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Antibiogram of Pathogenic bacteria isolated and identified from edible fish *Labeo rohita*

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Abstract

The microbiological studies on freshwater fish culture appear to be limited predominantly in India. Consequently a study was done to identify the bacterial populations and pathogens in the sample water and in some tissues of cultured carp *Labeo rohita*. Among the various organs analyzed, the maximum bacterial load was found in gills followed by intestine and skin surface of the infected *Labeo rohita*. In general, the bacteria isolated from fish samples appeared to be very similar to those obtained from water. The Bacterial isolates were confirmed based on the morphological, Physiological and Biochemical Characteristics using standard methods. In biochemical test it is observed, that the bacteria was 90 percentage confirmed as Klebsiella genus which is gram negative and rod shape. For further confirmation, the bacterial sample have been inoculated with specific selective medium (Mac Conkey Agar Medium) and confirmed that the bacteria as *Klebsiella pneumoniae* by the appearance of the colony and change in the colour of the medium. Among the 13 antibiotic discs, certain antibiotics were sensitive such as Gentamicin-32mm, Ofloxacin-29mm, kanamicin-28mm and tetracycline-25mm against the pathogenic bacteria *K. pneumoniae*. This present study shows that Gentamicin, Ofloxacin, Kanamycin and Tetracycline can be used as a drug against the pathogenic bacteria *K. pneumoniae* for the infected fish (*Labeo rohita*).The above mentioned antibiotic disc against *K. pneumoniae* induced the growth of *Labeo rohita* and promote the aquaculture production and increase the economic status of aquaculture.

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Introduction

Concomitant to the increasing world population, the aquaculture sector continuously strives to be more productive and sustainable in order to outspace the growing population demands (FAO, 2012). Aquaculture has emerged as one of the most promising and fastest-growing industries, and provides high-quality animal protein with total global production increasing to 66.63 million tonnes in 2012 from 63.6 million tonnes in 2011 (FAO, 2014). Globally, carp production is still the most important group of aquaculture species, contributing over 72% of freshwater production (Kuhlwein *et al.,* 2014). Recent reports suggest that the share of fisheries production for human consumption has increased dramatically from 10 million metric tonnes in 1980s to 136 million tonnes in 2012 (FAO, 2014). However, the industry faces substantial economic losses owing to the disease outbreaks like Aeromoniasis, Columnaris, Streptococcosis, Furunculosis and other epizootics (Leung and Bates, 2013). Although the use of antibiotics and other potential chemotherapeutants to mitigate such pathogenic infestations is very well documented, the purported resurgence of antibiotic resistance and associated environmental issues cannot be ignored (Romero *et al.,* 2012).

Carps stand the world's most important group of aquaculture species in terms of production. Rohu is one of the most popular species especially in Asia and fetches high price. Its total global production is over 1.5 million tons in 2012 (FAO, 2013). Unavailability of fry as a result of high mortality (70–80%) during early stages has been one of the major obstacles in expanding its production. Poor survival of fish larva is mostly due to pathogen and unavailability of suitable diet (Edwards, 2013).

In addition to feed quality, bacterial infection causes mass mortality (Vadstein *et al.,* 2012). Poor growth, malformation, sudden decrease in survival, and lack of reproductive capacity are other important causes for unavailability of quality seedlings (Mohapatra *et al., 2012*). Traditionally, antibiotics and chemotherapeutics are used as either preventative or curative agents with at least partial success.

Although application of antibiotics and chemotherapeutics is quite effective, drug resistance and residual effects of these chemical/drugs are of major public health concerns.

Therefore, the present study aimed to isolate and identify the pathogenic bacteria from the infected fish (*Labeo rohita*) and to identify antibiotics for the specific pathogenic bacteria which were isolated from infected fish *Labeo rohita*.

Materials and methods

Infected fishes (*Labeo rohita*) were collected from Tamil Nadu fisheries Development Corporation, Aliyar, Pollachi, Coimbatore District, Tamil Nadu, India. It was transported to the laboratory by using of thermocol ice box. The Length & weight of infected Fish (*Labeo rohita*) was measured.

Analysis of physico-chemical parameters of Water

The physico-chemical parameters viz., air and water temperature, pH, dissolved oxygen, total alkalinity, hardness, calcium, nitrates, nitrites, ammonia, phosphates, iron, fluorides, chlorides and residual chlorine were estimated in Aliyar Dam water.

Sample Preparation

Serial Dilution and Media Preparation

Sample was prepared by the method of Obi and Krakowiaka (1983). Each test tube containing 9 ml of distilled water as a stock. 1 ml of the stock (fish sample and distilled water) was collected using a pipette from the first test tube and then pipette out into the second test tube, the same process was repeated up to the fifth test tube respectively i.e. 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} respectively. 10^{-4} and 10^{-5} were used as the dilution factor and 1 ml was taken from each factor into a sterilized petridish in duplicate.

The media was prepared using nutrient agar dissolved in distilled water. It is then autoclaved at a temperature of 121^{0}C for 15 minutes, cool down and pour into each of the sterilized petridishes containing 1 ml of the diluents. It was then allowed to solidify.

Culture of Bacteria

A metal spreader should be flame sterilized by dipping in alcohol, shaking off the excess alcohol, and igniting the residue. The spreader is then allowed to cool.

The spreader is placed in contact with the inoculum on the surface of the plate and positioned to allow the inoculum to run evenly along the length of the spreader. Alternatively the spreader may be rotated over the agar surface. All plates were incubated at a temperature of 37^0C for 24 hrs, before colony counting and isolation procedures.

Bacterial Colony Count

After 24 hrs of incubation, Bacteria colonies were counted using colony machine (Digital Colony Counter). The number of colonies on the plate was multiplied and average count was taken for obtaining the total count

Identification of Bacteria

Gram's staining and Biochemical test

Gram staining procedure was carried out using Standard method to authenticate whether it is gram positive (or) gram negative. Biochemical test such as Carbohydrate fermentation (Durhams tube), Indole (Tryptophan degradation) Production test, Methyl red test (mixed fermentation), Voges - Proskauer test (Butanediol fermentation), Citrate utilization test, Catalase test, Oxidase test, Triple sugar iron (TSI) agar test, Urea hydrolysis test (Urease test), Starch hydrolysis test etc. were carried out for the identification of bacteria.

Antibiotic Susceptibility Test

Antibiotic susceptibility test was carried out by the disk diffusion technique using a commercially available disc (Hi-media).

The antimicrobial sensitivity of the test strains to thirteen antibacterial drugs was done using the Kirby-Bauer disk diffusion method (Bauer *et al.,* 1966).

The antibiotics used were ampicillin, chloramphenicol, erythromycin, gentamicin, kanamycin, methicillin, ofloxacin, penicillin G, polymyxin-B, rifampicin, tetracycline, streptomycin, vancomycin. A lawn of test pathogen (18 hours nutrient broth culture) was prepared by evenly spreading 100µl inoculums with the help of a sterilized spreader onto the entire surface of the agar plate.

The plates were allowed to dry before applying antibiotic disc. Some commercially available antibiotic discs were placed on the agar plates and left for 1 hour at room temperature for the diffusion of antibiotics into the agar medium. The Zone of inhibition in the plate indicates the presence of antimicrobial activity and it was measured in millimetre. When the inhibition zone was found to be above 19 mm, it indicates that the organisms are highly susceptible, intermediate between 15-18 mm and resistant when it is less than 13 mm.

Results and discussion

Water quality parameters

The Physico-chemical parameters have considerable influence on the growth of fishes. The Physico-chemical parameters, such as temperature, colour, pH, dissolved oxygen, alkalinity, hardness and other characters were tested in the water sample collected from the fish farm in Aliyar (Table 1).

At the same time the length and weight of the infected fish (*L. rohita*) were observed (Table 2) before the commencement of the experiment.

The pH lower than 4.5 and greater than 9.5 are generally hazardous to aquatic organisms which ultimately affects the growth, reproduction and other biological activities (RAMP 2016).

If there is an increase in the ammonia level in the aquatic ecosystem concomitantly there is a decreases in the DO and increase in the Co_2 (FAO, 1987). Russo *et al.,* (1981) reported that in contrast to ammonia, nitrite toxicity also increases at lower pH levels.

Table 1. Physico-chemical parameters of Aliyar Fish Farm water during the sampling day.

S. No.	Parameters	Units	Result
1	Atmospheric Temperature	^{0}C	32
2	Water Temperature	^{0}C	24
3	pH	-	7.5
4	Dissolved Oxygen	mg/l	6.5
5	Total alkalinity	ppm	190
6	Hardness	mg/l	110
7	Calcium	mg/l	30
8	Nitrates	mg/l	5
9	Nitrites	mg/l	0.5
10	Ammonium	mg/l	0.5
11	Phosphates	mg/l	0.5
12	Chlorides	mg/l	50
13	Fluoride	mg/l	1
14	Iron	mg/l	0.3
15	Residual Chlorine	mg/l	0.2

Table 2. Length and weight range of the infected fish (*Labeo rohita*).

Sl.No.	Name	Length	Weight
1	*Labeo rohita*	8.2cm	3.8g

Bacterial colony count

The bacterial counts in water and the various tissues of infected fish are shown in the Table 3 and 4. Comparisons of the bacterial count in various organs were analyzed. In this study, the maximum bacterial load was found in gills followed by intestine and skin surface. As per previous research work, they compared the bacterial count in the skin, among the four carps the *Cyprinus carpio* recorded the maximum count followed by *Cirrhinus mrigala, Labeo rohita* and *Catla catla*.

Table 3. Bacterial Counts in Pond water (per ml).

S. No.	Total Bacterial Count at Room Temperature	Total Bacterial Count at 37^{0}C
1.	2.5×10^4	1.65×10^4

Table 4. Bacterial Counts in Skin Surface, Gills and Intestine of Infected fish (*Labeo rohita*).

S. No.	Tissues	Total Bacterial Count at RT	Total Bacterial Count at 37^{0}C
1.	Skin surface	7.1×10^4	1.33×10^5
2.	Gills	6.7×10^4	1.69×10^5
3.	Intestine	9.7×10^4	1.57×10^5

RT - Room Temperature.

In gills, the maximum load was found in *C. catla* followed by *C. mrigala, L. rohita* and *C. carpio*. However, bacterial counts in intestine reveals that the maximum load was found in *C. mrigala* followed by *C. catla*, *L. rohita* and *C. carpio*. Thus among the various organs analyzed, the maximum bacterial load was found in skin followed by gills and intestine for all the fishes (Saraswathi *et al*., 2015).

This study showed the symptoms of infected fish in and around the skin, gills and intestine, due to increase in the secretion of mucous mass on the surface of skin, gills, inflammed areas on body, swollen abdomen, necrotic intestine, colouration to the skin and fins.

These observations are in correlation with the earlier studies made by several investigators (Cann and Taylor, 1982).

Gram's staining and Biochemical test

The bacterial isolates were confirmed, based on the morphological, physiological and biochemical characteristics using standard methods. The results of the gram stain and the biochemical tests were presented in Table 5.

Table 5. Biochemical Tests for Identification of Bacteria.

S.No	Test	Results
1.	Gram's stain	-ve
2.	Morphology	Rod
3.	Arrangement	Single
4.	Catalase	+ve
5.	Oxidase	-ve
6.	Indole	-ve
7.	Methyl Red	-ve
8.	Voges-Proskeur	+ve
9.	Gas Production	-ve
10.	Citrate Utilization	+ve
11.	Triple Sugar Iron Agar (H_2S production)	-ve
12.	Urea Hydrolysis	+ve
13.	Starch Hydrolysis	+ve

Table 6. Antibacterial susceptibility analysis against *Klebsiella pneumoniae.*

S.No	Antibiotics name		Reaction of Strain	Inhibition Zone (mm)
1.	Ampicillin	AMP	R	02
2.	Chloramphenical	C	S	25
3.	Erythromycin	E	R	08
4.	Gentamicin	GEN	S	32
5.	Kanamycin	K	S	28
6.	Methicillin	MET	R	02
7.	Ofloxacin	OF	S	29
8.	Penicillin G	P	R	03
9.	Polymyxin-B	PB	S	10
10.	Rifampicin	RIF	R	01
11.	Streptomycin	HLS	I	14
12.	Tetracycline	TE	S	25
13.	Vancomycin	VA	S	24

R - Resistant (<13mm), S – Sensitive (>19mm), I – Intermediate (14-18mm).

The result shows that, the bacteria was gram negative as well as rod shaped and in the biochemical test observation, the bacteria was 90 percentage confirmed as Klebsiella genus (Fig.1 and 2). For further confirmation, the bacterial sample have been inoculated with specific selective medium (Mac Conkey Agar Medium) and confirmed that the bacteria is *K. pneumoniae* by the appearance of the colony and change in the colour of the medium (Fig. 3).

In the present study, *K. pnemoniae* which belong to the family Enterobacteriaceae is a predominant pathogen isolated in the intestine of infected fish (*L. rohita*). These results correlate with many investigators, enteric bacteria have been reported to be the dominant Histamine-Producing Bacteria (HPB) in fishes (Taylor and Eitenmiller, 1986).

K. pneumonia were also identified in rainbow trout (*Oncorhynchus mykiss Walbaum*), gills and intestine of Tilapia zilli and smoked African catfish (*Clarias gariepinus)* (Daskalov *et al.*, 1998; Ogbonna *et al.,* 2008; Ayeloja *et al.*, 2011). All the strains of *K. pneumoniae* isolated from fishes, exhibited biochemical characters. They were catalase positive, utilised citrate, melanate and lysine, reduced nitrate to nitrites and hydrolysed urea. In previous study, they observed abnormal variations in biochemical composition in difference tissues of *N. japonicus* in relation of *klebsilla* infection (Diana and Ramulu, 2010).

IDENTIFICATION OF BACTERIA

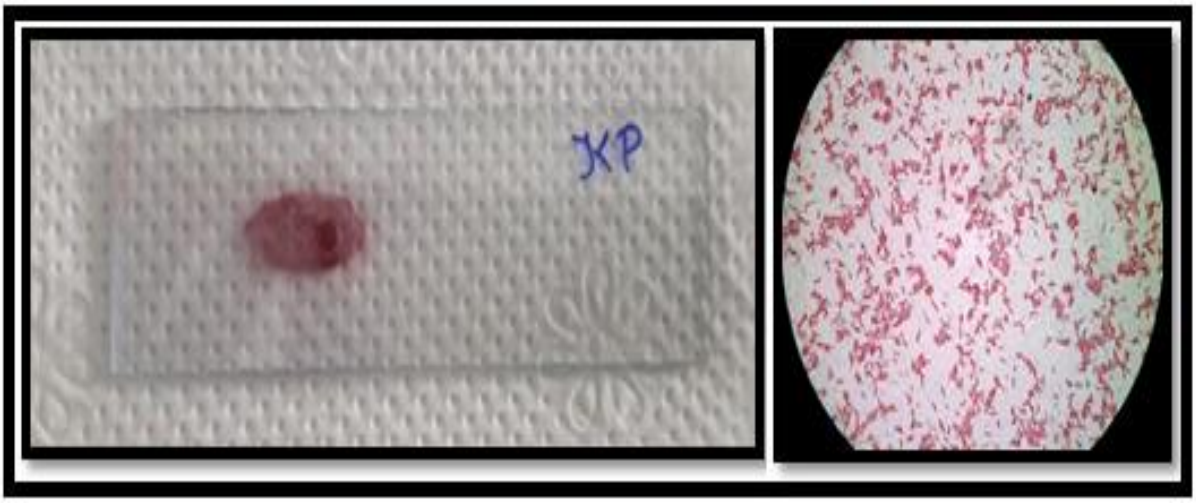

Fig. 1. Gram stain test- Gram negative Bacteria.

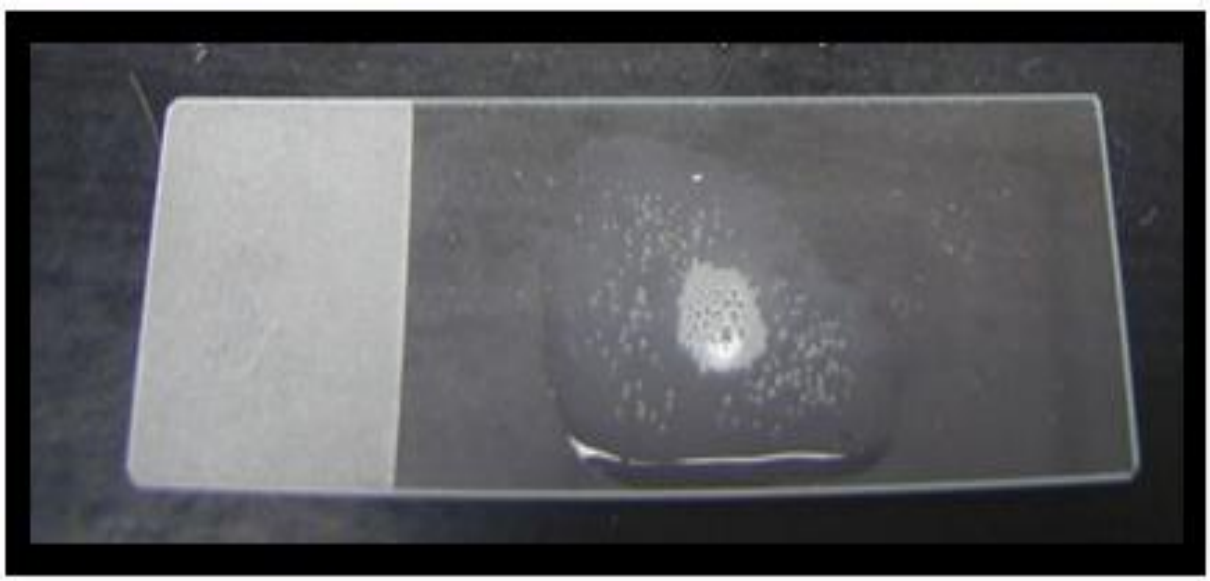

Fig. 2. Catalase Test - Positive.

Antibiotic Susceptibility Test

Antibacterial sensitivity testing of the confirmed *K. pneumoniae* isolates was done on nutrient agar plates. On the basis of resistance to bacteria, strains were categorized into three groups. In this study, one group of strains, were sensitive (over 85%) to chloramphenicol, gentamicin, kanamycin, vancomycin, ofloxacin, tetracycline and the zone of inhibition ranges from 23-32 mm (Fig. 1 and 2), the second group of strains were moderately susceptible (intermediate- 62%) to rifampicin, streptomycin, polymyxin-B, and observed zone of inhibition was in

range between 14-15 mm and third group of strains were resistant to ampicillin, methicilli, erythromycin, rifmpicin and penicillin G (Table 6). Similarly, Archana Singh Sikarwar and Harsh Vardhan Batra in their study reported that, the strains of *K. pneumonia* were found to be highly susceptible to quinolones, aminoglycoside, amykacin and gentamycin, were 60% of strains are resistant to chloramphenicol and tetracycline and remaining 28 to 76% are resistant to cephalosporins.

CONFIRMATION OF BACTERIA

Fig. 3. *Klebsiella pneumoniae* on Mac Conkey Agar Plate.

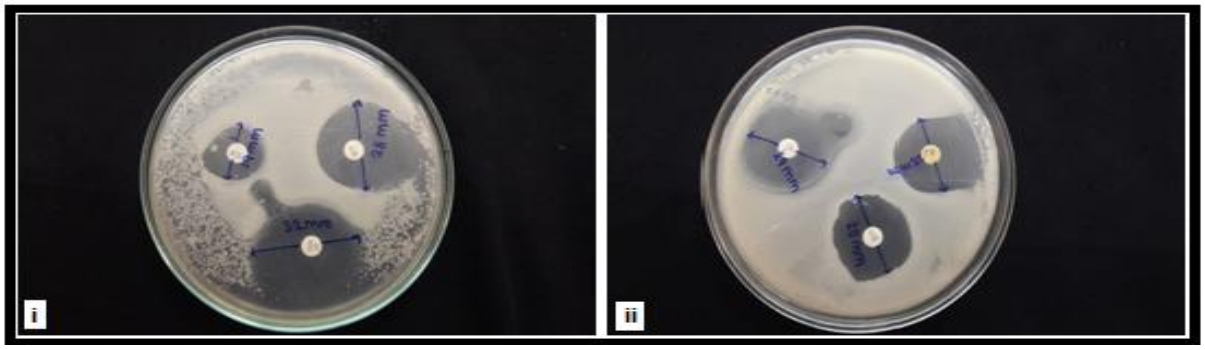

Fig. 4. i. Antibacterial susceptibility measuring zone (Vancomycin, Kanamycin, Gentamicin).
ii. Chloramphenical, Tetracycline, Ofloxacin.

Conclusion

The present result shows that the *K. pneumoniae* was identified and confirmed by biochemical test and by the specific selective medium (Mac Conkey Agar Medium) isolated from the infected fish *L. rohita*. Various antibiotic discs were treated against the *K. pneumoniae*, among them the best results were obtained from gentamicin, ofloxacin, kanamycin and tetracycline antibiotics. The above mentioned antibiotics not only inhibit the growth of *K. pneumoniae* but it also promotes the growth of *L. rohita* in order to increase the economic status of aquaculture.

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