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ISOLATION, IDENTIFICATION OF NOVEL VIRULENT BACTERIA *Enterobacter quasiroggenkampii AND Pseudomonas guezennei* sp. ASSOCIATED WITH FIN ROT DISEASE IN RED TAIL GOLDEN ASIAN AROWANA (Scleropages formosus)

Ankita Rajeshirke, Ujwala Jadhav
Research Scholar, Associate Professor
Department of Life Sciences
University of Mumbai, Vidyanagari, Santacruz (E), Mumbai-400098

ABSTRACT

Red Tail Golden Asian Arowana (*Scleropages formosus*) is a magnificent tropical freshwater fish commonly found in Thailand, Malaysia, Myanmar, Indonesia etc., and is considered as world's most expensive and demanding ornamental fish. Due to its prominent metallic golden shine, cycloid scales, elongated fins and undulating movements as it swims which is very much reminiscent to the Chinese lore ancient dragon, considered as an auspicious symbol giving rise to the belief that the fish brings good fortune, prosperity and success. Generally *S. formosus* are resistant to disease causing pathogens but occasionally they get infected by bacteria which lead to scale discoloration, loss of dorsal, caudal fin portions which causes fatal fin rot disease. Thus, in the current study chief virulent bacteria associated with fin erosion was isolated and identified from the Arowana maintained in laboratory conditions. The cause of the infection was attributed to the bacteria *Enterobacter quasiroggenkampii, Pseudomonas guezennei* that belongs to *Enterobacteriaceae* and *Pseudomonas* family i.e. cosmopolitan in nature and was responsible for mortality of majority different fish species. Accordingly, 16S rRNA gene sequencing, Antibiotic susceptibility test was done to identify the bacterial isolated from the infected fin of the fish. This is a pioneering study which reported *Enterobacter quasiroggenkampii* as a fish pathogen for the first time, a novel bacteria reclassified by Wu *et al*.2020 and also one of the chief causative agent for Red Tail Golden Asian Arowana fin rot disease. This study will also aid Arowana cultivators with the necessary information about fin rot disease and the ways for restricting or preventing the growth of virulent bacteria.

Keywords: ***Scleropages formosus, Enterobacter quasiroggenkampi,*** **Fin rot, Arowana,** ***Pseudomonas guezennei***

1. INTRODUCTION

In Aquaculture, commercial value of Red Tail Golden Asian Arowana (*Scleropages formosus*) is determined by its unique shape, elongated, slim steam lined body appearance and lustrous golden shine entitled for one of the most adored and precious fish reared in aquarium. *Scleropages formosus* has special cultural significance in many Asian countries and it is also known as "Chinese Ancient Dragon fish", which brings lucks and fortune,

due to this belief *Scleropages formosus* has become the leading choice for many aquarist throughout the world (Pouyaud *et at.*, 2003). Red Tail Golden Asian Arowana is generally considered as a resilient fish but disease outbreak may occur which flare up to a fatal condition. The biggest challenge while rearing these fish in captive is to grow a disease free and achieve bright coloration which result from good water parameters, low crowded area and proper filtration of leftover food and fish excretes. In aquarium, several types of bacteria are opportunistic pathogens and major infections are caused by *Enterobacter* sp, *Aeromonas* complex, *Flexibacter* sp., *Pseudomonas* sp., *Vibrio* sp., *Mycobacteria* sp etc.

Fin erosion disease is one of the major determinant for weaken health indicator of *Scleropages formosus* which bring about loss of appetite, erosion and shredding of fins and tail, dullness in the golden shine of the scales if not treated immediately. So changing the trends of rearing an exotic expensive fish especially for Asian countries like India where drastic seasonal changes occurs, due to which the domestication of this fish in such an environment becomes a big challenge in ornamental fish keeping sectors. Red Tail Golden Asian Arowana provides high income source to fish traders and diseased ornamental fish results into major economic losses. Fin rot disease is found in different fish aquariums and the rate of incidence of this type of disease is assumed to be increased in the recent years (Faruk *et al*., 2004).

*Enterobacte*r and *Pseudomonas* sp. are extensively spread in nature. *Enterobacter quasiroggenkampii* which was reclassified by Wu & others in 2020, is a genus comprising of Gram negative, facultative anaerobic, rod shaped, non-spore-forming bacteria of the family Enterobacteriaceae, is reported as a fish pathogen for the first time by us. In this study, bacteria from infected fin of the fish were isolated and characterized and identified using 16SrRNA gene sequence. Furthermore, Antibiotic susceptibility test were carried out to prevent the growth of microbes and it also gave an opportunity to treat the fishes and keep them healthy.

2.MATERIALS AND METHODS

2.1. Bacteria isolation

A live diseased fish with clear fin rot infection on the tail and fins was aseptically transferred to the nursing tank. Sample from the diseased fish was taken by rubbing the sterilized cotton swab over the infected fin of the fish in duplicates in sterile screw cap vials (Haftu R.*et al.*, 2012). Both the tubes were aseptically opened inside a laminar air flow cabinet and serial dilutions up to 10^{-6} dilution of sample were prepared in sterile saline solution. From each serial dilution 100 mL of suspension was spread over duplicate Nutrient Agar plates and incubated at 37 °C for 24 hrs. Following the incubation colonies that appeared with different morphological features were selected and separately inoculated on fresh nutrient agar plates. Cultures were maintained and stored at 4 °C for any additional work.

2.2. Amplification of 16S rRNA and sequencing

After isolation, phenotypic characteristics (colonial morphology, microscopic appearance), Gram's staining technique was performed. Then pure cultures of the isolates were identified using 16S rRNA gene sequencing using universal primers. DNA of bacterial strain was extracted by using M-N Nucleospin DNA kit. A partial 16S rRNA gene was amplified by using universal primers 8F and 806R, namely 8F 5'-AGAGTTTGATCCTGGCTCAG-3 'and 806R -5' TAATCTWTGGGVHCATCAGG- '. Then PCR was performed in Applied Biosystems Thermal Cycler with the initial denaturation for 5 min at 94ºC. First step of 40 cycles of denaturation at 94ºC for 1 min. followed by second step annealing at 55ºC for 1 min and extension at 72ºC for 2 min and final extension of 10 min at 72ºC. The amplification of 16S rRNA of the isolate was confirmed by running the amplification product on 2% agarose gel electrophoresis in 0.5X TBE buffer. Moreover sequencing of 16S rRNA gene amplification products was done on Applied Biosystems 3130 Genetic analyzer using Applied Biosystems Big Dye Terminator v3.1 Cycle sequencing Kit and then further comparison was made with previously available sequences in NCBI (National Center for Biotechnology Information) using BLAST (Blast Local Alignment Search Tool).

2.3. Antibiotic susceptibility test of the isolates

The standard Agar-disc-diffusion method was used to examine the Antibiotic susceptibility of the pathogenic bacteria on Nutrient Agar isolated directly from the fish samples (Au-Yeung. *et al.*, 2022) Antibiotic discs of commonly available antibiotics such as Gentamicin (SD016), Penicillin (SD028), Chloramphenicol (SD081),

Ciprofloxacin (SD080) and Amoxicilin (SD001) manufactured by Himedia were used and then Plates with LB Agar for rapidly growing aerobic organisms were prepared as per Bauer-Kirby Method. Around 4-5 similar colonies were inoculated with a wire loop to 5 ml Tryptone Soya Broth (M011) and incubate at 37°C for 8 hours until light to moderate turbidity develops. The inoculum turbidity was measured on spectrophotometer, as the inoculum achieved OD=0.2 with turbid suspension at 625 nm, it was further used for culturing by spread plate method. Discs were placed in the plate at appropriate distance, and plates were incubated immediately at 37°C and examine after 16-18 hours. The zones showing complete inhibition were measured and the diameters of the zones to the nearest millimeter were recorded using a calibrated scale.

3. RESULTS

3.1. Isolation of microorganism

A live diseased fish with clear fin rot infection on pectoral and caudal fin (Fig 1.b) was collected from Aquaculture Technology Laboratory, Department of Life Sciences, University of Mumbai aquarium for the isolation and identification of the virulent microbial species. Total two bacterial strains were isolated using 10^{-1} dilution method. Based on physiological, morphological, staining reactions, sub culturing, 16S rRNA gene sequencing *Enterobacter quasiroggenkampii* and *Pseudomonas guezennei* were identified as the main pathogens.

Figure 1.a: Live healthy Red Tail Golden Asian Arowana (*Scleropages formosus*) in aquarium with prominent golden colour.

Figure 1.b: Live diseased fish with 'fin rot' infection on pectoral and caudal fin along with dull colored scales.

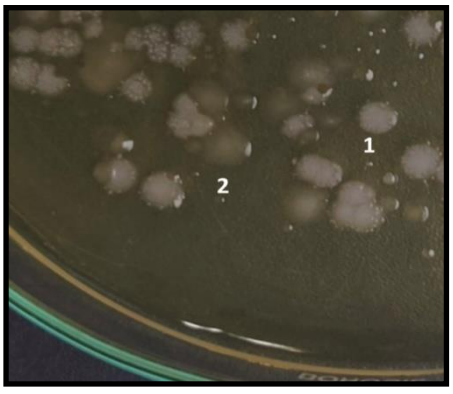

Figure 2: Colonies numbered (1) and (2) having different colony characteristics were selected for further identification.

Characteristics	Colony 1	Colony 2
Gram character	Gram negative rods	Gram negative rods
Morphology margin	Flat irregular	Elevated irregular
Motility	Motile	Motile
Shape of colony	Round	Round
Color	Off white	Yellow

Table 1: Colony Characteristics

3.2. Sub culturing results of isolates.

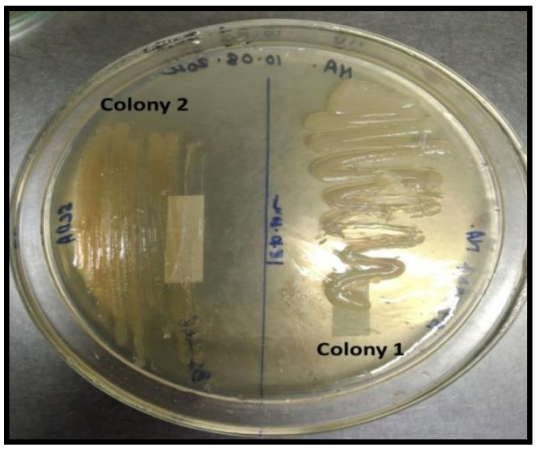

Figure 3: Isolated colonies were sub cultured to a fresh plate.

3.3. Identification of bacteria by 16S rRNA gene sequencing

a. Comparative analysis of the sequences (Fig. 4) with already available database showed that colony 1 strains were closed to the members of genus *Enterobacter*. However, colony 2 showed closest match with bacterial strain from genus *Pseudomonas*. The highest sequence similarities of wastewater bacteria as follows:

>Colony1_16S_seq_BI5847 (600 bp)

AAGTAGCTTGCTACTTTGCCGGCGAGCGGCGGACGGGTGAGTAATGTCTG
GGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACC
GCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCA
GATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGG
CGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAG
ACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATG
GGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGT
TGTAAAGTACTTTCAGCGGGGAGGAAGGTGTTGAGGTTAATAACCTCAGC
AATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCC
GCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGC
GCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTG
GGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAA

>Colony2_16S_seq_BI5848 (561 bp)

GGATGCAATTCCCAGGTTGAGCCCGGGGCTTTCACATCCAACTTGCTGAAC
CACCTACGCGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTTC
GTATTACCGCGGCTGCTGGCACGAAGTTAGCCGGTGCTTATTCTGTTGGTA
ACGTCAAAACAGCAAGGTATTAACTTACTGTCCTTCCTCCCAACTTAAAGT
GCTTTACAATCCGAAGACCTTCTTCACACACGCGGCATGGCTGGATCAGGC
TTTCGCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGA
CCGTGTCTCAGTTCCAGTGTGACTGATCATCCTCTCAGACCAGTTACGGAT
CGTAGCCTTGGTGGGCCATTACCCCACCAACTAGCTAATCCGACCTAGGCT
CATCTGATAGCGTGAGGTCCGAAGATCCCCCACTTTCTCCCGTAGGACGTA
TGCGGTATTAGCGTTCCTTTCGAAACGTTATCCCCCACTACCAGGCAGATT
CCTAGGCATTACTCACCCGTCCGCCGCTGAATCATGGAGCAAGCTCCACTC

Figure 4: Nucleotide sequence data generated using 16S rRNA gene sequencing

Culture	NCBI BLAST Result	Accesssion No.	% Identity
Colony1_BI5847	*Enterobacter quasiroggenkampii*	NR_179166.1	100%
Colony2_BI5848	*Pseudomonas guezennei*	NR_114957.1	99.82%

Table 2: Identification details of *Enterobacter quasiroggenkampii* and *Pseudomonas guezennei.*

b. The nucleotide sequences of both isolates were submitted to Genbank database through NCBI Bankit tool. Accession number are SUB12448509 BI5847 OQ076289 and SUB12448509 BI5848 OQ076290 for *Enterobacter quasiroggenkampii* and *Pseudomonas guezennei* respectively.

3.4. Phylogenetic analysis of isolates with closest matching organisms

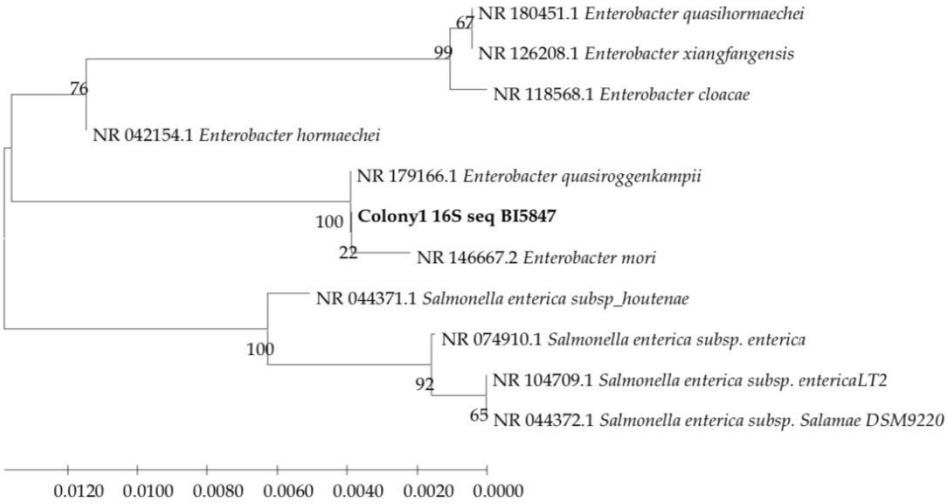

Figure 5: A Neighbour-joining tree constructed using MEGA-X software with 500 bootstraps has been shown in above figure. Colony 1 16S Sequence data shows closest phylogenetic relationship with NCBI Genbank sequence NR 179166.1 *Enterobacter quasiroggenkampii*

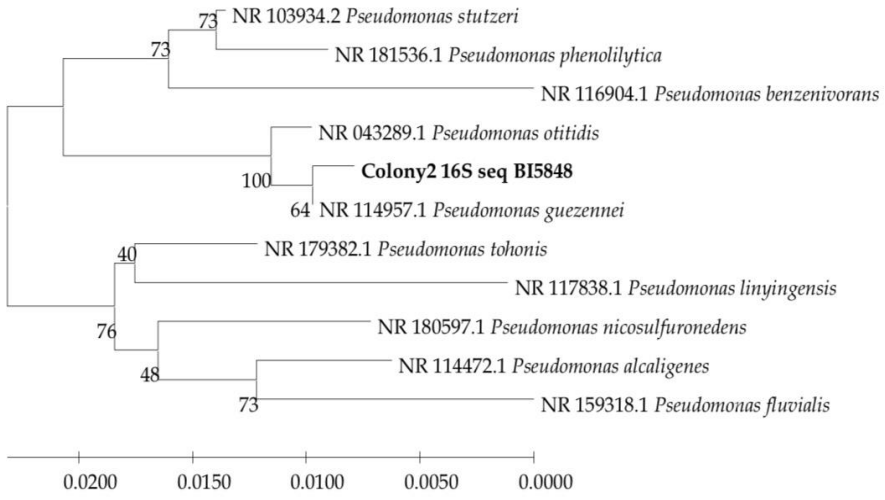

Figure 6: A Neighbour-joining tree constructed using MEGA-X software with 500 bootstraps has been shown in above figure. Colony 2 16S Sequence data shows closest phylogenetic relationship with NCBI Genbank sequence NR 114957.1 *Pseudomonas guezennei*

3.5. Results of antibiotic susceptibility testing

The zones of growth inhibition were recorded three times and the mean zones of inhibition were compared.

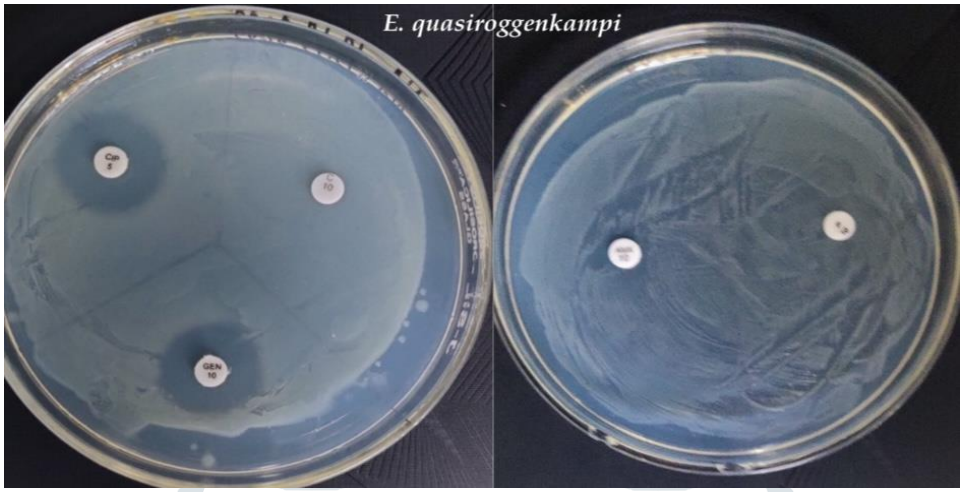

Figure 7: Culture plates after 16 hrs of incubation for *Enterobacter quasiroggenkampi* shows zone of inhibition developed for CIP -5 and GEN 10 antibiotic discs.

Disc Code	Antimicrobial agent	Zone of inhibition (nm)	Resistant	Intermediate	Susceptible
PG-10U	Penicillin	-	Yes	-	-
GEN-10mcg	Gentamicin	19	No (<12)	No (13-14)	Yes (>15)
Chl- 10mcg	Chloramphenicol	-	Yes	-	-
CIP-5mcg	Ciprofloxacin	22	No (<15)	No (16-20)	Yes (>21)
AMX-10mcg	Amoxicillin	-	Yes	-	-

Table 3: *Enterobacter quasiroggenkampi* shows zone of inhibition developed for CIP -5 and GEN 10 antibiotic discs.

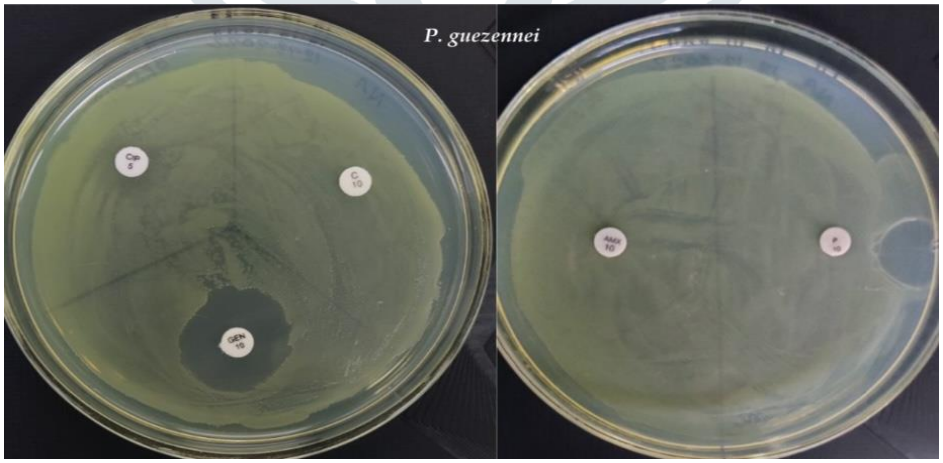

Figure 8: Culture plates after 16 hrs of incubation for *Pseudomonas guezennei* shows zone of inhibition developed for GEN 10 antibiotic discs only.

Disc Code	Antimicrobial agent	Zone of inhibition (nm)	Resistant	Intermediate	Susceptible
PG-10U	Penicillin	-	Yes	-	-
GEN-10mcg	Gentamicin	24	No (<12)	No (13-14)	Yes (>15)
Chl- 10mcg	Chloramphenicol	-	Yes	-	-
CIP-5mcg	Ciprofloxacin	12	No (<15)	No (16-20)	Yes (>21)
AMX-10mcg	Amoxicillin	-	Yes	-	-

Table 4: *Pseudomonas guezennei* shows zone of inhibition developed for GEN 10 antibiotic discs only.

Organism	Penicillin	**Gentamicin**	Chloramphenicol	**Ciprofloxacin**	Amoxicillin
Enterobacter quasiroggenkampi	Resistant	**Sensitive**	Resistant	**Sensitive**	Resistant
Pseudomonas guezennei	Resistant	**Sensitive**	Resistant	Resistant	Resistant

Table 5: Summary of antimicrobial susceptibility

It was observed that Colony1 16S sequence data showed the closest phylogenetic relationship with NCBI Genbank sequence NR179166.1 and Colony2 16S Sequence data shows the most intimate phylogenetic relationship with NCBI Genbank sequence NR 114957 which were closed to the members of genus *Enterobacter quasiroggenkampii* accession number SUB12448509 BI5847 OQ076289 accounting 100% identity followed by *Pseudomonas guezennei* accession number and SUB12448509 BI5848 OQ076290 accounting 99.82% identity respectively. The Antibiotic susceptibility test after 16 hrs of incubation for *Enterobacter quasiroggenkampi* shows zone of inhibition of 19.21mm for GEN 10 and CIP -5 antibiotic discs while *Pseudomonasguezennei* showed susceptibility towards GEN 10 antibiotic disc only with the development of about 24mm zone of inhibition on the agar plate.

4. Conclusion

In this study, we have isolated *Enterobacter quasiroggenkampii* and *Pseudomonas guezennei* from finrot infected Red Tail Golden Asian Arowana fish. Furthermore, Antibiotic susceptibility test were carried out to prevent the growth of microbes which also gave an opportunity to treat the fishes, keep them healthy and maintain its lustrous charm. The present study is the first to report *Enterobacter quasiroggenkampii* strain as a disease causative agent in fish.

5. References

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