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COMMUNICATION

A patho-microbiological study of tissue samples of the Greater Adjutant *Leptoptilos dubius* (Aves: Ciconiiformes: Ciconiidae) that died in Deeporbeel Wildlife Sanctuary, Assam, India

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Abstract: The Greater Adjutant is an IUCN Red List 'Endangered' scavenging stork. This study reports the findings of post-mortem, histopathology, and a series of microbiological tests conducted on the Greater Adjutant that died in Deeporbeel Wildlife Sanctuary, Assam. A post-mortem examination revealed extensive nodule forming parasitic (*Balfouria monogama*) infestations in the stomach and intestine. Generalised congestion and haemorrhages in multiple organs were also revealed by the histopathological findings. Bacteriological culture detected the presence of *Escherichia coli, Enterococcus* sp., and *Clostridium perfringens* (*C. perfringens* was confirmed by *cpa* gene PCR). Virus detection tests like HA and HI test for NDV and rapid antigen detection test for Avian Influenza virus were found to be negative; however, PCR of tissue samples from two Greater Adjutants for Flavivirus was found to be positive. Greater Adjutants may carry the above bacteria as commensals in their GI tract and may possibly act as a reservoir of Flavivirus. The actual cause of deaths, however, were confirmed by the forensic report to be due to organophosphorus toxicity.

Keywords: *Balfouria monogama*, *Clostridium perfringens*, Flavivirus, northeastern India, organophosphorus toxicity, stork.

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INTRODUCTION

The Greater Adjutant *Leptoptilos dubius* is a member of the stork family Ciconiidae. The world population was estimated at less than 1,000 individuals in 2008 and led to the Greater Adjutant being upgraded as 'Endangered' on the IUCN Red List of Threatened Species (BirdLife International 2016, 2019). The bird, has now been confined only to Cambodia and in Assam and Bihar in India. Kamrup District in Assam is known to be a stronghold of the species, with almost 75% of its population in Assam found in this district (BirdLife International 2016).

Greater Adjutants being massive in their stance, have sparse natural predators and the only recorded causes of premature mortality are due to the direct or indirect actions like nest falls, malicious man-made acts like poisoning, shooting, and rarely electrocution when the birds accidentally fly into overhead electricity wires (Singha et al. 2003; Birdlife International 2016). The Greater Adjutant feeds partly on carrion, especially at refuse dumps and also hunts small live animals in typical stork fashion, by walking slowly in marshes and shallow waters, lakes, and agricultural land (Grimmett et al. 2016).

There was an unprecedented death of about 30 numbers of Greater Adjutants in the Deeporbeel Wildlife Sanctuary, Assam, mainly confined to a garbage dumping site from 22 January to 3 February, 2017. A forensic report by the Directorate of Forensic Sciences, Govt. of Assam confirmed the cause of deaths to be due to organophosphorus (OP) toxicity (Report No. DFS.1192/164/Tox-61/17). Here, a patho-microbiological study of tissue samples of the Greater Adjutant was done for the screening of a possible association of bacteria and viruses to the cause of death of the Greater Adjutant, besides OP toxicity. Screening for the possibility of the presence of zoonotic viruses, especially Flavivirus in the Greater Adjutants was also carried out in this study.

MATERIAL AND METHODS

Post-mortem examination and sample collection: A post-mortem (PM) was done on six Greater Adjutants, and samples like heart, blood, and tissue samples from all the vital organs were collected aseptically for both bacteriological and virological screening. Tissue samples were preserved in 10% formalin for the histopathological studies. Appropriate tissue samples like intestinal loop, pieces of liver, pieces of brain, and body fats were collected in saturated salt solution and sent to the Directorate of Forensic Sciences, Govt. of Assam for examination.

Histopathology: Histopathological examination of the tissue samples were carried out with routine hematoxylin and eosin (H&E) stain as per the standard procedure (Culling 1974).

Microbial screening tests: For the bacteriological screening, PM samples from all the birds were subjected to aerobic (in brain heart infusion agar and eosin methylene blue agar) and anaerobic bacterial isolation (in blood agar), at 37˚C for 24 hours and observed for cultural characteristics and gram staining was done for differentiation of gram positive and negative bacteria. For the virological screening, homogenised tissue samples were inoculated in nine days old embryonated chicken eggs for isolation of probable viral etiology. Viral haemagglutination (HA) and haemagglutination inhibition (HI) test was carried out using known serum and 4HA unit of the antigen. Procedure for HA and HI test was done according to standard protocol (OIE terrestrial manual 2015a,b). Screening for avian influenza virus was done using rapid antigen detection technique from lung, spleen, and cloacal swabs (OIE terrestrial manual 2015a).

Molecular diagnosis

Polymerase chain reaction (PCR) for *Clostridium perfringens* targeting *cpa* gene**:** PCR was done for confirmation of the anaerobic bacterial culture using specific primers *cpa* (Titball et al. 1999) targeting alpha toxin of *Clostridium perfringens.* The sequence of the primers are Forward: 5'-GCTAATGTTACTGCCGTTGA-3', and Reverse: 5'- CCTCTGATACATCGTGTAAG-3'. PCR cycling conditions were: 95˚C for 5 min for 1 cycle, 94˚C for 30 sec, 53˚C for 1.30 min, 72˚C for 1.30 min for 40 cycles and final extension of 72˚C for 7 min, with 25µl of total PCR reaction volume comprising 12.5µl of PCR mastermix, 1µl (10 pmol) forward primer, 1µl (10 pmol) of reverse primer, 2µl of DNA template and 8.5 ul of nuclease free water (NFW). Bacterial colony DNA was extracted by using heat and cold lysis method.

PCR for screening of Flavivirus: Screening for flavivirus was done by PCR using universal primer targeting flavivirus genus. The flavivirus universal primer sequences are: DJS (+) : 5' –GACATGGGGTATTGGAT-3' and DJA (-) : 5'-TCCATCCCATACCTGCA-3' (Meiyu et al. 1997) with positive band size at 413bp. The PCR conditions were run according to Meiyu et al. (1997). RNA extraction from the suspected tissue samples (Table

no. 1) were done using Qiagen RNA extraction kit. cDNA was prepared by PCR in two steps, first step by using 11µl RNA sample, 1µl random hexamer primer and incubated at 65˚C for 7 minutes, then the second step by adding RT buffer (5x) 4µl, dNTP mix (10mM) 2µl, RT enzyme (200 units/ul) 1µl, RT inhibitor (40 units/ul) 0.5µl, NFW 0.5µl and incubated in PCR for one cycle each at 25˚C for 5 min, 42˚C for 1 hr and 72˚C for 10 minutes. The cDNA obtained was finally subjected to PCR using Flavivirus universal primer set. A 25µl reaction volume was made adding 6 ul cDNA, 12.5µl master mix (Thermoscientific), 1µl each of forward and reverse primer (25 pmol), 4.5µl NFW and then subjected to PCR conditions as following: one cycle of initial denaturation at 94˚C for 5 min, 30 cycles of subsequent denaturation, annealing and extension at 93˚C for 40 sec, 55˚C for 45 sec, 72˚C for 60 sec, respectively and a final extension step at 72˚C for 10 min.

RESULTS

None of the affected birds survived, despite the supportive treatment. Post-mortem findings of most of the dead Greater Adjutants (n= 6) showed congested brain (Image 1), mild hepatomegaly (Image 2), and splenomegaly, congestion, & haemorrhage of lungs (Image 3) & intestine (Image 4). There were presence of nodule forming trematode parasites (*Balfouria monogama*) inside the nodules under mucosal and sub-mucosal layer of proventriculus, gizzard, and intestine (Image 5, 6). The stomach also contained partially digested food materials.

Microscopically in the brain, there were purkinje cell degeneration, heterophilic infiltration in the parenchyma, severe congestion, haemorrhages and perivascular oedema (Image 7). In the liver, there was degeneration and necrosis of hepatocytes with congestion, focal haemorrhages, and hemosiderosis. The vascular walls were thickened with perivascular infiltration of lymphocytes and macrophages with lymphoid nodules formation at some places, and fibrous tissue proliferation were also observed (Image 8). In the lung, there was severe congestion and haemorrhages throughout the lung parenchyma (Image 9). In the intestine, necrotic desquamated epithelial debris of intestinal epithelium were seen. The mucosal and submucosal layer showed lymphoid proliferation. Some of the follicles showed lymphoid depletion. In some areas depleted follicles were replaced by reticular fibre. In the kidney, the renal tubular epithelial cells were severely necrotic with focal haemorrhage and atrophy of glomerulus were seen. Cystic dilation of some of the tubules in the medullary part were also observed (Image 10).

Bacterial culture in specific media showed bacterial growth in both aerobic and anaerobic bacterial cultures from different organs at 37˚C for 24 hours. Results from gram staining of the isolates from different organs has been given in Table 1; however, no bacterial growth was observed from heart blood. Bacterial cultures from stomach and intestinal contents were found to be positive for *C. perfringens* in PCR targeting *cpa* gene, giving a band size of 324bp (Image 11). Cultures of *E. coli* isolated from different organs showing characteristic metallic sheen in the EMB agar, also characteristic gram-

Image 1–6. Gross Lesion: 1—Congestion in brain (→) | 2—Enlarged liver (→rounded edge) | 3—Congestion and haemorrhage in lung (→) | 4—Congestion and haemorrhage in intestine (→) | 5 & 6—Parasitic infestation in gizzard (→). © B. Dutta. P. Kakati & D. Brahma

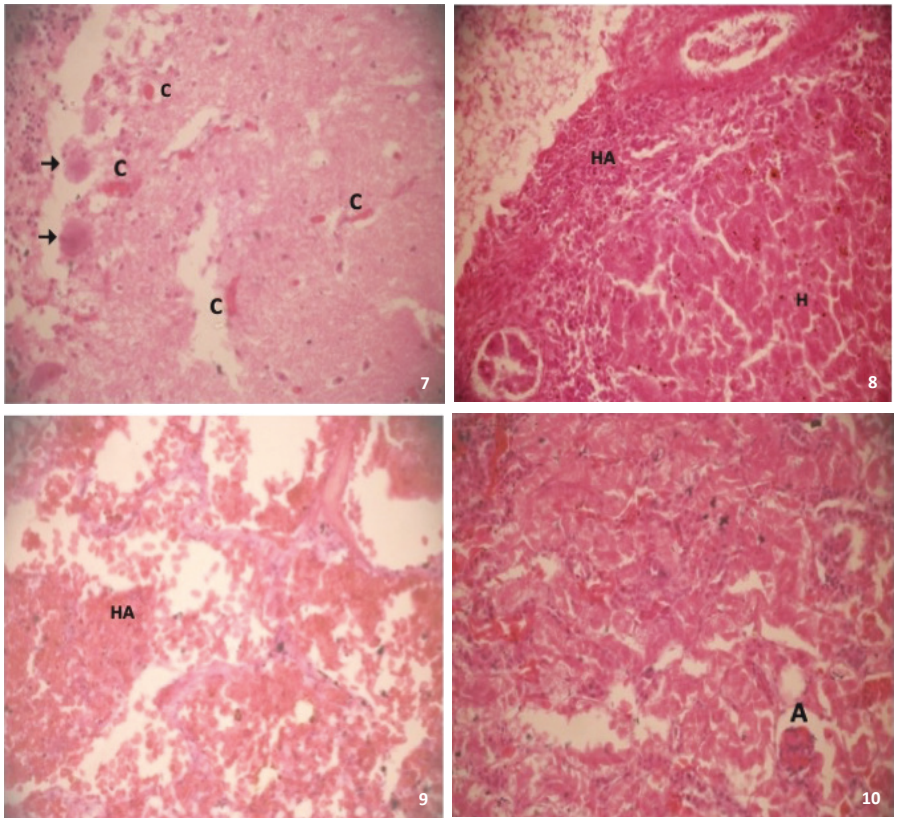

Image 7–10. Histopathological lesions (under 400x magnification): 7—Congestion (C) and degeneration of purkinje cells (→) in brain | 8—Degeneration and necrosis of hepatocytes with congestion, focal haemorrhages (HA) and hemosiderosis (H) in liver | 9—Congestion and haemorrhage (HA) in lung | 10—Atrophy of glomerulus (A), necrotic renal tubular epithelial cells with focal haemorrhage in kidney. © B. Dutta

Table 1. Results of organ-wise bacterial and viral detection tests.

Tests/Organisms		Samples (n= 6)							
		Brain	Lung	Spleen	Liver	Heart blood	Kidney	Intestine	Stomach content
Bacterial Culture	*Clostridium perfringens*	-ve	-ve	-ve	-ve	-ve	-ve	**2**	**2**
	Escherichia coli	-ve	**4**	**3**	**3**	-ve	-ve	6	6
	Enterococcus sp.	-ve	**2**	-ve	-ve	-ve	-ve	6	6
	Other unidentified bacteria	-ve	-ve	-ve	-ve	-ve	**2**	6	6
Virus detection tests	Egg inoculation	-ve	-ve	-ve	-ve	-ve	Not done	Not done	Not done
	HA/HI for NDV								
	Rapid antigen test for Avian influenza								
	PCR for Flavivirus	1	2	2	Not done	Not done	Not done	Not done	Not done

positive diplococci, i.e., *Enterococcus* spp. were detected in gram staining. Besides these, some other bacteria were also present which were unidentified.

Out of the tests for detection of virus, the samples from the two Greater Adjutants were found positive for genus *Flavivirus* by PCR using Flavivirus universal primer giving a band size at 413bp (Image 12). All the samples were found to be negative for avian influenza virus by rapid antigen detection test. Samples were also negative for New Castle Disease Virus (NDV) in Hemagglutinin (HA) and Hemagglutinin inhibition (HI) tests. Details of bacteria and virus detected from different organs are given in Table 1.

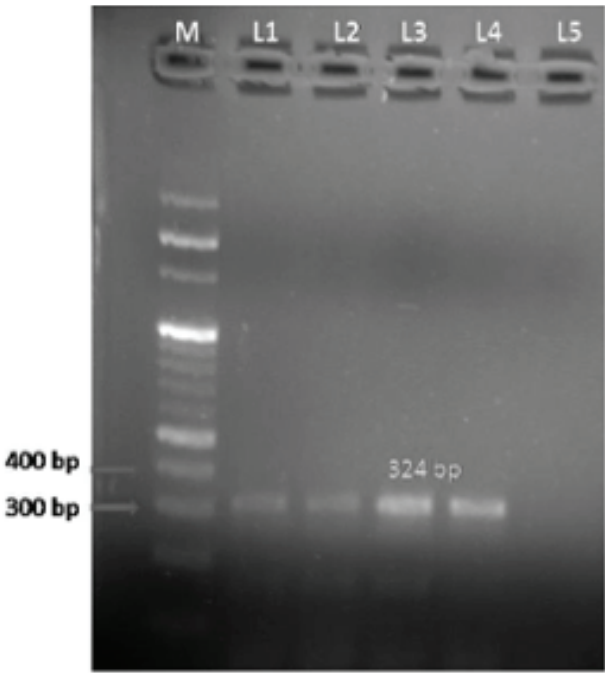

Image 11. PCR for *Cl. perfringens* (*cpa* gene) from tissue samples of Greater Adjutant Stork. M= Marker (1kb), L1–L4= bacterial culture from intestine and stomach content, L5= negative control

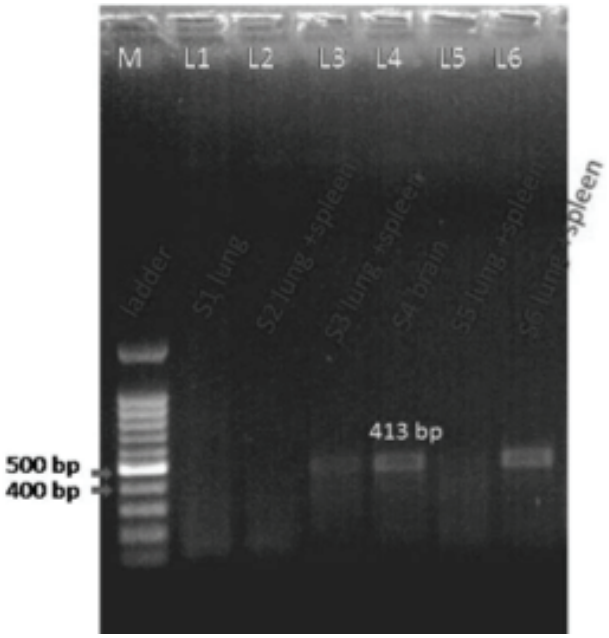

Image 12. RT-PCR for Flavivirus from tissue samples of Greater Adjutant Stork. M= marker (1kb), L1–L6= tissue samples.

DISCUSSION

Poisoning is a malicious act which causes toxicity and death of both domestic as well as wild animals. Accidental poisoning or toxicity cases may also occur due to consumption of contaminated food waste from garbage dumping sites. This study reports the case of OP toxicity in the Greater Adjutants, found dead in the Deeporbeel area, Guwahati, Assam. The histopathological findings of the multiple internal organs like the brain, lung, liver, kidney in our study, showing overall congestion and haemorrhages similar to the histopathological lesions of OP toxicity reported by other studies (Harith 2009) and in the literature (Smith et al. 1972). The storks that died of OP toxicity might have consumed some food waste from the garbage dumping site, contaminated by OP. Poisoning of small wetlands to catch fish in the dry forests of northern and eastern Cambodia potentially poses a significant threat, and in Guwahati, pesticide use at open rubbish dumps where storks flocked to feed led to several mortalities in 2005 (BirdLife International 2016).

The Greater Adjutants being natural scavengers, survive on the dead and decaying matters besides their feeding habits on amphibians and fishes in shallow water bodies and paddy fields (Grimmett et al. 2016). They have chances of exposure and infestation to intestinal parasites besides many pathogenic microorganisms. Similar to our case, Islam et al. (2009) reported *Balfouria monogama* as a highly pathogenic nodule forming parasite and caused extensive nodules on the wall of small intestine of a juvenile male Greater Adjutant, grossly visible from serosal surface, with presence of 1–2 adult parasites and necrotic masses in each nodule. Besides, some bacteria were also isolated and identified in our case study. The bacteria *C. perfringens*, a gram-positive, spore-forming, non-motile anaerobe ubiquitous in the environment, being found in the soil, in decaying organic matter and as a member of the normal gut flora of many animals that causes a variety of diseases in humans, including gas gangrene (*Clostridial myonecrosis*), enteritis necroticans (Pigbel), acute food poisoning, and antibiotic associated diarrhoea (Titball et al. 1999; O'Brien & Melville 2004). As detected in this case, *C. perfringens* may be found as commensal in these scavenging birds; however, the presence of the bacteria *C. perfringens* may have aggravated the condition of necrotic enteritis in the storks. Besides, *E. coli*, gram-negative bacteria and *Enterococcus* sp., gram-positive bacteria are also found as commensal in the GI tract of most animals and birds. These bacteria are also found in the environment as saprophytes/ coliforms, and may cause infection or food poisoning due to contamination of food and water with faecal materials (Farnleitner et al. 2010). These bacteria are

also associated with GI tract or secondary infections in immunocompromised conditions. The scavenger birds may be resistant to infections due to these bacteria and they may be found as commensals. Immune suppressed or diseased condition, however, may make the birds susceptible to infections. In fact, the bacteria like *E. Coli* and *Enterococcus* may have spread from the GI tract to other organs due to tissue damage due to the toxicity. Wild animals, especially wild birds are indirectly involved in the global transmission of antimicrobial resistant genes of the bacteria like *E.coli, K. pneumoniae* and *Enterobacter* spp. by acting as reservoirs and vectors, and are responsible for the interspecies transmission between humans, domestic animals, the environment, and wildlife (Wang et al. 2017). Thus, the bacteria like *C. perfringens, E. coli,* and *Enterococcus* sp., are capable of causing enteric infection in animals and birds (companion/domestic/wild) as well as humans indicating their zoonotic importance (Benskin et al. 2009; Kiu & Hall 2018; Ramos et al. 2019).

Many water and migratory birds are also important reservoirs of viruses like avian influenza, newcastle disease virus, and most of the important poultry viruses (Vandegrift et al. 2010; Snoeck et al. 2013; OIE 2018a,b). The flaviviruses (genus *Flavivirus*) are important pathogens of wild birds, domestic poultry and humans, and several members are zoonotically important (OIE 2018a). The viruses in the Japanese encephalitis group are related to birds and mostly transmitted by Culex mosquitoes. These viruses are distributed worldwide and cause widely diverse diseases varying from mild viral symptoms to severe and fatal hemorrhagic and neurological diseases (Meiyu et al. 1997; Davidson 2015). West Nile fever, caused by West Nile virus under the genus *Flavivirus*, is also a mosquito-borne viral disease that can affect birds, humans, and horses causing inapparent infection, mild febrile illness, meningitis, encephalitis, or death (OIE 2018b). Migratory birds could spread into densely populated urban areas (in places like urban parks) allowing introduction of a Flavivirus that could infect local Culex mosquitoes and produce disease after feeding on humans (Lopes et al. 2015). The Greater Adjutants living near the water bodies may get infected by Flavivirus from the bites of infected mosquitoes and, thus, there is a possibility of them serving as reservoirs of Flavivirus.

CONCLUSION

From this study of Greater Adjutants, we come to the conclusion that, the birds may carry bacteria like *E. coli*, *Enterococcus* sp., and *C. perfringens* and some other bacteria as commensals in their GI tract. Greater Adjutant Storks may also act as the reservoirs of Flavivirus; however, the forensic report confirmed the cause of their deaths to be due to organophosphate toxicity, which is also obviously suggestive from the post-mortem and histopathological findings. The presence of the bacteria and virus may have aggravated the condition of the Greater Adjutants during the acute phase of the toxicity.

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