]. Here we present a study of the compatibility between physical optimization and physiological robustness in the design of the human bronchial tree. We show**