

Isolation and Characterization of Bacteria from the Gut of Blue Gourami (*Trichogaster tricopters*) and its Role on Growth

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Abstract

Five variant groups were isolated from the gut of Blue gourami *Trichogaster tricopterus* through serial dilution, identified by using biochemical tests and bacteria were *Bacillus* sp., (G1) *Pseudomonas* sp., (G2) *Enterobacter* sp., (G3) *Aeromonas* sp., (G4) and *Escherichia* sp., (G5). Based on enzymatic productivity (Amylase, Cellulase, Lipase, and Protease) and antimicrobial activity *Bacillus* sp., *Enterobacter* sp., and *Aeromonas* sp., and mass multiplied using nutrient broth. Four different feeds such as feed I (Control), II (1ml *Bacillus* sp.), III (1ml each of *Bacillus* sp. and *Enterobacter* sp.) and IV (1ml each of *Bacillus* sp., *Enterobacter* sp. and *Aeromonas* sp.,) were prepared. Feed utilization parameters of blue gourami *Trichogaster tricopterus* were studied after 21 days of rearing and all feed utilization parameters were higher in feed IV.

Keywords: Gut bacteria, blue gourami, in vivo trial, growth parameters.

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INTRODUCTION

Ornamental fish rearing has emerged as the second most popular hobby next to photography due to its aesthetic beauty (Aly *et al.*, 2008). The trade-in ornamental fish and aquarium supplies are multi-million dollar industries that span the globe, with a retail value of approximately US\$ 500 million. Besides this, it also provides employment and revenue generation for the country (Balacazar, 2006). The major problem facing aquaculturists is the uncontrollable mortalities associated with disease and related disorders. Since the economic importance of aquarium fish is not less than that of the food fish. Ornamental fishes are susceptible to bacteria, viral, fungi, protozoa, and parasitic organisms and cause loss to the produce. Among the various pathogens affecting the cultured fish species, bacteria cause severe damage. Control of the bacterial disease is made possible by using drugs and antibiotics. The traditional use of antibiotics as growth promoters in aquaculture has been challenged because of the potential development of antibiotic-resistant bacteria. The use of vaccines is laborious, costly and highly stressful to the animals. Since these methods have certain limitations, alternative, productive methods must be examined to reduce the incidence of the pathogen in ornamental fish culture. The bacterial community in the gut of aquatic animals is much more crowded compared to terrestrial animals, as water serves an ideal medium for bacterial growth. The microbial network in the gastrointestinal tract of fish is very complex and plays a vital role in fish nutrition and disease prevention. The composition of the community of microbes in the fish gut is not constant and may change with nutritional status, age, surrounding water and other environmental conditions (Banerjee and Ray, 2017). However, the microbial balance in the gastrointestinal tract is crucial in response to host metabolism, disease prevention, and physiology. The microbial composition in the gut of vertebrates, including fish largely depends on the nutritional status of the host. Protease producing bacterial communities is dominant flora in carnivorous animals, which helps to degrade complex proteins to simple amino acids. Similarly, amylase and cellulose producing bacterial communities are reported to be highest in herbivorous animals (Ray *et*

al. 2012). In addition to host nutrition, the gastrointestinal microbiota serves a variety of other beneficial functions in the host such as preventing the colonization of infectious agents, energy homeostasis and maintenance of healthy mucosal immunity. The normal microflora in the intestinal tract of the fish includes *Pseudomonas* spp., *Aeromonas* spp., *Enterobacteriaceae* spp., *Micrococcus* spp., *Escherichia* spp, and *Bacillus* spp and these bacteria play a vital role in fish nutrition and disease prevention. Bairagi *et al.* (2002) have reported the existence of several enzymes producing bacterial strains, isolated from different freshwater fishes having different feeding habits. The study related to the isolation and characterization of bacteria from the gut of Blue Gourami *Trichogaster trichopterus* and its role in growth is entirely absent. So present research was undertaken.

MATERIALS AND METHODS

Fish collection

For the present study, Blue gourami, *Trichogaster trichopterus* were collected from Aqua Garden, Madurai, Tamil Nadu, India and transported to the laboratory in polyethylene bags filled with aerated water. Fishes were acclimated in glass aquaria (60 ' 45 ' 45 cm) for 15 days at $28 \pm 2^\circ\text{C}$. During acclimation fishes were fed with trainee feed containing fish meal, groundnut oil cake, wheat flour and rice bran in the form of dry Pellets.

Isolation of gut bacteria

After transportation to the laboratory the gut content of Blue gourami was collected, consequently diluted and 10^{-6} was selected for the separation of bacteria. The diluted sample was plated over sterilized nutrient agar medium and incubated at 37°C for 24 hours. (Bergey's Manual of Determinative Bacteriology, 1994). Nutrient Agar was used for the present study, different incubation temperature was used to obtain a wider range of isolation and the incubation time ranges from 24 hours, depending on the incubation temperature, groups were enumerated and separated.

Characterization of gut bacteria

The preponderant groups on the nutrient agar medium were selected and identified based on the cellular morphology, microscopic and

biochemical characteristics. The tests used for examining the colonies were Indole, Methyl Red, Vogues Prokauer, Citrate, Catalase, Gelatin hydrolysis, Starch, Oxidize test, Sucrose and lipase test and identified at the genus level of bacteria (Rajan and Selvi Christy, 2010). The gut bacteria of blue Gourami were examined for the production of digestive enzymes like Amylase, Cellulase, Lipase, and Protease using selective media. (Muge Aliye Hekimoglu et al., 2014). Selected gut bacteria were examined for Double-layer Screening Antibacterial activity using selective media. (Jawahar Abraham, 2008). The different pathogens selected are *Staphylococcus aureus*, *Shigella sonnei*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, and *Klebsiella pneumonia*. The isolated *Bacillus species*, *Enterobacter species*, *Aeromonas species* (10^{-6} Cells) were mass multiplied by inoculating into the nutrient broth.

Experimental Feed Preparation:

The raw materials such as fish meal, groundnut oil cake, wheat flour, and tapioca were used for preparing the feed. After knowing the protein content by Micro - Kjeldahl method (Jayaraman,1992) (Table 1), one control(without bacteria), three experimental feeds (Ali, 1980) were prepared by using different isolated bacteria. The components used for feed preparation were dried, powdered and sieved through 425- micron sieve. The ingredients were weighed and mixed thoroughly with 130 - 150 ml of distilled water.

The mixed feedstuff was put in an autoclave for 15 min at 100°C and cooled. After cooling, fish oil, sunflower oil, supplevite - mix, sodium chloride, sodium benzoate, and different isolated (1ml of *Bacillus sp.*, 1ml of *Bacillus sp.*, +*Enterobacter sp.*, and 1ml of *Bacillus sp.*, + *Enterobacter sp.*, and *Aeromonas sp.*) bacteria were mixed with the feed. And then it was extruded with the help of Pelletizer. The pellets were dried at room temperature. This formulated feed was kept in an airtight container in -20°C until used to prevent contamination (Table 2).

In vivo experimental design and growth study

For growth study, uniform size of Blue gourami *Trichogaster trichopterus* (3.66 ± 0.36 g) were selected and were introduced in rectangular glass tanks (45 cm L ' 22 cm B ' 22cm H) having a capacity of 18 liters. Five fishes were introduced in each tank and triplicates were maintained. During rearing, the fishes were fed on the ad – libitum diet of the prepared feed twice a day for 1 hour each from 9 – 10 am and 4 – 5 pm. The unfed were collected after one hour of feeding

Table 1. Protein level of Major ingredients

S.No.	Ingredients	Percentage of Protein
1	Fish meal	58
2	Groundnut oil cake	44
3	Wheat flour	11
4	Tapioca	03

Table 2. Composition of different ingredients in Experimental feed (g/100gm)

S.No.	Ingredients	Experimental Feeds			
		Feed I (Control)	Feed II	Feed III	Feed IV
1	Fishmeal	36.2	36.2	36.2	36.2
2	GNOC	36.2	36.2	36.2	36.2
3	Wheat flour	8.7	8.7	8.7	8.7
4	Tapioca	8.7	8.7	8.7	8.7
5	Fish oil	2	2	2	2
6	Sunflower oil	2	2	2	2
7	Supplevite mix	4	4	4	4
8	Sodium chloride	1	1	1	1
9	Sodium benzoate	1	1	1	1
10	Microbes	-	1ml <i>Bacillus sp.</i>	1ml <i>Bacillus sp.</i> 1ml <i>Enterobacter sp.</i>	1ml <i>Bacillus sp.</i> 1ml <i>Enterobacter sp.</i> 1ml <i>Aeromonas sp.</i>

Table 3. Biochemical characterization of intestinal bacteria of Blue Gourami

Test	G1	G2	G3	G4	G5
Simple staining	Rods	Cocci	Rods	Rods	Rods
Gram's staining	Negative	Positive	Negative	Positive	Negative
Mortality Test	Motile	Motile	Motile	Motile	Motile
Indole test	Negative	Positive	Positive	Positive	Positive
Methyl Red	Positive	Positive	Positive	positive	Positive
Voges Prokauer	Negative	Positive	Positive	Positive	Positive
Citrate test	Positive	Positive	Negative	Positive	Positive
Catalase test	Positive	Positive	Positive	Positive	Positive
Starch test	Positive	Negative	Negative	Positive	Negative
Gelatin hydrolysis	Positive	Negative	Negative	Positive	Negative
Oxidase Test	Positive	Negative	Negative	Positive	Negative
Lactose Test	Positive	Negative	Negative	Positive	Positive
Sucrose Test	Positive	Positive	Not -performed	Positive	Positive
Lipid Test	Positive	Positive	Positive	Positive	Positive
Identification result	<i>Bacillus</i> sp.	<i>Pseudomonas</i> sp.	<i>Enterobacter</i> sp.	<i>Aeromonas</i> sp.	<i>Escherichia</i> sp.

without disturbing the fishes. The unfed was dried to constant weight. The faecal matter was collected daily before changing the water with the least disturbance to the fishes and dried at 95°C. Approximately 70% of the water in the tank was replaced with tap water. The experiment was continued for 21 days. On the 21st day, the length and weight of the fishes were measured in live conditions. Feed Utilization Parameters such as condition factor (K), Feed consumption, Feed Conversion Efficiency, Feed Conversion Ratio, Growth, Percentage Growth Rate, Relative Growth Rate, Assimilation, Metabolism, Gross Growth Efficiency and Net Growth Efficiency were calculated.

RESULTS AND DISCUSSION

The organisms isolated from the intestinal content were identified *Bacillus* sp, *Enterobacter* sp, *Aeromonas* sp., using biochemical tests as

given in table 3. The selected intestinal bacteria were *Bacillus* sp., *Enterobacter* sp., *Aeromonas* sp., based on the Biochemical tests, Enzyme Production (Amylase, Cellulase, Lipase, and Protease) (Table 4). Based on the test the selected *Bacillus* sp., *Enterobacter* sp, *Aeromonas* sp., (G1), (G3), (G4) found to be producing a higher amount of digestive enzymes. Liu et al. (2016) reported that the gut micro biome of eight fish species comprising a relatively large number of bacteria predominantly Proteobacteria, Firmicutes, Bacteroidetes, and Acidobacteria. Ray et al. (2012) reported the isolation of amylase, cellulase, lipase and protease producing bacteria from the gut of different fishes. Banerjee et al. (2013) reported that the amylase producing bacteria were higher than cellulose producing bacteria in the gastrointestinal tract of two Indian air breathing fishes. Parmita Das et al. (2014) reported the isolation of amylase, cellulase, protease, and lipase

Table 4. Enzyme Productivity of Intestinal bacteria of Blue Gourami

S.No	Intestinal bacteria	Amylase	Cellulase	Lipase	Protease
1	G1(<i>Bacillus</i> sp.,)	+++	++	+++	+++
2	G2(<i>Pseudomonas</i> sp.,)	+	+	++	+
3	G3(<i>Enterobacter</i> sp.,)	++	++	++	+++
4	G4 (<i>Aeromonas</i> sp.,)	++	+++	++	++
5	G5 (<i>Escherichia</i> sp.,)	++	++	++	++

+ - Nil(Absent)or (Negative) ++ - Low productivity (Positive) +++ - Higher productivity (Positive).

producing bacterial strains in the digestive tract of 4 brackish water fish species.

Antibacterial Activity (Table 5), and enumeration of bacteria are given in Table 6. Based on antibacterial activity *Bacillus* sp., *Enterobacter* sp., *Aeromonas* sp., (G1), (G3), (G4) has shown higher inhibition against pathogens *Staphylococcus aureus*, *Shigella sonnei*, *Enterococcus faecalis*, *Pseudomonas aeruginosa* and *Klebsilla pneumoniae*. Zapata et al. (2013) reported that Lactic acid bacterial strains isolated from Nile Tilapia intestine showed the high ability to inhibit the growth of freshwater fish pathogen particularly *Vibrio* sp. and *Mycobacterium* sp. Shubhankar Ghosh et al. (2014) reported the antibacterial activity of Lactic acid bacteria isolated from the gut of marine fish *Rastrelliger kanagurta* against fish, shrimp and human pathogens. Banerjee et al. (2016) reported that the *Bacillus subtilis* isolated from the gastrointestinal tract of *Labeo rohita* showed inhibitory activity against

four fish pathogens such as *Bacillus mycoides*, *Aeromonas salmonicida*, *Pseudomonas fluorescens* and *Aeromonas hydrophila*. The intestinal bacteria *Enterobacter* sp., isolated from the intestine of *Labeo rohita* showed higher zone of inhibition of fish pathogens of *Staphylococcus aureus* (P1), *Shigella sonnei* (P2), *Enterococcus faecalis* (P3), *Pseudomonas aeruginosa* (P4) and *Klebsilla pneumonia* (P5) (Sivakumar et al. 2017). Agustina et al.(2018) reported that 19 bacteria isolated from the gut of Kelabau fish showed antibacterial activity against *Aeromonas hydrophila* and *Pseudomonas* sp. Mukherjee et al. (2019) reported that the gut bacteria *Bacillus methylophilicus*, *B. amyloliquefaciens*, *Pseudomonas fluorescens* and *B. licheniformis* isolated from rohu, *Labeo rohita* were antagonistic to fish pathogen *Aeromonas* spp.,

Condition factor of Blue gourami *Trichogaster trichopterus* reared in different feeds were presented in Table 7. Condition factor (K)

Table 5. Antibacterial Activity (Double layer screening) of intestinal bacteria of Blue Gourami

S.No.	Intestinal Bacteria	Zone of Inhibition in mm									
		P1	CA	P2	CA	P3	CA	P4	CA	P5	CA
1	G1(<i>Bacillus</i> sp.,)	14	10	14	09	08	05	13	07	11	08
2	G2(<i>Pseudomonas</i> sp.,)	07	05	07	05	05	04	06	04	06	04
3	G3(<i>Enterobacter</i> sp.,)	11	08	12	08	09	05	09	07	08	04
4	G4(<i>Aeromonas</i> sp.,)	13	07	10	06	07	04	08	05	09	06
5	G5(<i>Escherichia</i> sp.,)	10	06	09	07	06	03	06	04	06	03

CA - Commercial Antibiotic (Zendamycin) P1 - *Staphylococcus aureus* P2 - *Shigella sonnei*
 P3 - *Enterococcus faecalis* P4 - *Pseudomonas aeruginosa* P5 - *Klebsilla pneumonia*

Table 6. Enumeration of Intestinal bacteria of Blue Gourami

S.No	Intestinal Bacteria	Sample (Intestine of Blue Gourami)	No. of colonies	CFU/ml of the sample
1.	G1(<i>Bacillus</i> sp.,)	10 ⁻⁶ (O)	111	110 X 10 ⁶
		10 ⁻⁶ (R)	101	
2.	G2(<i>Pseudomonas</i> sp.,)	10 ⁻⁶ (O)	103	101 X 10 ⁶
		10 ⁻⁶ (R)	112	
3.	G3(<i>Enterobacter</i> sp.,)	10 ⁻⁶ (O)	114	111 X 10 ⁶
		10 ⁻⁶ (R)	108	
4.	G4 (<i>Aeromonas</i> sp.,)	10 ⁻⁶ (O)	109	106 X 10 ⁶
		10 ⁻⁶ (R)	111	
5.	G5 (<i>Escherichia</i> sp.,)	10 ⁻⁶ (O)	110	110 X 10 ⁶
		10 ⁻⁶ (R)	111	

O-Original, R- Replica.

of blue gourami *Trichogaster trichopterus* was estimated for comparative purposes to assess the feed. The average initial condition factor is 1.15 ± 0.13 and the final condition factor increased in feed IV (1.55 ± 0.18) and in all others, the final condition factor was decreased. Sivakumar et al., (2016) reported that the average initial condition factor of yellow molly was 1.84 and the final condition factor increased in feed V (2.65) when fed with *Escherichia fergusonii*. Suganya et al. (2018) also reported that the final condition factor was higher in feed containing 4ml of *Pseudomonas* sp. in the feed of Zebrafish.

Feed utilization parameters of blue gourami *Trichogaster trichopterus* is presented in Table 8. Feed consumption of Blue gourami was higher in feed IV (5.4 ± 0.13) containing every 1 ml each of *Bacillus* sp., *Enterobacter* sp., *Aeromonas* sp. and lower in feed I (control) (3.1 ± 0.41). Rajan and Revathi (2011) reported that the feed consumption of *Platy Xiphophorus maculatus* was higher in Ex. Feed V containing 10^4 cells

of *Bacillus subtilis*. Bisht et al., (2012) reported that the feed consumption in common carp was higher (95%) in diet D3 and lower (85%) in diet D1. Wang et al. (2015) reported that the feed intake was higher in juvenile *Pseudobagrus ussuriensis*. Feed Conversion Efficiency of Blue gourami was higher in feed IV (0.06 ± 0.01). But in the case of gold fish, the feed conversion efficiency gradually decreased from lower to a higher quantity of *Pseudomonas* sp. (Rajan and Jeyachristina Arokia Selvi, 2014). Feed Conversion Ratio (FCR) of Blue gourami was lower in feed IV (4.46 ± 0.53) and higher in feed I (8.41 ± 0.23). Suganya et al. (2018) reported that the Feed Conversion Ratio (FCR) of Zebra fish was lower in feed V (0.03 ± 0.01) and higher in feed I (0.13 ± 0.02). Somrudee Silarudee et al. (2019) reported such lower feed conversion ratio (FCR) in Black Eared Catfish (*Pangasius larnaudii*) fed with *Lactobacillus plantarum* in the feed than control. The Growth of Blue gourami was higher in feed IV (0.54 ± 0.04). Dhanraj et al., (2013) reported that the growth of Koi carp was higher in diet 3 (SCD) (0.32 ± 0.07) lower in control (0.19 ± 0.02). Roy et al. (2013) reported that the growth was higher in *Labeo rohita* fed with two phytase producing bacterial strains isolated from the gut. Pornthep Niamphithak et al. (2017) reported higher growth in Bocourti Catfish (*Pangasius bocourti* Sauvage, 1880) fed with *Lactobacillus plantarum* (1×10^7 cfug⁻¹) in

Table 7. Condition factor(K) of Blue Gourami

Feeds	Intial	Final
EX. Feed I	1.15 ± 0.13	1.21 ± 0.17
EX. Feed II	1.10 ± 0.05	1.33 ± 0.15
Ex. Feed III	1.15 ± 0.04	1.44 ± 0.16
Ex. Feed IV	1.36 ± 0.08	1.55 ± 0.18

Table 8. Feed utilization and Growth parameters of Blue Gourami in relation to different bacteria. Each value is the average (\pm SD) performance of 5 individuals in triplicates reared for 21 days

S.No.	Parameters	Experimental Feeds			
		Feed I	Feed II	Feed III	Feed IV
1.	Feed Consumption(FC)(g/g live wt/21days)	3.11 ± 0.41	3.33 ± 0.31	3.55 ± 0.16	5.44 ± 0.13
2.	Feed Conversion Efficiency(FCE)	0.03 ± 0.01	0.6 ± 0.02	0.08 ± 0.01	0.06 ± 0.01
3.	Feed Conversion Ratio (FCR)	8.41 ± 0.23	5.60 ± 0.33	4.4 ± 0.10	4.46 ± 0.53
4.	Growth (G) (g/g live wt/ 21 days)	0.22 ± 0.05	0.33 ± 0.08	0.44 ± 0.05	0.54 ± 0.04
5.	Percentage Growth (PG) (%)	5.46 ± 1.62	8.12 ± 1.05	10.25 ± 1.37	10.25 ± 0.09
6.	Relative Growth Rate (RGR)	0.11 ± 0.06	0.15 ± 0.02	0.19 ± 0.02	0.23 ± 0.20
7.	Assimilation (A) (g/g live wt/21days)	1.22 ± 0.05	1.36 ± 0.15	1.31 ± 0.30	1.44 ± 0.32
8.	Metabolism (M) (g/g live wt/21days)	0.43 ± 0.42	1.10 ± 0.11	1.42 ± 0.41	1.44 ± 0.22
9.	Gross Growth Efficiency (GGE) (%)	3.25 ± 1.32	5.15 ± 0.23	5.25 ± 1.02	6.40 ± 0.25
10.	Net Growth Efficiency (NGE) (%)	10.25 ± 0.38	10.22 ± 0.15	11.33 ± 2.13	12.46 ± 2.12

the diet. Dias *et al.* (2018) reported that *Bacillus cereus* improves growth performance of tambaqui *Colossoma macropomum*. This enhanced growth performance of fish supplemented with probiotics is probably due to an improvement in digestion, as well as an increase in the synthesis and absorption of nutrients (Hoseinifar *et al.* 2017). As the growth, the percentage growth of Blue gourami was higher in feed IV (10.25 ± 0.09). Sivakumar *et al.*, (2014) reported that the percentage growth of Common carp was higher in feed V (51.12 ± 22.30) and lower in the feed I control, (16.11 ± 9.53). The relative growth rate of Blue gourami was higher in feed IV (0.23 ± 0.20) and lower in feed I (0.11 ± 0.06). Roy *et al.* (2013) reported that the relative growth rate was higher in feed D3 containing two phytase producing bacterial strains isolated from the gut of rohu *Labeo rohita*. Suganya *et al.*, (2018) reported that the relative growth rate of Zebra fish was higher in feed V (0.08 ± 0.02) containing 4ml *Pseudomonas* sp. and lower in feed I (0.03 ± 0.01). The Assimilation of Blue gourami was higher in feed IV (1.44 ± 0.3) lower in feed I (1.22 ± 0.05). Assimilation of Platy was higher in Feed V containing about 104 cells of *Bacillus subtilis*. (Rajan and Revathi, 2011). Metabolism of Blue gourami was higher in feed IV (1.44 ± 0.22) lower in feed I (0.43 ± 0.42). The same result was also reported by Suganya *et al.* (2014) in goldfish. Gross and Net growth efficiency of Blue gourami was higher in feed IV and lower in feed I. Sunil Kumar and Vishnu (2011) reported a similar result when clownfish were fed with *Lactobacillus*. Pushparaj *et al.*, (2012) reported higher gross and net growth efficiency when Platy was fed with higher levels of *Bacillus subtilis* in the feed.

CONCLUSION

From this study, it was concluded that the combination of three isolated bacteria, such as 1ml *Bacillus* sp., 1ml *Enterobacter* sp., and 1ml. *Aeromonas* sp. can improve the growth of Blue gourami and act as a potential probiotic feed additive. So, it is suggested that the present research work is useful to the ornamental fish farmers.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

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None.

AUTHOR'S CONTRIBUTION

D.G. - Laboratory experiments were conducted starting from a collection of fish, separation and mass multiplication of bacteria, growth studies, and calculations.

P.S - Done experiments related to the production of enzymes by bacteria and antibacterial studies.

M.R.R - The research work was formulated and guidance was given to the first author for execution.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript and/or the Supplementary Files.

ETHICS STATEMENT

Fish used in the present research were in accordance with the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals [CPCSEA, Ministry of Environment & Forests (Animal Division), Government of India] on the care and use of animals in scientific research and also approved by the Institutional Ethical Committee for Research on Human and Animal Subject (IECRHAS) from The Gandhigram Rural Institute- Deemed to be University, Govt. of India, Gandhigram, Tamil Nadu, India

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