Asian J. Med. Biol. Res. 2019, 5 (1), 56-62; doi: 10.3329/ajmbr.v5i1.41046

Asian Journal of Medical and Biological Research
ISSN 2411-4472 (Print) 2412-5571 (Online)
www.ebupress.com/journal/ajmbr

Article

Identification of pathogenic bacteria from infected Thai koi (*Anabas testudineus*)

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Received: 04 March 2019/Accepted: 25 March 2019/ Published: 31 March 2019

Abstract: A study was conducted for identification of pathogenic bacteria from Thai koi (*Anabas testudineus*) and an experimental infection test was run for identifying the actual causative agent of the infection. Due to perform the experiment, the fish sample was collected from different fish farm located in Fulbari, Dinajpur to the Microbiology lab of the HSTU-Dnajpur campus and placed in tray for taking sample from different infected part such as gill, slime, muscle, fin of the fish by using wire loop then these were taking in the nutrient ager medium for observing the culture of bacteria. After then specific culture media, *Salmonella-Shigella* media, Mannitol salt agar media, Mac-Conkey (MaC) agar media and Eosin Methylene Blue media were used for observing specific bacterial characteristics. Then biochemical tests, Methyl red (MR), Voges-proskaure test, Triple sugar iron test, Indole test were performed for bacterial identification. As a result *Salmonella* spp., *Shigella* spp., *Klebshiella* spp. and *Staphylococcus* spp. were confirmed. Catalase test and Simon citrate test was also performed. Then Grams staining method was followed for microscopic observation of identified bacteria. Then experimental infection test was performed in Aquaculture lab by setting up 5 aquarium holding fresh fish. The fresh water and identified bacteria were added specifically to the aquarium and it was continued for 15 days for observing infectious symptoms. After 15 days the fish with *Salmonella* spp. and *Staphylococcus* spp. were showed infectious symptoms but other did not any change in physical appearance. So it can be said that *Staphylococcus* spp. and *Salmonella* spp. are able to show ulcerative symptoms in Thai koi (*Anabas testudineus*) that is a bacterial infection.

Keywords: Thai koi (*Anabas testudineus*); biochemical identification of bacteria; experimental infection test

1. Introduction

Bangladesh is the world leading fish producing country in the world. Bangladesh is now holding the 3rd position among the fish producing countries in the world after China and India (FRSS, 2017). At present the total fish production is about 41.34 lakh MT, where aquaculture contributes 56.44 percent to total production. Government is trying to sustain this growth performance, which eventually ensures to achieve the projected production target of 4.55 million MT by 2020-21. Now Bangladesh has achieved self-sufficiency in fish production that is a big achievement for the country. Over the last three decades, the fish production is increased more than five times. Inland aquaculture of indigenous and exotic carp species as well as tilapia, pangas and koi

expanded massively. Besides, new interest grew in farming of indigenous species like koi, singh, magur, pabda, gulsha, and mola. (DoF, 2017). The koi *(Anabas testudineus*) is known as small indigenous species in Bangladesh and it looks like greenish in appearance. In earlier period the native koi was very plentiful in almost all the freshwater body in our country (Mahmood, 2003). The Koi (*Anabas testudineus*) naturally found in Bangladesh, India, Pakistan, Ceylon, Myanmar, Srilanka, Thailand, Cochin-China, Tongking, Souththern China, Philippines, Polynesia and Malaysia (Sterba, 1983; Sen, 1985; Talwar and Jhingram, 1991). Once upon a time, climbing perch or Koi was very much abundant in almost all freshwater systems of Bangladesh (Mahmood, 2003). Koi (*Cyprinus carpio* koi) are well-known common carp (*C. carpio*) that belong to the *Cyprinidae* family. Thai koi *(Anabas testudineus)* has been brought in Bangladesh from Thailand in 2002 due to higher taste, growth, nutritious value and a high market price (Alam *et al*., 2006). The koi fish are usually known to the ornamental varieties of domesticated common carp that have high economic value. The culture of koi *(Anabas testudineus)* fish is a popular culture practice in Bangladesh because of the development of induced breeding and mass seed production. About 23,000 farmers are involved in koi production. It was assumed that the number of koi *(Anabas testudineus)* farmers would be increased at large number in near future. Most farmers (60%) produce koi under polyculture while the remainder (40%) practices monoculture. About 10% of farmers are involved in extensive or improved-extensive farming, while 60% and 30% practice in semi-extensive and intensive farming, respectively. The total annual koi production in Bangladesh was estimated at 22,989 tons in 2010-11. Now-a-days koi *(Anabas testudineus)* fish has been introduced to our culture arena. It has a great demand in the market forties nutritive value and taste. Many hatcheries have been established in our country with a view to producing koi (*Anabas testudineus*) fry. Especially greater Mymensingh, Gazipur and bogra are playing prominent role in producing koi fry (Roy *et al*., 2013). Culture of koi fish is faced with several problems on which disease infection is usual in winter season. The common disease of koi fish includes fin rot, gill rot, skin ulcer. Among of this skin ulcer is fatal to koi fish. It may be appeared due to several causes such as presence of pathogen especially bacteria, fungi, low water quality, presence of high content of ammonia etc. It is associated with an outbreak in the skin extending through all the layers that fails to heal and accompanied by inflammation. Bacterial infection in fish farm is crucial to the fish and consumer. The presence of bacteria belonging to the genus *Staphylococcus, Pseudomonas, Flavobacterium, Escherichia, Salmonella*, *Aeromonas* are majorly responsible for bacterial infection in koi fish. Due to bacterial infection fish loses it demand to consumer and sometime become totally unusable for consume and so great economic loss can be occurred. As there is no detailed information on the bacterial diseases in koi (*Anabas testudineus)* culture systemin Bangladesh, the present work was designed to study the diagnosis of bacterial diseases in koi (*Anabas testudineus)* culture and finally reduce the production loss of koi culture system in Bangladesh. So the main objective was to isolate and identify the responsible bacteria from Thai koi *(Anabas testudineus)* fish, and to observe the clinical signs produced by experimental infection.

2. Materials and Methods

2.1. Collection of samples

The infected experimental fish-Thai Koi *(Anabas testudineus)* sample (Figure 1) was collected from different fish farm in Phulbari, kalirhat, Dinajpur on January 2018 to the Bacteriological laboratory in the Department of Microbiology, at Hajee Mohammad Danesh Science and Technology University, Dinajpur-5200. During sample collecting period the range of Ph, dissolved oxygen, temperature was confirmed from farm manager. The temperature was 20°c, ph was 6 and dissolved oxygen was 5ppm.

Figure 1. Infected koi fish.

2.2. Preparation of sample

Firstly the collected fish was taken in a tray and it was anaesthetized by inserting forceps at the end of the head region. Then sample was taken from different infected body part of the fish-Muscle, Gill, and Fin, Slime and then kept in Petridis. A homogenized suspension was made with the help of mortar and pastel.

2.3. Techniques for the isolation and identification of associated bacteria

Culture of fish samples as soon as earlier and inoculated separately with nutrient agar media and were incubated at 37^{O}C for overnight. The colonies on primary cultures were repeatedly subcultured by streak plate method (Cheesbrough, 1985) until the pure culture with homogenous colonies were obtained. Media such as MacConkey agar, Eosin Methylene Blue agar, Salmonella Shigella (SS) agar, and Manitol Salt Agar (MSA) were used for sub-cultures and incubated at 37°C for 24 hours for growth. Identification of bacteria was performed on the basis of colony morphology Gram's staining reaction and biochemical test. Isolation the organism with supporting growth characteristics of *Klebshiella spp.* were maintained on EMB agar, *Salmonella spp.* were maintained *Salmonella-Shigella* agar and *Staphylococcus spp.* was maintained on MS agar. Biochemical tests, named as TSI, MR, VP, Indole and Catalase test were performed as per the standard methods (Cheesbrough, 1985).

2.4. Inoculation of isolated bacteria in experimental fish

For **e**xperimental infection, the fresh Thai koi (*Anabas testudineus*) species were collected from another fish farm 5 aquariums were set in which one was control and other contains specific bacteria that was performed in the Aquaculture lab with maintaining water quality. The inoculation of bacteria was done by using were loop that was used to take the bacteria in the aquariums in which aquarium 1 contains *Salmonella* spp., aquariums 2 contains *Shigella* spp., aquariums 3 contains *Staphyloccus* spp., and aquariums 4 contains *Klebshiella* spp., and aquarium 5 is the control. The experiment was run for 15 days to determine the infection and two time observation was continued daily for 15 days.

3. Results

3.1. Result of cultural characteristics

Cultural characteristics of each type of bacteria isolated from infected Thai koi were studied for the examination of size, shape, colony characteristics, and pigment production in various solid media. The pure cultures of the organism from each mixed culture were obtained by repeated streak plate method by using different selective solid media for study. The individual culture characteristics of bacterial isolates are presented in Table 1.

Table 1. Result of cultural characteristics of the organisms in selective media.

Isolated Organism	NA	SS agar	MS agar	MC agar	EMB agar	SCA agar
***Satphylococcus* spp.**	On nutrient ager isolates produce translucent, opaque, smooth, gray whitish colonies	-	yellowish colony	-	-	-
***Salmonella* spp.**	On nutrient ager isolates produce translucent, opaque, smooth, gray whitish colonies	black color in appearance	-	whitish growth colony	-	blue colony
***Shigella* spp.**	On nutrient ager isolates produce translucent, opaque, smooth, gray whitish colonies	whitish color in appearance	-	-	-	.
***Klebshiella* spp.**	On nutrient ager isolates produce translucent, opaque, smooth, gray whitish colonies	-	-	-	blackish color	-

Legends: NA = Nutrient agar, MC = MacConkey agar, EMB = Eosin Methylene Blue agar, SS = Salmonella Shigella agar, SCA= Simmons citrate agar, MS=Mannitol salt agar.

3.1.1. Nutrient agar test

Nurient agar plates streaked separately with the samples and the growth of bacteria were indicated by the circular, small smooth, and gray white colonies after 24 hours of incubation at 37^{0}C aerobically.

3.1.2. *Salmonella-Shigella* agar test

Salmonella-Shigella agar plates streaked separately with the samples and the growth of bacteria were indicated by the Clear, black center colony that refers to *Salmonella* spp., and whitish colony refers to *Shigella* spp., fter 24 hours of incubation at 37^{0}C aerobically.

3.1.3. Mannitol salt ager test
Mannitol salt agar plates streaked separately with the samples and the growth of bacteria were indicated by the yellowish colony after 24 hours of incubation at 37^0C aerobically that refers to *Staphylococcus* spp.

3.1.4. Mac Conkey ager test
Mac Conkey agar plates streaked separately with the samples and the growth of bacteria were indicated by the whitish colonies after 24 hours of incubation at 37^0C aerobically that refer to *Salmonella* spp.

3.1.5. Eosine methyline blue ager test
Isolates produce nonmetallic blackish color in EMB ager that refers to the growth of *Klebshiella* spp.

3.1.6. Simon citrate test
Isolated *Salmonella* spp. bacteria were poured into the prepared medium and it turned into blue color that was the positive reaction.

4. Result of biochemical test
Bacteria isolated from the sample subjected to various types of physiological tests such as Triple sugar iron test, methyl red test, Voges-Prostauer test, and indole test to determine their biochemical characters. The result was presented in Table 2.

Table 2. Result of biochemical tests of the isolated *Satphylococcus* sp., *Salmonella* sp., *Shigella* sp., *Klebshiella* sp., from infected parts of Thai koi.

Isolated bacteria	MR	VP	Indole	TSI
Satphylococcus sp.	+	+	-	+
Salmonella spp.	+	+	+	+
Shigella sp.	+	+	-	+
Klebshiella sp.	+	+	+	+

MR= Methyl Red, VP= Voges Proskaure, TSI= Triple Super Iron

4.1.1. Methyl red test (MR)
After 48hr. methyle red solution was added and it gave red color with ring for isolates bacteria that was positive reaction.

4.1.2. Voges-Prostauer test (VP)
In VP test after 72hr. alpha left hone and potassium hydroxide was added and it produced red color for identified bacteria that was positive reaction.

4.1.3. TSI test
In this test black color of hydrogen sulfide gas was produced for *Salmonella* spp. and *Klebshiella* spp. as well as red and yellowish color were produced for *Staphylococcus* spp. and *Shigella* spp. that refers to the positive reaction.

4.1.4. Indole test
After 24hr of incubating, kava's indole reagent was added in which red ring was observed for *Salmonella* spp., *Klebshiella* spp. that was positive reaction on the other hand *Staphylococcus* spp. and *Shigella* spp. did not produce any color that was negative.

4.1.5. Catalase test for *staphylococcus sp.*
After giving of 1-2 ml hydrogen peroxide solution in bacteria containing slide gas bubble was observed for *Staphylococcus* spp.

4.2. Microscopic observation though Gram's staining method
Microscopic observation was observed after preparing slide with the help of Grams staining method that was performed to observe the shape and gram reaction of the isolates. The isolates were found to be curved, chain, cluster, comma and rod shape.

4.3. Experimental infection

Among four isolated bacteria *Salmonella* and *Staphylococcus* containing fish in aquarium show disease sign but other bacteria containing fish do not show any change in physical appearance.

5. Discussion

Fish farming is an important sector that has been contributing progressively to our economy. It is one of the fastest growing and most promising industries with the brightest of future for our country. In spite of such potentiality, fish farming is confronted with acute problem of disease like fungal, bacterial, viral disease and also skin ulcer that can be caused by different factor can causes great harm to the production cycle. Majorly fungal and bacterial agents are responsible for skin ulcer. The study was aimed at determination the lethal effect of bacterial agent like *Salmonella spp., Shigella* spp., *Staphylococcus* spp., and *Klebshiella* spp., isolated from different part of infected body parts of koi fish. The research work was carried out at microbiology laboratory and aquaculture laboratory in university campus after collecting fish sample from a fish farm of Fulbari, Dinajpur. Due to identification of the bacteria specific culture media and biochemical tests were performed. After characterization of bacteria, an experimental test setting up 5 ordinary aquarium holding fresh fish with identified bacteria was run for 15 days to know the actual causative agent of skin ulcer of fish. By performing this test, it was confirmed that fish with *Salmonella* spp. and *Staphylococcus* spp. produced ulcerative symptoms but fish with *Shigella* spp. and *Klebshiella* spp. did not produce ulcerative symptoms. Aquatic environments are the major sources of *Salmonella* spp. and fishery products have been recognized as a major carrier of food-borne pathogen such as *Salmonella* spp. (Kamat *et al*., 2005). The major hazards of concern for aquaculture fish include pathogenic microorganisms, antimicrobial or drug residues and environmental contaminants. It has been reported that fish can serve as vehicle of *Salmonella* transmission to public (Hradecka *et al*., 2008). Prevalence of *Salmonella* spp. in chicken, eggs and feed has been reported by many researches previously (Arshad *et al*., 2006). Thus it was postulated that, this is one of the possible pathway how *Salmonella* spp. was introduced to catfish aquaculture in Kelantan. The feed made from chicken offal, spoiled eggs and commercial fish feed can transfer *Salmonella* spp. to the aquaculture environment and feed serves as a source for *Salmonella* spp. growth in fish and aquaculture water (Lunestad *et al*., 2007). Budiati *et al*. (2012) stated that, the prevalence of *Salmonella* spp. in catfish fed with chicken offal or homemade food was relatively higher than the fed with only commercial fish feed. The outbreak of fish-related *Salmonellosis* in human was believed to be due to improperly cook of contaminated fish. Novotny *et al.* (2004) reported that the fish could become a vector of *Salmonella* spp. infection and smoking process may not eliminate bacterial contamination from raw fish. Improper storage or cross-contamination of food by contaminated raw fish or utensil was also the factors of *Salmonellosis* outbreak in human. An epidemiological investigation proved that there was no estimate available for foodborne illness attributed to channel catfish meat because catfish are usually cooked to sufficient temperature to kill microbial pathogen. Several studies reported and demonstrated that raw or undercooked animal-source proteins such as catfish may be contaminated with a variety of pathogenic organism including *Salmonella* spp. (Finley *et al*., 2006). Therefore, fish-associated food borne *Salmonellosis* outbreak could happen primarily in animal and human due to consuming the improper cooking of contaminated raw catfish or improper handling of contaminated raw catfish, or, it could happen as secondary *Salmonellosis* outbreak in human (owner) from infected animals due to consumption of contaminated raw catfish. This is related to 'One-Health' issues. According to Thong (2006), the *Salmonella corvallis* and *Salmonella typhimurium* caused non-typhoidal *Salmonellosis* in human in Malaysia during the year 2005. From the above discussion it is clear that Thai koi fish farm could be highly infected by *Salmonella* spp. which may come through fish feed or culture environment or both and can causes a great harm to koi culture system in Bangladesh. The other bacteria *Staphylococcus* spp. is also responsible for fish bacterial diseases which are also reported by different scientists. Richards *et al.* (1999) found that *Staphylococcus aureus* is responsible for skin and soft tissue infections, pneumonia, bloodstream infections, osteomyelitis and endocarditis, as well as toxin-mediated syndromes like toxic shock and food poisoning. According to Shittu *et al*. (2009) *S. aureus* is an important pathogens in acquired infection. It has developed resistance to a wide range of antimicrobial drugs, which complicates the treatment of infections. In particular, methicillin-resistant *Staphylococcus aureus* has become a notorious etiologic agent for a wide variety of infections and it is one of the most important nosocomial pathogens worldwide. With the use of tissue Gram staining method, the presence of Gram-positive cocci shaped cells around the necrotic tissue cells and erythrocytes in the heart and liver tissues were detected which is related with present study. From the present study it could concluded that bacterial infection is the most important causes of skin ulcer of fish leading to high mortality of fishes in farm that can causes great economic losses for fish farmer. But all bacteria are not responsible for primary infection, like *Shigella* spp. *and Klebshiella* spp. do not

produce ulcerative symptoms and are mainly responsible for secondary infection. So before any bacterial disease treatment it is essential to identify the causative bacteria first. This was the main proposes of present study. Overall the selected farm should be periodically checked for the prevention of infection and positive reactor should be culled as well as bio-security plan of the farms should be followed strictly to reduce the risk of bacterial diseases.

6. Conclusions

The research work was conducted to isolate and identify pathogenic bacteria from Thai koi that was collected from Fulbari Upazilla of Dinajpur District. Among Economically important disease of koi (*Anabas testudineus*), skin ulcer is one of the most well-recognized and able to create a great loss in production that are causes by bacteria. Bacteria isolated from different body part of the infected Koi fish (*Anabas testudineus*), are *Staphylococcus* spp., *Salmonella* sp., *Klebshiella* spp., and *Shigella* spp. These bacteria are the normal inhabitant of the skin, gill, muscle, and the slime of diseased and healthy fishes. Among the identified bacteria, *Salmonella* spp. and *Staphylococcus* spp. was confirmed after completing experimental test are the actual causative agent of bacterial diseases in koi fish. They are also the important opportunist that can cause superficial to life-threatening illness in koi fish including ulcerative symptoms. The other two bacteria *Shigella* spp. and *Klebshiella* spp. may be occur secondary infection in Thai koi (*Anabas testudineus).* This is a preliminary work on bacterial diseases, especially for identification of causative agent of bacterial diseases for koi fish and based on biochemical examination. More works like molecular identification of bacteria are needed for rapid and affordable monitoring of bacterial diseases of koi fish culture in Bangladesh.

Acknowledgements

This research work was supported by Department of Microbiology and Department of Aquaculture, Hajee Mohammad Danesh Science & Technology University, Dinajpur, Bangladesh.

Conflict of interest

None to declare.

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