Emergence of epizootic ulcerative syndrome: large-scale mortalities of cultured and wild fish species in Uttar Pradesh, India

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Epizootic ulcerative syndrome (EUS), a disease listed by World Organisation for Animal Health (OIE) has been reported in 26 countries across 4 continents. Till date, 94 fish species have been found to be naturally infected with EUS and its host range is gradually expanding. In the year 2010–11, outbreaks resulting in heavy fish mortality were recorded in wetland districts of Uttar Pradesh, India, and EUS was confirmed as the cause of mortality on the basis of histopathology, isolation of *Aphanomyces invadans*, bioassay and PCR. A prevalence of ~69% (371/540) was recorded and 13 fish species were found to be infected. Interestingly, EUS was observed in seven new species (*Aristichthys nobilis*, *Channa punctatus*, *Wallago attu*, *Mastacembelus armatus*, *Mystus cavasius*, *Anabas testudineus* and *Puntius conchonius*) for the first time in natural outbreaks. Furthermore, the disease was observed even in the month of May when the mean water temperature was 31.6 ± 0.65°C. This suggests that the disease can result in severe losses even after two decades of its emergence.

Keywords: *Aphanomyces invadans*, epizootic ulcerative syndrome, wild fish species.

EPIZOOTIC ulcerative syndrome (EUS) has been reported to be one of the most destructive diseases both for farmed and wild fishes of fresh and brackishwater origin[1]. After its original report in cultured ayu, *Plecoglossus altivilis* from Japan in 1971, EUS has been found in more than 94 species of fishes spread across 26 countries[2–8]. Recognizing its potential impact on cultured and wild fisheries, EUS has been considered as a reportable disease by the Network of Aquaculture Centres in Asia-Pacific (NACA) and World Organisation for Animal Health (OIE). Therefore, OIE member countries are obliged to report any new incursions of EUS, whether it is in a new species or in a new area[1,5].

Once an outbreak of EUS occurs in a region, it generally re-occurs with less severity over the next 2 to 3 years and with a reduced frequency thereafter[9,10]. In India, EUS was first reported in 1988 in Tripura and by 1991 it had spread to several parts resulting in large-scale fish mortalities[2,11–16]. After the initial severe outbreaks during the 1990s, mortalities due to EUS in equivalent epidemic proportions have not been reported in spite of the endemic status of the disease[17,18]. Following reports of heavy mortalities of cultured and wild fish species in wetland districts of Uttar Pradesh (UP), India in 2011, a thorough survey of the fish farms was carried out with the objectives to confirm the cause of large-scale mortalities and to document the severity and prevalence of the disease.

Materials and methods

Collection of samples

Thirty fish farms in eleven villages of wetland districts of UP (lat. 27°40′N, long. 80°00′E), were included for this study. A total of 540 fishes, of which 375 exhibited ulcerative lesions were collected for laboratory examination (Tables 1 and 2). Infected fishes were euthanized with MS222 overdose and affected tissues were fixed in 10% neutral buffered formalin for histopathology and also preserved in 95% ethanol for detection of *Aphanomyces invadans* by PCR. Some of the infected fish species were used for the isolation of *A. invadans*. The water temperature, pH and dissolved oxygen (DO) were recorded at the time of sampling using an Orion 5-Star multiparameter (Thermo-Fisher Scientific, Beverly, USA).

Histopathology

The histopathological analysis was carried out according to the method described by Chinabut and Roberts[19]. The tissue sections were stained with haematoxylin and eosin (H&E) for general pathological investigation and with Grocott's methenamine silver nitrate for the presence of *A. invadans* hyphae.

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Table 1. Details of the cultured fish species affected by epizootic ulcerative syndrome (EUS) outbreak in Uttar Pradesh (UP), India during 2010–11*

Fish species	Body length (cm)	Raipur ($n = 3$)	Bajpei ($n = 3$)	Mitauli ($n = 3$)	JB Ganj ($n = 3$)	Dhandel ($n = 3$)	Bilhayia ($n = 3$)	Saketu ($n = 3$)	Deboria ($n = 3$)	Ahiorori ($n = 2$)	Mishrikh ($n = 2$)	Gajurala ($n = 2$)	Total ($n = 30$)	Percentage
Cirrhinus mrigala	16–27	34/34	13/13	11/14	5/7	7/10	3/3	4/6	–	17/28	12/20	15/21	121/156	88.5
Labeo rohita	17–34	4/10	–	3/20	3/5	4/10		2/5	3/6	3/9	4/8	5/12	31/85	36.4
Catla catla	15–33	2/7	–	2/6	2/4	4/8	–	3/7	5/14	8/13	4/9	3/5	33/73	45.2
Aristichthys nobilis	13–21	–	–	–	–	3/10	–	–	5/11	–	–	–	8/21	38.1
Total		40/51	13/13	16/40	10/16	18/38	3/3	9/18	13/31	28/50	20/37	23/38	193/335	57.6
Percentage		78.4	100	40	62.5	47.3	100	50	41.9	56	54.0	60.5	57.6	

*Values in parentheses indicate the number of fish farms surveyed.

Table 2. Details of the wild fish species affected by EUS outbreak in UP, during 2010–11*

Fish species	Body length (cm)	Raipur ($n = 3$)	Bajpei ($n = 3$)	Mitauli ($n = 3$)	JB Ganj ($n = 3$)	Dhandel ($n = 3$)	Bilhayia ($n = 3$)	Saketu ($n = 3$)	Deboria ($n = 3$)	Ahiorori ($n = 2$)	Mishrikh ($n = 2$)	Gajurala ($n = 2$)	Total ($n = 30$)	Percentage
Channa punctatus	8–13	2/2	4/4		2/2	–	6/7	7/10	12/15	10/12	9/10	11/14	63/76	82.8
Channa striatus	30–35	–	–	3/4	3/4	–	–	3/3	–	4/5	3/4	2/3	18/23	78.2
Wallago attu	22–55	2/2	2/2	–	–	3/5	6/6	6/6	–	3/4	4/5	3/3	29/33	87.9
Puntius conchonius	7.5–10	2/2	4/4	4/4	4/4	–	6/6	–	3/3	3/3	2/2	–	28/28	100
Mastacembelus armatus	34–45	2/2	–	–	–	–	–	–	–	–	–	–	2/2	100
Colisa fasciata	5–8	4/4	3/3	–	6/7	–	7/9	–	–	2/2	3/5	–	25/30	83.3
Glossogobius giuris	11–16.5	1/1	2/2	–	1/1	–	1/1	–	–	–	–	–	5/5	100
Mystus cavasius	10–14	2/2	2/2	–	–	–	–	–	2/2	–	–	–	6/6	100
Anabas testudineus	9–13	2/2	–	–	–	–	–	–	–	–	–	–	2/2	100
Total		17/17	17/17	7/8	14/16	3/5	26/29	16/19	17/20	22/26	21/26	16/20	178/205	86.8
Percentage		100	100	87.5	87.5	60	89.6	84.2	85	84.6	80.7	80	86.8	

*Values in parentheses indicate the number of fish farms surveyed.

Detection of A. invadans in infected tissue by PCR

DNA was extracted from 25 mg of infected tissue using DNeasy tissue kit (Qiagen, Hilden, Germany). PCR reaction was carried out as described by Oidtmann *et al.*[20]. Amplified products were visualized by electrophoresis on 1.5% agarose gel incorporated with ethidium bromide and analysed by a Gel Doc System (UVP BioDoc System, UK).

Isolation of A. invadans and its confirmatory identification by PCR, sporulation and bioassay

A. invadans was isolated from the infected fish (*Cirrihinus mrigala* and *Puntius conchonius*), as described by Willoughby and Roberts[21]. After isolation, the presumptive *A. invadans* isolates (INM20101 and INP20102) were grown on GPY broth for 4 days as described by Lilley *et al.*[10]. Part of the cultures of each of the isolates was used for DNA isolation and the rest for inducing sporulation. Extraction of DNA from the cultures and PCR were performed as described by Oidtmann *et al.*[20]. The PCR products were purified by PCR purification kit (Qiagen, Hilden, Germany), sequenced and the sequence output was analysed by BLASTn algorithm in NCBI.

Sporulation was induced according to Lilley *et al.*[2]; typical achlyoid cluster formation was observed and the secondary zoospores from the isolates were used in the bioassay. Juveniles of rohu (*Labeo rohita*) ($n = 30$, 65.8 ± 3.9 g) used in the bioassay were collected from the fish farms of the National Bureau of Fish Genetic Resources, Lucknow and maintained according to the prescribed guidelines[22]. They were divided in three groups (two test and one control), each comprising ten fishes and maintained separately in fibre reinforced plastic (FRP) tanks ($1.2 \times 1 \times 0.8$ m^3) containing 500 l water. Fishes from two of the test groups were injected with 0.1 ml of zoospore suspension (10^3 zoospores ml^{-1}) with two separate isolates by intramuscular route. The control fishes were injected with 0.1 ml of autoclaved pond water (APW). Each group of fishes was maintained separately in FRP tanks and the water quality parameters (temperature, DO and pH values) were regularly monitored. The fishes were examined daily for the development of clinical signs and symptoms. The moribund fishes were collected and EUS was confirmed through histopathology as well as re-isolation of *A. invadans* from muscle tissue, as described earlier.

Results

Gross pathology and histopathology

Gross pathology of the affected fishes varied from red haemorrhagic spots and scale losses to severe ulceration and exposure of underlying tissues (Figure 1). In histological examination, mycotic granulomas were demonstrated in the fishes showing gross lesions. In many of the affected fishes, below the dermal ulcers the underlying musculature was largely replaced by mycotic granulomas (Figure 2 *a*). Sarcolysis and myophagia were also observed at sites far away from the ulcer (Figure 2 *b*). In some of the severely affected fishes, the hyphae had penetrated deep into the kidney across peritoneum from the body cavity (Figure 2 *c*). However, in fishes without noticeable haemorrhages and ulcerations, the hyphae associated inflammatory changes were observed only in the epidermis, and the dermis and skeletal musculature were free of pathological lesions (Figure 2 *d*). The response was observed in Indian major carps of large size (Figure 1 *g* and *h*).

Detection of A. invadans from EUS-infected tissue by PCR

In addition to histology, PCR was used as the second confirmatory test. PCR performed on ulcerated muscle tissues collected during outbreaks was positive for *A. invadans* DNA (540 bp PCR product specific to *A. invadans*) in 13 fish species (Figure 3). Although four of the *Cyprinus carpio* (Linnaeus) samples had distinct haemorrhagic dermal lesions suggestive of EUS, *A. invadans* could not be detected in any of these samples.

Confirmatory identification of A. invadans isolates through culture characteristics, PCR, gene sequencing and bioassay

Two oomycete isolates were obtained from the EUS outbreaks. One isolate (INM20101) was from *C. mrigala*, while another isolate (INP20102) was from *P. conchonius*. The morphology of the two isolates was similar to that described for *A. invadans*[2]. Primary zoospore clusters were achlyoid and secondary zoospores were motile, biflagellated and oval. Sequence analysis of the PCR-amplified ITS region from INM20101 (KC137250) and INP20102 (KC137251) isolates revealed 100% homology with the known *A. invadans* sequences available in GenBank (Figure 4).

In the bioassay, there was 100% mortality in rohu juveniles injected with zoospores of both the isolates. The mortality started on 18 days post-injection (dpi) with 100% mortality by 24 dpi. The fishes developed severe swollen hemorrhagic areas on injected as well as non-injected sides (Figure 5 *a*). The tissue pathology in all the moribund fishes was typical of EUS and consisted of severe myonecrosis along with mycotic granulomas in skeletal muscles and internal organs (Figure 5 *b–d*). All the challenged fishes had consistent histopathological features and *A. invadans* could be reisolated from the

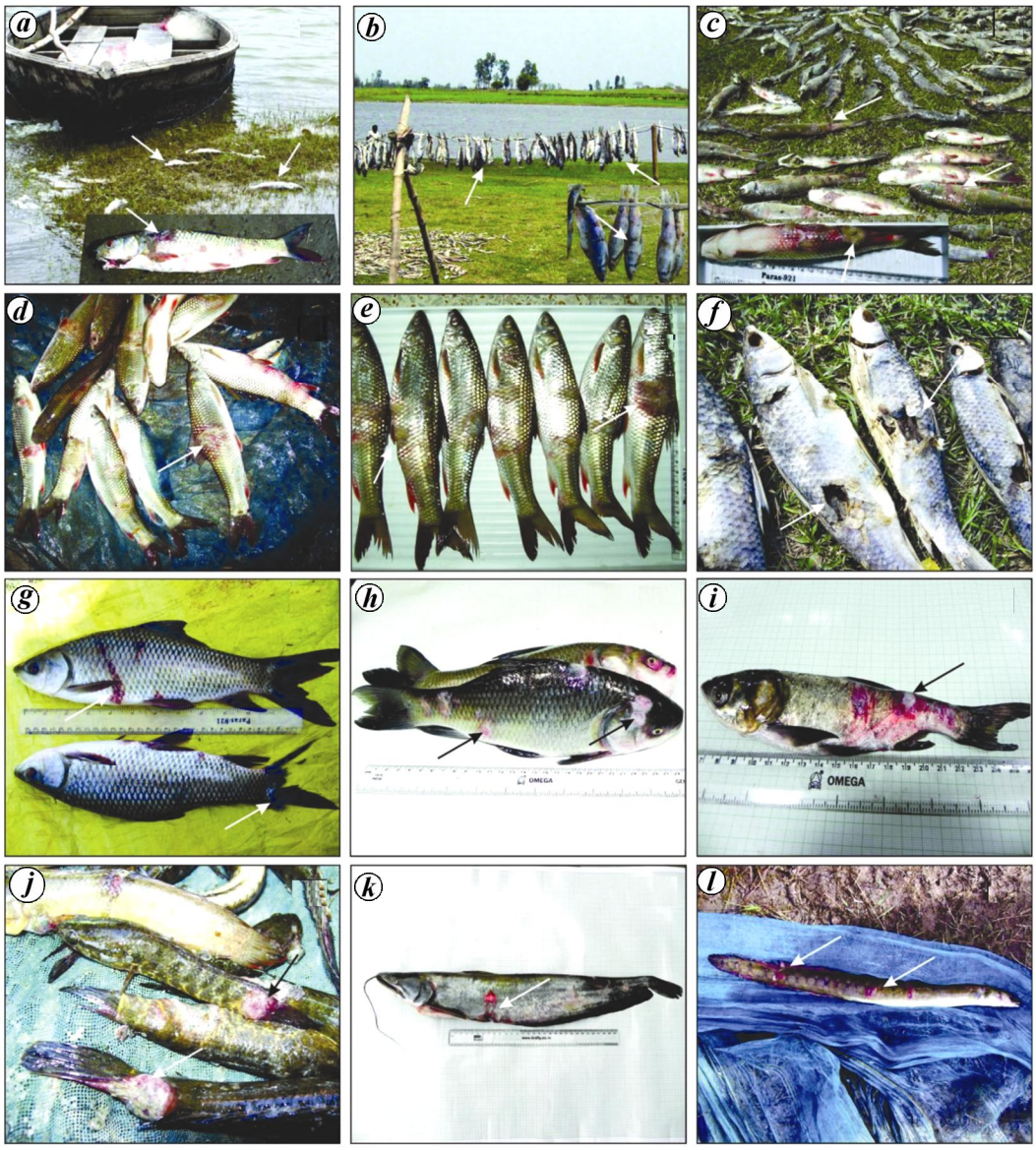

Figure 1. Epizootic ulcerative syndrome (EUS)-affected fishes. ***a***, Large-scale mortality of fishes in epidemic form. ***b***, ***c***, EUS-affected fishes being dried on the pondside. ***d–f***, Deeply ulcerated lesions in *Cirrhinus mrigala*. ***g***, Red spots and caudal fin necrosis in *Labeo rohita*. ***h***, Loss of scale and skin in *Catla catla*. ***i***, Deeply ulcerated *Aristichthys nobilis*. ***j***, Severely ulcerated *Channa punctatus*. ***k***, Exposed of peritoneal cavity in *Wallago attu*. ***l***, Ulcerated *Mastacembelus armatus*. Arrows indicate the EUS-affected fishes and/area of lesion.

moribund fishes. No lesions were observed in the control group injected with APW during the observation period. During the experimental period, temperature, DO and pH in the experimental tanks were 16.6 ± 1.2°C, 7.3 ± 0.7 mg l^{-1} and 7.1 ± 0.43 respectively.

Prevalence of epizootic ulcerative syndrome

Of the 540 fishes examined, 371 (≈69%) were confirmed to be infected with EUS. They belonged to 13 species and comprised of both cultured and wild fishes (Tables 1 and 2). Among the cultured Indian major carps, mrigal was found to be severely affected and in some of the fish farms, the prevalence was 100% in mrigal (Table 1) with large-scale mortalities (Figure 1 *a–f*). At the same time, prevalence in other two species, rohu (*L. rohita*) and catla (*Catla catla*) was 36.4% and 45.2% respectively. Though Chinese carps (*Aristichthys nobilis*) are considered to be resistant to EUS, 28.5% (6/21) of the examined samples was found to be infected with *A. invadans*. In addition, all the wild fish species in the affected farms were severely affected, with 78–100% prevalence. In four common carp (*C. carpio*) samples with distinct haemorrhagic lesions collected during the course of the study, hyphae of *A. invadans* were not observed (data not included in the tables).

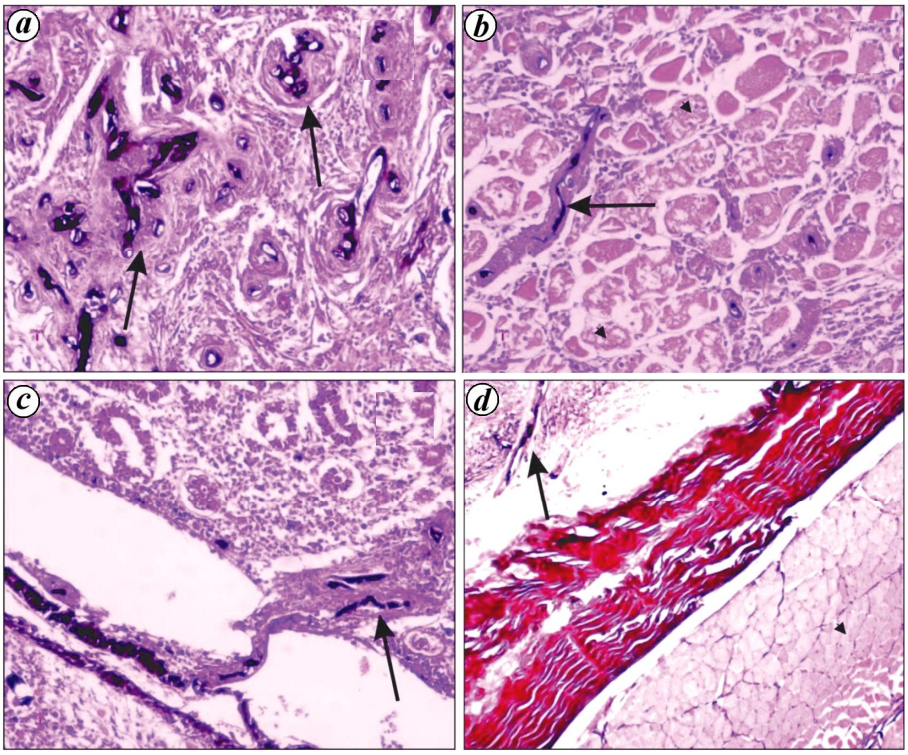

Figure 2. Histopathology of EUS-affected fishes. ***a***, Mycotic granulomas (arrows) replacing most of the host tissue in *C. catla* (400×). ***b***, Myonecrosis (arrow head) and *A. invadans* hyphae (arrow) in a section taken well away from the ulcer in *A. nobilis* (200×). ***c***, *Aphanomyces invadans* hyphae (arrow) penetrating across the peritoneum into kidney in *C. mrigala* (100×). ***d***, *A. invadans* hyphae (arrow) in the epidermis of *L. rohita*. Note the dermis and musculature are intact in large-sized *L. rohita* (100×).

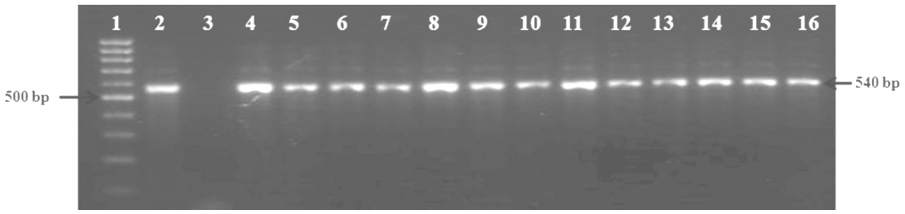

Figure 3. Detection of *A. invadans* DNA in EUS-infected tissue of different fish species by PCR. Lane 1, Marker (100 bp DNA ladder, Fermentas, USA); lane 2, Positive control (NJM9701); lane 3, Negative control; lane 4, *C. mrigala*; lane 5, *L. rohita*; lane 6, *C. catla*; lane 7, *A. nobilis*; lane 8, *C. punctatus*; lane 9, *Channa striatus*; lane 10, *W. attu*; lane 11, *Puntius conchonius*; lane 12, *M. armatus*; lane 13, *Colisa fasciata*; lane 14, *Glossogobius giuris*; lane 15, *Mystus cavasius*; lane 16, *Anabas testudineus*.

Environmental parameters associated with EUS outbreaks

Water quality parameters such as temperature, pH and DO estimated at the time of EUS outbreaks in fish farms of 11 different villages were 18.3°± 5.43°C; 8.5 ± 0.46 and 8.3 ± 1.42 mg l^{-1} respectively (Figure 6). The outbreaks were observed in 10 out of 11 villages during December 2010 and February 2011. However, one of the outbreaks started in March 2011 and lasted till the end of May 2011, when the mean water temperature, pH and DO were 31.6 ± 0.65°C, 9.1 ± 0.05 and 9.7 ± 0.4 mg l^{-1} respectively.

Discussion

The disease responsible for the present epizootics in the wetland districts of UP, was confirmed to be epizootic ulcerative syndrome using the OIE-recommended diagnostic tests (histopathology, PCR of infected tissue, isolation of *A. invadans* and its confirmatory identification by PCR, sequence analysis and bioassay). Large-scale mortality of fishes in this region due to EUS was only reported during the 1990s (refs 12, 13). Subsequently, only sporadic and low-level mortalities have been observed and the disease had been reported to be endemic[18,23]. The present findings indicate that EUS can

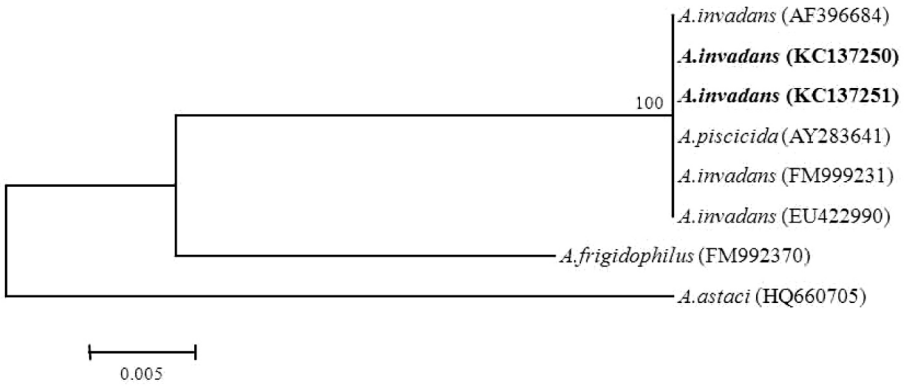

Figure 4. Neighbour-joining phylogenetic tree for two *Aphanomyces invadans* isolates, INM20101 (KC137250) and INP20102 (KC137251). Numbers at branch nodes are bootstrap percentages based on 1000 resamplings; only values greater than 50% are shown. The tree was rooted with *Aphanomyces astaci*. Scale bar represents 0.005 substitutions per nucleotide position. GenBank accession numbers are given in parentheses.

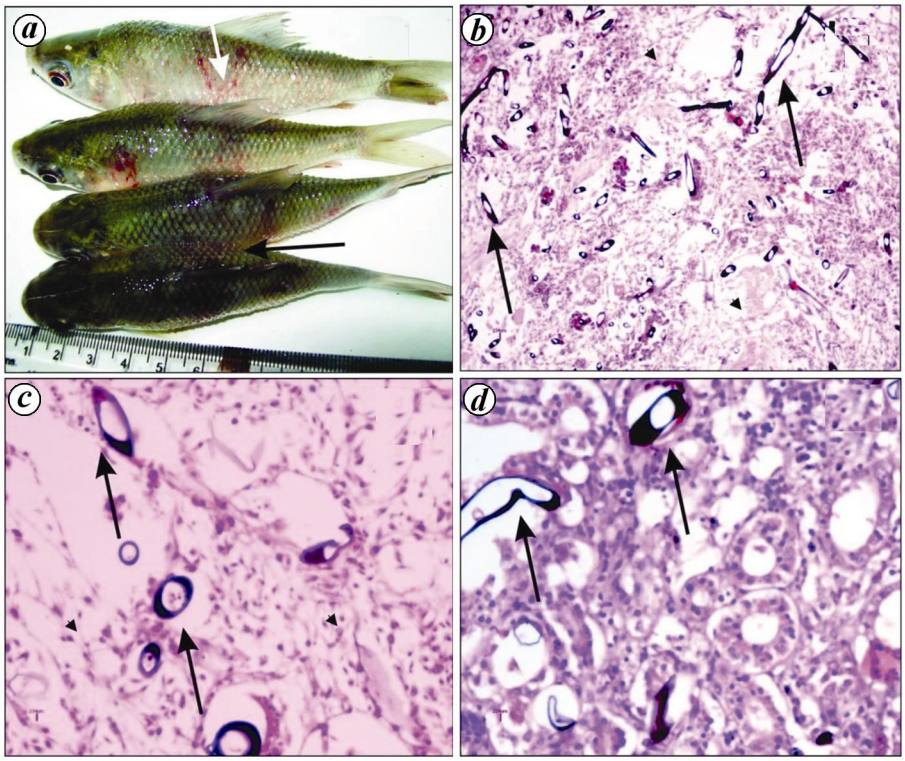

Figure 5. Gross and histopathological lesions of moribund *L. rohita* in bioassay. ***a***, Severe swollen haemorrhagic areas following experimental infection with *A. invadans* at 18 dpi. ***b***, Area of lesion showing myonecrosis (arrowheads) and *A. invadans* hyphae (arrows) (100×). ***c***, Higher magnification of the area of lesion showing severe myonecrosis (arrowheads) and hyphae (arrows) (400×). ***d***, *A. invadans* hypahe (arrows) invading the kidney tissue (400×).

once again result in large-scale mortalities both in cultured and wild fishes in spite of its endemic status. This observation is against the general perception that the severity of EUS goes down gradually in an endemic region and the disease is no longer supposed to be a problem.

Histopathological evidence documented here clearly demonstrated that *A. invadans* was highly pathogenic. Presence of hyphae away from the site of ulcers, in contra-lateral part of the ulcer underneath the intact skin as well as pathology in visceral organs, indicated the invasive ability of *A. invadans*. Although most of the fishes were severely affected, the severity of lesions was comparatively less in larger fishes. Histopathological examination of these fishes revealed that oomycete hyphae and granulomatous response were confined only to the dermis

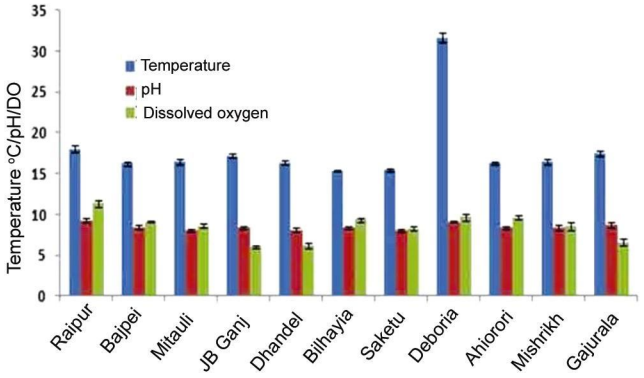

Figure 6. Water quality parameters (temperature, pH and dissolved oxygen) of EUS-affected fish farms during 2010–11 in Uttar Pradesh, India.

and epidermis with absence of extensive necrotic pathology. These results confirm the earlier reports that Indian major carps of higher age groups resist *A. invadans* infection both during natural outbreaks[11,23] and experimental infection[24].

In the present study, we were successful in culturing *A. invadans* from two infected fish hosts and both the isolates shared 100% homology with the *A. invadans* ribosomal sequences in GenBank. In the bioassay, both the isolates resulted in 100% mortality in juveniles of rohu with injection of 100 zoospores/fish. In another study, injection of 10 zoospores/fish also could result in 100% mortality[25]. These findings indicated that *A. invadans* isolated from the recent EUS outbreaks of UP is highly pathogenic. In accordance with our studies, Sosa *et al.*[26] reported that injection of five spores per fish could result in high mortality (80%) in grey mullet. Furthermore, Kiryu *et al.*[27] demonstrated that even injection of a single zoospore was capable of initiating ulcer that could lead to mortality in menhaden. Recently, Baruah *et al.*[23] have reported isolation of *A. invadans* from North East India, but histopathological lesions described by the authors do not represent typical EUS pathology.

Water temperature is an important factor in the development of EUS. It occurs when the water temperature is comparatively low during winter seasons[10,28,29]. In the recent epizootics of EUS, although the mean water temperature was 18.3° ± 5.43°C, in some of the fish farms the outbreak had occurred in March and it continued till end of May (peak summer season) when the mean temperature was 31.6° ± 0.65°C. In those farms, pH and DO were 9.13 ± 0.05 and 9.66 ± 0.4 mg l^{-1} respectively, which were above the trigger values. Therefore, it seems unlikely that any specific environmental factor is always associated with all EUS outbreaks. More epidemiological studies are required to get an insight into the role of various environmental risk factors responsible for EUS.

Till date, more than 100 fish species have been reported to be susceptible to EUS worldwide[30]. However, around 94 species of fishes have been confirmed to be naturally infected[1] and this list is expanding. In our study, we provide confirmed record of EUS in additional seven fish species, i.e. *A. nobilis*, *Channa punctatus* (Bloch), *Wallago attu* (Bloch & Schneider), *Mastacembelus armatus* (Lacepède), *Mystus cavasius* H, *Anabas testudineus* B. and *P. conchonius*. So far, the Chinese carps were considered to be unaffected by EUS[2]. However, in our study, 28.5% (6/21) of bighead fishes examined had lesions and *A. invadans* was detected both by histopathology and PCR. On the other hand, four common carps were found to have ulcerative lesions, but none of the samples was positive for *A. invadans* either in histopathology or through PCR. This is consistent with earlier reports that *C. carpio* is resistant to EUS[1,3].

In the present study, sampling was done during the outbreak situations and prevalence of the disease varied from 28.5% in bighead to 100% in wild fish species. Amongst Indian major carps, the prevalence of EUS was highest in mrigal (88.5%) followed by catla (45.2%) and rohu (36.4%). Importantly, in some of the locations, there was 100% prevalence in mrigal with large-scale mortalities (Figure 1 *a*). Such a high rate of prevalence (98.8%) in mrigal was observed only in early 1990s, during initial years of EUS outbreaks[13]. Similarly, in Bangladesh, 100% infection with high rates of mortality in certain fish species was only reported during initial years of EUS outbreaks[17,31]. There has been a decreasing occurrence of EUS in both farmed and wild fish in Bangladesh over the last 10 years[10]. Although EUS is considered to be endemic in India, outbreaks in epidemic proportion as observed in 2010–11 have not been reported in previous years, after initial outbreaks in the 1990s. Therefore, considering the severity of EUS outbreaks in 2010–11, it is assumed that there is emergence of this dreaded disease. Till date, EUS was regarded as a potential threat only to the new geographical regions. However, after the present findings, it is likely that EUS can be a threat even in the geographical regions where the disease is considered to be endemic. The present findings will have significant implications for the fisheries and aquaculture sectors of the world.

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