J. Microbiol. Biotechnol. (2017), 27(5), 995–1004
https://doi.org/10.4014/jmb.1612.12002

jmb

Profiling Total Viable Bacteria in a Hemodialysis Water Treatment System

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Received: December 12, 2016
Revised: March 8, 2017
Accepted: March 9, 2017

First published online
March 13, 2017

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pISSN 1017-7825, eISSN 1738-8872

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Culture-dependent methods, such as heterotrophic plate counting (HPC), are usually applied to evaluate the bacteriological quality of hemodialysis water. However, these methods cannot detect the uncultured or viable but non-culturable (VBNC) bacteria, both of which may be quantitatively predominant throughout the hemodialysis water treatment system. Therefore, propidium monoazide (PMA)-qPCR associated with HPC was used together to profile the distribution of the total viable bacteria in such a system. Moreover, high-throughput sequencing of 16S rRNA gene amplicons was utilized to analyze the microbial community structure and diversity. The HPC results indicated that the total bacterial counts conformed to the standards, yet the bacteria amounts were abruptly enhanced after carbon filter treatment. Nevertheless, the bacterial counts detected by PMA-qPCR, with the highest levels of 2.14×10^7 copies/100 ml in softener water, were much higher than the corresponding HPC results, which demonstrated the occurrence of numerous uncultured or VBNC bacteria among the entire system before reverse osmosis (RO). In addition, the microbial community structure was very different and the diversity was enhanced after the carbon filter. Although the diversity was minimized after RO treatment, pathogens such as *Escherichia* could still be detected in the RO effluent. In general, both the amounts of bacteria and the complexity of microbial community in the hemodialysis water treatment system revealed by molecular approaches were much higher than by traditional method. These results suggested the higher health risk potential for hemodialysis patients from the up-to-standard water. The treatment process could also be optimized, based on the results of this study.

Keywords: Dialysis water, viable bacteria, VBNC, PMA-qPCR, high-throughput sequencing, microbial community

Introduction

Hemodialysis, the necessity of uremia patients to maintain normal life activities, makes contact with more than 400 L of dialysis water a week, which is nearly 30 times the amount of water a week of normal people [1]. It is necessary to strictly monitor the dialysis water, especially its microbiological characteristics, since numerous cases of acute or chronic complications caused by microbial contamination were reported [2, 3].

The heterotrophic plate counting (HPC) method is widely used in the quantification of bacterial amounts in dialysis water. For example, the total bacterial number should be below 100 CFU/ml according to China's national standard, and 200 CFU/ml according to the Association for the Advancement of Medical Instrument standards [4, 5]. However, the HPC method can only include culturable bacteria, which is only a very small part of the total viable bacteria in the samples. It was reported that >99% bacteria are uncultured or in a viable but non-culturable (VBNC) state in tap water or similar oligotrophic environments [6]. Many environmental strains are unculturable because the

artificial medium cannot simulate the necessary growing conditions in their natural habitats. In addition, many culturable bacteria can be induced into a VBNC state. In this state, the bacteria maintain their essential viability but cannot divide to reproduce in the medium where they can originally. Low temperature and lack of nutrition are the common factors inducing bacteria into such a state [7]. Otherwise, the induction by disinfectants such as chlorine and chloramine were also reported [8,9]. It is noteworthy that VBNC bacteria can resuscitate with suitable conditions like rich nutrition, favorable temperature, removal of adverse pressure, and so on [10]. Obviously, the occurrence of uncultured and VBNC bacteria (in the following text, the two groups will be collectively referred as non-culturable bacteria for convenience) in the samples will result in a dramatic underestimation of the total viable bacterial amounts (the sum of culturable and non-culturable bacteria) and thus potentially poses a threat to patient health.

Therefore, in order to elucidate the bacterial community in dialysis water accurately, it is indispensable to apply protocols independent of culturing. Fortunately, various such methods have been developed. In this study, the propidium monoazide quantitative polymerase chain reaction (PMA-qPCR) method, which can count the viable bacterial biomass via target DNA copies, was used as well as HPC [11]. Hence, the distribution of total viable bacteria in the whole water treatment train could be obtained. In addition, high-throughput sequencing of 16S rRNA gene amplicons was also implemented so that the complicated structure of microbial composition could be revealed [12]. To our best knowledge, this is the first report discussing the quantitative distribution and community variation of the total viable bacteria in a hemodialysis water treatment system. Hopefully, a deeper insight into the impact of different treatment processes on the bacteria and their epidemiological risks would then become possible.

Materials and Methods

Water Sampling and Preparation

Water samples were collected in a hemodialysis water treatment system (Gambro, Sweden) in a hospital in Xiamen City, China. The sampling ports were as shown in Fig. 1, and the frequency was once every 2 weeks for 4 months continuously. The equipments, such as sampling buckets and bottles, were all sterilized at 121°C for 15 min by steam autoclaving (HVE-50; Hirayma, Japan). The sampling process was in a windless condition and sure to be sterile. The dissolved oxygen (DO), pH, and temperature were measured on site with a portable multi-parameter water quality meter (Muti 3420; WTW, Germany). About 10 L of the water was collected from each process, except RO water (50 L), for enriching the bacteria, primarily due to the different levels of bacteria in the water samples. The collected water samples were sent immediately to the laboratory for analysis. Approximately 100 ml of each sample was used to measure the physical and chemical parameters according to China's national standards. Another 100 ml of each sample was utilized for microbial culture. The remaining sample was filtered through a 0.22 μm mixed cellulose ester membrane (GPWP04700; Millipore, Ireland) for bacterial enrichment and follow-up PMA-qPCR test.

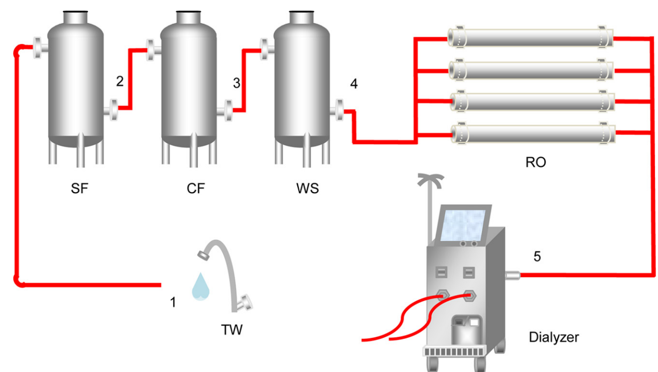

Fig. 1. Process flow diagram.
TW, tap water; SF, sand filter; CF, carbon filter; WS, water softener; RO, reverse osmosis.

Microbial Culture and Strain Identification

The membrane filter method was used for microbial culture. Briefly, 100 ml of the water samples were filtered with a 0.45 μm filter membrane (HAWG047S6; Millipore, France) [13], and incubated at 37°C for 48 h on nutrient agar (NA). All colonies in tap water, sand filter, and RO samples and a single colony with different strain morphological characteristics of carbon filter and water softener samples were picked for strain identification [14].

PMA Treatment

PMA, a nucleic acid dye that can inhibit DNA amplification in PCR through penetrating and damaging cell membranes to combine with cellular DNA after photo cross-linking, was used to quantify the total viable bacteria [15]. The PMA working solution was prepared by adding 25 μl of 1 mg/ml PMA stock solution (40013; Biotium, USA) into 475 μl of sterile water and then bringing the PMA final concentration to 100 μM [16]. After incubating for 5 min in the dark, the samples were irradiated for 4 min using a 650 W halogen lamp (220 V, 3,400 K; Osram, Germany). After photo cross-linking, the bacterial DNA was extracted using the FastDNA Spin kit according to the manufacturer's instructions (MP Biomedicals, USA).

PCR for Absolute Fluorescent Quantitation

The QuantStudio 6 Flex Real-Time PCR System (Applied

Biosystems, USA) was used to conduct the SYBR Green absolute fluorescent quantitation for the 16S rRNA gene [17]. The forward primer was designated 341F (CCTACGGGAGGCAGCAG) and the reverse primer was designated 534R (ATTACCGCGGCT GCTGG). The 20 µl qPCR contained 10 µl of 2× Taq Polymerase buffer solution, 0.4 µl of ROX Reference Dye, 0.4 µl each of forward and reverse primers (10 µM concentration), 6.8 µl of ddH_2O, and 2 µl of template. Samples were amplified at 94°C for 30 sec; and then 40 cycles of 94°C for 5 sec, and 60°C for 34 sec. After the final cycle was complete, we set up the melting curve. The standard curve was plotted for a series of serial dilutions of the plasmids (10-fold dilutions), and the results were analyzed.

Pyrosequencing and Data Analysis

Total bacterial DNA was extracted from five samples for further analysis. The V4 region of the 16S rDNA was amplified by using forward primer 515F 5′-GTGCCAGCMGCCGCGGTAA-3′ and reverse primer 806R 5′-GGACTACHVGGGTWTCTAAT-3′. The PCR was performed with the following conditions: 98°C for 1 min (1 cycle), 98°C for 10 sec/50°C for 30 sec/72°C for 30 sec (30 cycles), and a last step of 72°C for 5 min. The PCR products were purified by using the GeneJET Gel Extraction Kit (Thermo Scientific, USA) and then utilized for pyrosequencing on an Illumina HiSeq 2 × 250 platform according to protocols described by Caporaso *et al*. [18]. Sample reads were acquired by using Qiime V1.7.0 [19]. Chimeric sequences were removed by using the USEARCH software based on UCHIME algorithm. Sequences were clustered into operational taxonomic units (OTUs) at 97% sequence similarity by using Uparse v7.0.1001. Taxonomy assignment of the OTUs was performed by using the RDP Classifier V2.2 and GreenGene database [20]. Alpha diversity analysis including observed-species, Chao1, Simpson, abundance-based coverage estimator (ACE), and Goods-coverage were calculated by Qiime V1.7.0, and rarefaction curves were obtained using R package. Beta diversity, which illustrated the community diversity between samples based on phylogenetic information including weighted Unifrac distance, was calculated by Qiime, and these distances were visualized by Principal Coordinate Analysis (PCoA). Furthermore, the heatmap was acquired by R package heatmap.

Results

Performance of the Hemodialysis Water Treatment Processes: Physical/Chemical Parameters

The tested water treatment system has been in use for around 8 years. It could be drawn from Table 1 that all of the determined parameters showed expected trends along the treatment train. The temperature, DO, and pH fluctuated slightly in a narrow range since the system was hermetically operated. The chlorine residue in tap water was about 0.50 mg/l, and then dropped significantly to about 0.23 and 0.04 mg/l after sand and activated carbon filtration, respectively. The NO_2^--N, NH_4^+-N, and PO_4^{3-}-P in the tap water were already in very low levels, close to the detection limit, so they remained relatively stable in the treatment system. The other parameters, conductivity, turbidity, NO_3^--N, and TOC, almost remained constant in the system before RO membrane filtration. However, all of them were dramatically removed by this water quality guard process. For example, the conductivity was reduced from 142 to 1 µs/cm and the TOC from 1.73 to 0.08 mg/l, which proved its high efficiency in eliminating ions and organic matters from water.

Quantitative Distribution of the Bacteria in the Water Treatment System

Both the culturable and non-culturable bacterial amounts are shown in Fig. 2. Usually, the units for culturable bacteria is CFU/ml. However, because there were very few culturable

Table 1. Physical and chemical parameters of different technological processes.

	Tap water	Sand filter	Carbon filter	Water softener	Reverse osmosis
Temp (°C)	24 ± 2	24 ± 2	24 ± 2	24 ± 3	25 ± 2
DO (mg/l)	8.09 ± 0.36	8.41 ± 0.41	8.11 ± 0.48	7.76 ± 0.84	8.10 ± 0.39
Chlorine (mg/l)	0.50 ± 0.09	0.23 ± 0.06	0.04 ± 0.02	0.02 ± 0.01	0.03 ± 0.02
Conductivity (µs/cm)	152 ± 19	146 ± 14	142 ± 13	142 ± 14	1 ± 0
pH	6.70 ± 0.22	6.76 ± 0.19	6.68 ± 0.24	7.28 ± 0.36	7.54 ± 0.25
Turbidity (NTU)	0.18 ± 0.06	0.15 ± 0.06	0.13 ± 0.03	0.15 ± 0.08	0.07 ± 0.02
NO_3^--N (mg/l)	2.67 ± 0.49	2.31 ± 0.20	2.23 ± 0.15	2.27 ± 0.14	0.06 ± 0.02
NO_2^--N (mg/l)	0.002 ± 0.00	0.002 ± 0.00	0.003 ± 0.00	0.002 ± 0.00	0.002 ± 0.00
NH_4^+-N (mg/l)	0.021 ± 0.02	0.042 ± 0.09	0.041 ± 0.03	0.018 ± 0.2	0.017 ± 0.03
PO_4^{3-}-P (mg/l)	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.00	Not detected
TOC (mg/l)	1.72 ± 0.47	1.76 ± 0.39	1.67 ± 0.45	1.73 ± 0.46	0.08 ± 0.02

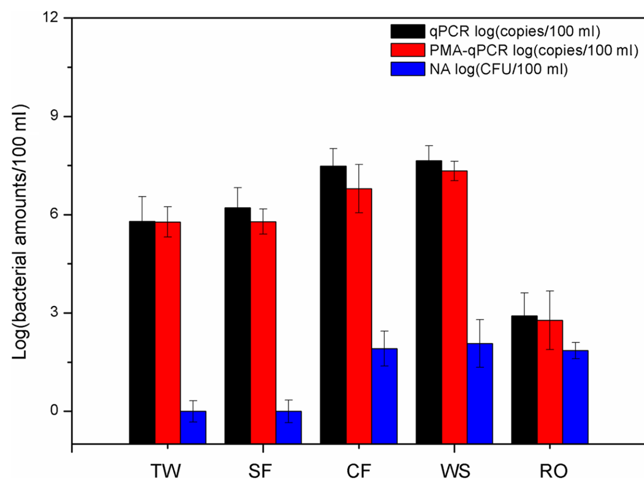

Fig. 2. Total number of viable and culturable bacteria in advanced treatment technological processes, by PMA-qPCR and heterotrophic plate counting.

The abbreviations of the samples are the same as used in Fig. 1.

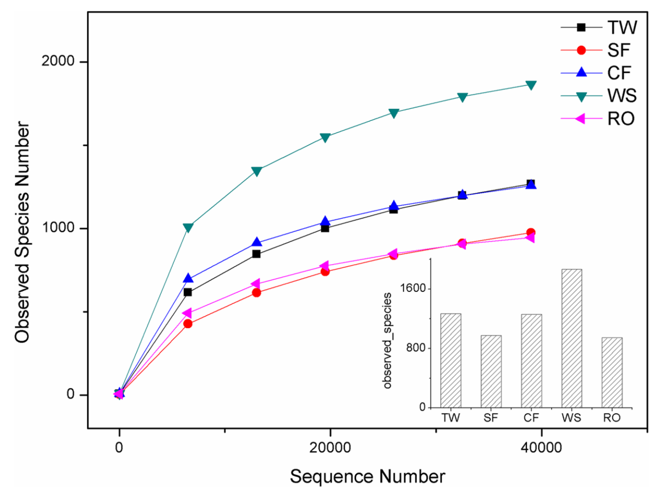

Fig. 3. Rarefaction curves of OTUs for bacteria and species number in each sample during all the processes.

The abbreviations of the samples are the same as used in Fig. 1.

bacteria in all the water samples, CFU/100 ml was applied in this study to make the results statistically significant. During the experiment, we set a blank control, the results of which were negative. In the tap water and sand filter effluent, the level of culturable bacteria was only approximately 1 CFU/100 ml on average. After carbon filtration, the number abruptly increased to about 82 CFU/100 ml. The highest bacterial biomass occurred in the softener effluent with an average of 118 and a peak of 420 CFU/100 ml. After RO treatment, the culturable bacterial amount dropped but was still close to 10^2 CFU/100 ml (71 CFU/100 ml). The culturable bacteria were also identified (Table 2). Similar to their amount distribution, the highest microbial diversities occurred in the effluent of the carbon filtration and softener. It was noteworthy that numerous pathogens, such as *Staphylococcus* and *Herbaspirillum*, were found in the system.

The total bacteria and total viable bacteria are also shown in Fig. 2. The viable ones as PMA-qPCR target copies (red column) were significantly higher than the corresponding culturable bacterial amounts, especially in the samples other than RO. Similar to the culturable bacteria, the total viable bacteria also climbed to higher levels of 6.31×10^6 and 2.14×10^7 copies/100 ml in effluents of the carbon filter and softener, respectively. The RO process exhibited excellent removals of viable bacteria, and the quantity dropped more than a $\log_{10}$ reduction factor of 4.56. The total bacterial amounts as qPCR (black column) showed the same tendency as the viable bacteria, except that their levels were always a little higher than the later.

Microbial Community Analysis through High-throughput Sequencing

Approximately 344,840 sequence reads of the 16S rRNA gene with an average length of 254 bp were obtained after trimming and chimera removal. A total of 6,315 OTUs were acquired of all samples (Table 3). The ACE and Chao1 indexes reached their maximum at the softener (2,114.978 and 2,025.865, respectively), and their minimum at the RO (1,099.241 and 1,051.55, respectively) (Table 3). Goods-coverage of different samples was all above 99%, which revealed that the sequencing results could represent the practical situation of the samples. Furthermore, most rarefaction curves failed to reach saturation (Fig. 3). As shown in Fig. 4, the RO unit was located in the farthest distance with the other processes, which had the lowest similarity of the others.

The microbial community structures were different from each other for the various treatments. Fig. 5A shows the top 10 phyla in each process after being normalized. As shown, the most abundant phylum was Proteobacteria (44.7%–82.8%) in all the samples. Proteobacteria (63.4%), Cyanobacteria (18.1%), Bacteroidetes (7.2%), and Firmicutes (5.7%) were predominant in tap water. In the sand filter effluent, the relative abundance of Proteobacteria (82.8%) and Firmicutes (7.6%) were increased, whereas Bacteroidetes (2.5%) and Cyanobacteria (2.1%) dramatically declined. After carbon filter treatment, seven phyla whose relative abundance was above 2% were detected, which was much more than the

Table 2. The results of strain identification.

Group	Genus	Closest described species	Frequency	GenBank Accesion No.
TW				
Firmicutes	*Bacillus*	*Bacillus cereus*	+	EU857430
	Paenibacillus	*Paenibacillus* sp. JCM 28314	+	LC133661
SF				
Firmicutes	*Staphylococcus*	*Staphylococcus epidermidis* ATCC 12228	++	AE015929- AE016752
		Staphylococcus pasteuri		HM130543,KT003275
	Paenibacillus	*Paenibacillus* sp. JCM 28314	+	LC133661,AB746175
Actinobacteria	*Streptomyces*	*Streptomyces caespitosus*	+	AB184320
	Micrococcus	*Micrococcus lylae*	+	HM209730
CF				
Firmicutes	*Bacillus*	*Bacillus beringensis*	++	JF895482
		Bacillus korlensis		KT720281,KC443095
	Exiguobacterium	*Exiguobacterium* sp. EH69	+	GU339294
Actinobacteria	*Rothia*	*Rothia mucilaginosa*	+	GQ456061
Proteobacteria	*Acidovorax*	*Acidovorax delafieldii*	+	GQ284421
	Hydrogenophaga	Uncultured *Hydrogenophaga* sp.	+	EU305579
	Acinetobacter	*Acinetobacter beijerinckii*	+	HQ425649
WS				
Actinobacteria	*Microbacterium*	*Microbacterium trichothecenolyticum*	+	EU714362
	Micrococcus	*Micrococcus luteus*	+	KT307978,JN644530
Proteobacteria	*Acinetobacter*	*Acinetobacter* sp. ST-01	+	EF566900
	Stappia	*Stappia indica*	+	KR697775
	Pelomonas	Uncultured *Pelomonas* sp.	+	LC093428
	Halomonas	*Halomonas* sp. YT2	+	KC953093
	Brevundimonas	*Brevundimonas aurantiaca*	+	KC494321
	Novosphingobium	*Novosphingobium* sp. THN1	+	HQ664117
Firmicutes	*Staphylococcus*	*Staphylococcus cohnii*	+	KT261250
	Bacillus	*Bacillus* sp. JBS-28	+	KC443095,JF895482
	Tumebacillus	*Tumebacillus algifaecis*	+	NR_136476
RO				
Proteobacteria	*Ralstonia*	*Ralstonia mannitolilytica*	+++	DQ239898
		Uncultured *Ralstonia* sp.		KP967487,AB743841
		Ralstonia pickettii		JX010987,LN681565
	Herbaspirillum	*Herbaspirillum huttiense* subsp. *putei* IAM 15032	+	NR_114068

"+" represents the frequency in the three appraisal results.
TW, tap water; SF, sand filter; CF, carbon filter; WS, water softener; RO, reverse osmosis.

Table 3. Diversity indicators of bacterial community using high-throughput sequencing.

Sample name	Reads	Obseversed-species	ACE	Chao1	Shannon	Simpson	Goods-Coverage
TW	77,088	1,268	1,609.665	1,576.062	5.772	0.911	0.990
SF	71,856	976	1,334.489	1,287.514	3.055	0.497	0.991
CF	49,697	1,258	1,529.262	1,511.614	7.172	0.977	0.992
WS	78,397	1,867	2,114.978	2,025.865	8.014	0.984	0.991
RO	67,802	946	1,099.241	1,051.55	5.557	0.91	0.995

TW, tap water; SF, sand filter; CF, carbon filter; WS, water softener; RO, reverse osmosis.

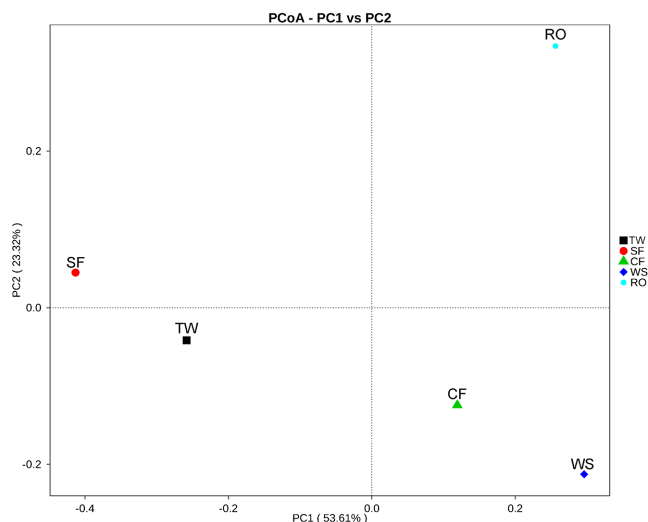

Fig. 4. Principal coordinate analysis of samples using weighted-UniFrac from pyrosequencing.
The abbreviations of the samples are the same as used in Fig. 1.

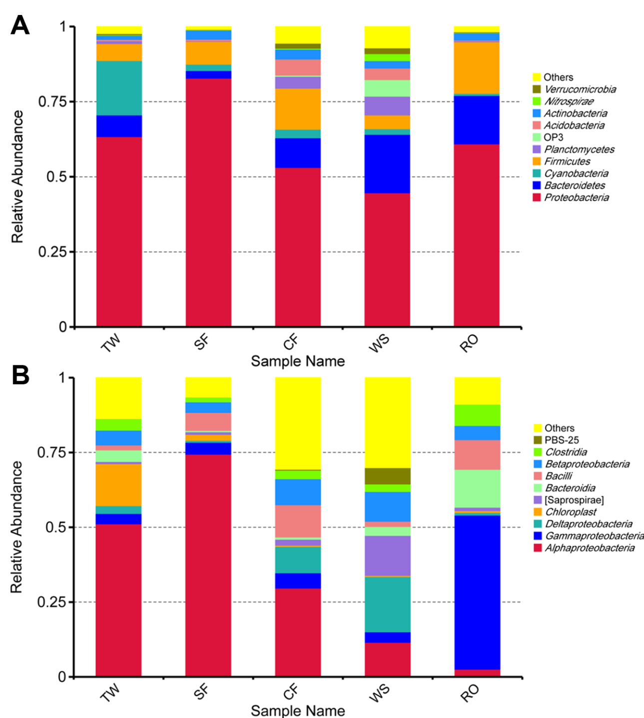

Fig. 5. Phylogenetic classification of bacterial communities for pyrosequencing at the phylum (**A**) and class (**B**) levels obtained from Ribosomal Database Project classifier analysis.
The abbreviations of the samples are the same as used in Fig. 1.

ahead processes. The microbial community structure continued to change after softener filter treatment, where the relative abundance of Proteobacteria (44.7%) and Firmicutes (4.6%) was decreased, whereas Bacteroidetes (19.4%) was apparently rising. After RO treatment, Proteobacteria (61.0%) and Firmicutes (17.2%) were incremental.

The heatmap shows the top 35 genera in all processes (Fig. 6). Similar to the tendency of strain identification, the relative abundance of genera was enhanced after the carbon filter treatment, where *Sphingomonas* and *Solibacillus* predominated. After RO treatment, *Escherichia* became the dominant genus.

Discussion

Performance with Respect to the Physicochemical Parameters

The water treatment processes used in this study are commonly used for blood purification in China or other parts of the world, whose core technology was RO membrane filtration [21, 22]. Filter media were critical factors influencing the treatment performance. Generally, the filter media replacement was not often undertaken and the media were usually utilized for years. Backwashing was a more frequent operation. However, previous reports have revealed that the filters might come to be steady soon after the backwashing [23]. Thus, the performance and the microbial community of the whole treatment train should be in a steady state. The sand filtration is primarily for removing particulate residues from tap water. Residual chlorine in tap water is corrosive to RO membranes and can shorten their service life, and excess chlorine entering the body of hemodialysis patients may lead to hemolytic anemia [24]; therefore, a carbon filtration unit is used to absorb it since there are abundant radicals on the surface of the activated carbon granules. The softening primarily reduces water hardness. The RO membrane has good retention function and can filter out 95−98% of salts, most of the particulates including microorganisms, and substances with a molecular mass greater than 200 Da [21]. The variations of the physicochemical parameters almost perfectly fitted the process functions, which confirmed that the processes were in a steady state. The above results demonstrated that the water treatment processes with a core of RO could optimize the physicochemical parameters effectively.

Occurrence and Variation of the Culturable Bacteria

Although the culturable bacteria amounts conformed to the standards, some tendency was worrying; that is, the culturable bacteria amounts increased after the carbon filtration and then remained at about 10^2 CFU/100 ml

Fig. 6. Heatmap showing the top 35 most abundant genera of the bacterial community for each sample.
The abbreviations of the samples are the same as used in Fig. 1.

which was about 2 orders of magnitude higher than that in tap water. This should be mostly attributed to the removal of chlorine residue by carbon filtration. The microorganisms were ready to reproduce with the absence of this growth inhibitor as well as the rough surfaces of carbon granules, which are a superior supporter of biofilm formation [25]. Thus, it was not strange that the dramatic rise in bacterial quantity occurred after carbon filtration. This situation might cause operating pressures to the latter units, especially to the RO membrane via biofouling, and increase the risks of microbial breakthrough. Therefore, it is essential to take measures to effectively control the growth of bacteria in the carbon filter, such as regular backwashing and disinfection. Totally, considering optimizing the technological parameter or appropriately adjusting the order of the processes, especially for the carbon filter, is necessary.

The RO effluent had a rather high culturable bacterial amount, which seemed contradictory to its particulate retention capacity. This was probably because it was not sampled directly after RO but after >10 m tube transportation and at the port of the dialysis machine. The microbes possess substrate uptake abilities beyond our imagination. Even in a habitat with a trace level (several μg/l) of organic matters like dialysis water, both the suspended bacterial

reproduction and biofilm formation on the tube wall might take place during this procedure. The cell density could even reach 10^6 CFU/cm^2 in the biofilm attached to the hemodialysis machine tubes [26, 27]. Previous reports have revealed that biofilm contributed more than 95% of the biomass in drinking water treatment distribution systems, which might be similar in hemodialysis water treatment [28]. The results suggested that a strengthened disinfectant for the tubes before the dialysis machine is important. Moreover, some detected culturable strains in the RO effluent, such as *Ralstonia* and *Herbaspirillum*, could release lipopolysaccharides responsible for the pyrogenic reaction and outbreaks of sterile peritonitis [29, 30], posing a direct threat to long-term dialysis patients.

Occurrence and Variation of the Total Viable Bacteria

As pointed out, the differences between the total bacteria and the total viable bacteria are slight, which indicated that most of the bacteria in the treatment system were viable. However, the differences between the total viable and culturable bacteria were huge. Even when considering the PCR methods may provide some false-positive results, these differences were still significant enough, which implied that most of the bacteria in the system (except the RO effluent) were non-culturable. The results also revealed that the HPC method, which was commonly used in many previous studies, could not accurately determine the bacteria [31]. It is necessary to profile the total viable bacteria for further understanding the dialysis water treatment and assessing the microbiological safety of dialysis water. In particular, it was reported that most of the human pathogenic bacteria can be induced into VBNC state, and the ultra-oligotrophic condition and the disinfectant residue, which were present in the dialysis water system, were well known as the inducers. Therefore, there were probably VBNC pathogenic bacteria in this system. The related risks would be amplified if it was considered that VBNC bacteria could still retain some toxic/infectious metabolic activity, and resuscitate under certain favorable conditions.

With respect to the quantitative distribution of the total viable bacteria, the two key impacting factors were chlorine residue and RO retention. As the most widely used disinfectant, chlorine can effectively repress bacterial growth. Therefore, before the chlorine residue reacted with the radicals in the activated carbon granular surface (*i.e.*, in the tap water and the sand filter effluent), the viable bacterial amounts remained stable. However, their amounts climbed 1.01 and 1.54 orders of magnitude higher in the carbon filter effluent and softener effluent when almost all of the chorine was consumed. The viable bacteria were reduced significantly by RO retention, confirming the superiority of this technology in removing particulates. In fact, the retention efficiency should be even higher. The bacterial cells occurring after RO should be mostly attributed to the regrowth in bulk water and release from the biofilm, as described above. The results warned us again that the health risks after RO treatment should bear more attention. However, most reports emphasized the water quality of the RO effluent but seldom considered the entire system [32], which was inadequate for the assessment of hemodialysis water quality safety from our results.

Structure and Diversity Analyses of Microbial Community

By comparing the results in Table 2 and in Figs. 3−6, the high-throughput sequencing of 16S rRNA gene amplicons evidently provided massive data, which made it possible to have a global view of the microbial community structure of the samples. Most of the rarefaction curves did not reach a plateau, indicating that the diversity of the microbial community structure was far higher than what we thought in the dialysis water treatment. Similar to the bacterial quantitative scenario, the microbial diversity increased after the carbon filter and dropped after the RO membrane steps. The microbial diversity of the samples could be categorized into three groups; that is, tap water and sand filter effluent, carbon filter and softener effluents, and RO effluent. The characteristics among the three groups were so distinctive that they were isolated clearly in three different areas in Fig. 4.

Our results suggested that Proteobacteria, which was ubiquitously distributed in freshwater, predominated in the whole process [33]. Previous studies have pointed out a similar conclusion that Proteobacteria led the diversity in RO water [14]. This phylum achieved the highest relative abundance in the tap water and sand filter effluent. Interestingly, there was no Proteobacteria in these samples in strain identification based on culturing, which inferred that most of the Proteobacteria might enter into the VBNC state in these samples, since chlorine and the oligotrophic condition, both of which are VBNC state inducers, occurred there. However, Proteobacteria appeared in the carbon filter effluent according to the strain identification data, suggesting that some might resuscitate in the carbon filter. It is noteworthy that alpha-Proteobacteria remained in high abundance although it gradually dropped off during the entire process. Because their genome generally contains a high CG content [34], it damaged the immune

milieu of the host and generated proinflammatory cytokines [35]. The risk of inflammation in long-term dialysis patients may be increased.

In terms of the genus level, *Ralstonia* and *Clostridium* occupied the highest abundance in the tap water and sand filter effluent, respectively, but they were undetected in strain identification tests. This indicated that they might have entered into the VBNC state with the oligotrophic and chlorine surroundings, and thus lost their culturability. *Sphingomonas* held the highest proportion in the carbon filter effluent. It was pointed out that these groups could produce slime and attach to the surfaces of other cells, facilitating the formation of biofilm [36]. This implied the formation of biofilm in the carbon filter and the downstream processes. Biofouling in RO is a special presentation of biofilm, which would reduce the lifetime of the RO membrane. In addition, *Escherichia* held the highest abundance in RO water but was not detected by strain identification, implying its VBNC state in the dialysis water. Moreover, a previous study revealed that Verocytoxigenic *Escherichia coli* O157:H7 could lead to hemolytic uremic syndrome, resulting in kidney failure [37]. Besides this, there still existed some pathogens such as *Staphylococcus* and *Clostridium* in RO water. *Staphylococcus* was also found to occur in RO water by Gomila *et al*. [38]. Moreover, it was revealed that *Staphylococcus aureus* could increase the risk of invasive infection of hemodialysis patients [39, 40]. The high level of these genera alerts the existence of certain occult microbial contaminations in RO effluent that could generate enormous risk to dialysis patients, and prompts the remedy of the insufficient traditional method that ignores the detection of uncultured or VBNC bacteria [38]. Therefore, it is necessary to utilize some culture-independent methods, such as high-throughput sequencing, to profile the complex bacterial community and comprehensively identify the bacteria in hemodialysis water. Meanwhile, profiling the microbiology of the entire hemodialysis water system, rather than only the RO water, is essential to better understand the potential risk of hemodialysis water.

Acknowledgments

This work was supported by the Fujian Provincial and Xiamen Municipal Departments of Science and Technology (2016Y0082, 3502Z20162003), Fujian Provincial Medical Innovation Project (2011-CXB-39), National Natural Science Foundation of China (51678551), and Haicang District Bureau of Science and Technology, Xiamen City (350205Z20134001).

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