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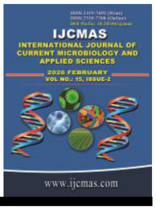

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Isolation and Characterization of Pathogenic Bacteria from Culture Fish, Rajshahi, Bangladesh

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ABSTRACT

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Bacterial disease of fresh water fish causes significant reduction of fish production leading to huge economic loss. This study aims to investigate and isolate bacterial pathogens from gills, kidney and intestine of the fresh water cultured fish and to study antibiotic resistance. The strains of *Proteus mirablis*, *Aeromonas veronii, Micrococcus luteus*, *Staphylococcus succinus* and *Bacillus* sp. have been isolated from the gills, kidney and intestine and which was later identified on the basis of biochemical tests and 16S rRNA gene sequencing. Antibiotic sensitivity patterns of the bacterial isolates were tested against eight commonly used antibiotics *viz*., ampicillin, amoxicillin, azithromycin, cefalexin, ciprofloxacin, kanamycin, streptomycin and tetracycline. *Proteus mirablis* showed resistance to ampicillin, amoxycillin, cefalexin, kanamycin, streptomycin and tetracycline, *Aeromonas veronii* and *Staphylococcus succinus* was highly resistant only to cefalexin and ampicillin while *Bacillus* sp. showed high to moderate resistance to ampicillin, amoxycillin and cefalexin. The presence of antibiotic-resistant pathogenic bacteria in cultivated fish is a very detrimental threat to human health. So, need for implementing measures that guard against the misuse of antimicrobial drugs in fish feeds is therefore emphasized.

Introduction

Fish is the most important source of protein providing about 16% of animal protein consumed by the world populace (Muhammad *et al.,* 2020). Fish are widely preferred food all over the globe that about one billion people rely on fish as their primary source of animal protein (Mahmoud and Mohammed, 2020). Bacteria are one of the important causative agents of fish disease in culture fishes and are responsible for serious economic loses. Fish farmers have been facing great problems due to bacterial fish disease that causes severe damage and mortality in cultured fish. Various types of diseases such as ulcer type disease including epizootic ulcerative syndrome, bacterial haemorrhagic septicaemia, tail rot, fin rot, bacterial gill rot, dropsy, columnaris disease, fungal disease and parasitic disease are important limiting factors for sustainable fish production (Chowdhury, 1997). *Aeromonas* sp. and *Pseudomonas* sp. are the important bacterial pathogens frequently isolated from the diseased fishes throughout the world as well as Bangladesh. Bacterial fish diseases, especially bacteria haemorrhagic septicaemia (Roberts, 1989) and motile *Aeromonnas* septicaemia (Lio-po *et al.,* 1992) in fresh water fish cause great losses. *Aeromonas spp.* are commonly found in a wide range of aquatic systems and have been isolated from waters, lakes, rivers and a variety of foods (Blaise and Armstrong, 1973; Buchanan *et al.,* 1985; Dumontet, 1990 and Santos *et al.,* 1993).

Aeromonas sp. and *Pseudomonas* sp. are the major bacterial fish pathogens which are widely distributed in aquatic organisms in nature (Banu, 1996 and Islam, 1996). In Bangladesh, *Aeromonas* spp. are frequently observed in farmed fishes as well as in the water (Iqbal *et al.,* 1996). It is also isolated from the lesions and kidney of EUS affected fishes (Chowdhury and Inglis, 1994).

Pathogenicity of different *Aeromonas* and *Pseudomonas* bacterial species has been detected (Sarker *et al.,* 1999; Alauddin *et al.,* 1999; Habib, 2001) in diseased fish in cultured and capture fisheries from the different regions of Bangladesh. Most fish pathogenic bacteria can reside in the environment or in apparently normal fish. Thus, infections are often precipitated by some stress that upsets the natural defenses against the agents (e.g. overcrowding, low DO, high ammonia).

A. hydrophila is the most common cause of bacterial haemorrhagic septicaemia. The disease occurs in three distinct forms: (a) abdominal dropsy, characterized by distension of the visceral cavity with fluid, (b) ulcerative, characterized by skin and muscle lesion, and (c) generalized bacterial haemorrhagic septicaemia. It has also been given several other names, e.g. infections, dropsy, red disease. The disease is worldwide affecting cultured cyprinids and other pond fishes. *A. hydrophila* caused a severe disease outbreak. Cultured fish suffering from *Aeromonas* sp. and *Pseudomonas* sp. infections with similar signs like dermal lesion, scale loss, frayed fins, tail and fin rot and dropsy. Red skin disease is caused by *Pseudomonas* sp. and red spot disease is caused by *Aeromonas* sp.

In Bangladesh, there is no proper scientific research on bacterial disease in fish. Study of aquatic bacteria associated with fish is very limited in Bangladesh. Few attempts have been taken in order to assess the bacterial population in aquatic environment and their involvement in causing diseases in fish. Chowdhury (1997) reported *Aeromonas* sp. and *Pseudomonas* sp. are very common in fish disease specially in carp and live fishes for these a successful investigation need to know, what kind of bacteria present associated with diseased fish, the quantity of bacteria present in different organs of diseased fish and pond water are harmful or not for fish culture. This study will also demonstrate the pathogenic potential of bacterial species associated with culture fishes suggesting the emerging threat to public health.

Materials and Methods

Fish select and sample collection

Labeo rohita, *Puntius sarana* and *Tilapia nilotica* are crucial for Bangladesh's fish culture due to their high market demand, adaptability to local environmental conditions, and ability to contribute to food security especially for low-income populations and economic growth. Tilapia and *Puntius sarana* is particularly important because it's a low-cost source of protein, relatively inexpensive, fast growth rate and readily available in market, both for local consumption and potential export. These three species can be cultured with other fish species, like *Labeo rohita*, in polyculture systems, maximizing resource utilization. These reasons, three fish species were chosen for the study.

A total of 45 healthy indigenous freshwater fish including 15 Rohu (*Labeo rohita*), 15 Punti (*Puntius sarana*) and 15 Tilapia (*Tilapia nilotica*), ranging in size from 300-500 grams were purchased from local retail fish market in Binodpur bazar, Rajshahi. Immediately after collections, the samples were transported to the Genetics and Molecular Biology Laboratory, Department of Zoology, University of Rajshahi, for isolation of pathogenic bacteria.

Sample processing and isolation of pathogenic bacteria

The collected fish samples were first sanitized externally with 70% alcohol. Dissecting tools (*viz*. forceps, scalpels, blades and scissors) were prepared in sterile conditions by autoclave at 123^0 C and 15 lb. pressure for 30 min. Fish was dissected from anus to operculum using already sterilized scissors. The samples of fish organ including gill and kidney were collected for isolation of microbes. The entire sampling process of organ was completed in an aseptic environment. One loopful of gill and kidney were directly transferred into nutrient broth media (Hi Media, India), which were incubated for 2 days at 37°C and subjected to shaking at 120 rpm on an orbital shaker.

Control flasks without inoculates were also prepared and incubated at 37°C with an orbital shaker. The cultures that were found turbid after a period of 0 up to 2 days were used as inocula in sub-sequent experiments.

Microscopic examination and identification of bacterial cells

For the identification of the pathogenic bacteria, microscopic observations followed by morphological characterizations, biochemical tests were performed. The microorganisms were identified according to *Bergey's Manual of Systematic Bacteriology* (Holt 2005).

Antibiotic susceptibility pattern of the isolates

Susceptibility pattern of the bacterial isolates to eight commonly used antibiotics was determined *in vitro* employing standard disk diffusion method (Mohanta *et al.,* 2012), and the susceptibility pattern of the isolates was interpreted using manufacturer's guidelines, where disc distances of 5-9 mm were considered resistance (R), 10-14 mm intermediate (I) and ≥15 mm sensitive (S). Antibiotics and their concentrations used were as follows: Amoxicillin (30µg/disc), Ampicillin (25µg/disc), Azithromycin (15µg/disc), Cefalexin (30µg/disc), Ciprofloxacin (5µg/disc), Kanamycin (30µg/disc), Streptomycin (10µg/disc) and Tetracycline (30µg/disc).

Identification of the pathogen by 16S rRNA gene sequence

Genomic DNA of the bacterial isolate was isolated according to Mohanta *et al.,* (2012). Gene fragments specific for the highly variable region of the bacterial 16S rRNA gene were amplified by PCR using universal PCR primer as described by Loffler *et al.,* (2000) (Sigma, USA) in a thermal cycler (MJ Research Inc., Watertown, USA). The sequence of the forward primer was 16SF 50-GAGTTTGATCCTGGCTCAG-30 and the sequence of the reverse primer was 16SR 50-GAAAGGAGGTGATCCAGCC-30. The PCR products were subjected to 1 % agarose gel electrophoresis, stained with ethidium bromide and visualized on a UV transilluminator for the presence of about 1,500 bp PCR products. Amplified 16S rRNA gene PCR products were purified using Strata Prep PCR purification kit (Stratagene, USA) according to the manufacturer's protocol. Sequencing reactions were carried out using ABI-Prism Big dye terminator cycle sequencing ready reaction kit and the PCR products were purified by a standard protocol. The purified cycle sequenced products were analyzed with an ABI Prism 310 genetic analyzer. The chromatogram sequencing files were edited using Chromas 2.32. The homology of the 16S rRNA gene sequences was checked with the 16S rRNA gene sequences of other organisms that had already been submitted to GenBank database using the BLASTN (http://www.ncbi.nih.gov/BLAST/) algorithm.

Results and Discussion

Isolation and identification of the bacteria

In this study, three fresh water fish species *Labeo rohita*, *Puntius sarana* and *Tilapia nilotica* were collected from the Binodpur fish market, Rajshahi. Bacteria were isolated from organs of sampling culture fish by plating onto an agar solidified nutrient medium. The plates were incubated at 37°C for 2 days and bacterial colonies were found to grow on the medium. Results of microscopic analysis of bacterial cells and their growth characteristics are presented in Table 1 while the biochemical and antibiotic sensitivity tests of the bacterium are presented in Table 2 and 3, respectively. Isolated bacterial strains were identified by both morphological and biochemical tests and this was further confirmed by 16S rRNA gene sequence analysis. The strains showed 99% homology with *Proteus mirablis*, *Aeromonas veronii, Micrococcus luteus*, *Staphylococcus succinus* and *Bacillus* sp. are presented in Table 4.

Fish farming is an important sector which has been contributing tremendously to our economy. Though this sector has such potentiality, fish farming is confronted with acute problem of disease like bacterial, fungal and viral disease and also skin ulcer which can be caused by different factor (Abedin *et al.,* 2020). Those factors can cause great harm to the production cycle in one hand and on the other hand, fish contain bacterial pathogens like *Vibrio*, *Aeromonas*, *E. coli*, *Bacillus* sp., *Mycobacterium* spp., and *Salmonella* leading to human diseases (Novotny *et al.,* 2004). Overuse of antibiotics in aquaculture and poor hygiene practices contribute to the presence of these pathogens and antibiotic-resistant strains in fish, posing a public health risk.

In the present study, two Gram-negative and three Gram-positive pathogenic bacteria *viz*., *Proteus mirabilis* strain

B1, *Aeromonas veronii*, *Micrococcus luteus*, *Staphylococcus succinus and Bacillus* spp. were isolated from organs of the cultured fishes that are sold live in the markets. Many previous studies reported isolation of pathogenic bacteria of different genus from cultured fresh fish organs, such as, *Vibrio cholerae*, *Salmonella spp.*, *Leclercia adecarboxylata, Aeromonas caviae* and *E. coli* (Khan *et al.,* 2023); *Staphylococcus aureus* and *E. coli* (Muhammad *et al.,* 2020); *Flavobacterium* spp., *Edwarsiella* spp., and *Aeromonas* spp. (Abedin *et al.,* 2020); *Aeromonas hydrophila*, *Pseudomonas* spp., *Enterobacter* spp., *Serratia* spp. and *Micrococcus* spp. (Manohar *et al.,* 2023); *Aeromonas hydrophila, A. sobria, A. schubertii, A. media, A. caviae, A. ecrenophila, Pseudomonas stutzeri, P. pseudoalcaligenes, P. aeruginosa, Plesiomonas shigelloides, Bacillus megaterium, Neisseria mucosa, Citrobacter freundii, Serratia marcescens, Staphylococcus epidermidis, Micrococcus luteus, Enterobacter aerogenes* and *Enterobacter sakazakii* (Geraldine *et al.,* 2020).

Proteus mirabilis is a Gram-negative facultative, zoonotic pathogen, the second most prevalent member of *Enterobacterales* (Reu *et al.,* 2018) and it serves not only as a commensal microbe in human gut microflora but also in environmental *viz*. wastewater and soil samples (Drzewiecka, 2016).

The occurrence of *Proteus mirabilis* strain B1, Gram-negative rod, motile, white, smooth and transparent on agar solidified nutrient media isolated from gills in *Labeo rohita* is being reported for the first time through this study. The isolate was positive for KOH, sulphur reduction and negative for oxidase, methyl red, indole production and voges-proskauer reaction. The strain was positive for glucose, fructose, sucrose and xylose showed yellow colouration. These findings are in close agreement with Shammah *et al.,* (2023) who reported that *Proteus mirabilis* was the most frequent extracted bacterium from fresh water fishes in FCT, Abuja-Nigeria. In Kirinyaga Country, Kenya, bacterial pathogen in farmed tilapia, catfish, goldfish and koi carp and source pond water were assessed, in which pathogenic *Proteus* spp. (14.9%), *Aeromonas hydrophila* (8.2%), *A. caviae* (6.3%), *A. sobria* (4.3%), *Plesiomonas* spp. (5.2%), *Flavobacterium* spp. (5.2%) and *Micrococcus* spp. (4.3%) were identified (Wanja *et al.,* 2019). In another report, a total of 225 samples from poultry, livestock and aquatic sources especially raw fish and fish market waste in Pakistan and the multidrug-resistant prevalent bacteria identified was *Proteus mirabilis* (Sarwar *et al.,* 2025). *Aeromonas veronii* is globally recognized as one of the dominant and virulent pathogenic agents in freshwater fishes (Zhang *et al.,* 2018; Sun *et al.,* 2016). Hemorrhagic septicemia, ulcerative lesion, pale body surface, dropsy, and tail and fin rot in different fish species caused by these species (Hoai *et al.,* 2019; Hassan *et al.,* 2017). In the present study, *Aeromonas veronii* was found in kidney of *Labeo rohita*. The biochemical tests of *A. veronii* was positive for KOH and negative for sulphur reduction, oxidase, methyl red, indole production and voges-proskauer reaction. The strain was positive for glucose, fructose, sucrose and xylose showed yellow colouration. Similar findings of the bacterium have been reported by many previous studies (Geraldine *et al.,* 2020; Ehsan *et al.,* 2023). Rahman *et al.,* (2004) identified several species of *Aeromonas*, including *A. veronii* based on phenotypic characteristics and 16S rRNA gene sequence analysis from fish suffering from Epizootic Ulcerative Syndrome (EUS) in Bangladesh and Thailand. Iqbal *et al.,* (1999) also identified *A. veronii* from fishes of Southeast Asian countries suffering from EUS through phenotypic characteristics and 16S rRNA gene sequence analysis.

Micrococcus luteus Gram-positive, round shaped cocci is naturally found in the aquatic environment and was isolated from the gill of *Puntius sarana*. Saleh *et al.,* (2021) reported that 14 isolates were identified as *Micrococcus luteus* from the examined *Oreochromis niloticus* in Egypt with prevalence of 9.3%. Similar findings have been noticed by Wanja *et al.,* (2020) that prevalence of *M. luteus* infection among the cultured *O. niloticus* in Kenya was 10%. Parra-Laca *et al.,* (2020) reported that prevalence of *M. luteus* infection among the cultured *O. niloticus* at Morelos in Mexico was 10%. In this study, *Micrococcus luteus* showed negative for KOH, sulphur reduction, oxidase, methyl red, indole production and voges-proskauer reaction similar to those reported by Aydin *et al.,* (2005) and Akayli *et al.,* (2020) for *M. luteus* isolated from the diseased fishes. *Staphylococcus* are Gram-positive, non-motile food-borne opportunistic bacteria isolated from cultured fish has been reported by many previous studies (Al-Obaidy *et al.,* 2011; Hamad and Eshak, 2012). In this study, *S. succinus* was isolated form gill organs of *Tilapia nilotica*. Ali, (2014); Ganaie and Sharma, (2022) examined that a total of five species of *Staphylococcus* including *S. saprophyticus*, *S. epidermidis*, *S. hyicus*, *S. aureus*, *S. intermedius* were detected in cultured fish. These findings are in close agreement with Lin *et al.,* (2007) and Ahmeed *et al.,* (2008).

Table.1 Colony characteristics of the isolated bacterial strain

Collected sample (Fish organ)	Fish Species	Colony morphology					Presumptive identification
		Margin	**Colour**	**Opacity**	**Surface**	**Shape**	
Gill	*L. rohita*	Circular	White	Transparent	Smooth	Rod	*Proteus mirabilis* Strain B1
	P. sarana	Circular	White	Transparent	Smooth	Rod	*Micrococcus luteus*
	T. nilotica	Circular	Creamy white	Opaque	Smooth	Round	*Staphylococcus succinus*
Kidney	*L. rohita*	Circular	Yellow	Opaque	Smooth	Round	*Aeromonas veronii*
	T. nilotica	Circular	White	Opaque	Smooth	Rod	*Bacillus sp.*

Table.2 Biochemical tests of the bacterial isolates from fish organ

Collected sample (Fish organ)	Fish Species	Gram staining	Motility tests	Biochemical tests						Carbohydrate utilization tests								Presumptive identification
				KOH	**Indole production**	**Sulpher reduction**	**Methyl red**	**Voges proskaure**	**Oxidase tests**	**Glucose**	**Fructose**	**Sucrose**	**Lactose**	**Maltose**	**Cellulose**	**Galactose**	**Xylose**	
Gill	*L. rohita*	-	+	+	-	+	-	-	-	+	+	+	-	-	-	-	+	*Proteus mirabilis* Strain B1
	P. sarana	+	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	*Micrococcus luteus*
	T. nilotica	+	-	-	-	-	-	-	-	+	-	+	-	+	-	-	+	*Staphylococcus succinus*
Kidney	*L. rohita*	-	-	+	-	-	-	-	-	+	+	+	-	-	-	-	+	*Aeromonas veronii*
	T. nilotica	+	+	-	+	-	-	-	+	-	+	+	-	+	-	-	-	*Bacillus sp.*

(+ sign indicate growth of the microorganisms while ‘-’sign indicates no growth)

Table.3 Antibiotic susceptibility patterns of the isolated bacterial strains

Antibiotics used	*Proteus mirabilis*	*Micrococcus luteus*	*Staphylococcus succinus*	*Aeromonas veronii*	*Bacillus sp.*
Ampicillin (25 µg)	R	S	R	I	R
Amoxycillin (30 µg)	R	S	I	S	S
Azithromycin (15 µg)	S	S	S	S	R
Cefalexin (30 µg)	R	S	I	R	S
Ciprofloxacin (5 µg)	I	S	S	S	S
Kanamycin (30 µg)	R	S	S	I	S
Streptomycin (10 µg)	R	S	S	S	S
Tetracycline (30 µg)	R	S	R	S	S

R= Resistant to antibiotics ((disc distances 5-9 mm)); I= Intermediate responses (10-14 mm); S= Sensitive to antibiotics (15-20 mm)

Table.4 Isolates with 16s rRNA sequences match results found in NCBI database

Fish organ	Fish species	Resembles with NCBI BLASTn			Family
		1st matching	Identities (%)	Sequence ID	
Gill	*L. rohita*	*Proteus mirabilis* strain B1	756/760(99%)	KC344360.1	**Enterobacteriaceae**
	P. sarana	*Micrococcus luteus*	747/752(99%)	AB079788.1	**Micrococcaceae**
	T. nilotica	*Staphylococcus succinus*	750/762(98%)	JN644525.1	**Staphylococcaceae**
Kidney	*L. rohita*	*Aeromonas veronii*	763/766(99%)	KU163442.1	**Aeromonadaceae**
	T. nilotica	*Bacillus* sp.	746/747(99%)	KF917158.1	**Bacillaceae**

Bacillus spp. is highly virulent (proteolytic and hemolytic properties) and they caused secondary infections in fishes and shrimp. In this study, a Gram-positive, rod shaped and motile *Bacillus* spp. were isolated and identified from kidney of *Tilapia nilotica*. The isolate was positive for oxidase, indole production and negative for KOH, sulphur reduction, methyl red and voges-proskauer reactions. The strain was positive for maltose, fructose, sucrose and showed yellow colouration. This result is supported by the finding of Annammal *et al.,* (2022). Antibiotics are used to find out their effectiveness against pathogenic bacteria. In this study, antibiotic sensitivity patterns of the bacterial isolates were tested against eight commonly used antibiotics *viz*., ampicillin, amoxycilin, azithromycin, cefalexin, ciprofloxacin, kanamycin, streptomycin and tetracycline. According to results of this study, *Micrococcus luteus* were 100% sensitive to test antibiotics. These findings agreed with findings of Saleh *et al.,* (2021) who found that *M. luteus* was sensitive to amoxicillin, penicillin, clavulanic acid, ampicillin, tetracycline and chroramphenicol and also with findings of Akayli *et al.,* (2020) who found that *M. luteus* was sensitive to tetracycline and chroramphenicol.

Antibiogram profile of the *Proteus mirablis* isolates revealed that the isolates showed resistance to ampicillin, amoxycillin, cefalexin, kanamycin, streptomycin and tetracycline, *Aeromonas veronii* and *Staphylococcus succinus* was highly resistant only to cefalexin and ampicillin while *Bacillus* sp. showed high to moderate resistance to ampicillin and azithromycin and sensitive to penicillin, clavulanic acid, ampicillin, tetracycline and chroramphenicol. Shammah *et al.,* (2023) examined that *P. mirablis* were 100% resistance to amoxicillin and 92.3% were resistance to erythromycin, this similar to the findings of Zafar *et al.,* (2019) who stated that *P. mirabilis* shows 93.7% resistance to amoxicillin and Umar *et al.,* (2016) reported 100% resistance to amoxicillin which agree with our findings. *Bacillus* spp. MSU1400 isolated from *L. lentjan* lesion was found to be highly sensitive to ampicillin, cephalexin and gentamycin, kanamycin and rifamycin (Annammal *et al.,* 2022).

According to Saavedra *et al.,* (2004), *Aeromonas* spp. are considered resistant to beta-lactam antibiotics such as ampicillin, amoxicillin, penicillin etc. (Zdanowicz *et al.,* 2020; Hasan *et al.,* 2017).

In conclusion, antibiotics are used to prevent and treat diseases, but unregulated and indiscriminate uses are observed in many parts of the world, especially in Asia and Africa. Most of the farmers could not recognize the actual diseases of cultured fish. Failure to recognize the disease is another cause for the development of antibiotic resistance as inappropriate antibiotics are used. However, the continuing use of aquaculture antibiotics has enhanced the natural selective pressures on microbial communities, leading to the emergence of antibiotic-resistant strains that are capable of widespread dissemination and causing severe infections. Consumption of farmed fish contaminated with antibiotic-resistant bacteria can result in public health consequences.

Because of their unique chemical structure, antibiotics are difficult to break down or degrade in the natural environment and can easily circulate in the food chain and accumulate in the human body. Additionally, antibiotic resistance genes (ARGs) can rapidly spread to other bacterial species by horizontal gene transfer and microbes gained resistance capability against antibiotics. This study indicates that antibiotic resistant bacteria are present in retail fish. The increasing presence of resistant strains in cultivated fish is a very detrimental threat to human health. So, need for implementing measures that guard against the misuse of antimicrobial drugs in fish feeds is therefore emphasized.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

Author Contributions

Sarmin Akther: Investigation, formal analysis, writing—original draft. M. Israil Haque: Validation, methodology, writing—reviewing. M. K. Mohanta:—Formal analysis, writing—review and editing.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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