Bacteria Associated with Fresh-water Aquaculture Tilapia Fish (*Oreochromisniloticus*) in Suez, Egypt

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ABSTRACT

Nile tilapia (Oreochromis niloticus) production is ranked on the top of aquaculture fish in Egypt. This study was aimed to screen bacteria associated with aquaculture fresh-water tilapia to determine the microbiological safety of these wide distributed fish in Egypt. The mean viable bacterial count from fish fillets with the skin samples revealed 5.6 ± 0.8 logs CFU/g. A total of eleven (11) bacterial species were isolated and identified including: E. coli, E. coli O157: H7, Salmonella enterica, Morganella morganii, Proteus mirabilis, Proteus vulgaris, Enterobacter cloacae, Enterobacter cancerogenus, Hafnia alvei, Aeromonas hydrophila, Photobacterium damaselae. The frequency of occurrences of the isolated bacteria indicated that Enterobacter cloacae had the highest frequency of occurrence (12%), while one isolate (2%) of Salmonella enterica, E coli O157:H7, and Aeromonas hydrophila were detected. These bacterial species are potentially pathogenic to humans. Therefore, hygienic handling methods and proper processing are needed before consumption of this fish.

Introduction

Aquaculture is considered one of the fastest growing aspects of agricultural industry over the world. With increasing demand for food fish and other seafood products, aquaculture has the potential of becoming an important alternative supply of these products (Lucas, 2003). Tilapia fish is widely grown farmed fish and is considered the second most important group after carps. In 2004, tilapia was ranked the eighth most popular among all seafood in the USA global. Production of tilapia (all species) was estimated 1.5 million tons in 2003 and increased to 2.5 million tons by 2010. Most of this enhanced production is expected to be attributed to Nile tilapia. Egypt is the world's second largest producer of farmed tilapia after China [1]. Egypt has the largest aquaculture industry in Africa that provides about 75.46 % of the country's fish production [2]. Nile tilapia production is ranked on the top of aquaculture fish in Egypt, its production increased from 557,049 tons in 2010 to 768,752 in 2012 [3].

Major pathogens that are affecting the aquaculture include bacteria, fungi, viruses, and parasites [4-6]. Bacterial diseases have become major concern to aquaculture, especially with warm water temperature [7]. Different bacterial species were reported pathogenic to aquatic tilapia, including *Aeromonas hydrophila, Edwardsiella tarda, Flavobacterium columnare, Francisella spp., Yersinia ruckeri, Staphylococcus epidermidis, Vibrio vulnificus,* and *Streptococcus agalactiae* [8-16].

According to data from the Centers for Disease Control and Prevention (CDC) [17] fish was linked to 24% of foodborne illness outbreaks and 6% of all food poisoning, or foodborne illness. Level of bacterial pathogens in tilapia fish was related to environment and handling prior to their arrival to food market and restaurants. Bacteria associated with tilapia fish could be transmitted to person in contact and result in foodborne illness. For example, handling tilapia was reported associated with *Vibrio vulnificus* outbreak in Seattle supermarket [18]. Other foodborne pathogenic bacteria including *Salmonella enterica*, enteropathogenic *Escherichia coli, Listeria monocytogenes, Yersinia enterocolitica*, and *Klebsiella*

pneumoniae were identified from fresh Nile tilapia in Kenyan fresh water fish chains [19]. Shigatoxigenic and enteropathogenic Escherichia coli were isolated from farmed tilapia fish (Oreochromis niloticus) in northeast region of Sao Paulo state [20]. Monitoring bacteria associated with aquaculture tilapia fish of great importance to public health because they help understand pathogen epidemiology in fish and demonstrate how they can transmit food borne illness related to fish to humans. The aim of this study was to screen bacteria associated with aquaculture tilapia to determine the microbiological food safety of these wide distributed fish in Egypt.

Materials and methods

A total of 50 healthy fresh water tilapia fish (*Oreochromis niloticus*) were randomly selected from a commercial food fish farms located in Suez, Egypt. All fish samples during collection were placed in sterile polypropylene bags, placed in polystyrene box containing crushed ice and the temperatures was between 4 °C and 6 °C during transportation. Ice was prepared in laboratory using sterile distilled deionized water. Samples were transported to the laboratory and examined immediately.

Bacteriological analysis

Fish samples were processed in complete aseptic condition. Fish were filleted where skin part were kept with the flesh using sterile knifes and forceps and placed on sterile tray. Samples (25 g) were homogenized for 2 min. in a sterile bag containing 225 ml of buffered peptone water (0.1%) (Lab M, UK) using a stomacher (Seward Stomacher 400 circulator, UK). After incubation for 18-24 h at 35 °C, 1 ml was transferred for further analysis from this nonselective pre-enrichment.

Aerobic plate count

Serial dilution 1:10 folds were then performed for total aerobic bacterial count. Dilutions were spread-plated onto plate count agar (lab M, UK) and incubated at 35 ±1°C overnight. Readings obtained within 25 to 250 colonies on a plate were used to calculate bacterial population numbers, and reported as logs of colony forming units (CFU/g). Experiments were repeated and results were represented as means ± standard deviations.

Isolation and identification of bacteria

E.coli and *E.coli* O157: H7 were identified by transfer pre-enrichment culture (1 ml) to Escherichia coli selective broth supplemented with novobiocin (EC+n) [21]. A loopful of culture was streaked onto a chromogenic selective agar (Lab M, Lancashire, UK), and sorbitol MacConkey agar (SMAC, Lab M, Lancashire, UK). Violet-colored colonies will be selected on the chromogenic selective agar. Non-sorbitol fermenting *E. coli* O157:H7 produce pale, colourless colonies will be selected from SMAC [22]. Suspect colonies are identified biochemically with indole kovac's reagent (Merck, Germany), Simmon citrate agar (Lab M, UK), Methyle red (MR, Lab M, UK), and Voges–Proskauer (VP, Lab M, UK) tests. Species were identification according to FDA bacteriological analytical manual (US-FDA, 2007). Further identification was done using API 20E diagnostic strips (Biomérieu, Marcy, France).

E. coli O157:H7 confirmed serologically using O157 and H7 antisera by direct agglutination tests.

Pre-enrichment culture (1 ml) were transferred into tetrathionate broth (TT, Lab M, UK) and incubated at 37°C for 24 h for *Salmonella spp.* isolation and identification. Culture were streaked onto Xylose lysine deoxycholate agar (XLD, Lab M, UK) and incubated at 37°C for 24 h. Red colonies with black center from XLD media were selected and streaked on trypticase soy agar slants (TSA, Lab M, UK). After incubation bacteria were subjected to biochemical tests: indole, citrate, triple sugar iron (TSI, Lab M, UK), urease (Lab M, UK), and identification with API 20E diagnostic strips as described in published reports (Food and Drug Administration, [18,23,24].

Colonies of different characteristics of shape, size, and color were selected randomly from plate count agar and incubated on additional Trypticase soy agar (TSA, Lab M, UK) slants. All the purified isolates were observed for Gram staining and cell morphology. The isolates were then identified biochemically with indole kovac's reagent, Simmon citrate agar, MR, VP tests, triple sugar iron (TSI, Lab M, UK), and H2S production for identification to genus or species level in parallel, the commercial API 20E strips were also used [25].

Identification by PCR and 16S rRNA gene sequencing

Randomly selected samples were used for this technique either for confirmation of API results or for identification of unknown samples. The technique was performed according to Azwai1 et al. [26]. DNA extraction was done using bacterial DNA preparation kit (Jena Bioscience, Thuringia, Germany). Partial 16S rDNA was amplified using the universal oligonucleotides primers forward 5′-GAGTTTGATCCTGGCTTAG-3′ and reverse 5′-GGTTACCTTGTTACGACTT-3′. Briefly, 2 µl DNA templates (20 ng/ µl) was added to 12.5 µl Master Mix (Qiagen, Hilden, Germany) and 10.5 µl deionized H2O for a total volume of 25 µl. The mixture was then amplified in a DNA Thermal Cycler (Techne Progene, Marshall Scientific, Hampton, NH) using the following program: one denaturation step at 94°C for 5 min; 37 cycles (30s at 94ºC, 30s at 51ºC, and 30s at 72ºC); and a final extension for 5 min at 72ºC. Gel analysis of the PCR products were performed by gel electrophoresis using 1.5% Agarose gel with 1X Tris-acetate-EDTA (TAE) buffer.

DNA Sequencing

QIA-quick Kit (Qiagen, Hilden, Germany), was used for purification of the PCR products. Second PCR was performed using BigDye Terminator v3.1 Cycle Sequencing Kit. Each reaction (20 µL) contained a terminator ready reaction mix (8 µL), Primer (3.2 pmol), DNA template quantized according to the PCR product size, and deionized water. Thermal profile for Cycle Sequencing PCR was 1 min at 96ºC; 25ºC cycles as follows: 10 s at 96ºC, 5 s at 50ºC, and 4 min at 60ºC. After an additional step of purification with CENTRI-SEP Columns (Princeton Separations, Freehold, NJ), DNA sequencing was carried out by 3500 Genetic Analyzer (Applied Biosystems, Massachusetts, USA). The

obtained consensus sequences were subjected to BLAST search through the Mega program (7.0.20) [27,28].

Results and Discussion

Quantitative estimation of aerobic bacteria in tilapia fish samples were estimated 5.6 ± 0.8 logs CFU/g. The total aerobic count were within the acceptable limits compared to the Egyptian Organization for Standardization, EOS (<10^6 CFU/g, EOS, 2005). Still, the bacterial load in all samples was high and one of the reasons may be that the high temperature as fish were collected in summer where temperature range from 30-35°C. This temperature was reflected on the water environment to be close to optimum for many mesophilic bacteria, and increase the bacterial load in fish [29]. In agreement with this results, tilapia (*Oreochromis niloticus*) were reported associated with high total bacterial count isolated from fish surface coming from the northern region of Costa Rica, and up to 27.3×10^8 CFU/g sold at Sokoto Central Market in Sokoto, Nigeria [30,31] reported even higher bacterial load (5.5×10^9 CFU/g) of tilapia fish than this study. The high bacterial count on the skin might be due to contamination by original aquatic species as well as commodity contamination during handling.

E.coli isolates (4/50, 8%) has been recovered from this study, and only one isolate (1/50, 2%) was identified as *E. coli* O157:H7 (Figure 1). *E. coli* O157:H7 was isolated and identified as gram negative sorbitol non fermenting colonies on SMAC plates. Isolates were confirmed by biochemical tests listed in table 1, and by using serological agglutination test against O157, and H7 specific antisera. For further confirmation, *E.coli* isolate was identified by PCR and 16S rDNA gene sequencing. The nucleotide sequence of *Escherichia coli* O157: H7 Ras4 has been submitted to the GenBank with accession number KY120324, and represented in a phylogenic tree in Figure 2. E.coli O157:H7 is not commonly reported associated with fish and seafood. *E. coli* were isolated in 414/484 finfish samples in India but typical *E. coli* O157 was absent, however MUG and sorbitol-negative strains were reported. Thampuran et al [32] concluded that, this result might suggest the existence of the strain. On the other hand, Surendraraj et al. [33] recovered *E. coli* O157:H7 from shellfish in seafood markets in India. Seven standard *E. coli* O157:H7 were identified and one shrimp sample was positive for 3 virulence markers. Another study conducted on Nile tilapia (*Oreochromis niloticus*) fish skin, gastrointestinal tract, and muscles from pay-to-fish ponds located in at the Córrego Rico watershed in São Paulo, Brazil isolates. Eight from 96 totals *E. coli* isolates (6.95%) from the fish gastrointestinal tract contained O157 gene, but *E. coli* O157 was not reported on fish skin [34]. Shiga toxin-producing *Escherichia coli* (STEC) O157 were reported recovered from processed salmon roe which had been a suspected food item in sporadic infections occurred in Japan, 1998 [35].

Wang and Doyle [36] reported that *E. coli* O157 can survive in water for several weeks. Therefore, faecal contamination of water sources or aquaculture environments by *E. coli* O157 can act as a vehicle of transmission of diarrheagenic enteric infections. Water supplies were reported contaminated by *E. coli* O157 in Brazil and

Scotland [37,38]. This may suggests that fish contamination may originate from bovine faeces, probably from the surrounding to aquaculture water. *E. coli* bacteria that can cause human diseases do not cause losses in aquaculture production. Therefore, fish farmers not feel the need to apply appropriate health control measures to ensure product quality. However, infected fish used as a food source can serve as means of transmission of these agents to humans. Therefore, good hygienic handling measures and proper processing are needed before consumption of fish products.
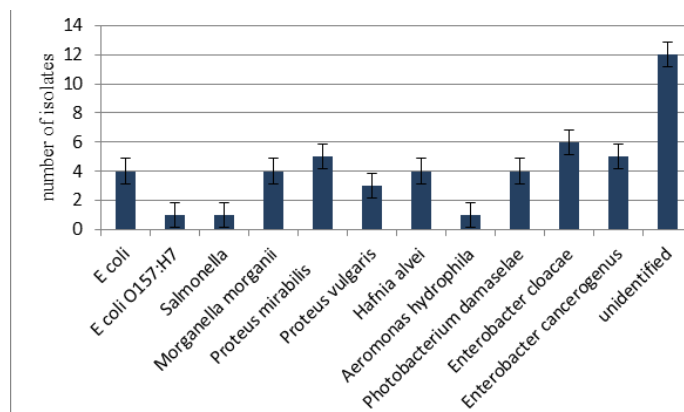

Figure 1: Number of occurrence of bacteria isolated from fresh-water acquaculture tilapia (Oreochromis niloticus) fish.
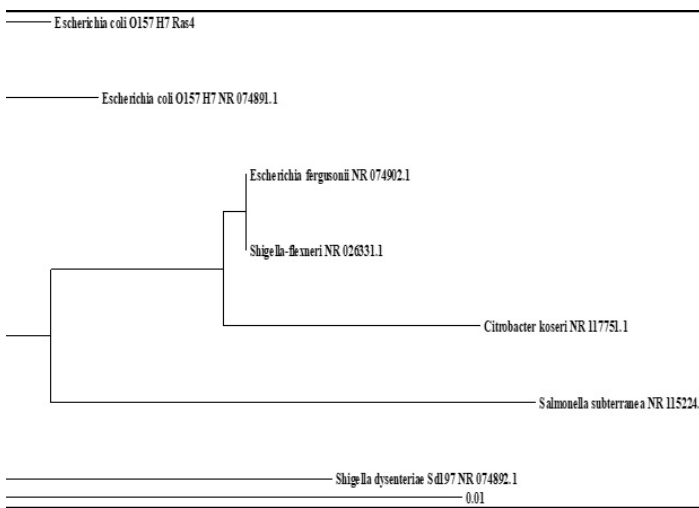

Figure 2: Phylogenic tree represented sequenced *Escherichia coli* O157: H7 Ras4 (GenBank accession number KY120324).

Salmonella spp. (1/50, 2%) was isolated and identified in this study (Figure 1). Isolates were gram negative black centered colonies on XLD media. Selected colonies were tested by indole, citrate, MR, VP, urease, and TSI biochemical tests and identified with API 20E as *Salmonella enterica* as listed in table 1. *Salmonella* causes foodborne illness associated with dehydration, reactive arthritis, septicemia, and can lead to death [39]. Therefore, Food safety standards have demanded the absence of Salmonella in chilled fresh fish [40]. *Salmonella* could be introduced to the aquatic system through many ways such as poor sanitation, inappropriate disposal of human and animal wastes [41]. The presence of *Salmonella enterica* in the present study suggested the existence of poor

hygienic measures and better control and monitoring is required.

Ellermeier and Slauch [42] revealed that cold-blooded animals such as tilapia by themselves are potential hosts for Salmonella species. Salmonella have been reported associated with tilapia fish in several studies. Different Salmonella serovars (*S. Corvallis, S. Bovis-mobificans, S. Agona, S. Mikawashima, and S. Typhimurium*) were isolated from tilapia (14/32, 43.8%) obtained from wet markets in Malaysia [43]. *Salmonella spp.* were found in fish mucus (20.0%) of a total of 20 tilapia. Salmonella spp. were reported in raw retail frozen imported fresh-water tilapia fish to eastern province of Saudi Arabia 64% (16/25) from Thailand, and 28% (14/50) from India [44]. Similar results to this study were revealed with fresh tilapia fish (*Oreochromis niloticus*) in Sokoto, Nigeria. Salmonella spp. Showed the least frequency of occurrence (1/31, 3.22%) [45].

Several bacteria isolated in this study have the potential of histamine formation. These bacteria include *Morganella morganii* (4/50, 8%), *Proteus mirabilis* (5/50, 10%), *Proteus vulgaris* (3/50, 6%), *Hafnia alvei* (4/50, 8%), *Aeromonas hydrophila* (1/50, 2%), *Photobacterium damaselae* (4/50, 8%), *Enterobacter cloacae* (6/50, 12%), and *Enterobacter cancerogenus* (5/50, 10%) (Figure1). All isolates were confirmed by biochemical tests listed in table 1, and for further confirmation, *Morganella morganii* isolate was identified by PCR and 16S rDNA gene sequencing. The nucleotide sequence of *Morganella morganii* Ras1 has been submitted to the GenBank with accession number (KY120325), and represented in a phylogenic tree in Figure 3. Same confirmation was done with *Proteus mirabilis* isolate, as it was identified by PCR and 16S rDNA gene sequencing. The nucleotide sequence of *Proteus mirabilis* Ras2 has been submitted to the GenBank with accession number (KY120326), and represented in a phylogenic tree in Figure 4.
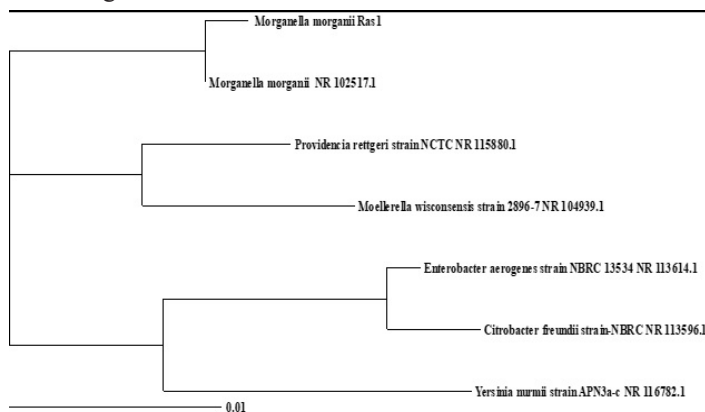

Figure 3: Phylogenic tree represented sequenced *Morganella morganii* Ras1 (GenBank accession number KY120325).

Histamine production is associated with scombroid poisoning, and its toxicity is enhanced by the presence of other biogenic amines in foods. Histamine is formed by decarboxylation of histidine, which is found at high levels in muscles of fish belonging to the Scombroidae family [46,47]. It generally results from proliferation of histamine producing bacteria, which possess histidine decarboxylase. Enterobacteriaceae has been reported to be the most important histamine-producing bacteria in fish [48]. *M. morganii*, *Proteus app.*, and *Hafnia alvei* are considered proliferating histamine forming bacteria and the quantity of histamine produced is varied among species [49-52] reported *Photobacterium spp.* and *Aeromonas spp.* as histamine producers. Tilapia did not belong to the Scombroidae family, but all food rich in protein are susceptible for histamine and other biogenic amines formation when desirable conditions are present for the microorganism [53]. Histamine was estimated in 52 tilapia fish in Bahir Dar town, Ethiopia. Mean level of histamine detected was between 3.8-290 mg/100 g, which exceed the accepted limit of histamine established by EU regulation and could cause histamine toxicity [54].
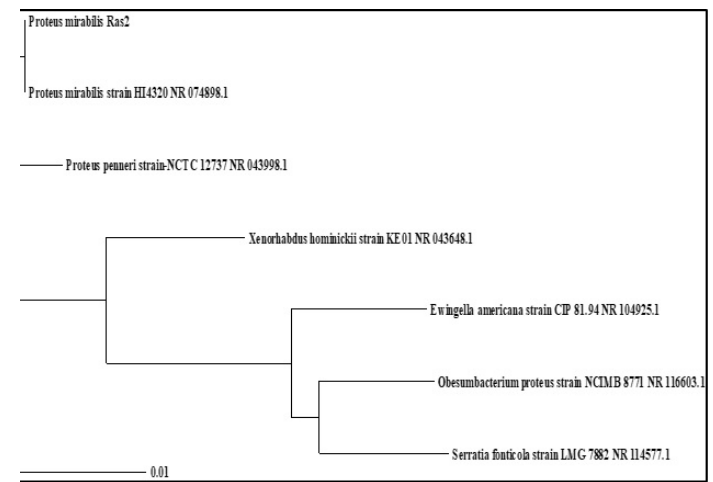

Figure 4: Phylogenic tree represented sequenced *Proteus mirabilis* Ras2 (GenBank accession number KY120326).

Bacteria isolated in this study associated with different complications that might affect the public health. *P. mirabilis* becomes an opportunistic pathogen where it causes urinary tract infections and other types of nosocomial infections [55]. *Hafnia alvei* reported associated with persistent septicemia [56]. According to Kirov [57,58]. *Aeromonas spp.* are pathogens which can cause bacteraemia, meningitis, pulmonary and wound infections. It might cause ''summer-diarrhoea'', which is a worldwide problem in children under five years old, the elderly, and travellers. *Photobacterium damaselae* was associated with infection after digestion of raw seafood, and urinary tract infection after exposure to contaminated water [59]. It was diagnosed with septicemia and hepatic dysfunction in a cirrhotic patient after ingestion of seafood [60]. *Photobacterium damsel* isolation from cultured fish with high economic value has made the bacterium as concern in aquaculture industry. Abdel-Aziz et al [61] identified Photobacterium damsel during mass mortalities of cultured seabream and European seabass in Egypt. *Enterobacter cloacae* are gram negative bacteria, can cause wound, respiratory and urinary tract infections. It is considered major human pathogen responsible for large outbreaks of nosocomial disease [62,63]. *Enterobacter cancerogenus* was associated with septicemia and wound infection especially with persons exposed to the organism during traumatic events [64].

	Morganella morganii	Proteus mirabilis	Proteus vulgaris	E. coli	Salmonella enterica	Enterobacter cloacae	Enterobacter cancerogenus	Hafnia alvei	Aeromonas hydrophila	Photobacterium damaselae
ONPG	-	-	-	+	+	+	+	+	+	-
Arginine dihydrolase	-	-	-	+	-	+	-	-	+	+
Lysine decarboxylase	-	-	-	+	-	-	-	+	+	-
Ornithine decarboxylase	+	+	-	-	+	+	-	+	-	-
Citrate	-	+	-	-	+	+	+	+	-	-
H 2S	-	+	+	-	+	-	-	-	-	-
Urease	+	+	+	-	+	-	-	-	-	+
TDA	+	+	+	-	-	-	-	-	-	-
Indole	+	-	+	+	+	-	-	-	-	-
Voges-Proskauer	+	-	-	-	-	+	+	+	+	-
Gelatinase	-	+	-	-	-	-	-	-	+	-
Acid from: glucose	+	+	+	+	+	+	+	+	+	+
Mannitol	+	-	-	+	+	+	+	+	+	-
Inositol	-	-	-	-	-	-	-	-	-	-
Sorbitol	-	-	-	+	+	+	-	-	-	-
Rhamnose	-	-	+	+	+	+	+	+	-	-
sucrose	-	-	+	+	+	+	-	-	+	-
Melibiose	-	-	-	+	+	+	-	-	-	-
Amylose	-	-	-	-	+	+	+	-	-	-
arabinose	-	-	-	+	+	+	+	+	-	-

Table 1: Biochemical test results of different isolated bacteria using API 20E diagnostic strips.

In conclusion, this research has indicated that the bacterial species associated with fresh aquaculture Tilapia fish and has shown that they are potentially pathogenic to humans. Therefore, adequate measures should be taken in handling and processing this wide distributed fish before consumption.

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