# RESEARCH COMMUNICATIONS

# Are conservation actions reducing the threat to India's vulture populations?

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**Veterinary use of the non-steroidal anti-inflammatory drug, diclofenac is responsible for the population collapse of resident vulture species in India. Conservation efforts, including a ban on veterinary diclofenac and the identification of a vulture-safe alternative (meloxicam), were introduced in 2006 in order to address the threat. Sampling of domesticated ungulate carcasses available to vultures in India was undertaken in three surveys prior to, around the time of, and 1–2 years after the ban in order to quantify the prevalence of diclofenac and meloxicam residues. A total of 1445, 1488 and 1251 liver tissue samples were collected from nine states and analysed with a validated LC-ESI/MS methodology. Overall diclofenac prevalence levels declined by almost a half over the three surveys, and there was an increase in meloxicam prevalence between the second and third surveys, although some states revealed little change. These surveys indicate that two of the key conservation actions to counter the threat faced by vultures – banning veterinary diclofenac and promoting meloxicam as a safe alternative – are beginning to take effect. However, because only a small proportion of diclofenac-contaminated carcasses is sufficient to cause vulture population declines, further efforts are needed to eliminate diclofenac from the food supply of India's vultures.**

**Keywords:** Carcass, conservation actions, non-steroidal anti-inflammatory drugs, vultures.

VULTURE populations in India have undergone a catastrophic collapse since the early 1990s with three resident species, the Oriental white-backed vulture (*Gyps bengalensis*), long-billed vulture (*G. indicus*) and slender-billed vulture (*G. tenuirostris*), which together used to number

tens of millions, now listed by the International Union for the Conservation of Nature (IUCN) as Critically Endangered[1]. Population decreases were first recorded to have started for the Oriental white-backed vulture between 1987 and 1996 at the Keoladeo National Park[2], and nationwide road-transect surveys showed that vultures had decreased across India between 1992 and 2000 (ref. 3). By 2007, the number of Oriental white-backed vultures had decreased by 99.9% in comparison to the early 1990s, with the long-billed vulture and slender-billed vulture decreasing by >98% over the same period[4]. An intensive research programme undertaken in India, Pakistan and Nepal has shown that veterinary use of the non-steroidal anti-inflammatory drug (NSAID), diclofenac is the main and perhaps the only cause of the population decline[5–9]. Vultures are exposed to diclofenac when they feed from carcasses of livestock that have died within a few days of treatment and so contain residues of the drug[6]. Birds that consume sufficient tissues from such carcasses die from kidney failure 1–2 days after exposure[6,10]. Population modelling has shown that just 0.8% of ungulate carcasses available to vultures would need to contain a lethal dose of diclofenac to cause the observed population declines[5]. However, surveys of ungulate carcasses collected from across northern India in 2004–2005 and 2006 revealed that 10–11% of carcasses contained detectable levels of diclofenac residues[11,12]. The prevalence and concentration of diclofenac in these carcasses was more than sufficient to account for the rapid vulture population declines[13].

In response to the threat faced by vultures, the Drug Controller General of India wrote to all State Drug Controllers in May 2006 withdrawing the licence to manufacture veterinary forms of diclofenac. This 2006 directive was strengthened in 2008 to make it an imprisonable offence to manufacture, retail or use diclofenac for veterinary purposes. To help address the problem being caused by diclofenac, researchers have now tested two other NSAIDs in an effort to identify alternatives to find other drugs that are both of low toxicity to vultures and also effective for treating livestock. This work, undertaken in India and southern Africa, revealed that the veterinary NSAID, meloxicam is of low toxicity to vultures and other scavenging birds[14–16], but that ketoprofen causes the same toxic effects as diclofenac in vultures[17], though fortunately its prevalence in ungulate carcasses in India is low[12]. Consequently, meloxicam has been promoted in India in an effort to replace veterinary use of diclofenac with this safe alternative[18]. However, ketoprofen is still licensed for veterinary use.

In this communication, we report the prevalence of veterinary NSAIDs in carcasses of domesticated ungulates sampled across northern and central India during the period January 2007 to December 2008, 1–2 years after the diclofenac ban and after the promotion of meloxicam. The results are compared with earlier pre-ban and immediately

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**Table 1.** Number of ungulate liver samples collected, the prevalence (% of samples) of detectable diclofenac residues in three surveys and the prevalence of meloxicam residues in two surveys, for nine states where repeat sampling occurred. In addition, total number of samples and overall prevalence are given for the entire dataset and for a sub-set of seven site-clusters (see text) sampled in all three surveys, adjusted for sampling effort in each cluster

| Location | Carcass samples ($n$) | | | Diclofenac (%) | | | Meloxicam (%) | | |
|---|---|---|---|---|---|---|---|---|---|
| | S1 | S2 | S3 | S1 | S2 | S3 | S1 | S2 | S3 |
| Andhra Pradesh | 154 | – | 143 | 2.6 | – | 1.4 | – | – | 0.7 |
| Gujarat | 65 | 222 | 159 | 9.2 | 4.1 | 1.9 | – | 0.0 | 4.4 |
| Jammu and Kashmir | 77 | 112 | – | 3.9 | 3.6 | – | – | 11.6 | – |
| Madhya Pradesh | 195 | 236 | 257 | 11.3 | 12.7 | 3.9 | – | 0.8 | 7.0 |
| Maharashtra | 194 | 241 | 262 | 5.7 | 11.2 | 5.3 | – | 4.6 | 7.3 |
| Punjab | 76 | 228 | – | 15.8 | 12.3 | – | – | 7.5 | – |
| Rajasthan | 310 | 339 | 303 | 17.1 | 14.5 | 12.2 | – | 4.7 | 5.9 |
| Uttar Pradesh | 280 | – | 127 | 11.8 | – | 3.1 | – | – | 3.1 |
| West Bengal | 94 | 110 | – | 9.6 | 16.4 | – | – | 0.9 | – |
| All states | 1445 | 1488 | 1251 | 10.6 | 11.1 | 5.6 | – | 4.0 | 5.4 |
| Seven site-clusters (adjusted for effort) | 518 | 728 | 981 | 13.5 | 11.4 | 8.9 | – | 3.0 | 6.0 |

post-ban data in order to assess the effectiveness of the banning of diclofenac and the promotion of meloxicam at reducing the threat to India's vulture populations.

Ungulate carcasses were sampled across India during three separate survey periods (hereafter referred to as Surveys 1–3). States selected for sampling were chosen on the basis of being representative of North, West, East and Central India, where vulture populations were formerly most abundant[7]. Survey 1 collected 1445 samples from 62 sites in nine states between May 2004 and July 2005, a period 1–2 years prior to the 2006 diclofenac ban. Survey 2 collected 1488 samples at 26 sites in seven states from May to December 2006, immediately after the announcement of the ban in May 2006 (at which point, a three-month period was given in order to implement the directive). Survey 3 collected 1251 samples from 15 sites in six states between January 2007 and December 2008. The coverage of states varied among surveys (Table 1). Samples were collected from carcass dumps managed by local government municipalities, co-operative and private companies, or from animal welfare charities and village carcass dumps. More than 99% of the samples were collected from carcass dumps, with a small number of additional samples (< 0.5%) collected opportunistically from carcasses encountered along roads and in fields. Previous publications[11,13,19] analysed 1848 samples collected from 67 sites and 12 states in Survey 1, a total that includes 279 samples collected from slaughterhouses in three states, and 121 and 3 samples from carcass dumps in Bihar and Jharkhand respectively. Neither Bihar and Jharkhand states were sampled, nor were the slaughterhouses visited in Surveys 2 or 3, and, these 403 samples were excluded from our analyses. Consequently, in this communication, changes in the prevalence of diclofenac in ungulate carcasses are only reported for samples collected at carcass dumps and for the nine states that were sampled on two or more occasions in the three surveys (Table 1).

Carcass dumps sampled within each state were those where access and permission to collect was obtained readily. Hence, these sites might not be a random sample of locations where ungulate carcasses were available to vultures. Where possible, samples were collected from the same carcass dump as for the previous surveys. Where this was no longer possible, samples were collected from the next nearest available site. The location of all dumps in all three surveys was recorded with a handheld GPS. At each site, liver samples were collected from all ungulate carcasses that were delivered to the dump on the day of sampling, thereby providing a representative sample of carcasses at the site on that day with no possible bias with regard to species, age or sex. At one site in Survey 1, the large number of animals arriving prevented all carcasses from being sampled, and only young, prime and mature animals were sampled[19], with infant, immature and old animals being excluded. Detailed protocols on the location of carcass dumps and collection of carcass samples have been reported elsewhere[19]. For each carcass, approximately 2 g of liver tissue was collected, immediately frozen and kept frozen during transportation to the laboratory for analysis[19]. Majority of carcasses (94–95%) were of cattle or buffalo, with occasional samples from sheep, goats, horses and camels.

In the laboratory, a sub-sample of each liver tissue was removed and weighed to between 0.45 to 0.55 g (to ± 0.0001 g). Each sub-sample was placed in a fresh glass test tube and homogenized with 2 ml of HPLC-grade Acetonitrile (Merck) using an Ultra Turrax IKA T8 homogenizer. The homogenate was centrifuged at 1000 $g$, then filtered through a 0.45 µm Nylon filter into a 2 ml LCMS vial and stored at –20°C until analysis. Analysis of diclofenac and meloxicam residues was undertaken with a validated liquid chromatography–electrospray ionization mass spectrometry (LC–ESI/MS) approach[11,12]. The analytical procedure is reported in detail elsewhere[11,12], and the same methods were followed in the present study. Values for meloxicam were only quantified in Surveys 2 and 3, following the development of a multi-NSAID methodology[12]. The limit of quantification for the LC–ESI/MS technique

(back-calculated in wet tissue concentration) for diclofenac and meloxicam was 0.01 ppm (0.01 mg kg$^{-1}$)[12].

We report the prevalence of detectable diclofenac and meloxicam residues over the whole period of each survey and separately by states. It is known from previous analyses that the prevalence varied geographically[11], so spurious differences among surveys in average prevalence might arise as a consequence of differences in the distribution of sampling. In order to avoid this potential bias all sampling locations were attributed to a site-cluster, with all sampling sites located within a 186 km radius of the geodesic centroid of the site-cluster. In Survey 3, all sampling sites could be attributed to the same 21 site-clusters as those for Surveys 1 and 2. Samples were obtained in all three surveys for seven site-clusters, with a further five site-clusters sampled in Surveys 1 and 2 only, and two site-clusters sampled in Surveys 1 and 3 only (Figure 1). To allow for potential effects caused by differences in the distribution of sampling, we estimated change in the prevalence of diclofenac using only the data for the seven site-clusters sampled in all three surveys, which were located in Gujarat ($n = 2$ clusters), Rajasthan ($n = 2$), Madhya Pradesh ($n = 2$) and Maharashtra ($n = 1$; Figure 1). For each survey, we calculated the negative cumulative distribution of diclofenac values for these seven site-clusters. We allowed for differences among surveys in the proportion of samples obtained at each of these seven site-clusters. We did this by assigning a weight $n_{i1}/(n_{ij}N_1)$ to each sample value, where $n_{i1}$ is the number of samples obtained in Survey 1 at the $i$th site-cluster, $n_{ij}$ is the number of samples obtained in the $j$th survey at the $i$th site-cluster and $N_1$ is the total number of samples obtained at all seven site-clusters sampled in all
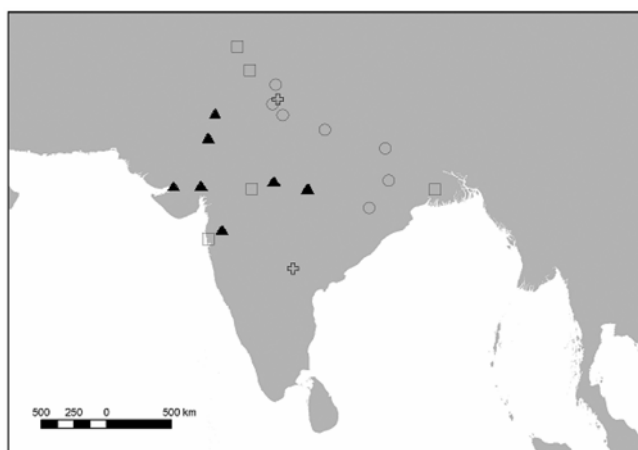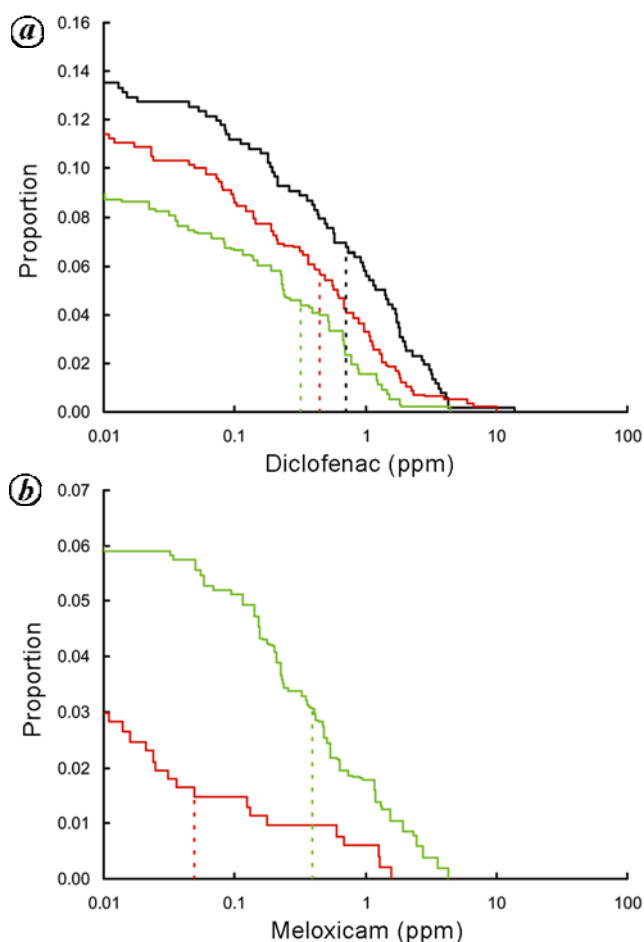


**Figure 1.** Map of the Indian subcontinent showing the location of centroids for the 21 site-clusters at which carcass sampling was undertaken. The seven site-clusters where samples were collected in all three surveys are indicated by filled triangles; squares indicate sites surveyed in Surveys 1 and 2 only; crosses indicate sites surveyed in Surveys 1 and 3 only, and circles show sites surveyed in Survey 1 only.

surveys. The negative cumulative distribution of diclofenac and meloxicam values was then obtained by accumulating these weighted values from each of the surveys in ascending order and then subtracting the result from one. This calculation produces negative cumulative distributions for Surveys 2 and 3, which simulate the expected distributions if the same proportions of samples had been taken in the seven site-clusters in Surveys 2 and 3 as were taken in these site-clusters in Survey 1. Statistical analysis on the significance of changes in the prevalence of diclofenac and meloxicam residues was undertaken through logistic regression, with the presence or absence of detectable drug residues as the binary dependent variable, survey as a factor with three levels (one each for Surveys 1–3) and state as a factor with nine or four levels (see below) as independent variables, and with a two-way interaction between these two factors. This is termed the full model. Simplified models in which the interaction term or the main effects were deleted were also fitted and likelihood ratio tests used to test whether these deletions resulted in a significant increase in residual deviance. The model with no effect of either of the explanatory factors is termed the null model. For diclofenac prevalence the first analysis used all available data from the nine states and three surveys, with a second analysis undertaken on the restricted set of data from the seven site-clusters and four states that were sampled in all three surveys. The same two analyses were undertaken for meloxicam, but only utilizing data from Surveys 2 and 3.

A total of 1445, 1488 and 1251 liver tissue samples were analysed from Surveys 1–3 respectively (Table 1). For diclofenac prevalence and using the dataset of all states, the full logistic regression model indicated that the survey factor, the state factor and their two-way interaction combined reduced the residual deviance to a highly significant extent when compared with the null model (difference in deviance between the full model and the null model = 135.8, difference in degrees of freedom = 21, $P < 0.0001$). Deletion of the two-way interaction term from the model caused no significant increase in residual deviance (difference in deviance = 19.1, difference in $df = 11$, $P = 0.059$), whereas deletion of either state or survey as a factor resulted in a highly significant increase in deviance ($P < 0.0001$ for both factors). We therefore concluded that the model with both survey and state included as main effects was the minimal adequate model of diclofenac prevalence for all states. The equivalent logistic regression analysis on diclofenac prevalence within the restricted set of seven site-clusters surveyed in all three surveys indicated a similar pattern to the analysis of data for all states. The full model reduced residual deviance to a highly significant extent when compared with the null model (difference in deviance = 76.6, difference in degrees of freedom = 11, $P < 0.0001$). However, deletion of the two-way interaction term from the model caused no significant increase in residual deviance
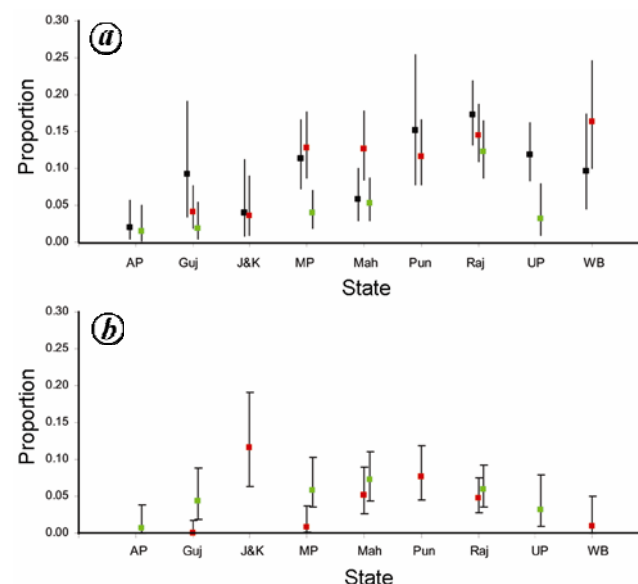
(difference in deviance = 9.3, difference in *df* = 6, *P* = 0.16) and the best-fit model was one with both survey and state being included as the main effects. Overall, the prevalence of diclofenac decreased markedly in Survey 3 in comparison with the first two surveys, with a similar pattern of change over time in the seven site-clusters as in the data from all states (Table 1, Figure 2 *a*). Diclofenac prevalence in Surveys 1–3 from the seven site-clusters was 13.5%, 11.4% and 8.9% respectively. These prevalence levels are higher than those indicated for all states that were sampled, as Rajasthan has a high diclofenac prevalence in comparison to the other states (Table 1) and a high proportion of samples (43%) were collected from this state. As well as indicating a decrease in diclofenac prevalence, the seven site-clusters data indicate a drop in the median value of concentration in those samples for which diclofenac was detected (Figure 2 *a*). State figures for diclofenac indicate a high degree of variation in diclofenac prevalence among the states (Figure 3 *a*).

For the meloxicam data there was significant reduction in residual deviance for the full model versus the null model for both the complete dataset from Surveys 2 and 3 (difference in deviance between the full model and the null model = 65.8, difference in degrees of freedom = 12, *P* < 0.0001) and the restricted seven site-clusters dataset (difference in deviance = 38.7, difference in degrees of freedom = 11, *P* < 0.0001). Deletion of the two-way interaction term from the model caused a significant increase in residual deviance for both the full dataset (difference in deviance = 14.7, difference in degrees of freedom = 3, *P* < 0.01) and the restricted seven site-clusters dataset (difference in deviance = 15.7, difference in degrees of freedom = 7, *P* < 0.05). We therefore conclude that for both datasets the full model with survey, state and their two-way interaction is the minimal adequate model of meloxicam prevalence. In the seven site-clusters, prevalence of meloxicam in carcass samples doubled from 3.0% to 6.0% from Surveys 2 to 3 (Table 1; Figure 2 *b*), along with an increase in the median value of positive samples (Figure 2 *b*). Prevalence figures for all four states sampled in Surveys 2 and 3 indicated an increase in meloxicam usage, and with Gujarat and Madhya Pradesh showing greater increase in comparison to Maharashtra and Rajasthan (Figure 3 *b*).

The changing patterns of diclofenac and meloxicam prevalence recorded by the three surveys analysed here indicate that there have been significant changes in veterinary use of these two NSAIDs in India since the



**Figure 2.** Negative cumulative distribution of diclofenac concentrations (*a*) and meloxicam concentration (*b*) for samples collected from the seven site-clusters subset in Survey 1 (black line), Survey 2 (red line) and Survey 3 (green line), indicating the overall decrease in diclofenac prevalence over the period covered by the three surveys (13.5%, 11.4% and 8.9% respectively), and the increase in meloxicam prevalence (3.0 and 6.0% respectively). Median values of diclofenac and meloxicam in the three surveys are indicated by the vertical dashed lines.



**Figure 3.** Proportion of samples positive for diclofenac (*a*) and meloxicam (*b*) in nine states (Andhra Pradesh, Gujarat, Jammu & Kashmir, Madhya Pradesh, Maharashtra, Rajasthan, Uttar Pradesh and West Bengal) from Survey 1 (black squares), Survey 2 (red squares) and Survey 3 (green squares). Vertical lines indicate the 95% binomial confidence limits around the proportion. Meloxicam residues were only analysed in Surveys 2 and 3.

ban on veterinary use of diclofenac in 2006. The direction of the changes, with an overall decrease in diclofenac prevalence and increase in meloxicam prevalence, indicates that two of the key conservation actions taken, namely the national ban on veterinary use of diclofenac and promotion of the vulture-safe alternative, meloxicam to counter the threat faced by vultures, are beginning to take effect. National figures, based on all nine states that were sampled, indicate that the prevalence of diclofenac has declined by almost half 1–2 years after the ban, with 5.6% of samples containing diclofenac in the third survey in comparison to 10–11% found prior to and around the time of the ban in 2006. Comparison of data from the seven site-clusters that were sampled in all three surveys and adjusted for sampling effort confirms this pattern of decrease, though with a somewhat smaller decrease: at these sites the decrease is from 13.5% prior to the ban to 8.9% after the ban. Values for meloxicam prevalence indicate an opposing trend to that recorded for diclofenac, with a doubling in the proportion of carcass samples with residues of this drug from Survey 2 to 3. The average concentrations of diclofenac and meloxicam also show opposite changes over time: the median concentration of diclofenac declined, while that of meloxicam increased.

Diclofenac is now widely recognized as the main driving force behind the rapid decline in India's vulture populations over recent times[6–9], and consequently evidence for a decline both in the prevalence and concentration of diclofenac residues in ungulate carcasses is important for India's threatened vulture populations. Comprehensive modelling[20], based on the results of these three surveys and the relationship between diclofenac levels and vulture toxicity, suggests that for the Oriental white-backed vulture the expected rate of population decline caused by diclofenac poisoning has decreased by around a third for Survey 3 in comparison to the rate predicted from Survey 1. This modelling indicates an annual decline rate of around 18% (ref. 20). While a reduction in population decline rate from 44% to 18% is a marked improvement in the rate of decline observed in the wild for Oriental white-backed between 2000 and 2007 (ref. 4), it is still a rapid rate of decrease. Because a small proportion ($< 0.8\%$) of ungulate carcasses containing lethal levels of diclofenac is sufficient to cause the observed rapid population declines[4], efforts to ban diclofenac and replace it with meloxicam will have to markedly increase in effectiveness to remove the threat currently faced by India's vulture populations. In the meantime, conservation initiatives, including captive breeding and preparation for reintroduction to the wild of threatened vultures remain vital in order to safeguard these critically endangered species.

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