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Rapid pathogen discovery in diseased turbot (*Scophthalmus maximus*) using 16S rRNA high throughput sequencing

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ABSTRACT

Pathogenic bacterium discovery using 16S rRNA high throughput sequencing has become more commonplace. It offers advantages of being high-throughput screening, cost-effective (less than $15 per sample), and rapid (less than one week). Fish pathogens causing aquatic bacterial diseases outbreak represent a major threat to aquaculture industry. A haemorrhagic disease in turbot (*Scophthalmus maximus*) outbreaks with unknown etiology in Haiyang, China. Initial analyses suggested it was a bacterial disease based on gross pathology of diseased fish containing ascitic fluid from the distended abdomen, a swollen intestine, an inflamed and red mouth. To fully understand the accurate etiology, distribution of pathogenic bacterium and comprehensive information on the diseased turbots is required. Tested tissues (ascites, heart, eye, mouth, intestine, spleen, and liver) with typical symptoms of diseased turbots were collected and 16 s rRNA sequencing was performed using the Illumina Mi-Seq platform. Sequencing results revealed generally low microbial diversity (< 50 %) in different infected tissues, but the microbiota did exhibit a little higher diversity in the intestine than in the other tissues. A key finding was that *Edwardsiella* was the most abundant genus detected in all the fish tissues. It was indicated that *Edwardsiella* spp. was the main causative pathogen in the diseased turbot. The evidence also suggested that *Edwardsiella* spp. as a severely pathogenic bacteria may cause disruption of the healthy balance in the host. Our study was a new attempt to investigate unknown pathogens in clinical marine fish specimens through the high-throughput next-generation sequencing technology.

1. Introduction

Traditionally, the causal microorganisms of fish diseases are isolated and determined by culture-dependent methods. This process for a pathogen's discovery mainly consists of its acquisition, separation, and cultivation followed by a conventional PCR assay. The PCR targeting the 16S rRNA gene has proven itself useful for the detection of bacterial pathogens of fish. Yet most of the aquatic pathogenic bacteria are actually opportunistic bacterium species. These opportunistic pathogenic bacteria are exposed to a range of changing environmental conditions during their life cycle, and they could utilize various strategies for living outside and inside a host (Zhu, 2020). Accordingly, it is quite difficult to identify the particular pathogenic bacterium that is the leading cause of death in aquatic animals based on the conventional PCR assay. To get around this problem, it is necessary to perform an artificial infection of the host fish with the candidate pathogenic bacteria; however, only about 1% of microorganisms can be cultured under laboratory conditions. Hence, given the many candidate pathogens, using the conventional identification approach is both time and labor consuming and could overlook novel pathogens.

Due to advances in next-generation sequencing (NGS) platforms, the massive parallel and short-read sequencing of nucleic acids spanning millions of intereactions could be achieved through a single instrument run (Brown et al., 2012; Cerda-Cuellar et al., 1997; Derakhshani et al.,

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2016; Patel, 2001). As noted above, the 16S rRNA (SSU rRNA) gene is currently the most widely used biomarker, since it is present in the genome of all bacteria (Grützke et al., 2019). Owing to its universal phylogenetic distribution, 16S rRNA is considered a key molecular target of NGS applications for characterizing the bacterial community and diversity of marine ecosystems, environmental microbiomes, or microbial ecology of fish gut (Frey et al., 2014; Grützke et al., 2019; Shokralla et al., 2012). Recently, 16S rRNA next-generation sequencing has proven itself valuable as a molecular diagnostic tool for detecting the pathogenic bacteria in the aquatic host animals, such as fish, shrimp, and shellfish (Alonso et al., 2012; Martínez-Porchas and Vargas-Albores, 2017; Stärk et al., 2019). Additionally, DNA sequencing techniques are becoming increasingly popular and less and less expensive. The methodology in this study currently costs less than $15 per sample and advances are made every year.

Turbot (*Scophthalmus maximus*) is an economically important mariculture fish that belongs to the family Scophthalmidae (order Pleuronectiformes). In its native range, turbot is widely distributed across Europe (Pereiro et al., 2016), and it was introduced to China in 1992. Nevertheless, the rapid development of a high-density industrial cultivation mode for rearing stocks of this fish has caused a parallel rise in the pathological conditions adversely affecting its production. Several constraints influence the farming of flatfish generally, namely a degraded genetic constitution and susceptibility to specific diseases that cause serious mortality and morbidity, resulting in substantial economic losses. Bacteria (*Vibrio* sp., *Streptococcus* sp., *Aeromonas* sp.), viruses (herpes virus, iridescent virus), and parasites (Scuticociliatid ciliate, *Cryptocaryon irritans*, *Trichodina* spp., *Paralembus digitiformis*, *Tetramicra brevifilum*, *Ichthyobodo* sp.) can all function as pathogens that diminish the health of such farmed fish (Gao et al., 2016; Pereiro et al., 2016). Among them, bacterial disease is probably the most destructive disease currently afflicting turbot aquaculture. In this respect, the pathogenic bacteria reportedly involved include *Edwardsiella tarda* (Padrós et al., 2006), *V. scophthalmi* (Cerda-Cuellar et al., 1997), *V. ichthyoenteri* (Montes et al., 2006), *V. anguillarum* (Westerdahl et al., 1991), *V. alginolyticus* (Munro et al., 1995), and *V. splendidus* (Thomson et al., 2005), among others. These various bacterial pathogens have been found to impact the turbot aquaculture with high frequency, high virulence, and high mortality, for which the associated outbreaks occur swiftly and widely, in both fish larvae and adult stages. The telltale symptom or clinical syndrome common to them is a swollen abdomen filled with a large amount of colorless or yellowish liquid. But in more severe cases, the visceral organs will also present lesions, in the form of an enlarged liver or intestine, or both.

In this study, clinical samples were collected from moribund turbots during an outbreak of an unknown disease that occurred in 2017, in a mariculture farm located in Haiyang (Shandong province), a northern coastal city of China. There are about 20 turbot farms distributed in Haiyang, whose industrial recirculating aquaculture and frozen-fish feeding results in frequent occurrence of disease in this area distinguished by apparent similar clinical symptoms. Despite much effort, the main etiology remains uncertain and no effective treatment yet exists. Considering that the investigation of pathogens using conventional methods is practically difficult to obtain the comprehensive information for pathogenic bacteriums, here we utilized high-throughput NGS to screen for potential pathogens responsible for disease in farmed turbot.

2. Materials and methods

2.1. Turbot culture and sampling

The turbots (body length: 15–18 cm) used in our study were reared and maintained in a mariculture farm in Haiyang (Shandong Province, China). A disease outbreak of unknown origin occurred among these cultured turbots, resulting in their mass mortality at the mariculture farm. Diseased turbot individuals were collected and transported to the laboratory (Shanghai Ocean University). Upon arrival of each specimen, its clinical symptoms were recorded and then all fish underwent a wet-mount examination to identify the presence of bacteria and possibly other microorganisms. For the 16S rRNA NGS analysis, tissues or organs were collected from twenty diseased turbots displaying the typical symptoms of the disease from two separate ponds, namely a red mouth, inflamed eyes, ascitic fluid, hepatomegaly, and intestinal swelling. Each tissue from the same pond was placed in a sterile centrifuge tube and frozen immediately at −80 °C for further study. Given the tissue type and turbot specimen (T1, 'Turbot pond 1' or T2, 'Turbot pond 2') examined, these samples were accordingly labeled as T1Ascite, T1Spleen, T1Liver, T1Intestine, T2Heart, T2Eye, T2Mouth, and T2Intestine.

To avoid viral infections, common viruses including the Turbot reovirus (SMReV) and lymphocystis disease virus (LCDV) were first detected in the diseased tissues. The methods to do this followed those previously described (Ke et al., 2011; Zhang et al., 2004).

2.2. DNA extraction

Total DNA was extracted from each sample using a TIANamp Bacteria DNA Kit (DP302, Tiangen, China), according to the manufacturer's protocol. All procedures were performed on ice, and the final elution volume used was 100 μL. The DNA concentration was measured by a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA), while the quality of extracted DNA was assessed by 1% agarose gel electrophoresis. All DNA extracts were stored at −80 °C until their further analysis.

2.3. Mi-Seq 16S rRNA sequencing

The V3–V4 hypervariable region of the bacterial 16S ribosomal RNA (rRNA) gene was amplified from genomic DNA samples, by using these primers: 338 F, 5′-ACTCCTACGGGAGGCAGCAG-3′ and 806R, 5′-GGACTACHVGGGTWTCTAAT-3′ (Mori et al. 2014). The product length was 469 bp. Briefly, PCR was performed in triplicate in a 50-μL reaction mixture that contained 100 ng of template DNA, 5 μL of 10 × *Pfu* DNA polymerase buffer with $MgSO_4$, 1 μL of each primer, 1 μL of 10 mM dNTPs, and 2 μL of *Pfu* DNA polymerase, with the final volume attained by adding ddH_2O (M774A, Promega, USA). The PCR conditions were as follows: 3 min of initial denaturation at 95 °C, followed by 35 cycles of denaturation at 95 °C for 45 s, annealing at 50 °C for 30 s, and an extension at 72 °C for 45 s, followed by a final elongation at 72 °C for 10 min. The ensuing PCR products were stored at −20 °C. The amplified products were analyzed with 1% agarose gel electrophoresis, using the DL2000 marker, for their quality examination and then purified with the QIAquick Gel Extraction Kit (28704, Qiagen, Germany). Magnetic beads were used to isolate the amplicons of interest. Finally, the libraries were sequenced using an Illumina Mi-Seq 2 × 300 platform (Illumina, Inc. San Diego, USA) housed at the Shanghai Majorbio Bio-pharm Technology Co., Ltd (Shanghai, China), by following an already described protocol (Caporaso et al., 2012).

2.4. Data analysis

Only high-quality data were obtained and used for the bioinformatics analyses of diseased turbot. The bacterial sequence reads were assembled using the Flash 1.2 tool (Magoč and Salzberg, 2011). To increase the analytical quality, all sequences of the samples were first filtered and classified in QIIME 1.9 software according to the SILVA taxonomic database (Caporaso et al., 2010). Next, the species-level operational taxonomic units (OTUs) for 16S rRNA gene were obtained using the threshold of a 97 % sequence identity. The microbial alpha diversity structures in the different tissue samples were analyzed by Mothur 1.30 (Schloss et al., 2009), and their corresponding alpha diversity indices—Sobs, Chao, ACE, Shannon, Simpson—were calculated from the OTU matrix, by using the R package 'phyloseq'. QIIME 1.9 software was

also used to analyze the microbial beta diversity structures across different samples, by using Python scripts (Caporaso et al., 2010). A Venn diagram was built based on the relative abundance of bacteria at the genus level. The taxonomic assignment of OTUs was investigated by comparing their sequences, using UPARSE 7.0 software, to generate a heatmap (Edgar, 2013). Principal components analysis (PCA) and principal coordinate analysis (PCoA) were performed using statistical software to determine the composition of the bacterial communities at the genus level. The original sequencing data of all obtained raw reads have been deposited in the Sequence Read Archive database of National Center of Biotechnology Information (under accession no. PRJNA613048).

3. Results

3.1. Gross pathology of diseased fish

Generalized congestion and abscess-like lesions filled with a purulent fluid were very typical symptoms in the diseased fish at approximately 10 fish farms surveyed in our study area. The macroscopic lesions of the diseased fish are shown and described in Fig. 1. Evidence of tumefaction was observed mainly in the head region, with inflamed eyes (Fig. 1A) and a red mouth (Fig. 1B). Infected turbots also exhibited hemorrhaging of their basal fin and the musculature in their abdominal area (Fig. 1C); in the latter respect, the abdomen was distended because of accumulated ascitic fluid (Fig. 1D). When these tumefacted areas were cut open, swelling of the intestine (Fig. 1E) and enlargement of the liver (Fig. 1F) were clearly evident.

3.2. Sample metadata and sequencing statistics

By applying the Illumina Mi-Seq sequencing platform to the 16S rRNA gene amplicons, a total of 305,895 high quality bacterial sequences with an average length of 469 bp (V3–V4338-806 bp) were obtained from all turbot samples (Table S1). These sequences were assigned to 99 OTUs (Table S2), ranging from 30 to 99 per sample, based on at least 97 % similarity (Table S3). After sampling the 15,000 reads, the number of newly discovered OTUs was eventually reduced, in that the rarefaction curves tended to attain their saturation plateau as sampled read number increased (Fig. 2). This figure demonstrates that the libraries of the eight specimen (eight different tissues from twenty diseased turbot) were large enough to reliably estimate the phylotype richness.

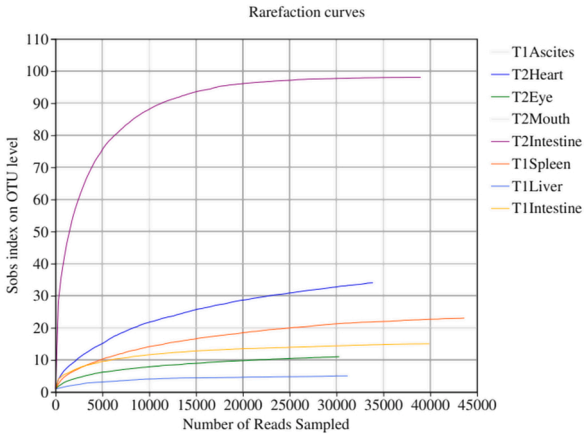

Fig. 2. Rarefaction curve analysis for the eight tissue microbiomes sampled from diseased turbot.

3.3. Abundance and diversity of bacterial microbiota

Fig. 3 shows the composition of the bacterial communities in the infected tissues of diseased turbots, at the genus level. Among those genera, *Edwardsiella* had a dominant proportion in all eight samples. Meanwhile, comparing the composition of the bacterial communities

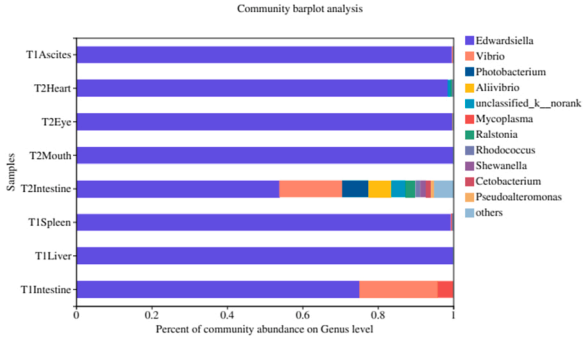

Fig. 3. The relative abundance of genus levels in the infected tissues of diseased turbot fish. The different coloring denotes different microbial species. The bar length represents the proportion of microbial species in a given sample.

Fig. 1. Pathological features of diseased turbot fish. (a) Evident tumefaction around the eyes; (b) an inflamed and red mouth; (c) abdominal areas with hemorrhaging; (d) ascitic fluid from the distended abdomen; (e) a swollen intestine; (f) an enlarged liver. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

revealed higher diversity in the intestines than the other samples. Additionally, based on this high-throughput sequencing data, a robust phylogenetic tree was constructed using maximum likelihood method (Fig. S1). Intuitively, the branching reflected the similarity and richness between the bacterial species. These results uncovered a cluster of *Edwardsiella* that appeared in every group, whose outstanding advantage in terms of the total number of its member species was indicated.

The PCA was applied to the OTU abundances (Fig. S2**)**. The microbial communities harbored by the T1Intestine and T2Intestine were separated from those of the other tissue samples taken from turbot. The microbiota of the T2Mouth and T1Spleen samples was relatively similar while the microbial communities of T1Ascites, T2Heart, T1Liver, and T2Eye were positioned very close to each other. In the corresponding PCoA, which used the UniFrac tool (Fig. S2B**)**, the above bacterial communities also displayed the same pattern of clustering.

We evaluated the alpha diversities (Sobs, Shannon, Simpson, ACE, and Chao) of the bacterial community in the infected tissues of turbot. All these indices were analyzed and calculated based on the OTUs. The Sobs, Chao, and ACE indices conveyed the species richness of the samples' communities, which respectively ranged from 5 to 98, 5.54–98, and 5–98. The Shannon and Simpson indices reflected the species diversity, which ranged from 0.006 to 2.026 and 0.311 to 0.999, respectively (Table S4); this uncovered discrepancies in diversity discrepancy among the infected tissues (Fig. 4). The ascites, heart, eye, mouth, spleen and liver harbored very low bacterial diversity, while the intestines of two samples sustained the highest levels of bacterial diversity.

3.4. Unique and shared OTUs in diseased turbot from two separate ponds and the cluster analysis of species compositions in different tissue samples

Along the lesions from the two infected fish specimens (i.e., Turbot pond 1 and Turbot pond 2), we were able to detect unique and shared bacterial OTUs at the species level in our sequencing data (Fig. S3). Those species whose average abundance > 0.1 % were analyzed through Venn diagrams (Fig. S3A). Sample turbot pond 1 and Sample turbot pond 2 shared a total of 30 OTUs, whose proportion was 96.77 % in Sample turbot pond 1 and 30.61 % in Sample turbot 2. Importantly, all 30 OTUs belonged to the *Edwardsiella* genus. Overall, 69 specific OTUs were found, but Sample turbot pond 1 had only 1 of these whereas and the rest (68 OTUs) were unique to Sample turbot pond 2. The classifications for the bacterial OTUs' relative abundances are summarized neatly in the heatmap (Fig. S3B). According to the taxonomic analyses, the heatmap for the eight tissue samples not only demonstrated the occurrence of every species in each single sample, but it also displayed the relative abundance of each microorganism in the sample. *Edwardsiella* (OTU10) was the most abundant genus in the composition of microbiota residing in the infected tissues of farmed turbot.

4. Discussion and conclusion

In our study, the data generated by this high-throughput sequence analysis showed that *Edwardsiella* spp. was the major pathogen associated with the high-mortality outbreak of ascites disease that happened in a mariculture farm in Haiyang, China. This finding is consistent with the typical symptoms caused by *Edwardsiella* spp. (Austin and Austin, 2016, Castro et al., 2006). Notably, all the tested tissues from the diseased turbots showed single-strain migration. These results also suggest that *Edwardsiella* spp. shows the ability to disrupt the host flora balance. *Edwardsiella* spp. can cause edwardsiellosis, which has emerged as one of the most severe diseases now threatening the turbot aquaculture industry, capable of causing serious economic losses. Well known for causing haemorrhagic septicaemia in fish, *Edwardsiella* spp. is an intracellular bacterium, so using antibiotics to kill its cells is difficult. Instead, many researchers are increasingly investigating safer, stable, and efficient vaccines. China started a bit late in its research of vaccines to protect its farmed fish stocks; but fortunately, the live vaccine against

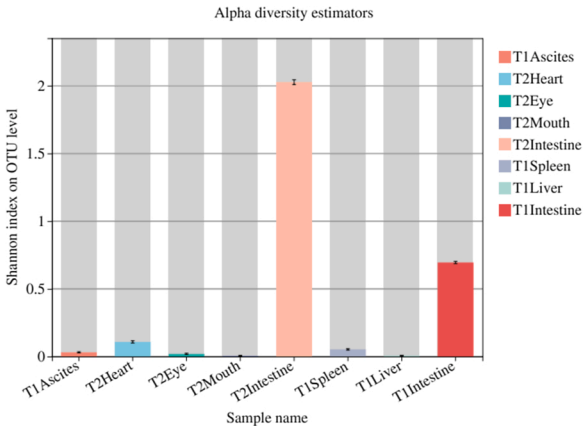

Fig. 4. Diversity of the microbial communities in the eight tissue samples from diseases turbot fish. The Shannon index showed the alpha diversity.

Edwardsiella tarda in turbot (*Scophthalmus maximus*) [EIBAV1 strain, (2016) 110576037] did receive a veterinary drug production license from the Chinese government (Gao et al., 2014). This made it the first live vaccine for mariculture animals in China and the first government-certified vaccine for use against *Edwardsiella tarda*. With the new aquatic vaccine products increasingly available, it should promote the establishment of a new model for the healthy aquaculture of marine fish in which the focus is on prevention and control strategies. Additionally, the candidate pathogens involved in this disease are extremely complicated. Two typical viruses, SMReV and LCDV, were tested for, but both had negative results.

Despite we failed to find a new pathogenic bacterium in the diseased turbots, these results also suggested that *Edwardsiella* spp. shows highly pathogenic to turbots. Finally, our study is also a new attempt to screen unknown pathogens in diseased fish through the high-throughput next-generation sequencing technology.

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Contributions

HW, and YS were responsible for the experimental design, analyses, and interpretation of the data. YS drafted the manuscript. YS, JW, YX, and RP performed the experiments. All authors read and approved the final version of the manuscript.

Data availability statement

The data that support the findings of this study are openly available in NCBI's SRA database at https://www.ncbi.nlm.nih.gov/bioproject/PRJNA613048/, under reference number PRJNA613048.

Declaration of Competing Interest

The authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aqrep.2021.100835.

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