

# Nimesulide Poisoning in White-Rumped Vulture *Gyps Bengalensis* in Gujarat, India

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## Research Article

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## Abstract

Catastrophic population decline of White-rumped Vulture due to use of a non-steroidal anti-inflammatory drug (NSAID), diclofenac throughout its distribution range is well documented. White-rumped Vulture was listed as Critically Endangered and only few thousands are remaining. During 2019, there were two incidents of White-rumped Vulture death in Gujarat. In February 2019, two vultures were reported dead in Sanand, Gujarat and the death was suspected to be poisoning. Another two vultures were also reported to have died in Wild Ass Sanctuary, Dhrangadhra in October 2019. Tissues and gut contents of all four vultures were received for toxicological investigation and checked whether these vultures died due to NSAIDs. The tissues were analysed for thirteen NSAIDs. Of all the NSAIDs, nimesulide was detected in all the tissues analyzed in high concentration (17 - 1395 ng/g). Subsequently, these tissues were also screened for a set of mostly used toxic pesticides in India, and none of them was in toxic level. Visceral gout was also observed in all the four vultures during post-mortem. Elevated levels of nimesulide in tissues with clear symptoms of gout, indicated that the vultures died due to nimesulide poisoning. Although, other than diclofenac, many NSAIDs are toxic/suspected to be toxic to White-rumped Vultures, only nimesulide is reported with clear symptom of gout in wild dead White-rumped Vultures similar to diclofenac consistently in recent past. Considering the fact that nimesulide also acts similar to diclofenac leading to death in White-rumped Vulture, it seems that nimesulide is replacing diclofenac in case of White-rumped Vulture in Gujarat. Nimesulide is cause of concern in conservation of White-rumped Vultures. Hence, nimesulide should also be banned by the government for veterinary use in addition to diclofenac to conserve White-rumped Vulture in Indian subcontinent. Further, an effective system is recommended to be put in place to collect the tissues of dead Vultures for toxicological investigations, and eventual conservation of the critically endangered species.

## Introduction

Catastrophic population decline in the White-rumped Vulture *Gyps bengalensis* in India as early as 90's was reported (Prakash et al. 2007). While diclofenac was reported to have caused the decline (Oaks et al. 2004; Shultz et al. 2004; Green et al. 2006), the population of White-rumped Vulture has not recovered even after the drug was banned for veterinary use in India. The species is still "at high risk of global extinction", listed as Critically Endangered (BirdLife International 2020), and categorized under Schedule I of Indian Wildlife Protection Act 1972; 2002 (Amended). According to Prakash et al. (2019) the population of the White-rumped Vulture in India as of 2015 was about 6,000. Despite efforts, population the species is yet to reach a healthy level. Mortality of White-rumped Vulture due to diclofenac was continued to be reported (Muralidharan and Dhananjayan 2010; Cuthbert et al. 2016; Nambirajan et al. 2018). The role of NSAIDs apart from diclofenac have also be monitored as use of NSAIDs in treating cattle has become inevitable to veterinarians. Hence, conservation initiatives to revive the population of White-backed Vulture has become an ongoing affair in India. It also became imperative to check if there was misuse of diclofenac and also if any other chemical was contributing to the population decline. Pesticide poisoning was also reported to have killed White-rumped Vulture in Sathiyamangalam Tiger Reserve, Tamil Nadu (Muralidharan S, Unpublished data).

Despite the ban on diclofenac, it is reported that diclofenac accounts for 10–46% of all NSAIDs available in the Indian market for veterinary use as on 2017 (Galligan et al. 2020). Studies revealed that other than diclofenac, NSAIDs, namely aceclofenac, ketoprofen, nimesulide, flunixin and carprofen are also reported to be toxic to Gyps vultures (Naidoo et al. 2010, 2018; Sharma 2012; Fourie et al. 2015; Zorrilla et al. 2015; Cuthbert et al. 2016; Galligan et al. 2016; Eleni et al. 2019) and the same are among the 11 NSAIDs available in India for veterinary use as on 2017 (Galligan et al. 2020). Hence, an investigation was carried out to assess whether any of these NSAIDs or pesticides was responsible for the death of four White-rumped Vultures received at the National Centre for Avian Ecotoxicology (NCAE), Sálim Ali Centre for Ornithology and Natural History (SACON), Coimbatore for toxicological investigation during 2019.

## Materials And Methods

### Sample collection

Opportunistic sampling was the strategy followed to collect samples of dead vultures. After post-mortem examination, tissues of organs, namely kidney, liver, intestine and gut contents were dissected out, wrapped in aluminum foil, labelled, frozen immediately and transported Centre for Avian Ecotoxicology laboratory, SACON, Coimbatore. Samples were stored in deep freezer at -20°C until chemical analyses were performed.

### Chemicals and reagents

Certified Reference Materials (CRMs) of Individual NSAID with purity higher than 98% were obtained from Sigma-Aldrich, USA. Individual pesticide CRMs with purity more than 97% were purchased from Agilent Technologies, USA. While chromatography grade acetonitrile and sodium chloride were purchased from Merck, India, gradient grade acetonitrile for LC and hypergrade acetonitrile for LC-MS were of Merck KGaA, Germany. Anhydrous magnesium sulphate was of Himedia. Clean up reagents, namely Primary Secondary Amine (PSA), C18 bulk sorbent and graphitized carbon block (GCB) were from Agilent Technologies, USA. Ultrapure water was produced with a water purification system (Purelab classic, Elga, UK) in the laboratory.

### Residue extraction

Residues of NSAIDs and pesticides were extracted from tissues such as liver, kidney and gut contents of the vultures using multi residue extraction method as adopted by Anastassiades et al. (2003) for vegetables and optimized by Nambirajan et al. (2018) for tissues samples. About two grams of homogenised tissue samples were accurately weighed and placed in a 50 mL centrifuge tube. 5 mL of purified water was added, hand shaken to homogenise well and kept for 30 minutes. Subsequently, 10 mL of acetonitrile was added to it and shaken again vigorously for a minute. Two grams of anhydrous magnesium sulphate (MgSO<sub>4</sub>) and half gram of sodium chloride were added to the centrifuge tube and shaken vigorously again for a minute, and centrifuged at 2,000 g for 5 min. After centrifugation, 2 mL of the organic layer of centrifugate was transferred into a 15 mL centrifuge tube which contained 100 mg of Primary Secondary Amine (PSA), 100 mg of C18 bulk sorbent and 250 mg of MgSO<sub>4</sub>, and shaken well. Then it was centrifuged at 1,000 g for 5 min, and 1 mL of the centrifugate was transferred into vials for instrumental analysis.

## Instrumental analyses

Residues of pesticides and NSAIDs were quantified using Shimadzu LCMS-8050 Ultra-Fast Triple Quadrupole Mass Spectrometer with Nexera X2 Ultra Performance Liquid Chromatography as a front end. Instrument conditions for estimation of NSAIDs were as follow; Shim-pack GISS C18 (3 $\mu$ m, 2.1 x 150 mm) column (Shimadzu, Japan) was used for separation. 5  $\mu$ l of sample was subjected to a binary gradient elution profile, which consisted 0.1% acetic acid in water (solution A) and 100% acetonitrile (solution B) as follows; starting conditions 70% A/30% B for 1.0 min, a 7-min linear gradient from 70% A/30% B to 10% A/90% B, followed by a 3-minute re-equilibration step with 70% A/30% B before the next injection. Flow rate was 0.4 ml minute<sup>-1</sup> and the column temperature was 40° C. Interface, heat block and dissolution line temperature were 300, 400 and 250° C respectively.

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## Quality assurance/quality control

Both the types of contaminants were analysed in Multiple Reaction Monitoring (MRM) mode. MRM experiments were carried out to obtain the maximum sensitivity for the detection of the target molecules. For confirmation, two MRM transitions and a correct ratio between the abundances of the two optimised MRM transitions (MRM2/MRM1) were used, along with retention time matching. A matrix-matched calibration curve was constructed for each compound at eight calibration levels using 1, 2.5, 5, 10, 25, 50, 100 and 200 ng/ml of mixed pesticide and NSAID standards. The criterion for the acceptance of the linearity ( $R^2 \geq 0.98$ ) was fulfilled for all analytes in the method and the estimated values are expressed in ng/g (wet weight basis). Limit of quantification of compounds ranged from 5 ng/g to 20 ng/g. Average recoveries of the all compounds from fortified samples were above 70%. Results were not corrected for per cent recovery. Analyses were done in batches of 10 samples plus three quality controls, namely blank, method blank and mid-range standard.

## Results And Discussion

During 2019, four White-rumped Vultures died in two separate incidents in Sanand and Dhrangadhra in Gujarat, and the deaths were suspected to be due to poisoning based on circumstantial evidences (Table 1). Towards identifying the reason for the death, tissues, namely kidney, liver and gut contents of the White-rumped Vultures were screened for thirty five most commonly used toxic pesticides in India, namely acephate, dimethoate, monocrotophos, phosphamidon, malathion, triazophos, quinalphos, phorate, profenofos, ethion, chlorpyrifos, pirimiphos-methyl, phosalone, fenthion, temephos, phenthoate, carbofuran, carbaryl, propoxur, carboxin, carbosulfan, fenobucarb, indoxacarb, thiodicarb, fenobucarb, methomyl, alachlor, butachlor, pretilachlor, pendimethalin, propanil, atrazine, metribuzin, propargite and fipronil. Residues of all the pesticides were below detection limit in all the tissues.

Table 1  
Details of White-rumped Vulture received dead from Gujarat for toxicological investigation from Gujarat during 2019.

| Date          | Location                                 | Causality | Post-mortem findings                        |
|---------------|--|-----------|---|
| February 2019 | Sanand, Gujarat                          | 2         | Uric acid crystal deposition in the viscera |
| October 2019  | Wild Ass Sanctuary, Dhrangadhra, Gujarat | 2         | Uric acid crystal deposition in the viscera |

Subsequently, the tissues were analysed for fourteen non-steroidal anti-inflammatory drugs (NSAIDs), namely diclofenac, aceclofenac, ketoprofen, ibuprofen, naproxen, paracetamol, mefenamic acid, meloxicam, nimesulide, piroxicam, tolfenamic acid, indomethacin, flunixin and carprofen. Of all the above-listed NSAIDs, only nimesulide was detected in all the tissues analysed in high concentrations (Table 2). Of all the four birds, the one received from Sanand had higher concentration of nimesulide residues ranged from 792.1 to 1395.4 ng/g. Among the three tissues kidney had higher level of nimesulide (1395.4 ng/g) followed by gut content (1340.7 ng/g) and liver had the lowest. It is to be noted that uric acid crystal deposition in the viscera in all the four vultures during post-mortem indicated visceral gout. Absence of diclofenac residues in all the birds investigated indicate that diclofenac is no longer a major threat in Gujarat. It may not be inappropriate to extrapolate the same to rest of India.

Table 2

Concentration of nimesulide (ng/g, wet weight basis) recorded in the tissues of White-rumped Vultures received dead from Gujarat during 2019.

| Incident / Date  | Vulture Id             | Organ       | Concentration of nimesulide (ng/g, wet weight) | Uric acid crystal / Visceral gout |
|--|------------------------|-------------|--|-----------------------------------|
| Incident 1<br>February 2019<br>Sanand, Gujarat                         | White-rumped Vulture 1 | Liver       | 792.1  | Present                           |
|  |                        | Kidney      | 1395.4   |                                   |
|  |                        | Gut content | 1108.2   |                                   |
|  | White-rumped Vulture 2 | Liver       | 112.5  |                                   |
|  |                        | Kidney      | 234.2  |                                   |
|  |                        | Gut content | 1340.7   |                                   |
| Incident 2<br>October 2019<br>Wild Ass Sanctuary, Dhrangadhra, Gujarat | White-rumped Vulture 1 | Liver       | 731.6  | Present                           |
|  |                        | Kidney      | 61.0   |                                   |
|  |                        | Gut content | 424.8  |                                   |
|  | White-rumped Vulture 2 | Liver       | 17.1   |                                   |
|  |                        | Kidney      | 107.7  |                                   |
|  |                        | Gut content | 195.9  |                                   |

Nimesulide (N-(4-Nitro-2-phenoxyphenyl)methanesulfonamide) is a nonsteroidal anti-inflammatory drug and has analgesic, antipyretic and anti-inflammatory properties. Data from a case study on drug induced liver injury of NSAIDs, namely nimesulide, ketoprofen, ibuprofen, diclofenac and paracetamol in Italy revealed nimesulide to be associated with higher, and ibuprofen and ketoprofen with moderate risk of hepatotoxicity in human (Donati et al. 2016). Due to the death of a six-year-old children because of concomitant hepatic and renal failure due to nimesulide in India (Gupta et al. 2012), controversy exists in the safety of nimesulide to human. Nevertheless it is being widely used in India (banned for use in under 12) and many other developing countries on human beings. However, in many developed countries, such as USA, UK, Canada, Australia, New Zealand and Japan, nimesulide is not in use due to its high risk of liver damage. Also the international society of drug bulletins (ISDB) on a press release dated December 2007 recommended withdrawal of nimesulide worldwide due to its potential to cause serious liver damage in humans (ISDB 2007). However, there is no such guideline on its use in cattle.

While nimesulide is legally approved for veterinary use in India, next to meloxicam, it was the most available NSAID (48%) for sale for veterinary use in eleven states in India between November 2007 and June 2010 (Cuthbert et al. 2011). A recent covert survey revealed that eleven NSAIDs were available for sale in pharmacies for veterinary use between 2012 and 2018 including nimesulide and diclofenac (Galligan et al. 2020). However, residues of nimesulide was recorded in none of the liver tissues of ungulates (n = 1,488) collected across seven states in India including Gujarat during April and December 2006 (Taggart et al. 2009). There is no recent study in India that reported NSAIDs in the carcasses available for wild White-rumped Vultures to feed. But, residues of nimesulide were recorded in the tissues of five White-rumped Vultures which were found dead in the wild in Gujarat after 2008, and also were associated with gout as observed in vultures those died due to diclofenac poisoning (Cuthbert et al. 2016).

Cuthbert et al. (2016) recorded elevated levels of nimesulide in kidney (14–2753 ng/g) and liver (156–573 ng/g) in four White-rumped Vultures collected dead from Gujarat between 2008 and 2011. All the four White-rumped Vultures had visceral gout and did not have diclofenac residues. Hence, it was concluded that nimesulide may have toxic effect like diclofenac and these four White-rumped Vultures might have died due to renal failure caused by nimesulide. Levels of nimesulide recorded in the current investigation in the kidney (61–1395 ng/g) and liver (17–792 ng/g) tissues of White-rumped Vultures received from two incidents were similar to the levels reported by Cuthbert et al. (2016). Moreover, the high levels of nimesulide in gut contents (196–1341 ng/g) of White-rumped Vultures from the both incidents clearly show that all the White-rumped Vultures were exposed to nimesulide through food they consumed before death. It is also to be noted that visceral gout was observed in all the four White-rumped Vultures. The elevated levels of nimesulide in tissues with clear symptoms of gout, confirmed that the vultures died due to nimesulide poisoning.

Reddy et al. (2006) suggested nimesulide is likely to be safe for vultures based on a study on Domestic Fowl *Gallus domesticus*. However, Hassan et al. (2018) reported that three species of birds, namely Japanese quails *Coturnix japonica*, Muscovy ducks *Cairina moschata* and Domestic pigeons *Columba livia* to be not suitable as surrogates for NSAID toxicity test in vultures, and the NSAID toxicity to Gyps Vultures to be peculiar. Since, there are large differences in the toxicity of NSAIDs among different species of birds (Cuthbert et al. 2006), we cannot consider the safety of nimesulide to Domestic fowls to the safety to vultures. Further, due to variation in toxicity of nimesulide to various species of birds, Cuthbert et al. (2006) suggested that safety of nimesulide to other species of birds cannot be considered as safe to vultures too. This is further substantiated by the findings of the present study.

It is now evident that nimesulide has the potential to harm vultures similar to diclofenac. Ban on the use of diclofenac for veterinary use in India as early as 2006, and the reduction in volume of diclofenac formulation for human use to restrict misuse for veterinary purpose in 2015, surely has slowed down the population decline of White-rumped Vulture. But the population is still at low levels (5,729) in India unlike Nepal and Pakistan where the population recovered subsequent to the ban on diclofenac (Chaudhry et al. 2012; Galligan et al. 2019; Prakash et al. 2019). This may be mainly because of illegal use of diclofenac, and use of other toxic NSAIDs such as nimesulide, aceclofenac, ketoprofen, flunixin and carprofen on animals. Although, other than diclofenac, many NSAIDs are toxic or suspected to be toxic to White-rumped Vulture, only nimesulide is reported with clear symptoms of gout in wild dead White-rumped Vultures similar to diclofenac consistently in the recent past (Cuthbert et al. 2016).

Further, aceclofenac administered to cattle also rapidly metabolises into diclofenac and harm White-rumped Vulture (Galligan et al. 2016). Diclofenac accounted for 10–46% of all NSAIDs available in the market for veterinary use as on 2017 (Galligan et al. 2020) and also diclofenac residues were recorded in 68.7% of White-rumped Vultures received dead after the ban (between 2011 and 2014) in India (Nambirajan et al. 2018). It appears at this junction that the ban on diclofenac alone will not help save vulture in the Indian subcontinent. In the light of available facts, in addition to diclofenac, at least nimesulide and aceclofenac should be banned for veterinary use to conserve White-rumped Vulture in Indian subcontinent. The volume of human formulations of these drugs also should be reduced like diclofenac to avoid misuse. As of now, only meloxicam is experimentally proved to be a safe NSAID to vultures (Swan et al. 2006; Swarup et al. 2007). Fortunately, the safe drug, meloxicam is freely available in Indian market for veterinary use (Cuthbert et al. 2011; Galligan et al. 2020). Veterinarians should be encouraged to use meloxicam, the only proven safe alternate NSAID to treat domestic cattle at least though out the distribution ranges of Gyps species.

Between March and April 2020, there were two other incidents of mortality of White-rumped Vulture in Assam and West Bengal. While 13 vultures died after eating carcass of pig in Jalpaiguri district, in West Bengal (Business standard, 13th April 2020, [https://www.business-standard.com/article/printer-friendly-version?article\\_id=120041301162\\_1https://](https://www.business-standard.com/article/printer-friendly-version?article_id=120041301162_1https://)), 19 vultures died in Sibsagar, Assam may be due to poisoning (Hindustan Times, Guwahati, 24th April 2020, <https://www.hindustantimes.com/india-news/19-vultures-die-in-a-day-in-assam-s-sibsagar-poisoning-suspected/story-mEr7EjPrsNjfEgc4hoMUip.html>). Unfortunately, we could not collect tissue samples from both the cases for laboratory investigation. However, the circumstantial evidences show both the cases to be poisoning. An effective system is recommended to be put in place to collect the tissues of dead Vultures for toxicological investigations, and document the reason for the death, for take further action to conserve the endangered species. Further monitoring and toxicity testing of NSAIDs, particularly ketoprofen, carprofen and flunixin on vultures are also suggested to save the Gyps vultures in Indian subcontinent.

## Declarations

**Ethical approval and Consent to participate:** Not applicable.

**Consent for publication:** Not applicable.

**Availability of data and materials:** The data generated during the current study are available from the corresponding author on request.

**Conflict of interest:** The authors declare that they have no conflict of interest.

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**Authors' contributions:** **Kanthan Nambirajan:** Conceptualization, Investigation, Methodology, Chemical analyses, Data curation, Writing - original draft.

**Subramanian Muralidharan:** Funding acquisition, Conceptualization, Resources, Supervision, Writing – review & editing. **Aditya Roy Ashimkumar:**

Conceptualization, sample collection, Writing – review & editing. **Shashikant Jadhav:** Sample collection and post-mortem.

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