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Application of digital PCR and next generation sequencing in the etiology investigation of a foodborne disease outbreak caused by *Vibrio parahaemolyticus*

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ABSTRACT

Globally, *V. parahaemolyticus* infection is a leading cause of bacterial diarrheal diseases. Pathogenic *V. parahaemolyticus* strains that produce hemolysins are responsible for these diseases. The composition of pathogenic and non-pathogenic *V. parahaemolyticus* and the change of the bacterial composition before and after traditional selective enrichment in a single sample associated with disease outbreak remain unclear. We investigated an outbreak by using next generation sequencing and digital PCR to address those questions. NGS showed that the *V. parahaemolyticus* caused the outbreak belonged to s single clone. In contrast, among the seven non-pathogenic *V. parahaemolyticus* isolated from the suspected food sample, 4 serotypes and 6 PFGE patterns were identified. And nearly 70,000 SNPs were identified among the non-pathogenic strains. This result confirmed that the outbreak was caused by *V. parahaemolyticus*. Furthermore, NGS results clearly showed the diversity of non-pathogenic *V. parahaemolyticus* in a single contaminated food sample. The ratios of non-pathogenic and pathogenic *V. parahaemolyticus* were 31.41 and 620.11 in the original and enriched food samples respectively showed by digital PCR. Meta-genomic data indicated the top 3 species were *Weissella cibaria*, *Weissella confusa,* and *Enterobacter cloacae* in the original food sample, and *Vibrio* sp *Ex25*, *Vibrio* sp *712i,* and *V. parahaemolyticus* in the enriched sample. Therefore, the combing of NGS and digital PCR results showed that traditional *Vibrio* selective enrichment media could facilitate the growth of *Vibrios*, however, it provided no advantages to pathogenic *V. parahaemolyticus*. Hence, our results indicated that the traditional culture methods alone may lead to wrong conclusions and so improvements in culture methods are needed.

1. Introduction

V. parahaemolyticus is a halophilic estuarine bacterium, usually isolated from seafood and marine environments. *V. parahaemolyticus* infection can lead to gastroenteritis, wound infection, and septicemia (Joseph et al., 1982; Nair et al., 2007), and food that is cross-contaminated by seafood could also serve as a vector. Thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) are the main virulence factors produced by *V. parahaemolyticus* (Shirai et al., 1990; Yamamoto et al., 1983). Pathogenic *V. parahaemolyticus* strains, which

Abbreviation: NGS, Next Generation Sequencing; WMS, Whole metagenome sequencing

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can cause infections in humans, usually produce one or both of these hemolysins (Nair et al., 2007). Nevertheless, a small portion of clinical *V. parahaemolyticus* produced none of these two hemolysins (Haendiges et al., 2015). Pathogenic *V. parahaemolyticus* could rarely (~3%) be isolated from the environment samples (Velazquez-Roman et al., 2012). Moreover, either in routine surveillance or in outbreak investigations, pathogenic *V. parahaemolyticus* were not frequently isolated from food sample although those foods were highly suspectable. Previous studies showed that the *tdh* and or *trh* positive strains could account for up to 52% of *V. parahaemolyticus* isolated from environmental samples (Kaufman et al., 2003; Velazquez-Roman et al., 2012). Overall, great diversity has been observed in *V. parahaemolyticus* strains isolated from seafood and the environment (Theethakaew et al., 2013; Urmersbach et al., 2014). However, the diversity of *V. parahaemolyticus* and the proportion of pathogenic *V. parahaemolyticus* in individual food sample have been rarely reported (Klein and Lovell, 2017).

Next generation sequencing (NGS) is one of the most significant technology advances in the biological sciences (Staley et al., 2018), and has significantly changed the workflow in clinical and public health labs. NGS has been used for identification and source tracking in infectious disease outbreak investigations (Haendiges et al., 2015; Harris et al., 2013; Pang et al., 2016; Whistler et al., 2015). Whole metagenome sequencing (WMS) is the application of NGS to nucleic acids extracted directly from raw clinical or environmental specimens (Li et al., 2014). Theoretically, WMS results could reveal the whole spectrum of microorganisms in a specimen. WMS is therefore an ideal method for the identification of pathogens in infectious disease outbreak investigation. However, researchers have encountered many problems while using WMS (Gargis et al., 2016).

Digital PCR is an approach to nucleic acid detection and quantification, which could be used for absolute quantification and rare allele detection. Digital PCR is based on traditional PCR and fluorescent-probe-based detection methods, but eliminates the need for standard curves; in this method, a PCR sample is divided into many droplets, which are each subjected to the traditional PCR process. After amplification, droplets containing target sequences are detected by fluorescence and scored as positive, and droplets without fluorescence are scored as negative. Poisson statistical analysis of the numbers of positive and negative droplets yields absolute quantitation of the target sequence. Digital PCR has been used in the detection and quantification of viruses and bacteria (Bartsch et al., 2018; Staley et al., 2018).

In this study, we employed NGS and digital PCR to investigate and confirm an outbreak caused by *V. parahaemolyticus*. Furthermore, we accurately showed the proportion of pathogenic *V. parahaemolyticus* in the entire *V. parahaemolyticus* population in a single food sample before and after enrichment, which explained the reason that pathogenic *V. parahaemolyticus* were hard to be isolated from suspected food sample in outbreak investigation. Moreover, we showed that the traditional selective enrichment media is not suitable for the isolation of pathogenic *V. parahaemolyticus* although it can facilitate the growth of *V. parahaemolyticus*.

2. Materials and methods

2.1. Sample collection

Three patients with watery diarrhea visited local hospital in August 2017 in Shunyi District, Beijing, China. Ten grams of fresh stool samples were collected from each of these patients. The samples were stocked in sterile containers at 4 °C and transported to the laboratory for bacterial isolation.

2.2. Isolation of V. parahaemolyticus from suspected contaminated food (tripe) and patient stool samples

Twenty-five grams of the suspected contaminated food (tripe) was grinded and added into 225 mL of alkaline peptone water (APW) with 3% NaCl. The samples were incubated at 37 °C for 18 h. One loop of the enriched cultures was then streaked on to 10 CHROMagar agars (CHROMagar, France) and incubated at 37 °C for 24 h. Ten suspected purple colonies on the plate were transferred onto triple sugar iron slants and incubated at 37 °C for 24 h. A loop of stool sample from the patients was inoculated into 5 mL of 3% Sodium Chloride APW and enriched at 37 °C for 18 h. One loop of enriched culture was streaked onto 3 Chromagar agars and incubated at 37 °C for 24 h. Five suspected *V. parahaemolyticus* colonies from each agar plate were transferred onto triple sugar iron slants and incubated at 37 °C for 24 h. Biochemical identification of the 115 isolates from food sample and patient stool samples were conducted using VITEKR 2 COMPACT (BioMerieux). Isolates with typical *V. parahaemolyticus* phenotypes were serotyped using a commercial kit (*V. parahaemolyticus* antisera, Denka Seiken, Japan).

2.3. Pathogen screening from food sample, patient stool samples, and isolated V. parahaemolyticus with TaqMan real time PCR

We collected 200 mg of original food sample, enriched food sample, and stool samples from the three patients for nucleic acid extraction. Bacterial and viral nucleic acids were extracted using the QIAamp Fast DNA Stool Mini Kit (Qiagen GmbH, Hilden, Germany) and High Pure Viral Nucleic Acid Kit (Roche Diagnostics, Mannheim, Germany), respectively. DNA of the *V. parahaemolyticus* strains was extracted by using Bacterial Genome Extraction Kit (YR02000-1, Beijing Land Bridge Technology Co., Ltd., Beijing, China). Real-Time PCR was used for the screening of pathogens including *V. cholerae, V. parahaemolyticus* (Park et al., 2013)*, Campylobacter. jejuni* (Zhang et al., 2013)*, Salmonella, Shigella,* Diarrheagenic *E. coli,* Norovirus, Rotavirus, and Enteric Adenovirus (Shen et al., 2016). The *ToxR*, *tdh,* and *trh* genes in *V. parahaemolyticus* were detected as described previously (Wang et al., 2009). The *tlh* gene in *V. parahaemolyticus* was detected with primers used in a previous paper (Bej et al., 1999).

2.4. Pulsed-field gel electrophoresis (PFGE) analysis of the V. parahaemolyticus strains isolated in suspected contaminated food and patients

PFGE was performed as described previously with minor modification (Kam et al., 2008). PFGE images were obtained using a Universal Hood II (Bio-RAD, USA) and analyzed using BioNumerics software version 7.1 (Applied Maths, Saint-Martens-Latem, Belgium). A clustering tree indicating relative genetic similarity was constructed using UPGMA (Unweighted Pair Group Method with Arithmetic Mean) and the Dice-predicted similarity value with a 1.0% pattern optimization and 1.5% band position tolerance.

2.5. Quantitative analysis of tdh and toxR genes in the original and enriched samples by digital PCR

The *tdh* and *toxR* genes of *V. parahaemolyticus* were used as target genes for digital PCR quantitative analysis. In order to improve the concentration of *tdh* gene in the samples, we used 10 sets of 200 mg of original and enriched triple samples to in the preparation of nucleotide acid. Before the elution step, the ten sets of original and enriched triple samples were proceed individually. At the elution step, we used 200 μl elution buffer to resolve nucleotide acid on the 10 membranes one after another for the original and enriched triple samples respectively. And then we concentrated the 200 μl nucleotide acid sample into 50 μl. The digital PCR quantitative analysis was performed on an ABI QuantStudio 3D Digital PCR System (Thermo Fisher Scientific, USA). Each reaction contained 7.5 μl of 2*mix premix (QuantStudioTM 3D Digital Chip Kit v2 A26316 LOT: 1706212), 0.4 μl (10 μmol/L) each of the *tdh* or *toxR* forward and reverse primers, 0.2 μl of the TaqMan probes for *tdh* or

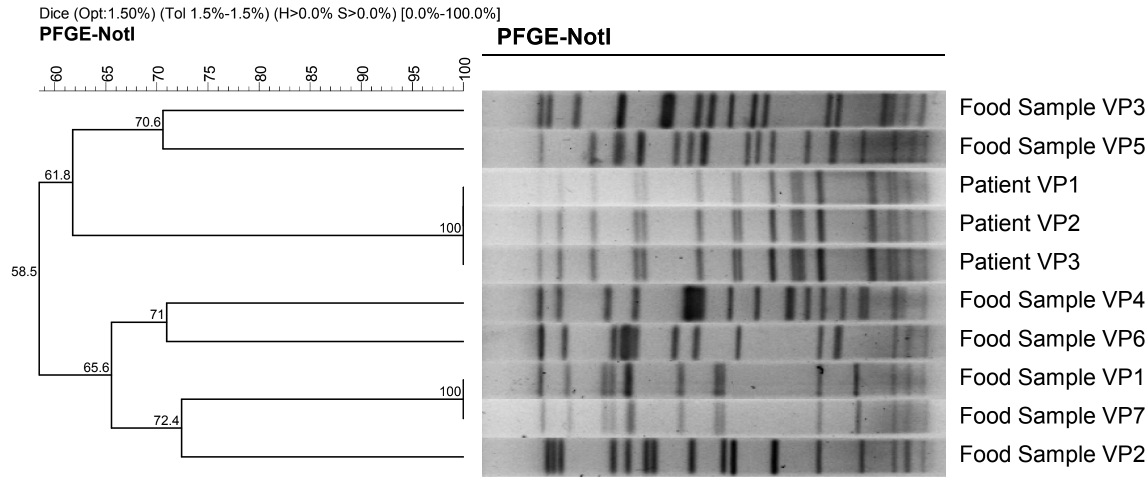

Fig. 1. Pulsed-field gel electrophoresis patterns of *V. parahaemolyticus* strains isolated from patient stool and food samples.

toxR genes (Wang et al., 2009), 3 µl of ddH2O and 3 µl of nucleic acid template in a total volume of 14.5 µl. The cycling conditions included an initiating stage of 3 min at 95 °C followed by 40 cycles of 10 s at 95 °C, 20 s at 55 °C, and 15 s at 72 °C.

2.6. Whole metagenome sequencing (WMS) analysis

2.6.1. DNA extraction and metagenomic sequencing

Genomic DNA was isolated from approximately 200 mg of original tripe sample by using the QIAamp Fast DNA Stool Mini Kit (Qiagen GmbH, Hilden, Germany) and following manufacturer's instruction.

DNA library construction was performed using a kit (KAPA Hyper Prep Kit, kk8504) by following the manufacturer's instruction. Owing to the low amount of input DNA, adapter-ligated DNA was enriched by 15 PCR cycles. One paired-end library with insert size 350 bp for each sample was constructed and sequenced with 150 bp read length from each end. The sequencing was carried out on an Illumina HiSeq X Ten instrument (Illumina, USA).

2.6.2. Data analysis

We obtained about 33.18 million of sequence raw data for each sample. We used the FASTX-Toolkit to remove the low quality raw data and SOAPdenovo2 to filter the host genome sequences (Luo et al., 2015). We eventually obtained 28.44 million high quality reads for each sample. We then used bowtie2 to map the reads to *toxR* and *tdh* respectively.

2.7. Whole genome sequencing

2.7.1. DNA preparation and genomic sequencing

DNA was prepared from 1 mL overnight cultures with the Wizard Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's instructions. Whole genome sequencing was performed on 10 genomes using an Illumina HiSeq X Ten with 250-bp paired-end library (BGI, China). All the data have been submitted to the European Nucleotide Archive.

2.7.2. Genome assembly and identification of SNPs

Short reads were assembled into contigs and scaffolds using SPAdes (V3.9). MuMMER (V3.0) was used to perform whole genome alignment for all of the genomes (Kurtz et al., 2004). The whole genome sequence of RIMD2210633 (Heidelberg et al., 2000) was used as the reference to call SNPs in MuMMER. SNPs with a quality score < 30 were excluded. SNPs in high-density clusters or in possible recombination segments were also discarded using a previously described method (Croucher et al., 2011).

3. Result

3.1. Pathogen screening of the patient stool and suspected food samples

All nucleic acid samples from the 3 patient stool samples and the suspected food sample were positive for *toxR*, *tlh*, and *tdh*, which indicated the existence of *V. parahaemolyticus* in these samples. In contrast, these nucleic acid samples were negative for *Salmonella, Shigella, V. cholerae, Campylobacter, Diarrheagenic E. coli,* Norovirus, Rotavirus, and Enteric Adenovirus.

3.2. Bacterial isolation, toxin detection, and PFGE analysis

In total, 15 *V. parahaemolyticus* were isolated from all the 3 patient stool samples. The strains were serotype O3: K6 and *toxR+/tdh+/trh-*. One strain isolated from each patient stool sample was subject to PFGE assay. The PFGE patterns of these strains were indistinguishable by both *Not*I and *Sfi*I digestion. Further, 7 *V. parahaemolyticus* strains were isolated from the suspected food sample and were all *toxR+/tdh-/trh-*. These strains exhibited 6 unique *Not*I-digested PFGE patterns (Fig. 1).

3.3. Quantitative analysis of the toxR and tdh genes in the original and enriched food samples by digital PCR

The copy numbers of the *toxR* and *tdh* genes in the original food sample were 2347.63 and 74.75 copies/µl, respectively and those in the enriched food sample were 4886.43 and 7.88 copies/µl, respectively. The ratio between the copy numbers of *toxR* and *tdh* (*toxR/tdh*) were 31.41 and 620.11 in the original and enriched food sample respectively (Table 1). The confidence interval (CI) and precision results are presented in Table 1.

3.4. Phylogenetic analysis of V. parahaemolyticus isolated from patients and food samples from the outbreak

In order to investigate the diversity of *V. parahaemolyticus* isolated in this outbreak we sequenced the genomes of all the 10 strains isolated from patients (3 strains) and food samples (7 strains). All the sequenced strains were assembled into less than 300 scaffolds. We compared these genomes with that of the O3:K6 *V. parahaemolyticus* RIMD 2210633 strain (Didelot et al., 2015) and identified 68,793 SNPs in the non-recombinant, non-mobile, non-repetitive core genome, which were then used to calculate the Neighbour-joining phylogeny. The genomes of the strains from the 3 patients and the reference RIMD 2210633 formed a tight cluster (Fig. 2). No SNPs were observed among the genomes of the strains from the patients, and 140 SNPs were identified between RIMD

Table 1
Copy numbers, confidence interval (CI), and ratio (*toxR/tdh*) of the *toxR* and *tdh* genes in the original and enriched food samples.

		toxR			*tdh*			Ratio
		Copies/μl	CI	Average	Copies/μl	CI	Average	
Original sample [a]	Replicate 1	2307.60	2201.20–2419.00	2347.63	85.861	68.47–107.67	74.75	31.41
	Replicate 2	2410.00	2305.00–2519.70		67.944	52.53–87.89		
	Replicate 3	2325.30	2224.60–2430.60		70.453	54.34–91.34		
Enriched sample [b]	Replicate 1	4961.6	4807.50–5120.60	4886.43	6.632	3.00–14.76	7.88	620.11
	Replicate 2	4898.2	4746.90–5054.30		10.105	5.26-19.42		
	Replicate 3	4799.5	4652.90–4950.70		6.905	3.10–15.37		
Negative control		0.08	0.01-0.59	NA	1.28	0.80-2.06	NA	NA

[a] Nucleic Acid extracted from 200 μl enrichment medium before an 18-h enrichment culture in 3% NaCl alkaline peptone.
[b] Nucleic Acid extracted from 200 μl enrichment medium after an 18-h enrichment culture in 3% NaCl alkaline peptone.

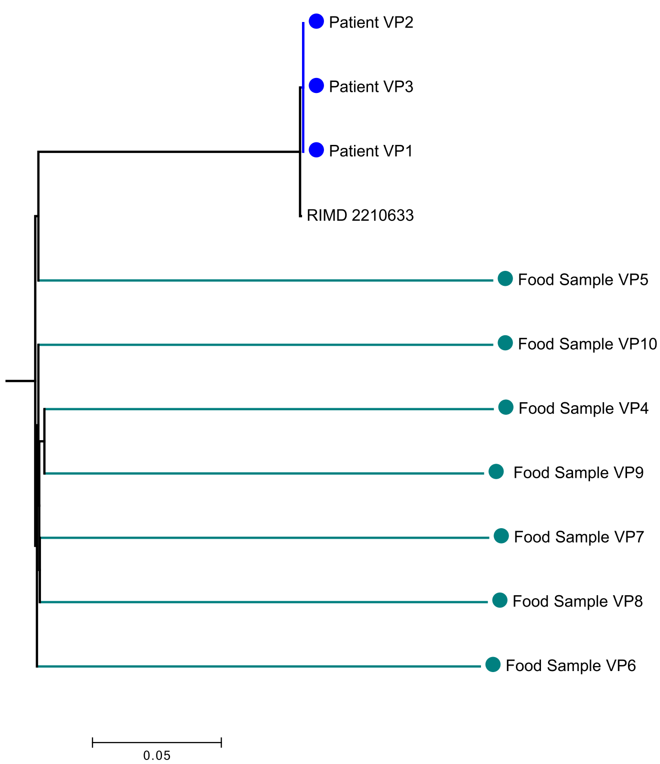

Fig. 2. Phylogenetic tree of *V. parahaemolyticus* isolated from patient stool and food samples based on the SNPs. Branches are colored according to the isolation source of the strains. Strains isolated from patients are shown in blue while those from food samples are shown in dark green. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2210633 and the genomes of the strains from the patients. In contrast, more than 9000 specific SNPs were identified in each of the *V. parahaemolyticus* strains isolated from the food sample.

3.5. Whole metagenome sequencing (WMS) analysis

In the original food sample, the top 3 species (based of nucleic acid analysis) were *Weissella cibaria*, *Weissella confusa,* and *Enterobacter cloacae* (absolute and relative abundance). In the enriched food sample, *Vibrio* sp *Ex25*, *Vibrio* sp *712i,* and *Vibrio parahaemolyticus* were the top 3 species (absolute and relative abundance). No reads were mapped to the *tdh* gene from the original or enriched food samples and no reads were mapped to the *toxR* gene from the original food sample. However, 112 reads were mapped to *toxR* gene from the enriched food sample.

4. Discussion

Diarrheal diseases caused by microorganisms remain a global issue, and *V. parahaemolyticus* is a leading cause of diarrheal disease in China. *V. parahaemolyticus* infection is associated with the consumption of many kinds of contaminated food—usually raw or undercooked seafood. However, in this study, the suspected food is triple, which is seldom related with *V. parahaemolyticus* infection. We were surprised that *V. parahaemolytcius* was detected by PCR in tripe. Epidemiological investigation showed that the patients did not eat any seafood in 3 days before they got sick this time. They bought the triple in a take-away shop. That shop also sold other kinds ready-to-eat food. And the shop owner purchased ready-to-eat food in bulk from a wholesale market. That market also wholesaled other ready-to-eat food including seafood. We speculate that there were cross-contamination in the wholesale market. The triple might be cross-contaminated by *V. parahaemolyticus* in seafood.

Multiple *V. parahaemolyticus* could exist in individual seafood (Huehn et al., 2014). However, the genetic diversity of the strains isolated from individual samples has rarely been investigated. Previous *V. parahaemolyticus* studies mainly focused on the genetic diversity of strains isolated from multiple samples (Theethakaew et al., 2013; Urmersbach et al., 2014). In this study, we showed that diverse non-*tdh* producing *V. parahaemolyticus* strains existed along with clonal *tdh* producing strains in a single food sample. Similar observations have been made in other bacteria , such as *Salmonella*, *Vibrio cholerae* (CDC, 2018; Kaper et al., 1995). Pathogenic *V. parahaemolyticus* comprise only a small proportion of the total *V. parahaemolyticus* population in environment and seafood samples. The detection and isolation of pathogenic *V. parahaemolyticus* against a background of the more abundant and genotypically diverse non-pathogenic *V. parahaemolyticus* strains has remained a challenge (Nair et al., 2007). Pathogenic strains are usually difficult to isolate from suspected food samples because of the small quantity. Our digital PCR data showed that the ratio between *tdh*-producing and non-*tdh*-producing strains in the original food sample was about 1:30. Since we quantified the ratio between *tdh*-producing and non-*tdh*-producing strains according to the percentage of the genes specific to these two categories of strains our result is more accurate than the previous studies that based on the isolation of *V. parahaemolyticus* (Lopatek et al., 2018; Xie et al., 2016). Another difference between the previous study and our study is in the sample; in the previous study (Lopatek et al., 2018; Xie et al., 2016), strains were isolated from different samples whereas the strains were isolated from a single sample in our study. Our data further showed that the absolute copy numbers of the *tdh* gene in the original and enriched food sample were very low, which indicated a low amount of *tdh*-producing strains in the samples. Moreover, the ratio of *tdh*-producing strains to non-*tdh*-producing strains was much lower in the enriched food sample than in the original food sample (~20 fold). This is likely the reason pathogenic *V. parahaemolyticus* has rarely been isolated from suspected food samples even after enrichment. These results indicated that more isolates must be picked up for further test if we want to get the pathogenic *V. parahaemolyticus*. Our WMS result showed that the traditional *Vibrio* selective enrichment media could facilitate the growth of *Vibrio* strains; however, it did not specifically facilitate the growth of *tdh*-producing strains. A media that can promote the growth of *tdh*-producing *V. parahaemolyticus* would be of great value in the isolation of *tdh*-producing strains.

NGS is increasingly used in the investigation of disease outbreaks, because the sequencing speed is adequate for emergency response, and the price has become affordable to public health labs (Gargis et al., 2016). In a public health lab, NGS could be used for pathogen identification and source tracing. At present, NGS of pure strain culture has been widely used in bacterial population genetics (Didelot et al., 2015; Morelli et al., 2010) and outbreak investigation (Chin et al., 2011; Pang et al., 2016). In this study, no SNP was detected among the genomes of the *V. parahaemolyticus* strains isolated from the patients, which clearly indicates an outbreak. WMS is promising in the identification of pathogens; however, it is associated with several challenges. The quality and quantity of input nucleic acid is one such challenge. In our test, no reads that mapped to *tdh* gene were detected in the nucleic acid of the original food sample despite amplification with 15 cycles before routine WMS. Digital PCR results showed that the copy number of *tdh* gene was 14.90 copies/ml in the original food sample. Thus, the input nucleic acid was insufficient for WMS. Increasing the input of nucleic acid could solve this problem. However, in some cases the specimen is not sufficient, which would necessitate the improvement of nucleic acid preparation.

5. Conclusion

In conclusion, we showed high genomic diversity among *V. parahaemolyticus* strains isolated in a single contaminated food sample, which involved in a diarrheal disease outbreak with NGS data. The digital PCR results showed a change in the ratio between pathogenic and non-pathogenic *V. parahaemolyticus* before and after enrichment of a single suspected food sample. Moreover, we used meta-genomic data to show a change in bacterial composition in the suspected food sample before and after enrichment. Taken together, our results indicated that the culture method alone might lead to wrong conclusion of outbreak investigation. Hence our results revealed the necessity of developing new media, which can selectively facilitate the growth of pathogenic *V. parahaemolyticus.* However, there is obvious limitation in our study. More samples or more outbreaks should be included in our future study.

YZ, SZ, and HJ were involved in the collection of samples. YL and SZ collected the clinical data. YL, YW, SZ, YZ, and MC performed the detection. YL, SZ, MC, JL and HZ performed the PFGE and Digital PCR tests. MC, MH, HM, YDL, LZ and BP and performed the data analysis. YL, BK, and BP designed this study, drafted and revised this manuscript.

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