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Co-infection of bacterial and parasitic pathogens including the myxosporean *Zschokkella auratis* infecting brain in farmed striped murrel *Channa striata* (Bloch, 1793) causing large scale mortality

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ABSTRACT

Co-infections in fish by the wide array of pathogens have been little researched, although such infections are commonly noticed in nature. A murrel farm recorded 100% mortality in adults of *Channa striata* within 3-4 days of infection during winter. The clinical signs included cloudy eyes, excess mucus secretion, haemorrhages on the body, lethargy and anorexia before mortality. Heavy infection with *Ichthyophthirius multifiliis* on the body surface and *Zschokkella auratis*, a new myxosporean to this country, in the brain tissue were detected by wet mount microscopy and 18s rDNA sequencing, respectively. Histological sections of the brain also demonstrated the presence of myxosporean cyst. Examination of eye fluid and eye tissue revealed the presence of bacterium *Acinetobacter baumannii* and blood samples containing *Aeromonas sobria* and *Klebsiella pneumoniae* were detected species-specific PCRs for 16s rDNA followed by sequencing. Interestingly, samples collected from infected fishes also revealed multiple parasitic pathogens *viz., Epistylis* sp., *Trichodina* sp., *Argulus siamensis* and *Ergasilus* sp. Thus, it was delineated that prolonged low water temperature might be playing one of the influential roles in creating stress in fish, thus making the fish susceptible to complicated or mixed bacterial and parasitic infections.

Keywords: *Acinetobacter baumannii*, *Channa striata*, Co-infections, Mass mortality, *Zschokkella auratis*

Introduction

Co-infections by multille pathogens in fish seems to be common in nature. However, very little is known or investigated on this important aspect to understand the disease prognosis. Co-infection is defined as infection of fish by two or more genetically different pathogens where each pathogen has a definite pathogenic effect on the host (Cox, 2001; Bakaletz, 2004; Kotob *et al.,* 2016). Many a times, the researchers give importance to single infection describing the other pathogen(s) as opportunistic (Kotob *et al*., 2016). However, interactions between or among the pathogens lead to varied outcome: the load or virulence of an individual or other pathogens may increase or decrease; duration, degree of pathogenicity and prognosis of infection in the host may be greatly influenced resulting in immunomodulation and mortality as the common outcome in many instances (Telfer *et al*., 2008; Kotob *et al*., 2016). Parasitic infections mostly increase the risk of secondary bacterial diseases by increasing stress in fish and making easy access for opportunistic bacterial pathogens (Bowers *et al*., 2000; Holzer *et al*., 2006). However, most of the research focused on a single bacterium or parasite (Kotob *et al*., 2016).

In the aquatic environment, fish are in constant interaction with a wide range of pathogenic and non-pathogenic environmental microorganisms (Subramanian *et al*., 2007). Many diseases are being reported to cause mortality in snakeheads and epizootic ulcerative syndrome (EUS) is one of the most devastating diseases reported till date (John and George, 2012). Among snakeheads, the effect of EUS is more in *Channa striata* compared to other species of the genus *Channa* (Frerichs *et al*., 1989). There are reports of the presence of several secondary pathogens *viz., Aeromonas veronii* biovar *sobria* association in *Channa* during EUS (Rahman *et al*., 2002). Rauta *et al*. (2011) reported *Acinetobacter baumannii* as a virulent bacterial pathogen in *C. striata* causing mortality in farm condition. Different myxosporean parasites have been found to cause diseases in fishes *viz., Myxobolus, Thelohanellus, Henneguya, Kudoa, Myxidium, Myxosoma* and several others (Kent *et al*., 2001). Brain myxoboliasis has been found mainly in

trouts causing whirling disease (El-Matbouli *et al*., 1995). Brain myxosporean has also been described in common carp (Dykova *et al*., 1986) and a few other fish (Dayoub *et al*., 2007; Cirkovic *et al*., 2010). Among various skin parasites, *Ichthyophthirius* is one of the most pathogenic and devastating fish parasites having broad host range and infection mainly occurs at the lower temperatures (<25^{0}C) (Paperna 1972). *Ichthyophthirius multifiliis* is a ciliated protozoan, responsible for considerable losses in freshwater fish worldwide that increases bacterial invasion and fish mortality by damaging the epithelium of the gills and skin (Matthews, 2005). Shoemaker *et al*. (2012) observed co-infection of *I. multifiliis* and *Edwardsiella ictaluri* leads to higher bacterial load in different internal organs with increased mortalities in the co-infected group as compared to only parasite infected fish. In another study, *I. multifiliis* parasitised catfish showed significantly higher mortality (80%) after being exposed to *Aeromonas hydrophila* and had a higher load of *A. hydrophila* in the internal organs (Xu *et al*., 2012). Similarly, in an experimental infection with *Gyrodactylus niloticus* in Nile tilapia followed by challenge with a pathogenic bacterium *Streptococcus iniae* showed high mortality in co-infected group as compared to the group infected with only *S. iniae* (Xu *et al*., 2007). *Dactylogyrus intermedius*, a monogenean, was also reported to increase the susceptibility of gold fish (*Carassius auratus*) to the bacterium *Flavobacterium columnare,* causing columnaris disease; resulting in higher mortality and increasing the bacterial loads in fish tissues when compared to non-parasitised fish (Zhang *et al*., 2015). Similarly, in rainbow trout, fish lice (*Argulus coregoni*) increased the susceptibility of fish to *F. columnare* and the cumulative mortality was significantly higher in the co-infected group when compared to the single pathogen infected group. In channel catfish, the susceptibility to *S. iniae* or *S. agalactiae* was greatly increased after concurrent parasitism with *Trichodina* spp. with mortalities reaching 100% (Evans *et al*., 2007). A mixed infection between *Myxobolus cerebralis*, the causative agent of whirling disease and *Yersinia ruckeri*, the causative agent of enteric red mouth disease was also reported by Densmore *et al*. (2004). The examples of co-infections with two pathogens are many, however, understanding of these macro and microparasite infections seem to be important and may help to formulate better prevention strategy.

Snakeheads are carnivores, can tolerate low dissolved oxygen (because of the presence of accessory respiratory organ) and relatively very poor water quality for longer periods of time (Kumar *et al*., 2011). *C. straita* commonly known as striped murrel is a highly priced fish all over India and recently *C. striata* has become a species of interest for the aquaculturists for their good keeping quality, unique flavour, nutritive, recuperative and medicinal properties (Sahu *et al*., 2012; Kumar *et al*., 2013). Global aquaculture production of *C. striata* has increased from 480 t in 1950 to 17847 t in 2014 (FAO, 2017).

An incidence of heavy mortality was recorded in air breathing fish breeding and culture unit of ICAR-Central Institute of Freshwater Aquaculture (CAR-CIFA) Farm, Kausalyaganga, Bhubaneswar, India, during winter of 2016-17. The mortality continued during winter in different murrel tanks and the mortality reached 100% in one of the tanks having monoculture of *C. striata* (Bloch, 1793) juveniles, which was subjected to detailed investigations. The tank was stocked with 20 nos. m^{-3} of *C. striata*. The fish samples examined were found to have co-infection with varieties of bacteria and parasites with the first record on new tissue tropism (in the brain), host environment (freshwater) and first-time report of the myxosporean *Zschokkella auratis* from India.

Materials and methods

Collection of diseased fish sample

High mortality due to a disease outbreak was reported during December (2016) in striped snakehead *C. striata*, in the air breathing fish breeding and culture unit farm of the ICAR-CIFA, Bhubaneswar. Moribund specimens were sampled and brought to the laboratory for diagnosis. The affected fish were of 229.9±18.2 g in weight and 30-35 cm in size. Water quality parameters recorded in the rearing tanks were as follows: temperature, 23-25ºC; pH 7.8; alkalinity 125-130 mg l^{-1} as $CaCO_3$ and dissolved oxygen 4-5 mg l^{-1}. Overt clinical signs observed were anorexia, sluggish movement, fish remains at the corner of the tanks, white spots over body surface, haemorrhages and cloudy eyes. As the mortality continued over a few days, the diseased samples were collected over a period of three days. In total, 30 nos. of moribund fish samples were examined during this study.

Wet mount microscopy, histopathology and bacteriology

Gill and skin scrapings were collected from the anaesthetised fish smaples and examined under light microscope. Squash preparations of different internal organs were also observed for the presence of parasites. Mucus scrapings and internal organs of the infected fish were preserved in absolute ethanol (EMSURE, Merck) and 10% neutral buffered formalin (NBF) for molecular identification of parasites and histopathology, respectively. Samples (eye fluid, blood, kidney tissues and swabs from ulcers or haemorrhagic areas) from infected fish were collected aseptically for bacterial isolation and characterisation. The samples were inoculated into tryptone soy broth (TSB) (HiMedia, Mumbai, India) and incubated at 28°C for 24 h. Further, the culture was streaked onto tryptone soya agar (TSA) (HiMedia, Mumbai)

plates to isolate single colonies for further phenotypic and molecular identification. Eye samples of infected fish were also collected in 10% NBF for histopathology. The collected samples of brain and eye including other organs preserved in 10% NBF were processed for histopathological analysis, embedded in paraffin wax, sectioned and stained with routine haematoxylin and eosin (H&E) as well as with Giemsa's stain followed by eosin as the counter stain. The stained sections were then analysed under a trinocular compound microscope (Nikon YS 100, Japan) and photomicrographed.

DNA isolation from bacteria and parasites

Pure colonies of bacteria obtained from the inoculum of the blood, kidney tissue and eye fluid of *C. striata* were processed for DNA isolation following phenol-chloroform extraction method of Sambrook and Russell (2001). Simultaneously, brain and skin mucus of infected *C. striata* were also processed for DNA isolation to identify parasites. Tissue samples were treated with proteinase K in lysis buffer [50 mM Tris/HCl, 100 mM NaCl, 100 mM EDTA, 1% (w/v) SDS, pH 8.0] and subjected to extraction with phenol/chloroform/isoamyl alcohol, followed by ethanol precipitation. The DNA pellet was diluted in TE (50 mM Tris/HCl, 1 mM EDTA, pH 7.5). Concentration and purity of the extracted DNA samples were determined by measuring OD at 260 and 280 nm using a NanoDropND1000 spectrophotometer (Nano-Drop Technologies Inc., USA). The DNA samples were stored at -20ºC for further analysis.

PCR amplification of 16S rDNA for bacterial pathogens

The DNA samples extracted were subjected to 16S rDNA PCR followed by sequencing to identify the individual isolates (Table 1) (Sahoo *et al*., 2016). The consensus 16S rRNA gene sequences obtained from the sequencing of three amplicons of each pathogen were analysed using BLAST (Basic Local Alignment Search Tool) algorithm provided by NCBI (National Centre for Biotechnology Information) for all three bacterial samples obtained from eye fluid and blood.

Based on the sequencing results, PCR for different toxic and species-specific genes were carried out for *A. baumannii, Aeromonas sobria* and *Klebsiella pneumoniae*. Amplification of OXA gene was performed using primer blaOXA-23F/R and blaOXA-51F/R (Hou *et al*., 2015) for confirmed detection of *A. baumannii*. One pair of *Klebsiella pneumoniae* specific primers Pf//Pr1 (Liu *et al*., 2008) was assigned for amplification of 130-bp fragment. Similarly, the ASA1 primer set was used to amplify a 249 bp fragment of *A. sobria* haemolysin gene (Wang *et al*., 2003) for further confirmation of organism and their virulence characteristics.

Table 1. Details of primers and PCR reaction conditions used in the study

Primer	Nucleotide base sequence (5'-3')	Product (bp)	PCR conditions
16S-F 16S-R	AGA GTT TGA TCA TGG CTC AG GGT TAC CTT GTT ACG ACT T	1500	Initial denaturation at 94ºC for 5 min; 35 cycles of: 45 sec at 95ºC; 45 sec at 47ºC. 1 min at 72ºC and a final extension step of 7 min at 72ºC
BlaOXA-23F BlaOXA-23R BlaOXA-51F BlaOXA-51R	ATGAATAAATATTTTACTTG TTAAATAATATTCAGCTGTT TAATGCTTTGATCGGCCTTG TGGATTGCACTTCATCTTGG	501 353 	Initial denaturation at 94ºC for 5 min; 30 cycles of: 30 sec at 95ºC; 30 sec at 55ºC. 1.30 min at 72ºC and a final extension step of 7 min at 72ºC
Im1F Im1R Im2F Im2R	GGTTCTGGGGGAAGTATGGT GCAGGTTAAGGTCTCGTTCG GGGGATCAAAGAC GATCAGA AGGTTCCACTTCTGGTGTGC	250 200 	Initial denaturation at 94°C for 5 min; 35 cycles of: 15 sec at 94°C, 30 sec at 58°C, 30 sec at 72°C and a final extension of 7 min at72°C
ERB1 ERB10	ACCTGGTTGATCCTGCCAG CCTCCGCAGGTTCACCTACGG	1800	Initial denaturation at 95°C for 10 min; 30 cycles of: 1 min at 95°C, 1 min at 48°C, 2 min at 72°C and a final extension step of 10 min at 72°C
H2 H9 Genmyxo3 H9	TTACCTGGTCCGGACATCAA CGACTTTTACTTCCTCGAAATTGC TGATTAAGAGGAGCGGTTGG CGACTTTTACTTCCTCGAAATTGC	650 900 	Initial denaturation at 95°C for 10 min; 30 cycles of: 1 min at 95°C, 1 min at 52°C, 2 min at 72°C and a final extension step of 10 min at 72°C
K. pneumoniae Pf *K. pneumoniae* Pr1	ATTTGAAGAGGTTGCAAACGAT TTCACTCTGAAGTTTTCTTGTGTTC	130	Initial denaturation at 94°C for 15 min; 30 cycles of: 30 sec at 95ºC; 30 sec at 55 ºC. 1.30 minute at 72ºC and a final extension of 7 min at 72ºC
ASA1F ASA1R	TAAAGGGAAATAATGACGGCG GGCTGTAGGTATCGGTTTTCG	249	Initial denaturation at 94°C for 5min; 50 cycles of: 0.5 min at 95°C, 0.5 min at 59°C, 0.5 min at 72°C and a final extension of 7 min at 72°C

The PCR reaction mixture (25 µl) was prepared which included 19.50 µl nuclease free water, 2.50 µl 10x assay buffer, 0.50 µl dNTPs, 0.50 µl forward primer and 0.50 µl reverse primer, 0.25 µl *Taq* DNA polymerase (Bangalore Genei, India) and 1.25 µl genomic DNA of 25-50 ng μl^{-1}. The PCR conditions employed for different primer sets are mentioned in Table 1. The PCR amplified products were subjected to electrophoresis in 1% agarose gel containing 2.5 µl ethidium bromide with an expected product size for respective pathogens, using standard molecular weight marker of 50 bp DNA ladder (Thermo Scientific, India). The gel was then photographed using gel documentation unit (Bio Rad, Germany).

PCR for diagnosis of parasites

Based on histopathological observations, DNA from infected brain tissues were processed for identification of myxosporean parasite**.** The 18S SSU rDNA gene was first amplified with the universal eukaryotic primers ERIB1 and ERIB10 (Barta *et al*., 1997; Fiala, 2006). Nested PCR reactions were carried out using the generic myxozoan primer sets H2-H9 and Genmyxo3-H2 as described by Hanson *et al*. (2001) and Griffin *et al*. (2008), respectively. The initial 25 µl PCR reaction mixture contained 2.5 µl of 10x PCR assay buffer (GeNei, India), 0.5 µl of 2 M deoxynucleotide triphosphates, 10 pmol of each primer (ERIB1 and ERIB10), 0.5 units of *Taq* polymerase (GeNei, India), 19.75 µl of nuclease-free water and 100 ng of DNA template. The reaction mixture was cycled on a Veriti thermal cycler (Applied Biosystem, United States) and reaction conditions are mentioned in Table 1. One µl of PCR product from the initial reaction was further used in nested PCR with the following primer combinations: H2-H9 and Genmyxo3-H2. All reaction components remained the same and PCR conditions are mentioned in Table 1.

I. multifiliis specific primers Im1F/R and Im2F/R were used at 10 pmol of each primer with similar reaction conditions as described earlier. All the amplified PCR products were run on a 1% agarose gel and stained with 10 mg ml^{-1} ethidium bromide stain (MP Biomedicals, India) to confirm the presence of DNA product using standard molecular weight marker of 100 bp DNA ladder (Thermo Scientific, India). The gel was then photographed using gel documentation unit (BioRad, Germany).

Gene sequencing and phylogenetic analysis

The DNA amplicons (using primers 16s F/R, 18s ERIB1/10 and H2-H9) from different organs of infected fish samples were purified using gel purification kit (Bangalore GeNei; Bangalore) and purified PCR products were commercially sequenced (Agri Genome Labs Pvt Ltd., Kochi, India). The nucleotide sequences were analysed using BLAST of NCBI (http://www.ncbi.nlm.nih.gov/blast) to find out the homology. Available nucleic acid sequences of 16s rDNA and 18s rDNA gene sequences were retrieved from the NCBI database and aligned with the consensus nucleic acid sequences of our samples. Multiple alignment was performed with MEGA6 by using ClustalW algorithm (Tamura *et al*., 2013). Phylogenetic analysis of 16s and 18s rDNA sequences were performed for bacterial isolates and parasites, respectively, through Maximum Likelihood method available in MEGA6 and the phylogenetic tree was constructed using Maximum Likelihood method.

Results

Gross and wet-mount observations

Large scale mortality in *C. striata* was observed in the tanks that reached 100% within a short span of 3-5 days. The moribund murrels revealed white nodules along with haemorrhagic skin. Cloudy eyes were also a very consistent clinical sign observed in most of the fish. The mucosal scrapings from body and gills revealed presence of several parasites *viz., Ichthyophthirius* spp., *Ergasilus* spp., *Trichodina* spp., *Argulus* spp. and *Epistylis* spp. (Fig. 1a-c) under the microscope.

Histopathological observation of brain and eye tissues of infected C. striata

In the cross sections of the brain of *C. striata,* spores of the myxosporeans were clearly visible in H&E staining (Fig. 2a and b) as well as in Giemsa's staining followed by eosin counter staining (Fig. 2c). Further, Giemsa's

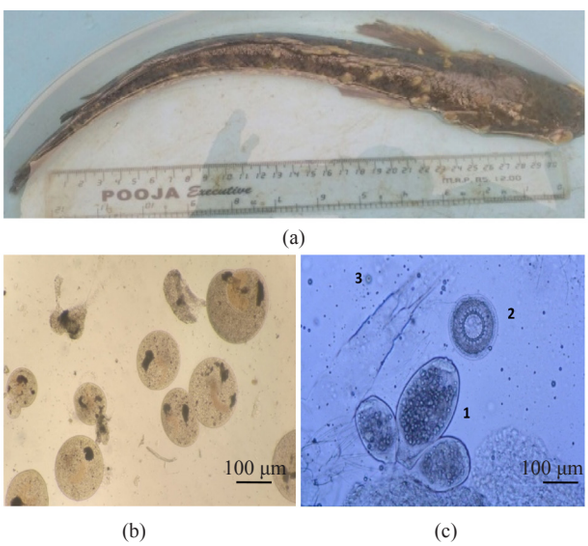

Fig. 1. (a) Heavily infected *C. striata*; (b) Wet-mount of skin mucous showing *Ichthyophthirius* spp. (10X); (c) Wet mount of gill mucous: mixed infections of (1) *Epistylis* spp., (2) *Trichodina* spp. and (3) *Ergasilus* spp. (10X)

stain followed by eosin staining of the eye sections of *C. striata* also demonstrated high bacterial load (Fig. 2d).

PCR amplification for isolated bacterial pathogens

In the present study, bacterial pathogens isolated from the diseased *C. striata* were confirmed to be *A. baumannii*, *K. pneumoniae* and *A. sobria* by 16s rDNA PCR followed by sequence analysis and using species-specific primers. *A. baumannii* was identified from the eye fluid of the diseased fish whereas *K. pneumoniae* and *A. sobria* were identified from the blood/kidney of the infected fish at later stage (5th day) of infection. The 16S rDNA gene was amplified from the genomic DNA of the isolated bacterium using universal primers amplifying 1500 bp products (Fig. 3a). After sequence confirmation, the bacteria identified were also subjected to several toxic gene PCRs *viz.,* amplification of OXA genes for *A. baumannii* using primers BlaOXA-23 F/R and BlaOXA-51F/R (Fig. 3b and c), *K. pneumoniae* specific primers Pf//Pr1 (Fig. 3d) and ASA gene using primers ASA 1F/R for *A. sobria* (Fig. 3e). The PCR products revealed desired size fragments of respective genes.

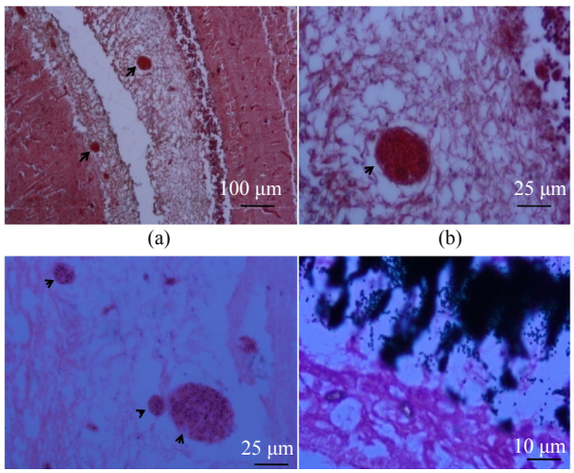

Fig. 2 . Infected *C. striata* brain with H&E stain showing cysts (a) 10X; (b) 40X; (c) Infected *C. striata* brain with Giemsa's stain followed by eosin stain showing cysts (40X): (d) Infected *C. striata* eye with Giemsa's stain followed by eosin stain showing bacterial colonies (100X)

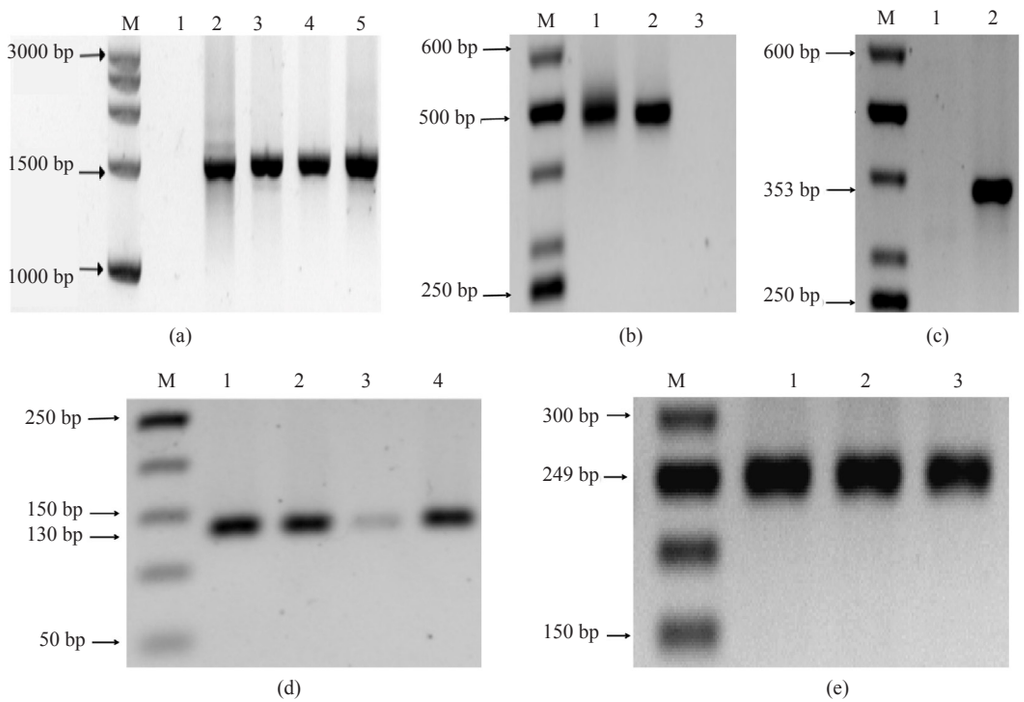

Fig. 3. Gel images of (a) PCR products (1500 bp) amplified with the primer set 16S F/R. Lane 1: Negative control; Lane 2: 16S rDNA gene of Positive control; Lane 3-5: Samples from different samplings; M: 50 bp Marker. (b) PCRproducts (501 bp) amplified with the primer OXA-23F/R. Lane 1 and 2: Samples in duplicate. Lane 3: Negative control. M: 50 bp Marker. (c) PCR products (353 bp) amplified with the primer OXA-51F/R. Lane 1: Samples; Lane 2: Negative control; M: 50 bp Marker. (d) PCR products (130 bp) amplified with the primer *K. pneumoniae* Pf /Pr1. Lane 1, 2, 3: Samples; M: 50 bp Marker. (e) Detection of *A. sobria* specific ASA gene (Primers ASA 1F/R, amplicon size 249 bp). Lane 1: +ve control; Lane 2-3: Samples collected from murrel blood; M: 50 bp Marker

PCR amplification for diagnosis of parasites

C. striata brain DNA samples were amplified by PCR using 18S rDNA universal primers and myxozoan species-specific nested primers and subsequently sequenced for further identification of suspected parasite. Universal 18S rDNA PCR produced a product size of 1800 bp (Fig. 4a). The second step PCR using two primer sets H2/H9 and Genmyxo3/H2 generated 650 bp and 900 bp amplicons, respectively (Fig. 4b). Sequencing of 650 bp amplicon identified the myxosporean as *Zschokkella auratis*. DNA extracted from skin scrapings of diseased fish used for amplification of 18S rDNA using universal primers produced expected product size of 1800 bp. The product was further sequenced, and the amplicons upon sequencing and blast search matched with *I. multifiliis.* Further, it was confirmed with species-specific PCR assay using primers Im1F/R and Im2F/R amplifying products of 250 bp and 200 bp, respectively (Fig. 4c).

Sequencing and phylogenetic analysis

The 16s rDNA amplicons of *A. baumannii*, *K. pneumoniae* and *A. sobria* obtained were sequenced and a BLAST search of the sequence revealed 100% identity with previously published 16s rDNA sequences of respective species. Amplicons of 1432 bp for *A. baumannii,* 1367 bp for *K. pneumoniae* and 1395 bp for *A. sobria* have been submitted to GenBank. A phylogenetic analysis of all three bacterial isolates obtained here *i.e., A. baumannii* (MF973079), *K. pneumoniae* (MF967214) and *A. sobria* (MF967213) revealed their close relationship with same species reported from elsewhere (Fig. 5). Simultaneously, 618 bp amplicon of 18s rDNA (GenBank accession number: MF978273) also revealed 100% identity with retrieved sequences of *Z. auratis* upon BLAST. Phylogenetic analyses of *Z. auratis* obtained here revealed its close relationship with the same species reported from elsewhere (Fig. 6).

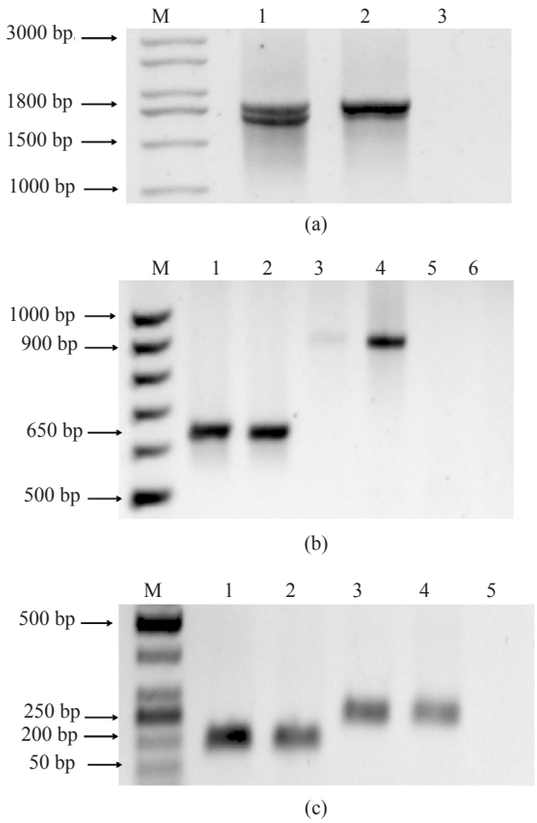

Fig. 4. Gel images of (a) PCRproducts (1800 bp) amplified with the primer ERB1/ERB10. Lane 1: Brain sample; Lane 2: Skin scrape sample; Lane 3: Negative control; M: 1kb Marker. (b) Nested PCR amplification for the 18S rDNA using Primers H2/H9 and Genmyxo3/H2 producing expected sizes of 650 bp and 900 bp, respectively. Lanes 1 and 2: 650 bp product amplified using primers H2/H9; Lanes 3 and 4: 900 bp product amplified using primers Genmyxo3/H2; Lanes 5 and 6: Negative controls for both, respectively; M: 50 bp Marker. (c) PCR products (250 bp and 200 bp) amplified with the primer Im1F/R and Im2F/R, respectively. Lane 1: Negative control; Lanes 2 and 3: Samples amplified with Im1F/R and samples 4 and 5 amplified with Im2F/R; M: 50 bp Marker

Discussion

The present study elaborates the potential threat to *C. striata* culture in terms of complexity of pathogens prevalent, causing large scale mortality. An unusual occurrence of several kinds of bacteria and parasites were noticed from a single outbreak. At the first instance, most prominent clinical signs observed were cloudy eyes and whitish nodules on the body surface. The eye fluid revealed presence of *A. baumannii* associated with the cloudiness of the eye, whereas the skin scrapings showed presence for *I. multifiliis*. Interestingly from the brain of the diseased fish, a new myxosporean parasite (*Z. auratis*) was diagnosed that seems to be the first report from India and also from the freshwater environment as well. In the later stage of the infection, other opportunistic bacterial and parasitic pathogens were also identified from the same group of diseased fishes.

Myxozoan parasites are one of the cosmopolitan parasites that can infect both vertebrates and invertebrates (Bassey, 2011) and found in different internal organs of fishes (Dykova and Lom, 1978, 1982; Dykova *et al*., 1986; Cirkovic *et al*., 2010; Abidi *et al*., 2015). Myxosporean parasite *M. cerebralis* is the most devastating parasite known to infect the brain of the rainbow trout

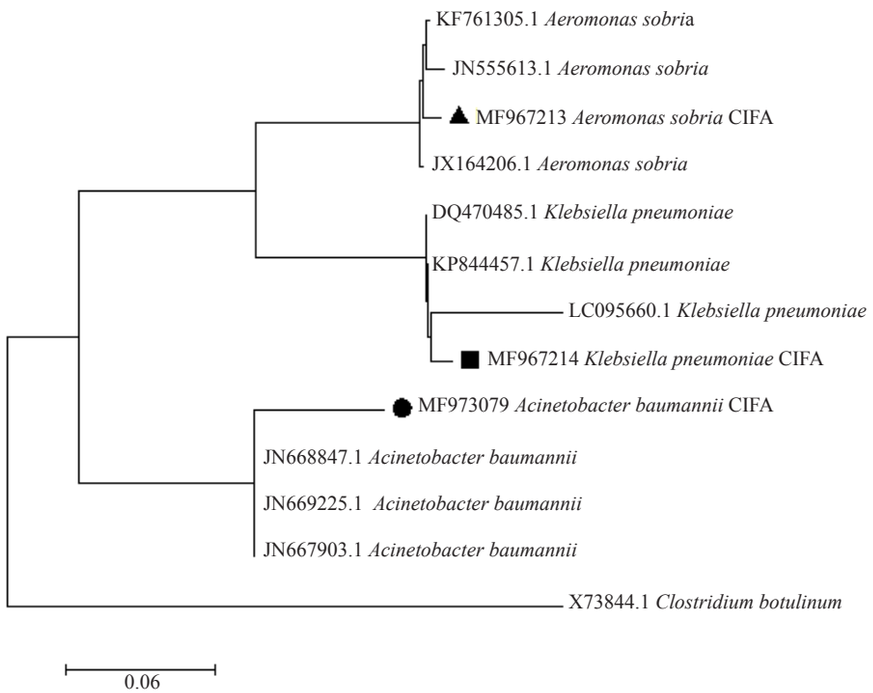

Fig. 5. Phylogenetic tree based on the nucleotide sequence of 16s rDNA amplicons of *A. baumannii* (MF973079), *K. pneumoniae* (MF967214) and *A. sobria* (MF967213) revealed their close relationship with same species reported from elsewhere

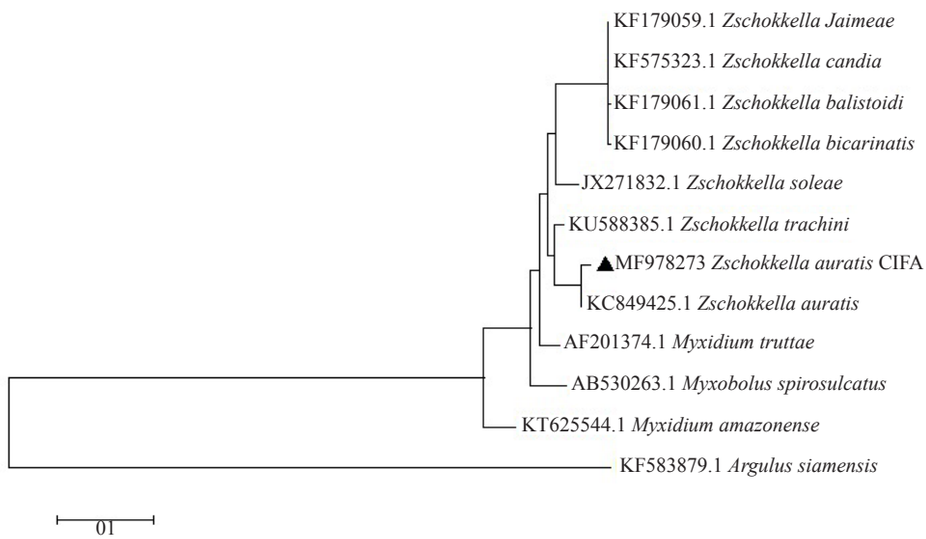

Fig. 6. Phylogenetic tree based on the nucleotide sequence of 18s rDNA amplicons of *Z. auratis* (MF978273) revealed close relationship with the same species reported from elsewhere

(Halliday, 1976; Markiw and Wolf, 1983). In the present investigation, the myxosporean, *Z. auratis* was noticed which was confirmed by histology and genus and species-specific PCRs and this froms the first report of this parasite from India. Rocha *et al*. (2013) reported *Z. auratis* infecting gall bladder of gilthead seabream (*Sparus aurata*) which is a marine fish species and hence, the presence of *Z. auratis* in *C. striata* disclosed its tolerance to both fresh and marine environments. Also it further established that the particular parasite could also infect brain tisuue of fishes. Further study is needed to confirm its exact tissue tropism and susceptible fish species.

A. baumannii was isolated from the eye fluid of the infected *C. striata* which was confirmed using 16s rDNA sequencing and blast analysis with 100% sequence similarity with the sequences of earlier reported *A. baumannii,* retrieved from NCBI. Although this is a clinically important bacteria for mainly human, there are instances of presence of this bacterium causing fish diseases (Gu *et al*., 1997; Xia *et al*., 2008). Recently, Rauta *et al*. (2011) reported *A. baumannii* causing mortality in *C. striata* from the same farm with similar clinical signs. Further, amplifications of two of the pathogenic genes of *A. baumannii*, *i.e.* BlaOXA 23 and BlaOXA51 indicated the probable pathogenicity of the bacterium and its pathogenic relevance. *A. sobria* and *K. pneumoniae* were also isolated from infected fish blood samples that were further confirmed by 16s rDNA sequencing and blast analysis as well as using ITS primer-based PCR. Blast analysis of these two bacterial pathogens in NCBI revealed 100% similarity with previously reported *A. sobria* and *K. pneumoniae* 16s rDNA sequences described from elsewhere. *K. pneumoniae* is known as a pathogenic organism to animals and humans and the bacterium is mainly associated with nosocomial infections in humans (Lery *et al*., 2014). There are some instances of involvement of this bacterium in fish diseases. Daskalov *et al*. (1998) isolated a pathogenic *K. pneumoniae* from rainbow trout causing mortality with remarkable fin and tail rot. Aoki (1999) mentioned that the susceptibility of aeromonad infection increases with overcrowding, rough handling, poor nutritional status, low dissolved oxygen and fungal or parasitic infections. The virulence of aeromonads largely depend on two major virulence factors namely hemolysin and aerolysin that play a major roles in the development of septicemic condition (Chopra *et al*., 1993; Nordmann and Poirel, 2002). In the present study, detection of *A. sobria* from the infected fish further complicated the cause of mortality and the same might have taken advantage of an opportunistic pathogen in the presence of parasite-induced skin or gill damages.

Ichthyophthiriasis is a potential threat to the aquaculture industry (Xu and Klesius, 2004). In our study, during the winter season (water temperature, 23-25^{0}C) a high intensity of infection with *I. multifiliis* was observed, which led to whitish discolouration of the body of *C. striata*. The life cycle of *I. multifiliis* largely depends on the temperature. Temperature ranging from 25-28^{0}C increases the chances of this infection and also the numbers of tomite production (Nigrelli *et al*., 1976; Noe and Dickerson, 1995). Interestingly at later stage of infection, the load of *I. multifiliis* was found to be comparatively low and there was higher infestation with other opportunistic parasitic pathogens *viz., Trichodina*, *Epistylis*, *Ergasilus* and *Argulus*. Several authors have reported aggressive innate and adaptive immune response of fish against *I. multifiliis* which may be one of the reasons for the load reduction of the parasite at the later stage (Hines and Spira, 1974; Buchmann *et al*., 2001; Dickerson and Findly. 2014). In this study, we also observed infection with *Argulus* in *C. striata,* which had not been reported earlier in this species.

Co-infections are very frequent in nature; however, very little is known about this, particularly in fish species. During any co-infection, one particular pathogen alters host's susceptibility to any further infection by modulating the immune system of the host and in many instances the pathology in host becomes synergistic (Bradley and Jackson, 2008; Telfer *et al*., 2008). Perhaps in the present case, *Ichthyophthirus* infection initially increased the susceptibility of the host to secondary bacterial infections along with other parasites, leading to mass mortality. Similar nature of high level of mortality has also been noticed in channel catfish experimentally infected with *Ichthyophthirius* followed by *E. ictaluri* (Shoemaker *et al*., 2012) or *A. hydrophila* leading to increased mortality compared to only parasite (Xu *et al*., 2012). Examples of parasitic infection followed by bacterial infection leading to increase in susceptibility and mortality in fish have also been reported in many instances (Bandilla *et al*., 2006; Evans *et al*., 2007; Xu *et al*., 2007; Zhang *et al*., 2015). Such interactions are important to be considered during designing any management or therapeutic measures. However, the present case is unique in the sense of detection of a wide array of pathogens from a single outbreak in one farm of cultured snakeheads.

Hence, as the culture practice of murrels will take up the gear, there may also be many pathogens waiting to take up the opportunity when the fishes are under stress. The recurrent occurrence of zoonotic bacteria *A. baumannii* in *C. striata* is alarming for the farming of this particular fish species. The myxosporean parasite (*Z. auratis*) is detected for the first time in brain tissue and its occurrence in freshwater fishes needs to be studied thoroughly with focus on its transmission, host range and pathogenicity. It is evident that concentrated research effort is needed to improve our understanding on the emerging issues of co-infections in fish and how the host immune system is being modulated during co-infections. This will strengthen our knowledge of the pathogenesis and pathology and prognosis of infections and will prove useful for fish disease management.

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References

Abidi, R., Fariya, N., Irshan, M. and Chauhan, U. K. 2015. A new species of myxozoan parasite, *Myxobolus lucknowii* sp. nov. in kidney of *Clarias batrachus* linn. from river Gomti at Lucknow. *Trends Parasitol. Res.*, 4(1): 5-11.

Aoki, T. 1999. Motile aeromonads (*Aeromonas hydrophila*). In: Woo, P. T. K. and Bruno, D. W. (Eds.), *Fish diseases and disorders*, *vol. 3: Viral, bacterial and fungal infections* CABI Publishing, Wallingford, UK.

Bakaletz, L. O. 2004. Developing animal models for polymicrobial diseases. *Nat. Rev. Microbiol*., 2(7): 552-558. https://doi.org/10.1038/nrmicro928.

Bandilla, M., Valtonen, E. T., Suomalainen, L. R., Aphalo, P. J. and Hakalahti, T. 2006. A link between ectoparasite infection and susceptibility to bacterial disease in rainbow trout. *Int. J. Parasitol*., 36(9): 987-99. doi: 10.1016/j.ijpara.2006.05.001.

Barta, J. R., Martin, D. S., Liberator, P. A., Dashkevicz, M., Anderson, J. W., Feighner, S. D., Elbrecht, A., Perkins-Barrow, A., Jenkins, M. C., Danforth, H. D. and Ruff, M. D. 1997. Phylogenetic relationships among eight *Eimeria* species infecting domestic fowl inferred using complete small subunit ribosomal DNA sequences. *J. Parasitol*., 83: 262-271. doi:10.2307 /3284453.

Bassey, S. E. 2011. *A concise dictionary of parasitology,* 1st edn. Zetus Concepts, Port Harcourt, 115 pp.

Bowers, J. M., Mustafa, A., Speare, D. J., Conboy, G. A., Brimacombe, M., Sims, D. E. and Burka, J. F. 2000. The physiological response of Atlantic salmon (*Salmo salar* L.) to a single experimental challenge with sea lice (*Lepeophtheirus salmonis*). *J. Fish. Dis*., 23(3): 165-172. doi: 10.1046/j.1365-2761.2000.00225.x.

Bradley, J. E. and Jackson, J. A. 2008. Measuring immune system variation to help understand host-pathogen community dynamics. *Parasitology*, 135(7): 807-823. doi:10.1017/s0031182008000322.

Buchmann, K., Sigh, J., Nielsen, C. V. and Dalgaard, M. 2001. Host responses against the fish parasitising ciliate *Ichthyophthirius multifiliis*. *Vet. Parasitol*., 100(1): 105-116. doi: 10.1016/s0304-4017(01)00487-3.

Chopra, A. K., Houston, C. W., Peterson, J. W. and Jin, G. F. 1993. Cloning, expression and sequence analysis of a cytolytic enterotoxin gene from *Aeromonas hydrophila*. *Canad, Int. J. Microbiol*., 39(5): 513-523.

Cirkovic, M., Milosevic, N., Markovic, M. and Potkonjak, A. 2010. Brain myxoboliasis of common carp. *Bulg. J. Agric. Sci*., 16: 263-265.

Cox, F. E. G. 2001. Concomitant infections, parasites and immune responses. *Parasitology*, 122(S1): S23-S3. doi:10.1017/s003118200001698x.

Daskalov, H., Stobie, M. and Anson, B. 1998. *Klebsiella pneumoniae*: A pathogen of rainbow trout (*Oncorhynchus mykiss*, Walbaum). *Bull. Eur. Ass. Fish Pathol*., 18(1): 26.

Dayoub, A., Molnar, K., Salman, H. and Al-Samman, A. and Szekely, C. 2007. *Myxobolus* infections of common carp (*Cyprinus carpio*) in Syrian fish farms. *Acta. Vet. Hung*., 55(4): 501-509. doi: 10.1556/avet.55.2007.4.9.

Densmore, C. L., Ottinger, C. A., Blazer, V. S., Iwanowicz, L. R. and Smith, D. R. 2004. Immunomodulation and disease resistance in post-yearling rainbow trout infected with *Myxobolus cerebralis*, the causative agent of whirling disease. *J. Aquat. Anim. Health*, 16(2): 73-82. doi: 10.1577/H03-039.1.

Dickerson, H. W. and Findly, R. C. 2014. Immunity to *Ichthyophthirius* infections in fish: A synopsis. *Dev. Comp. Immunol*., 43(2): 290-299. doi: 10.1016/j.dci.2013.06.004.

Dykova, I. and Lom, J. 1978. Histopathological changes in fish gills infected with myxosporidian parasites of the genus *Henneguya*. *J. Fish. Biol*., 12(3): 197-202. doi: 10.1111/j.1095-8649.1978.tb04165.x.

Dykova, I. and Lom, J. 1982. *Sphaerospora renicola* n. sp.: A myxosporean from carp kidney and its pathogenicity. *J. Parasitol. Res*., 68(3): 259-268. doi:10.1007/bf009274 04.

Dykova, I., Lom, J. and Cirkovic, M. 1986. Brain myxoboliasis of common carp (*Cyprinus carpio*) due to *Myxobolus encephalicus*. *Bull. Eur. Ass. Fish. Pathol*., 6: 10-12.

El-Matbouli, M., Hoffmann, R. W. and Mandok, C. 1995. Light and electron microscopic observations on the route of the triactinomyxon sporoplasm of *Myxobolus cerebralis* from epidermis into rainbow trout cartilage. *J. Fish. Biol*., 46(6): 919-935. doi:10.1111/j.1095-8649.1995.tb01397.x.

Evans, J. J., Klesius, P. H., Pasnik, D. J. and Shoemaker, C. A. 2007. Influence of natural *Trichodina* sp. parasitism on experimental *Streptococcus iniae* or *Streptococcus agalactiae* infection and survival of young channel catfish (*Ictalurus punctatus*) (Rafinesque). *Aquac. Res*., 38(6): 664-667. doi:10.1111/j.1365-2109.2007.01710.x.

FAO 2017. *FAO Publications Catalogue*. Food and Agriculture Organisation of the United Nations, Rome, Italy.

Fiala, I. 2006. The phylogeny of *Myxosporea* (Myxozoa) based on small subunit ribosomal RNA gene analysis. *Int. J. Parasitol. Parasites*, 36: 1521-1534. doi: 10.1016/j.ijpara.2006.06.016.

Frerichs, G. N., Millar, S. D. and Alexander, M. 1989. *Rhabdovirus infection of ulcerated fish in South-East Asia. Viruses of lower vertebrates*, Springer, Berlin, Heidelberg, p. 396-410. doi:10.1007/978-3-642-83727-2_36.

Griffin, M. J., Pote, L. M., Wise, D. J., Greenway, T. E., Mauel, M. J. and Camus, A. C. 2008. A novel *Henneguya* species from channel catfish described by morphological, histological, and molecular characterization. *J. Aquat. Anim. Health*, 20(3): 127-135. doi:10.1577/H07-001.1.

Gu, T. Z., Lu, C. P. and Chen, H. Q. 1997. *Acinetobacter baumannii*: A novel pathogen of acute epidemic in mandarin fish (*Siniperca chuatsi*). *Microbiology*, 2: 104-106.

Halliday, V. M. M. 1976. The biology of *Myxosoma cerebralis*: The causative organism of whirling disease of salmonids. *J. Fish Biol*., 9(4): 339-357. doi:10.1111/j.1095-8649.1976.tb04683.x.

Hanson, L. A., Lin, D., Pote, L. M. W. and Shivaji, R. 2001. Small subunit rRNA gene comparisons of four action sporean species to establish a polymerase chain reaction test for the causative agent of proliferative gill disease in channel catfish. *J. Aquat. Anim. Health*, 13: 117-123. doi:10.1577/1548-8667(2001)013<0117:SSRGCO>2.0.CO;2.

Hines, R. S. and Spira, D. T. 1974. Ichthyophthiriasis in the mirror carp (*Cyprinus carpio* L.) V. Acquired immunity. *J. Fish. Biol*., 6(4): 373-378. doi:10.1111/j.1095-8649.1974.tb04554.x.

Holzer, A. S., Sommerville, C. and Wootten, R. 2006. Molecular studies on the seasonal occurrence and development of five myxozoans in farmed *Salmo trutta* L. *Parasitology*, 132(2): 193-205. doi:10.1017/S0031182005008917.

Hou, C. and Yang, F. 2015. Drug-resistant gene of blaOXA-23, blaOXA-24, blaOXA-51 and blaOXA-58 in *Acinetobacter baumannii*. *Int. J. Clin. Exp. Med*., 8(8): 13859.

John, K. R. and George, M. R. 2012. Viruses associated with epizootic ulcerative syndrome: An update. *Indian J. Virol*., 23(2): 106-113. doi :10.1007/s13337-012-0108-x.

Kent, M. L., Andree, K. B., Bartholomew, J. L., EL-Matbouli, M., Desser, S. S., Devlin, R. H. and Hallett, S. L. 2001. Recent advances in our knowledge of the Myxozoa. *J. Eukaryot. Microbiol*., 48(4): 395-413.

Kotob, M. H., Menanteau-Ledouble, S., Kumar, G., Abdelzaher, M. and El-Matbouli, M. 2016. The impact of co-infections on fish: A review. *Vet. Res*., 47(1): 98. doi:10.1186/s13567-017-0432-7.

Kumar, K., Eknath, A. E., Sahu, A. K., Mohanty, U. L., Kumar, R., Sahoo, M. and Jahan, N. 2011. Snake heads: Challenging fish for diversification of fish farming. *Fishing Chimes*, 31(1): 110-113.

Kumar, K., Lalrinsanga, P. L., Sahoo, M., Mohanty, U. L., Rajesh, K. and Sahu, A. K. 2013. Length-weight relationship and condition factor of *Anabas testudineus* and *Channa* species under different culture systems. *World J. Fish. Mar. Sci.,* 5(1): 74-78.

Lery, L. M., Frangeul, L., Tomas, A., Passet, V., Almeida, A. S. and Bialek-Davenet Tournebize, R. 2014. Comparative analysis of *Klebsiella pneumoniae* genomes identifies a phospholipase D family protein as a novel virulence factor. *BMC Biol*., 12(1): 41. doi:10.1186/1741-7007-12-41.

Liu, Y., Liu, C., Zheng, W., Zhang, X., Yu, J., Gao, Q., Hou, Y. and Huang, X. 2008. PCR detection of *Klebsiella pneumoniae* in infant formula based on 16S-23S internal transcribed spacer. *Int. J. Food Microbiol*., 125(3): 230-235. doi:10.1016/j. ijfoodmicro.2008.03.005.

Markiw, M. E. and Wolf, K. 1983. *Myxosoma cerebralis* (Myxozoa: Myxosporea) etiologic agent of salmonid whirling disease requires tubificid worm (Annelida: Oligochaeta) in its life cycle. *J. Eukaryot. Microbiol*., 30(3): 561-564. doi:10.1111/j. 1550-7408.1983.tb01422.x.

Matthews, R. A. 2005. *Ichthyophthirius multifiliis* Fouquet and ichthyophthiriosis in freshwater teleosts. *Adv. Parasitol*., 59: 159-241. doi:10.1016/S0065-308X(05)59003-1.

Nigrelli, R. F., Pokorny, K. S. and Ruggieri, G. D. 1976. Notes on *Ichthyophthirius multifiliis*: A ciliate parasitic on freshwater fishes with some remarks on possible physiological races and species. *Trans. Amer Microscop. Soc*., 607-613. doi:10.2307 /3225383.

Noe, J. G. and Dickerson, H. W. 1995. Sustained growth of *Ichthyophthirius multifiliis* at low temperature in the laboratory. *J. Parasitol*., 81: 1022-1024. doi: 10.2307/3225383.

Nordmann, P. and Poirel, L. 2002. Emerging carbapenemases in Gram□negative aerobes. *Clin. Microbiol. Infect*., 8(6): 321-331. doi:10.1046/j.1469-0691.2002.00401.x.

Paperna, I. 1972. Infection by *Ichthyophthirius multifiliis* of fish in Uganda. *Prog. Fish Cult*., 34(3): 162-164. doi:10.1577/1548-8640(1972)34[162:ibimof]2.0.co;2.

Rahman, M., Colque-Navarro, P., Kuhn, I., Huys, G., Swings, J. and Mollby, R. 2002. Identification and characterisation of pathogenic *Aeromonas veronii* biovar *sobria* associated with epizootic ulcerative syndrome in fish in Bangladesh. *Appl. Environ. Microbiol*., 68(2): 650-655. doi:10.1128/aem.68.2.650-655.2002.

Rauta, P. R., Kumar, K. and Sahoo, P. K. 2011. Emerging new multi-drug resistant bacterial pathogen, *Acinetobacter baumannii* associated with snakehead (*Channa striatus*) eye infection. *Curr. Sci*., 548-553.

Rocha, S., Casal, G., Rangel, L., Severino, R., Castro, R., Azevedo, C. and Santos, M. J. 2013. Ultrastructural and phylogenetic description of *Zschokkella auratis* sp. nov. (Myxozoa): A parasite of the gilthead seabream *Sparus aurata*. *Dis. Aquat. Org*., 107(1): 19-30. doi:10.3354/dao02661.

Sambrook, J. and Russell, R. W. 2001. Molecular cloning: A laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA.

Sahoo, P. K., Swaminathan, T. R., Abraham, T. J., Kumar, R., Pattanayak, S., Mohapatra, A., Rath, S. S., Patra, A., Adikesavalu, H., Sood, N. and Pradhan, P. K. 2016. Detection of goldfish haematopoietic necrosis herpes virus (Cyprinid herpesvirus-2) with multi-drug resistant *Aeromonas hydrophila* infection in goldfish: First evidence of any viral disease outbreak in ornamental freshwater aquaculture farms in India. *Acta. Trop*., 161: 8-17. doi:10.1016/j.actatropica.2016.05.004.

Sahu, B. B., Kumar, K., Sahoo, A. K., Kumar, R., Mohanty, U. L., Sahoo, N. J. M. and Eknath, A. E. 2012. Carcass characteristics of marketable size striped murrel (*Channa striatus* Bloch,1793). *J. Appl. Ichthyol*., 28(2): 258-260. doi:10.1111/j.1439-0426.2011.01917.x.

Shoemaker, C. A., Martins, M. L., Xu, D. H. and Klesius, P. H. 2012. Effect of *Ichthyophthirius multifiliis* parasitism on the survival, hematology and bacterial load in channel catfish previously exposed to *Edwardsiella ictaluri*. *J. Parasitol. Res.,* 111(5): 2223-2228. doi:10.1007/s00436-012-2988-5.

Subramanian, S., MacKinnon, S. L. and Ross, N. W. 2007. A comparative study on innate immune parameters in the epidermal mucus of various fish species. *Com. Bioch. Physiol., B: Biochem. Mol. Biol.,* 148(3): 256-263. doi:10.1016/j.cbpb.2007.06.003.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis ver. 6.0. *Mol. Biol. Evol*., 30(12): 2725-2729. doi:10.1093/molbev/mst197.

Telfer, S., Birtles, R., Bennett, M., Lambin, X., Paterson, S. and Begon, M. 2008. Parasite interactions in natural populations: insights from longitudinal data. *Parasitology*, 135(7): 767-781. doi:10.1017/s0031182008000395.

Wang, G., Clark, C. G., Liu, C., Pucknell, C., Munro, C. K., Kruk, T. M., Caldeira, R., Woodward, D. L. and Rodgers, F. G. 2003. Detection and characterisation of the hemolysin genes in *Aeromonas hydrophila* and *Aeromonas sobria* by multiplex PCR. *J. Clin. Microbiol*., 41(3): 1048-1054. doi:10.1128/JCM.41.3.1048-1054.2003.

Xia, L., Xiong, D., Gu, Z., Xu, Z., Chen, C., Xie, J. and Xu, P. 2008. Recovery of *Acinetobacter baumannii* from diseased channel catfish (*Ictalurus punctatus*) in China. *Aquaculture*, 284: 285-288. doi:10.1016/j.aquaculture.2008.07.038.

Xu, D. H. and Klesius, P. H. 2004. Two year study on the infectivity of *Ichthyophthirius multifiliis* in channel catfish (*Ictalurus punctatus*). *Dis. Aquat. Org*., 59(2): 131-134. doi:org/10.3354/dao059131.

Xu, D. H., Shoemaker, C. A. and Klesius, P. H. 2007. Evaluation of the link between gyrodactylosis and streptococcosis of Nile tilapia (*Oreochromis niloticus* L.). *J. Fish Dis*., 30(4): 233-238. doi:10.1111/j.1365-2761.2007.00806.x.

Xu, D. H., Shoemaker, C. A., Martins, M. L., Pridgeon, J. W. and Klesius, P. H. 2012. Enhanced susceptibility of channel catfish to the bacterium *Edwardsiella ictaluri* after parasitism by *Ichthyophthirius multifiliis*. *Vet. Microbiol*., 158(1): 216-219. doi:10.1016/j.vetmic.2012.02.007.

Xu, D. H., Pridgeon, J. W., Klesius, P. H. and Shoemaker, C. A. 2012. Parasitism by protozoan *Ichthyophthirius multifiliis* enhanced invasion of *Aeromonas hydrophila* in tissues of channel catfish. *Vet. Parasitol*., 184(2): 101-107. doi:10.1016/j.vetpar.2011.09.020.

Zhang, C., Li, D. L., Chi, C., Ling, F. and Wang, G. X. 2015. *Dactylogyrus intermedius* parasitism enhances *Flavobacterium columnare* invasion and alters immune-related gene expression in *Carassius auratus*. *Dis. Aquat. Org*., 116(1): 11-21. doi:10.3354/dao02902.

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