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Original Research Article



Microbiological screening and antibiotics responsiveness of pathogenic bacteria isolated from pond cultivated pangas fishes (*Pangasius hypophthalmus*) in Bangladesh

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Abstract

Pangas fish (*Pangasius hypophthalmus*) is one of the most cultivated fishes in Bangladesh and exposed to various infectious diseases including bacterial diseases. An attempt was done to determine the most infectious bacterial pathogens and antibiotics susceptibilities of isolated bacterial pathogens. There are 52 infected pangas fishes that were collected from different ponds in the Mymensingh district for one year. The physicochemical parameters of water samples of fishes were noted, and it was found that the parameters did not show any significant impact on the disease development of pangas. *Aeromonas spp* (27%), *Flavobacterium spp* (25%), *Edw ar dsiella spp* (23%), *Vibrio spp* (2%), *Enterococcus spp* (2%), *Pseudomonas sp* (2%) were found from different body parts of pangas fish and rest of the isolates were normal flora. The antibiotic susceptibility test was performed by following Kirby- Bauer disc diffusion method, and it was found that the antibiotic susceptibility patterns of the bacterial pathogen differed slightly. *Edw ar dsiella spp* (67%) and *Vibrio spp* showed the most sensitivity to ciprofloxacin while other pathogens such as *Aeromonas spp* (57%), *Flavobacterium spp* (69%), and *Enterococcus spp* exerted the highest sensitivity to Levofloxacin. Additionally, 100% resistant cases were observed to amoxicillin by most of the isolates except *Flavobacter spp* (85%). Conclusively, levofloxacin was found as the most sensitive antibiotic (41%) for pathogens, but the isolates showed the most resistance against amoxicillin (41%) among all antibiotics. Multiple drugs resistant to *Pseudomonas spp* were also found, which paves the way to widen the use of the range of antibiotics and /or synergistic application of the most potential antibiotics.

Keywords Pangas fish (*Pangasius hypophthalmus*), bacteriological agents, antibiotics responsiveness

Introduction

Economy of Bangladesh is largely dependent upon agriculture and industry. Environmental stresses and diseases of crops and fishes cause a high cost which limits productivity and thus reduces the maximum economic outcome. Bangladesh is enriched with rivers, ponds, canals, swamps which comprise a large aquatic environment. Weather and

climatic factors are favorable for fish culture in Bangladesh, which makes it one of the leading fish-producing countries in the world. People in rural and urban areas fill their demand for animal protein from fish due to availability, high productivity, and lower cost than meat. Though different types of freshwater and marine fishes are available in Bangladesh.

Carp, tilapia, catfishes, climbing perches(koi), shrimps and Pangas catfishes are mostly cultured due to the ease of cultivation and their huge economic domestic and international demands (Abedin et al., 2020).

Pangasius (*Pangasianodon hypophthalmus*) is one of the most commonly cultured fishes due to its fast growth, high productivity, year-round production, resistance to diseases (Faruk et al., 2017). In 1989, *Pangasius spp* were imported from Thailand for aquaculture purposes due to its capability of contributing to increased production of fish and thereby meeting the demands of food-fish (Banglapedia, 2015). Different types of diseases, e.g., nutritional and environmental diseases, viral diseases, bacterial diseases, protozoan diseases make constraints to profitable commercial production and natural productivity of *Pangasius* (Chowdhury 1998, Faruk et al., 2004). But significant numbers of mortality due to infectious problems has been observed caused by bacterial pathogens. Diseases causing bacterial pathogens include *Edw ar sdsiella ictaluri*, *Aeromonas hydrophila*, *A. sobria* and *A. caviae*. Among them, *Aeromonas spp* has been found as major bacterial pathogens in the aquatic environment (Sarker and Faruk, 2016). *Aeromonas hydrophila* causes diseases in various cultured fishes for which the effectiveness of the antibiotics against such pathogens has been an important topic in fish research for prevention and proper treatment. However, protozoan parasites attack *Pangasius spp* includes *Microsporidium*, *Myxobolus*, *Henneguya*, *Trypanosoma*, *Ichthyonyctus*, *Trichodina*, *Balantidium* and *Epistylis*, as well as *Ichthyophthirius multifiliis*.

Farmed fishes have multi-factorial etiology for disease development (Hedrick 1998, Thorburn, 1996) and several factors such as pathogen virulence and environmental factors are also involved in this (Kumer, 2006). Moreover, resistance to antibiotics of a pathogen has now been a potential threat for the treatment of diseases. This study focused on the relationship between disease development of *Pangasius spp* and the physico-chemical factor of the aquatic environment in different seasons and different regions. Additionally, an attempt was done for the establishment of an antibiotic susceptibility profile for different pathogens.

Materials and methods

Collection of fish sample for bioassay

A total of 52 infected Pangas catfish (*Pangasius pangasius*) was collected from different regions of Bangladesh (Tarkanda, Trishal, Ishwargonj, Mymensingh sadar, Mukhtagacha, Kishorganj, Fulbaria, Bogar Bazar) between January 2020 to January 2021 (Fig.1). Sample collection was done by using an aseptic technique and low temperature was maintained by using ice-box before transporting to Quality Aqua Laboratory, Quality feeds Limited, Mymensingh. After collection and transportation, fresh samples were subjected to analysis for bacteriological culture and antibiotic responsiveness profiling.

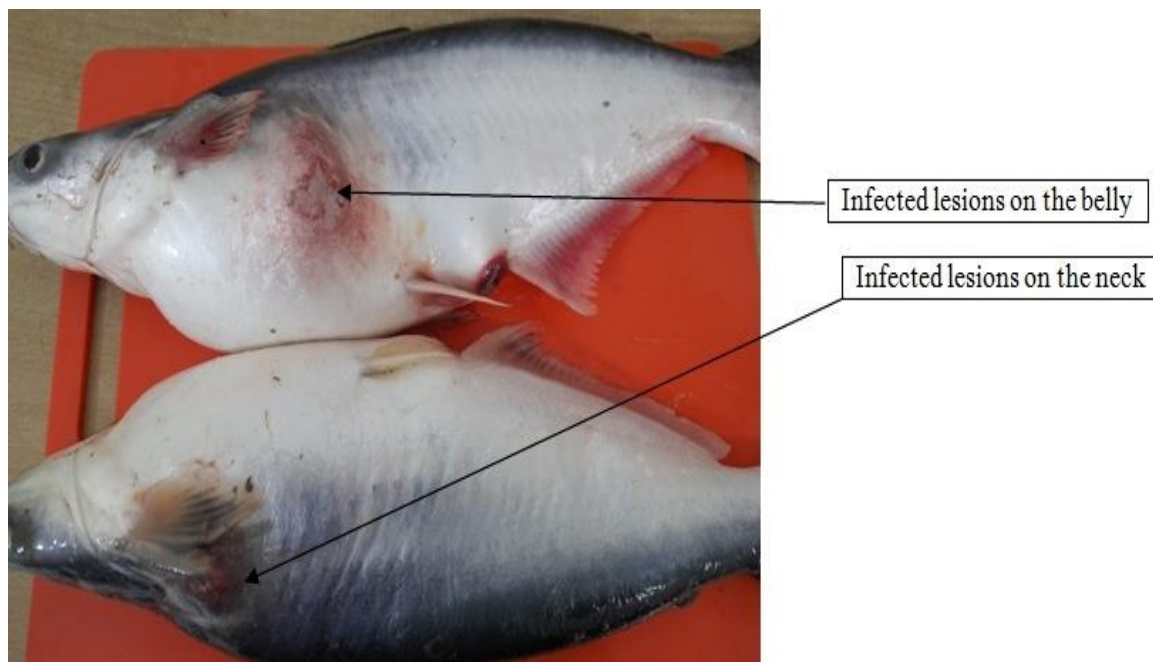


Fig 1. Infected Pangas (*Pangasius pangasius*) fish sample

Preparation of sample for analytical purpose and enrichment of bacteria

During sampling, the aseptic technique was followed to prevent cross contamination. Portion of specimens was taken from the intestine, skin, and gill of infected fishes for microbiological assay. After taking the specimens on a sterile chopping board, these were minced and grinded. For enrichment of bacteria, 10 gm of samples were homogenized with freshly prepared 90 ml of 0.1% peptone water and then 0.1 ml of homogenized samples were inoculated onto both selective and non-selective media according to standard plate count such as Rimler-Shotts Medium Base agar (for *Aeromonas spp.*), *Pseudomonas* Base agar (for *Pseudomonas spp.*), Thiosulfate citrate bile salts sucrose (TCBS) agar (for *Vibrio spp.*), Tryptic Soy Agar (for enrichment of bacterial isolates), blood agar, and MacConkey agar plates. The culture plates containing homogenized samples were then incubated at 37^o C for 24-48 hours.

Identification of bacterial pathogen

Bacterial pathogens were identified after an analysis of their morphological and biochemical characteristics and, Gram reaction. Colonies were found on selective and conventional agar media and then streaked on TSI agar slants, MIU medium, Simmons citrate agar slant, and then incubated overnight at 37^oC. Morphological characteristics of the subject isolates were analyzed by observing colony characteristics, size, and shape and so on.

Biochemical characteristics of the isolated pathogens were determined by various tests such as alkaline and acidic reaction, H₂S (hydrogen sulfide) production, gas production, MIU (motility, indole, urease), oxidase, catalase, Methyl Red (MR) test, and Voges-Proskauer (VP) test. Gram staining of the isolated pathogens was done to identify the organisms as either Gram-positive or Gram-negative bacteria. Suspension of a loopful bacterial culture was taken on a clean, grease-free slide and air dried to prepare a fix-smear. Then crystal violet, Iodine, alcohol, and safranin were added gradually for staining and counter-staining purposes. Before the addition of a reagent, the slide was washed and dried. After final washing and drying, the slide was taken under a light microscope (Nikon Co., Tokyo, Japan) and observed by 10X100 lenses with immersion oil. Morphological, characteristics, biochemical characteristics and Gram staining result of the isolates were used for identification by following Bergey's manual of Bacteriological classification (Chauhan and Jindal, 2020).

Antimicrobial responsiveness profiling

Antibiogram patterns of the isolated pathogen were prepared by following Kirby- Bauer disc diffusion method (Cockerill and CLSI, 2013). Amoxicillin (10µg), ciprofloxacin (5µg), colistin (25µg), chlortetracyclin (30µg), doxycycline (30µg), erythromycin (15µg), cotrimoxazole (25µg), and enrofloxacin (5µg) antibiotics were used in this study for the preparation of antibiotic susceptibility patterns.

Bacterial colonies were taken to sterile PBS (Phosphate buffer saline) water and then adjusted to 0.5 McFarland's turbidity standard. Then the bacterial suspensions were spread onto Mueller–Hinton agar and incubated at 37°C for 24 hours following the placement of impregnated antibiotics (Himedia, India). To determine antimicrobial responsiveness of the subject pathogens, the antibiotic inhibition zone was observed around discs and measured in diameter of millimeter(mm). During measurement, the antibiotic inhibition zone was scaled from the focal point of the antimicrobial plate as far as possible of the reasonable zone where microscopic organisms could be observed developing. Based on the diameter of the zone of inhibition, the antibiogram was interpreted as resistant, sensitive and intermediate according to the guideline of the producer.

Temperature and pH of the fishpond were measured by a thermometer and a pH meter respectively. Ammonia, nitrate, and nitrite concentration were determined by following the method of Ogbonna and Chinomso, 2010. Alkalinity and hardness were measured according to the method of Eaton et.al, 2005.

Results

Agro-aquatic environment (temperatures, pH, ammonia, nitrate, nitrite, alkaline, and hardness) showed no stressful condition which favored the infection of pathogens. In pond water, the range of temperature was 26-29^oC whereas pH was found neutral to alkaline. Moreover, the average ammonia, nitrite, nitrate, alkaline and hardness were 0.98, 0.11, 6.88, 121.21 and 91.69 respectively.

Pathogenic bacterial isolates were isolated from infected fish and identified as a result of biochemical tests by following the description of Bergey's manual of Determinative Bacteriology. However, after the identification of isolated from 52 pangas samples, pathogens including *Edw ar dsiella spp.*, *Aeromonas spp.*, *Flavobacterium spp.*, *Vibrio spp.*, *Enterococcus spp.* and *Pseudomonas spp.* and normal flora were found. The most predominant group of bacteria was *Aeromonas spp.* (27%) followed by *Flavobacterium spp.* (25%) and *Edw ar dsiella spp.* (23%). In some cases, *Vibrio spp.*, *Enterococcus spp.* and *Pseudomonas spp.* were found as pathogenic isolates, and the rest of the cases the subject isolates were normal flora (Table-1 & Fig-2). The antibiogram profile of these isolate were prepared to screen sensitive, moderate and resistant antibiotics.

Edw ar dsiella sp showed the most sensitivity against ciprofloxacin and resistance against amoxicillin (Table-2). The most moderate response towards doxycycline was observed. No sensitivity was observed against erythromycin, amoxicillin and chlortetracycline. However, no resistance was observed against ciprofloxacin, doxycycline, enrofloxacin, levofloxacin, and chlortetracycline. Additionally, no intermediate response was observed against amoxicillin.

Table 1. Frequency of pathogen isolated from pangas fish

Pathogen	Frequency	Prevalence (%)
<i>Edw ar dsiella spp.</i>	12	23%
<i>Aeromonas spp.</i>	14	27%
<i>Flavobacterium spp.</i>	13	25%
<i>Vibrio spp.</i>	01	02%
<i>Enterococcus spp.</i>	01	02%
<i>Pseudomonas spp.</i>	01	02%
Normal Flora	10	19%

Levofloxacin, chlortetracycline, amoxicillin antibiotics were found as the most sensitive, intermediate, and resistant

antibiotics respectively for *Aeromonas spp* (Table-3). *Aeromonas spp* showed no sensitivity against cotrimoxazole, erythromycin, amoxicillin and chlortetracycline. Ciprofloxacin, doxycycline, levofloxacin, and chlortetracycline antibiotics were not found to kill or inhibit the growth of *Aeromonas spp.* like *Edw ar dsiella spp.*, *Aeromonas spp.* showed no intermediate response against amoxicillin.

Flavobacterium spp. showed the most sensitivity to levofloxacin, most intermediate response to doxycycline, enrofloxacin, and resistance to amoxicillin (Table-4). No sensitivity was found against erythromycin, doxycycline and enrofloxacin. In contrast, *Flavobacterium spp.* did not show any resistance against ciprofloxacin, doxycycline, and enrofloxacin.

Vibrio spp. and *Enterococcus spp.* showed sensitivity to ciprofloxacin and levofloxacin respectively. Interestingly, *Pseudomonas spp.* did not show sensitivity to any antibiotics. All of these three isolates showed resistance to cotrimoxazole, erythromycin, and amoxicillin.

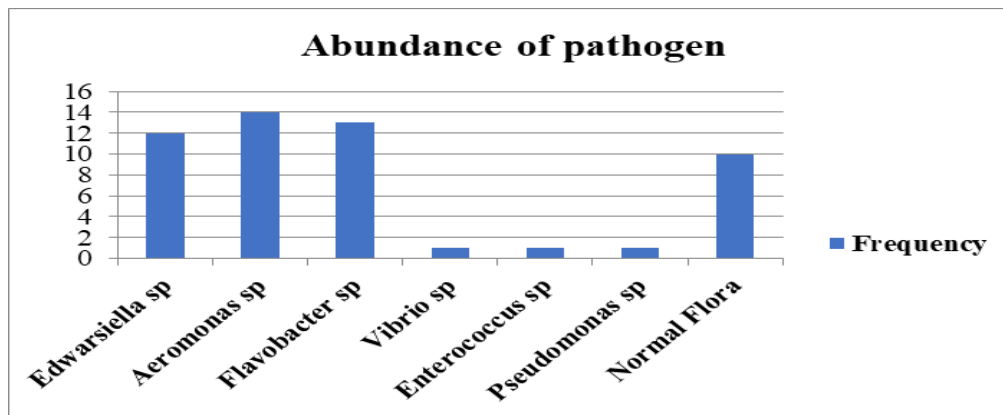


Fig 2. Graphical representation shows of the abundance of pathogen in pangas fish

Table 2. Antibiotic susceptibility pattern of *Edw ar dsiella spp.*

Antibiotic	Sensitivity	Moderate	Resistance
Ciprofloxacin	67%*	33%	0%
Doxycycline	0	100%*	0%
Enrofloxacin	17%	83%	0%
Cotrimoxazole	8%	67%	25%
Erythromycin	0%	25%	75%
Amoxicillin	0%	0%	100%*
Levofloxacin	25%	75%	0%
Chlortetracycline	0%	100%*	0%

* Most abundant

Table 3. Antibiotic susceptibility pattern of *Aeromonas* spp.

Antibiotic	Sensitivity	Moderate	Resistance
Ciprofloxacin	43%	57%	0
Doxycycline	14%	86%	0
Enrofloxacin	14%	79%	7%
Cotrimoxazole	0	86%	14%
Erythromycin	0	36%	64%
Amoxicillin	0	0	100%*
Levofloxacin	57%*	43%	0
Chlortetracycline	0	100%*	0

* Most abundant

Table 4. Antibiotic susceptibility pattern of *Flavobacterium* spp.

Antibiotic	Sensitivity	Moderate	Resistance
Ciprofloxacin	23%	77%	0
Doxycycline	0	100%*	0
Enrofloxacin	0	100%*	0
Cotrimoxazole	15%	62%	23%
Erythromycin	0	46%	54%
Amoxicillin	0	15%	85%*
Levofloxacin	69%*	31%	0
Chlortetracycline	23%	77%	0

* Most abundant

Overview of the antimicrobial susceptibility profiling of the subject pathogens

After the analysis of the antibiotic susceptibility pattern of the subject pathogenic isolates, levofloxacin and amoxicillin were found as the most sensitive and resistant antibiotic (Table-5). Doxycycline was found as the most moderate antibiotic. No sensitivity was found against the antibiotic erythromycin and amoxicillin among which the lowest numbers of moderate responses against amoxicillin were

reported. The findings observed from our study suggested that the use of amoxicillin is less effective treatment against the pathogenic isolates of pangas fish. Moreover, no resistance pattern was observed against ciprofloxacin, doxycycline, levofloxacin, and chlortetracycline. Among these four antibiotics, doxycycline and chlortetracycline showed a significant number of moderate responses which paved the way to choose ciprofloxacin and levofloxacin as the best options for the treatment of diseased pangas.

Table 5. Comparative analysis among antibiotics based on antimicrobial responsiveness exerted by the isolated bacterial pathogens

Antibiotics	Sensitivity	Moderate	Resistance
Ciprofloxacin	35%	11%	0%
Doxycycline	4%	18%	0%
Enrofloxacin	8%	16%	1%
Cotrimoxazole	6%	12%	11%
Erythromycin	0%	6%	28%
Amoxicillin	0%	1%	41%
Levofloxacin	41%	9%	0%
Chlortetracycline	6%	17%	0%

Discussion

Pangas fish is subjected to various diseases during cultivation. Among them, contagious diseases, more specifically bacterial diseases, pose a great threat due to the availability of bacterial pathogens in the agro-aquatic environment. Likewise, the treatment of human infectious diseases, antimicrobial resistance has now been a tremendous challenge for the treatment of infected plants, animals and fishes. The morbidity and mortality rate of diseased fishes can be controlled by the application of effective treatment of proper antibiotics. An attempt was made to assess the impact of environmental parameters on disease development and make an antimicrobial susceptibility pattern of the subject pathogens.

Aquatic environments from which infected pangas were detected, temperature was recorded above the room temperature (26-29⁰ C), and pH was found neutral to alkaline pH (7-9). However, other parameters such as: Ammonia, Nitrate, Alkalinity and hardness showed no significant impact. Mentioned parameters differ from one pond to another and recorded data revealed that there was a large difference between each maximum and minimum

Pathogens isolated from pangas fish included *Edw ar dsiella spp.*, *Aeromonas spp.*, *Flavobacterium spp.*, *Vibrio spp.*, *Enterococcus spp.* and *Pseudomonas spp.* among which *Edw a drsiella spp.*, *Aeromonas spp.*, *Flavobacterium spp.* were predominant. These pathogens were also found in Shing, Koi, Pabda fish (Abedin et al., 2020, Lee et al., 2010). Except *Enterococcus spp.*, all the pathogenic isolates were gram-negative bacteria which resembles the findings of Abedin et al; 2020. Temperature and pH of the fish pond water are favorable for microbial growth of pangas fishes as all of the pathogens are mesophiles and neutrophiles. Interestingly, in a study, Seongwei et al suggested to stop aquaculture activity in dry and hot season due to an increase in water temperature promote proliferation and infection of fishes (Chowdhury et al., 1989, Fernandes et al., 1997, Al-Harbi et al., 2004 and Hossain et al.,1999).

Pathogens found from the body parts of pangas fishes showed a difference in their antibiotic sensitivity pattern. Levofloxacin was found as the most sensitive antibiotics for all pathogens except *Edw ar dsiella spp.*, *Vibrio spp.*, and *Pseudomonas spp.*, *Edw ar dsiella spp.*, and *Vibrio spp.*, exhibited sensitivity against ciprofloxacin in most of the cases. However, no sensitive antibiotic was found against *Pseudomonas spp.* Abedin et al found different sensitivity patterns for pathogenic isolates isolated from pangas. According to their study, *Edw ar dsiella spp.* showed highest sensitivity to cotrimoxazole and enrofloxacin and poor sensitivity to ciprofloxacin, colistin and chlortetracycline. In our study, higher frequency of intermediate sensitivity to doxycycline, enrofloxacin, and cotrimoxazole was observed. Resistance against amoxicillin by most of the pathogens was observed. Additionally, subject isolates showed significant

cases of resistance against erythromycin. *Flavobacterium sp.* showed high frequency of intermediate sensitivity against doxycycline, enrofloxacin, chlortetracycline. Abedin et al. found reverse antimicrobial susceptibility pattern of *Flavobacterium spp.* which was isolated from Koi, Pabda, Singh and pangas fishes.

In our study, *Aeromonas spp.* showed moderate sensitivity against ciprofloxacin in most of the cases. Interestingly different *Aeromonas spp.* isolates collected from different infected fishes and chicken showed 100% sensitivity against ciprofloxacin (Abedin et al., 2020 and Igbinsosa 2014). Didugu et al found *Aeromonas* isolates showing significant frequency cases of moderate sensitivity against ciprofloxacin. Moreover, Yang et al found *Aeromonas species* collected from clinical samples of urinary tract infected patients showed higher cases of resistance against ciprofloxacin and Cotrimoxazole (Yang et al., 2019). But comparatively lower cases of resistance against Cotrimoxazole by *Aeromonas spp.* were observed in our study.

It can be concluded that physicochemical parameters were not found as the significant factors for the outbreak and spreading of infectious diseases among pangas fish. Together, levofloxacin and Amoxicillin were found as the most sensitive and resistant antibiotics, which is corresponded with other studies (Abedin et al., 2020). Other antibiotics exerted significant number of intermediate and resistant cases. Emergence of multiple drugs resistant (MDR) has been found in our study. Extension of the list of antibiotics can be a good option for the effective treatment of infectious diseases associated with fish. Combination of multiple antibiotics can be applied to observe microbial responses towards it.

Conclusion

In our study, we found common bacterial pathogens isolated from pangas fish. These bacterial pathogens showed lower but concerning frequency of moderate sensitivity and resistance against most of the antibiotics except levofloxacin and ciprofloxacin. Results found from our study and other studies suggested that applications of amoxicillin to kill bacterial pathogens are no longer effective. Multiple drugs resistant to bacteria were also found in our research. More antibiotics should be applied for effective treatment of bacterial pathogens and thus prevention of environmental and economic loss generated by the mortality and morbidity of crops, fishes, chickens, and livestock animals.

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Conflict of interests

The authors declare there are no conflicts with due respect to the publication of this article.

Ethical Statement

Institutional ethical clearance was taken from the ethical committee of Khwaja Yunus Ali University before conducting the research.

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