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Pathophysiology of diclofenac-associated hyperuricaemia in *Gyps* spp. vultures

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By

Bono Nethathe

Department of Paraclinical Sciences
Section of Pharmacology and Toxicology
Faculty of Veterinary Science
University of Pretoria.

Supervisor: Prof. Vinny Naidoo

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DECLARATION

I declare that this thesis, which I hereby submit for the degree Philosophiae Doctor at the University of Pretoria, is my own work except where the inputs of others are acknowledged. This thesis has not been submitted to another university for consideration.

I, Bono Nethathe, declare the above statement to be correct

_____ B Nethathe

_____ Prof. V. Naidoo

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DEDICATION

To my late maternal grandparents (Mr Gangashe Muvhi phillimon and Mrs Gangashe Munzhedzi Jane) may their soul continue to rest in internal peace and my smile keepers Maswime Vholuga Okhwethaho and Maswime Madammbini.

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LIST OF ABBREVIATIONS

A	adenine
ABC	ATP binding cassette
ATP	Adenosine triphosphate
AWB	African White-backed
BBC	British Broadcasting Corporation
BLAST	Basic Local Alignment Search Tool
bp	basepairs
C	cytosine
cq	Quantification cycle
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
cGMP	cyclic guanosine monophosphate
CH3CN	Acetonitrile
COX	Cyclo-oxygenase
DNA	deoxyribonucleic acid
G	guanine
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFR	Glomerular filtation rate
H	hour
HPLC	High performance liquid chromatography
IHC	Immunohistochemistry
IUCN	International Union of the Conservation of Nature
LD	Lethal dose
Ln	Natural logarithmic
LOQ	Limit of quatification
mRNA	Messenger Ribonucleic Acid
MRPs	Multiple resistance proteins
MW	Molecular weight
NBD	Non-binding domain
NCBI	National centre of biotechnology information
NGS	Next generation sequencing
NH4CL	Ammonium chloride
NRF	National research foundation
NSAIDs	Non-steroidal anti-inflammatory drug
OATs	Organic anion transporters
OWBV	Oriential White backed vulture
PAH	para-Amino-hippuric acid
PCR	Polymerase chain reaction
R	Rand
RNA	Ribonucleic acid
RT-Qpcr	Quantitative reverse transcription polymerase chain reaction
SETA	Sector Education and Training Authority
SLC	Solute carriers
T	thymine
TMD	Transmembrane domain
TMH	Transmembrane helice
TrSSP	The Transporter Substrate Specificity Prediction Server
UPBRC	University of Pretoria Biomedical Research Centre

URAT	Uric acid transporter
USA	United State of America
α- kg	α-ketoglutarate

THESIS SUMMARY

Candicate: Nethathe Bono

Supervisor: Prof Naidoo Vinny

Department: Paraclinical Sciences

PhD title: **Pathophysiology of diclofenac associated hyperuricemia in *Gyps* spp. vultures**

Abstract

Diclofenac toxicity in old world vultures is well described in the literature. As part of the toxicity of the drug, dead birds were generally found in the environment with signs of severe renal damage and gout. In birds that were tested before death, signs of hyperuricaemia and hyperkalaemia were also present. Although the clinical picture is very clear, the sequence of hyperuricaemia is not yet established with one possibility being drug induced renal damage leading to hyperuriceamia and the second being secondary kidney damage resulting from hyperuricaemic cellular damage. For this study we evaluated this sequence by assessing the effect of diclofenac on uric acid transporters in the chicken, a validated model for diclofenac toxicity. We speculated that diclofenac, a known uricosuric drug in people, inhibits the avian organic anionic transporters (OATs) with subsequent increase in plasma uric acid, precipitation and kidney damage.

As a first step, the impact of diclofenac was evaluated in healthy chicken as it was not justifiable to kill vultures through diclofenac administration. For this two-phase study heathy chickens were treated intravenous with para-amino hippuric acid (PAH) and iohexol (IOH) in combination in phase 1 or this same combination with diclofenac (10 mg/kg) in phase 2. In both phases blood and faeces were sequentially collected. In phase 1, birds showed no signs of ill health, moreover PAH, IOH and uric acid clearance was rapid. In phase 2, two chickens eventually died with hyperuricaemia being present as soon as 8 hours after exposure. Necropsy showed classic signs of renal damage and hyperuricaemia. Pharmacokinetic analysis revealed a rapid half-life of elimination of less than 2 hours indicating that toxicity was due to irreversible inhibition of a physiological process. In phase 2 all the birds had decrease uric acid, PAH and IOH clearance. While tubular excretory rates of PAH were reduced in all birds in phase 2, they were 98% reduced in the two birds that died. Based on the global change in clearance parameters, it is concluded that diclofenac alters both renal perfusion (IOH measures

glomerular filtration) and renal plasma flow. However death results from tubular secretion being reduced to negligible functionality for a prolonged period. With birds being highly reliant on tubular secretion of uric acid, we conclude that diclofenac hyperuricaemia is as a result of OAT inhibition and that cell death results secondarily from uric acid precipitation as described in gouty chicken.

In the final conclusive study, we used next generation sequencing of the transcriptome of the renal tissue from one African white backed vulture (AWB), to establish if these tissues expressed OATs and multidrug resistance protein (MRP) transporters. Both these channels are known to be involved with uric acid transport either basolaterially or apically respectively. The Trinity assembled transcriptome, was used to create a local blast database from which predicted sequences of OAT1 and 2 and MRP2 and 4 from the Golden Eagle were evaluated for similarity. The golden eagle was selected as it was the closest related species phylogenetically that also had a complete genome sequence published. OAT3 was not included in this study as no avian sequence was available on the NCBI database. From the search, all four channels were identified in the AWB vulture kidney with high similarity to the golden eagle. Sanger sequencing confirmed the presence of the OAT 1, 2 and MRP2, 4 related mRNA. The predictive amino acid sequence and predictive protein channel (Swiss-Prot) was also used to provide some evidence that the proteins in question shared the required characteristic and function of OAT and MRP channels. After in silico analysis revealed the similarity of only AWB OAT2 gene with other species i.e chicken, expression study was carried out. It revealed that chicken OAT2 gene was expressed more than the vulture, this maybe the reason to vulture sensitivity to diclofenac. With the genes showing the presence of the requisite uric acid transport proteins in the kidney, the distribution of the OAT channels in the vulture was confirmed by immunohistochemistry (IHC) using mouse polyclonal OAT3 antibodies as sequence analysis showed high similarity between vulture OAT1 and mouse OAT3. As expected the IHC showed the presence of OAT1 with good distribution along the renal tubules.

CHARPTER ONE

1. Introduction.

1.1. Vulture decline.

The Indian subcontinent experienced a catastrophe of *Gyps* vulture deaths in the early 1990s with three *Gyps* species (*Gyps indicus*, long-billed vulture, *Gyps Bengalensis*, Oriental white-backed and *Gyps tenuirostris*, slender-billed vulture) reaching numbers nearing extinction (Green et al., 2004; Prakash et al., 2003 and Schultz et al., 2004) . Over the period these species have been estimated to have declined by more than 97% (Green et al., 2004; Prakash et al., 2003 and Schultz et al., 2004), which prompted the International Union for Conservation of Nature (IUCN) to list the above mentioned vulture as critically endangered species (IUCN, 2006). While the initial cause of the vulture declines was unknown, research undertaken by Oaks et al. (2004), who linked the massive die-off of vultures to the presence of diclofenac residues in the meat they fed upon (Oaks et al., 2004). Most importantly the vultures were completely inadvertently exposed to the drug that happened to be in their food source as a residue, purely to the commonality of the drug being in use to treat cattle in the area (Oaks et al., 2004).

With diclofenac being identified as the cause of deaths, efforts were put into place to establish if other similar drugs, grouped as the non-steroidal anti-inflammatory drugs (NSAIDs), could also be toxic (Jackson and Morrow, 2001). In subsequent toxicity testing, ketoprofen and carprofen have since also been shown to be toxic, however meloxicam was shown to be safe (Cuthbert et al., 2006; Naidoo et al., 2008). This raised questions on the potential mechanism of toxicity which thus remains unknown. With all evaluations showing signs of severe visceral gout on post-mortem examinations, either in the wild and control experimental conditions (Oaks et al., 2004; Meteyer et al., 2005). Guided by the necropsy result, we believed that the mechanism of diclofenac toxicity may be at the level of the kidneys or its supportive circulatory system, as the kidneys are extremely important in clearing uric acid from the body. More specifically, this transport is under very finely control and reliant on apical and basolateral uric acid transport proteins on the cells of the proximal convoluted tubules.

1.2. Hypothesis

1. Diclofenac toxicity in renal proximal tubules of chicken kidney results from inhibition of uric acid Transport channels.
2. Diclofenac does not change blood flow to the chicken kidney.

1.3. Objectives

I. To establish functional mechanism of diclofenac toxicity using the chicken kidney as the model system.
II. To characterize the OATs and MRPs uric acid transporters in AWB vulture kidney.
III. To establish phylogeny of OATs and MRPs genes of AWB vulture with other avian species.

1.4. Thesis overview

Chapter 1: The purpose of this chapter is to provides the rational and objectives for this study.
Chapter 2: The aim of this chapter is to provide a detailed literature review of vulture, consequences of vulture declines, international efforts to protect vultures, current status of the vulture population, diclofenac and its mechanism of action , the solute carrier family, organic anion transporters and proposed mechanism of diclofenac`s toxicity in vultures.
Chapter 3: This chapter focused on establishing the mechanism of diclofenac toxicity in vulture using chicken as surrogate.
Chapter 4: The focus of this chapter was on determination of OAT1, OAT2 and OAT3 genes in AWB vulture and chicken expressed in the kidneys using molecular biology techniques and immunohistochemistry furthermore determine phylogeny of OATs in latter species with other avian OAT genes.
Chapter 5: The chapter focused on determining if MRPs channels are expressed in AWB vulture and explore phylogenetic relationship between AWB and other avian MRP genes.
Chapter 6: The last chapter provides a general discussion, conclusion and recommendations.

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CHARPTER TWO

2. Literature review.

2.1. Overview of vultures

Vultures are a convergently evolved scavenging group of birds (Wink, 1995), which are further broadly divided into two groups, the accipitridae (old world vultures) and the cathartidae (new world vultures) (Wink, 1995; Rodrigues, 2007; National Zoo Washington-USA, 2008; Valentini, 2011) (Table 2-1, Figure 2-1). Vultures are generally characterized by their large size, big featherless head, curved beaks and are known to be among the highest-flying birds (Wink, 1995) reaching heights of 10,000 feet (±3,000 m) above sea level (Rodrigues, 2007). They are different from other birds in that they predominantly eat carrion while only resorting to predation in times of extreme food shortage (Kumar et al., 2003). A major difference between the old and new world vultures is they are not considered to be closely related genetically even though they may look similar physically, they are placed in different orders which are Falconiformes and Ciconiiformes respectively (new world encyclopedia contributors, 2008).

Table 2-1: Some typical differences between old and new world vultures

Characteristics	Old world vulture	New world vulture
Family	Accipitridae	Cathartidae
Location	Europe, Africa and Asia	North and South America
Species within family	Fifteen species reported	Seven species reported
Ability to find carcasses	They find carcasses exclusively by sight.	They have a good sense of smell and can smell deceased animals from high altitudes up to a mile away.
General description	Their pervious nostrils are dived by septum	Their pervious nostrils are not divided hence you can see through the beak

Examples	Oriental white backed vulture (*Gyps Bengalensis*), slender-billed vulture (*Gyps* Tenuirostris) and Long- billed vulture (*Gyps* Indicus)	Californian condor, (*Gymnogyps californianus*) turkey vulture (*Cathartes aura*) and Andean Condors (*Vultur gryphus*)

(Picture A from Valentini (2011), picture number IBC985226 and B from National zoo washington-USA-8a.jpg (2008).
Figure 2-1: Examples of the two major families of vulture, A) Old world vulture (*Gyps Africanus*) and B) new world vulture (*Sarcoramphus papa*)

2.2. General endangered status of vultures

In the past 2 decades the world witnessed catastrophic declines of three *Gyps* species in South Asia and possibly other birds scavenging on the carcasses of livestock treated with diclofenac (Green et al., 2004; Prakash et al., 2012; Galligan et al., 2014, Sharma et al., 2014 and Paudel et al., 2016). A number of reasons are behind the global vulture decline, and include human persecution, injuries, muti trade, poisonings and loss of habitat (Houston, 1996; Gangoso et al., 2009).

2.2.1. Accidental poisonings

Accidental poisonings happen when scavengers get inadvertently exposed to poisons that are present within the food that they feed upon. This exposure can happen from intentional placement of poisons out in the environment as happens when people deliberately place out

poisons for carnivore control, and due to its commonality in practice features as the most common cause of vulture poisoning. The said practice is common in Europe and Africa (Bridgeford, 2001, 2002; Hernandez and Margalida 2008, 2009), and is used against predators to protect the livestock. In Africa, poisoning is used only to venge livestock killings, while in Europe it is viewed as the first choice to prevent carnivores from attacking livestock (Fajardo et al., 2012).

The second type of poisoning occurs when a toxin is more insidious and in most cases is unknown to be a toxin, until birds are found dead. This is best illustrated by the rapid and severe population decline in Asian *Gyps* vultures that were reported in the 1990's. The said poisoning was eventually linked to their exposure to the nonsteroidal anti-inflammatory drug diclofenac which was present as a veterinary residue in the livestock meat they fed upon. The said poisoning at as little as 0.8 mg/kg was sufficient to be the single greatest cause of vulture mortality thus far recorded (Oaks et al., 2004). Another case of insidious poisoning was reported in the Arizona condor (*Gymnogyps californianus*) population, which was devastated following the ingestion of lead based-bullets in hunter-killed carcasses with the result that the species nearly became extinct (Wiemeyer et al., 1988; Parish et al., 2007).

2.2.2. Malicious Poisonings and persecution

Deliberate persecution of vultures has also been practiced for centuries. Vultures have been victimized as a result of ignorance, superstition and retaliation (Boshoff et al., 1980). Among these beliefs vulture have been blamed for the spread of disease, being responsible for blowfly plagues, fouling the drinking water of livestock and representing evil spirits, all due to their close relationship to carion (Campbell, 2009). Furthermore the two primary types of persecution are shooting and intentional poisoning. Shooting vultures in the United States, Europe and North Africa have previously been reported as sports (Miller et al., 1965; Mingozzi and Estève, 1997; Mundy, 2000; Hunt et al., 2009) while intentional poisoning has been documented in cases such as retaliation for the alleged livestock killings; disguising the location of poacher activities and obtaining vulture body parts for muti (traditional medicine) in West, East and South Africa (For more on traditional medicine, see below) (Bridgeford, 2001; Verdoorn et al., 2004; Parish, 2009).

2.2.3. Traditional Medicines (Muti Trade)

Vulture body parts also feature in the practice of traditional medicine, commonly known as African witchcraft and forms a component of African traditional medicinal systems. Poachers and Sangomas (muti-men) place much value on certain parts of vulture especially adult vultures with the adults being more valuable than juveniles (Figure 2-2), with some sangomas willing to pay about R4 000 for an adult vulture. There is a belief that smoking, drinking, eating or smearing the brain of a vulture on one's body will produce powers of clairvoyance. The eyes are also meant to be a gateway to the future for clairvoyants. The foot and a beak of vulture are believed to claim good luck in gambling while covering oneself with vulture fat should help to boost one's courage (Sodeinde and Soewu, 1999; Nikolaus, 2001; McKean, 2004 and Mander et al., 2007). In South Africa, it has been reported that the use of vultures in the muti trade is more focused on sports betting or for playing the national lottery (Taylor, 2010).

Picture obtained from https://citizen.co.za/news/south-africa/1025942/game-slaughtered-for-muti/

Figure 2-2: Sangoma trading vulture in Johannesburg, South Africa.

2.2.4. Power line injuries, wind farms and drownings

Collision with power lines, electrocution and drowning has also been identified as a cause of high vulture mortalities (van Rooyen, 2000; Janss, 2000). While wind farms are commonly lauded for being a source of green energy production, they are fast becoming a major threat to vulture survival with current studies indicating that the vulture have suffered the highest level

of mortality from collision with wind turbine blades (Barrios and Rodriguez, 2004). These threats are also anticipated to rise in the coming years due to a rapid inrease in green technology development and electricity infrastructure worldwide (De Lucas et al., 2008).

Power line injuries still feature as a high cause of mortality in South Africa for Cape vulture population (*Gyps coprotheres*). It was estimated that the former population declined by 10% between 1994 and 1999 (Barnes 2000), and the species continued to decline by 60-70% in Eastern South Africa in 1992-2007 (McKean and Botha, 2007). The vultures are vulnerable to injuries, which commonly leads to mortality, since they tend to collide with overhead wires when trying to use power-line pylons as roosting sites in areas with few tree (www. ewt.org.za). It has also been suggested that due to their large sizes and area needed for their take off, they find it difficult to avoid flying into powerlines when the said lines are near a carcass the bird has fed upon. Lastly a fairly unique cause of death has been reported in South Africa where vultures have been found to drown in artificial reservoirs due to the high vertical reservoir walls (Anderson et al., 1999). This posed such a problem, that special mitigation measures such as net coverings on these dams had to be installed to minimise vulture deaths.

2.2.5. Air traffic

Large population of vulture became a hazard to air traffic in the 1980 and 90s in India. High impact of vulture to the plane mostly ends up in mortality due to broken neck and other injuries the vulture sustains. Due to their large bodies, this can lead to major damage to the windshields and engines of planes landing and taking off at airports, making flight in the area very unsafe. This is best highlighted with the declines in vulture numbers on the Asian subcontinent actually making flights in these areas safer and initially sparked conspiracy theories that the airline industry was somehow intentionally poisoning vultures (Satheesan and Satheesan, 2000). The damage bird strike was evident most recently with a British Airway plane that was surprisingly attacked by vultures in the air and completely damaging the aircraft and requiring much effort from the pilots to land the plane (Figure 2-3) (Pragativadi news service,2017).

Picture obtained from http://pragativadi.com/pic-strange-birds-attacks-british-airways-caused-serious-damages/

Figure 2-3: Vultures attacking British Airways in an incident that happened in China.

2.2.6. Loss of habitat and food shortage

Literature indicated that loss of habitat and food shortage has also played a significant role in vulture declines. This impact is mostly observed in West Africa where human population has grown very rapidly (Thiollay, 2006, 2007). Food shortage due to changes in livestock husbandry and overhunting also play a role in vulture decline in the area (Thiollay, 2007). A similar situation has been reported in Europe, where improved sanitary legislation to limit the spread of bovine spongiform encephalopathy placed a restriction on carcasses being placed out for vulture feeding. As such, vultures were unintentionally being starved (Don´azar et al., 2009). A similar situation has been occurring in South Africa over the last 30 years, with the result that supplementary feeding sites had to be established to protect the larger colonies (www.ewt.org.za).

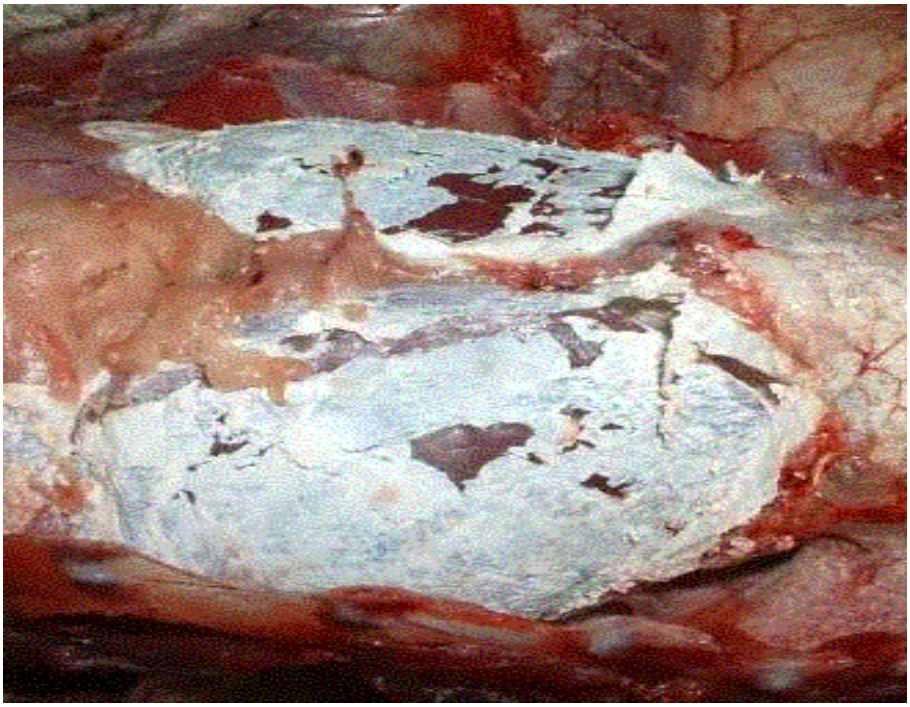
2.3. Diclofenac and vultures

2.3.1. Unprecedented deaths of vultures

Despite the above mentioned threats to vulture survival, nothing mentioned thus far rivals the devastation experienced by three *Gyps* species, the Oriental White-backed Vulture (*Gyps bengalensis*), Long- billed Vulture (*Gyps indicus*) and Slender-billed Vulture (*Gyps tenuirostris*) across the Indian subcontinent (Prakash, 1999 and Green et al., 2004). Initially

the population declined by 95%, within a 10-year period from the mid-1990s, with the first recorded incidence being in the Keoladeo National Park India, (Prakash, 1999). Since then continuing research and surveys have confirmed a summative decline above 97% for these three species in India, Pakistan and Nepal (Gilbert et al., 2004, Prakash et al., 2003; Shultz et al., 2004 and Cuthbert et al., 2006), with the result that the International Union for Conservation of Nature (IUCN) has classified these three vulture species as critically endangered (IUCN, 2006).

Despite vulture deaths being widely reported, more perplexing were the unusual clinical signs evident in the birds before death. Prakash (1999) reported birds dropping from roosts, perches, in branches or the nests, with afflicted vultures being sick for a long period that could extend to 32 days. Other signs reported were drowsiness with a limp and a dangling neck and reproductive failures resulting in the failure to produce eggs or for eggs to hatch (Prakash, 1999). The end results were always mortality with no reported cases of recovery. On necropsy, the only signs evident were the presence of urates (whitish crystals) on the kidney, heart, liver and spleen (Risebrough, 2000; 2004). Histopathology showed striking kidney lesions and urinary tubules had degenerative changes in the form of deep eosinophilic epithelial cells, many with absent nuclei in early stages of necrosis. Urate tophi presence in the glomeruli evident as radiating eosinophilic masses and mononuclear cell infiltration that is also found around the glomeruli particularly lymphocytes and monocytes. Urates (whitish deposits) were also present on the abdominal visceral organs including the heart, liver, kidney and spleen (Figure 2-4) (Risebrough, 2000; 2004 and Oaks et al., 2004).



(Picture from the following site: http://www.vulturerescue.org/index_files/Page712.htm)

Figure 2-4: Uric acid precipitates (white pasty material) covering the abdominal visceral.

It was only in 2004, that Oaks and his team finally related the mortalities in the Oriental White Backed Vultures (OWBV) to diclofenac residues in the carrion which the birds fed upon. Oak`s research team was able to demonstrate a link between histopathological lesions and visceral gout observed in twenty three (23) vultures that died in the field with trace presence of diclofenac concentrations within the same birds. This was eventually confirmed under controlled conditions in a study in which meat harvested buffalo or goats, treated by an intramuscularly dose of veterinary diclofenac (a non-steroidal anti-inflammatory drug, NSAID), were fed to ten OWBV's. All ten of these vultures died from renal failure with diclofenac residue being detectable in their kidneys. It was also established that the exposure to a rather small dose for ungulates of 0.8mg/kg could induce a lethal toxicity in vulture (Oaks et al., 2004, Green et al., 2004; Shultz et al., 2004).

2.3.2. Method of exposure

To understand the reason for the exposure of diclofenac to vulture, one needs to have a better understanding of the value of cattle across the Asian subcontinent. In India the predominant religion is Hinduism. This religion regards the cow as a holy animal for a number of reasons with the most important being the mother of life because it provide life sustaining milk which is mostly valued for its religious properties because it can be turned into ghee. In Hinduism,

Ghee symbolises purity and it's a common feature in all their religious ceremonies (Harris, 1978, Naidoo et al., 2009). Through this positive human-animal interaction, cattle are more important to the Hindu household than their status as meat producers as seen in the western world. As a result, every effort is made to care for the animals, especially in times of illness, until recovery or they succumb to the illness usually in the field (the latter in vast numbers due to the large population size) where there were left to decompose naturally. Through time, the decline of vulture populations soared as the cattle carcasses were an easily available food source (Harris et al., 1966). With modern veterinary practices advancing in India, as with other countries in the world, sick animals were increasingly being treated for pain with the anti-inflammatory drugs. Unfortunately one of the drugs chosen, from a cost basis, was diclofenac. Due to the principle of animals being treated until recovery or death, the unintended consequence was that cattle which failed to recover from treatment, often died with abundant amounts of diclofenac in their bodies (Simoons et al., 1979).

A different scenario was present in Pakistan, where cattle are kept for meat production according to Prophet Muhammad's teachings (Simmons et al., 1979; Kocturk, 2002). In accordance with one of his teachings, it is prohibited to slaughter sick animals for human consumption. As in India, every effort was made to treat sick animals with diclofenac also with the result that a large number of animals often succumbed while still on diclofenac. With vultures being opportunistic carrion feeders, this resulted in diclofenac ending up in their food chain (Figure 2-5) (Simmons et al., 1979; Baert and De Backer, 2002). Statistical modelling has since indicated that only less than 1% of the total carcasses in the environment needed to contain diclofenac during vulture consumption to cause the massive die off witnessed in South Asia (Green et al., 2004, Cuthbert et al., 2011). This as a result of the carcasses having sufficiently high concentrations of diclofenac (0.005 to 1.0 mg/kg) to be deadly in a vulture that consumes a single large meal (Green et al., 2004). Food intake of an avarage vulture of 5kg can be up to 1kg of meat in one feeding session.

Picture from www.bbc.co.uk/nature

Figure 2-5: Vultures are vulnerable to toxic substances due to their opportunistic diets

2.4. Consequences of vulture declines

The decrease in the vulture population had a major impact in India and the ecosystem. Due to their high population numbers in India, vultures could strip a carcass to the bone in under 30 minutes, making them a highly effective and cheap disposal system. With their population numbers going down, this has led to an increase in the number of carcasses that are unconsumed (Markandya et al., 2006). The knock-on effects of these are below:

- **Increase in dog and rat's population**

  In localized regions such as India where vultures are functionally extinct, there is an increase in the abundance of opportunistic species such as rats (*Rattus rattus*) and feral dogs at carcasses (Pain et al., 2003; Prakash et al., 2003). Feral dogs were also known to compete with vultures for carcasses prior to the population collapse. Rats and feral dogs are well-known disease carriers and their increase at carcasses increases rates of infectious disease transmission to people. The vulture declines have promoted an increase of diseases such as bubonic plague and rabies, for which rats and dogs are the primary reservoirs respectively. Livestock and wildlife are also at increased danger from diseases caused by rats and dogs such as *Leptospira*s pp. bacteria, canine distemper virus and canine parvovirus (Pain et al., 2003).

- **Carcasses rotting where they died**

  Vulture declines have limited the ability of these birds to dispose carcasses, resulting in an increase in the number of carcasses that have not been fed upon. Due to this, carcasses end up decomposing in the environment producing unpleasant smell in garbage dumps of the villages (Markandya et al., 2006). Moreover these dumping site attract pests which are transmitters of human diseases i.e. vultures through their rapid feeding activities prevented the accumulation of decomposing carcasses therefore moderating contact between mammalian scavengers with carcasses (Markandya et al., 2006).

- **Increase in incidence of anthrax**

  For a long period it has been generally accepted that vultures have a role in decreasing the total burden of spores in the environment as a result of the birds low stomach pH efficiently breaking down *Bacillus Anthrax* spores (Houston and Cooper, 1975). In contrast to vulture other predators are linked to spreading *Bacillus Anthrax* spores (Mudur, 2001), either from mechanically spreading of spores from contamination on feathers, hair and legs or from faecal excretion of undigested spores (Houston and Cooper, 1975). While the link is tenuous, since the vulture population decline, India has seen an increase in the number of Anthrax cases.

- **Loss of income**

  Rural areas in India are highly populated with poverty levels being very high. One of the jobs that were available to the poorest of the poor, was the collection of bones for processing as fertilizer after carcasses has been cleaned by the vultures (Simoons et al., 1979 and Markandya et al., 2006). The decline in the vulture population has made it difficult for these people to collect bones which has led to loss of wages.

- **Impact on the regional economy**

  Looking at it at an economist point of view human health costs have increased due to vulture declines with estimates of about $1.5 billion annually due to subsequent increase to rabies following the vulture declines in India (Markandya et al., 2008). In Nepal, the importance of vulture conservation in the economy was estimated at $6.9 million.

Moreover, surveys with people in important bird areas showed that they were deemed considerably useful to humans (Baral et al., 2007).

- **Parsi religion**

  Vultures hold an important social significance in South Asia. The Parsi community in India believed in the preservation of pure element such as fire, water, air, and earth. Due to this belief they built "Towers of Silence" on low mountains or hilltops inorder to lay their dead in the open to be disposed of by scavengers, mainly vultures (Markandya *et al*., 2008; Subramanian, 2008). This ancient custom is also called sky burial is also practiced by Tibetan Buddhists. For the last decade the practice has come to an end due to vulture declines. Until present day the Parsi community has unsuccessfully turned to solar reflectors in hope to concentrate solar radiation on bodies (SAPA-AFP, 2006).

2.5. International Efforts to protect the vulture.

While the problems associated with diclofenac were overwhelming and supported the outright removal of this drug from the Indian sub-continent, much resistance was received from the Indian government. While they acknowledged the death of vulture and the link to diclofenac, the value and need of the drug for cattle outweighed the plight of the vultures. They did, however agree, that if a suitable vulture-safe alternative anti-inflammatory could be found, especially one as effective as diclofenac, they would reconsider the ban of diclofenac (Swarup et al., 2007).

Research into a safe alternative drug was undertaken by the Royal Society for the Protection of Birds (RSPB), in partnership with the Bombay Natural History Society (BNHS), the Indian Veterinary Research Institute (IVRI) and the Faculty of Veterinary Science at the University of Pretoria (South Africa):

- As a first step: Swan (2006b) and his team validated the use of the less threatened African White-backed vulture (*Gyps africanus*) as a surrogate model for the endangered OWBV (Swan et al., 2006b).

- As the second step: the RSPB, undertook an international survey with raptor rehabilitation centres, veterinarians at zoos, and raptors collections in order to identify possible safe

alternative NSAID. Survey reports on the treatment of 79 scavenging birds and over 870 vultures were collected. More than four drugs were shown to be toxic in contrast with meloxicam which appeared to be safe for scavenging birds and raptors (Table 2) (Cuthbert et al., 2006).

- In the third part of the study: Swan et al. (2006a), selected meloxicam as their candidate and undertook a large multi-centric study in which they demonstrate the safety of meloxicam. In response to these findings on the 11th of May 2016, the Drug Controller General of India controllers withdrew the manufacturing of veterinary diclofenac. Nepal and Pakistan also banned the use of the drug in 2006, Bangladesh only prompted the ban in 2010 (Prakash et al., 2012).

Table 2-2: Survey results with an indication of the potential safety of NSAIDs in different vulture species and scavenging birds from the RSPB study.

Drug	**Toxicity**	**Number of birds**	**Dose (mg/kg/bw)**	**Species treated**
Aspirin	No	3	5.4-6.4	*Ciconia ciconia, Corvus corax, Aegypius monachus*
Ketoprofen	No	20	1.0-7.7	*Geranoaetus melanoleucus, Vultur gryphus, flammeus flammeus, Gyps fulvu, Aegypius monachus, Necrosyrtes monachus, Leptoptilos crumeniferus, Bubo virginianus, Otus asio, Gyps rueppellii, Buteo jamiacensis*
Meloxican	No	739	0.1-0.75	A total of 34 species were recorded as being treated, of which 46 were old world vultures including 39 *Gyps* species and for new world vulture species treated were 21

Ketoprofen & Meloxican	No	1	Ketoprofen 1.0 Meloxican 0.2	*Gyps africanus*
Diclofenac	Yes	28	0.1-2.5	*Gyps africanus, Gyp bengalensis, Gyps fulvus*
Diclofenac	No	8	0.25-0.6	*Gyp bengalensis*
Flunixin	Yes	7	1.0-4.5	*Leptoptilos crumeniferus, Platalea alba, Gyps rueppellii, Cariana cristata, Aegypius monachus*
Flunixin	No	16	0.5-12.0	*Gyps fulvus, Leptoptilos crumeniferus, Aegypius monachus, Vultur gryphus, Gyps rueppellii, Haliaeetus leucocephalus, Terathopius ecaudatus, Parabuteo unicinctus*
Flunixin & Ketoprofen	Yes	1	-	*Gyps africanus*
Carprofen	Yes	5	1.0-5.0	*Aegolius acadicus, Gyps fulvus, Parabuteo unicinctu,*
Carprofen	No	35	1.5-7.6	*Gyps africanus, Gyps bengalensis, Gyps fulvus, Gyps himalayensis, Gyps africanus, senegalensis, Ephippiorhynchus, Grus vipio, , Aegypius monachus, Necrosyrtes monachus, Haliaeetus leucocephalus, Bugeranus carunculatus, Ciconia Ciconia, Bugeranus carunculatus, Ardeotis kori*
Carprofen & Ketoprofen	Yes	1	Carprofen 7.2	*Gyps africanus*

			Ketoprofen 4.3	
Ibuprofen	Yes	1	-	*Aegypius monachus*
Phenylbutazone	Yes	1	-	*Torgus tracheliotus*

Modified from Cuthbert et al., 2006

Since the discovery of diclofenac as a vulture toxin, testing has continued on a range of anti-inflammatory drugs currently in use for domestic livestock. Thus far five non-steroidal anti-inflammatory drugs were identified as being toxic to vultures being aceclofenac, carprofen, flunixin-meglumine, phenylbutazone and ketoprofen (Cuthbert et al., 2006), albeit with various levels of sensitivity of the birds to the toxic effect of the drugs mentioned.

2.6. Current status of the vulture population

In 2012, Prakash et al, was able to show that of *Gyps* vultures in India and Nepal seems to be slowly improving following the ban of diclofenac after previous survey in 2007, which showed the species to still be on the decline (Prakash et al., 2007). The results also unfortunately indicate that the population of *Gyps* species albeit on the rise is unlikely to reach its former population size (Figure 2-6) (Prakash et al., 2012)

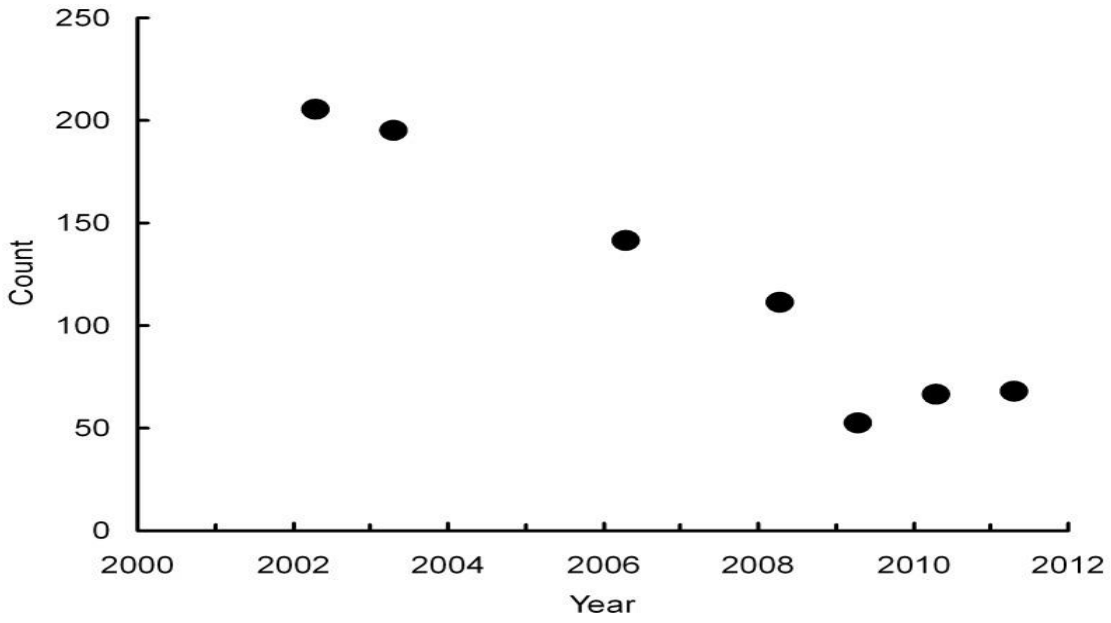


Figure 2-6: Gyps bengalensis counts on a road transect through western Nepal (Prakash et al., 2012).

Most recently a study by Paudel et al., 2016 reported that despite most of a district in Nepal being diclofenac free (Figure 2-7), there has been very little population changes between 2009 and 2015 for *Gyps Bengalansis*, with the species still remaining vulnerable (Figure 2-8), this possibly due to additional threats e.g. other NSAIDs used to treat livestock which are toxic to birds, human persecution and electrocution and poisoning (Paudel et al., 2016).

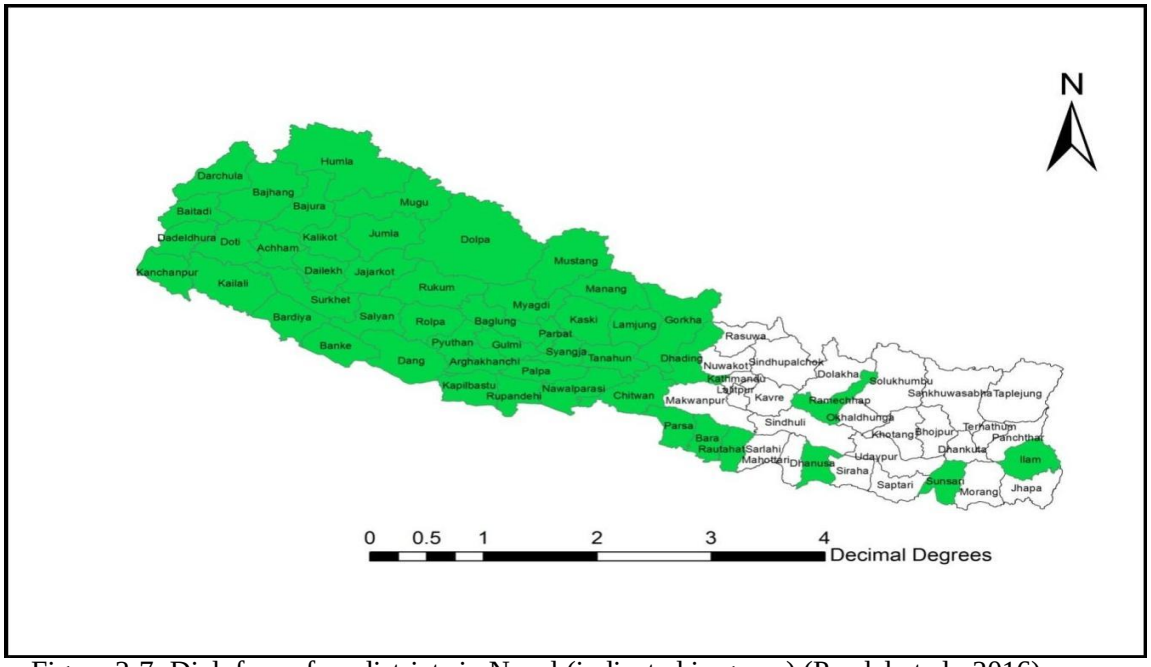


Figure 2-7: Diclofenac free districts in Nepal (indicated in green) (Paudel et al., 2016).

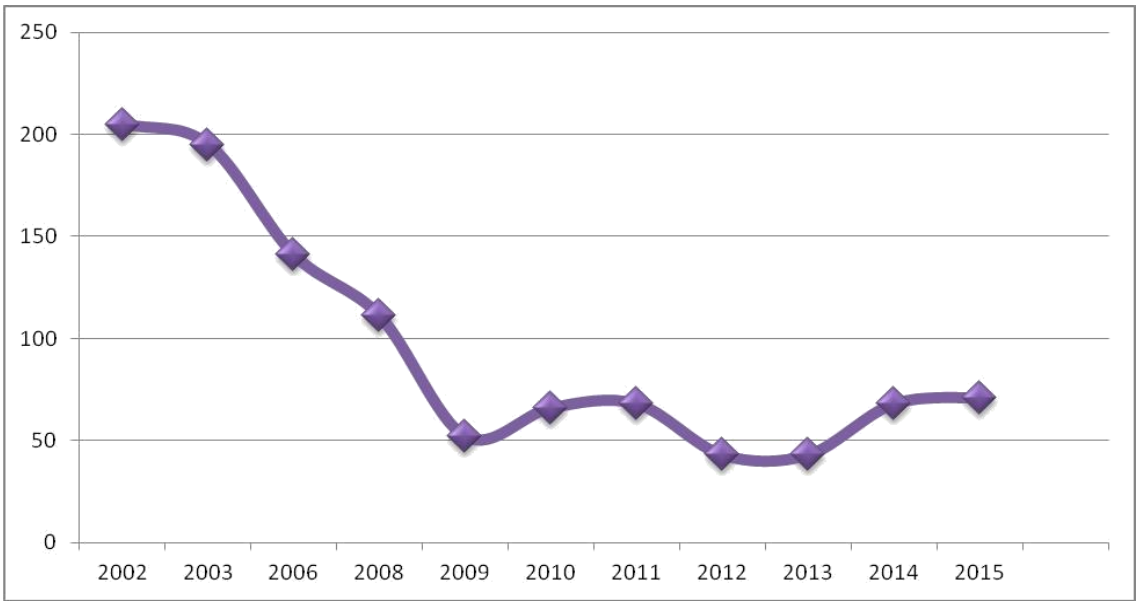
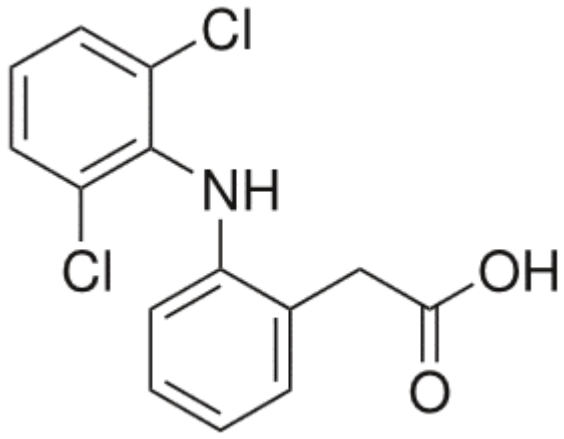


Figure 2-8: Gyps bengalensis absolute counts during road transect survey in Nepal (2002-2015) (Data from Paudel et al., 2016).

2.7. Diclofenac

2.7.1. General use of diclofenac in people and animals

Diclofenac, also known as (2- [2-(2,6-dicholorophenyl amino)phenyl]acetic acid)(Figure 2-9), is derived from phenylacetic acid and is part of NSAIDs group (MW 296.1g/Mol, CAS Registry: 15307-86-5) (Martindale,2007). Diclofenac was first synthesized by Rudolf Pfister and Alfred Sallmann. Ciba-Geigy now known as Novartis introduced diclofenac as Voltaren in 1973 onto the market for use in people.



(https://pubchem.ncbi.nlm.nih.gov/compound/diclofenac#section=2D-Structure)

Figure 2-9: Molecular structure of Diclofenac

Diclofenac is used to treat inflammatory disorders, algesia and dysmenorrhea in humans (Cheng et al., 2004; Jackson and Morrow, 2001). This drug is also an agent for the management of gout. It is typically used for the management of inflammatory disorders that may include musculoskeletal complaints include arthritis, osteoarthritis, polymyositis, rheumatoid arthritis dermatomyositis, dental pain, pain management in cases of kidney stones, gout attacks gallstones, dysmenorrhea (menstrual pains) and acute migraines in humans. It is also commonly used to treat mild to moderate post-operative or post-traumatic pain, especially in animals with inflammation (Cheng et al., 2004; Jackson and Morrow, 2001).

2.7.2. Diclofenac adverse effects

Diclofenac is among the better-tolerated NSAIDs by people and is characterized by a lower incidence of gastric ulceration in contrast with older generation of NSAIDs, known as non-selective agents, hence its wide use in animals and people. Nonetheless with the drug functioning as a cyclooxygenase (COX) enzyme inhibitor, it does decrease prostaglandins in the epithelium of the stomach, making the epithelium more sensitive to corrosion effect of the gastric acid. Thus the chronic use of diclofenac is associated with gastrointestinal complaints such as ulceration or bleeding that may requires immediate termination of treatment (Ulubay et al., 2018).

The use of diclofenac is also associated with an increased risk of cardiovascular death as seen with the coxibs, which are derived from diclofenac (Fosbøl et al., 2010). In Britain (June 2013), the Medicines and Healthcare Products Regulatory Agency (MHRA) said that people with serious underlying heart conditions, heart disease, stroke and suffered heart failure to be advised to stop using the drug completely (BBC, 2013). However a study by Graham (2006) indicated that diclofenac did not increase the risk of myocardial infarction which is a complete opposite to the latter findings (Graham, 2006).

It is also known to induce hepatotoxicity **(**liver damage) due to toxic activation of diclofenac by enzyme cytochrome (CYP) 2C8 and 3A4 (Gomez-Lechon et al., 2003 and Park et al., 2005). Hepatotoxicity clinical signs are cholestasis, cholestatic injury, hepatocellular damage, a mixed hepatocellular and cholestatic and steatosis (Schapira et al., 1986). Liver function monitoring is recommended during long-term treatment with diclofenac to avoid hepatotoxicity. In December 2009, Endo, Novartis, and United States Food and Drug Administration (US FDA)

notified healthcare professionals that with all products containing diclofenac sodium, they should add new warnings and precautions about the potential for elevation in liver function tests during treatment (Graham, 2006). These clinical signs relate to the molecular mechanisms of inactivation of the drug which is invoked in hepatocyte through the liver`s physiological function of metabolizing and detoxifying xenobiotics, which in this case results in a toxic metabolite formation (Gomez-Lechon et al., 2003a).

The molecular mechanisms have two metabolic phases. In phase 1 of metabolism, diclofenac gets converted enzymatically into 5-OH-diclofenac and the minor metabolite, $N,5(OH)_2$-diclofenac. Thereafter the body go into substrate cycle in which the metabolite ($N,5(OH)_2$-diclofenac) gets continuously converted into 5-OH-diclofenac and vice versa leading to oxidation of NADPH by oxygen and reactive oxygen species (ROS) formation. The ROS decreases mitochondria membrane permeability (MMP) from the oxidation of mitochondrial membrane proteins (Fleury et al., 2002 and Simon et al., 2000). The end results are mitochondrial membrane being damaged with subsequent proteins efflux from mitochondria into the cytoplasm and activation of caspase proteins (2, 8 and 9) and finally cellular apoptosis (Green and Reed et al., 1998).

Diclofenac is also associated with kidney damage from to ischaemic necrosis in people. Diclofenac inhibit COX-I enzyme leading to inhibition of Prostaglandin E_2 (PGE_2) synthesis. PGE2 maintains renal haemodynamics and modulates the vasoconstrictor effects of endogenous mediators. It`s inhibition promotes renal ischaemia resulting in renal papillary necrosis (Murray and Braxter, 1993; Dunn and Hood, 1977; Burke et al., 2005). Moreover the former drug also inhibits uric acid transporters (OAT1, OAT3, OAT4 and URAT1) which can result in accumulation of uric acid in the blood leading to kidney damage (Khandang et al., 2002).

A similar mechanism has been suggested for the vulture. Ng suggested that the suppression of ATP generation results in the inhibition of the uric acid transporter (multiple drug resistance pump channel) leading to accumulation of uric acid within the cells and causes necrosis (Ng et al., 2006). In contrast Meteyer et. al. (2005) proposed that diclofenac causes nephrotoxicity by inhibiting of renal prostaglandins as mentioned above leading to ischaemia as seen in people. They came to this conclusion after observing histopathological lesions and necrosis in the proximal convoluted tubules in vultures that had succumb to diclofenac toxicity (Meteyer et

al., 2005). Naidoo et. al. (2009), were, however, able to show using isolated renal vasculature, that diclofenac did not induce venous vasoconstriction (Naidoo et al., 2009).

2.7.3. Mechanism of action of diclofenac

2.7.3.1. Anti-inflammatory, Antipyretic and Analgesic effects

The exact mechanism of action for the drug's anti-inflammatory, antipyretic and analgesic actions is not known, but is believed to be due to inhibition of the two cylooxygenase enzymes (COX-1 and COX-2), leading to inhibition of prostaglandin synthesis (Kirchheiner et al., 2003). Diclofenac achieves its anti-inflammatory effects by decreasing the prostaglandins concentrations in the tissues, which are the mediators of inflammation. Their effects include increased vessel permeability, vasodilatation and chemotaxis of inflammatory cells into the injured region (Boothe, 2001). For the analgesic effect, it is believed that diclofenac function is mediated by decreasing local concentration of prostaglandins sensitising and activating local pain receptors i.e. inhibition of prostaglandins synthesis indirectly results in an analgesic effect by blunting pain transmission. The antipyretic effects appear to be on the hypothalamic set-point through COX inhibition, with resultant peripheral dilation and increased cutaneous blood flow and subsequent heat dissipation in combination with the blunting of other heat increasing mechanism (Blomme et al., 2003). Diclofenac is also a unique member amongst the NSAIDs because there's evidence that indicates it inhibits the lipoxygenase pathways thus inhibiting the synthesis of the leukotrienes (Murray and Brater, 1993).

2.7.3.2. Uricouric Effect

In addition to the traditional effect of the diclofenac as expected for the NSAID class of drugs, diclofenac is known to be highly effective as a uricosuric drug, in that it can decrease the plasma concentration of uric acid in humans. The drugs function via the inhibition of specific uric acid transporters in the kidney (see below for more details)

2.7.3.3. Gout: A common disease in humans.

Gout is a disorder of purine metabolism and it is usually characterized by recurrent attacks of acute inflammatory arthritis (Eggebeen, 2007), from high concentrations of uric acid (hyperuricemia) in the blood. Once the uric acid saturation point of blood (dependent on temperature and sodium concentrations) is exceeded, uric acid crystals are deposited in joints,

tendons, and surrounding tissues in the form of monosodium urate (Terkeltaub, 2010; Richette and Bardin, 2010). Long-standing hyperuricemia usually results in tophi (painless deposits of uric acid crystals), which when extensive tophi can lead to chronic arthritis due to bone erosion and joint damage (Terkeltaub, 2010). Elevated levels of uric acid may also lead to kidney stone (crystals precipitating in the kidneys, resulting in stone formation) and urate nephropathy (Tausche et al., 2009).

The high concentration of uric acid in the blood results from a combination dietary factors as well as metabolic constraints. Diets rich in purines, such as bacon, turkey, liver, alcohol and venison result in the formation of high concentrations of uric acid. Under normal conditions, the uricase enzyme should convert uric acid into allantoin, which allows for excretion. In addition the uric acid is cleared by the uric acid transporters in the kidney (see below) (Klemp et al., 1997; Doherty, 2009; Rider and Jordan, 2010).

In certain people, an evolutionary loss of uricase enzyme, is the main reason that drives hyperuricaemia and the development of gout especially when combined with poor eating habits and lifestyles (Richette and Bardin, 2010, Riches et al., 2009, Doherty, 2009 and Feig et al., 2008). Other cause has also been attributed to genetic polymorhism of renal uric acid transporters (Vitart et al., 2008; Doherty, 2009 and Richette and Bardin, 2010). The Mairo tribe of New Zealand is the clear example, as before the 18th century they had no mention/record of gout probability linked to their diet (sweet potato, taro, fern root, birds and fish). However in the early 1990s an epidemic of obesity and gout developed, which appeared to have resulted from a change in their diet to one low in dairy products, high in fat meats and carbohydrates (Johnson and Rideout, 2004). This change in diet and an adoption of new lifestyle has consequently resulted in this community having the highest gout prevalence in the world (Klemp et al., 1997; Doherty, 2009 and Rider and Jordan, 2010).

With the high prevalence of gout, with 58 million people being diagnosed with the illness from 1990 to 2013 globally (Global Burden of Disease Study 2013 Collaborators, 2015; Vos et al., 2015), numerous treatments are currently available for the management of gout. The drugs that are used in the treatment of gout, works generally by two mechanisms:

- Enzyme Inhibitors: NSAIDs are commonly used in treatment of gout. They work by inhibiting an enzyme called cyclooxygenase (COX) enzyme. This enzyme is important

for synthesis of prostaglandins which causes inflammation, so by inhibiting the enzyme fewer prostaglandins are produced leading to less inflammation and pain. They are classified in selective and non-selective inhibitors. The older generation of NSAIDs are non-selective inhibitors because they inhibit both COX 1 and COX 2 e.g aspirin and ibuprofen while the most selective drugs only inhibit COX 2 and are characterized by lower gastric ulceration incidence e.g diclofenac. COX 1 plays a role in making platelets stick together (clotting process), control blood flow and protecting gastric mucosa from stomach acids through the production of the gastric mucous layer; while the COX 2 enzyme is responsible for control of inflammation and fever (Clark, 2006; Coruzzi et al., 2007). Other drugs like allopurinol and benzobromarone are also commonly used to treat gout by inhibiting xanthine oxidase decreaing uric acid production. (Elion et al., 1978; Rundles, 1966). Oxidation of hypoxanthine to xanthine is catalyze by xanthine oxidase enzyme, the same enzyme continues to catalyze the oxidation of xanthine to uric acid (Harrison, 2002; Hille, 2005).

- Transport inhibitors: The NSAID group of drugs typically functions through this mechanism with diclofenac being an example of a commonly used drug in the treatment of uricaemia (Table 2-3) (Khandang et al., 2002; Anzai et al., 2006; El-sheik et al., 2007). Diclofenac primarily inhibits the Organic anion transporter 1 (OAT1) to Organic anion transporter 3 (OAT3) excretion channels on the basal surface of the proximal convoluted tubular cells in the kidney. This initially results in a build-up of uric acid in the blood as tubular uric acid excretion via this route gets inhibited. However, this increase uric acid then undergoes complete excretion via the glomerulus as glomerular filtration of uric acid in man is a very efficient process. Under normal circumstances the uric acid present in the tubular filtrate would be exposed to a reabsorptive process through the URAT1 uric acid channel present in the apical surface of renal proximal tubular cells. It is at this point, that diclofenac and the other NSAIDs appear to most effect as they also inhibiting the URAT1 channel which result in total higher excretion of uric acid (For more information on uric acid excretion, see below) into the urine. The ability of the different NSAID to influence OAT function is presented in table 2-3.

Table 2-3: Interaction of NSAIDs with human OAT1-3.

Drug	IC50 OAT1 (µm)	IC50 OAT2 (µm)	IC50 OAT3 (µm)
Diclofenac	4.0[e]; 4.46[a]; 6.1[d]	14.3[a]; 18.7[d]	6.57[b]; 7.78[a]
Ketoprofen	0.89[b];1.3[e]; 1.4[f]; 4.43[a]	272.8[d]; 400[a]	5.04[b]; 5.98[a]
Ibuprofen	1.38[b]; 8.0[d]; 55.6[a]	692[a]	3.7[h]; 5.11[b]; 6.0[a]
Loxoprofen	27.1[g]	n.d	8.7[g]
Naproxen	1.18[b]; 5.67[a]; 5.8[e]	486[a]	4.67[a]; 7.15[b]
Phenylbutazone	71.6[b]	n.d	6.82[b]
Flurbiprofen	1.5[e]	n.d	n.d
Indomethacin	3.0[e];3.83[a]; 4.4[d]; 6.72[b]	49.5[d]; 64.1[a]	0.61[a]; 0.98[b]
Mefenamate	0.14[d]; 0.83[a]	20.6[d]; 21.7[a]	0.78[a]
Phenacetin	200[e]; 275[a]	1878[a]	19.4[a]
Piroxicam	19.8[j]; 20.5[e]; 62.8[a]	70.3[a]	2.52[a]; 4.83[b]; 4.88[j]
Salicylate	1.573[i];280[f];325[a]; 407[b]	No inhib	50[a]; 111[b]
Sulindac	36.2[a]; 77.8[b]	440[a]	3.62[a]; 6.89[b]
Tolmetin	5.08[b]	n.d	n.d
Diflunisal	0.85[e]	n.d	n.d
Etodolac	50[e]; 103[b]	n.d	12.0[b]
Flufenamate	Inhib	n.d	Inhib
Acetylsalicylate	769[a]	No inhib	717[a]
Acetaminophen	639[a]	Inhib[c]; no inhib[a]	No inhib[a]

Inhib., inhibition was shown but IC_{50} was not indicated; n.d., not determined; no inhib., no inhibition.

[a] Khandang et al., 2002

[b] Nozaki et al., 2007

[c] Kobayashi et al., 2005b

[d] Kimura et al., 2007

[e] Mulato et al., 2000

[f] Cihlar and Ho 2000

[g] Uwai et al., 2004

[h] chu et al., 2007

[I] Ichida et al., 2003

[J] Jung et al 2001

2.8. The SLC family with transporters of anions, cation and zwitterions

The kidneys play an important role in maintaining homeostasis in the body, via glomerular filtration, tubular secretion and re-absorption. Of these two areas, the glomerulus functions as a filter system while the tubules are dependent on a more active process, as the proximal tubule cells have a plasma membrane which a barrier to passive diffusion (Wang and Sweet, 2013). This active process is facilitated by a transport protein that falls into two main superfamilies, the ATP binding cassette and the solute carriers transport proteins.

- The ATP binding cassette (ABC) as the name implies requires ATP as a driving force for the unidirectional transport of substrates across cell membranes (Van Wert et al., 2010). The substrates that can be transported by ABC include ions, sugars, amino acids, peptides and hydrophilic molecules (Davidson et al., 2008).

- The solute carriers (SLC) (facilitated transporters) transport proteins that are indirectly coupled to cellular energy and utilize the membrane potential difference or the stored energy of concentration gradients as driving forces (He et al., 2009). They participate in hepatic, small intestinal absorption and renal excretion and renal absorption of drugs, endogenous and xenobiotics compounds. Main endogenous substrate they transport include urate, α-ketoglutarate, cAMP, cGMP, L-carnitine, neurotransmitters, prostaglandins and monoamine (Koepsell, 2013).

Of these transporters the SLC22 can be further divided into three subfamilies, of which, only one is involved in the pathology of gout (Sweet, 2005, 2010; Van wert et al., 2010).

- The organic anionic transported (OATs), which is responsible for the transport and excretion of anionic substances such as uric acid (Explained below in section 2.8.1).

- The organic cation transporters (OCTs) facilitate the transport of large number of cationic substrates that are both endogenous and exogenous. There are three OCTs isoforms that have been identified in humans: OCT1 encoded by SLC22A1, is primarily localized in the liver where it is expressed in the basolateral membrane of hepatocytes (Nies et al., 2009). They are involved with the hepatic metabolism/biliary excretion of many catoinic drugs such as berberine, lamuvidine, acyclovir and metformin and

xenobiotics (Ahlin et al., 2011; Nies et al., 2009). OCT2 encoded by SLC22A2, is commonly distributed in the kidneys and expressed in the basolateral membrane of proximal tubules. It takes up cations from the basolateral membrane of renal proximal tubules (Motohashi et al., 2002). OCT3 is encoded by SLC22A3, is expressed in different tissues including liver, heart, placenta and e.t.c (Table 4). It has higher substrate selectivity for monoamines (Zhang et al., 2008).

The zwitterion transporters are transporters commonly known for they role in transporting zwitterions (molecules with both a positive and negative change), L-carnitine and organic cations (Koepsell et al., 2007). There are two OCTNs isoforms that have been identified in humans and rats (Sekine et al., 1998; Tamai et al., 1998 and Wu et al., 1998). OCTN1 is encoded by SLC22A4 while OCTN2 is encoded by SLC22A5. They are both expressed in kidney and other organs (Table 4) (Meier et al., 2007; Gilchrist and Alcorn, 2010; Lamhonwah et al., 2011 and Horvath et al., 2007).

Table 2-4: SLC22-organic cations, zwitterion and anion transporter family.

Human gene type	Protein name	Predominate substrates	Tissue distribution and cellular and subcellular expression
SLC22A1	OCT1	Organic cations	Kidney, liver (sinusoidal membrane of hepatocytes), small intestine, lung, skeletal muscle, brain (endothelial cells of blood brain barrier), adipose tissue, immune cells.
SLC22A2	OCT2	Organic cations	Kidney (basolateral membrane of proximal tubules), small intestine, lung, placenta, thymus, brain (neurons, blood brain barrier) and inner ear
SLC22A3	OCT3	Organic cations	Kidney, liver, heart, small intestine, lung, skeletal muscle, brain (neurons, glial cells, and plexus choroideus), urinary bladder, mammary gland and skin blood vessels.
SLC22A4	OCTN1	Zwitterions, organic cations	Spleen, heart, skeletal muscle, brain, intestine, kidney, mammary gland, thymus, prostate, testis, airways, eye and fetal liver
SLC22A5	OCTN2	Zwitterions (L-carnitine), organic cations	Prostate, lung, pancreas, heart small intestine, skeletal muscle, kidney (apical membrane of the proximal tubules), adrenal gland, thyroid gland and liver
SLC22A6	OAT1	Organic anions	Kidney (basolateral membrane of the proximal tubules), placenta, brain

SLC22A7	OAT2	Organic anions	Kidney (basolateral membrane of proximal tubules), liver (sinusoidal membrane of hepatocytes)
SLC22A8	OAT3	Organic anions	Kidney (basolateral membrane of proximal tubules), brain (luminal membrane of choroid plexus), skeletal muscle, developing bone

Modified from: www.bioparadigms.org

2.8.1. Organic Anion Transporters

In man, the Organic Anion Transporter 1, 2 and 3 (OAT1, OAT2 and OAT3) are located in the basolateral membrane of renal proximal tubule cells in contact with blood vessels. They are accountable for the active transport of uric acid via dicarboxylate/organic anion exchange from the efferent blood vessels into the intracellular environment (Sweet, 2005, 2010; Van wert et al., 2010). As a result of this coupled mechanism, OAT1 & 3-positive cells are at risk of exhausting their supply of dicarboxylates due to constant removal of dicarboxylic acid which leads to loss of function. To prevent the former outcome sodium-dicarboxylate cotransporter (NaDC3) is expressed by OAT1& 3-positive cells. The latter cotransporter is used to transport dicarboxylates back into the former cells moreover to drive this process sodium is required. Thus an absence of a sodium gradient across the cell membrane can also results in the NaDC3 co-transporter ceasing to function this leads to dicarboxylates been depleted and ultimately transporters failure (Fig: 2-10) (Sweet, 2005, 2010; Van wert et al., 2010).

Apical Multiple Resistance Protein (MRP 1 and 4) channels then excrete uric acid into renal tubule from intracellular environment. Also within the apical membrane are the OAT4 and Uric Acid Transporter 1 (URAT1) which mediate reabsorption of substrates from urine through exchange with monocarboxylate and dicarboxylates or hydroxyl ions respectively (Fig 2-10). In essence mammalian plasma uric acid gets excreted into the glomerular filtrate via active tubular excretion by the OAT1-3 channels. The glomerular filtrate is also exposed to tubular reabsorption via the OAT4 and URAT1 channel, in controlled manner to maintain homeostasis.

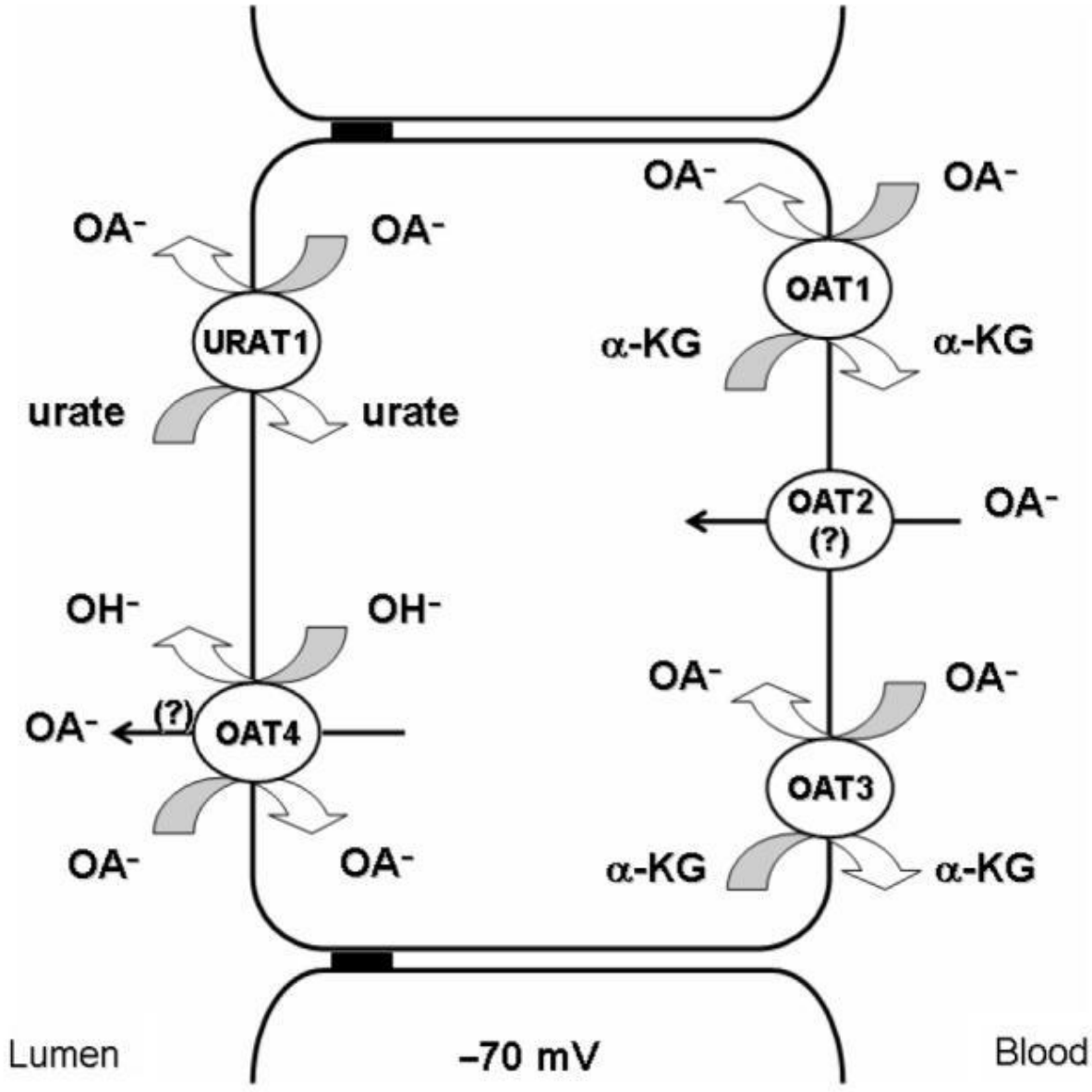


Figure 2-10: A schematic presentation of channels involved in uric acid excretion and reabsorption in man, with arrows indicating directional flow of organic ion.
OAT1: Organic Anion Transporter; OAT2: Organic Anion Transporter 2; OAT3: Organic Anion Transporter 3; OA^-: negatively charged Organic Anions; α-KG: alpha- Keto- glutarate (dicarboxylates); URAT1: Uric Acid Transporter 1 and OAT4: Organic Anion Transporter 4.

2.9. Proposed mechanism of diclofenac's toxicity in vultures

Uric acid excretion mechanism in birds differs from that of mammals. In contrast with mammals, birds are uricotelic meaning they don't conserve uric acid (Long and Skadhauge, 1983; Dudas et al., 2005; Sinclair et al., 2006). The reason for this resides in the development of the embryo in the enclosed environment of the egg. If the bird was to use urea for nitrogen

excretion, the foetus (avian embryo) could be harmed due to its dehydrating effect. The avian system has decreased the importance of urea as a nitrogenous waste product to a less dehydrating uric acid to prevent dehydration (Dudas et al., 2005; Sinclair et al., 2006).

At present the mechanisms of excretion of uric acid in the vulture is unknown. However, a large body of literature is available in the chicken. With the common understanding that uric acid excretion as a process is highly conserved in avians, the chicken serves as the general model for uric acid excretion for all birds. Firstly, the chicken is a net excretory of uric acid. Studies have demonstrated that the chicken kidney makes use of a two-stage excretory mechanism as per man (Marys, 1924; Dudas et al., 2005). More recently the chicken was shown to make use of the OAT channel (OAT 1 and 3) basal and the MRP (MRP 2) apically for the excretion of uric acid. In conrast to human physiology, a major difference between people and birds, is at the level of the glomerulus with glomerular filtration playing a minor role in the excretion of uric acid in birds. In a study by Berger et al. (1960), it was found that the tubules were responsible for 80% of the urinary excreted uric acid. Birds also do not appear to have a process to reabsorb tubular uric acid and thus far the URAT1 channel has not been described to occur either, making the chicken a net uric acid excretor (Dantzler and Braun, 1980). Most importantly the chicken kidney handles a substantially higher load of uric acid than mammalian kidney, as uric acid is the predominant nitrogenous waste in birds.

Based on the mechanism by which diclofenac functions in people, we speculate that the same mechanism results in the vulture death i.e. diclofenac inhibits the tubular excretion of uric acid with resultant hyperuricaemia. However, unlike man, with negligible glomerular clearance of uric acid, up to 90% being excreted via the tubules (Frazier et al., 1995), and the absence of a URAT1 channel, the bird has no alternate mechanism for the excretion of increase plasma uric acid concentration. This results in a catastrophic hyperuricaemia over time, visceral gout and death.

2.10. Conclusion

Research has conclusively proven the toxicity of diclofenac on Oriental white-back vultures. Despite this, the mechanism underlying this toxicity is not fully understood. The latter is important to understand wheather other drugs in the class could be equally as toxic. The

following study will be aimed at a further understanding of the uric acid transport mechanism present in the vulture.

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CHAPTER THREE

3. The mechanism by which uric acid clearance is inhibited by diclofenac: Further understand of the toxicity of diclofenac in vultures

B Nethathe[1], J Chipangura[2], I Hassan[1], N Duncan[3], E Adawaran[1], LN Havenga[2], V Naidoo,[1,2]

[1] Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, Pretoria, South Africa.

[2] Biomedical Research Centre, Faculty of Veterinary Science, University of Pretoria, South Africa

[3] Department of Pathology, Faculty of Veterinary Science, University of Pretoria, Pretoria, South Africa.

3.1. Introduction.

In India, birds belonging to the *Gyps* genus (Oriental white-backed vulture, *Gyps bengalensis*; long-billed vulture, *G. indicus* and slender-billed vulture, *G.tenuirostris*) were in grave danger of extinction in the 1990s following their inadvertent exposure to veterinary diclofenac that found its way into their food chain (Oaks et al., 2004). Following their single exposure to quantities of drug that equated to a dose in the region of 0.8 mg/kg, birds were found dead within 48 hours of said exposure with signs of severe visceral gout and associated renal and hepatic damage (Oaks et al., 2004, Green et al., 2004; Shultz et al., 2004; Swan et al., 2006b). Despite the diclofenac induced deaths being largely recognised as one of the worst environment intoxications in recent times, the mechanism behind the evident gout is still incompletely understood. One of the theories put forward, suggests that toxicity is due to the inhibition of the uric acid transporter channels in the renal tubular epithelial cells (Naidoo et al., 2007). This would be consistent with diclofenac being a uric acid channel inhibitor in mammals (Khamdang et al., 2002; Nozaki et al., 2007; Burckhardt, 2012).

Uric acid is the final product of exogenous and endogenous purines metabolism, through the action of xanthine dehydrogenase (oxidase) which synthesizes uric acid from xanthine and hypoxanthine (Landon et al., 1960). Due to its poor solubility, if left to accumulate in the

plasma, uric acid precipitates into the tissue resulting in the formation of gout and tissue damage (Lumeij, 2008). To facilitate proper excretion both the kidney and the liver have become the major excretory organs for the elimination of uric acid in mammalian species, with the three renal processes of glomerular filtration, tubular secretion and tubular reabsorption being most important (Chin and Quebbemann, 1978). The following studies, just to mention a few, Yu and Gutman (1959); Long and Skadhauge (1983); Wang and Sweet (2013) have exhaustedly studied tubular secretion of uric acid in mammals (more details in chapter 2 page 42-43).

Although uric acid excretion in birds is not as extensively studied as in mammals, there is conclusive evidence that uric acid is eliminated predominantly by renal tubular secretion (Mayrs, 1924; Smith, 1955) in the same way as humans (tubular secretion). A major difference is however present in that excretion in birds occurs in the absence of any reabsorption, as birds are uricotelic and don't conserve uric acid through reabsorption and more specifically don't have the URAT1 reabsorptive channels (Long and Skadhauge, 1983; Dudas et al., 2005; Sinclair et al., 2006; Berger et al., 1960). The absence of reabsorptive channels has been related to the development of the foetus within an enclosed environment and urea being highly hygroscopic. If the bird was to use urea as their main form of nitrogenous waste excretion as for mammals, the avian embryo could be harmed due to the dehydrating effect of urea. In comparison, the avian system has adapted to the use of less dehydrating uric acid (Dudas et al., 2005; Sinclair et al., 2006). By producing insoluble uric acid and not water-soluble urea the foetus can grow well in a shelled-egg without been poisoned by nitrogenous waste products (Skadhauge, 1981; Harcourt-Brown, 2009). In avian species, uric acid constitutes 68-80% of the total nitrogenous wastes present in the ureteral urine, with the remaining nitrogenous waste being in the form of ammonia, urea and creatine. Uric acid is excreted as a colloidal solution with approximately 80% being excreted by the proximal tubule while urea and other water-soluble products are predominantly filtered via the glomerulus (Moorthy and Blichfeldt, 2009).

For this study we investigated the mechanism through which diclofenac interferes with uric acid excretion in the domestic chicken, which has been previously validated as a suitable model for the evaluation of mechanism of the toxicity of diclofenac (Naidoo et al., 2007). From previous studies, it has been demonstrated that the time to toxic effects, clinical signs and pathological changes seen with diclofenac are identical between the chicken and the numerous vulture species, albeit at a higher 10-fold LD_{50} of 9.8 mg/kg (Naidoo et al., 2007; 2009). The

other reason for using surrogate species is also due to the highly endangered status of vulture species (IUCN, 2006). For this study we exposed chickens to diclofenac in the presence of para-aminohippuric acid (PAH) and iohexol (Omnipaque). The former is a very specific transport substrate of the OAT channel while omnipaque is a non-radioactive radiographic marker of renal blood flow and glomerular filtration. In combination, these two substances have been previously used in the dog to non-lethally evaluate changes in renal functionality (Laroute et al., 2005). Further, Smeets et al. (2004) showed the MRPs were not involved in PAH excretion in rat kidney slice cultures (Smeets et al., 2004).

3.2. Materials and methods

3.2.1. Animals welfare

Before the commencement of experiments, ethical clearance (V108-16) was obtained from the Animal Ethics committee of University of Pretoria. Ross Broiler chickens (*Gallus gallus domesticus*) (n = 6) were used in this study. The chickens were grouped housed at University of Pretoria Biomedical Research Centre (UPBRC) and fed commercial broiler foods and an antibiotic-free ration ad libitum. The birds were allowed one week acclimatization period and were 4 weeks old at the start of the study. On the morning of the study, the birds were removed from the pens and transferred to individual cages (size: 45cm x 45cm x 40cm) where they were housed for the first 24 hours of the study, to allow for individual animal faecal collection. After 24 hours, the birds were returned to their pens.

3.2.2. Treatment and sample collection

In phase 1 (control phase), birds were treated with a single intravenous dose of PAH (10 mg/kg) and iohexol (Omnipaque, 1.25 mg/kg), mixed in one syringe immediately prior to administration. Faecal samples were collected at 0, 2, 6, 8 and 12 hrs and stored at -80 ^{0}C until further analysis. Blood was collected from the branchial or jugular vein, at before treatment and 2, 4, 6, 8, 12 and 24 hrs thereafter, into a syringe and immediately transferred into 5 ml lithium heparinised vacutainer (BD vacutainers). Blood samples collected were centrifuge at 3000 x g at 4 ^{0}C for 15 min and the supernatant of each sample was transferred into a labelled polycarbonate tubes and stored at -80 ^{0}C until further analysis.

The birds were allowed a two-week recovery period before phase 2 starts. In phase 2 all the birds received a single dose of diclofenac (10 mg/kg). Two hours after administration of diclofenac, the mixture of PAH and omnipaque was administered and the same methodology was applied as per phase 1 for sample collection.

Stored faeces and plasma samples from a previous study of two Cape griffon vultures (*Gyps coprotheres*) treated with meloxicam (2 mg/kg) were also analysed for uric acid concentrations as a control for species specific uric acid clearance. The faecal and plasma samples were selected for the 10 h time points, since plasma analysis showed both the absence of meloxicam (after 10 half-lives) and that uric acid concentrations were at pre-meloxicam exposure concentrations (Adawaran et al., 2019).

3.2.3. Sample analysis

3.2.3.1. Diclofenac analysis in domestic chicken plasma samples

Diclofenac in plasma samples, was analysed by a validated HPLC method as previously described (Naidoo et al., 2007). In brief, plasma samples (200 µl) were mixed with diethyl ether (400 µl), 0.3 M potassium dihydrogen phosphate (400 µl) and vortexed. The organic layer was separated in an ice bath (methanol/solid carbon dioxide), evaporated to dryness and dissolved in 400 µl mobile phase. Samples were analysed on a Beckman System Gold HPLC consisting of an autosampler module 507, programmable solvent module 126, diode array detector module (DAD) 168, and 32 Karat™ software package was used (Beckman Instruments, Fullerton, California, USA). Separation was achieved with a Thermo Scientific C18 column (250mm × 4.6mm × 5µm; Thermo Scientific, Runcorn, UK). The mobile phase consisted of 0.05 M sodium dihydrogen phosphate (pH = 4.85 to 4.89):CH_3CN, 42.5:57.5. One hundred (100) µl of the reconstituted samples was injected onto the HPLC column at 1 ml/min in an isocratic run. Detection of diclofenac (internal standard) was carried out at 275 nm. The total runtime per sample was 8 min with retention times of 4.9 min. Control values (100- 0.39 µg/ml) showed a mean accuracy of 99% and regression coefficients greater than 0.99 for the analytical run. When sufficient time points were available, samples were analysed by non-compartmental pharmacokinetic analysis (Thermo Kinetica) using the standardised programme settings.

3.2.3.2. Diclofenac analysis in faecal samples.

For the method validation, 25 mg of faeces (the white urine and solid components mixed in natural proportions) were transferred into 5 ml tube and spiked with 200 µl of diclofenac standard concentrations ranging from 6.25-200 µg/ml in triplicate. One millilitre of acetonitrile was added to the spiked excreta sample and homogenised using a manual homogenizer. The homogenate was transferred into 2 ml microcentrifuge tube and centrifuged at 10,000 rpm for 10 min at 4 ^{0}C. After centrifugation, the supernatant was filtered into a clean marked glass tube and evaporated to dryness using zymark turbovalp LV evaporator at 60 ^{0}C under nitrogen. This was followed by adding 200 µl of the mobile phase (acetonitrile: sodium dihydrogen phosphate; 50:50) into the dried sample and vortex for 2 min to mix. 30 µl of the reconstituted sample was injected onto the HPLC column, using the same method and equipment as above, with the exception of the mobile phase. After optimizing different ratios of mobile phase, the ratio that yielded clearer separation of peak of interest was (acetonitrile: sodium dihydrogen phosphate; 40:60). The method was validated for linearity, precision and accuracy. After validation 25 mg of the excreta were homogenized using 1 ml of acetonitrile and centrifuged at 10,000 rpm for 10 min at 4 ^{0}C. The supernatant of the treated samples was filtered and also dried under nitrogen at 60 ^{0}C. The dried residue reconstituted with 200 µl of mobile phase at the mixture ratio described above for diclofenac standard. Also, 30 µl of the reconstituted sample was injected onto the HPLC column using the same method and equipment mentioned above.

The elimination constant and half-life of elimination was determined from the cumulative excretion over time period of evaluation. For the calculation, the total concentration of diclofenac for each time period of measurement was converted to the ARE (amount remaining to be excreted) as the total cumulative amount excreted over the total period less the total amount excreted per time point in an iterative manner starting from the first time point of measure. The slope (Kel) of the ARE versus time period of collection on the logarithmic scale was the elimination constant. The half-life of elimination was determined as Ln(2)/Kel. Renal clearance was measured by diving total faecal excretion of uric acid divided by total time period of evaluation divided by drug concentration in plasma (Gibaldi and Perrier, 1982).

3.2.3.3. PAH and iohexol analysis in chicken plasma samples

The samples were analysed at the commercial PharmOVS Parexel laboratory in Bloemfontein, South Africa. Samples were thawed in a water bath at ~22 ^{0}C and vortexed briefly. Centrifuged for 5 min at 1300 x g and 50 µl of plasma was aliquoted into microcentrifuge tubes. Added 950 and 400 µl of methanol to the samples containing internal standards 250 and 5 ng/ml for PAH and iohexol respectively. The samples were vortex for 30 sec and centrifuged for 5 min at 500 x g. The supernatant (100 µl) was transferred into microcentrifuge tube and diluted with 0.1% formic acid (1:8; 1:3; v/v) for PAH and iohexol respectively. Transferred the extracts to a 96-well plate.

Samples were analysed by Liquid chromatography tandem mass spectrometry (LC/MS/MS) using an Agilent 1100 series high pressure liquid chromatography with temperature controlled autosampler (model G1367B) and a diode array detector coupled to a Sciex API4000 QTRAP mass spectrometer fitted with a "Turbo V" electrospray ionization (ESI) source. The analytical column used was Supelco Discovery column (C18) column (150 x 2.1 mm, 4 µl particle size) fitted with a phenomenex security guard system containing C18 (4 x 2 mm) pre-column. The mobile phase consisted of methanol: 0.1% formic acid solution (1:1, v/v). The pump delivered the mobile phase at the flow rate of 250 µl/min. The sample injection volume used was 4 and 3 µl for iohexol and PAH respectively. The autosampler was equipped with a 96-well plate tray and it was fitted with a cooling device to keep the samples at ~5 ^{0}C.

The mass spectrometer at unit resolution in the multiple reaction monitoring (MRM) mode was used to monitor the transition of the protonated precursor ions *m/z* 195.0 and 152.0 to the product ions *m/z* 120.0 and 110.0 for PAH and the internal standard respectively. For iohexol and its internal standard, protonated ions *m/z* 822.0 and 256.0 to the product ions *m/z* 804.0 and 110.0 respectively. Electro Spray Ionisation (ESI) was used for ion production. The instrument was interfaced to a workstation running Analyst version 1.6.2 software. Additionally, the extracted wavelength diode array chromatograms were used to confirm retention time of PAH, iohexol and their internal standards. The retention times were as follows: PAH at ~1.70 min, iohexol at ~1.67 min and both internal standards at ~1.80 min.

The clearance of iohexol (CL_{IO}) and PAH (CL_{PAH}) were determined using standard pharamacokinetic equation of Dose/Area under curve (AUClast), where AUClast was determined using the linear trapezoidal rule for the visible portion of the plasma concentration versus time curve. The tubular clearance of PAH (Tm_{PAH}) was determined as $CL_{PAH} - CL_{IO}$, CL_{IO} represent glomerular filtration and CL_{PAH} the total PAH clearance.

3.2.3.4. Uric Acid analysis in Chicken and Cape vulture plasma samples

Plasma uric acid concentrations was determined by a commercial veterinary clinical pathology laboratory (University of Pretoria, South Africa). Samples were analysed using Cobas Integra 400 plus anayser (Roche, USA). It is a fully automated machine using a colorimetric model. The samples were initially incubated with peroxidase (POD) and 4-aminophenazone (TOOS), which reacted with peroxide in the sample to form quinoneimine dye. The intensity of the red colour formed was proportional to the uric acid concentration and it was determined by measuring the increase in absorbance at 552 nm. The total exposure to uric acid to the last time point was determined as area under curve (AUC_{last}-UA) using the liner trapezoidal rule.

3.2.3.5. Uric acid determination in domestic chicken and cape vulture faecal samples.

The uric acid concentration in faeces was determined using the method of Marquardt, 1986. The total faeces collected per period was dried in an oven. Ten milligrams of the faeces was ground and added to 10 ml of 0.1 M glycine buffer, pH 9.3 and extracted at 40 ^{o}C with constant mixing for 1 hr. The suspension was allowed to settle, and an aliquot was diluted 15-fold with 14 volumes of 5.35% perchloric acid (PCA). The samples were centrifuged at 20.000 x g for 5 min. The absorbance of the samples was determined at 285 nm at a path-length of 1 cm. The concentration of uric acid in the faecal sample were determined against a standard curve for uric acid (MW, 168.1). For the standard curve a known concentration of uric acid was dissolved into a 0.1 M glycine buffer, PH 9.3. The standard curve was diluted with different amount of water, prior use, with equal volume of 10% PCA. Absorbance was determined against the appropriate blank at the same wavelength as the unknown samples (285 nm). Cloacal faecal samples from untreated chickens obtained opportunistically post-mortem were used to establish the standard curve. The total uric acid concentrations in the faeces were used to determine uric acid clearance with $Cl_{UA=}$ Total Faecal Uric Acid/Plasma Uric acid for the same time point. The faecal clearance hereby calculated becomes a reasonable estimate of urinary

clearance as previous studies have shown nearly 98 to 99% of uric acid in the faeces is of urinary origin and the remainder of cloacal origin (Beck and Chang, 1980) i.e. the cloacal samples used in establishment of the standard curve allows for this 1% correction. Turate was calculated as the clearance of iohexol (CL_{IOH}) multiplied by the plasma uric acid (P_{UA}) concentration at 12 hours, subtracted from total excreted uric acid over the preceding 4 hours.

Total urate (Turate) = $T_{EUA\ over\ 4hrs}$ - CL_{IOH} x $P_{UA[]\ at\ 12hrs.}$

Moreover $Tm_{UA\%}$ was determined as Turate divided by corrected uric acid excreted ($E_{UA(corrected)}$) multiple by 100%

Tubular secretion of uric acid ($Tm_{UA\%}$) = Turate/ $E_{UA(corrected)}$ x 100%

while GFR was calculated as corrected plasma uric acid minus iohexol clearance.

Glomerulus filtration rate (GFR) = $P_{UA\ (corrected)}$ - CL_{IOH}

3.2.4. Radiographic analysis

Five minutes following the administration of iohexol, all birds were radiographed and visually evaluated to ascertain if the different lobes of the kidneys were visible. Radiographs were taken using a diagnostic radiographic unit and a computed radiographic system on a Carestream digital analyser (UPBRC).

3.2.5. Pathology and histopathology

All birds, either 48 hrs after treatment or unscheduled deaths were subject to full necropsy. Birds were euthanized with a pentobarbitone overdose. Necropsy was conducted and lesions seen were recorded. For histopathology, tissue samples collected for residue analysis (kidney, liver, heart, spleen, intestine and lungs) were preserved in buffered formalin. Samples preserved in formalin were trimmed, embedded in paraffin, sectioned and stained using standard methods with hematoxylin and eosin.

3.3. Results

3.3.1. Clinical Signs and histopathology

No ill-health was observed on birds in phase 1 however in phase 2, two birds (9192 and 9194) died with the remaining four birds surviving till the termination point. The birds that died were diagnosed with severe renal-glomerulo-tubular necrosis and urate nephropathy. The birds had dilation of mammalian glomeruli and some renal tubules, with complete loss of internal structure and only the basement membrane remaining (moderate-to-severe glomerulo-tubular necrosis). Multifocal renal tubules in between were filled with fine fibrillar eosinophilic material (urate deposits), with complete loss of tubular epithelium. The urate deposits were surrounded by multinucleated giant cells and macrophages. Moreover, on the spleen the presence of multifocal fibrin deposits was observed. For the birds that survived all the organs were normal expect the lungs and one bird (9189) had early signs of urate nephropathy, albeit less severe than the birds that died.

3.3.2 Pharmacokinetics.

Following administration of a single dose of diclofenac (10 mg/kg, LD_{50}) to 6 domestic chickens by intravenous route, four of the treated birds diclofenac had barely detectable low concentrations at the first sampling point (0.29 ± 0.1395 µg/ml; first sampling was 4 hrs after administration of the diclofenac) which was below the lower standard tested (0.4 µg/ml) and completely non-detectable thereafter. Two of the latter birds died within 48 hrs. Guided by the above findings, the half-life elimination was much shorter than 2 hrs. For the remaining two birds (9189 and 9190), diclofenac concentrations were detectable at a level which allowed for pharmacokinetic analysis (Fig 3-1). The half-life, AUC_{last} and V_d were 2.4 ± 1.8 h, 10.79 ± 2.55 µg/ml*h and 3.65 ± 3.27 l/kg respectively (Table 3-1).

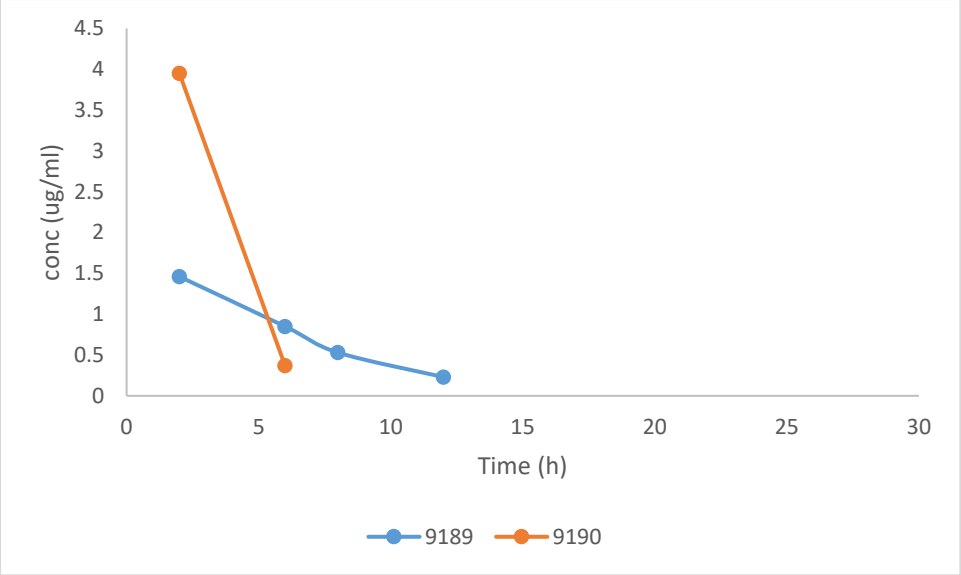

Figure 3-1: Diclofenac plasma vs time profile for bird 9189 and 9190 following dosing at 10 mg/kg (Sampling points based on PAH administration times).

Table 3-1: Pivotal pharmacokinetics parameters for diclofenac following intravenous administration in domestic chicken plasma using a one compartmental analysis.

Pk parameters	**Units**	**Bird 9189**	**Bird 9190**	**Mean**	**SD**
Cmax	**µg/ml**	1.46	3.95	2.705	1.76.696
Tmax	**h**	2	2	2	0
Vz (Vd)	**l/kg**	5.9613798	1.34169117	3.651535485	3.266613
Lz (ke)	**h^{-1}**	0.1868	0.592	0.3894	0.28652
T1/2	**h**	3.71063801	1.17085672	2.440747365	1.795897
AUClast	**µg/ml*h**	8.98	12.59	10.785	2.552655
AUCext	**µg/ml*h**	1.23126338	0.625	0.92813169	0.428693
AUCinf	**µg/ml*h**	10.2112634	13.215	11.7131317	2.123963
%AUC	**%**	87.9421053	95.2705259	91.6063156	5.181976

AUMC	**µg/ml*h2**	42.3	28.14	35.22	10.01263
Cl	**l/kg*kg**	1.11358575	0.79428118	0.953933465	0.225782

Cmax – maximum concentration; Tmax – time to maximum concentrations ; Vz (Vd) – volume of distribution; Lz (ke) – lambda (elimination constant) ; T1/2 –half-life ; AUClast – area under curve last; AUCext –area under curve extension ; AUCinf – area under curve infinity; %AUC- area under curve percentage; AUMC –area under movement curve ; Cl- clearance.

3.3.3. Method validation and faecal diclofenac clearance.

To ascertain the elimination constant of the drug, faecal samples were analysed for diclofenac concentrations. To analyse the samples a method was developed for diclofenac and it yielded sharp and well resolved curves. Peak identification of faecal samples spiked with diclofenac was performed by comparing the retention time (4.9) with pure standard of diclofenac and confirmed by characteristic spectra obtained from the photodiode array detector, which also permitted the conformation of the purity of the peak (Fig 3-2). The method was found to be linear at the range of 6.25-200µg/ml for three independent curves with the co-efficient of determination (r^2) above 0.99 (Table 3-2). The developed method had LOD and LOQ values of 6.25 and 12.5µg/ml respectively.

Table 3-2: Validation parameters of diclofenac in the faeces samples.

Calibration parameters	**Experiment 1**	**Experiment 2**	**Experiment 3**	
Wavelength (nm)	275	275	275	
No. of data points	6	6	6	
Range (µg/ml)	6.25-200	6.25-200	6.25-200	
Regression coefficient	0.99	0.99	0.99	
Slope	32926	32363	33599	
Accuracy (%)	98.50	96.30	99.00	
Intra-day and inter-day precision				
	Intra day		Inter-day	
Sample ((µg/ml)	Calculated concentration (µg/ml)	%Recovery	Calculated Concentration (µg/ml)	%Recovery
6.25	6.27	100.28	5.82	93.08
12.5	10.23	81.87	12.05	96.41
25	25.91	103.66	23.23	92.93
50	51.27	102.56	51.64	103.28
100	103.97	103.97	95.49	95.49
200	197.72	98.86	202.08	101.04

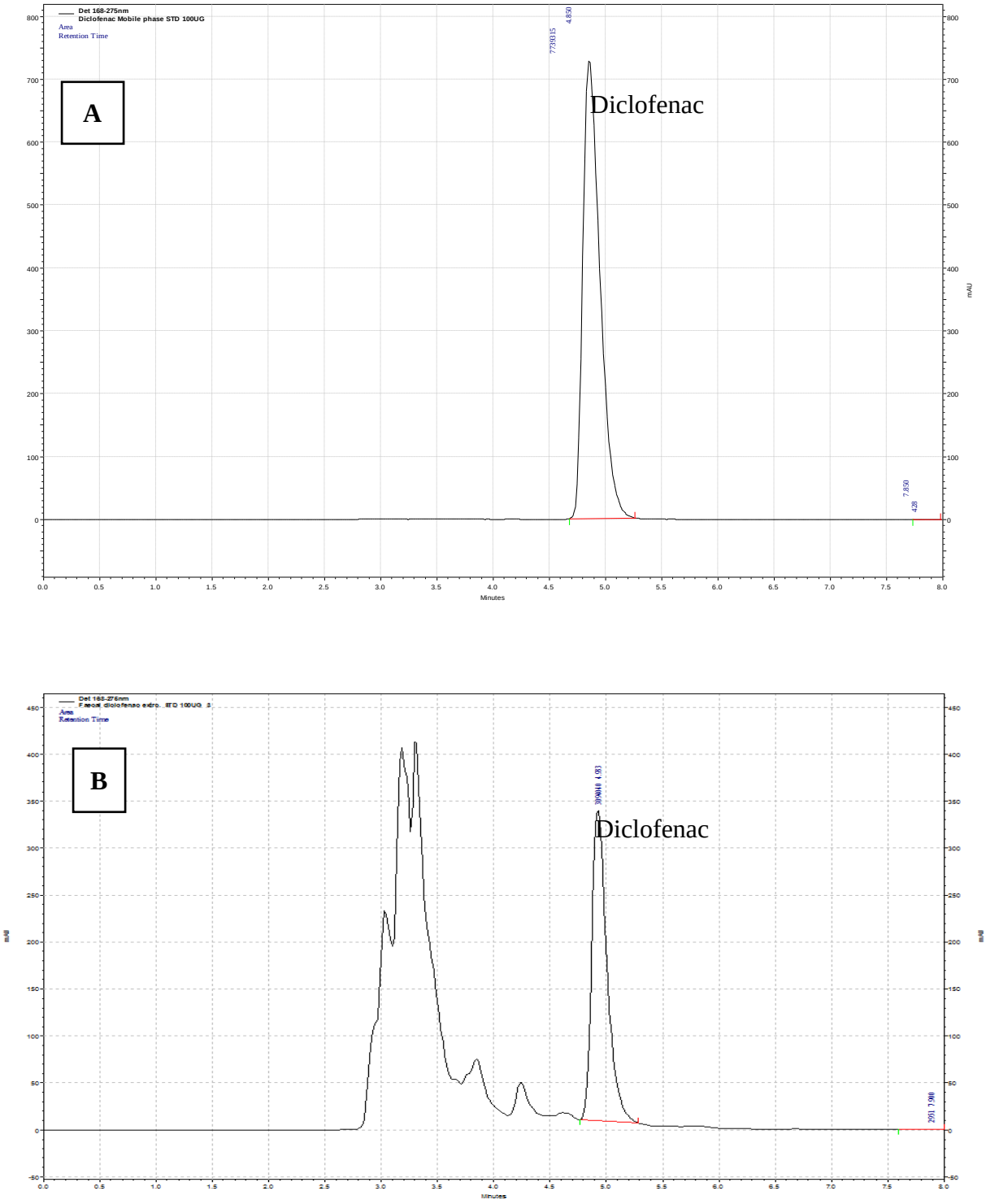

Figure 3-2: A) Representative of chromatogram of pure diclofenac standard and B) spiked faecal sample with 100 µg/ml of diclofenac

After method validation faecal samples were analysed and the elimination constant (kel) and half-life (h) of elimination for diclofenac was 1.92 ± 2.67 h and the Lz was 1.16 ± 0.85 h^{-1} (Table 3-3). Despite all the birds having quantifiable half-life of elimination, there was poor correlation between the plasma and faecal half-life of elimination for bird 9189 and 9190.

Table 3-3: PK parameters of diclofenac concentration in the chicken faecal samples

Animal	Weight	Clearance (l/kg*kg)	Kel (h^{-1})	Half-life (h)
9189	2.16	0.16	1.04	0.67
9190	2.99	0.14	0.60	1.16
9191	2.80	0.27	0.15	4.57
9192	2.54	0.19	0.48	1.45
9194	2.67	0.51	0.14	5.02
9195	2.92	0.30	0.70	1.00
Average	2.68	0.26	0.52	2.31
SD	0.30	0.14	0.34	1.95

Kel (Lz) = elimination constant

3.3.4. Plasma and faecal uric acid

No changes in plasma uric acid concentrations were noted in phase 1, with five of the birds excreting more than 85.28 % of uric acid by the tubules ($Tm_{uricacid}$) (Table 3-4). After the administration of diclofenac, plasma uric acid concentrations increased rapidly over a period of time, even though the concentrations at zero hour at the start of each phase were within the normal ranges (0.217-0.403 mmol/l) expected for the species. The change in plasma uric acid for phase 2 was evident as early as the 2 hrs post exposure, and for most birds had peaked by 8 hrs (Times based on PAH/Iohexol administration, which was 2 h after diclofenac administration) (Fig 3-3). The birds that died (9192, 9194) had very high levels of plasma uric acid, which was more than 10 times the basal concentration as well as a more prolonged increase in uric acid concentrations (Table 3-5). Concurrent to the plasma uric acid increase, faecal uric acid concentrations of the treated birds and plasma uric acid clearance decreased compared to the untreated birds, with four diclofenac treated birds showing a 99% decrease (Table 3-5) i.e. the increase in plasma uric acid occurred concurrently with a decrease in the uric acid clearance. Of the four birds in which the excretion of uric acid had decreases, two had died. For these two birds the uric acid faecal concentrations were reduced to almost 0. While $Tm_{uricacid}$ had decreased for all birds, it had was virtually absent for three birds including the two that died (Table 3-4). There was poor correlation between plasma and faecal changes. The area under the curve last (AUC_{last}) showed that the birds that died had at minimum a two-fold higher total exposure to uric acid than the birds that survived and a ten-fold increase from when

healthy. The cumulative result thus showed a strong relationship between death and absence of uric acid excretion, mainly at the level of the tubules.

Table 3-4: Clearance of the PAH and Iohexol, and related calculation of the PAH tubular excretion

Animal	$Cl_{PAH(L/kg*kg)}$			$CL_{iohexol}$ (GFR)$_{(L/kg*kg)}$			Tm (PAH)		
	Ctrl	DF	% Inhibition	Ctrl	DF	% Inhibition	Ctrl	DF	% Inhibition
9189	4.48	6.29	no inhibition	0.13	0.04	72.9	4.35	6.25	no inhibition
9190	80.16	3.56	95.56	0.18	0.02	87.01	79.98	3.53	95.58
9191	79.39	43.16	45.63	0.25	0.07	70.76	79.14	43.09	45.56
9192	11.65	0.19	98.35	0.13	0.02	87.54	11.52	0.18	98.47
9194	12.38	0.24	98.08	0.2	0.03	83.79	12.18	0.2	98.32
9195	6.01	0.48	91.98	0.14	0.05	64.99	5.87	0.43	92.63
Mean	32.34	8.99	71.6	0.17	0.04	77.83	32.17	8.95	71.76
SEM	15.05	6.91	16.54	0.02	0.01	3.89	15.04	6.9	16.59

Cluric acid= Uric acid clearance calculated from plasma and faecal uric acid levels; Cl_{PAH}= PAH clearance; $Cl_{iohexol}$= iohexol clearance; Tm (PAH)= maximum tubular transport rate of PAH; Ctrl= control; Highlighted cells are the results for the two birds that died.

Table 3-5: Pivotal uric acid parameters calculated for the study

Animal	$F_{uric\ acid\ (\mu g)}$			$P_{uric\ acid\ (mmol/L)}$			$Cl_{uric\ acid\ (L/kg*kg)}$			$AUC_{uric\ acid\ (\mu g/ml*h)}$		
	Ctrl	DF	% decrease	Ctrl	DF	% increase	Ctrl	DF	% Inhibition	Ctrl	DF	Increase
9189	208.48	161.56	22.5	0.3	2.98	1002	1.97	0.18	no inhibition	568.88	2123.57	3.73
9190	211.86	134.46	36.53	0.26	3.39	1288	4.13	0.14	96.54	717.66	3592.17	5.01
9191	2588.93	84.14	96.75	0.32	0.33	101	26.53	0.77	97.1	571.41	881.23	1.54
9192	58.01	0.72	98.76	0.24	4.83	2057	7.85	0	99.99	576.62	6768.78	11.74
9194	284.21	4.6	98.38	0.19	0.32	168	27.34	0.05	99.83	484.83	6381.96	13.16
9195	3905.54	21.29	99.45	0.36	6.2	1721	33.5	0.06	99.81	889.89	3161.98	3.55
Mean	1209.5	67.79	75.34	0.28	3.01	1056.12	16.89	0.2	82.21	634.88	3818.28	6.01
SEM	667.11	28.38	14.63	0.02	0.97	326.68	5.61	0.12	16.45	59.49	953.21	1.96

Furic acid=faecal uric acid; Puric acid= Plasma uric acid; Cluric acid= Uric acid clearance calculated from plasma and faecal uric acid levels; AUC – area under the uric acid plasma curve; Ctrl= control; Highlighted cells are the results for the two birds that died.

Table 3-6: Uric acid secretion by the glomerulus and renal tubule at the 12 hrs sample point, and collected total faecal output for 4hrs

Animal	Excreted UA (mg)	Body weight(kg)	Normalized by BW UA excreted mg/min	Plasma UA (mmol/L)	Plasma (mg/mL)	GFR (ml/min	Turate (mg/h)	%tubular Cl
Phase 1								
9189	208.48	1.28	0.87	0.30	0.05	0.11	0.76	87.35
9190	211.86	1.50	0.88	0.26	0.04	0.13	0.75	85.28
9191	2588.93	1.44	10.79	0.32	0.05	0.22	10.56	97.94
9192	58.01	1.27	0.24	0.24	0.04	0.09	0.16	64.24
9194	284.21	1.33	1.18	0.19	0.03	0.11	1.08	90.90
9195	3905.54	1.30	16.27	0.36	0.06	0.14	16.13	99.12
Mean	**1209.50**	**1.35**	**5.04**	**0.28**	**0.05**	**0.13**	**4.91**	**87.47**
SD	**1634.07**	**0.10**	**6.81**	**0.06**	**0.01**	**0.05**	**6.78**	**12.67**
Phase 2								
9189	161.56	2.16	0.67	2.98	0.50	0.30	0.37	55.70
9190	134.46	2.99	0.56	3.39	0.57	0.22	0.34	61.20
9191	84.14	2.80	0.35	0.33	0.05	0.07	0.28	81.19
9192	0.72	2.54	0.00	4.83	0.81	0.22	0	
9194	4.60	2.67	0.02	0.32	0.05	0.03	0	
9195	21.29	2.92	0.09	6.20	1.04	0.86	0	
Mean	**67.79**	**2.68**	**0.28**	**3.01**	**0.51**	**0.28**	**0.17**	**33.02**
SD	**69.51**	**0.30**	**0.29**	**2.37**	**0.40**	**0.30**	**0.19**	**37.15**

UA = uric acid; Bw= body weight

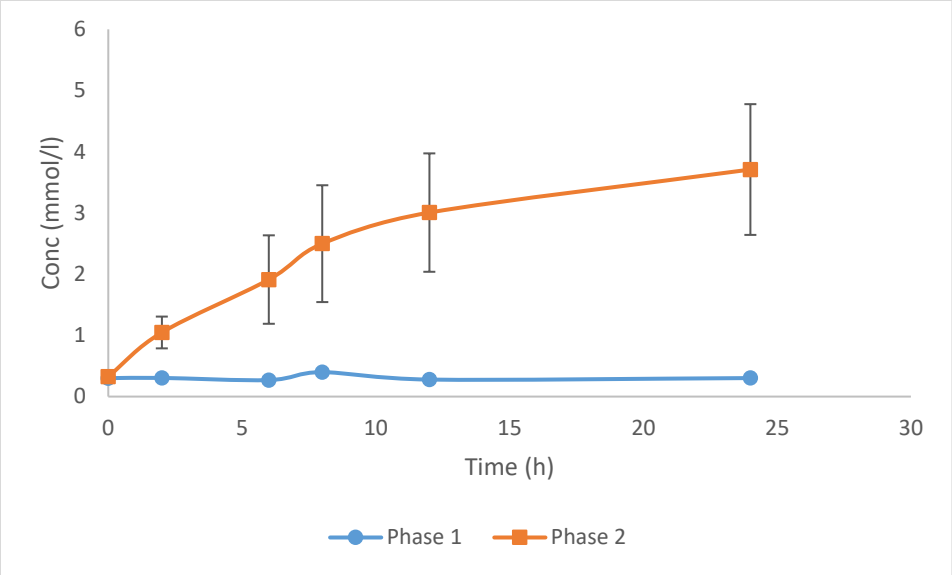

Fig 3-3: Plasma uric acid of the chicken over the period of time indicating the time taken to reach maximum. Phase 1 =untreated birds; Phase 2 =treated birds.

3.3.5. PAH and iohexol clearance

For phase 1, PAH was not detectable in the plasma for two birds (9190 and 9191) and for two time points for the remaining four (9189, 9192, 9194, and 9195). As a result, it is concluded that PAH is rapidly cleared in the chickens under normal conditions (Fig 3-4 for mean changes while Fig 3-5 illustrates the individual bird changes). The clearance was estimated as presented in Table 3-6, using the LOQ as the first point value. The results were also highly variable in phase 2 between the birds. The PAH clearance decreased from phase 1 to 2 with an average clearance of 32.34 ± 15.05 and 8.99 ± 6.91 L/h*kg respectively (Table 3-6). For five of the treated birds, diclofenac induced an increase in the total exposure to PAH, as seen by the $AUCl_{ast}$ (Table: 3-5). The increase in PAH plasma concentrations was associated with a decrease in plasma clearance by 71.60 ±16.54 % (Table: 3-6). For the two birds (9192, 9194) that succumbed to the toxicity, PAH clearance decreased by 98% (Table 3-6). The plasma concentrations for iohexol was more quantifiable (Fig 3-6). The same trend was evident for iohexol, however with clearance decreased by up to 87%. The highest inhibition in the clearance of iohexol was present in both a bird that died and for one that survived (Table 3-6).

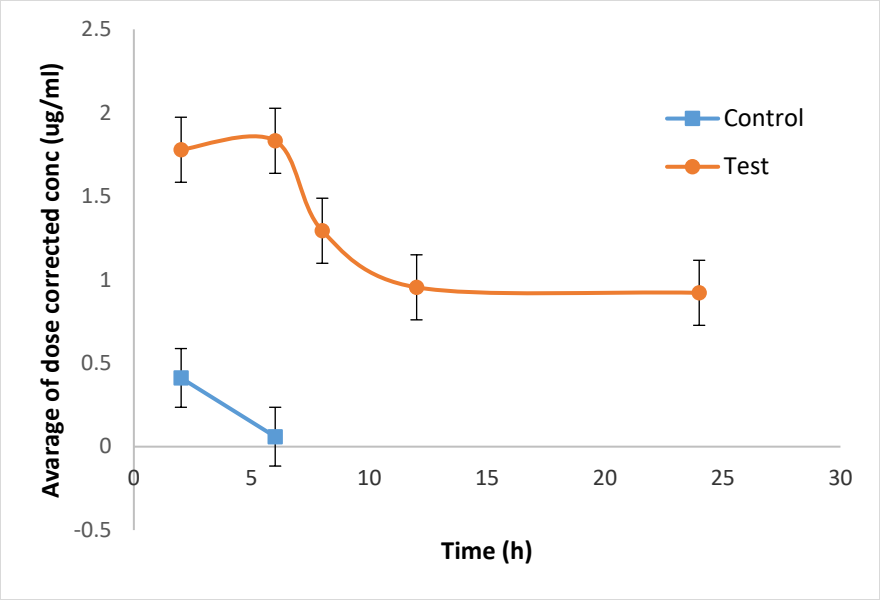

Figure 3-4: Mean PAH concentration vs time as an indication of uric acid transporter inhibition over a period of 24 hrs, error bars= standard error.

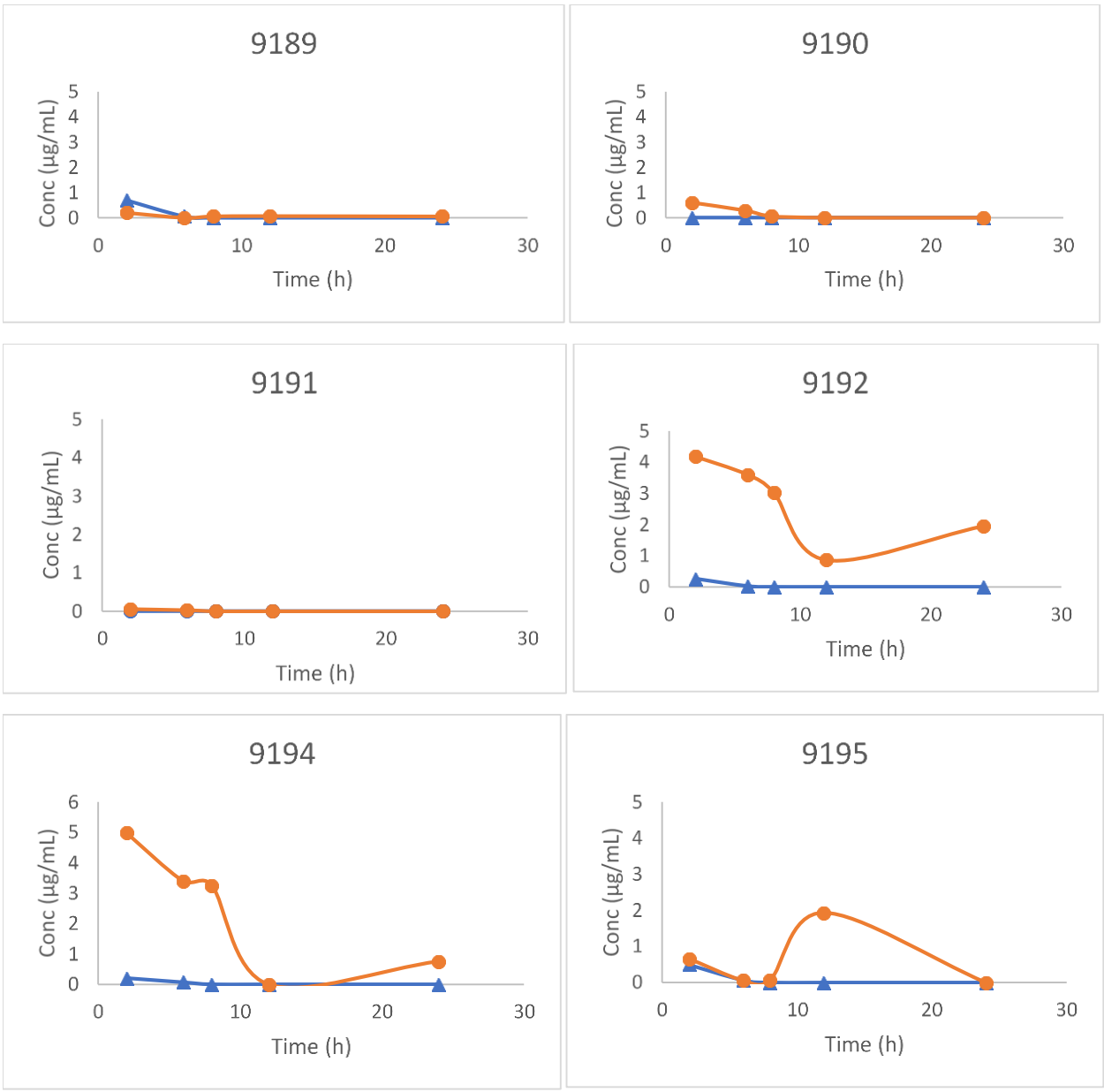

Figure 3-5: PAH concentration over time for the individual birds as an indication of uric acid transporter inhibition over a period of 24 hrs period in phase 1 (triangle) and phase 2 (circle). Birds 9194 and 9192 died with serve visceral gout.

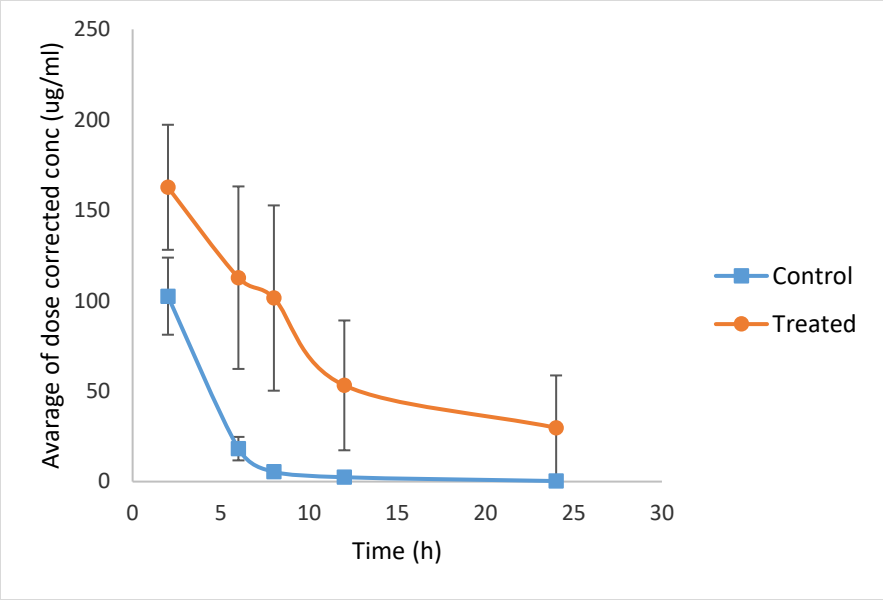

Figure 3-6: Mean iohexol concentration over time as an indication of glomerular filtration rate over a period of 24 hrs, error bars= standard error.

3.3.6. Change in tubular clearance of uric acid at the first signs of toxicity.

The tubular secretion of PAH (Tm_{PAH}) were reduced by more than 90% for all the birds except one bird which was reduced by 45%. Furthermore, it was also interesting to note that the birds that died the Tm_{PAH} was reduced by more than 98% (Table 3-6). Tubular contribution to excretion, based on area under curve for uric acid, decreased significantly after exposure to diclofenac from 20 477.58 ± 9 893.21 to 11 302.62 ± 5 824.65 L/h*kg with the percentage decrease of 50% of the control. The GFR contribution varied per individual, with GFR decreasing for only some birds (Table 3-5, 3-6).

3.3.7. Comparing uric acid level of domestic chicken untreated/treated with diclofenac and cape vulture

Plasma uric acid concentrations of domestic chicken (untreated and treated) and cape vulture ranged from 0.202-0.543, 0.250-6.196 and 0.108-0.258 mmol/l respectively. As mentioned above uric acid clearance of chicken treated with diclofenac significantly decreased over time, and at 12 hrs was 0.026 ± 0.41 ml/min while the control was 15.37 ± 19.01 ml/min. In comparison the two vultures evaluated had uric acid clearance at 10 hrs of 1.58 and 18 ml/min respectively. Considering that there likely is diurnal variation and that the lower value may be indicatory of a

point of inactivity, at the upper end the two species have similar clearance (Table 3-7). However considering that the vultures were approximately 7.5kg in body weight, their uric acid clearance by size is roughly half that of the chicken at 2.41 and 5.71 ml/min/kg respectively.

Table 3-7: Average uric acid clearance of the plasma and faecal uric acid in both groups of domestic chicken.

		Plasma UA (µMol/mL)		Faecal UA (µMol)		Cl (mL/min)		Plasma AUClast-UA (mg/L)	
Time point	Bird ID	P1	P2	P1	P2	P1	P2	P1	P2
8hrs	9189.00	0.36	0.97	1.76	31.47	0.04	0.27		
	9190.00	0.54	2.47	179.57	40.26	2.76	0.14		
	9191.00	0.39	0.46	486.83	6.06	10.54	0.11		
	9192.00	0.42	5.25	606.16	0.51	12.14	0.00		
	9194.00	0.25	5.46	1586.07	0.88	52.66	0.00		
	9195.00	0.44	0.39	436.50	122.65	8.33	2.65		
	Mean	**0.40**	**2.50**	**3296.88**	**201.84**	**14.41**	**0.53**		
	SD	**0.10**	**2.34**	**553.4**	**46.7**	**19.29**	**1.05**		
12hrs	9189.00	0.30	2.98	208.48	161.56	2.92	0.23	568.88	2123.57
	9190.00	0.26	3.39	211.86	134.46	3.36	0.17	717.66	3592.17
	9191.00	0.32	0.33	2588.93	84.14	33.50	1.08	571.41	881.23
	9192.00	0.24	4.83	58.01	0.72	1.03	0.00	576.62	6768.78
	9194.00	0.19	0.32	284.21	4.60	6.23	0.06	484.83	6381.96
	9195.00	0.36	6.20	3905.54	21.29	45.20	0.01	889.89	3161.98
	Mean	**0.28**	**3.01**	**7257.02**	**406.77**	**15.37**	**0.26**	**634.88**	**3818.28**
	SD	**0.06**	**2.37**	**1634.1**	**69.5**	**19.01**	**0.41**	**145.7**	**2334.87**

UA= Uric acid; Cl= clearance; P1= untreated phase; P2= treated phase; AUClast-UA= Area under the curve to the last quantifiable time point for plasma uric acid. The Faecal uric acid is the total uric acid in faeces excreted over a 2h period.

3.3.8. Radiography

The kidneys were not visible in any of the treated birds irrespective of the phase of the study, which was not too unexpected since the kidneys are firmly attached to the renal fossae (Havenga et al, 2016). The iohexol was already evident in the cloaca after 5 min. While subjective, the amount of iohexol appeared to present at a lower concentration in the cloaca in the birds in phase 2, 5 min after its administration, with exception of 9189. The results for bird 9192, which died,

was most clearly evident as seen in the Figure 3-7. In the figure, the cloaca of the bird in the absence of diclofenac is more densely strained than in its presence.

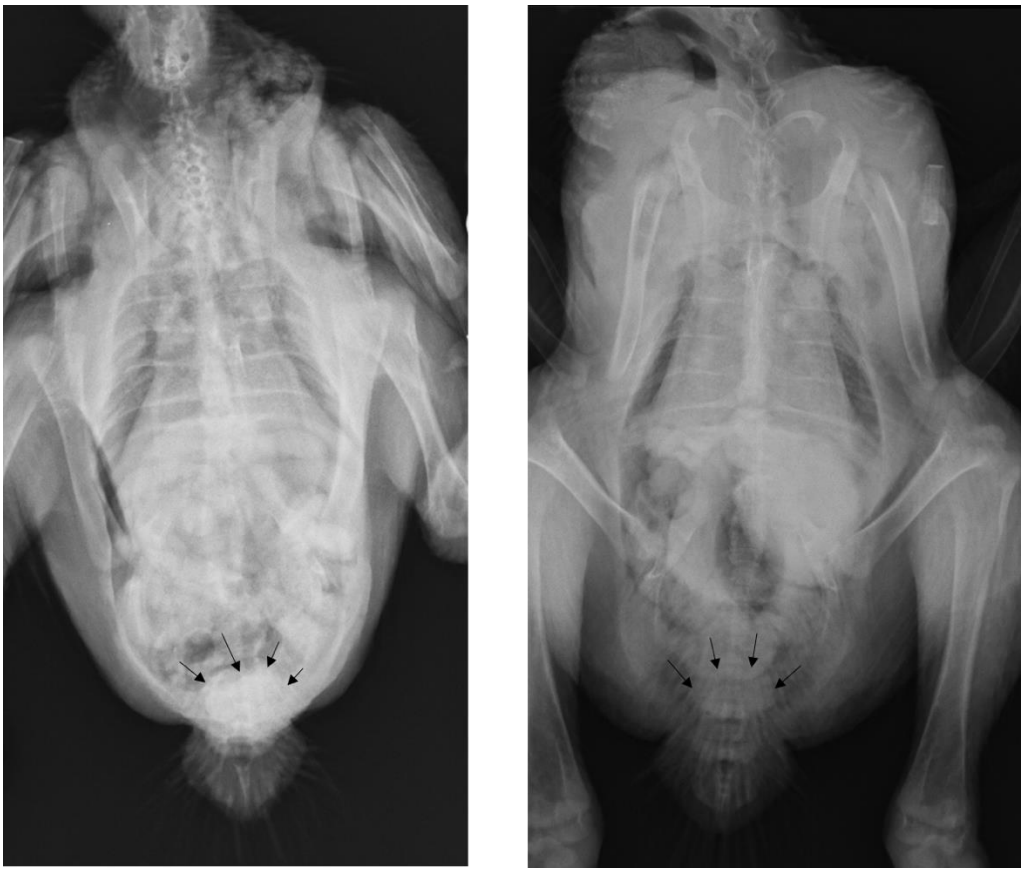

Fig 3-7: Radiographs of chicken 9192 five minutes after iohexol administration. The iohexol is more dense in the cloaca (arrows) in the absence of diclofenac (left) than with (right).

3.4. Discussion

Diclofenac toxicity in the vultures and chickens is characterized by major increases in plasma uric acid and subsequent gout (Oaks et al., 2004; Meteyer et al., 2005; Jain et al., 2009; Reddy et al., 2006; Naidoo et al 2007). With uric acid excretion known to be facilitated by active tubular transport via the OAT channels, for this study we investigated the effects of diclofenac on the functionality of the OAT channel using a channel specific marker (Sweet, 2005, 2010; Van wert et al., 2010). From mammalian physiology it is known that diclofenac has the ability to inhibit both the functions of the OAT channel, reduce blood supply to the kidney and alter glomerulus filtration (Khamdang et al., 2002; Anzai et al., 2006; Katzung et al., 2009). However unlike in mammals where glomerular filtration of uric acid is very efficient, birds excrete more than 80% of

their total uric acid using the OATs channels (Berger et al., 1960). This aligns with the findings of this study where 5 birds where excreting more than 85% of their total uric acid using former channels expect for one bird that was somewhat lower at 64.24%. Further for the efficient plasma clearance of uric acid by the OAT channels, adequate blood supply needs to be maintained to the renal tubules. From previous studies the glomerulus receives only arterial blood supply, while the avian renal tubules received an equal admixture of venous and arterial blood as ascertained through PAH clearance studies (Shideman et al., 1981). With diclofenac known to inhibit both tubular excretion and blood supply, both these mechanisms could explain the diclofenac induced increase evident in the chicken and vulture. Specifically, for this study we investigate the pathophysiology of diclofenac's toxicity using iohexol and PAH as marker substances. PAH is a substrate for OATs channels which are located on the basolateral membrane of the renal proximal tubules and iohexol is a marker for renal blood flow and glomerulus filtration.

Following the administration of 10mg/kg diclofenac as a single dose, mortality of 33% within 48 hrs were recorded, which was similar to previous mortalities of 40% evident in white leghorn after 12 days of exposure to 10 mg/kg of diclofenac (Jain et al., 2009 and Reddy et al., 2006) and the LD_{50} of 10mg/kg reported after a single dose exposure in layer hens (Naidoo et al 2007). Furthermore histopathology changes observed in 50% of the domestic chicken following diclofenac exposure were similar to those reported in the Leghorn and *Gyps* species (Meteyer et al., 2005; Oaks et al., 2004; Naidoo et al., 2007, 2009; Jain et al., 2009; Swan et al., 2006b). The pathological changes seen in the birds that died were identical to what had been reported previously in the vulture. The results obtained in this study thus confirmed the applicability of the study to evaluate the pathophysiology of diclofenac. It was interesting to notice that one bird which had mild urate necrosis did not succumb to toxicity within the 48 hrs monitoring period. This implies that the difference observed in the former and other surviving birds are subject to a degree of intra-subject variation which may be attributable to genetic, environmental and epigenetic differences (Fusco and Minelli, 2010; Frésard et al., 2013).

The iohexol clearance of 0.17 L/h*kg (2.83 ml/min*kg) for phase 1 of the study was similar to the iohexol clearance of 2.94 ml/min*kg reported by Dhondt et al. (2019), which supports the results from this study being accurate. Following diclofenac exposure, the latter birds showed a decrease

in iohexol clearance compared to the untreated birds. Iohexol clearance ranged from 0.17 ± 0.02 to 0.04 ± 0.01 L/h*kg in untreated and treated respectively with the highest level (being present in a bird each that died or survived). It was also interesting to note that due to the higher plasma concentration, despite the lower GFR, the predicted total uric acid filtered was higher than for phase 2 for some of the birds. Nonetheless this change in GFR for birds in phase 2, indicates that diclofenac consistently decreased the blood supply to the kidneys in all the birds, and thus alter the rate of delivery of uric acid to the excretion points. This finding is not unusual as evident in elderly people on oral doses of diclofenac (50 mg three times a day), where diclofenac was reported to impair the renal blood flow and glomerular filtration rate leading to renal failure (Katzung et al., 2009). Diclofenac suppresses the synthesis of prostaglandin by inhibiting enzyme COX. Prostaglandins play a physiologic role in maintaining glomerular filtration rate (GFR). Prostaglandins predominately function to regulate renal blood flow primarily supplied via the afferent arterioles and as a renal vasodilator (Verlander, 1997; Oates et al., 1998). Naidoo et al. (2007) were able to demonstrate using chicken venous tissue that diclofenac was also a venodialatory substance. Based on the similarity between human and chicken COX2 enzymes, it is likely that COX inhibition drives this process in the chicken (Botting, 2006).

For the four birds in which PAH clearance was properly quantifiable, their PAH clearance of 143.8 ml/min*kg compared well with Dhondt et al. (2019) who described PAH clearance of 117.5 ml/min*kg. As for iohexol, the PAH clearance of the treated birds decreased following diclofenac administration from 32.34 ±15.05 to 8.99 ± 6.91 L/h*kg. This change for PAH was however, not at a constant level of change in treated birds and was very markedly changed in the two birds that died with a decrease in the rate of PAH clearance before 12 hrs. The necropsy of these dead birds further revealed urate deposits on kidney and other organs. Urate deposits were associated with very high plasma uric acid concentration which had multiplied by more than 10-fold from its original concentration, reaching maximum of 6,196 mmol/l from 0.250 mmol/l in one bird. With iohexol clearance being constantly decreased and PAH clearance being almost negligible (Tm_{PAH} and Cl_{UA} both reduced by more than 98% furthermore with $Tm_{uricacid}$ been abesent) in only the two birds that died, it appears the total inhibition of OATs channels for a prolonged period when it does occurs, would explain the high level of plasma uric acid and subsequent death. The results partially also support the in vitro findings of Naidoo et al. (2009) in which it was demonstrated that renal tubular secretory cells were susceptible to the toxic effect of diclofenac because of

inhibition of the PAH transporters independent of blood supply (Naidoo et al., 2009). The effect of the diclofenac on PAH clearance was not surprising as both sodium salicylate and phenylbutazone have previously shown to inhibit PAH clearance in the chicken by 90 and 50% respectively, albeit at much higher doses (Berger et al., 1960).

With uric acid clearance mediated by PAH transporters being the point of diclofenac inhibition, the results from this study also offer some insight into the sensitivity of the vulture over the chicken, as the uric clearance in the chicken was two-fold higher than the vulture despite the high protein diet of the vulture when clearance was corrected for body mass . In general, it is accepted that the main sources of uric acid in protein diet is red meat especially beef, lamb, veal and pork which are the main meals for vultures (Teng et al., 2015).

The unexpected finding in the pharmacokinetic study, was that for the majority of the birds diclofenac was completely metabolized before the 2 hrs sampling point indicating that the half-life was under 0.5 hrs, as five half-lives are required for complete elimination. This was markedly different for the vulture with a half-life of 14 hrs as recorded by Naidoo et al. (2007) in adult layers. The first implication of this is that the toxicity in the two chickens that died, was only evident quite a substantial time after the drug was removed from the system which can only be indicative of irreversible channel inhibition and would explain why a recovery model (hysteresis) could not be fitted to the vulture data. More so, it would indicate that the channel sensitivity in the vulture and chicken are likely to have an inhibitory level somewhere above 4 µg/ml (assuming a half-life circa 30 min), based on a sample being at half the limit of quantification at 0.125 µg/ml at 2 hrs and five half-lives to reach this level. Using plasma uric acid concentrations as a marker, with some of the birds showing recovery by 8 hrs, we estimated that the lowest period of inhibition is 8 hrs and for fatal cases inhibition needs to be longer than 12 hrs. The latter is not surprising as only prolonged high concentration of uric acid in the plasma would eventually result in tissue precipitation and cellular death (Lumeij, 2008). When comparing the chicken to the vulture, the maximum plasma concentration for the vultures that died were 7.41 and 4.6 ug/ml respectively (Naidoo et al., 2009), which is above the concentration 4 µg/ml estimated for the chicken. This despite the vulture being exposed to a dose of only 0.8 mg/kg as opposed to the 10 mg/kg in the chicken. Based on the difference in dose between the species, this would once again support metabolic constraint in

vulture resulting in plasma concentration that inhibit uric acid excretion despite the significantly lower dose.

3.5. Conclusion

From this study we conclude that diclofenac mechanism of toxicity is due to the inhibition of both renal blood supply and the organic anionic transporters as seen in mammals. However the effect is only fatal when the inhibition or tubular uric acid secretion is near 100%. For the actual inhibition of the OAT channels, the effect involves a degree of non-competitiveness since the uric acid increased despite diclofenac being non-detectable in the plasma.

3.6. Acknowledgments.

This study was approved by animal ethics committee of the University of Pretoria. The use of vulture tissue, a CITES protected species. was approved by the South African Department of Environmental affairs. The research was partially financed by the National Research Foundation (NRF), Health and Welfare SETA and the University of Pretoria. The authors would like to express gratitude to Ms A van Wyk and the other staff at the UPBRC for caring for the chickens.

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CHAPTER FOUR

4. Molecular characterization of *Gyps Africanus* (African white-backed vulture) Organic Anion Transporter 1 and 2 expressed in the kidney.

Bono Nethathe[1], Phaswana Rephima[2], Aron Abera[3], Vinny Naidoo[1]

[1] Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, Pretoria, South Africa.

[2] Department of Pathology, Faculty of Veterinary Science, University of Pretoria, Pretoria, South Africa.

[3]Inqaba Biotechnology, Pretoria, South Africa

4.1. Introduction.

In birds as for other species, the kidneys contribute to the maintenance of homeostasis by removing exogenous and endogenous organic anions including several clinically used drugs (with diverse chemical structures) with tubular secretion being an important process (Muller and Jansen, 1997; Meier, 1995; Ullrich and Rumrich, 1993 and Pritchard and Miller, 1993). The proximal tubules in the kidney are essential for the latter (Wang and Sweet, 2013). Despite their important role in homeostasis, the proximal tubule cells are a physiological barrier to the passive diffusion of charged, hydrophilic molecules from blood into the glomerular filtrate. To overcome this, the cells make use of dozens of membrane-bound transporter proteins. These transport proteins fall into two main superfamilies, the ATP binding cassette and the solute carriers (SLC) transport proteins (Sweet, 2005; He et al., 2009; Van Wert et al., 2010). For this study I focus on the organic anion transporters (OATs) that falls under SLC22A family (Sweet, 2005, 2010; Van wert et al., 2010), which is responsible for the cellular uptake of organic anions (Kusuhara et al., 1999; Sekine et al., 1997). At least four SLC22A genes have been identified, which encode the OATs: SLC22A6 which encodes OAT1, SLC22A7 which encodes OAT2; SLC22A8 encoding OAT3 and lastly SLC22A11 which encodes OAT4 ((Burckhardt, 2012; Pavlova et al., 2000; Sweet et al., 1997; VanWert et al., 2010).

Through the cloning of the various OAT isoforms in humans, studies have provided some insight of function, tissue distribution, regulation and energy dependence within the OAT family (Pritchard and Miller, 1993; Sweet and Pritchard, 1999; Wright and Dantzler, 2004; Burckhardt and Wolff, 2000; Burckhardt and Burckhardt, 2003). In humans OAT1 and OAT3 are localized in the basolateral membrane of renal proximal tubule cells. They mediate the movement of organic anions like uric acid from the blood into the cells through dicarboxylate/organic anion exchange. At the same time a sodium-dependent dicarboxylate (NADC-3) co-transporter facilitates the transfer of the excreted dicarboxylate back into the cells. From within the cellular cytoplasm uric acid is secreted by multiple resistance protein (MRP2 and MRP4) channels located on the apical cell membrane of the renal tubule. Substrate and urate reabsorption via dicarboxylate or hydroxyl ions and monocarboxylate exchange is also facilitated by OAT4 and URAT1 respectively which are also localized in the apical membrane (Sweet, 2005, 2010; Van wert et al., 2010). In birds the process is the same as humans, with the exception that the later reabsorption of uric acid does not occur. While OAT2 transport mechanism is not as fully understood, the few available studies indicate that OAT2 mediated organic anion uptake, is Na^+-independent and was *trans*-stimulated by dicarboxylates like OAT1 and 3, which led to the argument that OAT2 is not energetically coupled to the Na^+ and α-KG gradients as part of the tertiary active basolateral organic anion uptake system. It was thus concluded that OAT2 functions as an apical facilitated-diffusion, efflux carrier in renal proximal tubular cells (Kobayashi et al., 2002b, Sekine et al., 1998).

In addition to the natural anionic substances, the OATs are also involved with the excretion of a number of drugs. OAT1 substrates include nonsteroidal anti-inflammatory drugs, antiviral drugs, β-lactam antibiotics, N-acetyl-L-cysteine conjugates, uremic toxins, diuretics, vitamins and hormones (Burckhardt and Burckhardt, 2003). Moreover OAT3 isoform also share many of the above substrates and inhibitors as OAT1 (Burckhardt and Burckhardt, 2003). Studies have also revealed that probenecid and other uricosuric drug used in the prevention of gout, inhibits OAT1 mediated transport of many organic anions (Cuthbert et al., 2006). As a result of their functioning in the maintenance of homeostasis, factors interfering with these channel functioning can lead to metabolic imbalances and toxicity.

After establishing the mechanism of toxicity of diclofenac in vulture using the chicken as the model species (see chapter three), it is evident that toxicity results from the inhibition of PAH molecular transporter, which is a specific substrate for OAT1, OAT2 and OAT3. As such is it not surprising that diclofenac, and other NSAIDs, induce visceral and articular gout in Gyps vulture. What was not clear from the chicken study, was wheather the uric acid transporters are present as the same type and in the same concentration as in the vulture. The OAT channels in the *Gyps Africanus* vulture were evaluated in comparison to published information for the chicken.

4.2. Material and methods.

4.2.1. Sample collection

Before the commencement of experiments, ethical clearance was obtained from the Animal Ethics committee of University of Pretoria (V108-16, V027-18). Two AWB (*Gyps africanus*) vulture of unknown age with a badly fractured wing, five healthy mice (*Mus musculus*) and two healthy chicken (*Gallus gallus domesticus*) were euthanized at Faculty of Veterinary Science, University of Pretoria. The latter animals were never treated with any analgesic before. The kidneys were harvested immediately after euthanization and stored in cryogenic vials containing RNA*later* (Whitehead Scientific, Cape Town, South Africa) for molecular assays or in buffered formalin for immunohistochemistry. The vials were stored at -80°C freezer until analysed.

4.2.2. OATs studies on AWB vulture and domestic chicken kidneys using published primers.

Total RNA was extracted from kidney samples of one of the AWB vulture and a chicken using the RNeasy plus mini kit (Qiagen) according to the manufacturer's instructions. The RNA was eluted in 50 µl RNase-Free water and stored at -80°C for reverse transcription. Total RNA was checked for quality and integrity using Nanodrop spectrophotometer and gel electrophoresis (1 % agarose gels with ethidium bromide) respectively. RNA was subsequently denatured to remove secondary structure before cDNA synthesis. Reverse transcription was conducted using ClontechSmart MMLV Reverse Transciptase kit according to the manufacture`s instruction. The integrity and quality of the cDNA was confirmed by gel visualisation and a nanodrop spectrophotometer. cDNA of vulture and chicken were used as a template and OAT3 chicken primers forward

(5`CCCTTCTTCCTCTTCTTCCTCG-3` and reverse 5`-TGGATCAGATAAATGCTGACCCC-3`) used by Dudas et al. (2005) for the partial amplification of OAT3-like gene in the chicken proximal epithelial cells (Primer supplied by Integrated DNA Technologies, South Africa). PCR reactions were prepared using Fermentors kit following manufacturer's instructions. The amplification conditions were as follows: 38 cycles with a denaturing temperature of 94°C, an annealing temperature 61.6°C and an elongating temperature of 72°C (Dudas et al., 2005). One (1) % agarose gel stained with ethidium bromide was used to separate PCR products. Amplicons were sliced out and submitted to Inqaba Biotechnology (Pretotia, South Africa) for sequencing using ABI 3500X1 genetic analyser. BioEdit (Hall, 1999) and Clustal omega (Sievers et al., 2011) were used to analyse the forward and the reverse sequences obtained. Alignment comparison of the obtained consensus sequences and the sequence (BBSRC Chick EST ID 603812145F1) used by Dudas et al. (2005) was carried out using Clustal omega (Sievers et al., 2011). The obtained sequence was also further analysed using BLASTn algorithm on National Centre of Biotechnology (NCBI) (Altschul et al., 1990).

4.2.3. Next Generation Sequencing

Fresh Total RNA was extracted from the above AWB vulture kidney as previously mentioned and transported to Agricultural Research Council (ARC), (Onderstepoort, Pretoria, South Africa) on dry ice for sequencing. DNA contamination was removed from total RNA using DNase. The RNA fragments were reverse transcribed into cDNA and sequencing adaptors were ligated. Finally, the ends of the cDNAs were sequenced using Illuminia Truseq mRNA stranded Ran preparation kit on Hiseq 2500 v4 2x125bp chemistry model. The result obtained from sequencing was 50 million short reads which were 125 nucleotides long each. Prior assembly of the raw reads, several quality control steps were conducted on the raw reads using the online Galaxy platform (Blankenberg et al., 2010) and fast quality check (FASTQC) (Andrews, 2014) to check overall quality of the sequences, duplication and adapters. After checking the quality of the sequences, the adapters and PCR duplicates were removed using the programme Trimmomatic (Bolger et al., 2014). The pre-processed reads were assembled into transcripts using TRINITY (Grabherr et al., 2011). The assembled transcriptome was converted to a local blast database NCBI (Altschul et al., 1990) and OAT1 and OAT2 sequences were identified based on the predicted sequences from the Golden Eagle OAT1(XM_011601043.1); OAT2 (XM_011585794.1) and chicken (OAT2)

(>NM_001199438.1). The Golden Eagle was selected as another study completed in our laboratory has shown them to be closely related species from liver mitochondrial analysis (Unpublished data).

4.2.4. Immunohistochemistry Analysis

Immunohistochemistry was conducted on the kidneys following method previously described by Hwang et al., 2010. The kidneys were briefly perfused with phosphate buffered saline (PBS) at an osmolality of 298 mOsm/kg H2O (pH 7.4) to remove all blood, followed by perfusion with a periodate-lysine-percent paraformaldehyde (PLP) solution for 10 min. After perfusion, the kidneys were removed and sliced into sections (1-2 mm thick), which were further fixed by immersion in the same fixative overnight at 4°C. After fixation, kidneys were embedded in wax and cut transversely at a thickness of 4 µm using a microtome. Sections were processed for immunohistochemistry using an indirect immune peroxidase method. All sections were washed three times in PBS containing 50 mM NH4Cl for 15 min. The sections were first treated with a graded series of ethanol, and then incubated for 4 h with solution A (PBS containing 1 percent bovine serum albumin (BSA), 0.05 percent saponin, and 0.2 percent gelatin). The tissue sections were thereafter incubated overnight at 4°C with the antibodies (1:3000) diluted in solution A. Prior to incubation with the primary antibodies sequence analysis was used to first show similarity between the polyclonal rabbit anti-OAT3 antibody (Whitehead scientific, South Africa) binding site, corresponding to immunogen sequence KKEEGERLSL EELKLNLQKE ISLAKAKYTA SDLFRIPMLR.

After numerous washes in PBS containing 0.1percent BSA, 0.05 percent saponin, and 0.2 percent gelatin known as solution B, the tissue sections were incubated for 2 hours in avidin-biotin-peroxidase-conjugated Goat anti-rabbit IgG (H+L) Fab fragment (White head Scientific, South Africa) diluted 1:100 in solution C (PBS containing 1 percent BSA). The latter sections were rinsed in solution B, thereafter in 0.05 M Tris buffer (pH 7.6). To detect avidin-biotin-peroxidase, the sections were incubated in 0.1 percent 3,3'diaminobenzidine (DAB, Sigma) in 0.05 M Tris buffer for 5 min; H_2O_2 was added to a final concentration of 0.01 percent and the incubation continued for 10 min. The sections were washed three times with 0.05 M Tris buffer, dehydrated with a graded series of ethanol. All samples were examined with a light microscope. Mouse kidney samples were used to validate the method and as a positive control.

4.2.5. Confirmation of transcriptome OAT1 and OAT2 genes using Sanger sequencing.

Fresh total RNA was extracted from the recently harvested AWB vulture kidney tissue to prevent the effect of storage using Quick RNA Miniprep kit following instructions from the manufacturer (Zymo Research, USA). cDNA synthesis was performed using Lunascript RT supermix kit from New England Biolabs (NEB), (USA) according to the manufacturer`s instructions. Primers of OAT1 and OAT2 designed based on the transcriptome sequences generated from illuminia next generation sequencing. The special primers were synthesized at Inqaba Biotechnology, Pretoria, South Africa. Primers of OAT1 and 2 were as follows:

Table 4-1: Primers sequences used to amplify OAT1 and OAT2 gene from AWB vulture`s kidney.

OATs Primers	**Predicted amplicon length**
OAT1 Forward: GACCTTGTCTGCAGCTACCG	1453bp
OAT1 Reverse: CCAGAGCTGCTTTATTCCTCCAAG	
OAT2 Forward: CTCATGTTGCTGCTCCTTAGTACA	1776bp
OAT2 Reverse: CTAGGTGGACAGTAAAGGCTCTTT	

Using the latter set of primers, PCR was performed using One taq polymerase kit from NEB and AWB vulture`s kidney cDNA as a template. The amplification protocol was as follows: Initial denature 94^0C for 30 sec, 40 cycles of (denature 94^0C for 30sec; annealing 55^0C for 30sec and elongation 68^0C for 2min) and final elongation 68^0C for 5min. The PCR products was run on 1% agarose gel. The gel was viewed for desired bands using gel documentation system and PCR products of the desired bands was further cleaned up using Exopsup from NEB and sequences were generated using ABI 3500X1 genetic analyser.

4.2.6. In Silico analysis

The obtained forward and reverse OATs sequences of AWB vulture, from the primers listed in table 4-1, were aligned using Clustal Omega (Sievers et al., 2011) to obtain consensus sequences for both genes. The consensus sequences were also aligned with their respective NGS OATs sequence to determine their similarity.To identity evolutionary patterns of the obtained Sanger OAT1 and OAT2 sequences with other avian species, phylogenetic analyses were conducted. For the construction of the phylogenetic tree, 26 (OAT1) and 39 (OAT2) closely related avian species with more than 85% similarities with the AWB vulture OAT1 and 2 were retrieved from Genbank (NCBI) (Altschul et al., 1990). The avian species downloaded from Genbank (NCBI) belongs to the following orders: Papaeognathae, Galoanseres and majority of the species were Neoaves. The downloaded taxa were aligned using muscle algorithm (Molecular Evolutionary Genetics Analysis version 7.0 (MEGA7) software package (Edgar, 2004; Kumar et al., 2016). After alignments, the gaps were classified as missing data. The genetic distance and the statistics of the nucleotide composition of all the taxa were computed in MEGA7.0 version. The phylogenetic relationships were constructed using maximum likelihood (ML) estimate of Tamura-Nei model and General Time Reversible model (Tamura and Nei, 1993; Nei and Kumar, 2000). To assess nodal reliability, bootstrap analysis was conducted with 1000 replicates for the phylogenetic tree topologies (Felsenstein, 1985). The accession numbers of OATs (1 and 2) from different avian species included in the phylogenetic tree are listed in (Table 4-2).

Table 4-2: Avian OAT1 and 2 sequences used for phylogenetic analysis.

Species	**Common name**	**OAT 1**	**OAT 2**
Anas platyrhynchos	Mallard duck		XM_027453544.1
Anser cygnoides domesticus	Domestic goose	XM_013202223.1	XM_013194917.1
Aptenodytes forsteri	Emperor penguin	XM_019470643.1	XM_009272605.1
Aquila chrysaetos canadensis	Golden eagle	XM_011601043.1	XM_011585794.1
Balearica regulorum gibbericeps	East African grey crowned crane	XM_010301204.1	XM_010301419.1
Calidris pugnax	Ruff	XM_014966557.1	XM_014962397.1
Calypte anna	Anna`s hummingbird	XM_008496106.1	XM_008504985.1
Caprimulgus carolinensis	Chuck-will`s widow	XM_010173171.1	XM_010168880.1
Charadrius vociferus	Killdeer	XM_009883135.1	XM_009894862.1
Columba livia	Rock pigeon	XM_021301243.1	XM_005512038.2

Corvus brachyrhynchos	American crow	XM_017747256.1	XM_008635764.2
Falco cherrug	Saker falcon	XM_027811268.1	
Falco peregrinus	Peregrine falcon	XM_027792445.1	
Fulmarus glacialis	Northern fulmar	XM_009583211.1	XM_009587322.1
Gallus gallus	Chicken		NM_001199438.1
Gavia stellata	Red throated loon	XM_009814693.1	XM_009815441.1
Geospiza fortis	Medium ground-finch	XM_014311357.1	XM_005422609.1
Haliaeetus leucocephalus	Bald eagle	XM_010570679.1	
Lepidothrix coronate	Blue-crowned manakin	XM_017840174.1	XM_017818790.1
Lonchura striata domestica	Society Finch	XM_021545734.1	
Nipponia nippon	Crested ibis	XM_009460680.1	XM_009468586.1
Opisthocomus hoazin	Hoatzin	XM_009937221.1	XM_009939956.1
Pelecanus crispus	Dalmatian pelican	XM_009489468.1	XM_009478709.1
Phaethon lepturus	White-tailed trophic bird	XM_010292254.1	XM_010285480.1
Pseudopodoces humilis	Tibetan ground tit	XM_005533638.1	
Serinus canaria	Common canary	XM_018914013.1	XM_009096157.2
Struthio camelus australis	South African ostrich	XM_009671879.1	XM_009673139.1
Sturnus vulgaris	Common starling	XM_014893033.1	XM_014891915.1
Tinamus guttatus	White- throated tinamou	XM_010227345.1	XM_010217054.1
Tyto alba	Barn Owl	XM_009972042.1	
Apteryx australis mantelli	North island brown kiwi	XM_013942757.1	XM_013954347.1
Dromaius novaehollandiae	Emus	XM_026122546.1	
Chaetura pelagica	Chimney swift		XM_010005012.1
Chlamydotis macqueenii	MacQueen bustard		XM_010123992.1
Corvus cornix cornix	Hooded crow		XM_010411086.2
Egretta garzetta	Little egret		XM_009641620.1
Eurypyga helias	Sunbittern		XM_010161130.1
Ficedula albicollis	Collard flycatcher		XM_005042781.2
Haliaeetus albicilla	White-tailed eagle		XM_009922646.1
Leptosomus discolor	Cuckoo roller		XM_009961411.1
Meleagris gallopavo	Turkey		XM_003203777.3
Melopsittacus undulatus	Budgerigar		XM_005152033.2
Mesitornis unicolor	Brown roatelo		XM_010182673.1
Nestor notabilis	Kea		XM_010016946.1

Numida meleagris	Helmeted guineafowl		XM_021389777.1
Parus major	Great tit		XM_015620291.2
Phalacrocorax carbo	Great cormorant		XM_009502511.1
Picoides pubescens	Downy woodpecker		XM_009903833.1
Pterocles gutturalis	Yellow-throated sandgrouse		XM_010077045.1
Pygoscelis adeliae	Adélie penguin		XM_009331354.1
Tauraco erythrolophus	Red-crested turaco		XM_009990757.1
Zonotrichia albicollis	White-throated sparrow		XM_005486767.1

4.2.7. Protein Structure and modelling

The obtained Sanger OAT1 and OAT2 sequences were converted to protein sequences using Expasy Translate (Gasteiger et al., 2003) and the open reading frame was ascertained. Thereafter the deduced amino acids sequences were analysed by Swiss model Protparam (Gasteiger et al., 2003) and Phyre2 (Kelley et al., 2015) for prediction of OATs structures. The PROTTER sequence database was used to predict N-glycosylation sites (Omasits et al., 2013) moreover Expasy database was also used to investigate other possible post-glycosylation and phosphorylation sites (http://www.expasy.ch/ prosite). The protein sequences were also analysed according to the method of Mishra et al. (2014) using TrSSP (The Transporter Substrate Specificity Prediction Server) to ascertain if the predicted protein was likely an anionic transporter.

4.2.8. Expression of vulture and chicken OAT2 MRNA using Real time PCR (qPCR).

Total RNA was isolated from the kidney of the chicken and AWB vulture using Quick RNA Miniprep kit from Zymo Research (USA) following instructions from the manufacturer and reverse transcribed the former into cDNA using Lunascript RT supermix kit from New England Biolabs (NEB) (USA) following manufactures instructions. The chicken and vulture primers were designed and the reverse primer was designed to span exon-exon junction and were synthesized at Inqaba Biotechnology (Pretoria, South Africa) to amplify 128 and 126bp for chicken and vulture OAT2 gene respectively. Reverse transciptase (RT) qPCR was performed on AWB vulture and chicken cDNA (template) targeting GAPDH (housekeeping gene) and for negative control no template was added. Moreover, AWB vulture and chicken cDNA (template) were also run

targeting OAT2 conserved region and negative control was included as above. RT-qPCR reactions were performed using Luna Universal qPCR Mastermix (NEB) using gene specific primers: chickenOAT2_F: ACCATCTCCACTGAGTGGGAC, chickenOAT2_R: CGGCCGAACCTGTCTGAAAG and vultureOAT2_F: CATCTCCACGCAGTGGGAC, vultureOAT2_R: CGTCCGAACCTGTCTGAAAGG. All reactions were performed on CFX96 Real-Time PCR Detection System. The RT-qPCR thermal cycling conditions were as follows: 1 cycle at 95°C for 60 sec, 40 cycles of amplification at 95°C for 15 sec and annealing at 60°C for 30 sec. The average of the quantification cycle (Cq) was determined using manual quantification settings and was normalized using GAPDH Cq values. The formula used was as follows: normalized OAT2 Cq= OAT2 Cq - GAPDH Cq (Livak et al., 2001).

4.3. Results

4.3.1. cDNA identification of chicken and vulture OAT3 gene using published chicken OAT3 primers.

Amplification was achieved for the desired band in chicken while no amplification was achieved for the AWB vulture. The sequences of the amplicons were aligned and revealed a consensus sequence of 348 bp (Fig 4-1) while Dudas`s results had 344 bp of OAT3-like partial sequence (Dudas et al., 2005). The consensus sequence generated was aligned with OAT3-like partial sequence (BBSRC Chick EST ID 603812145F1, 556bp) using Clustal Omega with 97.11% similarity (Fig 4-2), indicating that the primers amplified the correct segment. In contrast the obtained consensus sequence analysed using the blastn algorithm (NCBI) revealed alignment with OAT1 gene of other avian species with a highest similarity of 83% with the *Pelecanus crispus* (the dalmatian pelican) (Fig 4-3). Furthermore Golden eagle also showed similarity of 81.40%. No chicken OAT3 sequence was present in the NCBI database. Moreover the OAT3 transporter, as far as we could ascertain has not been described in the Golden Eagle (*Aquila chrysaetos canadensis*) or any other birds species in the NCBI database.

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f       ------------------------------------GCATCTGCGCGCTGGTTGGT  20
r       TCCCTTCTTCCTCTTCTTCCTCGTCTCCTGGTGGTTGGCGGAATCGGCGCGCTGGTTGGT  60
                                           * *** **************

f       GCTGGCGGGGAAGGAAKAGAAGGCGGTGCGGATCCTACGGAGGGTGGCCAAAATCAACGG  80
r       GCTGGCGGGGAAGGAAGAGAAGGCGGTGCGGATCCTACGGAGGGTGGCCAAAATCAACGG  120
        ***************** ****************************************

f       CAGGGAGGAGGAGGGGGAGAAGATCACCGTGGAGAACCTGAAGTCCAGCATGAAGGAGGA  140
r       CAGGGAGGAGGAGGGGGAGAAGATCACCGTGGAGAACCTGAAGTCCAGCATGAAGGAGGA  180
        ************************************************************

f       GTTGGCAGCTCTGAAGTCCTCCTACACCGTTTCGGATCTGATCCGCACGCCCGTCATCCG  200
r       GTTGGCAGCTCTGAAGTCCTCCTACACCGTTTCGGATCTGATCCGCACGCCCGTCAWCMR  240
        ********************************************************** *

f       CCACATCTTCCTCTGCCTGTCCATCGTTTGGTTCTCCATCAGTTTCTCGTACTACAGCTT  260
r       SCACATCTTCCTCTGCCTGTCCATCGWWTGGTTCTCCATCAGTTTCTCGTACTACAGCCT  300
         ************************  ****************************** *

f       GGCCATGGACCTCCAGAACTTTGGGGTCAGCATTTATCTATCCAAAG       307
r       GSGCCCRTKWYT---------------------------------       312
        *  *       *
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Pane A

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TCCCTTCTTC  CTCTTCTTCC  TCGTCTCCTG  GTGGTTGGCG  GMATCKGCGC
GCTGGTTGGT  GCTGGCGGGG  AAGGAAGAGA  AGGCGGTGCG  GATCCTACGG
AGGGTGGCCA  AAATCAACGG  CAGGGAGGAG  GAGGGGGAGA  AGATCACCGT
GGAGAACCTG  AAGTCCAGCA  TGAAGGAGGA  GTTGGCAGCT  CTGAAGTCCT
CCTACACCGT  TTCGGATCTG  ATCCGCACGC  CCGTCATCCG  CCACATCTTC
CTCTGCCTGT  CCATCGTTTG  GTTCTCCATC  AGTTTCTCGT  ACTACAGCYT
GGSCMYGKAC CTCCAGAACT TTGGGGTCAG CATTTATCTG ATCCAAAG
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Pane B

Figure 4-1: A) Alignment of chicken OAT3-like forward and reserve sequence, F= forward sequence; R=reserve sequence. B) The consensus sequence, highlighted bases are degenerate bases.

Fig 4-2: Alignment of the obtained chicken partial OAT3 like gene and OAT3-like partial sequence (BBSRC Chick EST ID 603812145F1) using Clustal. D= BBSRC Chick EST ID 603812145F1; C= Chicken partial sequence from this study.

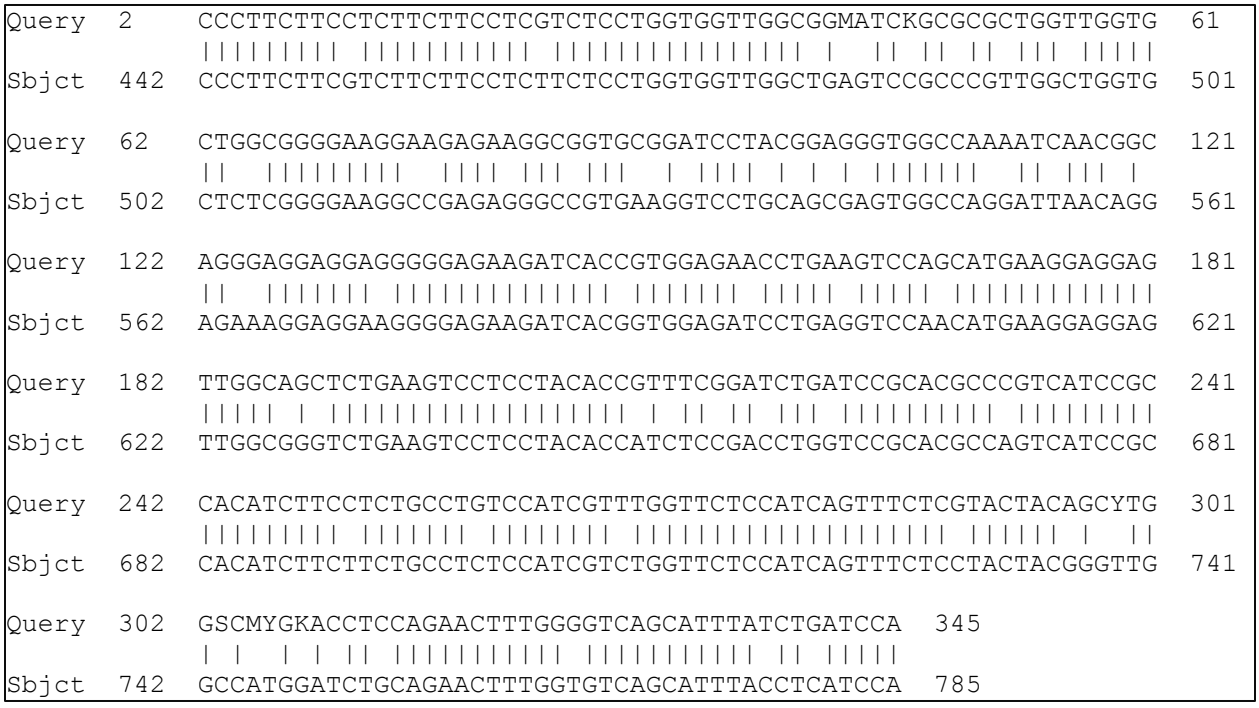
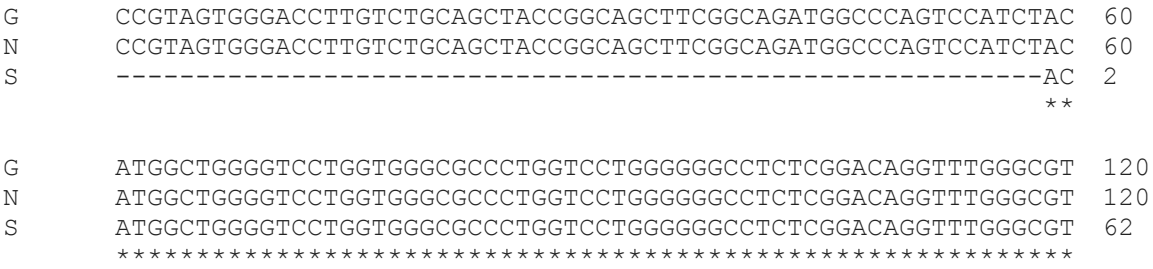


Figure 4-3: Alignment of predicted *Pelecanus crispus* OAT1- like MRNA (XM_009489468.1) and obtained chicken OAT3- like partial gene using blastn algorithm.

4.3.2. Next-Generation sequencing and Sanger sequencing results

After no amplification was achieved for AWB vulture, NGS analysis was undertaken. Quality control on the raw reads was good after PCR duplicates and adapters were removed and the transcriptome reads were submitted to NCBI and allocated sequence read archive (SRA) accession number PRJNA560189 thereafter assembled into a transcriptome using Trinity software. From the local blastn, using the sequences for OAT1 and OAT2 published for the golden eagle, hits against both sequences were identified in the transcriptome with similarity of 98.89% and 98.07% respectively (Fig 4-4). From this result it was concluded that OAT1 and OAT2 mRNAs are expressed in the AWB vulture kidneys.



Pane A

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G       AAGGCCATGTTGATGTGGTCCTACCTGCAGTTGGGGGTGATGGGGACGTGCACGGCCTTC  180
N       AAGGCCATGTTGATGTGGTCCTACCTGCAGTTGGGGGTGATGGGGACGTGCACGGCCTTC  180
S       AAGCCCATGTTGATGTGGTCCTACCTGCAGCTGGGGGTGATGGGGACGTGCACGGCCTTC  122
        *** ************************* ****************************

G       GCCCCCAACTACGCGTCCTACTGCGTCTTCCGCTTCGCCGGTGGCATGGCCCTCTCTGGC  240
N       GCCCCCAACTACGCGTCCTACTGCGTCTTCCGCTTCGCCGGTGGCATGGCCCTCTCTGGC  240
S       GCCCCCAACTACGCGTCCTACTGCGTCTTCCGCTTCGCCGGTGGCATGGCCCTCTCTGGC  182
        ************************************************************

G       TTCGGCCTCAGCATCGCCTGCCTCGTGGTGGAGTGGATCCCCACGCCCTACCGCGCCATC  300
N       TTCGGCCTCAGCATCGCCTGCCTCGTGGTGGAGTGGATCCCCACGCCCTACCGCGCCATC  300
S       TTCGGCCTCAGCATCACCTGCCTCGTGGTGGAGTGGATCCCCACGCCCTACCGCGCCGTC  242
        *************** ****************************************** **

G       ACCGTGGCCATCACCGGCTTCGCCTACACCTTGGGCCAGATTCTGCTGGCTGGCGTGGCC  360
N       ACCGTGGCCATCACCGGCTTCGCCTACACCTTGGGCCAGATTCTGCTGGCTGGCGTGGCC  360
S       ACCGTGGCCATCACCGGCTTCGCCTACACCTTGGGCCAGATTCTGCTGGCTGGCGTGGCC  302
        ************************************************************

G       TACGCTGTCCCCCACTGGCGCTGGCTCCAGCTCGCCGTCTCCCTGCCCTTCTTCATCTTC  420
N       TACGCTGTCCCCCACTGGCGCTGGCTCCAGCTCGCCGTCTCCCTGCCCTTCTTCATCTTC  420
S       TACGCTGTCCCCCACTGGCGCTGGCTCCAGCTCGCCGTCTCCCTGCCCTTCTTCGTCTTC  362
        ************************************************** *****

G       CTCCTCTACTCCTGGTGGTTGGCTGAGTCTGCCCGTTGGCTGGTGCTCTCGGGGAAGGCC  480
N       CTCCTCTACTCCTGGTGGTTGGCTGAGTCTGCCCGTTGGCTGGTGCTCTCGGGGAAGGCC  480
S       CTCCTCTACTCCTGGTGGTTGGCTGAGTCTGCCCGTTGGCTAGTGCTCTCGGGGAAGGCC  422
        ************************************** ******************

G       GAGAGGGCTGTGAAGGTCCTGCAGCGAGTGGCCAGGATTAACAAGAGAAAGGAGGAAGGG  540
N       GAGAGGGCTGTGAAGGTCCTGCAGCGAGTGGCCAGGATTAACAAGAGAAAGGAGGAAGGG  540
S       GAGAGGGCTGTGAAGGTCCTGCAGCGAGTGGCCAGGATTAACAAGAGAAAGGAGGAAGGG  482
        ************************************************************

G       GAGAAGATCACGGTCGAGATCCTGAAGTCCAACATGAAGGAGGAGTTGGCTGGTCTGAAG  600
N       GAGAAGATCACGGTCGAGATCCTGAAGTCCAACATGAAGGAGGAGTTGGCTGGTCTGAAG  600
S       GAGAAGATCACGGTCGAGATCCTGAAGTCCAACATGAAGGAGGAGTTGGCTGGGCTGAAG  542
        ************************************************** ******

G       TCCTCCTACACCATCTCCGACCTGGTCCGGACCCCAGTCATCCGCCACATCTTCTTCTGC  660
N       TCCTCCTACACCATCTCCGACCTGGTCCGGACCCCAGTCATCCGCCACATCTTCTTCTGC  660
S       TCCTCCTACACCATCTCCGACCTGGTCCGGACCCCAGTCATCCGCCACATCTTCTTCTGC  602
        ************************************************************

G       CTCTCCATCGTCTGGTTCTCCATCAGTTTCTCCTACTACGGGCTGGCCATGGATCTGCAA  720
N       CTCTCCATCGTCTGGTTCTCCATCAGTTTCTCCTACTACGGGCTGGCCATGGATCTGCAA  720
S       CTCTCCATCGTCTGGTTCTCCATCAGTTTCTCCTACTACGGGCTGGCCATGGATCTGCAA  662
        ************************************************************

G       AACTTTGGTGTCAGCATTTACCTCATCCAGGTGATTTTCGGCGCCGTTGACTTCCCGGCC  780
N       AACTTTGGTGTCAGCATTTACCTCATCCAGGTGATTTTCGGCGCCGTTGACTTCCCGGCC  780
S       AACTTTGGTGTCAGCATTTACCTCATCCAGGTGATTTTCGGCGCCGTTGACTTCCCGGCC  722
        ************************************************************

G       AAAGTGGTGGTGACTGTCTCCCTGAGCTACATTGGCCGGCGGGTGTCACTCATGGTGGCC  840
N       AAAGTGGTGGTGACTGTCTCCCTGAGCTACATTGGCCGGCGGGTGTCACTCATGGTGGCC  840
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S       AAAGTGGTGGTGACTGTCTCCCTGAGCTACATTGGCCGGCGGGTGTCACTCATGGTGGCC  782
        ************************************************************

G       CTCTTCTTGGCGGGGCTGGTCATCATTGCCAACATCTTTGTCTCCACAGAGCTGCAGACG  900
N       CTCTTCTTGGCGGGGCTGGTCATCATTGCCAACATCTTTGTCTCCACAGAGCTGCAGACG  900
S       CTCTTCTTGGCGGGGCTGGTCATCATTGCCAACATCTTTGTCTCCACAGAGCTGCAGACG  842
        ************************************************************

G       GTGCGCACGGCGCTGGCCGTCATCGGCAAGGGTTGCCTCTCCGCCTCCTTCAACTGCGTC  960
N       GTGCGCACGGCGCTGGCCGTCATCGGCAAGGGTTGCCTCTCCGCCTCCTTCAACTGCGTC  960
S       GTGCGCACGGCRCTGGCCGTCATCGGCAAGGGTTGCCTCTCCGCCTCCTTCAACTGCGTC  902
        *********** ************************************************

G       TTCCTCTACACCACCGAGCTCTACCCCACTCCCATCAGGCAGACCGGGCTGGGCTTTGGC  1020
N       TTCCTCTACACCACCGAGCTCTACCCCACTCCCATCAGGCAGACCGGGCTGGGCTTTGGC  1020
S       TTCCTCTACACCACCGAGCTCTACCCCACTCCCATCAGGCAGACCGGGCTGGGCTTTGGC  962
        ************************************************************

G       AGCACCATGGCCCGAGTGGGTGGCATCGTGGCACCGCTGGTGAAGATGATGGATGAGTAC  1080
N       AGCACCATGGCCCGAGTGGGTGGCATCGTGGCACCGCTGGTGAAGATGATGGATGAGTAC  1080
S       AGCACCATGGCCCGAGTGGGTGGCATCGTGGCGCCGCTGGTGAAGATGATGGACGAGTAC  1022
        ******************************** ****************** ******

G       TACCCCTTCTTGCCCCCTGCGGTCTATGGGGTGGCCCCGGTGGTGGCAGCCGTGGCGGCC  1140
N       TACCCCTTCTTGCCCCCTGCGGTCTATGGGGTGGCCCCGGTGGTGGCAGCCGTGGCGGCC  1140
S       TACCCCTTCTTGCCCCCTGCGGTCTACGGGGTGGCCCCGGTGGTGGCAGCCGTGGCGGCC  1082
        ************************** ********************************

G       GGGTTCCTCCCAGAGACCCTCAACACGCCGCTGCCCGACACCATCGAGGAGGTGGAGAGC  1200
N       GGGTTCCTCCCAGAGACCCTCAACACGCCGCTGCCCGACACCATCGAGGAGGTGGAGAGC  1200
S       GGGTTCCTCCCGGAGACCCTCAACACGCCGCTGCCCGACACCATCGAGGAGGTGGAGAGC  1142
        *********** ************************************************

G       AGGGCCAAGCGGAAGAAGACGGATGACCCCAAAGAGAAGATCCCCCTCCAGCCCCAGGAC  1260
N       AGGGCCAAGCGGAAGAAGACGGATGACCCCAAAGAGAAGATCCCCCTCCAGCCCCAGGAC  1260
S       AGGGCCAAGCGGAAGAAGACGGATGACCCCAAAGAGAAGATCCCCCTCCAGCCCCAGGAC  1202
        ************************************************************

G       AAGGCTCCACAGAAGGAGGCCTGAAGGACCACGGCGGGCTGTGGGGGGACAGCACCCTAT  1320
N       AAGGCTCCACAGAAGGAGGCCTGAAGGACCACGGCGGGCTGTGGGGGGACAGCACCCTAT  1320
S       AAGGCTCCACAGAAGGAGGCCTGAAGGACCACGGGGGGGTGTGGGGGGACAGCACCCTAC  1262
        ********************************** *** ********************

G       GCCCGGCCACCAGCAACCACCCTTCCCCACGGGTCCCCACAGCCTTGTTTTGGGGACGGG  1380
N       GCCCGGCCACCAGCAACCACCCTTCCCCACGGGTCCCCACAGCCTTGTTTTGGGGACGGG  1380
S       GCCCGGCCACCAGCAACCACCCTTCCCCACGGGTCCCCACAGCCTTGTTTTGGGGACGGG  1322
        ************************************************************

G       TACAAATTCGCTTCCTGCCCCACGGCTGGTGACCTCCCTGTGTGGGGCAGGGGACGTCCT  1440
N       TACAAATTCGCTTCCTGCCCCACGGCTGGTGACCTCCCTGTGTGGGGCAGGGGACGTCCT  1440
S       TACAAATTCGCTTCCTGCCCCACGGCCG------------------------------  1350
        ************************** *

G       TGGAGGAATAAAGCAGCTCTGGTGGGGA   1468
N       TGGAGGAATAAAGCAGCTCTGGTGGGGA   1468
S       ---------------------------   1350
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OAT1

Pane B

```
G       TCAAGCTCATGTTGCTGCTCCTTAGTACACAGATCCTCTGAATGAGCAAACTCCACAAAA  60
N       TCAAGCTCATGTTGCTGCTCCTTAGTACACAGCTGCTCTGAATGAGCAAACTCCACAGAA  60
S       ------------------------------------------------------------  0

G       AAAAAAATATGAAATTTGAGGATCTCTTGCTGGAAATCGATGGCTTTGGCAAATTCCAGA  120
N       AAA----AATGAAATTTGAGGATCTCTTGCTGGAAATCAATGGCTTTGGCAAATTCCAGA  116
S       --------------TTTGAGGATCTCTTGCTGGAAATCAATGGCTTTGACCATTTCCAGA  46
                      ************************ ********* * * *******

G       TTTTGATTCTGTTTATCCTCTGCCTTCCAAGAATGAACCTTCCCATGCATTTCCTGCTGC  180
N       TTTTGATTCTGTTTATCCTCTGCCTTCCAAGAATGAACCTTCCCATGCATTTCCTGCTGC  176
S       TTTTGATTCTGTTTATCCTCTGCCTTCCAAGAATGAACCTTCCCATGCATTTCCTGCTGC  106
        ************************************************************

G       ACAATTTTCTTGCTGCTACCCCCTCTCATCACTGTGCAATTCCACACCAAGAGGCATTTG  240
N       ACAATTTTCTTGCTGCTACCCCCTCTCATCACTGTGCAATTCCACACCAAGAGGCATTTG  236
S       ACAATTTTCTTGCTGCTACCCCCTCTCATCGCTGTGCAATTCCACACCAAGAGGCATTTG  166
        ****************************** ******************************

G       TGAACCTCACCATGGAGGAAGTTCTGCTCATCAGCATCCCCCGGGAGCCCGATGGCACTT  300
N       TGAACCTCACCATGGAGGAAGTTCTGCTCATCAGCATCCCCCGGGAGCCCGATGGCACTT  296
S       TGAACCTCACCATGGAGGAAGTTCTGCTCATCAGCATCCCCCGGGAGCCCGATGGCACTT  226
        ************************************************************

G       TCAAGTCCTGCGAGATGTTCTCGCAGCCTCAGTTTCACCTGCTGCTCAATTCCTCTCTGC  360
N       TCAAGTCCTGCGAGATGTTCTCGCAGCCTCAGTTTCACCTGCTGCTCAATTCCTCTCTGC  356
S       TCAAGTCCTGCGAGATGTTCTCGCAGCCTCAGTTTCACCTGCTGCTCAATTCCTCTCTGC  286
        ************************************************************

G       AACCAGAAAACAACTCCATCATCCAGGAGTGCCAGCACGGATGGGTCTATGACCACTCGC  420
N       AACCAGAAAACAACTCCATCATCCAGGAATGCCAGCACGGATGGGTCTATGACCACTTGC  416
S       AACCAGAAAACAACTCCATCATCCAGGAATGCCAGCACGGATGGGTCTATGACCACTCGC  346
        *************************** ***************************** **

G       AGTTCACCTCCACCATCTCCACGCAGTGGGACCTCGTATGCGAGCAGCGCGGACTGAACC  480
N       AGTTCACCTCCACCATCTCCACGCAGTGGGACCTCGTATGCGAGCAGCGCGGACTGAACC  476
S       AGTTCACCTCCACCATCTCCACGCAGTGGGACCTCGTATGCGAGCAGCGCGGACTGAACC  406
        ************************************************************

G       AGGCGACTGCAACCTTCTTCTTCATCGGCGTTACGATGGGGGCCGTGGTATTCGGATACC  540
N       AGGCGACTGCAACCTTCTTCTTCATCGGCGTTACGATGGGGGCCGTGGTATTCGGATACC  536
S       AGGCGACTGCAACCTTCTTCTTCATCGGCGTTACGATGGGGGCCGTGGTATTCGGATACC  466
        ************************************************************

G       TTTCAGACAGGTTCGGACGGAAAGCCATGCTCCTGCTGTCGCTTGTGTGCGCTGTCATAT  600
N       TTTCAGACAGGTTCGGACGGAAAGCCATGCTCCTGCTGTCGCTTGTGTGCGCCGTCATAT  596
S       TTTCAGACAGGTTCGGACGGAAAGCCATGCTCCTGCTGTCGCTTGTGTGCGCCGTCATAT  526
        ************************************************** *******

G       TTGGGATGCTGAGTGCCGCCTCCGTCTCCTACAGCATGCTGGCCGTCACGCGGACCCTCA  660
N       TTGGGATGCTGAGTGCCGCCTCCGTCTCCTACAGCATGCTGGCCATCACGCGGACCCTCA  656
S       TTGGGATGCTGAGTGCCGCCTCCGTCTCCTACAGCATGCTGGCCATCACGCGGACCCTCA  586
        ****************************************** ***************
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G       CCGGGGTGGCCCTGAGCGGCGTCTCCCTTATTGTATTGCCTTTGGGGATGGAGTGGGTGG  720
N       CCGGGGTGGCCCTGAGCGGCGTCTCCCTTATTGTATTGCCTTTGGGGATGGAGTGGGTGG  716
S       CCGGGGTGGCCCTGAGCGGCGTCTCCCTTATTGTATTGCCTTTGGGGATGGAGTGGGTGG  646
        ************************************************************

G       ACGTAGAGCACCGCACCTTCTCCGGGATCCTGACTAGCATGTTCTGGAGCGTCGGGAACA  780
N       ACGTAGAGCACCGCACTTTCTCCGGGATCCTGACTAGCATGTTCTGGAGCATCGGGAACA  776
S       ACGTAGAGCACCGCACCTTCTCCGGGATCCTGACTAGCATGTTCTGGAGCATCGGGAACA  706
        *************** ********************************* *********

G       TGCTGCTGGCCGCCGTAGCGTACTTGGTGCGGGAATGGCGCTGGTTATTAGTGGCCGTAA  840
N       TGCTGCTGGCCGCGGTAGCGTACTTGGTGCGGGAATGGCGCTGGTTATTAGTGGCCGTAA  836
S       TGCTGCTGGCCGCGGTAGCATACTTGGTGCGGGAATGGCGCTGGTTATTAGTGGCCGTAA  766
        ************* ***** ***************************************

G       CAGGACCGTGTCTTCTGAGCATCGTCTGCCTGTGGTGGGTCCCAGAGTCTGCCCGGTGGC  900
N       CGGGACCGTGTCTTCTGAGCATTGTCTGCCTGTGGTGGGTCCCAGAGTCTGCCCGGTGGC  896
S       CGGGACCGTGTCTTCTGAGCGTTGTCTGCCTGTGGTGGGTCCCAGAGTCTGCCCGGTGGC  826
        * ****************** * ************************************

G       TCATAGCCAACGGCAAAGTGAAACGAGCTCACAGGCATCTGCTTAGATGTGCAAGAATGA  960
N       TCATAGCCAACGGCAAAGTGGAACGAGCTCACAGGCATCTGCTTAGATGTGCAAGAATGA  956
S       TCATAGCCAACGGCAAAGTGGAACGAGCTCACAGGCATCTGCTTAGATGTGCAAGAATGA  886
        ******************** ***************************************

G       ACGGAAGGAAAGACTTTGCTGTCTCACCAGAGGACCTCAGAAGGATGACAACAGACAAAA 1020
N       ACGGAAGGAAAGACTTTGCTGTCTCACCAGAGGACCTCAGAAGGATGACAACAGACAAAA 1016
S       ACGGAAGGAAAGACTTTGCTGTCTCACCAGAGGACCTCAGAAGGATGACAACAGACAAAA  946
        ************************************************************

G       AGCCAGGAGAGAATTACTCTTACATCACCTTGTTCAGGACACCAGTCCTGCGGAAGATCT 1080
N       AGCCAGGAGAGAATTACTCTTACATCACCTTGTTCAGGACACCAGTCCTGCGGAAGATCT 1076
S       AGCCAGGAGAGAATTACTCTTACATCACCTTGTTCAGGACACCAGTCCTGCGGAAGATCT 1006
        ************************************************************

G       CTCTGTGTTCTGGGACTCTGTGGTTTGGTGTTGCCTTCTCTTATTACGGCATGAGCATGA 1140
N       CTCTGTGTTCTGGGACTCTGTGGTTTGGTGTTGCCTTCTCTTATTACGGCATGAGCATGA 1136
S       CTCTGTGTTCTGGGACTCTGTGGTTTGGTGTTGCCTTCTCTTATTACGGCATGAGCATGA 1066
        ************************************************************

G       ACCTAACTGGCTTTGGGCTCAACATGTATCTCTCCCAGTTTGTCTTTGGCTTCATTGAGA 1200
N       ACCTAACTGGCTTTGGGCTCAACATGTATCTCTCCCAGTTTGTCTTTGGCTTCATTGAGA 1196
S       ACCTAACTGGCTTTGGGCTCAACATGTATCTCTCCCAGTTTGTCTTTGGCTTCATTGAGA 1126
        ************************************************************

G       TTCCAGCCAAGATGATCATGTACGTGCTGGTGAATCGAGTTGGACGACGGCAGAGTCAGG 1260
N       TTCCAGCCAAGATGATCATGTACGTGCTGGTGAATCGAGTTGGACGACGGCAGAGTCAGG 1256
S       TTCCAGCCAAGATGATCATGTACGTGCTGGTGAATCGAGTTGGACGACGGCAGAGTCAGG 1186
        ************************************************************

G       CGTGGGCACTCATCCTGACCGGACTATGCATAGGAGCCAACATCGTTATTCCCAAGCCCT 1320
N       CGTGGGCACTCATCCTGACCGGACTATGCATAGGAGCCAACATCGTCATTCCCAAGTCCT 1316
S       CGTGGGCACTCATCCTGACCGGACTATGCATAGGAGCCAACATCGTCATTCCCAAGTCCT 1246
        ******************************************** ********* ***

G       TCACCCCCCTGCGCTCTGCAGTAGCCATTATGGGCAAAGGTTTCTCAGAAGCTGCGTTCA 1380
N       TCACCCCCTTGCGCTCTGCAGTAGCCATTATGGGCAAAGGTTTCTCAGAAGCTGCGTTCA 1376
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S      TCACCCCCTTGCGCTCTGCGGTAGCCATTATGGGCAAAGGTTTCTCAGAARCTGCGTTCA  1306
       ******** ********** ****************************** *********

G      CTACCGCCTTCTTGTACACCTGTGAGCTCTACCCCACCGTACTGAGGCAGAACGGGATGG  1440
N      CTACCGCCTTCGTGTACACCTGTGAGCTCTACCCCACCGTACTGAGGCAGAACGGGATGG  1436
S      CTACCGCCTTCGTGTACACCTGTGAGCTCTACCCCACCGTACTGAGGCAGAACGGGATGG  1366
       *********** ************************************************

G      GGTACAGCTCCTTCATGGCGCGCCTCGGCGGGGCTTTGGCCCCACTTGTGTTTCTGCTGG  1500
N      GGTACAGCTCCTTCATGGCACGCCTCGGCGGGGCTTTGGCCCCACTTGTGTTTCTGCTGG  1496
S      GGTACAGCTCCTTCATGGCACGCCTCGGCGGGGCTTTGGCCCCACTTGTGTTTCTGCTGG  1426
       ****************** ****************************************

G      ATGAGGTGTGGAGGTCTCTGCCCGAGGTGACGTACTGCGGCGTAGCAGTGTGCTGCGGCT  1560
N      ATGAGGTGTGGAGGTCTCTGCCCGAGGTGACGTACTGTGGCGTAGCAGTGTGCTGCGGCT  1556
S      ATGAGGTGTGGAGGTCTCTGCCCGAGGTGACGTACTGTGGCGTAGCAGTGTGCTGCGGCT  1486
       ********************************** **********************

G      TGGTGGCCTTCCTGCTCCCGGAGACGCTCCACGTGCGTTTGCCTGAGGGCATCGAGGACA  1620
N      TGGTGGCTTTCCTGCTCCCGGAGACGCTCCACGTGCGCTTGCCCGAGGGCATCGAGGACA  1616
S      TGGTGGCTTTCCTGCTCCCGGAGACGCTCCACGTGCGCTTGCCCGAGGGCATCGAGGACA  1546
       ******* **************************** ***** ****************

G      TCGAGAAGACACAAGTGAAAGGGCCGCCACAAATCGGTGGTCCCAAGGGCATGCCGCTAC  1680
N      TCGAGAAGACACAAGCGAAAGGGCCGCCACAAATCGGTGCTCCCAAGGGCACGCCGCTAC  1676
S      TCGAGAAGACACAAGCGAAAGGGCCGCCACAAATCAGTGCTCCCAAGGGCACGCCGCTAC  1606
       *************** ****************** *** *********** ********

G      AGCCTCTGCTGAAGTGAGGGTCCCCGTGGGACAGCCACTGCGTAGGACTGCAAAACTGAG  1740
N      AGTCTCTGCTGAAGTGAGGGTCCCCGTGGGACAGCCACTACGTAGGACTGCAAAACTGAG  1736
S      AGTCTCTGCTGAAGTGAGGGTCCCCGTGGGACAGCCACTACGTAGGACTGCAAAAC----  1662
       ** ********************************** ****************

G      CCGTGCTCCGACCCGTCAATAAAAGAGCCTTTACGGTCCACCTAGTCTCTCTCTGCAGGG  1800
N      CCGTGCTCCGACCCGTCAATAAAAGAGCCTTTACTGTCCACCTAGTCTCTCTCTGCAGGG  1796
S      ------------------------------------------------------------  1662

G      AAGTGGGGCTGAGGACATTGGAGGAAGGAGGCGGATCACGAAACCGGCTGGAGGGAGCTG  1860
N      AAGTGGGGCTGAGGACATTGGAGGAAGGAGGCGGATCACGAAACCAGCTGGAGGGAGCTA  1856
S      ------------------------------------------------------------  1662

G      CCCCACTCTGTAGTGTGGCCAGGAGCAAGGATGCAGAGGTCTAAGAGGATGTGGAAATAA  1920
N      CCCCACTCTGTGGTGTGGCCAGGAGCAAGGATGCAGAGGTCTAAAAGGATGTGGAAATAA  1916
S      ------------------------------------------------------------  1662

G      AAGCACCCCGTGCTAAGCTCTTTCCAGCCTTTGGCCTTGTTGGACTAGCAGCACCTTTCA  1980
N      AAGCATCCCGTGCTAAGCTCTTTCCAGCCTTTGGCCTTGTTGGACTAGCAGCACCTTGCA  1976
S      ------------------------------------------------------------  1662

G      GATCACTGCACAGCCTTCCAGAAACTACAATCCATCGGTTTACACACCAGAAGGAACGCA  2040
N      GATCACTGCACAGCCTTCCAGAAACTACAATCCATCGGTTTACACACCGCAAGGAACGCA  2036
S      ------------------------------------------------------------  1662
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G      ATGCAGCAATAAAAAATACTGGGTGTGAATCGTGCAAAACCAAATAATCTCAAATAAATC  2100
N      ATGCAGCAATAAAAAATACTGAGTGTGAATCGTACAAAACCAAATAATCTCAAATAAATC  2096
S      ------------------------------------------------------------  1662


G      TAACTGGATGGTGGATAAACCATAAACTGTAGCTACAAATGCTTCTTGAATTTGGAAATG  2160
N      TAACCGGATGGTGGATAAACCATAAACTGTAGCTACAAATGCTTCTTGAATTTGGAAATG  2156
S      ------------------------------------------------------------  1662


G      GTCTTGACGTTTTTCACACTGAGGCTCAAAGCCAGCAAAAAGCACACGCAGGAATCTCAC  2220
N      GTCTTGACATTTTTCACACTGAGGCTCAAAGCCAGCAAAAAGCACATGCAGGAATCTCAC  2216
S      ------------------------------------------------------------  1662


G      TGAGATGCTGACAAATCACTGACAAATGCTTGCATTTGTCCCACGTACACAAAGAAGAAT  2280
N      TGAGATGCTGACAAATCACTGACAAATGCTTGCATTTGTCCCACATACACAAAGAAGAAT  2276
S      ------------------------------------------------------------  1662


G      AAAAGCTTTTGCAAATTATTTAAA       2304
N      AAAAGCTTTTGCAAATTATTTAAA       2300
S      -------------------------      1662
```

OAT2

Fig 4-4: Clustal comparison of A) Golden eagle predicted OAT1 (XM_011601043.1), NGS and Sanger for OAT1 sequences. B) Golden eagle predicted OAT2 (XM_009272605.1), NGS and Sanger for OAT1 sequences. G= Golden eagle Predicted gene, N= NGS sequences, S= Sanger sequences.

To confirm NGS data, Sanger sequencing was conducted. The purity of the RNA was 2.02 at 206nm/280nm moreover RNA was synthesized into cDNA. After completion of PCR, gel electrophoresis revealed desired amplicons for OAT1 at 1453bp and OAT2 at 1776bp (Fig 4-5). The PCR products were cut, cleaned and sequenced. The sequences generated were analysed to achieve consensus sequence and the latter were compared with the NGS sequences using Clustal Omega (Fig 4-4). After alignment of NGS and Sanger sequences, minor differences in the both sequences were present with similarity of 98.81 for OAT1 however OAT2 had similarity of 99.34% (Fig 4-4). The few differences maybe because the AWB vulture used for Sanger was not the same as the one used for NGS leading to a conclusion of individual variation. Since Sanger sequences are regarded as more accurate, these sequences were subsequently deposited to GenBank and allocated the following accession numbers MK854995 and MK879652 for OAT1 and OAT2 respectively.

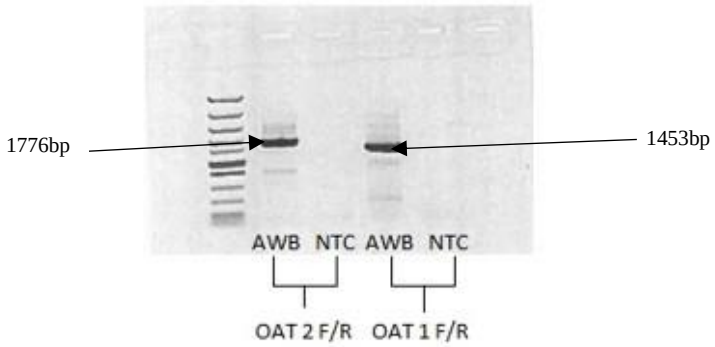


Fig 4-5: Conventional PCR amplified OAT1 and OAT2 gene from AWB vulture`s kidney, no PCR product was attained when the template was absent, Molecular size (100bp) is indicated on the left. NTC= no template control.

4.3.3. Immunohistochemistry results

With the NGS and Sanger sequence data revealing similarity of the mouse OAT3 polyclonal antibodies and vulture OAT1 for the binding sites of 72.09% (Fig 4-6), immunohistochemistry was conducted to ascertain the location of OAT1 distribution in the AWB kidney. The mouse kidney was used as a control, as the polyclonal antibodies were described for the mouse. It was decided not to include the chicken since its OAT1 sequence was not present in the NCBI database. Light microscopy of 4µm wax sections demonstrated immunostaining of AWB vulture`s kidney within the basolateral membrane of the proximal convoluted tubules (PCT) (Fig 4-7) as also observed for the mouse kidney. Some immunoreactivity was also seen on the latter kidneys on the distal convoluted tubule cells and the cortical collecting duct (Fig 4-7). While not quantifiable, the staining appears to be less intense for the vulture tissue.

Fig 4-6: Amino acids comparison between OAT3 antibody used and vulture OAT1 using Clustal Omega.

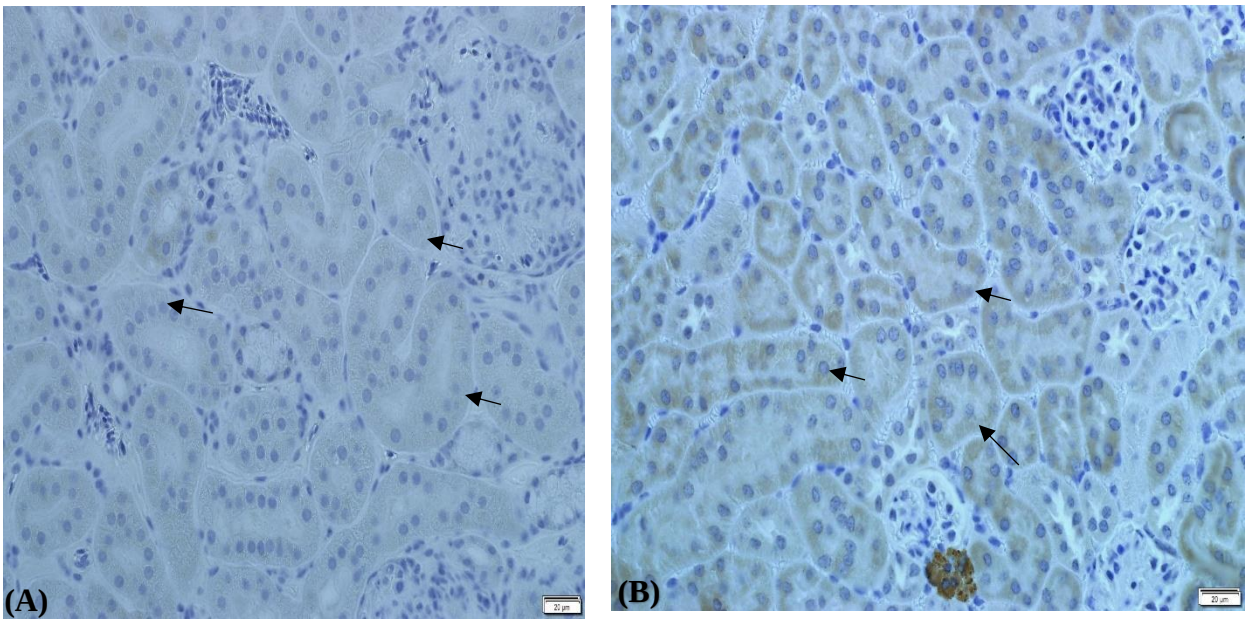
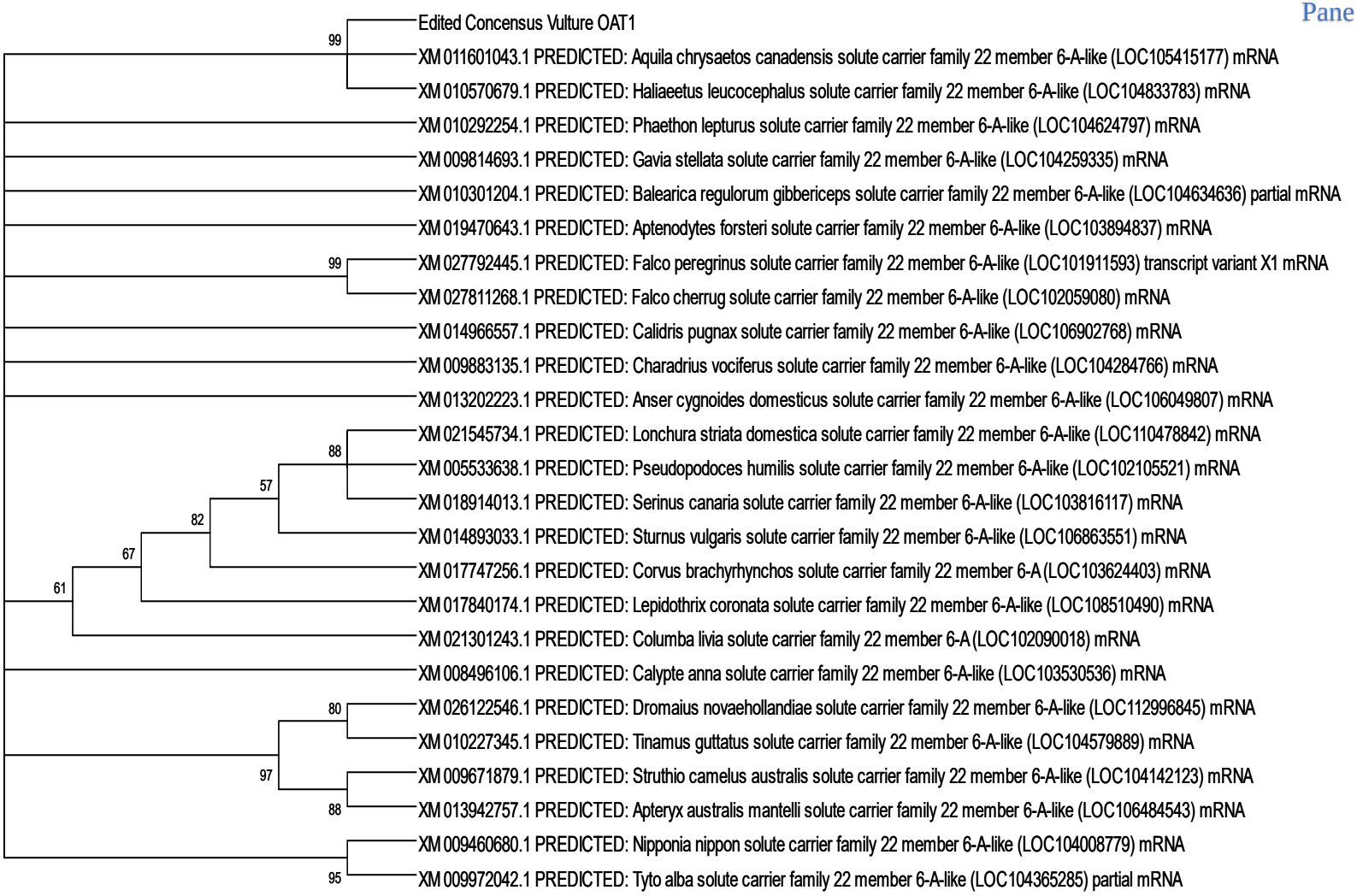


Fig 4-7: Light micrographs illustrating (A) immunostaining of OAT proteins in AWB kidneys (B) immunostaining of OAT3 protein mouse kidneys. For the AWB the area of staining was identified as PCT cells by the cells being columnar and indistinct cellular borders.

4.3.4. Phylogenetic Relationship based on the Sanger Sequence.

Maximum likelihood trees were based on OAT1 and OAT2 nucleotide sequences. The Phylogenetic trees revealed a close relationship between AWB vulture and eagle's taxa belonging to the same clade however AWB vulture and chicken did not share the same clade (Fig 4-8). By applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach, initial trees for the heuristic search were obtained and then selected topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.5011 and 0.4500)) for OAT1 and OAT2 respectively. The analysis involved 26 and 39 nucleotide sequences and there were a total of 1427 and 1626 positions in the final dataset for OAT1 and OAT2 in that order. The similarity of former species is represented by the number on the internal nodes of the branches (Fig 4-8).

Pane A


Edited Concensus Vulture OAT1
XM 011601043.1 PREDICTED: Aquila chrysaetos canadensis solute carrier family 22 member 6-A-like (LOC105415177) mRNA
XM 010570679.1 PREDICTED: Haliaeetus leucocephalus solute carrier family 22 member 6-A-like (LOC104833783) mRNA
XM 010292254.1 PREDICTED: Phaethon lepturus solute carrier family 22 member 6-A-like (LOC104624797) mRNA
XM 009814693.1 PREDICTED: Gavia stellata solute carrier family 22 member 6-A-like (LOC104259335) mRNA
XM 010301204.1 PREDICTED: Balearica regulorum gibbericeps solute carrier family 22 member 6-A-like (LOC104634636) partial mRNA
XM 019470643.1 PREDICTED: Aptenodytes forsteri solute carrier family 22 member 6-A-like (LOC103894837) mRNA
XM 027792445.1 PREDICTED: Falco peregrinus solute carrier family 22 member 6-A-like (LOC101911593) transcript variant X1 mRNA
XM 027811268.1 PREDICTED: Falco cherrug solute carrier family 22 member 6-A-like (LOC102059080) mRNA
XM 014966557.1 PREDICTED: Calidris pugnax solute carrier family 22 member 6-A-like (LOC106902768) mRNA
XM 009883135.1 PREDICTED: Charadrius vociferus solute carrier family 22 member 6-A-like (LOC104284766) mRNA
XM 013202223.1 PREDICTED: Anser cygnoides domesticus solute carrier family 22 member 6-A-like (LOC106049807) mRNA
XM 021545734.1 PREDICTED: Lonchura striata domestica solute carrier family 22 member 6-A-like (LOC110478842) mRNA
XM 005533638.1 PREDICTED: Pseudopodoces humilis solute carrier family 22 member 6-A-like (LOC102105521) mRNA
XM 018914013.1 PREDICTED: Serinus canaria solute carrier family 22 member 6-A-like (LOC103816117) mRNA
XM 014893033.1 PREDICTED: Sturnus vulgaris solute carrier family 22 member 6-A-like (LOC106863551) mRNA
XM 017747256.1 PREDICTED: Corvus brachyrhynchos solute carrier family 22 member 6-A (LOC103624403) mRNA
XM 017840174.1 PREDICTED: Lepidothrix coronata solute carrier family 22 member 6-A-like (LOC108510490) mRNA
XM 021301243.1 PREDICTED: Columba livia solute carrier family 22 member 6-A (LOC102090018) mRNA
XM 008496106.1 PREDICTED: Calypte anna solute carrier family 22 member 6-A-like (LOC103530536) mRNA
XM 026122546.1 PREDICTED: Dromaius novaehollandiae solute carrier family 22 member 6-A-like (LOC112996845) mRNA
XM 010227345.1 PREDICTED: Tinamus guttatus solute carrier family 22 member 6-A-like (LOC104579889) mRNA
XM 009671879.1 PREDICTED: Struthio camelus australis solute carrier family 22 member 6-A-like (LOC104142123) mRNA
XM 013942757.1 PREDICTED: Apteryx australis mantelli solute carrier family 22 member 6-A-like (LOC106484543) mRNA
XM 009460680.1 PREDICTED: Nipponia nippon solute carrier family 22 member 6-A-like (LOC104008779) mRNA
XM 009972042.1 PREDICTED: Tyto alba solute carrier family 22 member 6-A-like (LOC104365285) partial mRNA
99
99
88
57
82
67
61
80
97
88
95

Pane B

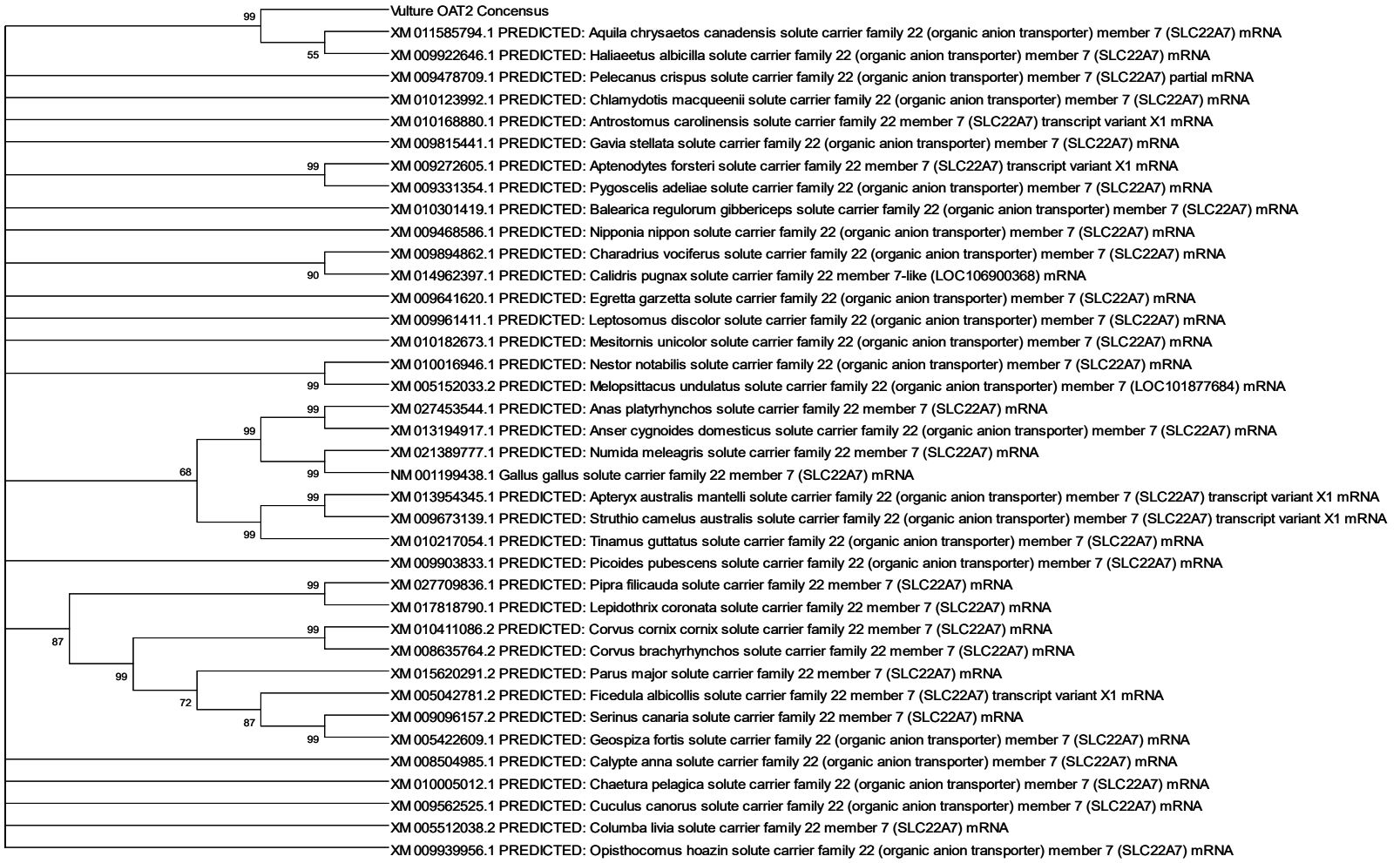


Figure 4-8: Reconstruction of phylogenetic relationships between A) OAT1 and B) OAT2 genes of avian species by maximum likelihood best model: bootstrap.

4.3.5. Protein Structure

The OAT1 protein was made up of 449 amino acids with molecular weight of 44748.10 while OAT2 consisted of 553 amino acids with molecular weight of 57281.49 (Fig 4-9). Functional prediction with TrSSP showed the both proteins to be functional transporter of amino acids. In contrast, only OAT2 was deemed to be a functional anionic transporter (Table 4-4). Domain analysis with scan prosite revealed the presence of a major facilitator superfamily which transport sugar while protein predict indicated molecular functionality of a transmembrane transporter activity aimed at anionic substances. PROTTER sequence database used for prediction of N-glycosylation sites revealed that OAT1 had 10 putative transmembrane helices and no N-glycosylation sites as for the Golden Eagle and AWB vulture while OAT2 had 11 transmembrane helices comprised of five N- glycosylation sites found at positions 31, 66, 73, 294, 330 while for Golden eagle had 12 transmembrane helices as well as 5 N-glycosylation sites at 68, 103, 110, 331, 3367 (Fig 4-10; Fig 4-11), which may be attributed to the sequence variation (Fig 4-12).

Transmembrane topology revealed AWB vulture OAT1 structures with the presence of intra and extracellular loops while human comprised of only one intracellular loop while OAT2 for the latter species only comprised of intracellular loop of the following amino acids (Table 4-3).

Table 4-3: Intra/extracellular loops of AWB, chicken and human.

Loop ID	Amino acid
OAT1 vulture external loop:	Prdlrtlrtalavfgkgclaasfncvflytgelyptvirqtgmglantmarlgsitaplvkmagev
OAT1 vulture internal loop:	Tesarwlvmvgkshqalkelqkvaringkkeegdkldievlrsymqkemassrshhtvvdlvrtpvvrr
Human OAT1 internal loop:	esarwhsssgrldltlralqrvaringkreegaklsmevlraslqkeltmgkgqasamellrcpt
OAT2 vulture internal loop:	esarwliangkverahrhllrcarmngrkdfavspedlrrmttdkkpgenysyitlfrtp
OAT2 chicken internal loop	esarwliangkvkqahkhllrcarmngrkdftispealrgmtteknpgesfsyislfrtp
OAT2 human internal loop	Pahrcalpgapanfshqdvwleahlprepdgtlssclrfaypqalpnttlgeerqsrgeledepatvpcsqgweydhsefsstiatesqwdlvceqkg

The alignment of the intracellular loop amino acid for the latter species for OAT1 revealed similarity of 46.69% (Fig 4.3-13). In contrast, the OAT2 had intra-cellular loop with similarity of 80% between chicken and vulture while similarity of both species with human was less than 21% (Fig 4-14). Phyre2, modelling was performed taking c6h7da protein as template having 97% and 83% identity for OAT1 and 2 respectively with 100% confidence. Phyre2 modelling supported the OAT1 PROTTER having 10 transmembrane helices while OAT2 had 12 transmembrane helices for which is different to PROTTER (Fig 4-15).

```
>OAT1 PROTEIN                                                    Pane 1

M A G V L V G A L V L G G L S D R F G R K P M L M W S Y L Q L G V M G T C T A
F A P N Y A S Y C V F R F A G G M A L S G F G L S I T C L V V E W I P T P Y R
A V T V A I T G F A Y T L G Q I L L A G V A Y A V P H W R W L Q L A V S L P F
F V F L L Y S W W L A E S A R W L V L S G K A E R A V K V L Q R V A R I N K R
K E E G E K I T V E I L K S N M K E E L A G L K S S Y T I S D L V R T P V I R
H I F F C L S I V W F S I S F S Y Y G L A M D L Q N F G V S I Y L I Q V I F G
A V D F P A K V V V T V S L S Y I G R R V S L M V A L F L A G L V I I A N I F
V S T E L Q T V R T X L A V I G K G C L S A S F N C V F L Y T T E L Y P T P I
R Q T G L G F G S T M A R V G G I V A P L V K M M D E Y Y P F L P P A V Y G V
A P V V A A V A A G F L P E T L N T P L P D T I E E V E S R A K R K K T D D P
K E K I P L Q P Q D K A P Q K E A - R T T G G C G G T A P Y A R P P A T T L P
H G S P Q P C F G D G Y K F A S C P T A
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>OAT2 PROTEIN                                                    Pane 2

F E D L L L E I N G F D H F Q I L I L F I L C L P R M N L P M H F L L H N F L
A A T P S H R C A I P H Q E A F V N L T M E E V L L I S I P R E P D G T F K S
C E M F S Q P Q F H L L L N S S L Q P E N N S I I Q E C Q H G W V Y D H S Q F
T S T I S T Q W D L V C E Q R G L N Q A T A T F F F I G V T M G A V V F G Y L
S D R F G R K A M L L L S L V C A V I F G M L S A A S V S Y S M L A I T R T L
T G V A L S G V S L I V L P L G M E W V D V E H R T F S G I L T S M F W S I G
N M L L A A V A Y L V R E W R W L L V A V T G P C L L S V V C L W W V P E S A
R W L I A N G K V E R A H R H L L R C A R M N G R K D F A V S P E D L R R M T
T D K K P G E N Y S Y I T L F R T P V L R K I S L C S G T L W F G V A F S Y Y
G M S M N L T G F G L N M Y L S Q F V F G F I E I P A K M I M Y V L V N R V G
R R Q S Q A W A L I L T G L C I G A N I V I P K S F T P L R S A V A I M G K G
F S E X A F T T A F V Y T C E L Y P T V L R Q N G M G Y S S F M A R L G G A L
A P L V F L L D E V W R S L P E V T Y C G V A V C C G L V A F L L P E T L H V
R L P E G I E D I E K T Q A K G P P Q I S A P K G T P L Q S L L K- G S P W D
S H Y V G L Q N
```

Fig 4-9: Protein sequence of AWB Pane 1) OAT1 gene containing 449 amino acids Pane 2) OAT2 gene containing 553 amino acids.

Table 4-4: Protein prediction scores of predicted AWB OAT1 and OAT2 using TrSSP software.

Protein ID	Amino Acid	Anion	Cation	Electron	Proteins/mRNA	Sugar	Other	TRANSPORTER
VULTURE_OAT1	yes	No	no	No	No	Yes	Yes	Yes
VULTURE_OAT2	Yes	yes	No	No	No	Yes	Yes	Yes

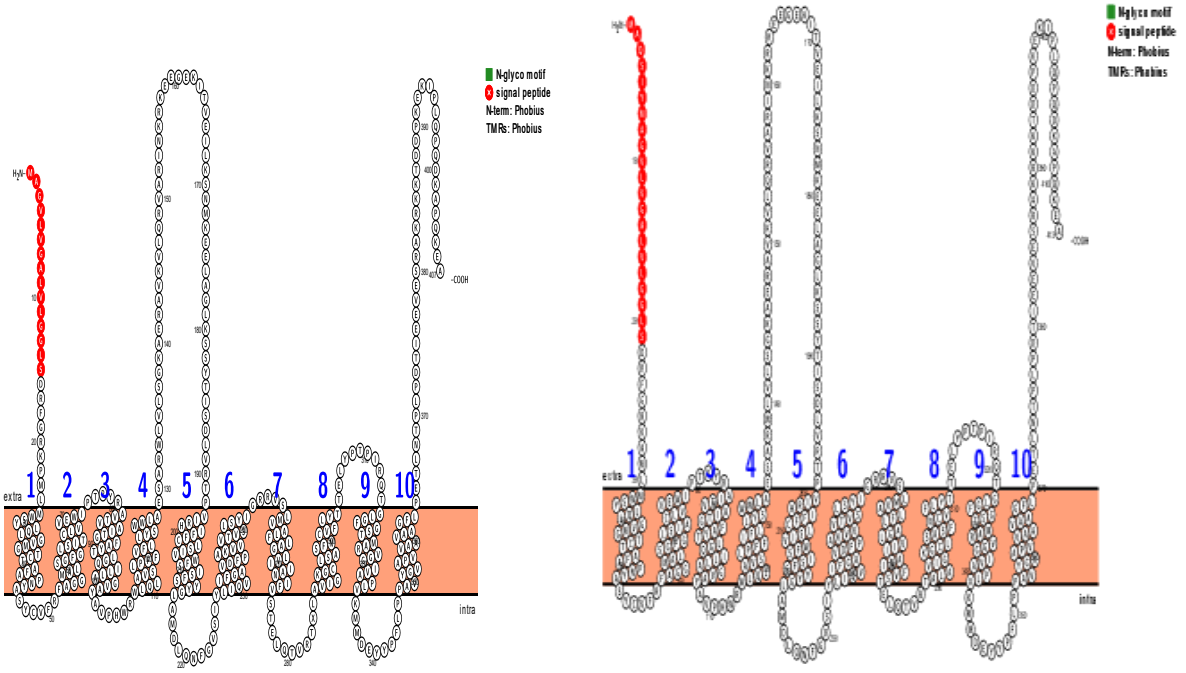


Figure 4-10: Pictorial presentation of OAT1 representing 10 transmembrane helices and no N-glycan motif sites of AWB and predicted Golden Eagle OAT1 gene (from left to right).

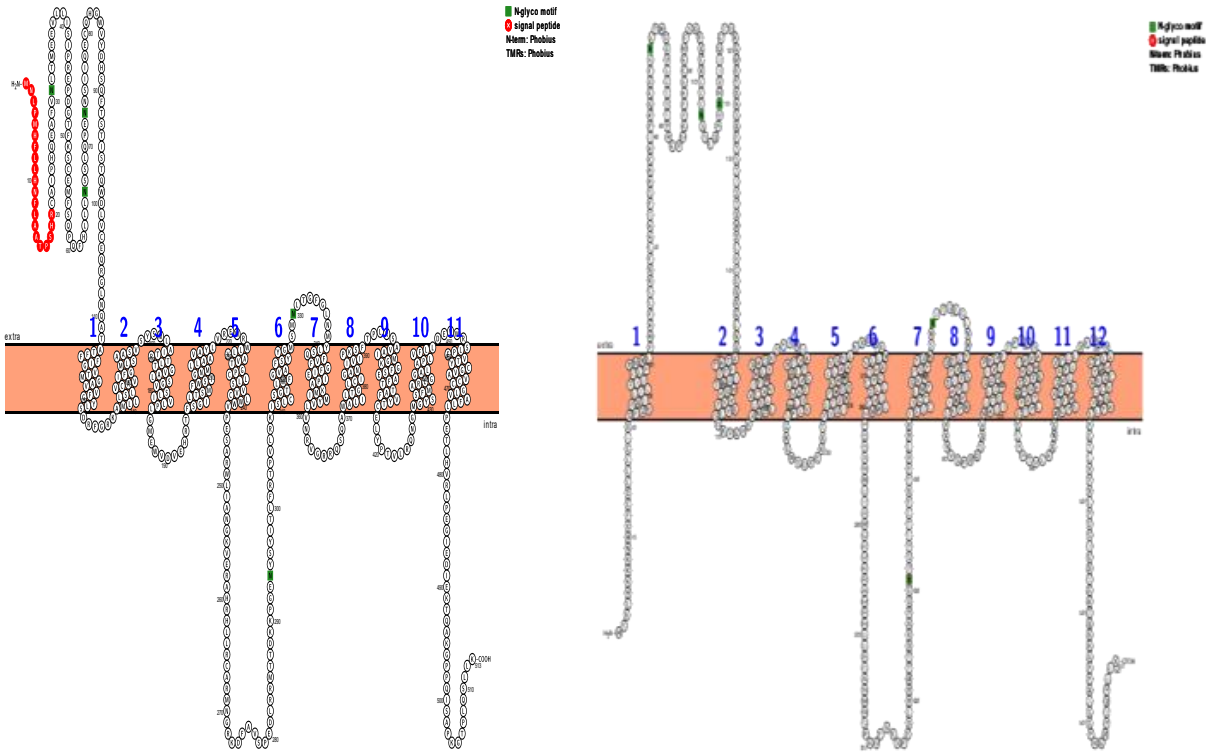


Figure 4-11: Pictorial presentation of OAT2 representing 11 transmembrane helices and 5 N-glycan motif sites of AWB OAT2 and 12 transmembrane helices and 5 N-glycan motif sites of predicted Golden Eagle OAT2 (from left to right).

.

Alignment of AWB and predicted Golden eagle proteins (97%).

```
Query  1    MNLPMHFLLHNFLAATPSHRCAIPHQEAFVNLTMEEVLLISIPREPDGTFKSCEMFSQPQ  60
            MNLPMHFLLHNFLAATPSH CAIPHQEAFVNLTMEEVLLISIPREPDGTFKSCEMFSQPQ
Sbjct  38   MNLPMHFLLHNFLAATPSHHCAIPHQEAFVNLTMEEVLLISIPREPDGTFKSCEMFSQPQ  97

Query  61   FHLLLNSSLQPENNSIIQECQHGWVYDHSQFTSTISTQWDLVCEQRGLNQATATFFFIGV  120
            FHLLLNSSLQPENNSIIQECQHGWVYDHSQFTSTISTQWDLVCEQRGLNQATATFFFIGV
Sbjct  98   FHLLLNSSLQPENNSIIQECQHGWVYDHSQFTSTISTQWDLVCEQRGLNQATATFFFIGV  157

Query  121  TMGAVVFGYLSDRFGRKAMLLLSLVCAVIFGMLSAASVSYSMLAITRTLTGVALSGVSLI  180
            TMGAVVFGYLSDRFGRKAMLLLSLVCAVIFGMLSAASVSYSMLA+TRTLTGVALSGVSLI
Sbjct  158  TMGAVVFGYLSDRFGRKAMLLLSLVCAVIFGMLSAASVSYSMLAVTRTLTGVALSGVSLI  217

Query  181  VLPLGMEWVDVEHRTFSGILTSMFWSIGNMLLAAVAYLVREWRWLLVAVTGPCLLSVVCL  240
            VLPLGMEWVDVEHRTFSGILTSMFWS+GNMLLAAVAYLVREWRWLLVAVTGPCLLS+VCL
Sbjct  218  VLPLGMEWVDVEHRTFSGILTSMFWSVGNMLLAAVAYLVREWRWLLVAVTGPCLLSIVCL  277

Query  241  WWVPESARWLIANGKVERAHRHLLRCARMNGRKDFAVSPEDLRRMTTDKKPGENYSYITL  300
            WWVPESARWLIANGKV+RAHRHLLRCARMNGRKDFAVSPEDLRRMTTDKKPGENYSYITL
Sbjct  278  WWVPESARWLIANGKVKRAHRHLLRCARMNGRKDFAVSPEDLRRMTTDKKPGENYSYITL  337

Query  301  FRTPVLRKISLCSGTLWFGVAFSYYGMSMNLTGFGLNMYLSQFVFGFIEIPAKMIMYVLV  360
            FRTPVLRKISLCSGTLWFGVAFSYYGMSMNLTGFGLNMYLSQFVFGFIEIPAKMIMYVLV
Sbjct  338  FRTPVLRKISLCSGTLWFGVAFSYYGMSMNLTGFGLNMYLSQFVFGFIEIPAKMIMYVLV  397

Query  361  NRVGRRQSQAWALILTGLCIGANIVIPKSFTPLRSAVAIMGKGFSEXAFTTAFVYTCELY  420
            NRVGRRQSQAWALILTGLCIGANIVIPK FTPLRSAVAIMGKGFSE AFTTAF+YTCELY
Sbjct  398  NRVGRRQSQAWALILTGLCIGANIVIPKPFTPLRSAVAIMGKGFSEAAFTTAFLYTCELY  457

Query  421  PTVLRQNGMGYSSFMARLGGALAPLVFLLDEVWRSLPEVTYCGVAVCCGLVAFLLPETLH  480
            PTVLRQNGMGYSSFMARLGGALAPLVFLLDEVWRSLPEVTYCGVAVCCGLVAFLLPETLH
Sbjct  458  PTVLRQNGMGYSSFMARLGGALAPLVFLLDEVWRSLPEVTYCGVAVCCGLVAFLLPETLH  517

Query  481  VRLPEGIEDIEKTQAKGPPQISAPKGTPLQSLLK  514
            VRLPEGIEDIEKTQ KGPPQI  PKG PLQ LLK
Sbjct  518  VRLPEGIEDIEKTQVKGPPQIGGPKGMPLQPLLK  551
```

Figure 4-12: Alignment of AWB and Golden eagle OAT2 protein, highlighted amino acids are the differences between the later species.

Pane A

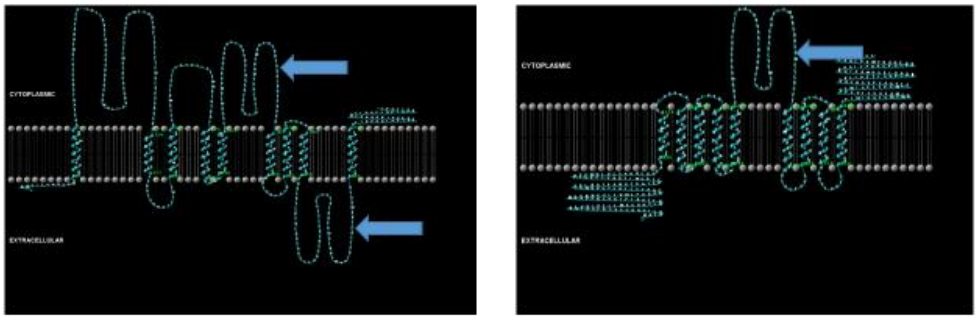


Fig: Vulture and human OAT1 (From left to right)

```
HOAT1       -esarwhsssgrldltlralqrvaringkreegaklsmevlraslqkeltmgkgqasame
         59
VOAT1       Tesarwlvmvgkshqalkelqkvaringkkeegdkldievlrsymqkemassrshhtvvd
         60
             *****    *: . :*: **:********:*** **.:****: :***:: .:.: :.::

HOAT1       llrcpt---   65
VOAT1       lvrtpvvrr   69
            *:* *.
```

Pane B

Fig 4-13: A) Transmembrane topology model of vulture and human organic anion transporter 1 indicating intracellular and extracellular loops for the later species B) Multiple sequence alignment using CLUSTAL O (1.2.4) of human and vulture intracellular loops revealing 47.69% similarity.

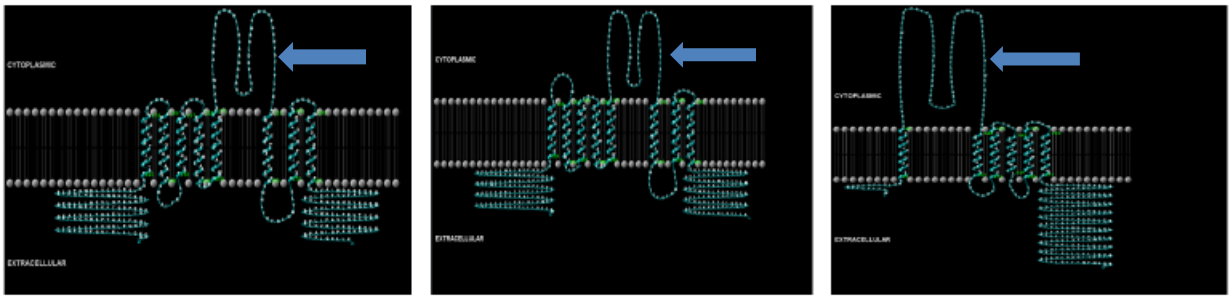


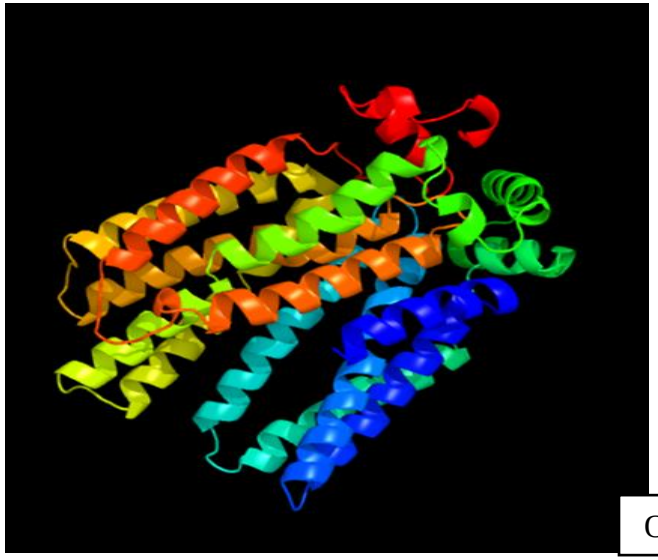
Fig: Vulture, chicken and human OAT2 (From left to right)

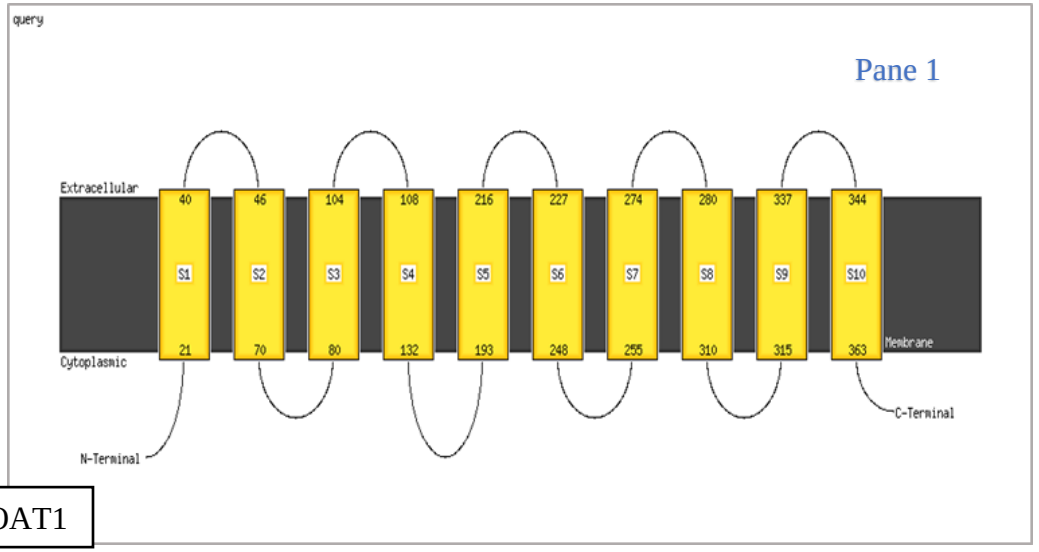
Pane A

```
CLUSTAL O(1.2.4) multiple sequence alignment (VOAT2 VS COAT2, 80% alignment)


Chicken         ESARWLIANGKVKQAHKHLLRCARMNGRKDFTISPEALRGMTTEKNPGESFSYISLFRTP
          60
VULTURE         ESARWLIANGKVERAHRHLLRCARMNGRKDFAVSPEDLRRMTTDKKPGENYSYITLFRTP
          60
                ************::**:***************::*** ** ***:*:***.:***:*****



CLUSTAL O(1.2.4) multiple sequence alignment (human and chicken OAT2 (20.34%
and human and vulture OAT2 18.64%)


HUMAN           PAHRCALPGAPANFSHQDVWLEAHLPR-EPDGTLSSCLRFAYPQALPNTTLGEERQSRGE
          59
Chicken         ---------------ESARWLIANGKVKQAHKHLLRCARMNGRKDFT---ISPEALRGMT
          42
VULTURE         ---------------ESARWLIANGKVERAHRHLLRCARMNGRKDFA---VSPEDLRRMT
          42
                               ..  ** *:    . .  *  * *:   : :    :. *

HUMAN           LEDEPATVPCSQGWEYDHSEFSSTIATESQWDLVCEQKG  98
Chicken         TEKNPG-----ESFSYI-----SLFRTP-----------  60
VULTURE         TDKKPG-----ENYSYI-----TLFRTP-----------  60
                 :.:*.     :.:.*      : : *
```

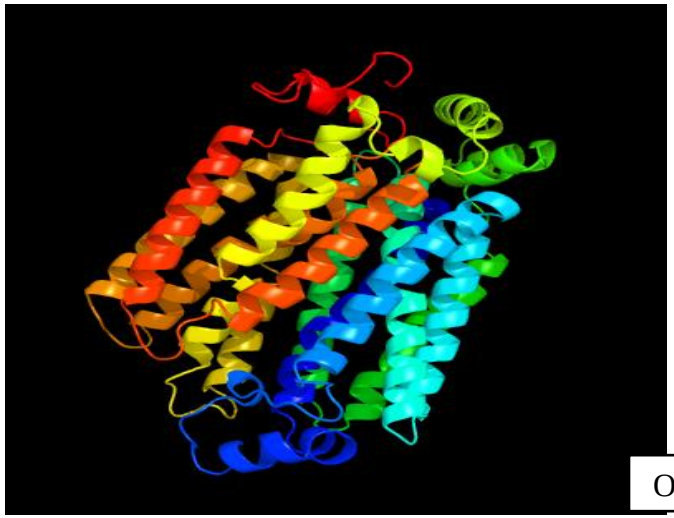
Pane B

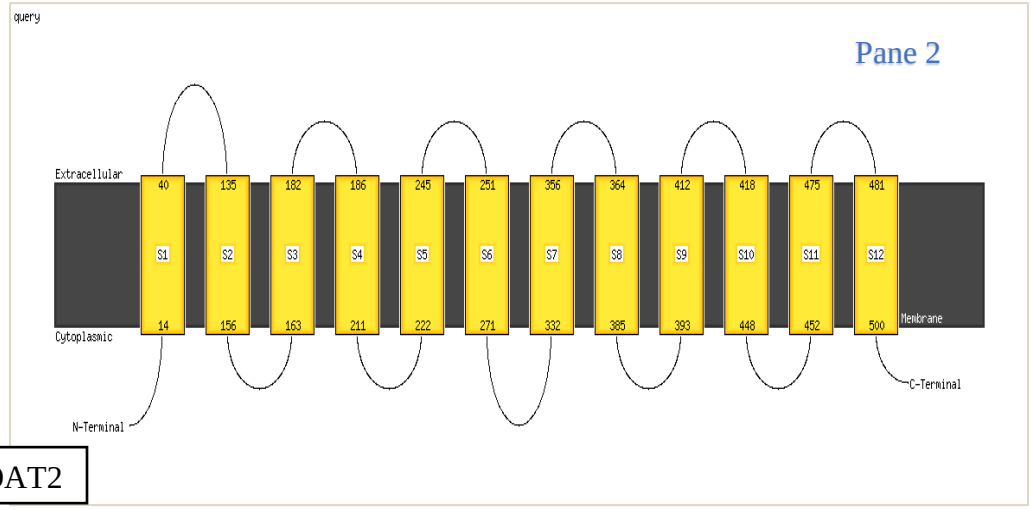
Fig 4-14: A) Transmembrane topology model of vulture, chicken and human organic anion transporter 2 indicating intracellular loop. B) Multiple sequence alignment using CLUSTAL O (1.2.4) of vulture, chicken and human intracellular loops revealing 80% similarity for vulture and chicken and less than 21% for the latter birds with human.





OAT1





OAT2

Figure: 4-15: 3D and 2D structures showing 1) OAT1 with 10 transmembrane helices (TMH) and 2) OAT2 with 12 TMH.

4.3.5. AWB and chicken OAT2 expression.

There was a slight difference in OAT2 amplification peaks between chicken and AWB vulture been expressed at cycle 20.53 and 22.17 respectively (Fig 4-16). To eliminate biasness a melting curve following RT-qPCR of housekeeping gene (GAPDH) was also evaluated for both species. The melting curve results for OAT2 of both birds confirmed that only a slight difference was present (Fig 4-16). The normalised OAT2 expression levels for chicken and AWB vulture was observed at quantification cycles (cq) of 6.609 and 6.947 respectively.

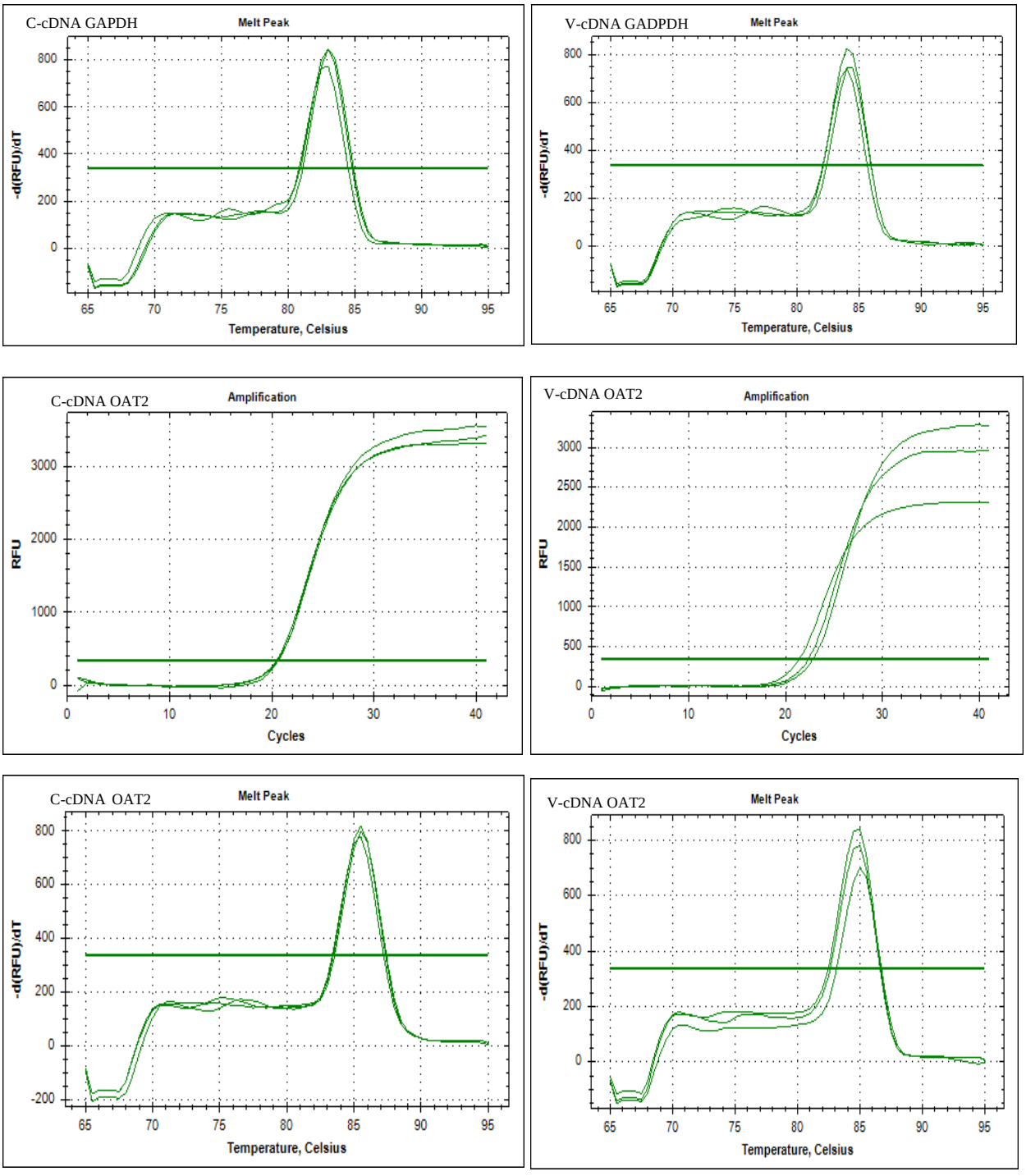


Figure 4-16: real time PCR results revealing 1) melting curve of the AWB and chicken cDNA targeting GAPDH 2) Amplification of AWB and chicken targeting OAT2 gene 3) Melting curve of the later species revealing their differences. C- chicken, V- AWB vulture.

4.4. Discussion

The cloning and understanding of the function of the OATs transport channels started with OAT1 from the rat (Rattus) and the winter flounder (*Pseudopleuronectes americanus*) in 1997. This helped to explain the molecular mechanisms of OAT1 and its functional characteristics in the uptake of organic anions like uric acid at the basolateral membrane of the proximal tubule cells. This allowed for further research of the other OAT genes (OAT2-10) and as well their interspecies differences with expression level, localization and substrate specificity (Duan and You, 2010; Sweet et al., 1997, Sekine et al., 1997; Wolff et al., 1997; Cha et al., 2000; Kusuhara et al., 1999; Youngboold and Sweet , 2004; Schnabolk et al., 2006; Shin et al., 2007; Bahn et al., 2000, 2004; Jacobsson et al., 2007; Simonson et al., 1994; Sekine et al., 1998, Sun et al., 2001; Kobayashi et al., 2002). Nonetheless this is the first study on the characterization of cDNA encoding organic anion transporter genes in *Gyps* vulture.

Mammals have three major OATs channels in the kidney with OAT3 being the most highly expressed in kidneys (Nishimura and Naito, 2005; Bleasby et al., 2006). In this study, the partial "OAT3" gene was successfully identified in the chicken as described by Dudas et al. (2005), with no amplification achieved for the vulture. It was however, surprising that the blast analysis only demonstrated similarity to the OAT1 genes of other avian species. This led to the conclusion that the OAT3-like partial sequences used by Dudas et al. (2005) was probably misclassified and that the study identified OAT1. Also as far as we could ascertain, OAT3 have not been identified in birds in other studies. Also it is unknown why the OAT1 partial sequences identified for the chicken by Dudas et al. (2005) was not present in the NCBI database.

To further evaluate the OATs present in the vulture kidney, next generation sequencing and Trinity assembly revealed the presence of OAT1 with 1468bp and OAT2 with 2300bp genes, both with greater than 98% similar to the Golden eagle. With the NGS assembly demonstrating the presence of the OAT 1 and 2 transporters, this finding was confirmed by Sanger sequencing, which is a gold standard of sequencing with accuracy of 99.99% (Sanger, 1975). The generation of OATs sequences from the amplicons supported the presence of the OAT1 and OAT2 transporters at 1350bp and 1662bp respectively, with up to 99% similarities to the NGS assembled sequences. The evident difference was likely a result of different bird being used for the analysis.

Subsequent phylogenetic analysis showed a major diversity in the OAT channels described thus far in various bird species may be attributable to evolution, mutation; gender and environment. The diversity in the sequence would likely also explain why the chicken primer of Dudas et al. (2005) didn't amplify vulture`s OAT3/OAT1 gene. Moreover, Eagles and AWB vulture OAT1-2 genes shared the same clade, likely attributable to them belonging to the family Accipitrid and their similarity in diet and also their sensitivity to diclofenac. A Study by Sharma et al. (2014) revealed gross, microscopic lesions and diclofenac tissue levels in Steppe Eagles (*Aquila nipalensis*). The former birds were found in Rajasthan, India at a cattle carcass dump showing evidence of the diclofenac toxicity (Sharma et al., 2014). Sharma concluded that diclofenac could be toxic to other Accipitrid raptors. It can be argued that this study revealed insight in phylogeny of OAT1-2 genes in avian species which can help in further molecular research studies in predicting other susceptible avian species.

Lastly in describing the OAT transporters, their location in the renal tubule was determined through the use of immunohistochemistry. For this we used the rabbit polyclonal antibody that is described to bind to mouse OAT3. A polyclonal antibody was specifically chosen as this made it more likely to bind to species different to the mouse. The OAT3 antibody was selected as the binding site showed good similarity with the vulture OAT1 NGS assembled sequence. Following staining, the distribution of the OAT1 transporter was localised to the proximal convoluted tubule. This result aligns with studies by Sekine et al. (1997); Sweet et al. (1997); Lu et al. (1999); Race et al. (1999) indicating that OAT1 is localised to basolateral membrane of the proximal convoluted tubular cells in other species which leads us to speculate that it may function the same way as other species.

The functionality of the obtained protein was hereafter predicted using in silico modelling. After translation, the predicted OAT1 and OAT2 protein was classified as a transporter protein with the likely substrates being anionic for OAT2. Despite the protein showing transporter activity, further simulations indicated that while the AWB OAT1 contained 10 putative transmembrane helices like its reference gene (Golden eagle OAT1) both proteins had no N-glycosylation sites. While this was an unexpected finding, studies by Tanaka et al. (2004), showed that glycosylation played no role in the functionality of the protein however it plays an important role in the attachment of

OAT1 onto the plasma membrane. These proteins are vesicular intracellular and need to be moved to the membrane to be functional. Tanaka et al. (2004) and Zhou et al. (2005) conducted a study of mutagenesis OAT1 and OAT4 in cultured cells respectively. Their results indicated that the removal of all glycosylation sites caused the transporters to be trapped in an intracellular compartment. Using this finding it is likely that OAT1 is inactive in the vulture or even likely that the predicted sequence for the Eagle is not truly an OAT1 transporter. The latter may explain why *in silico* by TrSSP predicted no anionic activity for this protein as well as why no OAT1 has been identified in the chicken either.

In contrast Protter modelling indicated that the OAT2 had 11 transmembrane helices and a large hydrophilic extracellular loop on transmembrane helice 1, which contains three N-glycosylation sites and another two N-glycosylation sites on the intracellular loop on transmembrane helices 5 and 6. This was not too different to the Golden eagle which also had the presence of large hydrophilic extracellular loop between transmembrane helices 1 and 2 which contained three potential N-glycosylation sites; moreover, two more N-glycosylation sites on intracellular loop on transmembrane helices 6 and 7, albeit with 12 putative transmembrane helices. The latter is the same as NGS AWB OAT2 sequence. The accuracy of the prediction of the transmembrane helices does require careful interpretation, as Phyre 2 analysis showed the vulture OAT2 to rather consist of 12 putative transmembrane helices. When compared to results from other species, OATs has previously shown to consist of 12 putative transmembrane helices and a large hydrophilic extracellular loop between transmembrane helices 1 and 2 containing several potential N-glycosylation sites and intracellular loop containing N-glycosylation sites between the 6 and the 7 transmembrane helices (Duan and You, 2010). The latter sites within the protein plays different roles including modulation of biological activity, protein folding, and maintenance of protein stability and regulation of intracellular targeting (Perry et al., 2006; Tanaka et al., 2004; Cha et al., 2000).

From the results we can conclude that OAT2 is the most important channel in excreting organic anions in the vulture compared to OAT1 and OAT3 channels. From the OAT3 results or lack thereof, one can conclude that its absence can contribute to sensitivity to NSAIDs in *Gyps* vulture because thus far the gene have not been characterised in any avian species and failure to

characterise it in this study drew conclusion of its absence. Moreover, the absence of N-glycosylation sites in OAT1 gene can also contribute to the latter toxicity because the former mediates the attachment of the transporter to the plasma membrane for the transporter to function. To add on to the above assumption, our results (chapter three) indicated that diclofenac interact with the renal organic anion transport system in vulture. This result aligns with study by Khamdang et al. (2002), in his study they tested diclofenac as an inhibitor of OATs and revealed that OAT3 had similar affinity towards most NSAIDs in comparison with OAT1 however OAT2 had lower affinity towards NSAIDs compared to former. The OAT1&3 proteins also share the molecular requirements for NSAIDs interaction whereas OAT2 differs. They further estimated the degree of *in vivo* inhibition of OAT1, 2 and 3 by diclofenac and the results revealed average IC_{50} of 4.46μm, 14.3μm, 7.78μm respectively (Khamdang et al., 2002). Furthermore extensive inhibition analyses in human, mouse, and vulture OAT1, OAT2 and OAT3 was carried out and it demonstrated that the later channels can be inhibited by diclofenac, ketoprofen, acetaminophen, naproxen, ibuprofen, indomethacin, mefenamic acid, piroxicam and phenacetin (for more NSAIDs refer to chapter 2, Table 2-3) (Khamdang et al., 2002; Nozaki et al., 2007; Kimura et al., 2007; Naidoo et al., 2009).

As OAT2 functional shape was conserved between the vulture and chicken, it is likely that they function the same way. Using RT-qPCR we were able to demonstrate that the chicken had a greater expression of OAT2 channels in comparison to the vulture per kg of body weight. The lower number of channels in vulture would firstly explain the sensitivity of the latter species to diclofenac compared to chicken because chicken OAT2 expression levels were at cq of 3.67 while vulture was 1.54 per kg of body weight affirming the results on chapter three. From the results from the in vivo studies, we were able to show that diclofenac inhibits the PAH transporters, which are better known as the OAT transporters. With the vulture kidney having a lower total number of transporters, it would explain the sensitivity to the effects of diclofenac.

4.5. Conclusion

In this study we conclude that AWB vulture `s kidney expresses OAT1 and OAT2 mRNA however OAT3 was not ascertained. It was an interesting surprise to establish that OAT1 of the latter specie and its refence gene (eagle) had no N-glycosylation sites unlike OAT2 which had five sites just

like other OATs gene of other species. This finding lead to a conclusion that OAT2 is the main transporter in AWB vulture and expression study revealed a slightly greater expression levels of chicken OAT2 compared to vulture which explain its sensitivity to diclofenac at as little as of 0.8 mg/kg per body mass of bird.

4.6. Acknowledgement

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CHAPTER FIVE

5. Analysis of expression of African White Backed vulture MRP2, MRP4 genes and phylogeny of MRPs in avian species.

Bono Nethathe[1], Aron Abera[2], Vinny Naidoo[1]

[1]Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, Pretoria, South Africa.

[2]Inqaba Biotechnology, Pretoria, South Africa

5.1. Introduction.

The multidrug resistance protein (MRP) family also known as ATP-binding cassette subfamily C (ABCC) was first described as a drug resistance protein by Cole and Deeley (1992) when they managed to clone the MRP1 gene in human (Cole and Deeley, 1992). They first associated it with transporting anti-cancer drugs (Haimeur et al., 2004). MRP1 is generally regarded as the godfather of the family and further research has since described five homologs named MRP2-6 (Kool et al., 1997, 1998, 1999). The MRP family have the highest homology between MRP1, 2, 3 and 6 genes as a result of a similar structure characterized by presence of extra N -terminal extension with five membrane (TMD_0) connected to P-glycoprotein (Pgp)-like core by a cytoplasmic linker (L_0) however the opposite can be said for MRP4 and 5 which are more similar because they lack TMD_0 domain but have intracellur L_0 in addition of Pgp-like core (Fig 5-1) (Bakos et al., 1996, 1997; Tusnady et al., 1997).

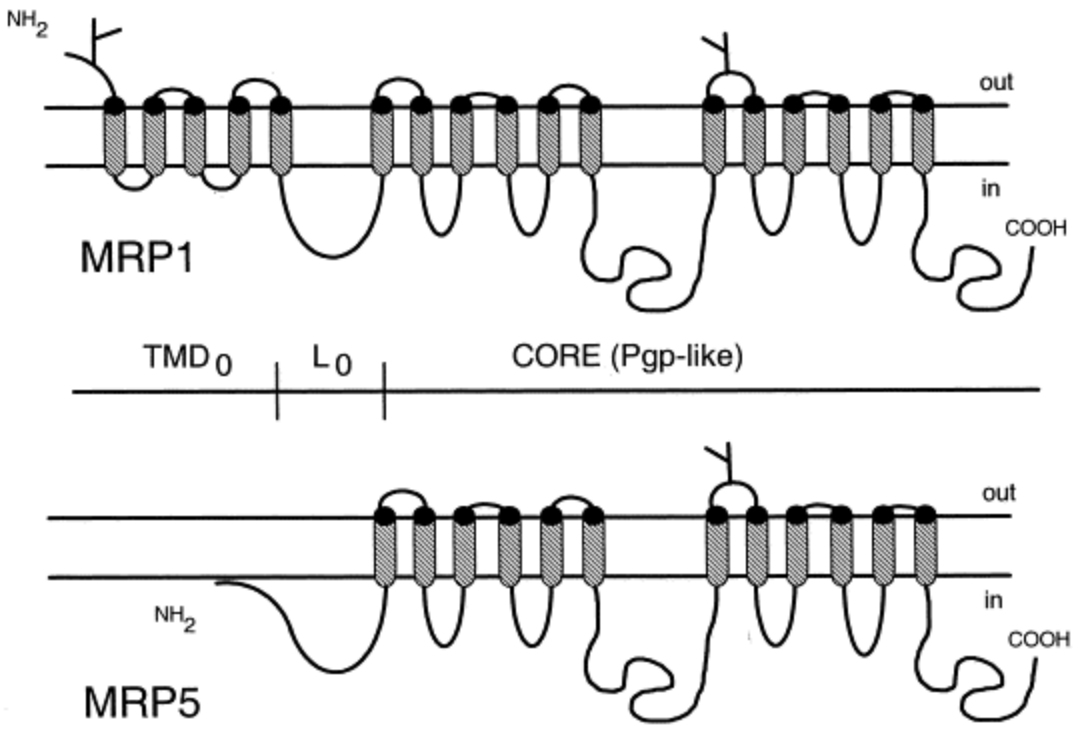

Fig 5-1: Two-dimensional membrane topology models for MRP1 and MRP5 according to Bakos et al., 1998).

In general the MRP protein functions as extrusion pump system responsible for moving substances from within the cell into the extracellular environment e.g. In biliary transport, it moves substances into the biliary tract and bile (Taniguchi et al., 1996; Kool et al., 1997), while MRP2 expressed in the proximal renal tubule endothelial cells found in apical membrane, play a role in the excretion of small intracellular organic anions (Sekine et al., 2006). Examples of substance transported by renal MRP2 are lipophilic substances conjugated to glutathione, glucuronate, or sulfate (König et al., 1999). The MRP channels are distributed throughout the body such MRP3 and MRP4 found in the pancreas, bladder, gut, lung, prostate, ovary, muscle, testis and gallbladder respectively while MRP5 is ubiquitous (Kool et al., 1997, 1999; Lee et al., 1998; Kiuchi et al., 1998; KÖnig et al., 1999). Nonetheless, the main MRPs channels are located on the liver and kidney.

In the kidney, the MRPs play an important role in the excretion of uric acid from the intracellular environment, following their movement therein by the Organic Anion Transporters in all species, as well by the Uric Acid Transporter in mammals (Sweet, 2005, 2010; Van wert et al., 2010). With diclofenac having the ability to induce severe hyperuricemia and gout in the vultures (Naidoo et

al 2009), this study focuses on characterising the last step in uric acid transport. In this study we focused on MRP2 and MRP4 channels that are localised in the kidney of AWB (African white-backed) vulture. In addition to being known to transport organic anions, it is believed that these channels function exactly the same as in mammals (for more details in chapter 2 page 42-43). Moreover we will also use a recent study by Barik et al. (2019) which characterised MRP4 gene on *Gyps Himalyanesis* for comparison with obtained *Gyps Africanus* MRP4 gene in this study (Barik et al., 2019).

5.2. Material and methods.

5.2.1. Sample collection

Before the commencement of experiments, ethical clearance for collection of samples was conducted according to the guidelines approved by University of Pretoria Animal Ethics committee (V108-16). Two AWB (*Gyps africanus*) vultures of unknown age with a badly fractured wing that had not been treated with any analgesic previously were euthanized at Faculty of Veterinary Science, University of Pretoria. The kidneys were harvested immediately after euthanasia and stored in cryogenic vials containing RNA*later* (Whitehead Scientific, Cape Town, South Africa) and stored at -80°C freezer until analysed, the same birds as chapter four.

5.2.2. Next generation sequencing (NGS)

Fresh Total RNA was extracted from the AWB kidney as previously described in section 4.2.3 of chapter 4 and transported to Agricultural Research Council (ARC, Onderstepoort, Pretoria, South Africa) on dry ice for sequencing. DNA contamination was removed from total RNA using DNase. The RNA fragments were reverse transcribed into cDNA and sequencing adaptors were ligated. Finally, the ends of the cDNAs were sequenced using Illuminia Truseq mRNA stranded Ran preparation kit on Hiseq 2500 v4 2x125bp chemistry model. The result obtained from sequencing was 50 million short reads which were 125 nucleotides long each. Prior assembly of the raw reads, several quality control steps were conducted on the raw reads using the online Galaxy platform (Blankenberg et al., 2010) and fast quality check (FASTQC) (Andrews, 2014) to check overall quality of the sequences, duplication and adapters. After checking the quality of the sequences, the adapters and PCR duplicates were removed using the programme Trimmomatic

(Bolger et al., 2014). The pre-processed reads were assembled into transcripts using TRINITY (Grabherr et al., 2013). The assembled transcriptome was converted to a local blast database NCBI (Altschul et al., 1990) and sequences were identified based on the predicted MRP2 (XM_011576169.1) and MRP4 (XM_011586368.1) sequences from the Golden eagle genome. The Golden Eagle was selected as another study completed in our laboratory has shown them to be closely related species as well as having both channels present. Transcript quantification was undertaken using Sailfish with reference transcripts from the de novo analysis. Alignment of AWB MRP4 and *Gyps Himalyanesis* was also conducted using Clusta omega to reveal similarity.

5.2.3. Confirmation of transcriptome MRP2 and MRP4 using Sanger sequencing.

Fresh total RNA was extracted from AWB kidney using Quick RNA Miniprep kit following instructions from the manufacturer (Zymo Research, USA). cDNA synthesis was performed using Lunascript RT supermix kit from New England Biolabs (NEB), (USA) according to the manufacturer`s instructions. Primers of MRP2 and MRP4 were designed based on the transcriptome sequences generated from illuminia next generation sequencing. The special primers were synthesized at Inqaba Biotechnology, Pretoria, South Africa. Primers of MRP2 and 4 were as follows:

Table 5-1: Primers sequences used to amplify OAT1 and OAT2 gene from AWB`s kidney.

MRPs Primers	**Predicted amplicon length**
MRP2 F(S1): CTGTGTGTCATCAGGGATTTTGTC	2754bp
MRP2-IN-R(S1): ACAAGTTCAGGAATTGGGCAAAG	
MRP2-IN-F(S2): CAGAGATTGGAGAGAAGGGCATT	2550bp
MRP2 R(S2): AAGGTTAACAGCTCACTCAAGTCC	
MRP4 F(S1): TATTGGCCATAAACGGAAGCTTGA	2241bp
MRP4-IN-R(S1): GCCACCGTTAAACCTGCATAAAT	

MRP4 S1: TCCTGTCCAGACCTGCATTG	To cover the sequences that were not covered by the original sequence
MRP4-IN- F(S2): GAACTCCCAACCTGAAGTCTGTC	2136bp
MRP4 R(S2): CACTTGCAAACATTGTCACTGAGT	
MRP4 S2: ATCCACAACTCGAAGTCCAGT	To cover the sequences that were not covered by the original sequence.

F-forward primer, R- reverse primer, S1-segment 1, S2-segment 2.

Using the latter set of primers, PCR was performed using One taq polymerase kit from NEB and AWB`s kidney cDNA as a template. The amplification protocol for all the targets was as follows: Initial denature 94^0C 30 sec, 40 cycles of (denature 94^0C for 30sec; annealing 55^0C for 30sec and elongation 68^0C for 2min) and final elongation 68^0C 5min. However for MRP2 segment 1, the former did not work so the following cycling conditions where used, Initial denature 98^0C 30 sec, 40 cycles of (denature 98^0C for 30sec; annealing 65^0C for 30sec and elongation 72^0C for 2min) and final elongation 72^0C 2min. The PCR products was run on 1% agarose gel. The gel was viewed for desired bands using gel documentation system and PCR products of the desired bands was further cleaned up using Exopsup from NEB and sequences were generated using ABI 3500X1 genetic analyser.

5.2.4. Phylogenetic analyses

Searches of database for homologous sequences were performed using BLASTn (Altschul et al., 1990). Thirty-five (MRP2) and 36 (MRP4) genes that were 85% and above identical to our obtained sequences were downloaded from GenBank (NCBI). A multiple sequence alignment was performed along with the downloaded sequences (Table 4-2), using muscle algorithm (Molecular Evolutionary Genetics Analysis version 7.0 (MEGA7)) software package (Edgar, 2004; Kumar et al., 2016). The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model for MRP2 (Tamura and Nei, 1993) while MRP4 was Maximum Likelihood method and General Time Reversible model (Nei and Kumar, 2000). The bootstrap consensus tree

inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). Evolutionary analyses was performed using MEGA 7 (Kumar et al., 2016).

Table 5-2: Avian MRP2 and 4 sequences accession number used for phylogenetic analysis

Species	**Common name**	**MRP2**	**MRP4**
Acanthisitta chloris	Rifleman	XM_009079998.1	
Anas platyrhynchos	Mallard		XM_021269067.1
Anser cygnoides domesticus	Domestic geese	XM_013196310.1	XM_013195781.1
Apaloderma vittatum	Bar-toiled trogon		XM_009864407.1
Aptenodytes forsteri	Emperor penguin	XM_009278032.1	
Apteryx australis mantelli	North Island Brown Kiwi		XM_013951288.1
Aquila chrysaetos Canadensis	Golden eagle	XM_011576169.1	XM_011586368.1
Balearica regulorum gibbericeps	East-African grey crowned-crane	XM_010301460.1	
Calidris pugnax	Ruff	XM_014957963.1	XM_014955633.1
Caprimulgus carolinensis	chuck-will`s widow	XM_010175463.1	XM_010169845.1
Cariama cristata	Red-legged seriema	XM_009708646.1	XM_009699864.1
Chaetura pelagica	Chimney Swift		XM_010006355.1
Charadrius vociferous	Killdeer	XM_009880472.1	XM_009891916.1
Chlamydotis macqueenii	Macqueen`s bustard	XM_010129192.1	
Colius striatus	Speckled mousebird		XM_010201078.1
Columba livia	Rock Pigeon	XM_005506981.3	XM_005505863.3
Corvus brachyrhynchos	American crow	XM_017726096.1	XM_017732634.1
Cuculus canorus	Common Cuckoo		XM_009555936.1
Egretta garzetta	Little egret	XM_009641936.1	XM_009634600.1
Eurypyga helias	Sun bittern	XM_010147273.1	XM_010157385.1
Falco cherrug	Saker falcon	XM_014276741.1	XM_005443961.2

Falco peregrinus	Peregrine falcon	XM_013300438.1	XM_005235621.2
Fulmarus glacialis	Northern Fulmar	XM_009575606.1	
Gallus gallus	Chicken	XM_015288821.1	NM_001030819.1
Gavia stellata	Red-throated loon	XM_009812133.1	
Gyps himalayensis	Himalayan Vulture		KX168697.1
Haliaeetus leucocephalus	Bald eagle	XM_010564882.1	XM_010578329.1
Leptosomus discolor	Cuckoo roller	XM_009958793.1	
Manacus vitellinus	Golden-Collared Manakin		XM_018071273.1
Meleagris gallopavo	Turkey	XM_010714591.2	XM_019612046.1
Melopsittacus undulates	Budgerigar		XM_005144756.1
Nestor notabilis	Kea		XM_010019073.1
Nipponia Nippon	Crested ibis	XM_009465232.1	XM_009467038.1
Numida meleagris	Helmeted guinea fowl	XM_021398589.1	XM_021416653.1
Opisthocomus hoazin	Stinkbird		XM_009942362.1
Parus major	Great tit	XM_015632687.2	XR_001520195.2
Pelecanus crispus	Dalmatian pelican	XM_009485052.1	XM_009488769.1
Phaethon lepturus	White-tailed trophicbird	XM_010294140.1	
Phalacrocorax carbo	Great cormorant	XM_009500518.1	
Pygoscelis adeliae	Adelie Penguin	XM_009323318.1	XM_009325566.1
Struthio camelus australis	Southern Ostrich	XM_009677757.1	XM_009667376.1
Sturnus vulgaris	Common starling	XM_014885931.1	XM_014881347.1
Tinamus guttatus	white-throated tinamou	XM_010213115.1	XM_010219632.1
Tyto alba	Barn owl	XM_009971968.1	XM_009963037.1
Zonotrichia albicollis	White-throated sparrow	XM_014275581.1	

5.2.5. Protein Structure and modelling

The obtained MRP2 and MRP4 NGS sequences were converted in Expasy (Gasteiger et al., 2003) to protein sequences and the open reading frame was developed. After the deduced amino acids sequences were analysed by following software; Transmembrane helix prediction using Markov

model (Sonnhammer et al., 1998), Swiss model Protparam for functional prediction of the protein (De Castro et al., 2006) and Phyre2 (Kelly, 2015) for prediction of 2-D and 3-D structures. PROTTER sequence database was used to predict N-glycosylation sites (Omasits et al., 2014) and Expasy database was also used to examine other possible post-glycosylation and phosphorylation (http://www.expasy.ch/ prosite).

5.3. Results.

5.3.1. Next generation sequencing.

The assembled transcriptomes were predicted against Golden Eagle genome. The predicted AWB MRP2 and MRP4 genes consisted of 5212 and 4061bp respectively. The MRP2 and 4 alignment of the AWB vulture and the golden eagle were 98.12% and 99.14% respectively (Figure 5-2 and 5-3). Moreover AWB and *Gyps Himalyanesis* alignment of MRP4 gene revealed 94.06% similarity (Fig 7-1).

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Query  1     TGCAATAGCACCAAGTTAACAATTAACTGTGTGTCATCAGGGATTTTGTCCCAACACCAC  60
             ||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct  521   TGCAATAGCACCAAGTTAACAATTAACGGTGTGTCATCAGGGATTTTGTCCCAACACCAC  580

Query  61    TTGAGGGTGTGTGGGGACTTTTGTCCCAAGCATCCATCCAGGACTCACAGCCAGGCAGAG  120
             |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||
Sbjct  581   TTGAGGGTGTGTGGGGACTTTTGTCCCAAGCATCCACCCAGGACTCACAGCCAGGCAGAG  640

Query  121   CGAGGGGAAGGAGCTGTCGTCTCTTCCCCCCTGAGCCATGTCGGCAGCCCTGGAGGAGTT  180
             |||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct  641   CGAGGGGAAGGAGCTGTCGTCTCTTCTCCCCTGAGCCATGTCGGCAGCCCTGGAGGAGTT  700

Query  181   CTGTGGCTCCGTCTTTTGGAATGCATCCTACCTCACTCGTCCAGATGCCGACCTGCCCGT  240
             |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct  701   CTGTGGCTCCGTCTTTTGGAATGCCTCCTACCTCACTCGTCCAGATGCCGACCTGCCCGT  760

Query  241   GTGCTTCCAGCAGACTGTGCTGGTCTGGGTCCCCCTTGGCTTCTTCTGGATTTTGGCTCC  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  761   GTGCTTCCAGCAGACTGTGCTGGTCTGGGTCCCCCTTGGCTTCTTCTGGATTTTGGCTCC  820

Query  301   ATGGCAGCTCCTGCCCATGTGCAAATCCAGAGCCAAGAAATCATCTGTGACCAAACTCTA  360
             ||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  821   ATGGCAGATCCTGCCCATGTGCAAATCCAGAGCCAAGAAATCATCTGTGACCAAACTCTA  880

Query  361   CATCATCAAACAGGTGCTGGCTACCTTGCTGATGCTGACGGCAGCAGCGGAGTTGGCCTT  420
             |||||||||||||||||||||||||||||||||||| ||||| || ||||||||| |
Sbjct  881   CATCATCAAACAGGTGCTGGCTACCTTGCTGATGCTGACAGCAGCGGCTGAGTTGGCCCT  940

Query  421   GGCGTTTGTAGAGGACACAGAGCAGGACCCCCTGCCAGCTGTCCAGTACACAAACCCCAG  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  941   GGCGTTTGTAGAGGACACAGAGCAGGACCCCCTGCCAGCTGTCCAGTACACAAACCCCAG  1000
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Query  481   CCTGTACATTGCCACCTGGCTCCTGGTCCTGCTGATCCATGATGCACGACGCTTCTGCTT  540
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |
Sbjct  1001  CCTGTACATTGCCACCTGGCTCCTGGTCCTGCTGATCCATGATGCACGACGCTTCTGCCT  1060

Query  541   GCGCAGAGACTCGGGGATACTTTTCTGCTTCTGGACACTGTCCCTGCTCTGTGGGATATT  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1061  GCGCAGAGACTCGGGGATACTTTTCTGCTTCTGGACACTGTCCCTGCTCTGTGGGATATT  1120

Query  601   GCCATTCCAGTCACTCCTCCGGAAAGCCCTGCAGGCACCAATCTCTGACGTGCCACGGTT  660
             |||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  1121  GCCATTCCAGTCACTCCTCCGGAGAGCCCTGCAGGCACCAATCTCTGACGTGCCACGGTT  1180

Query  661   TGTCCTTTTCTTCACCTCCTACGGGCTCCAGCTGCTGCTTTTTCTTGTCTCGGGCTTCTC  720
             |||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1181  TGTCCTTTTCTTCATCTCCTACGGGCTCCAGCTGCTGCTTTTTCTTGTCTCGGGCTTCTC  1240

Query  721   AGACGTTGCCCCAGAAACAAAGGAAATCACGAAGAAGAACCCACAGGTGACAGCCTCCTT  780
             |||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
Sbjct  1241  AGACGTTGCCCCAGAAACAAAGGAAATCACGAAGAAGAACCCAGAGGTGACAGCCTCCTT  1300

Query  781   CCTGAGCTCCATCACCTTTGAATGGTACACCAGCATGGTTTTCAAGGGCTATCGCAAACC  840
             |||||||||||||||||| |  ||||||||||||||||||||||||||||||||||||||
Sbjct  1301  CCTGAGCTCCATCACCTTCGCGTGGTACACCAGCATGGTTTTCAAGGGCTATCGCAAACC  1360

Query  841   CTTGGAGATAGAGGATATCTGGGAATTGAAAGGTAAAGACAAGACGCAGGCTATTTATGC  900
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||
Sbjct  1361  CTTGGAGATAGAGGATATCTGGGAATTGAAAGGTAAAGACAAGACGCAGGCTATTTACGC  1420

Query  901   TGTTTTGGAGAATAACATGAAGACTGCGGTGAGGAAGGCCCAAGCAGAACTGGAGAAACG  960
             |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1421  TGTTTTGGAGAAGAACATGAAGACTGCGGTGAGGAAGGCCCAAGCAGAACTGGAGAAACG  1480

Query  961   GAAACGCAAGAAAAGACGCCGGGAAGGTGACCCAGACCATGGGAACAACATGAGCAAGGC  1020
             |||||||||||||||||||||||||||||||||||||||||||||||||  ||||||||||
Sbjct  1481  GAAACGCAAGAAAAGACGCCGGGAAGGTGACCCAGACCATGGGAACAATGTGAGCAAGGC  1540

Query  1021  CCAGAGCCAAGACATCCTGGTGCTGGAGGAAAAGCAGCTGAAGAGGAAGAAGAAGGGAGA  1080
             |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||
Sbjct  1541  CCAGAGCCAAGACATCCTGGTGCTGGAGGAAAAGCAGCCGAAGAGGAAGAAGAAGGGAGA  1600

Query  1081  CAAAGGGGACTCTGGCCCTCACAAGGATTTCCCCCGGGGCTGGTTGGTGAAAACCCTGTG  1140
             |||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  1601  CAAAGGGGACTCTGGCCCTCGCAAGGATTTCCCCCGGGGCTGGTTGGTGAAAACCCTGTG  1660

Query  1141  CAAGACCTTCTGGCAGAACCTCCTGCTATCGGTGGCTTTCAAGCTGGTGCATGACGGACT  1200
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||||
Sbjct  1661  CAAGACCTTCTGGCAGAACCTCCTGCTATCGGTGGCTTTCAAGCTGGTGCATGATGGACT  1720

Query  1201  TGTGTTCGTCAGCCCCCAGCTGCTGAAGCTGCTGATCGCCTTTGTGTCAGATGAGGAGTC  1260
             |||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct  1721  TGTGTTCGTCAGCCCCCAGCTGCTGAAGCTGCTGATCGCCTTTGTGTCGGATGAGGAGTC  1780

Query  1261  CTTTGCCTGGCAAGGCTATCTGTATGCCATCCTGCTCTTCCTGACGGCACTGATCCAGTC  1320
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1781  CTTTGCCTGGCAAGGCTATCTGTATGCCATCCTGCTCTTCCTGACGGCACTGATCCAGTC  1840

Query  1321  CCTCTGCCTGCAGCAGTACTTCAGCTTGTGCTTCCAGCTTGGCATAAATGTGCGTGCCAG  1380
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1841  CCTCTGCCTGCAGCAGTACTTCAGCTTGTGCTTCCAGCTTGGCATAAATGTGCGTGCCAG  1900

Query  1381  TCTCATTGCTGCCATCTACAAGAAGGCACTCACCATGTCCAGTGCCACCCGCAAGGAGTC  1440
             ||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1901  TCTCACTGCTGCCATCTACAAGAAGGCACTCACCATGTCCAGTGCCACCCGCAAGGAGTC  1960
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Query  1441  CACGGTGGGAGAGACTGTGAATCTGATGTCAGCTGATGCCCAGAGGTTCATGGACACGGC  1500
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||
Sbjct  1961  CACGGTGGGAGAGACTGTGAATCTGATGTCAGCTGATGCCCAGAGGTTCATGGACACAGC  2020

Query  1501  CAACTTCGTTCACCAGCTGTGGTCATCCCCCCTGCAAATTATCCTGTCCATTGTCTTCCT  1560
              |||||| |||||||||||||||||||||||||||||||||||| ||||||||||||||
Sbjct  2021  CAACTTTGTTCACCAGCTGTGGTCATCCCCCCTGCAAATTATCCTGGCCATTGTCTTCCT  2080

Query  1561  CTGGGGAGAGCTGGGCCCCTCTGTTCTGGCTGGCATCGCAGTTATGGTGCTGCTCATCCC  1620
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2081  CTGGGGAGAGCTGGGCCCCTCTGTTCTGGCTGGCATCGCAGTTATGGTGCTGCTCATCCC  2140

Query  1621  CATAAATGGGTTCCTGGTTGCCAAGGCCAAAACCATCCAGGTGAGGAACATGAAGAACAA  1680
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2141  CATAAATGGGTTCCTGGTTGCCAAGGCCAAAACCATCCAGGTGAGGAACATGAAGAACAA  2200

Query  1681  GGATGAACGCATGAAAATAATGAGTGAAATCCTCAATGGAATCAAGATCCTGAAGCTTTT  1740
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2201  GGATGAACGCATGAAAATAATGAGTGAAATCCTCAATGGAATCAAGATCCTGAAGCTTTT  2260

Query  1741  TGCCTGGGAGCCCTCATTTGAGAAGCGAGTCAATGAGATCCGGGCACGTGAGCTCAAGGA  1800
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2261  TGCCTGGGAGCCCTCATTTGAGAAGCGAGTCAATGAGATCCGGGCACGTGAGCTCAAGGA  2320

Query  1801  CTTGGTGAACTTCAGTTACCTGCAGTCAATCTCTATCTTCGTGTTCACGTGTGCGCCCTT  1860
              |||||||||||||||||||||||||||||||||||||||||||||||||||||| |||||
Sbjct  2321  CTTGGTGAACTTCAGTTACCTGCAGTCAATCTCTATCTTCGTGTTCACGTGTGCCCCCTT  2380

Query  1861  CCTGGTCTCCTTGGCCAGCTTTGCTGTTTACATGCTGGTGGATGAGAACAACATCCTGGA  1920
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2381  CCTGGTCTCCTTGGCCAGCTTTGCTGTTTACATGCTGGTGGATGAGAACAACATCCTGGA  2440

Query  1921  TGCACAGAAAGCCTTTACTGCCATCTCCCTTTTCAACGTGCTGCGCTTCCCCATGGCCAT  1980
              |||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2441  TGCACAAAAAGCCTTTACTGCCATCTCCCTTTTCAACGTGCTGCGCTTCCCCATGGCCAT  2500

Query  1981  GCTGCCCTTGGTCCTTTCTTCCTTGGTGCAGACCAACGTGTCGACTGCGAGGCTGGAGCG  2040
              |||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||
Sbjct  2501  GCTGCCCTTGGTCCTTTCTTCCTTGGTGCAGACCAATGTGTCGACTGCGAGGCTGGAGCG  2560

Query  2041  CTACCTGGGCAGAGAAGACCTGGACACCTCGGCTATCCACCACAACCCCATTGCAGGCAG  2100
              |||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||
Sbjct  2561  CTACCTGGGCAGAGAAGACCTGGACACCTCGGCCATCCACCACAACCCCATTGCAGGCAG  2620

Query  2101  CGCTGTGCGTTTCTCGGAGGCCACCTTTGCCTGGGAGCAGGACGGCAATGCTGCGATAAG  2160
              |||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||
Sbjct  2621  CGCTGTGCGTTTCTCGGAGGCCACCTTTGCCTGGGAGCAGGACGGCAATGCTGTGATAAG  2680

Query  2161  AGATGTCACCCTGGACATCGCACCTGGGAGCCTGGTGGCCGTGGTGGGGGGCTGTGGGCTC  2220
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2681  AGATGTCACCCTGGACATCGCACCTGGGAGCCTGGTGGCCGTGGTGGGGGGCTGTGGGCTC  2740

Query  2221  AGGCAAATCTTCGCTGGTGTCAGCCATGCTCGGGGAGATGGAGAATATCAAGGGACACAT  2280
              |||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
Sbjct  2741  AGGCAAATCTTCGCTGGTGTCAGCCATGCTCGGGGAGATGGAGAGTATCAAGGGACACAT  2800

Query  2281  CAACATCCAGGGCTCCCTGGCCTATGTACCCCAGCAGGCCTGGATCCAGAATGCCACACT  2340
              |||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||
Sbjct  2801  CAACATCCAGGGCTCCCTGGCCTATGTACCCCAGCAGGCCTGGATCCAGAACGCCACACT  2860

Query  2341  GAAAGACAACATCCTTTTTGGGTCAGAACTGGATGAAGCCAGGTATCAGCAGGTCATCAA  2400
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Sbjct  2861  GAAAGACAACATCCTTTTTGGGTCAGAACTGGATGAAGCCAGGTATCAGCAGGTCATCAG  2920

Query  2401  GGCCTGCGCCCTCCTTCCAGACCTGGAACTGCTGCCTGCGGGTGACCAGACAGAGATTGG  2460
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2921  GGCCTGCGCCCTCCTTCCAGACCTGGAACTGCTGCCTGCGGGTGACCAGACAGAGATTGG  2980

Query  2461  AGAGAAGGGCATTAACCTGAGCGGGGGCCAGAAGCAGCGAGTCAGCCTGGCCCGGGCAGT  2520
              |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||
Sbjct  2981  AGAGAAGGGCATTAACCTGAGCGGGGGCCAGAAGCAGAGAGTCAGCCTGGCCCGGGCAGT  3040

Query  2521  GTACAGCAACGCAGACATCTACATCCTGGATGACCCCCTGTCTGCCGTGGATGCTCATGT  2580
              ||||||||||||||||||||  ||||||||||||||||||||||||||||||||||||||
Sbjct  3041  GTACAGCAACGCAGACATCTATGTCCTGGATGACCCCCTGTCTGCCGTGGATGCTCATGT  3100

Query  2581  CGGCAAGTACCTCTTCGAGCATGTGCTGGGGCCAAAAGGGCTGCTGCAAAAGAAGACACG  2640
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3101  CGGCAAGTACCTCTTCGAGCATGTGCTGGGGCCAAAAGGGCTGCTGCAAAAGAAGACACG  3160

Query  2641  GATCTTGGTGACGCACAGTATCAGTTTCCTGCCCCAGGTCGATAACATCGTGGTGCTGGT  2700
              |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||
Sbjct  3161  GATCTTGGTGACGCACAGTATCAGTTTCCTGCCCCAGGTTGATAACATCGTGGTGCTGGT  3220

Query  2701  GGCAGGAACAGTGTCTGAGCATGGCTCCTACAGCACCCTGCTTGCAAACAGGGGGGCCTT  2760
              ||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3221  GGCAGGAGCAGTGTCTGAGCATGGCTCCTACAGCACCCTGCTTGCAAACAGGGGGGCCTT  3280

Query  2761  TGCCCAATTCCTGAACTTGTACGGCAGCCAGGAGGAGGATGCTTCAGAGAAGAATACCAC  2820
              |||||| |||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  3281  TGCCCAGTTCCTGAACTTTTACGGCAGCCAGGAGGAGGATGCTTCAGAGAAGAATACCAC  3340

Query  2821  AGCTGTTGCTTTAGCTGGGGATGAAGAGCAGGGTGATGAAGACATTGAGCCTTGTGTGGA  2880
              |||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
Sbjct  3341  AGCTGTTGCTTTAGCTGGGGATGAAGAGCAGGGTGATGAAGACACTGAGCCTTGTGTGGA  3400

Query  2881  GGAGGGTCCTGATGATGTGGTGACCATGACCCTGAAGCGCGACGCCAGCATCCGTCAGAG  2940
              |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||
Sbjct  3401  GGAGGGTCCTGATGATGTGGTGACCATGACCCTGAAACGCGACGCCAGCATCCGTCAGAG  3460

Query  2941  AGAGTTCAGTCGCAGCCTTAGTAAAAGCAGCACCAATTCCTGGAAGAAGGCCCAGGAGGA  3000
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3461  AGAGTTCAGTCGCAGCCTTAGTAAAAGCAGCACCAATTCCTGGAAGAAGGCCCAGGAGGA  3520

Query  3001  GCCCCCCAAGAAGCTGAAAGGCCAGCAGCTGATTGAGAAAGAAGCTGTGGAAACCGGCAA  3060
              |||||| |||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct  3521  GCCCCCAAAGAAGCTGAAAGGCCAGCAACTGATTGAGAAAGAAGCTGTGGAAACCGGCAA  3580

Query  3061  GGTGAAGTTCTCCATGTACCTGCGGTACCTGCATGCCGTTGGCTTGTGGTATTCTTTCTG  3120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3581  GGTGAAGTTCTCCATGTACCTGCGGTACCTGCATGCCGTTGGCTTGTGGTATTCTTTCTG  3640

Query  3121  GGTTGCCATGGGCTACGTTGGACAGTACGTCGCCTTCGTGGGGACTAACCTGTGGCTCAG  3180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3641  GGTTGCCATGGGCTACGTTGGACAGTACGTCGCCTTCGTGGGGACTAACCTGTGGCTCAG  3700

Query  3181  TGCCTGGACTGACGATGCGCAGCACTACCTGAACCAGACCTATCCCACAGAGCAGCGGGA  3240
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3701  CGCCTGGACTGACGATGCGCAGCACTACCTGAACCAGACCTATCCCACAGAGCAGCGGGA  3760

Query  3241  CCTGCGGATCGGTGTCTTTGGGGCACTGGGAGTGTCACAAGCTCTCTTCCTGCTCCTTGC  3300
              |||||||||||| |||||||||||||||| |||||||||||||||||||||| ||||
Sbjct  3761  CCTGCGGATCGGCGTCTTTGGGGCACTGGGGGTGTCACAAGCTCTCTTCCTGCTCTTTGC  3820

Query  3301  AACCCTCCTGTCTGCTCGTGGTGCCATGCGAGCCTCGCGGGTTATGCATCAGCAACTGCT  3360
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            |||| |||||||||||||||||||||||||||||| ||||||||||||||||||||||||
Sbjct  3821 AACCATCCTGTCTGCTCGTGGTGCCATGCGAGCCTCCCGGGTTATGCATCAGCAACTGCT  3880

Query  3361 CAGCAACATCCTGCGTGTGCCCATGAGCTTTTTTGACACAACCCCGACTGGCCGCATTGT  3420
            |||||||||||||||||||||||||||||||||||||| ||||||||||||||||||||
Sbjct  3881 CAGCAACATCCTGCGTGTGCCCATGAGCTTTTTTGACACGACCCCGACTGGCCGCATTGT  3940

Query  3421 GAATAGGTTTGCCAAGGACATCTTCACGATAGATGAGACCATTCCCATGTCCTTCCGCAG  3480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3941 GAATAGGTTTGCCAAGGACATCTTCACGATAGATGAGACCATTCCCATGTCCTTCCGCAG  4000

Query  3481 CTGGCTCTCCTGTTTCATGGCCATCATTAGCACATTGCTCATGATCTCCCTGGCCACCCC  3540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4001 CTGGCTCTCCTGTTTCATGGCCATCATTAGCACATTGCTCATGATCTCCCTGGCCACCCC  4060

Query  3541 ATTCTTCACTCTCGTTATCATTCCCTTGAGCATCTTCTACTATTTTGTGCTGCGCTTCTA  3600
            |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct  4061 ATTCTTCACTCTCGTTATCATTCCCTTGAGCATCTTCTACTATTTTGTCCTGCGCTTCTA  4120

Query  3601 TGTCTCCACATCACGCCAGCTAAGGCGTCTGGACTCTGTAACTAGGTCTCCCATCTACTC  3660
            ||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4121 TGTCTCCACGTCACGCCAGCTAAGGCGTCTGGACTCTGTAACTAGGTCTCCCATCTACTC  4180

Query  3661 CCACTTTGGCGAGACAGTGTCAGGGCTTTCTGTGATCCGTGCCTTCGGACACCAAGAACG  3720
            ||||||||| ||||||||||||| ||||||||||||| ||||||||||||||||||||||
Sbjct  4181 CCACTTTGGTGAGACAGTGTCAGGACTTTCTGTGATCCGCGCCTTCGGACACCAAGAACG  4240

Query  3721 ATTCCTGCAGCAGAATGAGAGCACCATGGACGTCAATCAAAAAAGTGTTTACTCCTGGAT  3780
            |||||||||||||||||||||||||||||| ||||||| ||||||||||||||||||||||
Sbjct  4241 ATTCCTGCAGCAGAATGAGAGCACCATGGACATCAATCAGAAAAGTGTTTACTCCTGGAT  4300

Query  3781 AGTCTCAAATAGGTGGCTGGCCATCCGTCTGGAGTTCGTTGGGAGCCTGGTGGTCTTCTT  3840
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4301 AGTCTCAAATAGGTGGCTGGCCATCCGTCTGGAGTTCGTTGGGAGCCTGGTGGTCTTCTT  4360

Query  3841 CTCTGCGCTTCTAGCTGTGATTTCAAAGGGCACTTTGGAGGGCGGCATCGTGGGTCTTTC  3900
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4361 CTCTGCGCTTCTAGCTGTGATTTCAAAGGGCACTTTGGAGGGCGGCATCGTGGGTCTTTC  4420

Query  3901 TGTCTCCTCTGCCCTCAATGTGACCCAGACACTGAACTGGCTGGTGCGGACGTCTTCGGA  3960
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||
Sbjct  4421 TGTCTCCTCTGCCCTCAATGTGACCCAGACACTGAACTGGCTGGTGCGGACGTCTTCAGA  4480

Query  3961 GCTGGAGACAAACATTGTGGCTGTGGAGCGGGTACATGAGTACACGAAGGTGAAGAATGA  4020
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||
Sbjct  4481 GCTGGAGACAAACATTGTGGCTGTGGAGCGGGTACATGAGTACACGAAGGTGAAGAACGA  4540

Query  4021 GGCTCCGTGGGTGACAGAAAAGCGTCCACCCCATGGCTGGCCCAGCAAAGGTGAGATCCA  4080
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4541 GGCTCCGTGGGTGACAGAAAAGCGTCCACCCCATGGCTGGCCCAGCAAAGGTGAGATCCA  4600

Query  4081 GTTTGTTGACTACAAAGTTCGTTACCGACCTGAACTGGAGCTGGTTCTTCAGGGGATCAC  4140
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4601 GTTTGTTGACTACAAAGTTCGTTACCGACCTGAACTGGAGCTGGTTCTTCAGGGGATCAC  4660

Query  4141 CTGCAATATTGGGAGCACGGAGAAGGTTGGGGTTGTGGGCCGGACTGGGGCTGGAAAATC  4200
            |||||||||| ||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct  4661 CTGCAATATTCGGAGCACTGAGAAGGTTGGGGTTGTGGGCCGGACTGGGGCTGGAAAATC  4720

Query  4201 TTCCCTCACCAACTGCCTCTTCCGGGTGCTGGAGGCCGCTGGAGGGACGATCATCATCGA  4260
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4721 CTCCCTCACCAACTGCCTCTTCCGGGTGCTGGAGGCCGCTGGAGGGACGATCATCATCGA  4780
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Query  4261  CGAGGTGGATATAGCAACGATCGGCCTCCATGACCTGCGCCAGAACCTCACCATCATCCC  4320
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4781  CGAGGTGGATATAGCAACGATCGGCCTCCATGACCTGCGCCAGAACCTCACCATCATCCC  4840

Query  4321  TCAGGACCCCGTGCTCTTTACTGGCACCCTGCGGATGAACCTGGATCCCTTTGACCAGTA  4380
              |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
Sbjct  4841  TCAGGACCCCGTGCTCTTTACTGGCACCCTGCGGATGAACCTGGACCCCTTTGACCAGTA  4900

Query  4381  CATGGATGAGGAGGTCTGGAAGGCCCTTGAGCTGGCCCACCTGAAGACATATGTGCAAGA  4440
              || ||| |||||||||||||||||||||||||||||||||||||| ||||||||||||||
Sbjct  4901  CACGGACGAGGAGGTCTGGAAGGCCCTTGAGCTGGCCCACCTGAAGTCATATGTGCAAGA  4960

Query  4441  CCTTCCCGAGGGGCTGCTGCATCTTGTGAGCGAGGCGGGGGAGAACCTGAGTGTTGGGCA  4500
              |||||||||||||||||||||||||||||| ||||| || ||||||||||||||||||||
Sbjct  4961  CCTTCCCGAGGGGCTGCTGCATCTTGTGAGTGAGGCAGGCGAGAACCTGAGTGTTGGGCA  5020

Query  4501  GAGGCAGCTGGTGTGCCTGGCCCGGGCCCTCCTTCGCAAAGCCAAGATCCTCATCCTGGA  4560
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  5021  GAGGCAGCTGGTGTGCCTGGCCCGGGCCCTCCTTCGCAAAGCCAAGATCCTCATCCTGGA  5080

Query  4561  CGAAGCGACAGCAGCCGTAGATCTAGAAACTGATCATTTAATCCAGACAACGATCCGGAG  4620
               |||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  5081  TGAAGCGACAGCAGCCGTAGATCTGGAAACTGATCATTTAATCCAGACAACGATCCGGAG  5140

Query  4621  TGAGTTTGCTGACTGCACTGTCCTTACTATTGCCCACCGCCTCCACACCATCATGGACAG  4680
               ||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  5141  CGAGTTTGTTGACTGCACTGTCCTTACTATTGCCCACCGCCTCCACACCATCATGGACAG  5200

Query  4681  CAACAGGGTGATGGTGCTGCAGGCTGGGAGGATTGTGGAATACGACAGCCCTGAGGAGCT  4740
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  5201  CAACAGGGTGATGGTGCTGCAGGCTGGGAGGATTGTGGAATACGACAGCCCTGAGGAGCT  5260

Query  4741  GCTCAAGAAGCACGGTGTCTTCTCCGCAATGGCAAAGGACGCTGGCATCACGAATATAGA  4800
              |||||||||||||||||||||||||||||||||||| |||||||||  ||||| |||
Sbjct  5261  GCTCAAGAAGCACGGTGTCTTCTCCGCAATGGCAAAGGATGCTGGCATCGTGAATACAGA  5320

Query  4801  AACCACTGTGCTGTAGGTGGAGCAGAGCAGTGTGCGGGTGTGTGCGTTGGCAGCTCCCTC  4860
              |||||||||||||||||||||||||||||||||||||||||||||  ||||||||||||||
Sbjct  5321  AACCACTGTGCTGTAGGTGGAGCAGAGCAGTGTGCGGGTGTGTGCAGTGGCAGCTCCCTC  5380

Query  4861  CCACTGGCACTCACCAAGCAGGCAGCAGCTTCTCCCTGCTGCCGGGCTGCCCAGGAAATT  4920
              |||||||||||||||| |||||||||||||||||||||||||||| |||||||||||||| ||
Sbjct  5381  CCACTGGCACTCACCAGGCAGGCAGCAGCTTCTCCCTGCTGCCAGGCTGCCCAGGAATTT  5440

Query  4921  CTCTCTGCAGCTGGGAAGCAGAGAGAGTGGCTTCTCTGGCCAGGACAGAGGATCTGGACT  4980
              |||||||||||||||||| |||||||||||||||||||| ||||||||||||||||||||
Sbjct  5441  CTCTCTGCAGCTGGGAAGCGGAGAGAGTGGCTTCTCTGGCCGGGACAGAGGATCTGGACT  5500

Query  4981  TGAGTGAGCTGTTAACCTTGCTACCCCACCCTGCTTGCTGTGCGCATGAGGGTCTGGAGC  5040
              |||||||||||||||||||||||||| ||||||||||||||| ||||||||||||||||||
Sbjct  5501  TGAGTGAGCTGTTAACCTTGCTACCCTACCCTGCTTGCTGTGCACATGAGGGTCTGGAGC  5560

Query  5041  TGCATAATTTATTCCAGTATAGAGGTGAAAAGTCTGCCATGGGAGACCATGACCCCCGTG  5100
              |||||||||||||||||||||||||| ||||| |||||||||||||||||||||||||| ||
Sbjct  5561  TGCATAATTTATTCCAGTATAGAGGGGAAAATTCTGCCATGGGAGACCATGACCCCCATG  5620

Query  5101  GGGGTCTTAGTTTTTGTACTTCACCATGCCAGGGG-AACCTAGCTGAGATATGCTTTAGC  5159
               ||||| |||||||||||||||||||||||||| |||||||||||||| ||||||||
Sbjct  5621  AGGGTCCTAGTTTTTGTACTTCACCATGCCAGGGGAAACCTAGCTGAGATAAGCTTTAGC  5680

Query  5160  ACTACGGAATGAAATTTACAGTTTAATCTAAGAGTGTTATAAACTTTTGTAAC  5212
              |||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  5681  ACTATGGAATGAAATTTACAGTTTAATCTAAGAGTGTTATAAACTTTTGTAAC  5733
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Figure 5-2: Blastn results revealing the alignment of MRP2 genes between the AWB vulture and Golden eagle with similarity of 98.12%. Query- AWB vulture and Sbjct- Golden Eagle.

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Query  1     GGTGGTTGAATCCTTTATTTATTATTGGCCATAAACGGAAGCTTGAAGAAGATGATATGT  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  36    GGTGGTTGAATCCTTTATTTATTATTGGCCATAAACGGAAGCTTGAAGAAGATGATATGT  95

Query  61    ATAAAGTGCTGCCAGAAGATTCCTCAGAGAAGCTTGGAGAGGAATTGCAGTGGTACTGGG  120
             |||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
Sbjct  96    ATAAAGTGCTGCCAGAAGATTCCTCAGAGAAGCTTGGAGAGGAGTTGCAGTGGTACTGGG  155

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Sbjct  156   ATAAAGAGGTGCAAAAAGCAAAAAAGAGAGGAAAAACGCCACATTTAACAAAAGCCATTA  215

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Sbjct  336   CCTCAGATGAGGTAGCTTTGAATTTTGCATATTGCTACGCAGCTGCTCTGTCTGTGTGCA  395

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Sbjct  396   CGCTTATTCTAGCTATAATGCACCACTTATACTTCTATCATGTACAGCGGGCTGGCATGA  455

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Sbjct  636   TAACAGTACTTCTCTGGATGGAGATAGGCCCATCATGTCTTGCAGGAATGGCAGTTCTGA  695

Query  661   TTATTCTTCTTCCTGTCCAGACCTGCATTGGGAGGCTTTTTTCTTCCCTAAGAAGCAAGA  720
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Sbjct  696   TTATTCTTCTTCCCGTCCAGACCTGCATTGGGAGGCTTTTTTCTTCCCTAAGAAGCAAGA  755

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Sbjct  936   TTGTGGCAAGCAAAATAACAGTGTTCATGACTTTCATGGCATATGTACTGCTTGGCAATG  995

Query  961   TTATCTCTGCAAGTCGGGTGTTTGTTGCAGTGTCCCTGTATGGTGCAGTAAGACTGACAG  1020
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Sbjct  996   TTATCTCTGCAAGTCGGGTGTTTGTTGCAGTGTCCCTGTATGGTGCAGTAAGACTGACAG  1055

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Query  1081  GAATCAAGAACTTTCTGATACTTGATGAGATCTCACCCTTCAAGCCACAACTGCATGGTA  1140
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Sbjct  1176  ATAATGAGAATGTCGTTCTTCATGTTCAGGATTTGACTTGCTATTGGGATAAGAGTTCAG  1235

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Sbjct  1596  ATCTGGCCAGAGCTGTGTATCAAGATGCAGACATCTATCTTTTGGACGATCCACTGAGTG  1655

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Sbjct  1656  CAGTAGATGCTGAAGTTGGAAGACATTTGTTTGAAAAATGTATTTGTCAGGCCTTACATC  1715

Query  1681  AGAAGATCTCTGTTTTGGTTACTCACCAGTTGCAGTATCTCCGTGCTGCAAATCAGATTC  1740
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Query  1801  GCATCGACTTTGCTTCCCTTTTGAAAAAAGATGAGGAGGTAGAACAGCTGTCAGTTCCAG  1860
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Query  2281  TAGTAAGAAGTCTTTTGGTGTTTCAAGTTCTTGTTAATTCTGGTCAGACTTTGCACAACA  2340
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Sbjct  2376  AAATGTTTCAATCCATTTTGAAAGCTCCCGTCTTGTTTTTTGACAGAAATCCTATAGGAA  2435

Query  2401  GAATCTTAAATCGTTTCTCCAAAGATATTGGCCACCTGGATGACTTGCTTCCATTGACAT  2460
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Sbjct  2436  GAATCTTAAATCGTTTCTCCAAAGATATTGGCCACCTGGATGACTTGCTTCCATTGACAT  2495

Query  2461  TTTTGGACTTCATGCAGACTCTCCTACAGATTTTTGGTGTGGTGGCTGTGGCTGTGGCAG  2520
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Sbjct  2496  TTTTGGACTTCATGCAGACTCTCCTACAGATTTTTGGTGTGGTGGCTGTGGCTGTGGCAG  2555

Query  2521  TGATTCCTTGGATACTCCTCCCCTTAATTCCACTATTTATTCTTTTCATTTTCCTTCGAC  2580
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Sbjct  2556  TGATTCCTTGGATACTCATCCCCTTAATTCCACTATTTATTCTTTTCATTTTCCTTCGAC  2615

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Sbjct  2676  TGTTCTCCCACTTGTCGTCATCCCTCCAGGGACTTTGGACTATTCGGGCTTTGAAAGCAG  2735

Query  2701  AGGAAAGATTTCAAAAATTATTTGATGCACACCAAGACCTCCACTCAGAGGCCTGGTTTC  2760
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Query  3301  TACCTCAGGAGCCTGTTTTATTCACTGGAACTATGAGGAAAAACTTAGATCCTTTCAATG  3360
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Query  3601  GTGAAAAGTTTGCTCACTGCACAGTGCTGACCATTGCACACCGCTTGAACACCATTATTG  3660
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Query  3661  ACAGTGACAGGATTATGGTTTTAGATGAAGGAAGAGTGAAAGAATATGGTGAACCTTACA  3720
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Query  3721 TTTTGCTGCAAGAACAAGATGGCTTGTTTTACAAAATGGTGCAACAAGTGGGCAAGACTG 3780
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Sbjct  3756 TTTTGCTGCAAGAACAAGATGGCTTGTTTTACAAAATGGTGCAACAAGTGGGCAAGACTG 3815

Query  3781 AAGCAGCTTCTCTGATTGAAACAGCAAAACGGGTGTACTTCAGTAAGAATTACCCAGAAG 3840
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Query  3901 TAACCGAAACTGCACTGTGATTCCTAATAACCTTAACTGTTTTCCATTGAATGTAAACCT 3960
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Query  3961 GAGATCATCTAAACTCAGTGACAATGTTTGCAAGTGTCAGCAGGAGAGGAAAGGGAGGGG 4020
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Query  4021 GCGATTCTTTGCACTGGACATCCTTCCTATTTAATACTGAG  4061
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Sbjct  4056 GCGATTCTTTGCACTGGACATCCTTCCTATTTAATACTGAG  4096
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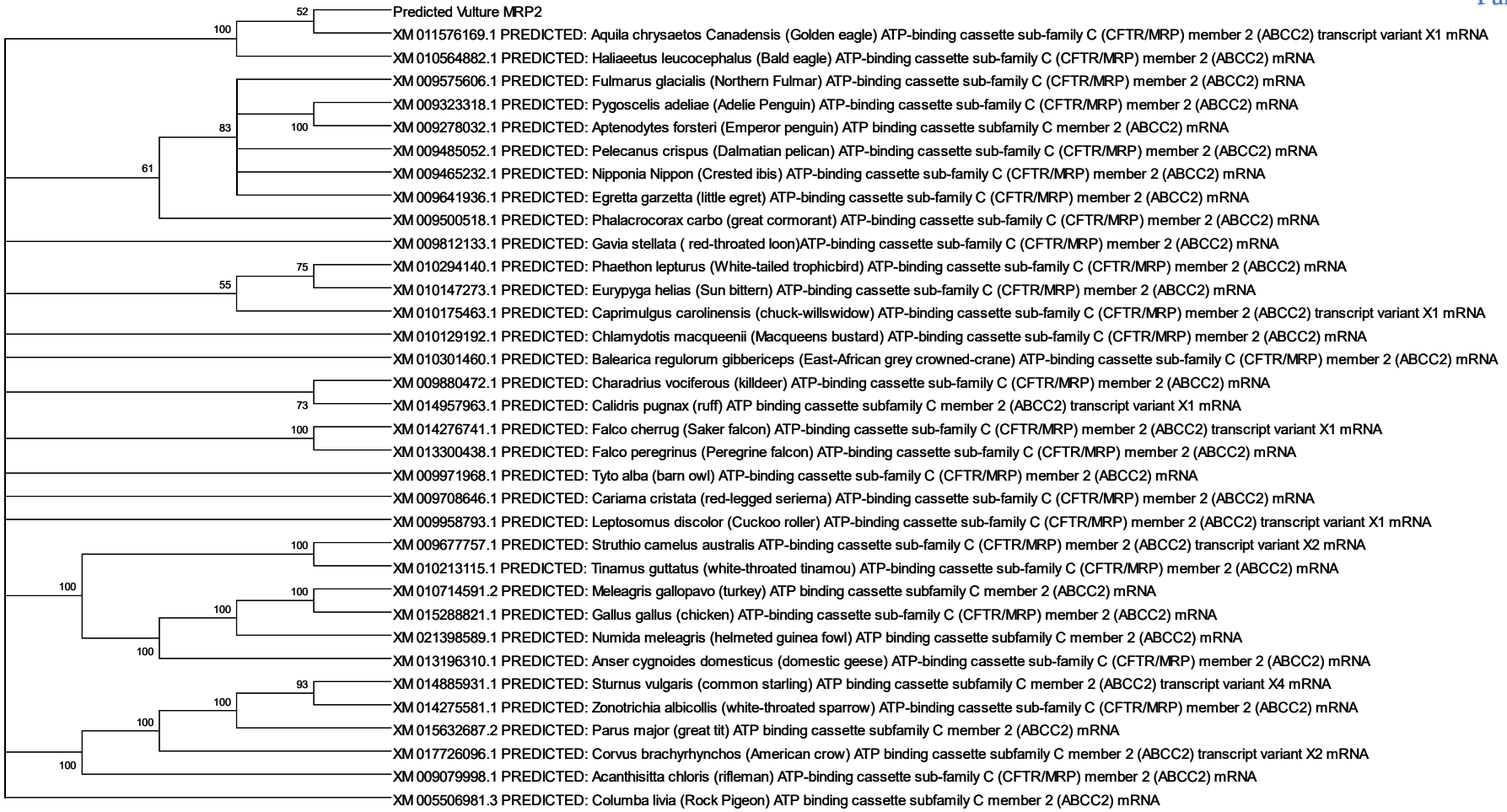
Figure 5-3: Blastn results revealing the alignment of MRP4 genes between the AWB vulture and Golden eagle indicating similarity of 99.14%. Query- AWB vulture and Sbjct- Golden eagle.

5.3.2. Phylogenetic analysis.

The AWB predicted genes were further analysed to confirm its homogeneity compared to MRPs gene of other avian species. The phylogenetic tree topology revealed 2 distinct clades between the vulture and chicken however the golden eagle, bald eagle and AWB vulture belonged to the same clade. The percentage similarity between vulture, eagles and chicken is stated on the internal nodes numbers representing the percentage of 1000 replicates for which the same branching patterns were attained. (Fig 5-4).

Pane A


Predicted Vulture MRP2
XM 011576169.1 PREDICTED: Aquila chrysaetos Canadensis (Golden eagle) ATP-binding cassette sub-family C (CFTR/MRP) member 2 (ABCC2) transcript variant X1 mRNA
XM 010564882.1 PREDICTED: Haliaeetus leucocephalus (Bald eagle) ATP-binding cassette sub-family C (CFTR/MRP) member 2 (ABCC2) mRNA
XM 009575606.1 PREDICTED: Fulmarus glacialis (Northern Fulmar) ATP-binding cassette sub-family C (CFTR/MRP) member 2 (ABCC2) mRNA
XM 009323318.1 PREDICTED: Pygoscelis adeliae (Adelie Penguin) ATP-binding cassette sub-family C (CFTR/MRP) member 2 (ABCC2) mRNA
XM 009278032.1 PREDICTED: Aptenodytes forsteri (Emperor penguin) ATP binding cassette subfamily C member 2 (ABCC2) mRNA
XM 009485052.1 PREDICTED: Pelecanus crispus (Dalmatian pelican) ATP-binding cassette sub-family C (CFTR/MRP) member 2 (ABCC2) mRNA
XM 009465232.1 PREDICTED: Nipponia Nippon (Crested ibis) ATP-binding cassette sub-family C (CFTR/MRP) member 2 (ABCC2) mRNA
XM 009641936.1 PREDICTED: Egretta garzetta (little egret) ATP-binding cassette sub-family C (CFTR/MRP) member 2 (ABCC2) mRNA
XM 009500518.1 PREDICTED: Phalacrocorax carbo (great cormorant) ATP-binding cassette sub-family C (CFTR/MRP) member 2 (ABCC2) mRNA
XM 009812133.1 PREDICTED: Gavia stellata (red-throated loon)ATP-binding cassette sub-family C (CFTR/MRP) member 2 (ABCC2) mRNA
XM 010294140.1 PREDICTED: Phaethon lepturus (White-tailed trophicbird) ATP-binding cassette sub-family C (CFTR/MRP) member 2 (ABCC2) mRNA
XM 010147273.1 PREDICTED: Eurypyga helias (Sun bittern) ATP-binding cassette sub-family C (CFTR/MRP) member 2 (ABCC2) mRNA
XM 010175463.1 PREDICTED: Caprimulgus carolinensis (chuck-willswidow) ATP-binding cassette sub-family C (CFTR/MRP) member 2 (ABCC2) transcript variant X1 mRNA
XM 010129192.1 PREDICTED: Chlamydotis macqueenii (Macqueens bustard) ATP-binding cassette sub-family C (CFTR/MRP) member 2 (ABCC2) mRNA
XM 010301460.1 PREDICTED: Balearica regulorum gibbericeps (East-African grey crowned-crane) ATP-binding cassette sub-family C (CFTR/MRP) member 2 (ABCC2) mRNA
XM 009880472.1 PREDICTED: Charadrius vociferous (killdeer) ATP-binding cassette sub-family C (CFTR/MRP) member 2 (ABCC2) mRNA
XM 014957963.1 PREDICTED: Calidris pugnax (ruff) ATP binding cassette subfamily C member 2 (ABCC2) transcript variant X1 mRNA
XM 014276741.1 PREDICTED: Falco cherrug (Saker falcon) ATP-binding cassette sub-family C (CFTR/MRP) member 2 (ABCC2) transcript variant X1 mRNA
XM 013300438.1 PREDICTED: Falco peregrinus (Peregrine falcon) ATP-binding cassette sub-family C (CFTR/MRP) member 2 (ABCC2) mRNA
XM 009971968.1 PREDICTED: Tyto alba (barn owl) ATP-binding cassette sub-family C (CFTR/MRP) member 2 (ABCC2) mRNA
XM 009708646.1 PREDICTED: Cariama cristata (red-legged seriema) ATP-binding cassette sub-family C (CFTR/MRP) member 2 (ABCC2) mRNA
XM 009958793.1 PREDICTED: Leptosomus discolor (Cuckoo roller) ATP-binding cassette sub-family C (CFTR/MRP) member 2 (ABCC2) transcript variant X1 mRNA
XM 009677757.1 PREDICTED: Struthio camelus australis ATP-binding cassette sub-family C (CFTR/MRP) member 2 (ABCC2) transcript variant X2 mRNA
XM 010213115.1 PREDICTED: Tinamus guttatus (white-throated tinamou) ATP-binding cassette sub-family C (CFTR/MRP) member 2 (ABCC2) mRNA
XM 010714591.2 PREDICTED: Meleagris gallopavo (turkey) ATP binding cassette subfamily C member 2 (ABCC2) mRNA
XM 015288821.1 PREDICTED: Gallus gallus (chicken) ATP-binding cassette sub-family C (CFTR/MRP) member 2 (ABCC2) mRNA
XM 021398589.1 PREDICTED: Numida meleagris (helmeted guinea fowl) ATP binding cassette subfamily C member 2 (ABCC2) mRNA
XM 013196310.1 PREDICTED: Anser cygnoides domesticus (domestic geese) ATP-binding cassette sub-family C (CFTR/MRP) member 2 (ABCC2) mRNA
XM 014885931.1 PREDICTED: Sturnus vulgaris (common starling) ATP binding cassette subfamily C member 2 (ABCC2) transcript variant X4 mRNA
XM 014275581.1 PREDICTED: Zonotrichia albicollis (white-throated sparrow) ATP-binding cassette sub-family C (CFTR/MRP) member 2 (ABCC2) mRNA
XM 015632687.2 PREDICTED: Parus major (great tit) ATP binding cassette subfamily C member 2 (ABCC2) mRNA
XM 017726096.1 PREDICTED: Corvus brachyrhynchos (American crow) ATP binding cassette subfamily C member 2 (ABCC2) transcript variant X2 mRNA
XM 009079998.1 PREDICTED: Acanthisitta chloris (rifleman) ATP-binding cassette sub-family C (CFTR/MRP) member 2 (ABCC2) mRNA
XM 005506981.3 PREDICTED: Columba livia (Rock Pigeon) ATP binding cassette subfamily C member 2 (ABCC2) mRNA
52
100
83
100
61
75
55
73
100
100
100
100
100
100
93
100
100
100

Pane B

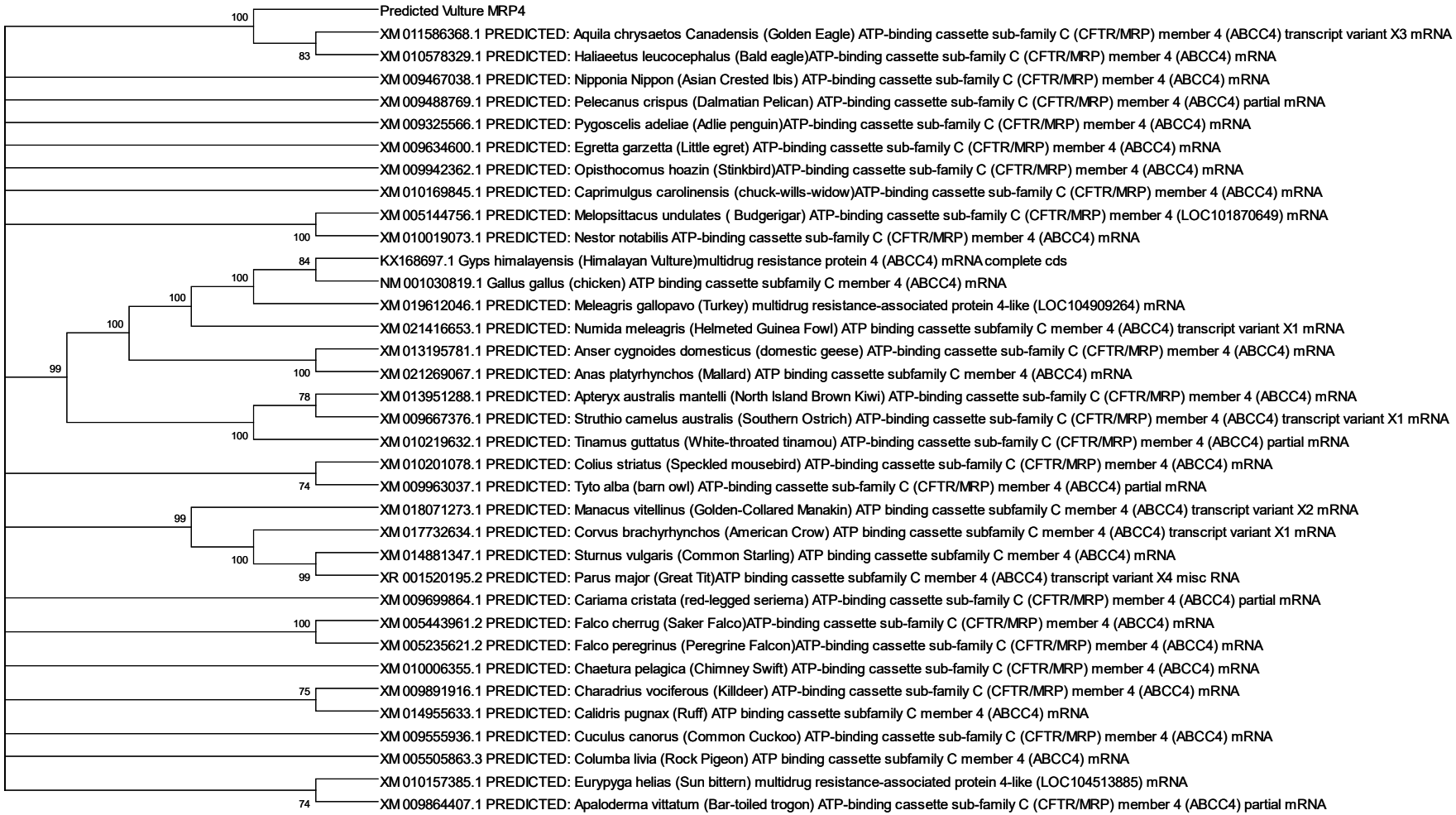


Figure 5-4: Reconstruction of phylogenetic relationships between A) MRP2 and B) MRP4 genes of different avian species by maximum likelihood best model: bootstrap.

5.3.3. Sequence analysis and in silico predictions.

The predicted AWB MRP2 sequence generated open reading frame (ORF) containing 1552 amino acids while MRP4 ORF contained 1287 amino acids with the following molecular weight 173610.33 and 145113.90 respectively. Scan prosite results revealed that the above proteins are likely ABC transporter integral membrane type 1 fused domain (ABC-TMF1) and ABC transporter 2. Secondary structure prediction with PHYRE2 revealed that the predicted MRP2 amino acids contain 62% of alpha-helix, 7% of beta- strand and 30% transmembrane helix. While MRP4 PHYRE2 results showed the former to contain 61% alpha-helix, 9% of beta- helix and 26 to be coiled in nature. TMHMM software analysis for MRP2 revealed the presence of two nucleotide binding domains (NBDs) and two transmembrane domains (TMDs) consisting of 6 and 5 transmembrane helices (TMH) respectively with an extra transmembrane domain (TMD) with 5 TMH and for MRP4 it revealed their presence of two NBDs and two TMDs containing 5 and 6 TMH each (Fig 5-5). PROTTER sequence database was used for prediction of N-glycosylation sites for MRP2 and were found at position 15, 106, 284, 551, 620, 725, 773, 886, 1019, 1193, 1254, 1383, 1443 and MRP4 sites were at position 428, 706, 716, 1009, 1138 for both AWB vulture and golden eagle moreover confirming the above results, MRP2 contained 16 TMH and MRP4 had 11 TMH (Fig 5-6). The quaternary structures predicted by PHYRE2 from MRPs amino acids revealed the presence of two NBDs for both MRPs protein containing 13 and 19 transmembrane helices for MRP4 and MRP2 respectively which contradict the above results (Fig 5-7).

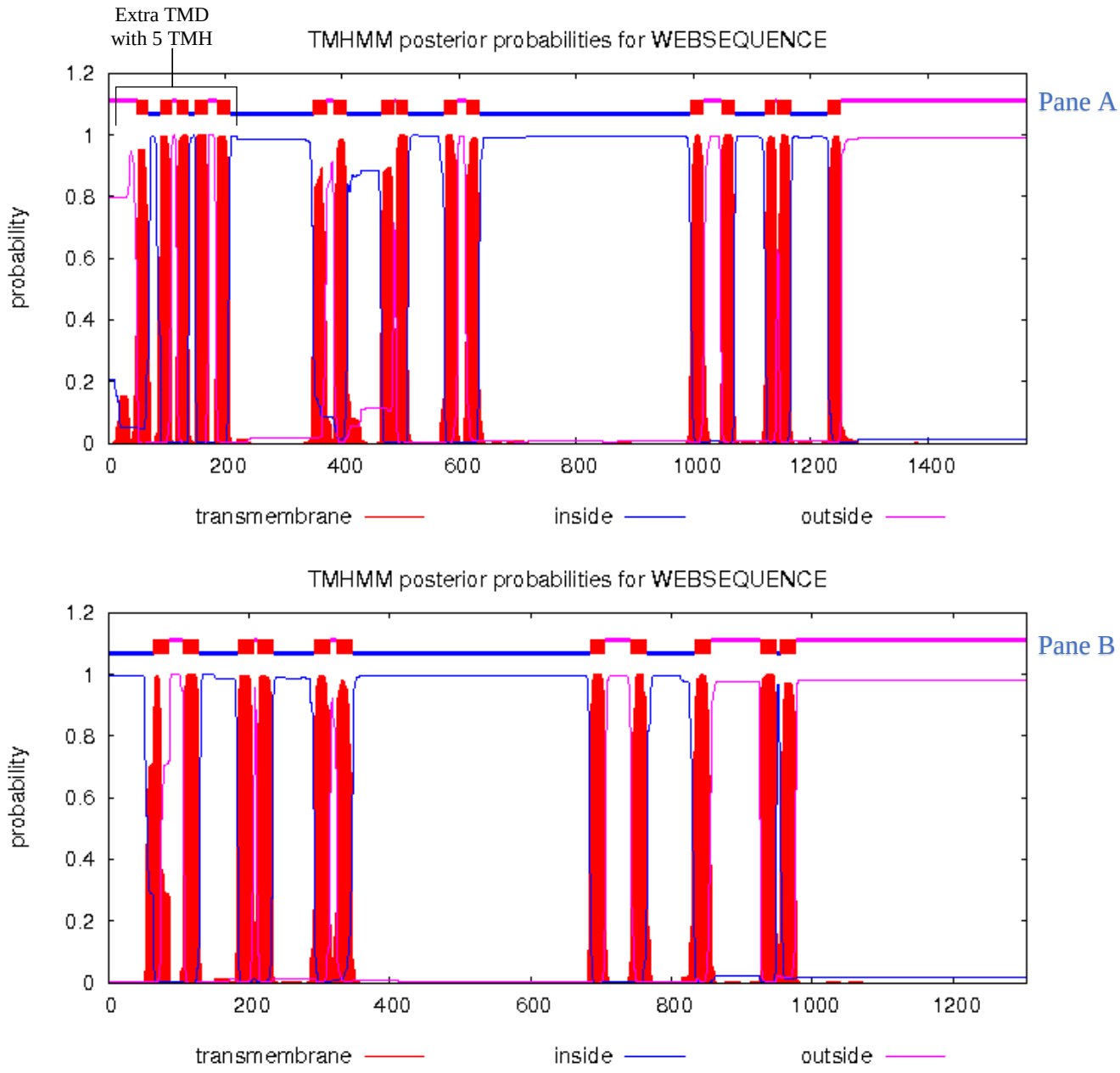
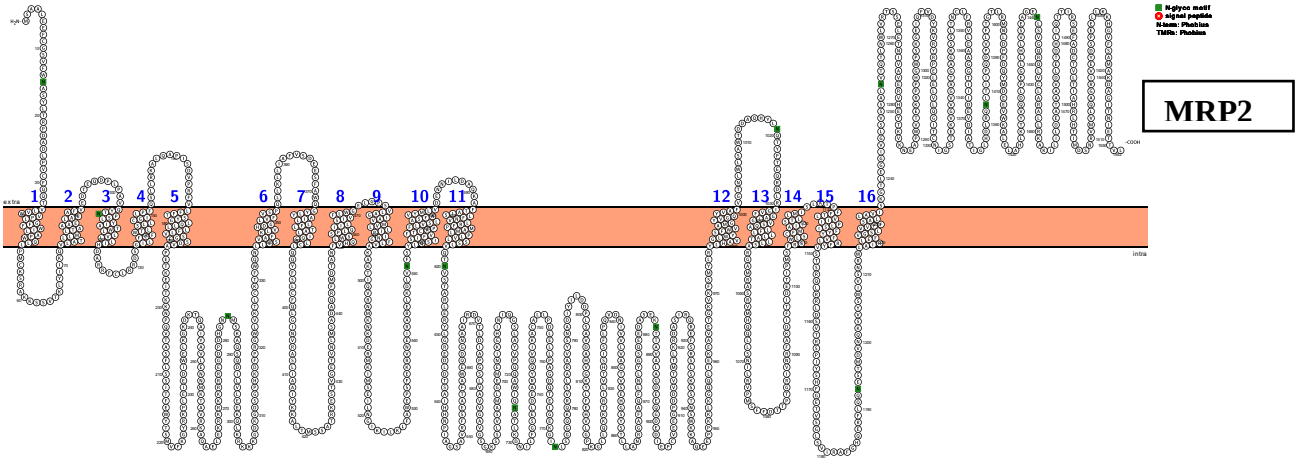


Fig 5-5: Prediction of transmembrane helices in A) MRP2 revealing the presence of 16 TMH and B) MRP4 showing the presence of 11 TMH.

Pane A



Pane B

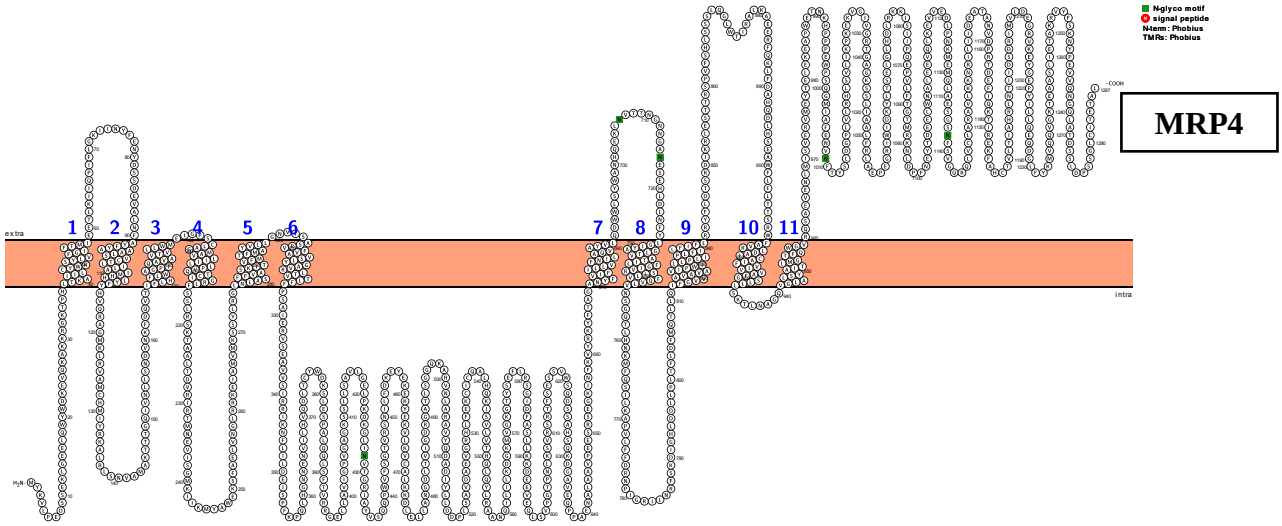
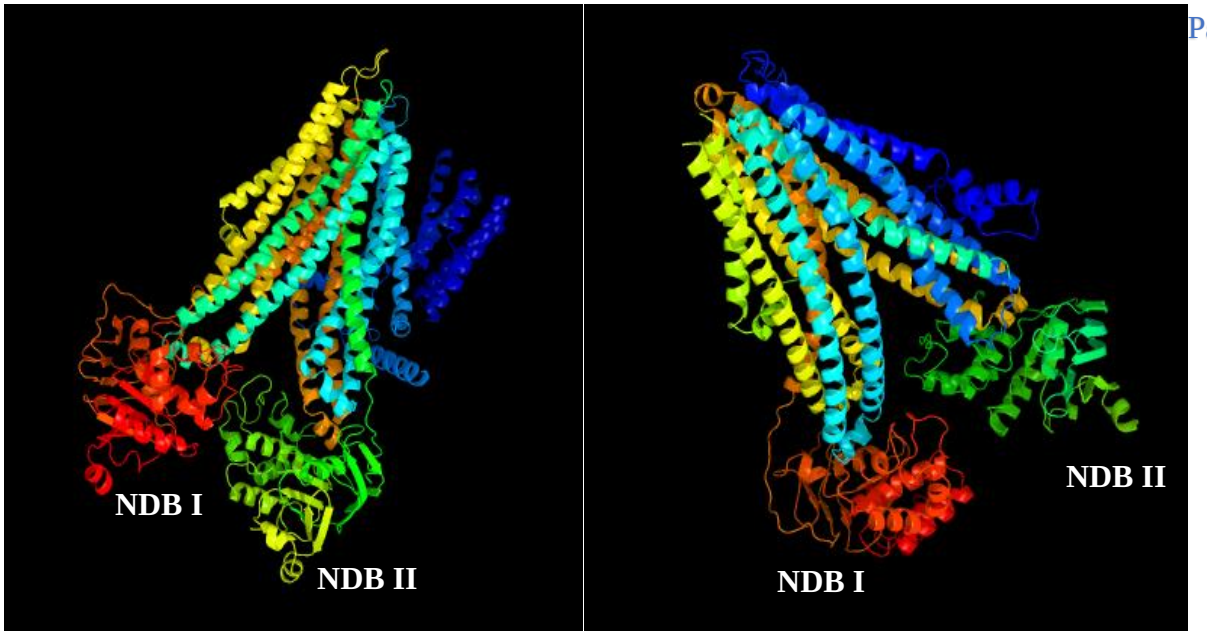


Fig 5-6: Pictorial presentation of transmembrane helices and N-gyclan motif sites from A) MRP2 containing 13 N-glycan motif sites with 16 TMH and B) MRP4 comprising of 5 N-glycan motif sites with 11 TMH.



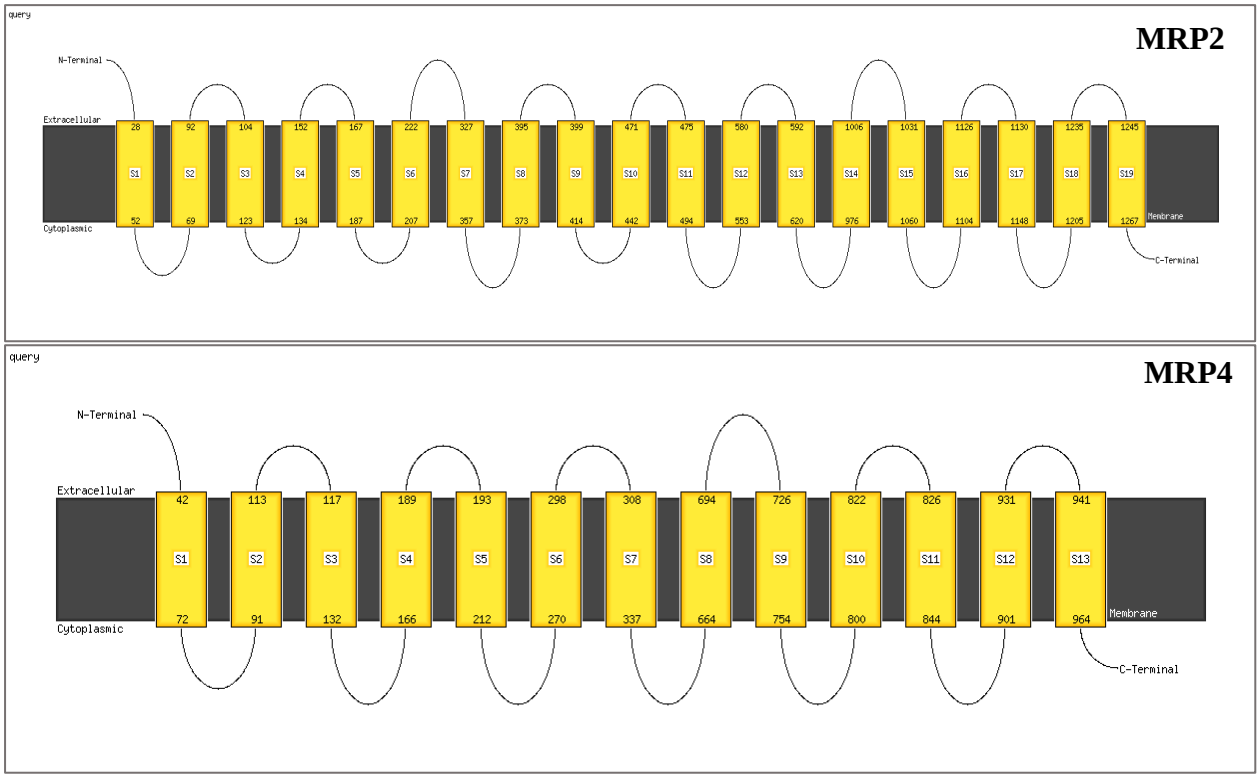
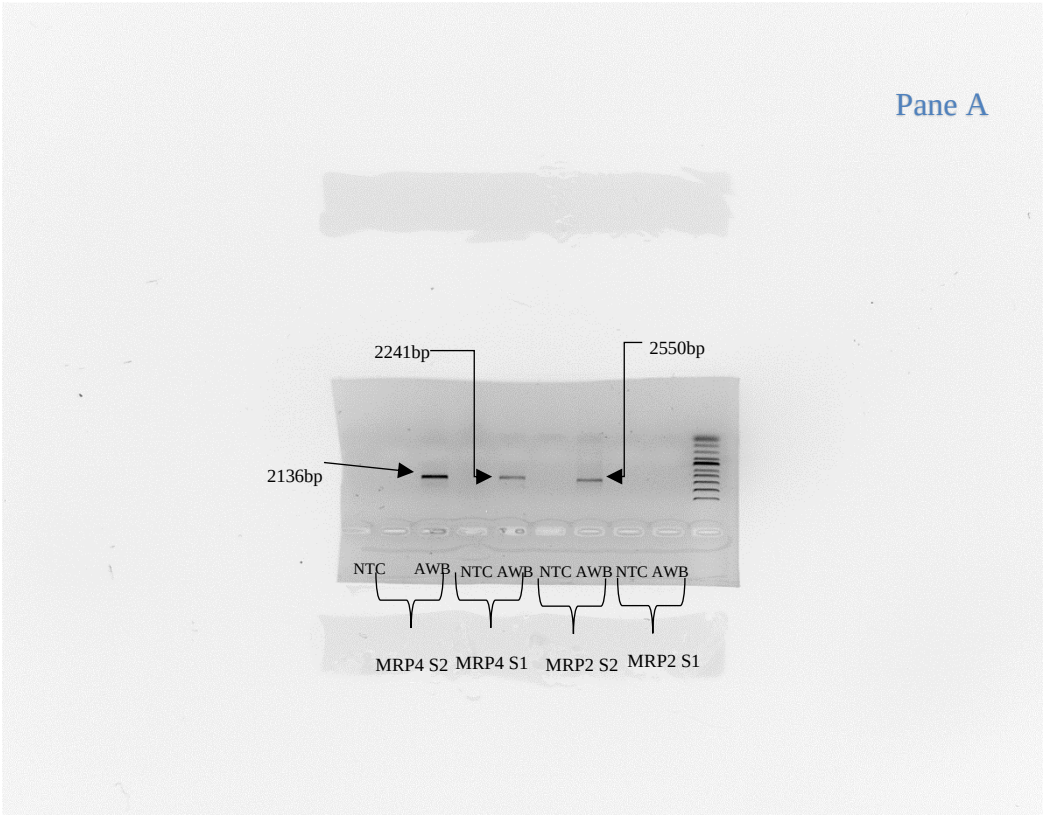


Fig 5-7: Predicted MRPs structure by PHYRE2 software representing A) 3-D structures of MRP2 (left) and MRP4 (right) proteins indicating that both proteins have two NDBs and B) 2-D structures of the latter proteins revealing 19 TMH for MRP2 and 13 TMH for MRP4.

5.3.4. Sanger sequencing of data.

To confirm the above NGS results, Sanger sequencing was conducted. The cDNA from the AWB kidney was used as template. MRP2 was amplified into two segments using primers

design based on NGS data. The PCR products were viewed on a gel under UV documentation and it revealed product size of 2754bp and 2550bp for segment 1 and 2 respectively (Fig 5-9). Moreover amplification of MRP4 was also divided into two segments and PCR products were 2241bp and 2136bp for segment 1 and 2 in that order (Fig 5-8). The above PCR products were sequenced and the sequences were analysed for consensus which revealed 4537bp for MRP2 and 3897bp for MRP4 however sequences were poor (alot of degenerate bases) and primers did not ampify the full segments. While full coverage was not possible, based on the degree of coverage and similarity between NGS and Sanger of 99.76% and 99.43% for MRP2 and MRP4 respectively was observed (Fig 5-9 and 5-10). We were convinced that the MRP2 and MRP4 NGS sequence is an accurate reflection of the channel in the AWBV furthermore the Sanger also confirm that genes obtained are correct genes.



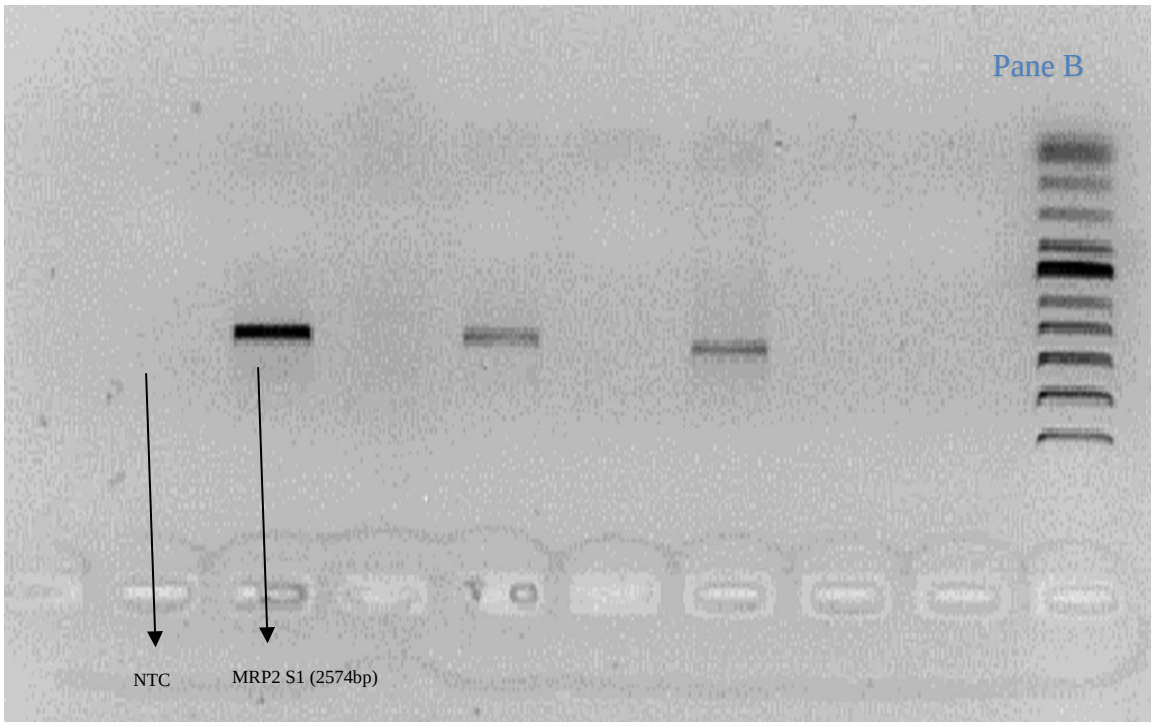


Fig 5-8: Conventional PCR amplified MRP2 S2, MRP4 S1 and S2 (Pane A) and MRP2 S1 (Pane B) genes from AWB`s kidney, no product was obtained when the template was omitted, Molecular size (100bp) is indicated on the right. NTC= no template control.

```
MRP2_gene          ------------------------------------------------------------      0
Predicted_MRP2     tgcaatagcaccaagttaacaattaactgtgtgtcatcagggattttgtcccaacaccac      60

MRP2_gene          ------------------------------------------------------------      0
Predicted_MRP2     ttgagggtgtgtggggacttttgtcccaagcatccatccaggactcacagccaggcagag      120

MRP2_gene          ---------ggagctgtcgtctcttcccccctgagccatgtcggcagccctggaggagtt      51
Predicted_MRP2     cgaggggaaggagctgtcgtctcttcccccctgagccatgtcggcagccctggaggagtt      180
                             **************************************************

MRP2_gene          ctgtggctccgkcttttggaatgcatcctacctcactcgtccagatgccgacctgcccgt      111
Predicted_MRP2     ctgtggctccgtcttttggaatgcatcctacctcactcgtccagatgccgacctgcccgt      240
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MRP2_gene          gtgcttccagcagactgtgctggtctgggtccccctggcttcttctggattttggctcc      171
Predicted_MRP2     gtgcttccagcagactgtgctggtctgggtccccctggcttcttctggattttggctcc      300
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MRP2_gene          atggcagctcctgcccatgtgcaaatccagagccaagaaatcatctgtgaccaaactcta      231
Predicted_MRP2     atggcagctcctgcccatgtgcaaatccagagccaagaaatcatctgtgaccaaactcta      360
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MRP2_gene          catcatcaaacaggtgctggctaccttgctgatgctgacggcagcagcggagttggcctt      291
Predicted_MRP2     catcatcaaacaggtgctggctaccttgctgatgctgacggcagcagcggagttggcctt      420
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MRP2_gene          ggcgtttgtagaggacacagagcaggacccccctgccagctgtccagtacacaaaccccag      351
Predicted_MRP2     ggcgtttgtagaggacacagagcaggacccccctgccagctgtccagtacacaaaccccag      480
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MRP2_gene          cctgtacattgccacctg------------------------------------------      369
Predicted_MRP2     cctgtacattgccacctggctcctggtcctgctgatccatgatgcacgacgcttctgctt      540
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MRP2_gene          ------------------------------------------------------------      369
Predicted_MRP2     gcgcagagactcggggatacttttctgcttctggacactgtccctgctctgtgggatatt      600

MRP2_gene          ---------------------------------ggcaccaatctctgacgtgccacggtt      396
Predicted_MRP2     gccattccagtcactcctccggaaagccctgcaggcaccaatctctgacgtgccacggtt      660
                                                   ****************************

MRP2_gene          tgtccttttcttcacctcctacgggctccagctgctgctttttcttgtctcgggcttctc      456
Predicted_MRP2     tgtccttttcttcacctcctacgggctccagctgctgctttttcttgtctcgggcttctc      720
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MRP2_gene          agacgttgccccagaaacaaaggaaatcacgaagaagaacccacaggtgacagcctcctt      516
Predicted_MRP2     agacgttgccccagaaacaaaggaaatcacgaagaagaacccacaggtgacagcctcctt      780
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MRP2_gene          cctgagctccatcacctttgaatggtacaccagcatggttttcaagggctatcgcaaacc      576
Predicted_MRP2     cctgagctccatcacctttgaatggtacaccagcatggttttcaagggctatcgcaaacc      840
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MRP2_gene          cttggagatagaggatatctgggaattgaaaggtaaagacaagacgcaggctatttatgc      636
Predicted_MRP2     cttggagatagaggatatctgggaattgaaaggtaaagacaagacgcaggctatttatgc      900
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MRP2_gene          tgttttggagaataacatgaagactgcggtgaggaaggcccaagcagaactggagaaacg      696
Predicted_MRP2     tgttttggagaataacatgaagactgcggtgaggaaggcccaagcagaactggagaaacg      960
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MRP2_gene          gaaacgcaagaaaagacgccgggaaggtgacccagaccatgggaacaacatgagcaaggc      756
Predicted_MRP2     gaaacgcaagaaaagacgccgggaaggtgacccagaccatgggaacaacatgagcaaggc      1020
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MRP2_gene          ccagagccaagacatcctggtgctggaggaaaagcagctgaagaggaagaagaaagggag      816
Predicted_MRP2     ccagagccaagacatcctggtgctggaggaaaagcagctgaagaggaagaaga-agggag      1079
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MRP2_gene          acaaaggggactctggccctcacaaggatttcccccggggctggttggtgaaaaccctgt      876
Predicted_MRP2     acaaaggggactctggccctcacaaggatttcccccggggctggttggtgaaaaccctgt      1139
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MRP2_gene          gcaagaccttctggcagaacctcc-------------------------------------      900
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Predicted_MRP2      ttgtgttcgtcagcccccagctgctgaagctgctgatcgcctttgtgtcagatgaggagt      1259

MRP2_gene           ------------------------------------------------------------      900
Predicted_MRP2      cctttgcctggcaaggctatctgtatgccatcctgctcttcctgacggcactgatccagt      1319

MRP2_gene           ------------------------------------------------------------      900
Predicted_MRP2      ccctctgcctgcagcagtacttcagcttgtgcttccagcttggcataaatgtgcgtgcca      1379

MRP2_gene           ------------------------------------------------------------      900
Predicted_MRP2      gtctcattgctgccatctacaagaaggcactcaccatgtccagtgccacccgcaaggagt      1439

MRP2_gene           ------------------------------------------------------------      900
Predicted_MRP2      ccacggtgggagagactgtgaatctgatgtcagctgatgcccagaggttcatggacacgg      1499

MRP2_gene           ------------------------------------------------------------      900
Predicted_MRP2      ccaacttcgttcaccagctgtggtcatccccccctgcaaattatcctgtccattgtcttcc      1559

MRP2_gene           ----------------------------------------cagttatggtgctgctcatcc      921
Predicted_MRP2      tctggggagagctgggcccctctgttctggctggcatcgcagttatggtgctgctcatcc      1619
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Predicted_MRP2      ttcctggtctccttggccagctttgctgtttacatgctggtggatgagaacaacatcctg      1918
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MRP2_gene           gatgcacagaaagcctttactgccatctccctttcaacgtgctgcgcttccccatggcc      1281
Predicted_MRP2      gatgcacagaaagcctttactgccatctccctttcaacgtgctgcgcttccccatggcc      1978
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MRP2_gene           atgctgcccttggtcctttcttccttggtgcagaccaacgtgtcgactgcgaggctggag      1341
Predicted_MRP2      atgctgcccttggtcctttcttccttggtgcagaccaacgtgtcgactgcgaggctggag      2038
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MRP2_gene           cgctacctgggcagagaagacctggacacctcggctatccaccacaaccccattgcaggc      1401
Predicted_MRP2      cgctacctgggcagagaagacctggacacctcggctatccaccacaaccccattgcag--      2096
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MRP2_gene           gggctcaggcaaatcttcgctggtgtcagccatgctcggggagatggagaatatcaaggg      1581
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Predicted_MRP2      gctcagtgcctggactgacgatgcgcagcactacctgaaccagacctatcccacagagca      3234
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MRP2_gene           gcgggacctgcggatcggtgtctttggggcactgggggtgtcacaagctctcttcctgct      2040
Predicted_MRP2      gcgggacctgcggatcggtgtctttggggcactgggagtgtcacaagctctcttcctgct      3294
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MRP2_gene           ccttgcaaccctcctgtctgctcgtggtgccatgcgagcctcgcgggttatgcatcagca      2100
Predicted_MRP2      ccttgcaaccctcctgtctgctcgtggtgccatgcgagcctcgcgggttatgcatcagca      3354
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MRP2_gene           actgctcagcaacatcctgcgtgtgcccatgagctttttgacacaaccccgactggccg      2160
Predicted_MRP2      actgctcagcaacatcctgcgtgtgcccatgagctttttgacacaaccccgactggccg      3414
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MRP2_gene           cattgtgaataggtttgccaaggacatcttcacgatagatgagaccattcctatgtcctt      2220
Predicted_MRP2      cattgtgaataggtttgccaaggacatcttcacgatagatgagaccattcccatgtcctt      3474
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MRP2_gene          caccccattcttcactctcgttatcattcccttgagcatcttctactattttgtgctgcg      2340
Predicted_MRP2     caccccattcttcactctcgttatcattcccttgagcatcttctactattttgtgctgcg      3594
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MRP2_gene          cttctatgtctccacatcacgccagctaaggcgtctggactctgtaactaggtctcccat      2400
Predicted_MRP2     cttctatgtctccacatcacgccagctaaggcgtctggactctgtaactaggtctcccat      3654
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MRP2_gene          ctactcccactttggcgagacagtgtcagggctttctgtgatccgtgccttcggacacca      2460
Predicted_MRP2     ctactcccactttggcgagacagtgtcagggctttctgtgatccgtgccttcggacacca      3714
                   ************************************************************

MRP2_gene          agaacgattcctgcagcagaatgagagcaccatggacgtcaatcaaaaaagtgtttactc      2520
Predicted_MRP2     agaacgattcctgcagcagaatgagagcaccatggacgtcaatcaaaaaagtgtttactc      3774
                   ************************************************************

MRP2_gene          ctggatagtctcaaataggtggctggccatccgtctggagttcgttgggagcctggtggt      2580
Predicted_MRP2     ctggatagtctcaaataggtggctggccatccgtctggagttcgttgggagcctggtggt      3834
                   ************************************************************

MRP2_gene          cttcttctctgcgcttctagctgtgatttcaaagggcactttggagggcggcatcgtggg      2640
Predicted_MRP2     cttcttctctgcgcttctagctgtgatttcaaagggcactttggagggcggcatcgtggg      3894
                   ************************************************************

MRP2_gene          tctttctgtctcctctgccctcaatgtgacccagacactgaactggctggtgcggacgtc      2700
Predicted_MRP2     tctttctgtctcctctgccctcaatgtgacccagacactgaactggctggtgcggacgtc      3954
                   ************************************************************

MRP2_gene          ttcggagctggagacaaacattgtggctgtggagcgggtacatgagtacacgaaggtgaa      2760
Predicted_MRP2     ttcggagctggagacaaacattgtggctgtggagcgggtacatgagtacacgaaggtgaa      4014
                   ************************************************************

MRP2_gene          gaatgaggctccgtgggtgacagaaaagcgtccaccccatggctggcccagcaaaggtga      2820
Predicted_MRP2     gaatgaggctccgtgggtgacagaaaagcgtccaccccatggctggcccagcaaaggtga      4074
                   ************************************************************

MRP2_gene          gatccagtttgttgactacaaagttcgttaccgacctgaactggagctggttcttcaggg      2880
Predicted_MRP2     gatccagtttgttgactacaaagttcgttaccgacctgaactggagctggttcttcaggg      4134
                   ************************************************************

MRP2_gene          gatcacctgcaatattgggagcacggagaaggttggggttgtgggccggactggggctgg      2940
Predicted_MRP2     gatcacctgcaatattgggagcacggagaaggttggggttgtgggccggactggggctgg      4194
                   ************************************************************

MRP2_gene          aaaatcttccctcaccaactgcctcttccgggtgctggaggccgctggagggacgatcat      3000
Predicted_MRP2     aaaatcttccctcaccaactgcctcttccgggtgctggaggccgctggagggacgatcat      4254
                   ************************************************************

MRP2_gene          catcgacgaagtggatatagcaacgatcggcctccatgacctgcgccagaacctcaccat      3060
Predicted_MRP2     catcgacgaggtggatatagcaacgatcggcctccatgacctgcgccagaacctcaccat      4314
                   ********* ************************************************

MRP2_gene          catccctcaggaccccgtgctctttactggcaccctgcggatgaacctggatccctttga      3120
Predicted_MRP2     catccctcaggaccccgtgctctttactggcaccctgcggatgaacctggatccctttga      4374
                   ************************************************************

MRP2_gene          ccagtacatggatgaggaggtctggaaggcccttgagctggcccacctgaagacatatgt      3180
Predicted_MRP2     ccagtacatggatgaggaggtctggaaggcccttgagctggcccacctgaagacatatgt      4434
                   ************************************************************

MRP2_gene          gcaagaccttcccgaggggctgctgcatcttgtgagcgaggcgggggagaacctgagtgt      3240
Predicted_MRP2     gcaagaccttcccgaggggctgctgcatcttgtgagcgaggcgggggagaacctgagtgt      4494
                   ************************************************************

MRP2_gene          tgggcagaggcagctggtgtgcctggcccgggcncctccttcgcaaagccaagatcctca      3300
Predicted_MRP2     tgggcagaggcagctggtgtgcctggcccgggc-cctccttcgcaaagccaagatcctca      4553
                   ********************************* ************************

MRP2_gene          tcctggacgaagcgacagcagccgtagat--------------------------------      3329
Predicted_MRP2     tcctggacgaagcgacagcagccgtagatctagaaactgatcatttaatccagacaacga      4613
                   ******************************
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MRP2_gene          ------------------------------------------------------------      3329
Predicted_MRP2     tccggagtgagtttgctgactgcactgtccttactattgcccaccgcctccacaccatca      4673

MRP2_gene          ------------------------------------------------------------      3329
Predicted_MRP2     tggacagcaacagggtgatggtgctgcaggctgggaggattgtggaatacgacagccctg      4733

MRP2_gene          ------------------------------------------------------------      3329
Predicted_MRP2     aggagctgctcaagaagcacggtgtcttctccgcaatggcaaaggacgctggcatcacga      4793

MRP2_gene          ------------------------------------------------------------      3329
Predicted_MRP2     atatagaaaccactgtgctgtaggtggagcagagcagtgtgcgggtgtgtgcgttggcag      4853

MRP2_gene          ------------------------------------------------------------      3329
Predicted_MRP2     ctccctcccactggcactcaccaagcaggcagcagcttctccctgctgccgggctgccca      4913

MRP2_gene          ------------------------------------------------------------      3329
Predicted_MRP2     ggaaattctctctgcagctgggaagcagagagagtggcttctctggccaggacagaggat      4973

MRP2_gene          ------------------------------------------------------------      3329
Predicted_MRP2     ctggacttgagtgagctgttaaccttgctaccccaccctgcttgctgtgcgcatgagggt      5033

MRP2_gene          ------------------------------------------------------------      3329
Predicted_MRP2     ctggagctgcataatttattccagtatagaggtgaaaagtctgccatgggagaccatgac      5093

MRP2_gene          ------------------------------------------------------------      3329
Predicted_MRP2     ccccgtgggggtcttagtttttgtacttcaccatgccaggggaacctagctgagatatgc      5153

MRP2_gene          ------------------------------------------------------------      3329
Predicted_MRP2     tttagcactacggaatgaaatttacagtttaatctaagagtgttataaacttttgtaac      5212
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Figure 5-9: Alignment of MRP2 gene of AWB vulture using Sanger and next generation sequencing revealing similarity of 99.76% with clusta omega software. MRP2_gene = sanger; Predicted_MRP2 = NGS

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 MRP4_gene          ------------------------------------------------------------      0
Predicted_MRP4     ggtggttgaatcctttatttattattggccataaacggaagcttgaagaagatgatatgt      60

MRP4_gene          --------------GAAGATTCCTCAGAGAAGCTTGGAGAGGAATTGCAGTGGTACTGGG      46
Predicted_MRP4     ataaagtgctgccagaagattcctcagagaagcttggagaggaattgcagtggtactggg      120
                               *******************************************

MRP4_gene          MTAAARAGGTGCAAAWAGCAWAAAAKAGAGGAAAAACGCCACGTTTAACAAAAGCCATTA      106
Predicted_MRP4     ataaagaggtgcaaaaagcaaaaaagagaggaaaaacgccacatttaacaaaagccatta      180
                    **** ********* **** **** ********** ******************

MRP4_gene          TTCTTTGTTACTGGAAATCCTATTTAKTTTTTGGAATTTTCACAATGATTGAGGAAACCC      166
Predicted_MRP4     ttctttgttactggaaatcctatttagtttttggaattttcacaatgattgaggaaaccc      240
                   ************************** ***********************************

MRP4_gene          TCAAAATAATTCAGCCAATATTTTTGGGAAAAATTATTAATTATTTTGAAAACTATGAT-      225
Predicted_MRP4     tcaaaataattcagccaatatttttgggaaaaattattaattattttgaaaactatgatt      300
                   ************************************************************

MRP4_gene          CCTCAGATGAGGTAGCTTTGAATTTTGCATATTTCTACGCAGCTGCTCTGTCTGTGTGCA      285
Predicted_MRP4     cctcagatgaggtagctttgaattttgcatatttctacgcagctgctctgtctgtgtgca      360
                   ************************************************************

MRP4_gene          CGCTTATTCTAGCTATAATGCACCACTTATACTTCTATCATGTACAGCGGGCTGGCATGA      345
Predicted_MRP4     cgcttattctagctataatgcaccacttatacttctatcatgtacagcgggctggcatga      420
                   ************************************************************

MRP4_gene          AGCTGAGGGTAGCTATGTGTCACATGATTTATCGRAAGGCACTTCGTCTCAGTAACGTAG      405
Predicted_MRP4     agctgagggtagctatgtgtcacatgatttatcggaaggcacttcgtctcagtaacgtag      480
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                  ********************************** ************************

MRP4_gene         CTATGGCAAAAACTACCACTGGKCAAATAGTGAATCTTCTGTCAAATGATGTGAACAAAT      465
Predicted_MRP4    ctatggcaaaaactaccactggtcaaatagtgaatcttctgtcaaatgatgtgaacaaat      540
                  ********************** ************************************

MRP4_gene         TTGATCAGGTAACAATCTTCTTGCACTTCTTGTGGGCTGGACCAATTCAAGCTGTARCAG      525
Predicted_MRP4    ttgatcaggtaacaatcttcttgcacttcttgtgggctggaccaattcaagctgtagcag      600
                  ********************************************************* ***

MRP4_gene         TAACAGTACTTCTCTGGATGGAGATAGGCCCATCATGTCTTGCAGGAATGGCAGYTCTGA      585
Predicted_MRP4    taacagtacttctctggatggagataggcccatcatgtcttgcaggaatggcagttctga      660
                  ********************************************************* *****

MRP4_gene         TTATTCTTCTTCCTGTCCAGACCTGCATTGGGAGGCTTTTTTCTTCCCTAAGAAGCAAGA      645
Predicted_MRP4    ttattcttcttcctgtccagacctgcattgggaggctttttttcttccctaagaagcaaga      720
                  ************************************************************

MRP4_gene         CAGCTGCCTTARCAGATGTCAGGATTAGGACCATGAATGAAGTCATAAGTGGTATGAAGA      705
Predicted_MRP4    cagctgccttaacagatgtcaggattaggaccatgaatgaagtcataagtggtatgaaga      780
                  *********** ************************************************

MRP4_gene         TAAATAAAAGATGTATGCTTGGGAAAAATCATTTGCGGAACTTGTGAATGGTTTAAGAAG      765
Predicted_MRP4    taat--aaagatgtatgcttgggaaaaatcatttgcggaacttgtgaatggtttaagaag      838
                  ***   ****************************************************

MRP4_gene         GAAGGAGATTTGCCATGGTTATGAAAAAGCTCCTACCTTCGAGGACTGAACTTAACCTCA      825
Predicted_MRP4    gaaggagat-tgccatggttatgaa-aagctcctaccttcgaggactgaacttagcctca      896
                  ********* *************** *************************** *****

MRP4_gene         TTTTTTGTGGCAAGCAAAATAACAGTGTTCATGACTTTCATGGCATATGTACTACTTGGC      885
Predicted_MRP4    ttttttgtggcaagcaaaataacagtgttcatgactttcatggcatatgtactacttggc      956
                  ************************************************************

MRP4_gene         AATGTTATCTCTGCAAGTCGGGTGTTTGTTGCAGTGTCCCTGTATGGTGCAGTAAGACTG      945
Predicted_MRP4    aatgttatctctgcaagtcgggtgtttgttgcagtgtccctgtatggtgcagtaagactg      1016
                  ************************************************************

MRP4_gene         ACAGTAACTCTGTTCTTCCCTTCGGCTATTGAGAGAGTATCCGAGGCAGTGGTTAGCATA      1005
Predicted_MRP4    acagtaactctgttcttcccttcggctattgagagagtatccgaggcagtggttagcata      1076
                  ************************************************************

MRP4_gene         CGACGAATCAAGAACTTTCTGATACTTGATGAGATCTCACCCTTCAAGCCACAACTGCAT      1065
Predicted_MRP4    cgacgaatcaagaactttctgatacttgatgagatctcacccttcaagccacaactgcat      1136
                  ************************************************************

MRP4_gene         GGTAATAATGAGAATGTCATTCTTCATGTTCAGGATTTGACTTGCTATTGGGATAAGAGT      1125
Predicted_MRP4    ggtaataatgagaatgtcattcttcatgttcaggatttgacttgctattgggataagagt      1196
                  ************************************************************

MRP4_gene         TTAGAAAGCCCAGCACTTCAACAACTTTCATTTACTGTCAGACGAGGGGAATTATTGGCT      1185
Predicted_MRP4    ttagaaagcccagcacttcaacaactttcatttactgtcagacgaggggaattattggct      1256
                  ************************************************************

MRP4_gene         GTGATTGGTCCTGTAGGAGCTGGCAAATCTTCACTCTTAAGTGCTGTGCTTGGTGAGCTA      1245
Predicted_MRP4    gtgattggtcctgtaggagctggcaaatcttcactcttaagtgctgtgcttggtgagcta      1316
                  ************************************************************

MRP4_gene         CCTAAAGACAAAGGTTTGATAAATGTTACTGGAAGAATTGCCTATGTTTCTCAGCAGCCT      1305
Predicted_MRP4    cctaaagacaaaggtttgataaatgttactggaagaattgcctatgtttctcagcagcct      1376
                  ************************************************************

MRP4_gene         TGGGTGTTTTCTGGTACAGTAAGAAGTAATATACTGTTTGACAAGGNAATATGAAAAAGA      1365
Predicted_MRP4    tgggtgttttctggtacagtaagaagtaatatactgtttgacaagg-aatatgaaaaaga      1435
                  ********************************************* **************

MRP4_gene         AAAATACGAAAAAGTTTTAAAAGTCTGTGCTCTTAAAAAGGACTTGGAATTATTAGCRAA      1425
Predicted_MRP4    aaaatacgaaaaagttttaaaagtctgtgctcttaaaaaggacttggaattattagcaaa      1495
                  ************************************************************ **

MRP4_gene         TGGTGACCTAACAGTAATAGGAGATCGTGGAGCTACGCTGAGTGGGGGACAGAAAGCCCG      1485
Predicted_MRP4    tggtgacctaacagtaataggagatcgtggagctacgctgagtgggggacagaaagcccg      1555
                  ************************************************************

MRP4_gene         TGTAAATCTGGCCAGAGCTGTGTATCAAGATGCAGACATCTATCTTTTTGGATGATCCAC      1545
Predicted_MRP4    tgtaaatctggccagagctgtgtatcaagatgcagacatctatctt-ttggatgatccac      1614
                  ******************************************* **************
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MRP4_gene          TGAGTGCAGTAGATGCTGAAGTTGGAAGACATTTGTTTGAAAAATGTATTTGTCAGGCCT      1605
Predicted_MRP4     tgagtgcagtagatgctgaagttggaagacatttgtttgaaaaatgtatttgtcaggcct      1674
                   ************************************************************

MRP4_gene          TWCATCAGAAGATCTCTGTTTTGGTTACTCACCAGTTGCAGTATCTCCGTGCTGCAAATC      1665
Predicted_MRP4     tacatcagaagatctctgttttggttactcaccagttgcagtatctccgtgctgcaaatc      1734
                   * ************************************************************

MRP4_gene          AGATTCTAATTTTAAAAGATGGTAAAATGGTGGGGAAAGGTACCTATTCAGAGTTCCTGA      1725
Predicted_MRP4     agattctaattttaaaagatggtaaaatggtggggaaaggtacctattcagagttcctga      1794
                   ************************************************************

MRP4_gene          GATCTGGCATCGACTTTGCTTCCCTTTTGAAAAAAGATGAGGAGGTAGAACAGCCGTCAG      1785
Predicted_MRP4     gatctggcatcgactttgcttccctttgaaaaaagatgaggaggtagaacagctgtcag      1854
                   ************************************************************ *****

MRP4_gene          TTCCAGGAACTCCCAACCTGAAGTCTGTCCGGAGCCGAACCTTCTCAGAGTCCTCTGTCT      1845
Predicted_MRP4     ttccaggaactcccaacctgaagtctgtccggagccgaaccttctcagagtcctctgtct      1914
                   ************************************************************

MRP4_gene          GGTCCCAGGATTCTTCTGCCCACTCACAGAAAGATGGAGCAGTGGAGCAACCACCTGCTG      1905
Predicted_MRP4     ggtcccaggattcttctgcccactcacagaaagatggagcagtggagcaaccacctgctg      1974
                   ************************************************************

MRP4_gene          AAAACGCACTGGCTGCAGTGCCAGAGGAGAGTCGCTCTGAGGGAAAAATAAACTTTAAGG      1965
Predicted_MRP4     aaaacgcactggctgcagtgccagaggagagtcgctctgagggaaaaataaactttaagg      2034
                   ************************************************************

MRP4_gene          TTTACAGAAAATATTTCACTGCAGGAGCAAACTACTTTGTGATTTTCATACTTCTAGTAT      2025
Predicted_MRP4     tttacagaaaatatttcactgcaggagcaaactactttgtgattttcatacttctagtat      2094
                   ************************************************************

MRP4_gene          TCAATATTTTGGCACAGGTGGCATACGTGCTCCAGGACTGGTGGCTTTCTTACTGGGCAA      2085
Predicted_MRP4     tcaatattttggcacaggtggcatacgtgctccaggactggtggctttcttactgggcaa      2154
                   ************************************************************

MRP4_gene          ATCATCAAGAAAAGTTGAACGTCACAACAAATGGAAATAATGGAGCAAATGAGAGTGAAC      2145
Predicted_MRP4     atcatcaagaaaagttgaacgtcacaacaaatggaaataatggagcaaatgagagtgaac      2214
                   ************************************************************

MRP4_gene          ATCTAGACCTTAACTTTTATTTGGGAATTTATGCAGGTTTAACGGTGGCTACAATACTGT      2205
Predicted_MRP4     atctagaccttaacttttatttgggaatttatgcaggtttaacggtggctacaatactgt      2274
                   ************************************************************

MRP4_gene          TTGGCATAGTAAGAAGTCTTTTGGTGTTTCAAGTTCTTGTTAATTCTGGTCAGACTTTGC      2265
Predicted_MRP4     ttggcatagtaagaagtcttttggtgtttcaagttcttgttaattctggtcagactttgc      2334
                   ************************************************************

MRP4_gene          ACAACAAAATGTTTCAATCCATTTTGAAAGCTCCCGTCTTGTTTTTTGACAGAAATCCTA      2325
Predicted_MRP4     acaacaaaatgtttcaatccattttgaaagctcccgtcttgtttttgacagaaatcctа      2394
                   ************************************************************

MRP4_gene          TAGGAAGAATCTTAAATCGTTTCTCCAAAGATATTGGCCACCTGGATGACTTGCTTCCAT      2385
Predicted_MRP4     taggaagaatcttaaatcgtttctccaaagatattggccacctggatgacttgcttccat      2454
                   ************************************************************

MRP4_gene          TGACATTTTTGGACTTCATGCAGACTCTCCTACAGATTTTTGGTGTGGTGGCTGTGGCTG      2445
Predicted_MRP4     tgacatttttggacttcatgcagactctcctacagattttttggtgtggtggctgtggctg      2514
                   ************************************************************

MRP4_gene          TGGCAGTGATTCCTTGGATACTCCTCCCCTTAATTCCACTATTTATTCTTTTCATTTTCC      2505
Predicted_MRP4     tggcagtgattccttggatactcctcccctaattccactatttattcttttcattttcc      2574
                   ************************************************************

MRP4_gene          TTCGACGATATTTCTTAGACACTTCAAGAGATATTAAACGTCTAGAATCCACAACTCGAA      2565
Predicted_MRP4     ttcgacgatatttcttagacacttcaagagatattaaacgtctagaatccacaactcgaa      2634
                   ************************************************************

MRP4_gene          GTCCAGTGTTCTCCCACTTGTCGTCATCCCTCCAGGGACTTTGGACTATTCGGGCTTTGA      2625
Predicted_MRP4     gtccagtgttctcccacttgtcgtcatccctccagggactttggactattcgggctttga      2694
                   ************************************************************

MRP4_gene          AAGCAGAGGAAAGATTTCAAAAATTATTTGATGCACACCAAGACCTCCACTCAGAGGCCT      2685
Predicted_MRP4     aagcagaggaaagatttcaaaaattatttgatgcacaccaagacctccactcagaggcct      2754
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MRP4_gene          GGTTTCTATTTTTGACGACCTCGAGGTGGTTTGCTGTGCGTCTGGATGCCATCTGTGCCA      2745
Predicted_MRP4     ggtttctatttttgacgacctcgaggtggtttgctgtgcgtctggatgccatctgtgcca      2814
                   ************************************************************

MRP4_gene          TTTTTGTTATAGTGGTTGCTTTTGGTTCCCTGCTTCTCKCCAAGACTTTGAATGCAGGGC      2805
Predicted_MRP4     tttttgttatagtggttgcttttggttccctgcttctctccaagactttgaatgcagggc      2874
                   ************************************ ********************

MRP4_gene          AGGTTGGTTTGGCACTATCCTATGCAATCACCCTCATGGGAACATTCCAGTGGGGTGTTA      2865
Predicted_MRP4     aggttggtttggcactatcctatgcaatcaccctcatgggaacattccagtggggtgtta      2934
                   ************************************************************

MRP4_gene          GACAAAGTGCTGAAGTTGAAAACCTGATGATATCAGTAGAAAGAGTAATGGAATACACAG      2925
Predicted_MRP4     gacaaagtgctgaagttgaaaacctgatgatatcagtagaaagagtaatggaatacacag      2994
                   ************************************************************

MRP4_gene          AACTTGAAAAAGAAGCTCCTTGGGAGACCAACAAGCATCCACCACCTGAATGGCCAAGCC      2985
Predicted_MRP4     aacttgaaaaagaagctccttgggagaccaacaagcatccaccacctgaatggccaagcc      3054
                   ************************************************************

MRP4_gene          AAGGAATGATAGCATTTGAAAATGTTAACTTCACTTACAGTCTAGATGGACCTTTGGTGT      3045
Predicted_MRP4     aaggaatgatagcatttgaaaatgttaacttcacttacagtctagatggacctttggtgt      3114
                   ************************************************************

MRP4_gene          TAAGACATTTGTCTGTTTTAATTAAACCAAAAGAAAAGGTTGGAATAGTGGGAAGAACTG      3105
Predicted_MRP4     taagacatttgtctgttttaattaaaccaaaagaaaaggttggaatagtgggaagaactg      3174
                   ************************************************************

MRP4_gene          GAGCTGGGAAAAGCTCTCTGATAGCAGCCCTCTTTCGCTTGGCGGAACCCGAAGGAAGGA      3165
Predicted_MRP4     gagctgggaaaagctctctgatagcagccctcttccgcttggcggaacccgaaggaagga      3234
                   ********************************** ************************

MRP4_gene          TTTGGATTGATAAGTACTTGACGTCAGAGCTAGGACTCCATGACTTGCGGAAGAAAATTT      3225
Predicted_MRP4     tttggattgataagtacttgacgtcagagctaggactccatgacttgcggaagaaaattt      3294
                   ************************************************************

MRP4_gene          CAATTATACCTCAGGAGCCTGTTTTATTCACTGGAACTATGAGGAAAAACTTAGATCCTT      3285
Predicted_MRP4     caattatacctcaggagcctgttttattcactggaactatgaggaaaaacttagatcctt      3354
                   ************************************************************

MRP4_gene          TCAATGAATACACTGATGAGGAGCTGTGGAATGCCTTGGAAGAGGTGCAACTGAAGGAGG      3345
Predicted_MRP4     tcaatgaatacactgatgaggagctgtggaatgccttggaagaggtgcaactgaaggagg      3414
                   ************************************************************

MRP4_gene          TTGTGGAAGATCTACCTAATAAAATGGAGATGCAGCTGGCAGAAATCTGGGTCTAATTTT      3405
Predicted_MRP4     ttgtggaagatctacctaataaaatggagatgcagctggcaga-atctgggtctaatttt      3473
                   ****************************************** ****************

MRP4_gene          AGTGTTGGTCAGAGACAGCTGGTGTGTCTTGCCAGAGCAGTTCTAAAAAAAAAATCGGAT      3465
Predicted_MRP4     agtgttggtcagagacagctggtgtgtcttgccagagcagttctaaaa-aaaaatcggat      3532
                   ************************************************ ************

MRP4_gene          CCTTATCATTGATGAAGCAACAGCAAATGTGGACCCAAGAACAGATGAGTTYATTCAAAA      3525
Predicted_MRP4     ccttatcattgatgaagcaacagcaaatgtggacccaagaacagatgagtttattcaaaa      3592
                   ************************************************** ********

MRP4_gene          GACGATCCGTGAAAAGTTTGCTCACTGCACAGTGCTGACCATTGCACACCGCTTGAACAC      3585
Predicted_MRP4     gacgatccgtgaaaagtttgctcactgcacagtgctgaccattgcacaccgcttgaacac      3652
                   ************************************************************

MRP4_gene          CATTATTGACAGTGACAGGATTATGGTTTTAGATGAAGGAAGAGTGAAAGAATATGGTGA      3645
Predicted_MRP4     cattattgacagtgacaggattatggttttagatgaaggaagagtgaaagaatatggtga      3712
                   ************************************************************

MRP4_gene          ACCTTACATTTTGCTGCAAGAACAAGATGGCTTGTTTTACAAAATGGTGCAACAAGTGGG      3705
Predicted_MRP4     accttacattttgctgcaagaacaagatggcttgttttacaaaatggtgcaacaagtggg      3772
                   ************************************************************

MRP4_gene          CAAGACTGAAGCAGCTTCTYTGATTGAAACAGCAAAACGGGTGTACTTCAGTAAGAATTA      3765
Predicted_MRP4     caagactgaagcagcttctctgattgaaacagcaaaacgggtgtacttcagtaagaatta      3832
                   ******************* ****************************************

MRP4_gene          CCCAGAAGTTGTTCAGAATGGTCAACTTGCCACAGACTCCTCCTTGGATCCTTCCTCAGG      3825
Predicted_MRP4     cccagaagttgttcagaatggtcaacttgccacagactcctccttggatccttcctcagg      3892
                   ************************************************************

MRP4_gene          ATTAKGCATAACCGAAACTGCACTGTGATTCCTAATAA----------------------      3863
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Predicted_MRP4      attatgcataaccgaaactgcactgtgattcctaataaccttaactgttttccattgaat      3952
                    **** ********************************

MRP4_gene           ------------------------------------------------------------      3863
Predicted_MRP4      gtaaacctgagatcatctaaactcagtgacaatgtttgcaagtgtcagcaggagaggaaa      4012


MRP4_gene           --------------------------------------------------       3863
Predicted_MRP4      gggaggggggcgattctttgcactggacatccttcctatttaatactgag       4061
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Figure 5-10: Alignment of MRP4 gene of AWB vulture using Sanger and next generation sequencing revealing similarity of 99.43% with clusta omega software. . MRP4_gene = sanger; Predicted_MRP4 = NGS

5.4. Discussion.

Previous studies revealed that the *Gyps* species found dead in India was due to the residue of diclofenac in the carcass they feed upon (Oaks et al., 2004), with signs of abnormally high level of plasma uric acid (Naidoo et al., 2009). Our results thus far have revealed that the mechanism of diclofenac toxicity in chicken, a proven surrogate for *Gyps* species, resulted through the complete inhibition of OAT transporters among other things. However to fully understand the excretion of uric acid in the vulture, this study was undertaken to demonstrate that the abnormally high uric acid evident in the birds was not as a result of problems with MRP channels.

To date expression of the MRPs gene have been intensively studied in mammals and avian species, and most recently MRP4 was characterised on one *Gyps* vulture species (Gyps *Himalyanesis*) (Barik et al., 2019). The presence of the transporter protein in *Gyps* vulture is important in excretion of its naturally high uric acid resulted from an all meat diet, especially since avian species do not conserve uric acid and are predominantly reliant on these transporters for excretion. In this study, we characterized a complete coding sequences of MRP2 and MRP4 in AWB vulture and it's the first study to characterized MRP2 in *Gyps* vulture. This aligns with previous findings by Haritova et al. (2010), who conducted expression studies of drug efflux transporters on poultry tissues and he revealed that MRP2 mRNA is highly expressed on kidneys, liver, duodenum and jejunum (Haritova et al., 2010) moreover Bataille et al. (2008) also revealed the presence on MRP4 gene in chicken`s kidney (Bataille et al., 2008).

The phylogenetic tree revealed level of similarity (100% identical) between vulture and eagle for MRP2 and MRP4 channels. The chicken and vulture were shown to belong to two distinct clades, which was in accordance with previous findings by Zhang et al. (2014) who explored macroevolution. In their evaluation using the full genomes of 48 avian species representing all main extant clades, their phylogenetic tree revealed that vulture and eagles share the same clade but not with chickens. Jarvis (2014) revealed that vulture and eagles also belonged to the same family called accipitrimorphae (Jarvis et al., 2014). The similarity of vulture and eagle is likely attributed to their foraging behaviour because eagles and vultures share a carnivorous diet and chicken is more an omnivore, this may explain why they evolved into different uric acid channels moreover eagles and vulture have skeleton which are strong and light hence they follow under the highest flying birds (Louchart and Viriot, 2011; Dumont, 2010) meaning that they have same ancestral gene.

In silico analysis with Expasy revealed ORFs of MRP2 and MRP4 proteins consisting of 1552 and 1287 amino acids respectively in both AWB vulture and golden eagle. This finding is similar to human MRP2 and MRP4 proteins which comprises of 1545 and 1325 amino acids in that order (Borst et al., 2000). Moreover a recent study by Barik et al., 2019 also revealed that chicken and *Gyps Himalyanesis* MRP4 consisted of 1349 amino acids while Expasy results of *Gallus gallus* MRP2 (accession number XM_015288821.1) and MRP4 (accession number NM_001030819) consisted of 1550 and 1330 amino acids respectively. The similarity in the above protein sequences of MRP2 and MRP4 in size and length maybe revealing that their structures are conserved, and likely of similar functionality.

TMHMM and PROTTER predicted transmembrane helices and the N-glycosylation sites of the latter proteins are found to be the same between AWB vulture and golden eagle, with MRP2 having 16 TMH and MRP4 having 11 TMH with two NBDs. MRP2 N-glycosation sites were 13 at position: 15, 106, 284, 551, 620, 725, 773, 886, 1019, 1193, 1254, 1383, 1443 in AWB and golden eagle however for chicken it was 12 at position: 16, 106, 495, 620, 725, 773, 1017, 1252, 1381, 1441 with 4 conserved sites (106, 620, 725, 773) for the three above species confirming the above speculation. MRP4 for AWB and golden eagle N-glycosation sites were 5 and observed at position 428, 706, 716, 1009, 1138 while chicken and *Gyps himalyanesis* where at position 471, 749, 759, 1052, 1200 (Barik et al., 2019). The chicken and *Gyps himalyanesis* findings contradict with our results, are likely due to different channel sensitivity, an adaption to different environmental factors and diets. This will however require further

study, which may be facilitated through the use of specific markers and in vivo excretion studies.

PHYRE2 results confirmed the two TMDs and NBDs for both proteins however the TMH for both proteins contradict with the results from PROTTER and TMHHM indicating TMH for MRP2 to be 19 and MRP4 consisting of 13 TMH. This finding is unusual because studies by Bakos et al. (1996, 1998); Tussnady et al. (1997); Dean et al. (2001); Barik et al. (2019) on human, fish and avian species revealed MRP2 with two NBDs and TMDs consisting of 12 TMH and an extra transmembrane domain (TMD) with 5 TMH while the MRP4 also had two NBDs and TMDs with 12 TMH (Bakos et al., 1996, 1997, 1998; Tusnady et al., 1997; Dean et al., 2001; Barik et al., 2019). The TMDs and NBDs elucidate how these proteins functions, since MRPs are ATP-binding cassettes (ABC) transporters they require the use of ATP to facilitate the movement of extensive range substance within the cells (Linton and Higgins, 1998). Research on few high-resolution structures of ABC transporters revealed that the former has two TMDs and two NBDs (also known as ABCs) which aligns with our results (Hollenstein et al., 2007; Oldham et al., 2008; Rees et al., 2009). Where TMDs binds the transported substrates and NBDs provide the transport energy by binding and hydrolyzing up to two molecules of ATP (Newstead et al., 2009; Khare et al., 2009; Oldham et al., 2007; Ward et al., 2007). However some ABCs transporter functions differently where the substrate-binding site is open to different sides of the membrane and when the substrate bound, energy is needed to reorient the TMDs and that energy is provided by the NBDs (Jardetzky, 1966; Smith et al., 2002).

In summary, the presence of MRP2 and MRP4 in avian species is essential because they defend against toxic compounds especially organic anions. Their presence in the vulture supports the presence of the described uric acid pathways as for other species. The importance of the channels is evident from a study by Konig (1999, 2003) who showed that rats' mutants and human lacking MRP2 gene had compromised excretion of organic anions from the liver leading to mild liver disease and inherited jaundice (Konig et al., 1999; 2003). When comparing it to MRP1, it plays a more significant role in drug and metabolite absorption, disposition and elimination. The significance of MRP4 channel was also highlighted by the following studies: an *Abcc4*−/− mice study revealed that the lack of the latter channel did not induce clincally obvious abnormalities in the healthy animals but did result in acute 9-(2-phosphonylmethoxyethyl) adenine (PMEA) toxicity. The latter suggestive of a protective role of MRP4 more in the bone marrow, gastrointestinal tract, thymus and spleen (Abla et al., 2008).

A similar study on humans was comparing two antiviral agents, adefovir (PMEA) and azidothymidine (AZT) by measuring the intracellular accumulation and there was no proof for a complete loss of function allele. However, some variants compared to reference with both substrates showed a significantly reduced function, as demonstrated by higher intracellular accumulation of PMEA and AZT compared to the reference (Alba et al., 2008). To further support MRP4 functionality, a study by Bakos et al., 1997; Ren et al., 2004 showed that the replacement of a highly conserved glycine in NBD1 with an aspartate, resulted in the intracellular accumulations similar to the empty vector-transfected cells, confirming their role in efflux by MRP4 (Bakos et al., 1997; Ren et al., 2004).

5.5. Conclusion

For this study we show the presence of MRP2 and MRP4 in AWB vulture`s kidney, supporting the presence of the expected cellular pathways in uric acid excretion. Phylogenetic analysis also confirmed that vulture and eagle are on the same clade in contrast to chicken.

5.6. Acknowledgement

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5.7. References

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CHAPTER SIX

6. General Discussion and Conclusions.

6.1. General Discussion

6.1.1. Hypothesis 1: Mechanism of diclofenac toxicity on OAT uric acid transporters

A two phased single intravenous dose study of 10 mg/kg (diclofenac), 10 mg/kg (PAH) and 1.25 mg/kg (iohexol) was undertaken to establish the mechanism of diclofenac toxicity in domestic chicken, a validated surrogate for vulture. Faecal and plasma samples were analysed for diclofeanc, PAH, iohexol and uric acid. The results were analysed with noncompartmental pharmacokinetic analyses using Kinetica were possible. In phase 1 no ill heath was noted on the birds but in phase 2 within 48 hrs, 33% of the birds died thereafter diagnosed with urate nephropathy. The histopathological lesions were not attributed to the long-term exposure because the drug was barely detectable at 2 hrs meaning that the drug half-life was very short. To fully understand the mechanism of diclofenac toxicity, iohexol was used to monitor glomerulus filtration rate. The results indicated that iohexol clearance (glomerular filtration rate) of the untreated birds was fast and most cleared within 6hrs. However for the treated group iohexol clearance decreased by up to 87%, leading to a conclusion that diclofenac interferes with renal arterial blood flow. However this was deemed to unlikely be the only mechanism causing the catastrophic diclofenac associated hyperuricaemia in birds as they used uric acid transport to excrete 80% of their uric acid load.

To fully understand the cause of high level of plasma uric acid, PAH was used because it is a substrate for OATs channels that transport uric acid and other drugs to their excretion points. PAH clearance of the untreated birds was rapid and non-detectable with up to 99.90% excreted while for the treated birds, diclofenac induced an increase in the total exposure to PAH, (Tm_{PAH} reduced by more than 98%). The latter occurred in conjunction with plasma uric acid levels rapidly increasing over 24 hrs, with the birds that died showing the highest level of plasma uric acid which increased by more than 10 fold compared to the untreated. Moreover, the faecal uric acid levels decreased over the period of time for some birds with $Tm_{uricacid}$ completely absent for the birds that died. This leads to the conclusion that OAT inhibition is the pivotal step in ultimate toxicity. The latter is not surprising and similar to studies with sodium salicylate, also in chickens (Berger et al., 1960). The sensitivity of the vulture over chickens is

also likely due to the vulture having an overall lower mass specific uric acid clearance, despite having a higher exposure to uric acid.

6.1.2. Hypothesis 2: African White backed vulture make use of uric acid transporters (OAT1, OAT2, OAT3, MRP2 and MRP4).

Uric acid transporters meditate the excretion of uric acid and other drugs just like other animals e.g humans (Sweet, 2005, 2010; Van wert et al., 2010; Sekine et al., 1997; Kusuhara et al., 1999; Burckhardt and Burckhardt, 2011; Wang and Sweet, 2012). These transporters are located on the basolateral and apical membrane of the kidney`s proximal tubules (Wang and Sweet, 2013) hence kidney was a sample of choice for this study. Moreover, it is also in the kidney where excretion of xenobiotics and endogenous compounds occur (Wang and Sweet, 2013). Total RNA was extracted from the AWB vulture and chicken kidneys and converted into cDNA and evaluated using conventional PCR with specific chicken OAT3 primers as described by Dudas et al. (2005). Unfortunately, there was no amplification for the AWB vulture. The chicken amplicons were sequenced and the obtained consensus chicken sequence revealed more similarity to OAT1 gene of other avian species when blasted and surprisingly with no match to the chicken OAT3. This led to a conclusion that the said Chicken OAT3 gene (accession number: BBSRC Chick EST ID 603812145F1) was likely misclassified, which would explain why the latter gene has been removed from NCBI.

In an effort to amplify the AWB vulture uric acid transporters, next generation sequencing was employed and the generated transcriptome raw sequences were submitted to NCBI and allocated SRA (sequence read archive) accession number: PRJNA560189. The latter transcriptomes were assemble using trinity software and compared to the Golden eagle based on phylogenetic studies in our laboratory indicating close species relatedness (OAT1->XM_011601043.1; OAT2->XM_011585794.1; MRP2- XM_011576169.1; MRP4- XM_011586368.1) found in a local blast database (NCBI). AWB and golden eagle genes alignment results revealed similarity of 98.89, 98.07, 98.12 and 99.14% respectively. OAT3 was not evaluated as no sequence could be found for any bird species. Furthermore primers were designed using obtained predicted AWB OAT1, OAT2, MRP2 and MRP4 sequences for Sanger sequencing to be carried out. The consensus sequences generated analysed using Clustal Omega revealed good similarity to NGS as follows: 98.81, 99.34, 94.75 and 97.97% for OAT1, OAT2, MRP2 and MRP4 respectively moreover the OAT1 and OAT2 Sanger sequences were

deposited to NCBI and given the following accession numbers: MK854995 and MK879652 in that order. The above results further confirm that the uric acid transporters characterised by NGS was correct. To affirm the latter statement, Sanger sequences (OAT2, MRP2 and MRP4) similarity with chicken were 88.05, 84.82 and 92.82% respectively.

With the transcriptome indicating, expression of the required mRNA, attempts were made to localize the OAT proteins. For this we undertook immunohistochemistry with a rabbit polyclonal human OAT3 antibody, as the binding domain of the antibody showed high similarity to the vulture OAT1 sequence. The location of OAT1, albeit nonquantifiable, was visually located in the AWB proximal tubules of the basolateral membrane and for mouse the staining were intense on the same location as the AWB, together with the mouse apparently unique immunoreactivity present on distal convoluted tubule cells and cortical collecting duct. The location of OAT2 could not be determined as no antibody could be found with good similarity.

Expression study was also conducted using RT-qPCR comparing AWB and chicken expression levels of OAT2 gene. The results revealed that AWB vulture expression levels were slightly lower than chicken, this may explain the low AWB vulture basal concentration of 0.06 mmol/l/ kg per body weight compared to 0.30 mmo/l/kg per body weight of chicken (hypothesis 1). The above findings lead to a conclusion that vulture had lower number of transporters compared to chicken hence its sensitivity to diclofenac. This finding was also supported by the clearance findings between vultures and chicken.

Lastly in silico analysis using protein predict and scan prosite indicated that the latter proteins were transporter proteins with OAT2 showing anionic transport capacity. Protter results also confirmed the above by revealing the presence of 10 THs with no N-glycosylation sites for OAT1 while for OAT2 it showed 11 TMs with 5 N-glycosylation sites furthermore two NBDs, two TMDs with an extra TMDs with 13 N-glycosylation sites for MRP2 and for MRP4 it had two NBDs and two TMDs with 5 N- glycosylation sites. However the absence of N-glycosylation sites on OAT1 protein is concerning because the latter sites within the protein plays an important role including modulation of biological activity, protein folding, and maintenance of protein stability and regulation of intracellular targeting (Perry et al., 2006; Tanaka et al., 2004; Cha et al., 2000).

6.2. Conclusion

Our study offers the first insight characterization of AWB vulture OAT1, OAT2, MRP2, MRP4 and the absence of OAT3 genes. The importance of OATs and MRPs in excreting uric acid in birds is well documented because they are the major route of uric acid (80%) excretion. A large body of literature stated that diclofenac is the major cause of *Gyps* vulture decline by increase in uric acid in their blood leading to mortalities however the mechanism of toxicity is not well understood. In this study we have concluded that the increase of uric acid that cause nephropathic death, was caused by a more than 98% inhibition of OAT channel functionality by diclofenac. With gene expression levels and uric acid clearance comparisons between the vulture and chicken also indicating that vulture had lower OAT functionality, this is likely the main reason behind the vulture's sensitivity to diclofenac.

As mentioned above the toxicity was due to pharmacology of diclofenac on the function of OATs proteins. Sensitivity of birds to different NSAIDs was due to individual variation and the difference in genetic makeup i.e in this study there were difference in chicken, eagle and vulture OATs gene as well as difference in OATs expression levels when comparing the AWBV to the chickens..

6.3. Further studies

Invitro studies of diclofenac on the OATs transporters should be conducted using the sequences generated on this study by cloning the latter transporters into appropriate cell lines in order to have more routine screening and to rank the NSAIDs by their inhibitory levels. Furthermore research on how the vulture and eagle OAT1 genes function without the N-glycosylation sites.

7. Annexes

```
Gyps-africanus        ------------------------------------------------------------  0
Gyps-himalayensis     ATGGAAGCGGTGCCCCGCGAGGAGAAACCCAACCCGCTGCGGGATGCCAACCTCTGCTCC  60

Gyps-africanus        -------------GGTGGTTGAATCCTTTATTTATTATTGGCCATAAACGGAAGCTTGAA  47
Gyps-himalayensis     CGGCTCTTCTTCTGGTGGCTGAATCCATTATTTATTATTGGCCACAAACGGAAACTTGAA  120
                                  ***** *******:****************** ********.******

Gyps-africanus        GAAGATGATATGTATAAAGTGCTGCCAGAAGATTCCTCAGAGAAGCTTGGAGAGGAATTG  107
Gyps-himalayensis     GAAGATGATATGTACAAAGTGCTGCCAGAAGATTCCTCAAAGAAGCTTGGGGAGGACTTA  180
                      ************** ************************.**********.*****.**.

Gyps-africanus        CAGTGGTACTGGGATAAAGAGGTGCAAAAAGCAAAAAAGAGAGGAAAAACGCCACATTTA  167
Gyps-himalayensis     CAGTGGTACTGGGATAAAGAGGTGCAAAAAGCAAAAAAGAGAGGAAAAACACCCCATTTA  240
                      ************************************************.**.******

Gyps-africanus        ACAAAAGCCATTATTCTTTGTTACTGGAAATCCTATTTAGTTTTTGGAATTTTCACAATG  227
Gyps-himalayensis     ACAAAAGCAATTATTCTGTGTTACTGGAAATCCTATTTAGTTCTTGGAATTTTCACAATG  300
                      ********.******** ************************ *****************

Gyps-africanus        ATTGAGGAAACCCTCAAAATAATTCAGCCAATATTTTTGGGAAAAATTATTAATTATTTT  287
Gyps-himalayensis     ATTGAGGAAACCCTCAAAATAGTTCAGCCAATATTTTTGGGAAAAATTATTACTTATTTT  360
                      **********************.*******************************.*******

Gyps-africanus        GAAAACTATGATTCCTCAGATGAGGTAGCTTTGAATTTTGCATATTTCTACGCAGCTGCT  347
Gyps-himalayensis     GAAAACTATGATGCCTCAGATGAGGTGGCTTTGAATGTTGCATATTGCTATGCAGCTGCT  420
                      ************ *************.********* ********* *** *********

Gyps-africanus        CTGTCTGTGTGCACGCTTATTCTAGCTATAATGCACCACTTATACTTCTATCATGTACAG  407
Gyps-himalayensis     CTATCTGTGTGCACACTTATTCTAGCTATAATGCACCACTTATACTTCTATCATGTACAG  480
                      **.************.**************************************************

Gyps-africanus        CGGGCTGGCATGAAGCTGAGGGTAGCTATGTGTCACATGATTTATCGGAAGGCACTTCGT  467
Gyps-himalayensis     CGGGCTGGCATGAAGCTAAGAGTGGCTATGTGTCATATGATTTATCGGAAGGCACTTCGT  540
                      ******************.**.**.************ ************************

Gyps-africanus        CTCAGTAACGTAGCTATGGCAAAAACTACCACTGGTCAAATAGTGAATCTTCTGTCAAAT  527
Gyps-himalayensis     CTCAGTAATGTAGCTATGGCAAAAACTACCACTGGACAAATAGTGAATCTTCTCTCAAAT  600
                      ******** **************************:***************** ******

Gyps-africanus        GATGTGAACAAATTTGATCAGGTAACAATCTTCTTGCACTTCTTGTGGGCTGGACCAATT  587
Gyps-himalayensis     GATGTGAACAAATTTGATCAGGTAACAATTTTCTTGCACTTCCTCTGGGCCGGACCAATT  660
                      ***************************** ************ * ***** *********

Gyps-africanus        CAAGCTGTAGCAGTAACAGTACTTCTCTGGATGGAGATAGGCCCATCATGTCTTGCAGGA  647
Gyps-himalayensis     CAAGCTGTAGCAGTAACAGTACTTCTCTGGATGGAAATAGGCCCATCATGTCTTGCAGGA  720
                      ********************************.***********************

Gyps-africanus        ATGGCAGTTCTGATTATTCTTCTTCCTGTCCAGACCTGCATTGGGAGGCTTTTTTCTTCC  707
Gyps-himalayensis     ATGGCAGTACTGATTATTCTTCTGCCTATACAAACCTGCATTGGGAGGCTTTTTTCTTCT  780
                      ********:*************** ***.*.**.*************************

Gyps-africanus        CTAAGAAGCAAGACAGCTGCCTTAACAGATGTCAGGATTAGGACCATGAATGAAGTCATA  767
Gyps-himalayensis     TTAAGGAGCAAGACAGCTGCCCTTACAGATGTCAGGATTAGGACCATGAATGAAGTCATA  840
                       ****.*************** *:************************************

Gyps-africanus        AGTGGTATGAAGATAATAAAGATGTATGCTTGGGAAAAATCATTTGCGGAACTTGTGAAT  827
Gyps-himalayensis     AGTGGTATGAAGATAATAAAGATGTATGCTTGGGAAAAATCATTTGCGGAACTTGTGAGT  900
                      ************************************************************.*

Gyps-africanus        GGTTTAAGAAGGAAGGAGATTGCCATGGTTATGAAAAGCTCCTACCTTCGAGGACTGAAC  887
Gyps-himalayensis     GGTTTGAGAAGGAAGGAGATTGCCATGGTTTTGAAAAGCTCCTACCTTCGAGGATTGAAC  960
                      *****.************************:********************** *****

Gyps-africanus        TTAGCCTCATTTTTTGTGGCAAGCAAAATAACAGTGTTCATGACTTTCATGGCATATGTA  947
Gyps-himalayensis     TTAGCCTCTTTCTTTGTGGCAAGCAAAATAACAGTGTTCATGACTTTCATGGCATATGTA  1020
                      ********:** ************************************************

Gyps-africanus        CTACTTGGCAATGTTATCTCTGCAAGTCGGGTGTTTGTTGCAGTGTCCCTGTATGGTGCA  1007
Gyps-himalayensis     CTACTTGGCAATGTTATCTCTGCAAGTCGGGTGTTTGTTGCAGTGTCCCTGTATGGCGCA  1080
                      ************************************************************ ***

Gyps-africanus        GTAAGACTGACAGTAACTCTGTTCTTCCCTTCGGCTATTGAGAGAGTATCCGAGGCAGTG  1067
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Gyps-himalayensis      GTCAGACTGACAGTAACTCTGTTCTTCCCTTCGGCTGTTGAGAGAGTATCCGAGGCAGTG   1140
                       **.**********************************.**********************

Gyps-africanus         GTTAGCATACGACGAATCAAGAACTTTCTGATACTTGATGAGATCTCACCCTTCAAGCCA   1127
Gyps-himalayensis      GTTAGCATACGACGAATCAAGAACTTCCTGATACTTGATGAGGTCTCACACTTCAAACCA   1200
                       ************************* ***************.******.******.***

Gyps-africanus         CAACTGCATGGTAATAATGAGAATGTCATTCTTCATGTTCAGGATTTGACTTGCTATTGG   1187
Gyps-himalayensis      CAACTGCATGATAATAATGAGAACGTCATTCTTCATGTTCAGGATTTGACTTGCTACTGG   1260
                       **********.************ ********************************* ***

Gyps-africanus         GATAAGAGTTTAGAAAGCCCAGCACTTCAACAACTTTCATTTACTGTCAGACGAGGGGAA   1247
Gyps-himalayensis      GATAAGAGTTTAGAAAGCCCAGCACTCCAGCAGCTTTCATTTACCGTCAGACAAGGGGAA   1320
                       ************************* **.**.*********** *******.*******

Gyps-africanus         TTATTGGCTGTGATTGGTCCTGTAGGAGCTGGCAAATCTTCACTCTTAAGTGCTGTGCTT   1307
Gyps-himalayensis      TTACTGGCTGTGATCGGTCCTGTGGGAGCTGGAAAATCTTCACTCCTAAGTGCTGTGCTT   1380
                       *** ********** ********.********.************ **************

Gyps-africanus         GGTGAGCTACCTAAAGACAAAGGTTTGATAAATGTTACTGGAAGAATTGCCTATGTTTCT   1367
Gyps-himalayensis      GGTGAGCTGCCTAAAGAGAAAGGTTTGATAAATGTTTCTGGAAGAATTGCCTATGTTTCT   1440
                       ********.******** ******************:**********************

Gyps-africanus         CAGCAGCCTTGGGTGTTTTCTGGTACAGTAAGAAGTAATATACTGTTTGACAAGGAATAT   1427
Gyps-himalayensis      CAGCAGCCTTGGGTGTTTTCTGGTACTGTAAGAAGTAACATACTGTTTGACAAGGAGTAT   1500
                       **************************:*********** *****************.***

Gyps-africanus         GAAAAAGAAAAATACGAAAAAGTTTTAAAAGTCTGTGCTCTTAAAAAGGACTTGGAATTA   1487
Gyps-himalayensis      GAGAGAGAAAAATATGAGAAAGTTTTAAAAGTCTGTGCTCTTAAAAAGGACTTGGACTTA   1560
                       **.*.********* **.**************************************.***

Gyps-africanus         TTAGCAAATGGTGACCTAACAGTAATAGGAGATCGTGGAGCTACGCTGAGTGGGGGACAG   1547
Gyps-himalayensis      CTAGCAAATGGTGACCTAACAGTTATAGGAGATCGTGGAGCTACACTGAGTGGGGGACAG   1620
                        **********************:*******************.***************

Gyps-africanus         AAAGCCCGTGTAAATCTGGCCAGAGCTGTGTATCAAGATGCAGACATCTATCTTTTGGAT   1607
Gyps-himalayensis      AAAGCCCGTGTAAATCTAGCCAGAGCTGTATATCAAGATGCAGATATCTATCTTTTGGAT   1680
                       *****************.***********.************** ***************

Gyps-africanus         GATCCACTGAGTGCAGTAGATGCTGAAGTTGGAAGACATTTGTTTGAAAAATGTATTTGT   1667
Gyps-himalayensis      GATCCACTGAGTGCAGTAGATGCTGAAGTTGGAAGACATTTGTTTGAAAAGTGCATTTGT   1740
                       ************************************************.** ******

Gyps-africanus         CAGGCCTTACATCAGAAGATCTCTGTTTTGGTTACTCACCAGTTGCAGTATCTCCGTGCT   1727
Gyps-himalayensis      CAGGCTTTACATCAGAAGATCTGTGTTTTGGTGACTCACCAGTTGCAATATCTCCGTGCT   1800
                       ***** **************** ********* **************.*************

Gyps-africanus         GCAAATCAGATTCTAATTTTAAAAGATGGTAAAATGGTGGGGAAAGGTACCTATTCAGAG   1787
Gyps-himalayensis      GCAACTCAGATTCTAATATTAAAAGATGGTAAAATGGTGGGAAAAGGGACCTATTCAGAG   1860
                       ****.************:************************.***** *************

Gyps-africanus         TTCCTGAGATCTGGCATCGACTTTGCTTCCCTTTTGAAAAAAGATGAGGAGGTAGAACAG   1847
Gyps-himalayensis      TTCCTGAGGTCAGGCATCGACTTTGCTTCGCTTCTGAAAAAAGAGGAGGAGGTAGAACAG   1920
                       ********.**:****************** *** ********** ***************

Gyps-africanus         CTGTCAGTTCCAGGAACTCCCAACCTGAAGTCTGTCCGGAGCCGAACCTTCTCAGAGTCC   1907
Gyps-himalayensis      CCATCAGTTCCAGGCACCCCCAACCTGAAGTCCTCCCGGAGCCGTACCTTTTCAGAGTCC   1980
                       * .***********.** **************   *********:***** *********

Gyps-africanus         TCTGTCTGGTCCCAGGATTCTTCTGCCCACTCACAGAAAGATGGAGCAGTGGAGCAACCA   1967
Gyps-himalayensis      TCTGTCTGGTCCCAGGATTCTTCTATCCACTCAGTGAAAGATGGAGCAGTGGAGCAGCCA   2040
                       ************************. ******* :**********************.***

Gyps-africanus         CCTGCTGAAAACGCACTGGCTGCAGTGCCAGAGGAGAGTCGCTCTGAGGGAAAAATAAAC   2027
Gyps-himalayensis      CCTACTGAAAATGCACTGGCTGCCGTGCCAGAGGAGAGTCGTTCTGAGGGAAAAATAAGC   2100
                       ***.******* ***********.****************** ****************.*

Gyps-africanus         TTTAAGGTTTACAGAAAATATTTCACTGCAGGAGCAAACTACTTTGTGATTTTCATACTT   2087
Gyps-himalayensis      TTTAAGGTTTACAGGAAATATTTCACTGCAGGAGCAAACTACTTTGTGATTTTTATACTT   2160
                       **************.****************************************** ******

Gyps-africanus         CTAGTATTCAATATTTTGGCACAGGTGGCATACGTGCTCCAGGACTGGTGGCTTTCTTAC   2147
Gyps-himalayensis      GTATTGTTTAATATTTTGGCACAGGTGGCGTATGTGCTCCAGGATTGGTGGCTTTCCTAC   2220
                        ** *.** ********************.** *********** *********** ***

Gyps-africanus         TGGGCAAATCATCAAGAAAAGTTGAACGTCACAACAAATGGAAATAATGGAGCAAATGAG   2207
Gyps-himalayensis      TGGGCAAATCATCAAGGAAAGCTGAATGTTACAACAAATGGAAATAATGGAGCAAATGAG   2280
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                  ****************.**** **** ** ******************************

Gyps-africanus    AGTGAACATCTAGACCTTAACTTTTATTTGGGAATTTATGCAGGTTTAACGGTGGCTACA  2267
Gyps-himalayensis ACTGAACATCTAGACCTTGCCTTTTATTTGGGAATTTACGCAGGTTTAACAGTGGCTACA  2340
                  * ****************..****************** ***********.*********

Gyps-africanus    ATACTGTTTGGCATAGTAAGAAGTCTTTTGGTGTTTCAAGTTCTTGTTAATTCTGGTCAG  2327
Gyps-himalayensis ATACTGTTTGGCATAATAAGAAGTCTTCTGGTCTTTCAAGTTCTTGTTAATTCTGGTCAG  2400
                  ***************.*********** **** *************************

Gyps-africanus    ACTTTGCACAACAAAATGTTTCAATCCATTTTGAAAGCTCCCGTCTTGTTTTTTGACAGA  2387
Gyps-himalayensis ACCTTGCACAACAAAATGTTTAAATCCATTTTGAAAGCTCCTGTCCTGTTTTTTGACAGA  2460
                  ** ******************.******************* *** ***************

Gyps-africanus    AATCCTATAGGAAGAATCTTAAATCGTTTCTCCAAAGATATTGGCCACCTGGATGACTTG  2447
Gyps-himalayensis AATCCTATAGGAAGAATCTTAAATCGTTTCTCCAAAGATATTGGCCATCTGGATGACCTG  2520
                  ********************************************* ********* **

Gyps-africanus    CTTCCATTGACATTTTTGGACTTCATGCAGACTCTCCTACAGATTTTTGGTGTGGTGGCT  2507
Gyps-himalayensis CTTCCATTGACGTTTTTGGATTTTGTGCAGACTCTTCTACAGATTTTTGGTGTGGTAGCT  2580
                  ***********.******** ** .********** ********************.***

Gyps-africanus    GTGGCTGTGGCAGTGATTCCTTGGATACTCCTCCCCTTAATTCCACTATTTATTCTTTTC  2567
Gyps-himalayensis GTGGCTGTGGCAGTGATTCCTTGGATACTCATTCCCTTAATTCCACTATTTATTCTTTTC  2640
                  ******************************.* ***************************

Gyps-africanus    ATTTTCCTTCGACGATATTTCTTAGACACTTCAAGAGATATTAAACGTCTAGAATCCACA  2627
Gyps-himalayensis ATTTTTCTTCGACGATATTTCTTAGATACTTCAAGAGATATTAAACGTCTAGAATCCACA  2700
                  ***** ******************** ********************************

Gyps-africanus    ACTCGAAGTCCAGTGTTCTCCCACTTGTCGTCATCCCTCCAGGGACTTTGGACTATTCGG  2687
Gyps-himalayensis ACTCGGAGTCCAGTGTTCTCCCACTTGTCATCATCCCTCCAGGGACTTTGGACTATTCGG  2760
                  *****.************************.******************************

Gyps-africanus    GCTTTGAAAGCAGAGGAAAGATTTCAAAAATTATTTGATGCACACCAAGACCTCCACTCA  2747
Gyps-himalayensis GCTTTGAAAGCAGAAGATAGATTTCAAAAACTATTTGATGCACATCAAGACCTCCACTCA  2820
                  **************.**:************ ************* ***************

Gyps-africanus    GAGGCCTGGTTTCTATTTTTGACGACCTCGAGGTGGTTTGCTGTGCGTCTGGATGCCATC  2807
Gyps-himalayensis GAGGCCTGGTTTCTATTTTTGACGACCTCAAGGTGGTTTGCTGTGCGTCTGGATGCCATC  2880
                  ******************************.******************************

Gyps-africanus    TGTGCCATTTTTGTTATAGTGGTTGCTTTTGGTTCCCTGCTTCTCTCCAAGACTTTGAAT  2867
Gyps-himalayensis TGTGCCATTTTTGTTATAGTGGTTGCTTTTGGTTCCCTGCTCCTTGCCAAGACTTTGAAT  2940
                  ************************************** **  ***************

Gyps-africanus    GCAGGGCAGGTTGGTTTGGCACTATCCTATGCAATCACCCTCATGGGAACATTCCAGTGG  2927
Gyps-himalayensis GCGGGGCAAGTTGGTTTGGCACTGTCCTATGCAATCACTCTCATGGGAACATTCCAGTGG  3000
                  **.*****.***************.*************** **********************

Gyps-africanus    GGTGTTAGACAAAGTGCTGAAGTTGAAAACCTGATGATATCAGTAGAAAGAGTAATGGAA  2987
Gyps-himalayensis GGTGTTAGACAAAGTGCTGAAGTTGAAAACCTGATGATATCAGTAGAAAGAGTAATGGAA  3060
                  ************************************************************

Gyps-africanus    TACACAGAACTTGAAAAAGAAGCTCCTTGGGAGACCAACAAGCATCCACCACCTGAATGG  3047
Gyps-himalayensis TACACAGATCTTGAAAAAGAAGCCCCCTGGGAGACTAACAAGCATCCACCACCTGAATGG  3120
                  ********:*************** ** ******** ************************

Gyps-africanus    CCAAGCCAAGGAATGATAGCATTTGAAAATGTTAACTTCACTTACAGTCTAGATGGACCT  3107
Gyps-himalayensis CCAAGCCATGGAATGATAGCATTTGAAAATGTTAACTTCACTTACAGTCTAGATGGACCT  3180
                  ********:****************************************************

Gyps-africanus    TTGGTGTTAAGACATTTGTCTGTTTTAATTAAACCAAAAGAAA-----------------  3150
Gyps-himalayensis TTGGTGTTAAGACATTTGTCTGTTGTTATTAAACCAGAAGAAAAGCAGTCATTACTGCAT  3240
                  ************************ *:*********.******

Gyps-africanus    ----------------------------------------AGGTTGGAATAGTGGGAAGA  3170
Gyps-himalayensis ACATCTGAGTCTCAGGCAGTGGGTAATTGCCCTCTGGTGTCTGTTGGAATTGTGGGAAGA  3300
                                                        . ********:*********

Gyps-africanus    ACTGGAGCTGGGAAAAGCTCTCTGATAGCAGCCCTCTTCCGCTTGGCGGAACCCGAAGGA  3230
Gyps-himalayensis ACAGGAGCTGGGAAGAGCTCTTTGATAGCAGCCCTCTTTCGGTTGGCTGAACCTGAAGGA  3360
                  **:***********.****** *************** ** ***** ***** ******

Gyps-africanus    AGGATTTGGATTGATAAGTACTTGACGTCAGAGCTAGGACTCCATGACTTGCGGAAGAAA  3290
Gyps-himalayensis AGGATCTGGATTGATAAGTACTTGACATCAGAGCTAGGCCTCCATGACTTAAGGAAGAAA  3420
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Gyps-africanus          ATTTCAATTATACCTCAGGAGCCTGTTTTATTCACTGGAACTATGAGGAAAAACTTAGAT   3350
Gyps-himalayensis       ATTTCAATTATACCTCAGGAGCCTGTATTGTTCACCGGGACGATGAGGAAAAACTTAGAT   3480
                        *************************:**.***** **.** ******************

Gyps-africanus          CCTTTCAATGAATACACTGATGAGGAGCTGTGGAATGCCTTGGAAGAGGTGCAACTGAAG   3410
Gyps-himalayensis       CCTTTCAATGAATACACTGACGAAGAACTGTGGAATGCCTTGGAAGAGGTGCAACTGAAA   3540
                        ******************* **.**.*********************************.

Gyps-africanus          GAGGTTGTGGAAGATCTACCTAATAAAATGGAGATGCAGCTGGCAGAATCTGGGTCTAAT   3470
Gyps-himalayensis       GAGGCTGTGGAAGATCTACCTAATAAAATGGAGACACAGCTGGCAGAATCTGGGTCTAAT   3600
                        **** ***************************** .************************

Gyps-africanus          TTTAGTGTTGGTCAGAGACAGCTGGTGTGTCTTGCCAGAGCAGTTCTAAAAAAAAAATCGG   3530
Gyps-himalayensis       TTTAGTGTTGGTCAGAGACAGCTGGTGTGTCTTGCCAGAGCGGTTCTAAAAAAAAATCGG   3660
                        ****************************************.******************

Gyps-africanus          ATCCTTATCATTGATGAAGCAACAGCAAATGTGGACCCAAGAACAGATGAGTTTATTCAA   3590
Gyps-himalayensis       ATCCTTATCATTGATGAAGCAACAGCAAATGTGGACCCAAGAACAGATGAGTTCATTCAA   3720
                        ************************************************** ******

Gyps-africanus          AAGACGATCCGTGAAAAGTTTGCTCACTGCACAGTGCTGACCATTGCACACCGCTTGAAC   3650
Gyps-himalayensis       AAGACAATCCGTGAAAAGTTTGCTCATTGCACAGTTCTGACCATTGCACACCGCTTGAAC   3780
                        *****.******************** ******** ************************

Gyps-africanus          ACCATTATTGACAGTGACAGGATTATGGTTTTAGATGAAGGAAGAGTGAAAGAATATGGT   3710
Gyps-himalayensis       ACCATTATCGACAGTGACAGAATTATGGTTTTAGATGCAGGAAGACTAAAAGAATATGGT   3840
                        ******** ***********.****************.******* *.*************

Gyps-africanus          GAACCTTACATTTTGCTGCAAGAACAAGATGGCTTGTTTTACAAAATGGTGCAACAAGTG   3770
Gyps-himalayensis       GAGCCTTACATTTTGCTGCAAGAAAAGGATGGCTTGTTTTACAAAATGGTGCAACAAGTG   3900
                        **.**********************.*.**********************************

Gyps-africanus          GGCAAGACTGAAGCAGCTTCTCTGATTGAAACAGCAAAACGGGTGTACTTCAGTAAGAAT   3830
Gyps-himalayensis       GGCAAGACAGAAGCAGCTTCTTTGATGGAAACAGCAAAACGGGTTTACTTCAGGAAGAAT   3960
                        ********:************ **** ***************** ******** ******

Gyps-africanus          TACCCAGAAGTTGTTCAGAATGGTCAACTTGCCACAGACTCCTCCTTGGATCCTTCCTCA   3890
Gyps-himalayensis       TACCCAGAAGTTGTTCAGAATGGTCAGCTCGCCACAGACTCCTCACTGGATCCTTCTTCA   4020
                        *************************.** **************. ********** ***

Gyps-africanus          GGATTATGCATAACCGAAACTGCACTGTGATTCCTAATAACCTTAACTGTTTTCCATTGA   3950
Gyps-himalayensis       GGATTATGCATAACTGAAACTGCACTGTGA------------------------------   4050
                        ************** ***************

Gyps-africanus          ATGTAAACCTGAGATCATCTAAACTCAGTGACAATGTTTGCAAGTGTCAGCAGGAGAGGA   4010
Gyps-himalayensis       ------------------------------------------------------------   4050


Gyps-africanus          AAGGGAGGGGGCGATTCTTTGCACTGGACATCCTTCCTATTTAATACTGAG  4061
Gyps-himalayensis       ---------------------------------------------------  4050
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Figure 7-1: Alignment of MRP4 gene between *Gyps africanus* and *Gyps humalayensis* revealing similarity of 94.06% using clusta omega software.