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Isolation and identification of pathogenic bacteria and its antibacterial susceptibility in edible fish *Cirrhinus mrigala*

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Abstract
In the present study, the pathogenic bacteria was isolated from the infected fish *Cirrhinus mrigala*. *Citrobacter freundii* species were identified from intestine of infected fish. Antibacterial discs were treated against the *Citrobacter* species, various zone of inhibition was recorded to conform the sensitive and resistance against the *Citrobacter freundii*. Among the 13 antibacterial discs, certain antibiotic were sensitivesuch Chloramphenical -32mm, Streptomycin -28mm, Gentamicin -26mm, Vancomycin -19mm etc… against the pathogenic bacteria, *Citrobacter freundii.* Results of the present study showed that Chloramphenical, Streptomycin, Gentamicin, Vancomycin can be used as a drug against the pathogenic bacteria *C.freundii* for the infected fish (*Cirrhinus mrigala*).The above mentioned Antibacterial disc against *C.freundii* induced the growth of *Cirrhinus mrigala* and promote the aquaculture production and increase the Economic status of Aquaculture.

Keywords: *chirrhinus mirgala, citrobacter freundii,* streptomycin

1. Introduction
Fisheries in India are a very important economic activity and a flourishing sector with varied resources and potentials. Only after the Indian Independence, has fisheries together with agriculture been recognized as an important sector. The freshwater aquaculture comprises of the culture of carp fishes, culture of catfishes, freshwater prawns, pangasius, and tilapia. Thus, the production of carp in freshwater from the bulk of major areas of aquaculture activity. The three Indian major carps, namely catla (*Catla catla*), rohu (*Labeo rohita*) and mrigal (*Cirrhinus mrigala*) contribute the bulk of production to the extent of 70 to 75 percent of the total fresh water fish production [1].

Mrigal is popular food fish and an important aquacultured freshwater species throughout South Asia. Indian major carp, *Cirhinus mrigala* is one of the commercially and highly cultivable species among aquaculture species. However, bacterial infections are the major constraint for rapid cultivation of these species and moreover, it is believed that contaminated water is one of the root cause for the origin of bacterial-borne infection in fishes.

Major pathogens that are affecting the aquaculture industry include: bacteria, fungi, viruses and parasites.Bacterial species belonging to 13 genera have been reported to be pathogenic to aquatic animals, including: (1) Gram-negative bacteria such as *Aeromonas, Edwardsiella, Flavobacterium, Francisella,* Photo bacterium, *Piscirickettsia*, *Pseudomonas*, *Tenacibaculum, Vibrio* and *Yersinia, Klebsiella*. (2) Gram positive bacteria such as *Lactococcus, Renibacterium* and *Streptococcus*. Major pathogenic Gram negative and Gram-positive bacterial species reported.

Antibiotics are among the most-used drugs in veterinary medicine [2]. The Principle reasons behind the control of infectious diseases in hatcheries are to prevent losses in production; to prevent the introduction of pathogens to new facilities when eggs, fry, or broodstock are moved; to prevent the spread of disease to wild fish via the hatchery effluent or when hatchery fish are released or stocked out; and to prevent the amplification of pathogens already endemic in a watershed [3, 4, 5]. Antibacterials are drugs of natural or synthetic origin that have the capacity to kill or to inhibit the growth of micro-organisms. Antibiotics that are sufficiently non-toxic to the host are used as chemotherapeutic agents in the treatment of infectious diseases amongst humans, animals and plants.

2. Materials and Methods
In the present study, *Cirrhinus mrigal* is used as an experimental animal

2.1 Scientific classification of fish
Taxonomy position

Kingdom	-	Animalia
Phylum	-	Chordata
Class	-	Oesteichthyes
Order	-	Cypriniformes
Family	-	Cyprinidae
Genus	-	*Cirrhinus*
Species	-	*mrigala*

2.2 Collection site
Infected fish (*Cirrhinus mrigal*) were collected from Tamil Nadu fisheries Development Corporation. Aliyar, Pollachi. Coimbatore district Tamil Nadu. India. It was transported to the laboratory.

2.3 Analysis of physico-chemical parameters of Water
The seasonal wise physico-chemical parameters viz., air and water temperature, pH, total alkalinity, hardness, calcium, nitrates, nitrites, ammonia, phosphates, irons, flurides, chlorides, and total resudial chloride. The water samples were analysed for the physio-chemical parameters was analysed by [6, 7] and Dissolved Oxygen was analysed by [8]. The Length and

weight of infected fish (*Cirrhinus mrigal*) was measured by using ruler scale and weighing balance respectively.

2.4 Culture and isolation of Bacteria

For the culture and isolation of the pathogenic bacteria, Methods suggested by OIE were followed. The specemens of diseased fish were collected and dissected; the affected tissue (Gill /Intestine) was taken in a test tube, homogenized mannually and spread over the nutrient agar medium in Petri plates under aseptic conditions. These plates were incubated in B.O.D at 30 ± 1C for 24 h. Bacterial growth on the nutrient agar plate was observed after 24 h.Pure colonies of bacteria were isolated and obtained further by sub-culturing of the single colonies on nutrient agar by proper streaking method [9]. These pure culture were stored at -20 C for further investigations/tests.

2.5 Bacterial colony counting

Bacteria colonies were counted using colony machine. The number of colonies on the plate is multiplied by the reciprocal of the dilution factor and calculation is done for 1 ml of original sample, and plating was done in duplicate foe each dilution. An average count was taken to obtain the total count.

2.6 Identification of Bacteria

Isolated pure cultures of bacteria were subjected to standard biochemical tests (primary and secondary) for identification aqs reported by Krieg and Holt [10]. The confirmation test of these bacteria was done with the help of selective media used for culturing that particular bacterium.

2.7 Grams Stainig

A smear is prepared on a clean slide using a sterile wire loop and fixed by heat or allowed to air dry. The smear is covered with crystal violet for 20 sec and rinsed with water to remove excess crystal violet. It is then covered with Gram's iodine for 1 min and rinsed with water to remove excess iodine solution. It is decolorized with 95% ethanol by holding the slide at 45° angle while adding decolorizing reagent drop by drop until the color stops running. The slide is immediately rinsed to remove the decolorizing agent and the smear covered with safranin for 1 min and rinsed again with water. The water from the slide is blotted out with filter paper, and dried. The smear is scanned under the microscope using oil immersion lens.

2.8 Antibiotic Susceptibility Test

Antibiotic susceptibility test is 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 [11]. The antibiotics used were ampicillin, chloramphenicol, erythromycin, gentamicin, kanamycin, methicillin, ofloxacin, penicillin G, polymyxin-B, rifampicin, tetracycline, streptomycin, and 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. Then, some commercially available antibiotic discs were gently and firmly placed on the agar plates, which were then left at room temperature for 1 hour to allow diffusion of the antibiotics into the agar medium. The plates were then incubated at 37°C for 24 hours. If an antimicrobial activity was present on the plates, it was indicated by an inhibition zone. The diameter of the inhibition zones was measured in millimeter at 24 hours using a scale. An organism is interpreted as highly susceptible if the diameter of inhibition zone is more than 19 mm, intermediate if diameter was 15-18 mm and resistant if the diameter was less than 13 mm. The intermediate readings were considered as sensitive in the assessment of the data.

3. Results

The physico-chemical parameters such as Temperature, pH, Dissolved oxygen, Alkalinity, Calcium hardness, Ammonia, Nitrite, Nitrate, Chlorine were studied and showed in Table-1. Table-2 showed the length and weight ranges of the infected fish (*Cirrhinus mrigal*) used for the present study.

Tables 3 and 4 showed the bacterial counts in water and among the various tissues of infected fish. A comparison of the bacterial count in various organs was analyzed, the maximum bacterial load was found in gills followed by intestine and skin surface.

The Bacterial isolates were confirmed based on the morphological, Physiological and Biochemical Characteristics of the isolates following Standard Method. The results of the gram stain and the biochemical tests were presented in Table 5. The result showed that, the bacteria was gram negative as well as Rod shaped and in the biochemical test observation, the bacteria was 90 percentage confirmed as *Citrobacter* genus. For further confirmation, the bacterial sample have been inoculated with specific medium (MacConkey Agar) and Confirmed this bacteria as *Citrobacter freundii.*

Table 6 showed the antibiotics used were ampicillin, chloramphenicol, erythromycin, gentamicin, kanamycin, methicillin, ofloxacin, penicillin G, polymyxin-B, rifampicin, tetracycline, streptomycin, and 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.

Citrobacter freundii species are facultative, anaerobic Gram negative bacilli of the Enterobacteriaceae family. The bacteria are rod in shape with a typical length of 1–5µm.Most *C.freundii* cells generally have several flagella used for locomotion. *C. freundii*is a soil organism, but can also be found in water, sewage, food and in the intestinal tracts of fishes, animals and humans. As an opportunistic pathogen, *C. freundii*is responsible for a number of significant infections. It is known to be the cause of nosocomial infections of the respiratory tract, urinary tract, blood, and many other normally sterile sites in patients. *C. freundii* represents about 29% of all opportunistic infections.

4. Discussion

Aquaculture is the farming of aquatic organisms such as fish, crustaceans, molluscs and aquatic plants. Aquaculture involves cultivating freshwater and saltwater populations under controlled conditions, and can be contrasted with commercial fishing, which is the harvesting of wild fish [12]. Aquaculture is globally expanding into new directions. A constant goal of global aquaculture is to maximize the effectiveness of production to optimize profitability.

The bacteria are transmitted by fish that have made contact with other diseased fish. Bacterial fish disease and infections are very common and are one of the most difficult health problems to Deal with [13].

Table-1 showed that various physio-chemical parameters such as pH-8.5, Dissolved Oxygen and Nitrite, Nitrate, Ammonia also shows the constant values. Similarly, the previous study showed that the physio-chemical parameters were significantly constant, such as pH-8.6, Dissolved Oxygen-8.79mg/l, Ammonia, Calcium, Phosphates, Total Solids etc [14].

In our present study, we have been taken the bacterial count from the various organs, such as Gills, Skin surface and Intestine of the infected *Cirrhinus mrigal*. Among this various organs, the highest amount of bacterial count was observed in Gills followed by Intestine and Skin surface. As per research work, they compared the bacterial count in the skin among the four carps reveals that *Cyprinus carpio* recorded the maximum count followed by *Cirrhinus mrigala, Labeo rohita* and *Catla catla.* Amongst the 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, in general, among the various organs analyzed, the maximum bacterial load was found in skin followed by gills and intestine for all the fishes.

The most predominant organism isolated from the skin surface, intestine and gills of fishes belonged to the Enterobacteriaceae family as has also been reported previous studies showed that on the bacterial microflora of some fresh water fishes in tropical water [15, 16, 17, 18, 19, 20].

The bacterial composition in all the fish species appeared to be a reflection of the bacteria found in the pond water. Several authors have been also reported that the bacterial flora of fish is a reflection of their respective environments [21, 22, 23, 24].

In this present study, we took an infected *Cirrhinus mrigal* and isolate the bacteria from the intestine. By the help of the biochemical test and by applying specific medium, we identified that the bacteria present in the intestine was *Citrobacter freundii*.

Before the 80's of the last century, there were indications that *Citrobacter freundii* can cause disease in fish. However, definite data were not obtained until the papers [25], when the organism was proved to be pathogenic for aquarium fish, and in the 90's for farmed fish. *Citrobacter freundii* was isolated from diseased Atlantic salmonids in Spain [26] and the USA [27], and from carp in India [28]. *Citrobacter freundii* belongs to Enterobacteriaceae family comprehending Gram negative bacilli, catalase positive and oxidase negative organisms. It is an important infectious agent recognized as opportunistic pathogen in medical and veterinary science [29, 30], responsible for systemic hemorrhages and gastroenteritis in animals [31] and human patients [32].

In our study, Figure 2 showed that infected fishes which disease causes symptoms of encompassing the skin, gills, and intestine, and examination of natural openings revealed an increase in the quantity of mucous mass on the surface of the skin and gills. Erosion and dropping off of scales were established on the skin. The gills were pale due to anemia. Diffuse bleeding was established on the ventral part of the abdomen. This important disease causes a generalized infection with hemorrhagic gastroenteritis that has been reported in sunfish Molamola rainbow trout Oncorhynchus mykiss carp *Cyprinus carpio* doctor fish Garrarufaobtusa [33] and cachara P. reticulatumas here described.

Fig: 1 Fish culturing site

Fig 2: Infected Fish (*Cirrhinus mrigala*)

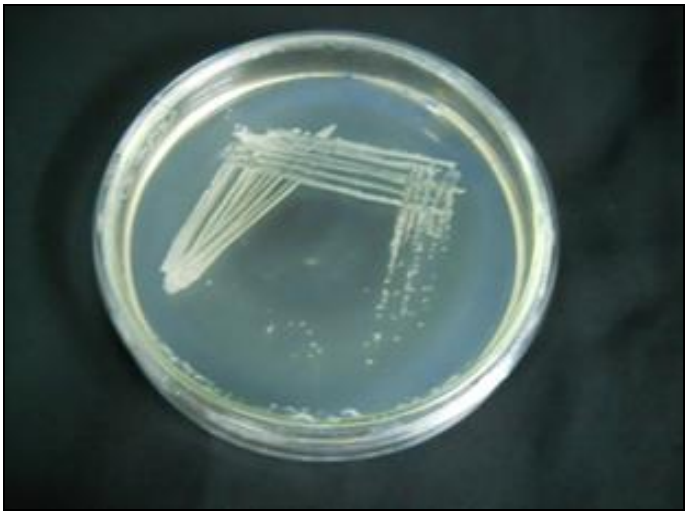

Fig 3: Pure Culture of Bacteria

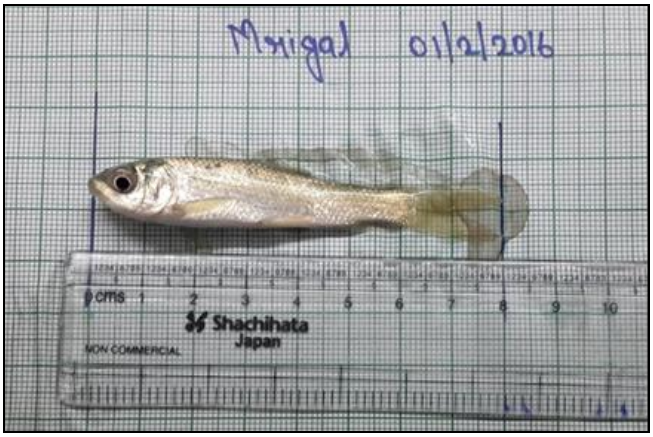

Fig 4: Length measurement of infected fish

Fig 5: Weight measurement of infected fish

Table 1: Physiochemical water quality parameters in Aliyar dam

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	0.5

Tables 2: Length and weight of the infected fish (*Cirrhinus mrigal*)

Sl. No.	Name	Size	Weight
1	*Cirrhinus mrigal*	7.8 cm	4.0 g

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 (*Cirrhinus mrigala*)

S. No.	Tissues	Total Bacterial Count at RT	Total Bacterial Count at 37^{0}C
1.	Skin surface	6.5×10^4	1.16×10^5
2.	Gills	6.5×10^4	1.49×10^5
3.	Intestine	8.8×10^4	1.42×10^5

RT - Room Temperature

Table 5: Biochemical Test for Identification

S. No	Test	Result
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.	Carbohydrate Fermentation	+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 *Citrobacter freundii*

S. No	Antibiotics name		Reaction of Strain	Inhibition Zone (mm)
1.	Ampicillin	AMP	R	02
2.	Chloramphenical	C	S	32
3.	Erythromycin	E	R	02
4.	Gentamicin	GEN	S	26
5.	Kanamycin	K	R	12
6.	Methicillin	MET	R	02
7.	Ofloxacin	OF	R	10
8.	Penicillin G	P	R	01
9.	Polymyxin-B	PB	I	14
10.	Rifampicin	RIF	R	03
11.	Streptomycin	HLS	S	28
12.	Tetracycline	TE	R	10
13.	Vancomycin	VA	S	19

R-Resistant (<13mm), S-Sensitive (>19mm), I-Intermediate (15-18mm)

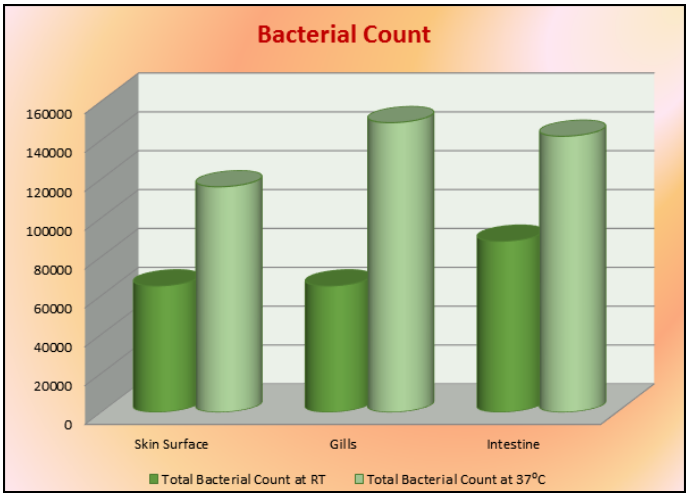

Fig 6: Bacterial Counts in Skin Surface, Gills and Intestine in Infected Fish (*Cirrhinus mrigal*)

Identification of bacteria
Gram stain

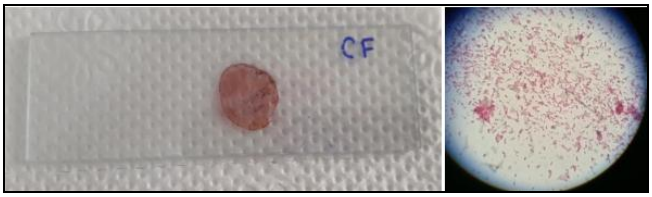

Fig 7: Gram stain test- Gram negative Bacteria

Catalase Test

Fig 8: Catalase Test – Positive

Biochemical Test

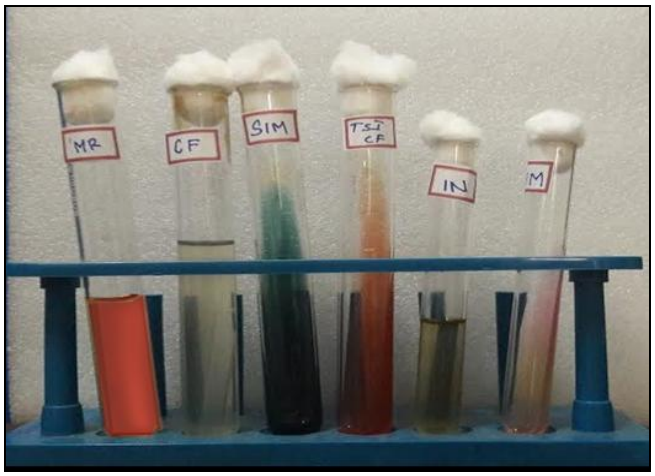

Fig 9: Biochemical Test

Confirmation of bacteria

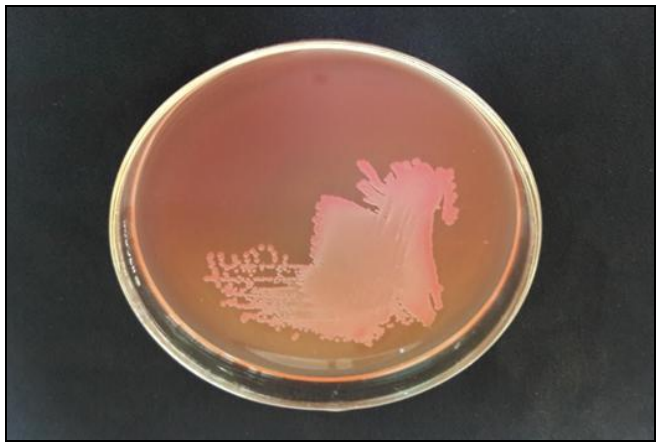

Fig 10: *Citrobacter freundii* on MacConkey Agar Plate

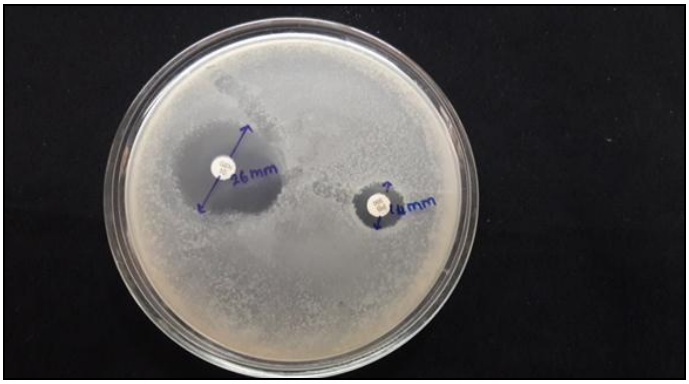

Fig 11: Antibacterial Susceptibility Measuring Zone. (Gentamicin, Polymyxin-B)

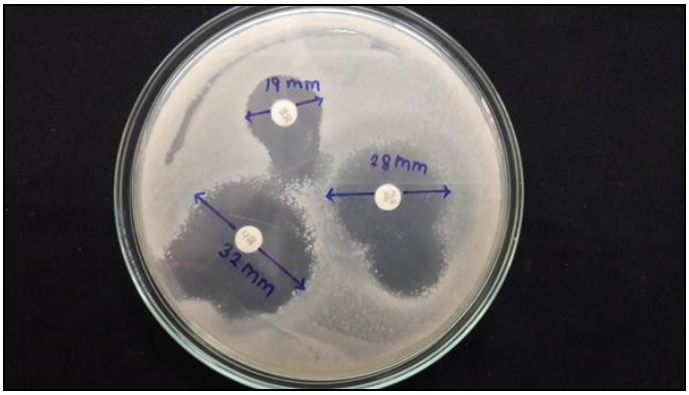

Fig 12: Antibacterial Susceptibility Measuring Zone (Chloramphenical, Vancomycin, Streptomycin)

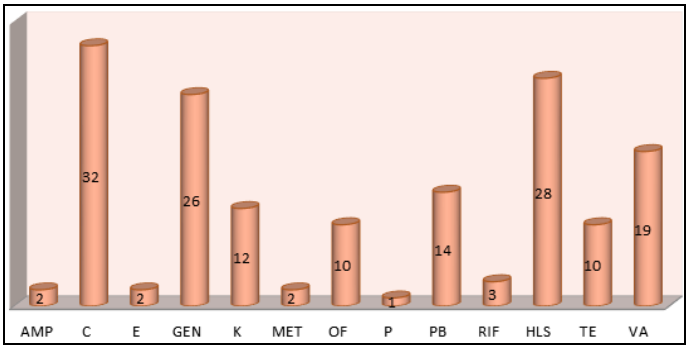

Fig 13: Antibacterial susceptibility analysis against *Citrobacter freundii*

5. Conclusion

Addressing the syndrome is very important in Fish culture as there is a rising require for them in both domestic and worldwide markets. A essential preventive measure has to be taken like quarantining the new fishes before introducing them with the existing stock. Prevention by means of good farming and maintaining water quality are keys to prevent the diseases.

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