Detecting diclofenac in livestock carcasses in India with an ELISA: A tool to prevent widespread vulture poisoning

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ABSTRACT

Diclofenac, a non-steroidal anti-inflammatory drug (NSAID), has caused catastrophic vulture declines across the Indian sub-continent. Here, an indirect ELISA is used to detect and quantify diclofenac in 1251 liver samples from livestock carcasses collected across India between August 2007 and June 2008, one to two years after a ban on diclofenac manufacture and distribution for veterinary use was implemented. The ELISAs applicability was authenticated with independent data obtained using LC–ESI/MS. Of 1251 samples, 1150 (91.9%) were negative for diclofenac using both methods, and 60 (4.8%) were positive at 10–4348 and 10–4441 µg kg^{-1} when analysed by ELISA and LC–ESI/MS, respectively. The residue level relationship in the 60 positive samples was highly significant ($p < 0.001$, $r^2 = 0.644$). Data suggest that this immunological assay could be used not only for cost effective sample screening, but also for residue level semi-quantification.

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1. Introduction

Vultures are at the apex of the terrestrial food web, and like other avian species are considered to be sentinel biomonitors of prevailing environmental conditions and quality (Sekercioglu et al., 2004). Recently, three species of *Gyps* vulture endemic to South Asia (*Gyps bengalensis*, *G. indicus* and *G. tenuirostris*) have undergone catastrophic declines across the Indian sub-continent, and are now listed as Critically Endangered (Prakash et al., 2007; IUCN, 2010). These declines have been clearly linked with the extensive use of the non-steroidal anti-inflammatory drug (NSAID) diclofenac (Oaks et al., 2004; Shultz et al., 2004; Cuthbert et al., 2009). Widely used (until recent restrictions were put in place) to treat ailing livestock across the Indian sub-continent, residues of this drug are commonly present in the carcasses of domesticated ungulates (buffalo, cow, goat, camel, horse) that provide a principal food source for these scavengers (Taggart et al., 2007, 2009). Diclofenac

is now known to be extremely toxic towards all *Gyps* species tested so far (Oaks et al., 2004; Swan et al., 2006a; Naidoo et al., 2009; Das et al., 2011), causing fatal extensive visceral gout in exposed individuals. Another NSAID, ketoprofen, is now also known to be toxic to vultures, causing the same toxicological symptoms as diclofenac (Naidoo et al., 2010a, 2010b).

In 2006, following extensive safety testing on meloxicam, another NSAID now known to be "vulture safe" (Swan et al., 2006b; Swarup et al., 2007), an India wide ban on the manufacture and distribution of veterinary diclofenac was imposed by the Drug Controller General in India (Kumar, 2006). Similar restrictions were also put in place in the same year in Pakistan and Nepal. In India, regulations were tightened further in 2008, and it is now an imprisonable offence to sell diclofenac for veterinary use or to use it on livestock (Singh, 2008). In order to assess how prevalent diclofenac residues are in vulture food sources, and to monitor the eventual affect these usage restrictions may have within India, two surveys have been published which have documented diclofenac levels and prevalence in livestock carcasses across the region (Taggart et al., 2007, 2009). Liquid chromatography – electrospray ionisation mass spectrometry (LC–ESI/MS) analytical methods

have been applied to samples taken during these surveys (undertaken in 2004–2005 and 2006) which in combination determined diclofenac concentrations and prevalence in >3000 individual ungulate carcass liver extracts. These surveys revealed that the prevalence of detectable diclofenac (>10 µg kg^{-1}) in these vulture food sources was between 10 and 11% during both periods (Taggart et al., 2007, 2009). Detailed modeling using this and other existing data (Green et al., 2004, 2006, 2007), has further demonstrated that only 0.13–0.76% of carcasses need contain a lethal dose of diclofenac in order to cause the extreme rates of vulture decline in India that have been recorded over the last one to two decades. The most common *Gyps* vulture in the region (*G. bengalensis*), for example, is now thought to have declined by >99.9% since 1992 (Prakash et al., 2007), having previously been considered to be probably the most abundant large raptor in the world (Houston, 1985).

Whilst the LC–ESI/MS methodology used to undertake diclofenac monitoring across India thus far is precise, accurate and sensitive (Taggart et al., 2007, 2009), it is also expensive, time consuming, and of limited availability across India, Pakistan and Nepal. As an alternative analytical option, an enzyme-linked immuno-sorbant assay (or ELISA) was recently proposed and pre-validated for such an application (Knopp et al., 2007). ELISAs are powerful immunological techniques that have been shown to have potential in monitoring drug residues such as antibiotics, in a range of matrices. ELISAs have been used (for example) to monitor amoxicillin in pigeon serum (Yeh et al., 2008), ciprofloxacin in meat destined for human consumption (Duan and Yuan, 2001), furazolidone in swine liver and muscle tissue (Chang et al., 2008), and marbofloxacin in beef and pork (Sheng et al., 2009). Such assays are often very sensitive, compound specific, reasonably easy to use, and are designed such that a large number of samples can be screened in a relatively short time period (using 96 well plate formats). A diclofenac specific ELISA originally designed for use in water/waste water applications (Deng et al., 2003), has (in trials) been shown to have potential as an assay which could be applied to diclofenac monitoring in tissue samples in India (Knopp et al., 2007), however, extensive validation has not so far been undertaken. The aim of the present study was to investigate whether such an ELISA could be reliably used as a screening tool for large scale diclofenac monitoring in the field. Thus, we present diclofenac residue data produced for 1251 extracts of liver tissue. Samples were collected during monitoring surveys which took place across India between August 2007 and June 2008. The results are compared to independently collated data generated using an existing validated LC–ESI/MS methodology. These data give an important first indication as to the effectiveness of restrictions on veterinary diclofenac use that are now meant to be in place across India.

2. Methods

2.1. Field sampling of livers from domesticated livestock carcasses

Samples of liver tissue from 1251 individual carcasses were collected over a ten-month period between the 18th of August 2007 and the 20th of June 2008 (15–25 months after the 2006 veterinary diclofenac ban was announced, and 12–22 months after it was theoretically implemented (Kumar, 2006)) using procedures described previously (Senacha et al., 2008). The majority of samples were taken from carcasses of cow (*Bos indicus*, *Bos taurus* and hybrids; $n = 752$) and buffalo (*Bubalus bubalis*; $n = 437$). The remainder were from sheep (*Ovis aris*; $n = 30$), goat (*Capra hircus*; $n = 27$), horse (*Equus caballus*; $n = 2$) and camel (*Camelus dromedarius*; $n = 3$); $n = 62$ in total.

Samples were frozen upon collection using a portable field freezer (Engel, Japan), and then transported whilst frozen to laboratory based freezers (-20 °C) where they were stored until extraction. Samples were collected from carcass dumps in six Indian states, i.e., in Rajasthan ($n = 303$), Gujarat ($n = 159$), Maharashtra ($n = 262$), Andhra Pradesh ($n = 143$), Madhya Pradesh ($n = 257$) and Uttar Pradesh ($n = 127$). Fig. 1 shows a map of the approximate locations of the sample sites used across India.
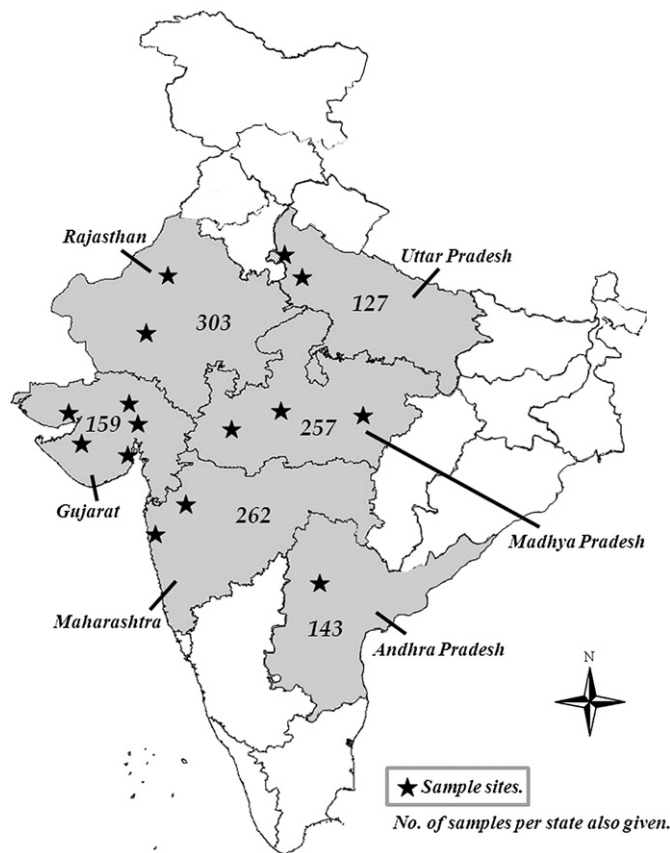

Fig. 1. Map showing the states sampled within India, the number of samples taken per state, and the approximate sample site locations. Liver samples were taken from dead livestock arriving at carcass dumps at these locations between the 18th of August 2007 and the 20th of June 2008.

2.2. Diclofenac extraction

A sub-sample from each liver sample was removed using a stainless steel scalpel, and weighed to an accuracy of ±0.0001 g, to a recorded wet weight between 0.45 and 0.55 g. Each liver tissue sample was weighed into a new glass test tube, and homogenized with 2 ml of HPLC grade acetonitrile using an Ultra Turrax IKA T8 homogenizer. The homogenate was then centrifuged at 1000g for 10 min, and the supernatant filtered through a 0.45 µm nylon disposable syringe filter unit. Filtrates were passed directly into 2 ml glass LC–MS vials, and stored as two aliquots (one for ELISA, one for LC–ESI/MS) at -20 °C until analysis. Each aliquot was analysed independently for the presence of diclofenac using an indirect competitive ELISA at the Indian Veterinary Research Institute (IVRI) in India and by LC–ESI/MS at the Instituto de Investigación en Recursos Cinegéticos (IREC) in Spain.

2.3. Analysis by ELISA

Prior to ELISA analysis, extracts were thawed, shaken, and then stored overnight at 4 °C. Extracts were then screened for the presence of diclofenac using 1:50 extract dilutions, and then (depending on the concentration of the drug detected at screening), quantified using 1:50, 1:100 and 1:200 dilutions in water (HPLC grade). For calibration curve construction on each ELISA plate, a stock solution containing 50 mg l^{-1} diclofenac (2-[(2,6-dichlorophenyl)amino] benzeneacetic acid sodium salt; Sigma Aldrich, D6899) was first prepared in 1:1 water:acetonitrile. Working calibration standards ranging from 0.01 to 10 µg l^{-1} were then prepared in an acetonitrile:water ratio which reflected the sample extract dilution being used on any particular plate (i.e., 1:50, 1:100 or 1:200).

Coating antigen synthesis, the production of the polyclonal serum against diclofenac, and the development of the indirect competitive ELISA used here has been described in detail previously (Deng et al., 2003; Knopp et al., 2007). In brief, lyophilized powder containing diclofenac–thyroglobulin or diclofenac–ovalbumin hapten conjugate was prepared using the mixed anhydride method, and was reconstituted in 0.05 M sodium carbonate buffer (pH 9.6) and then used as the plate coating antigen. Diclofenac–bovine serum albumin conjugate was used as an