# Microbiological And Physicochemical Analysis Of Water From Empurau Fish (Tor Tambroides) Farm In Kuching, Sarawak, Malaysian Borneo

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**Abstract**: The fish Tor tambroides locally known as empurau or kelah, is one of the most valuable and high commercial value fish species in the Cyprinidae (carps) family. The fish can grow up to the size of human in its natural habitats like cool, clean, fast moving waters and availability of riverine fruits from trees growing by the bank of upstream river. In aquaculture ponds where they are bred for the market, the fish have yet to get the right feed and appropriate water environment condition for their optimized growth, resulting in the slow production of the fish. The optimal parameter value related physicochemical and microbiological quality of water is important for the optimal growth of the fish. The Enterobacteriaceae group of bacteria has been commonly used as the main indicator for the microbiological water quality. This study aims to determine the water quality of empurau farm with regards to the physicochemical parameter value and microbiological indicator. The occurrence of Enterobacteriaceae as indicator organism and the physicochemical parameter value of the water used for the empurau farming were determined. Water samples from inlet and outlet were collected from seven empurau ponds at Indigenous Fish Research & Production Centre (IFRPC) in Tarat, Serian, Sarawak, Malaysian Borneo. Physicochemical parameters such as temperature, pH, biochemical oxygen demand (BOD), dissolved oxygen (DO) and total ammonia nitrogen (TAN) of water samples were determined. As for the microbiological analysis, water samples from the ponds were subjected to standard serial dilution followed by plating the samples on eosin methylene blue agar (EMBA) for isolation of Enterobacteriaceae. Gram staining was then performed on the isolates to determine their Gram's characteristics. Twenty five Gram-negative isolates were further analyzed with **(**$(GTG)_5$-PCR to screen for clonal isolates to be used for the identification by API 20E identification system. Dendrogram constructed from the **(**$(GTG)_5$-PCR analysis revealed that the 25 isolates were genetically diverged resulting in 4 major clusters (G1, G2, G3, G4) and 11 sub-clusters. Based on the dendrogram, 11 representative isolates of the Enterobactericeae were selected from different clusters and these isolates were identification by API 20E kit. The result of the API 20E identification revealed that the 11 presumptive Enterobacteriaceae isolates were belonged to ten different species within nine genera of Enterobacteriaceae. The Enterobacteriaceae species confirmed were Brucella spp., Enterobacter cloacae, Citrobacter braakii, Erwinia spp., Vibrio fluvialis, Serratia odorifera, Citrobacter freundii, Butiaxella agrestis, Proteus vulgaris group and Cedecea davisae. The Enterobactericeae isolated in this study were mostly human pathogens with few fish pathogens, suggesting human activities may be affecting the water quality. Physicochemical parameters may affect the microbial population in ecology, thus indirectly affecting the development of the fish.

**Index Terms**: Empurau farm water, Enterobacteriaceae, $(GTG)_5$-PCR analysis, API 20E kit, Physicochemical factors, Water quality

## 1 INTRODUCTION

The fish Tor tambroides or locally known as empurau or kelah in Malaysia, is one of the most expensive and delicious fish species within the family Cyprinidae (carps) [1]. Because of its high price and demand, Tor has been recognized to have high potential in aquaculture [2]. The fish can grow up with normal growth rate in its natural habitats like the cool, clean, fast moving waters and availability of riverine fruits from trees growing by the river bank of upstream river.


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However, in the ponds where they are bred for the market, the fish have yet to get the right feed and appropriate water environment for their optimized growth, resulting in the slow production of the fish. Good quality water is essential for sustaining the life of all living organisms in the aquaculture environment including fish [3], [4]. Enterobacteriaceae is non-sporing gram-negative bacteria that act as the main indicator for the bacteriological water quality. In fact, the bacteriological quality of the water reflects the bacterial flora of the fish [5]. Fish farmers should be able to understand the importance of managing the proper bacteriological quality of water, since bacteriological quality play an important role in the spreading of farmed fish diseases. Bacterial pathogens can cause infections and death of fish in aquatic environments [6]. Besides, the bacterial flora of the fish is the primary significant source of occupational disease on fish handlers [5]. In addition, bacteria, such as Enterobacteriaceae are closely related with many ecological factors in fish ponds. Some of the factors are the dissolved oxygen, suspended matter, organic detritus, transparency and nutrient salt. These factors show either positive or negative correlation in the pond management. Hence, the pond management has a very strong influence on bacterial number in the pond ecosystem [5]. The availability of information on microbiological aspect of empurau fish grown in ponds are still lacking. To date, there are still lacking of scientific studies regarding the suitable environment for Tor habitats, however, it is said that Tor lives in a very clean water environment. Thus, assessment of physicochemical and microbiological water quality needed to be done in order to determine the criteria for the suitable environment for rearing Tor species. Both the physicochemical parameters and microbiological parameters play a crucial role

in identifying and setting the indicator for the most suitable habitual environment for the quality development of Tor species. Meanwhile, the analysis of microbiological indicator can further enhance the current understanding of microbiological activity that occurs in the aquatic environment. Therefore, this study aims to determine the occurrence of Enterobacteriaceae and the physicochemical parameter of water of empurau ponds.

## 2 MATERIALS AND METHODS

### 2.1 Samples collection and physicochemical measurement

Water samples were collected from seven randomly picked empurau ponds at Fisheries Research & Production Centre (IFRPC) in Tarat, Serian, Sarawak, Malaysia. The water samples were collected in sterile 50 ml Falcon tubes from the inlet and outlet of each pond. The pH, temperature and dissolved oxygen (DO) of the water were measured using pH meter, thermometer and dissolved oxygen meter, respectively. The total ammonia nitrogen (TAN) of homogenized water sample from each pond was tested using Hach Test Kit. The water samples were kept at 4$^{o}$C in an ice box and transported to the laboratory within 24 hours for immediate samples processing and analysis.

### 2.2 Bacterial isolation

The water samples collected from outlet and inlet of each pond were homogenized. Approximately 1 ml of the homogenized water was diluted with 9 ml of 0.85% saline solution. The diluted water samples were plated on both NA and EMBA followed by incubation at 37$^{o}$C for 24 hours. The bacterial colonies growing on EMBA were isolated, purified and subjected to gram staining. The gram-negative isolates were further analyzed with $(GTG)_5$-PCR.

### 2.3 DNA extraction

Bacterial DNA was extracted by boiled cell method as described by Freschi et al. [7] with slight modifications. Briefly, a colony was picked from the freshly grown plate and then inoculated into 5 ml LB broth before incubation at 27$^{o}$C for overnight. From the LB broth culture, a 3 ml was centrifuged at 10,000 rpm for 5 minutes. The supernatant was discarded and the pellet was resuspended in 500 µl of sterile distilled water. The suspension was boiled for 10 minutes and placed on ice immediately for 10 minutes followed by centrifugation at 10,000 rpm for 10 minutes. The supernatant was used for the $(GTG)_5$-PCR analysis.

### 2.4 $(GTG)_5$-PCR fingerprinting

$(GTG)_5$-PCR was conducted according to Matsheka et al. [8] with slight modifications. The $(GTG)_5$-PCR was carried out in a 25 µl volume containing 5 µl of 10x PCR buffer solution, 3 µl of 25mM MgCl2, 0.8 µl of 25mM dNTPs, 1 µl of 25µM $(GTG)_5$ primer (5'-GTGGTGGTGGTGGTG-3'), 9.9 µl of sterile distilled water, 5 µl of DNA template and 0.3 µl of Taq DNA polymerase (Promega, USA). The $(GTG)_5$-PCR amplification was conducted with pre-denaturation at 95°C (7 minutes), followed by 4 cycles of denaturation, annealing and extension at 95°C (2 minutes), 36°C (2 minutes) and 72°C (2 minutes), respectively. This was followed by another 30 cycles of denaturation at 95°C (1 minute), annealing at 50°C (1 minute) and extension at 72°C (1 minute). Final extension at 72°C for 5 minutes was also included. The amplification products were electrophoresed on 1.5% agarose gel in 1x TBE buffer at 100V for 1 hour. The agarose gel was pre-stained with ethidium bromide and a 1 kb DNA ladder (Promega, USA) was used as a DNA size marker. The electrophoresed agarose gel was then visualized with UV transilluminator.

### 2.5 DNA fragment analysis

The fingerprint profiles obtained from the $(GTG)_5$-PCR were analyzed using the RAPDistance package (version 1.04). Scoring was done based on the banding patterns obtained using binary data format. Presence of band was scored "1", whereas absence of band was scored "0". The genetic distances between banding profiles were determined based on Dice formulation [9]. From the calculated genetic distances using Dice formula, a dendrogram of neighbor joining tree (NJTREE) was constructed.

### 2.6 Analytical Profile Index (API) Test System

Based on the dendrogram constructed, 11 isolates were selected for the API 20E kit identification. The identification using the API 20E identification kit (BioMerieux) was conducted according to manufacturer's instructions in the insert. Briefly, the test began with inoculation of saline suspension of pure culture into each well on the API 20E test strip then followed by incubation of the bacteria at 37$^{o}$C for 18-24 hours. The results of the test were analyzed through the on-line database, apiweb$^{TM}$.

## 3 RESULTS AND DISCUSSION

Several decades ago, empurau fish can be found abundantly in the upper reaches of many big rivers in the central of Sarawak, Malaysian Borneo when the rivers were still unpolluted. However, environmental degradation (i.e., river pollution, deforestation, logging road construction, etc.) of rural areas and upper streams has led to the rapid destruction of natural habitats of empurau. Furthermore, excessive demand for the highly priced empurau flesh which can reach up to RM750 locally and between RM800 and RM850 per kg for the export market, has led to overfishing of rivers by locals and illegal fish poachers. These practices have caused drastic dwindling of the fish population size and the catch from rivers become very limited [1]. Due to the high price of the fish, many investors are interested in empurau fish culture in ponds. However, slow breeding and growth and scarce of information on the optimal condition for the fish production in pond or aquaculture environment causes the low production of the fish. The management of the pond had a very strong influence on physicochemical and bacterial number in the pond ecosystem. Any type of alterations in the surrounding environment of the pond will add stress to pond ecosystem as well as the fish [10]. Some of the factors correlated with the pond management are the dissolved oxygen (DO), temperature, suspended matter, organic detritus, transparency and nutrient salt [5]. A stress on a pond ecosystem could be affected by water quality. The water quality includes the biological, chemical and physical characteristics of water [11]. Physical alterations include changes in water temperature, water flow and light availability whereas chemical alterations include changes in the loading rates of biostimulatory nutrients, oxygen consuming materials, pH and toxins. The microbiological alterations include introduction of new species of microorganisms as a result of human and animal activities.

In this study, the physicochemical parameters such as pH, temperature, dissolved oxygen (DO), Biochemical Oxygen Demand (BOD) and Total Ammonia Nitrogen (TAN) were analyzed. The reading of the parameters from seven randomly selected empurau ponds is shown in Table 1. These parameters were the commonly used as indicators for water quality [12].

**Table 1.** The readings of pH, temperature, dissolved oxygen (DO), biochemical oxygen demand (BOD) and total ammonia nitrogen content (TAN) for seven randomly selected Empurau ponds.

| Pond | pH | Temperature ($^{o}$C) | Dissolved Oxygen, DO (mg/L) | Biochemical Oxygen Demand, BOD (mg/L) | Total Ammonia Nitrogen Content, TAN (mg/L) |
|---|---|---|---|---|---|
| P1 | 7.95 | 28.9 | 5.6 | 3.2 | 0.00 |
| P2 | 9.15 | 29.5 | 5.2 | 3.4 | 0.46 |
| P3 | 8.40 | 27.7 | 4.8 | 2.8 | 2.14 |
| P4 | 7.41 | 28.6 | 5.4 | 3.2 | 0.14 |
| P5 | 9.16 | 28.5 | 5.7 | 3.2 | 0.04 |
| P6 | 9.00 | 29.2 | 5.5 | 4.2 | 0.00 |
| P7 | 7.62 | 26.4 | 5.6 | 3.3 | 1.01 |
| **Average** | 8.38 | 28.4 | 5.4 | 3.33 | 0.54 |

In this study, the average pH reading between the seven ponds was pH 8.38 which was still within the range of good quality. In fact, the largest fish crops were usually produced in water with pH ranging between pH 7.0 and pH 9.0 [14]. On the other hand, any pH value lower than pH 4.8 or higher than pH 10.8 might bring harmful effects to the aquatic organisms in a pond [14], [15]. The temperature played a significant role in some physiological processes, including the release of stimuli for breeding mechanism in fish, both under natural and artificial conditions [13]. The average temperature recorded was 28.4. Temperature between 30$^{o}$C and 35$^{o}$C might be responsible for high productivity [13]. Based on the results recorded, the average DO reading from seven ponds was 5.4 ppm. Generally, a pond with DO range in between 3.0-5.0 ppm is unproductive, whereas a good production pond should have DO concentration above 5.0 ppm [16]. However, a very high concentration of oxygen might lead to a state of super saturation that eventually leads to the lethal of fish in the pond [13]. Biochemical Oxygen Demand (BOD) is the total amount of dissolved oxygen required by microorganisms for biodegradation of organic matters [10]. It is a common indicator used to measure organic water pollutants. In this study, BOD was determined by measuring the DO level in the freshly collected water samples and then measured the same water samples again after 5 days of incubation period. The final BOD reading was recorded in units of mg/L. From the results obtained, the highest BOD recorded was 4.2 mg/L and the average between seven ponds was 3.33 mg/L. Murdoch et al. [17] described that unpolluted natural water should have a BOD reading of 5 mg/L or less. TAN value exceeding 2.2 mg/L would significantly reduce the growth rate, fecundity and disease resistance of the fish. Besides, it might increase the gill ventilation and metabolic rate, which results in the death of the fish. TAN value recorded in this study was 0.5. The presence of Enterobactericeae in water has been associated with water polluted with fecal. In this study, 30 isolates of bacterial were isolated from the water samples by using EMBA. The bacteria that were able to grow on EMBA were presumed as Enterobacteriaceae, which is a family in the gram-negative bacteria. In fact, EMBA contained sucrose and lactose with indicator dyes (eosin Y and methylene blue), which could use to differentiate between lactose fermenting and non-fermenting bacteria [18]. From the gram-staining, 28 isolates were shown to be gram-negative bacteria, whereas another 2 isolates were gram-positive bacteria. Out of the 28 isolates, three isolates were unable to survive after storage. Hence, all the 25 isolates of gram-negative bacteria were subjected to **(**GTG)$_5$-PCR analysis.

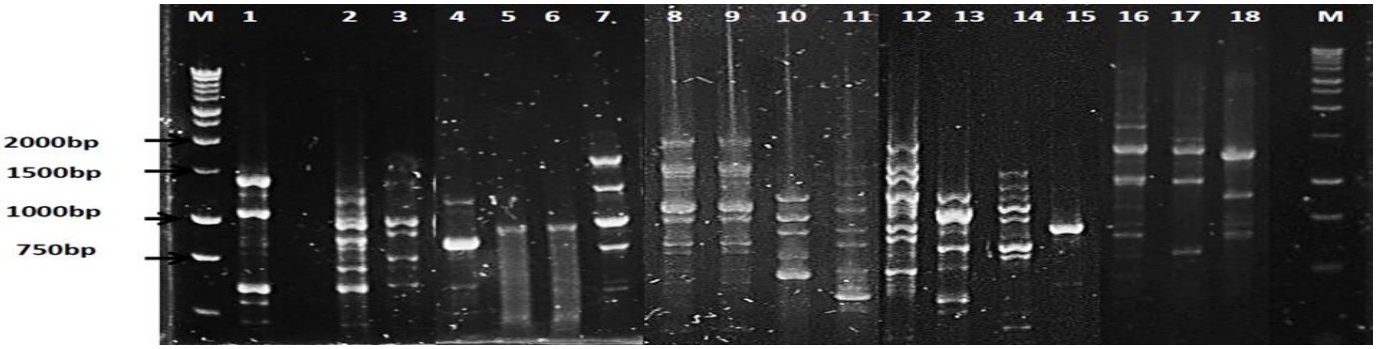


**Fig.1a.** $(GTG)_5$-PCR fingerprinting of Enterobacteriaceae isolates electrophoresed on 1.5% agarose gel. Lane M: 1kb DNA Ladder (Promega, USA); Lane 1: WP1-1; Lane 2: WP3-3; Lane 3: WP3-4; Lane 4: WP7-2; Lane 5: WP7-4; Lane 6: WP2-3; Lane 7: WP3-1; Lane 8: WP3-6; Lane 9: WP3-8; Lane 10: WP3-9; Lane 11: WP2-2; Lane 12: WP5-1; Lane 13: WP4-1; Lane 14: WP5-2; Lane 15: WP7-1; Lane 16: WP7-9; Lane 17: WP3-5; Lane 18: WP3-10.

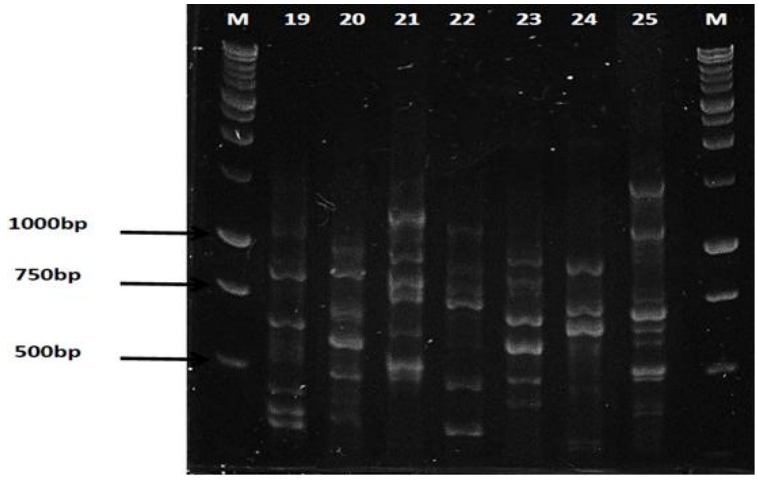


**Fig.1b.** $(GTG)_5$-PCR fingerprinting of Enterobacteriaceae isolates electrophoresed on 1.5% agarose gel. Lane M: 1kb DNA Ladder (Promega, USA); Lane 19: WP3-7; Lane 20: WP4-2; Lane 21: WP4-3; Lane 22: WP7-6; Lane 23: WP7-7; Lane 24: WP7-8; Lane 25: WP3-2.

The genetic relatedness among the 25 Enterobacteriaceae isolates was distinguished by the $(GTG)_5$-PCR fingerprinting. DNA fingerprinting using $(GTG)_5$-PCR technique is based on the principle of amplification of repetitive segments on genomic DNA with a short nucleotide sequence (primer). Besides, $(GTG)_5$-PCR could be used to determine the pedigree analysis of bacterial strain from the unrelated genetically diverse strain [8]. In addition, $(GTG)_5$-PCR has been also used for taxonomical studies [19]. The main purpose of using $(GTG)_5$-PCR in this study was to established the genetic relatedness among the Enterobacteriaceae isolates so that the representative from closely related strains or clones can be selected for further identification, in this case is by API kit. By selecting representative strains or clones from closely related strains in the group, one would save cost for the identification by avoiding repeated identification of the same clones or strains. Fig. 1a and Fig. 1b showed the $(GTG)_5$-PCR gel patterns obtained with $(GTG)_5$ primer. The molecular sizes of the DNA bands produced ranging from 250bp - 1000bp. The $(GTG)_5$-PCR analysis was performed for the 25 isolates of Enterobacteriaceae and the result shows that they were genetically distinct. Fig. 2 shows the dendrogram generated from the $(GTG)_5$-PCR bands of the isolates. The dendrogram generated clearly established the genetic relatedness among the Enterobacteriaceae strains. From the dendrogram, it was found that the Enterobacteriaceae isolates were divided into 4 major clusters and 11 sub-clusters. Isolates that located on the same sub-cluster were assumed to be closely related, hence, a representative was selected from each sub cluster for further identification using API kit identification system (Biomerieux). For example, WP7-4, WP2-3 and WP7-1 were clustered under same cluster so, only WP7-1 was randomly selected to be identified by API kit. The purpose of selecting representative is to avoid repeated identification of the same clones or strains, hence the cost for the identification could be saved. Eleven isolates were randomly selected from each sub-clusters to be used for the API kit identification. API 20E identification system (Biomerieux) is a standardized identification system of Enterobacteriaceae species and other non-fastidious gram-negative bacteria. Fig. 3 shows the API 20E identification system (Biomerieux) profiles of the representative isolates. The results of the identification are shown in Table 2.

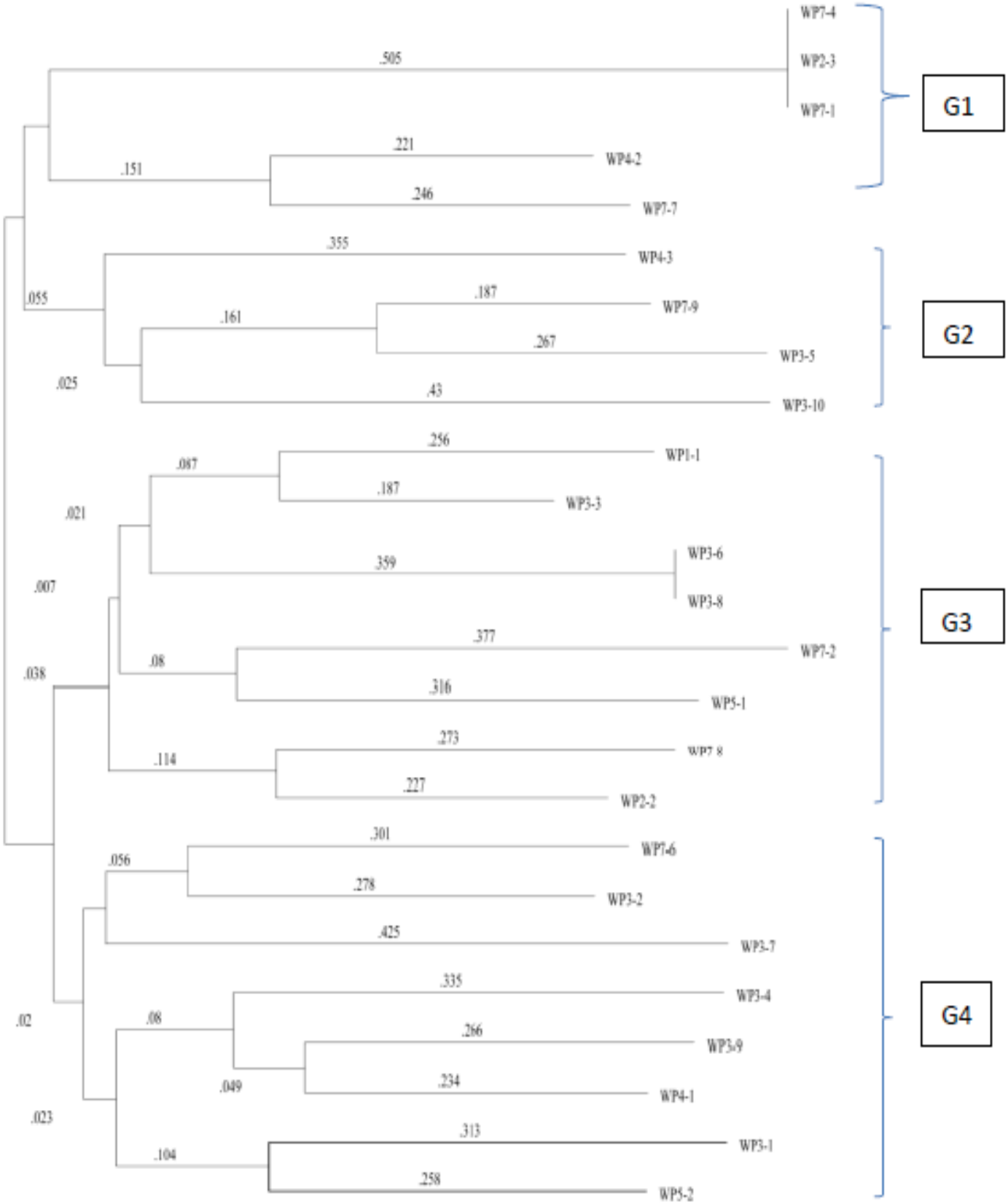


**Fig. 2:** Dendrogram generated from the $(GTG)_5$-PCR fingerprinting among 25 isolates of the Enterobacteriaceae

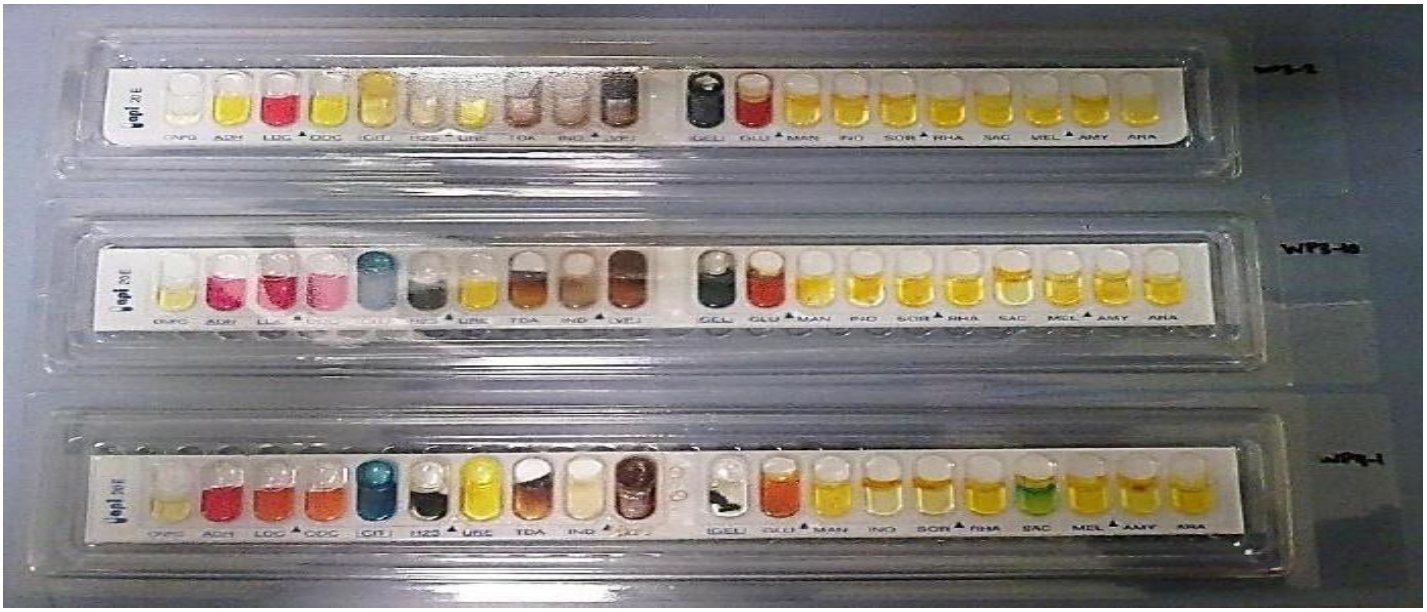

**Fig.3:** Representative profile of the API 20E test strips showing colour reactions after incubation at 27°C for about 18-24 hours.

**Table 2:** The identity of Enterobacteriaceae isolates identified by API 20E identification kit.

| Isolates | Identification |
|---|---|
| WP1-1 | *Enterobacter cloacae* |
| WP7-8 | *Erwinia* spp. |
| WP3-10 | *Serratia odorifera* |
| WP4-1 | *Citrobacter freundii* |
| WP5-1 | *Citrobacter braakii* |
| WP7-9 | *Buttiaxella agrestis* |
| WP3-4 | *Enterobacter cloacae* |
| WP4-3 | *Proteus vulgaris* |
| WP3-2 | *Vibrio fluvialis* |
| WP7-1 | *Brucella* spp. |
| WP3-7 | *Cedecea davisae* |

The API 20E identification kit successfully identified the 11 isolates as Brucella spp., Enterobacter cloacae, Citrobacter braakii, Erwinia spp., Vibrio fluvialis, Serratia odorifera, Citrobacter freundii, Butiaxella agrestis, Proteus vulgaris group and Cedecea davisae. From the identified species of Enterobacteriaceae, Brucella spp. was belonged to G1, and Serratia odorifera, Buttiaxella agrestis, Proteus vulgaris group were grouped in the same cluster, which was G2. Besides, Enterobacter cloacae, Erwinia spp. and Citrobacter braakii were grouped in the G3. On the other hand, the remaining of identified species (Citrobacter freundii, Enterobacter cloacae, Vibrio fluvialis and Cedecea davisae) were all clustered in the G4. This showed that there was a high genetic diversity among the Enterobacteriaceae species isolated from the pond water at IFRPC in Tarat, Sarawak. In fact, the presence of certain bacteria, such as Citrobacter freundii was related to public health issues since they are potentially pathogenic microorganisms that have been implicated as aetiological agent of animal and human diarrhea [5]. In addition, Citrobacter freundii could cause disease in fishes [20] and was shown to be pathogenic for both farmed fishes and aquarium fishes [21]. Besides, Escherichia, Enterobacter and Citrobacter were the main coliform group of bacteria that could cause health hazard to human through fishes [20], [22]. Moreover, Sekar et al. [23] found that Enterobacter cloacae was the causative agent of mortality in fishes, such as Mugil capito. On the other hand, the presence of Enterobacter cloacae, Citrobacter freundii, Citrobacter braackii and Proteus vulgaris may have implied the cross contamination degree from the handlers [24] and the presence of these microorganisms indicated a potential hazard, particularly to the immunocompromised individuals [20].

## 4 Conclusion

In conclusion, this study provided an overview of the physicochemical parameters and the microbiological water quality of Empurau fish ponds. The physicochemical parameters of the water samples collected from fish ponds such as the pH, temperature, DO, BOD and TAN were within the range of proper quality for fish production. $(GTG)_5$-PCR and phylogenetic analysis were useful in screening for representative clonal isolates for further identification of the Enterobactericeae isolates. The water of empurau fish aquaculture could be a reservoir for Enterobacteriaceae and the characteristics of these bacteria should be further studied in the future.

## 5 Acknowledgments

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