Note

Detection Method for Aquatic Bacteria of the Fingers, as a Potential Origin of the Aqueous Solution Contamination

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Received 24 December, 2014/Accepted 1 October, 2016

Aquatic bacteria were isolated from the hands of working staffs by an adapted culture protocol. When the sample solution obtained by the “glove juice method” was incubated for 3 days at room temperature, viable cell counts increased up to 10⁵-fold, and the majority of the isolated colonies were shown to be Gram-negative aquatic bacteria, which carry the risk of contaminating water. Using R2A medium, coagulase-negative staphylococci were the dominant microbes immediately after recovery from the hands. Here it was revealed that bacteria of the phylum Proteobacteria isolated from the hand can be the causative bacteria of aqueous contamination. This modification in the GJ method may be useful as an effective training protocol to demonstrate the importance of hand hygiene and clean operation for aseptic manufacturing.

Key words : Dialysate / Endotoxin / Hemodialysis / Touch contamination / Water pollution.

In aseptic manufacturing products, the prevention of microbial contamination from humans is of the highest importance. As in the food and pharmaceuticals, preparation of dialysate for use in hemodialysis therapy must be kept sanitary, as well as sterile (ISO 2014, Kawanishi et al. 2009), due to its contact with blood through a semi-permeable membrane. Previously, we reported that the incidence of dialysate contamination increased, as shown by an increase in endotoxin value, when maintenance of the central dialysate delivery system (CDDS) was performed without hand hygiene (Honda et al. 2010). In DNA sequencing analysis of skin microbes, independent of culture method, the main bacteria of the hands have been reported to be Gram-negative aquatic bacteria, such as Proteobacteria (Fierer

et al. 2008, Grice et al. 2009, Kong 2011), which have been shown to be able to contaminate dialysate (Oie et al. 2003). However, when bacteria from the hands were isolated and cultured, microbes from the phylum Firmicutes, such as coagulase-negative staphylococci (CNS), predominate despite using a medium suitable for aquatic bacterial culture (Cook 2007).

We attempted to isolate the aquatic bacteria inhabiting the hands by bacterial culture to demonstrate that hand hygiene associated with contamination of water-based materials, such as dialysate. Sampling was conducted on 11 dialysis room staff members (doctors, nurses, and clinical engineers) after treatment or operation in the dialysis room. To collect bacteria, the dominant hand was washed in a 100-ml sterile water in a sterile bag (glove juice method (GJ) by Kampf et al. 2006). The GJ method was done using sterile saline for six samples (Fuso Pharmaceutical Industries, Ltd.,

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Osaka, Japan), by the original method, or using dialysate treated by 0.22- μ m microfiltration (Ringer's solution containing 100 mg/dl glucose and divalent ion Ca in sodium bicarbonate buffer (pH 7.4) prepared by the CDDS method (approved by ISO23500, ISO 2014)) for eight samples. The non-dominant hands were also tested on a different day (Fierer et al. 2008) for three staff members. Bacteria on the hands were investigated on the collected sample solutions immediately after sampling, defined as "at GJ", as well as collected sample solutions that were sealed in a bag and incu-

bated for three days at room temperature in the dark, defined as "incubation" samples.

The samples were diluted with sterile distilled water for preparation of a 10-fold dilution series for viable cell counting on R2A medium (Merck Millipore Japan, Tokyo), which is suitable for culturing aquatic and heterotrophic bacteria in dialysate (ISO 2014, Kawanishi et al. 2009). The dilution series (0.1 ml each) were inoculated to the medium in two dishes, followed by a 5-day incubation period at 25°C. Viable cell counts were determined using the dilution rate of the plate containing

FIG. 1. Changes in viable cell counts at immediately after GJ and after incubation.

Viable cell counts were based on a 10-fold dilution series of the plate containing a range of 30-300 CFU and the actual number of colonies. The dashed lines shows the samples obtained in saline, and the solid line shows those in dialysates; the thin line shows samples from the non-dominant hand.

a range of 30-300 CFU to calculate the actual number of colonies. Colonies were categorized according to their shape, color, and gram staining, and individual colony counts were recorded to reflect the ratios of bacterial species; differences within a dilution series was identified as logarithmic, and differences within the same dilution plate was linear. After 2 to 3 passages of the isolated colonies on R2A medium, purity was checked by isolating bacterial DNA (QIAMP DNA mini kit, Qiagen, Tokyo, Japan) and amplifying by PCR using universal primers for 16S rRNA gene analysis (E. coli 8F and 1510r, Weisburg et al. 1991). The obtained bands were purified (Takara easy filtration kit, Takara, Osaka, Japan), and sequenced using an automatic analyzer (Applied Biosystems 3730 DNA analyzer, AB, Life Technologies Japan, Tokyo) with the 5'-side 8F primer. Bacterial species were identified based on the homology of the nucleotide sequences between nucleotides 8-825 using DDBJ BLAST and NCBI GenBank Blast. Of the candidate species, those with characteristics during culture that corresponded most to the descriptions of

bacterial species and genera in Bergey's Manual of systemic Bacteriology 2nd edition were selected. Statistical processing was performed with EZR software.

All results are shown in Figure 1. Bacteria were isolated from all collected solutions. The number of colonies from the "at GJ" samples were 10^3 - 10^5 CFU/ one hand for the saline samples (dashed line) and 10^4 - 10^7 CFU/ one hand for the dialysate samples (solid line). After a 3 day-incubation of the solutions at room temperature, the number of bacteria significantly increased 10 to 10^5 -fold (Students' paired *t*-test, $P < 0.0001$). The bacteria from both the "at GJ" and "incubation" samples were divided into 1-4 categories based on bacterial shape. All bacteria were able to be grown in pure culture using R2A medium. A total of 81 colonies were isolated. The microscopic images of Gram-stained samples demonstrated that the majority of the colonies present in the "at GJ" samples were Gram-positive cocci: 96.5% in saline and 85.6% in dialysate (Figure 2a). Gram-negative cocci, Gram-positive bacilli, and Gram-negative bacilli comprised 0 to 7.6% of the

FIG. 2. Proportions and distributions of isolated bacteria species cultured from "at GJ" and "incubation" samples.

a): Gram staining. The proportion of bacterial species was significantly different between the "at GJ" and "incubation" samples in both saline (upper, $n = 6$) and dialysate (lower, $n = 11$, χ -square test, $P < 0.0001$). Here, GPC, GPR, GNC, and GNR mean gram-positive coccus gram-positive bacillus (rod), and gram-negative cocci and gram-negative bacillus (rod), respectively.

b): Distribution of Phyla by 16S rRNA genome analysis of each sample. Distributions are significantly different between the "at GJ" and "incubation" samples in both saline and dialysate (Predominance of phylum Firmicutes by Fisher's exact probability, $P < 0.0001$).

TABLE 1. Changes in bacterial strains between immediately after GJ and after incubation.

at GJ		incubation		at GJ		incubation		
a)	Dialysate			f)	Saline			
	<i>Staphylococcus pasteurii</i>	10	<i>Bacillus subtilis</i>		<i>Staphylococcus pasteurii</i>	10	<i>Enterobacter</i> sp.	10
	<i>Staphylococcus warneri</i>	10			<i>Paracoccus</i> sp.	2	<i>Erwinia tasmaniensis</i>	1
	<i>Delftia lacustris</i>	1			<i>Corynebacterium</i>			
	<i>Rothia amarae</i>	1			<i>tuberculo</i> <i>stearicum</i>	1		
	Saline			g)	Dialysate			
	<i>Staphylococcus capitis</i>		<i>Staphylococcus pasteurii</i>		<i>Staphylococcus haemolyticus</i>	1	<i>Staphylococcus pasteurii</i>	
			<i>Staphylococcus epidermidis</i>		<i>Staphylococcus pasteurii</i>	1		
b)	Saline			h)	Dialysate			
	<i>Staphylococcus epidermidis</i>		<i>Corynebacterium propinquum</i>		<i>Staphylococcus haemolyticus</i>	3	<i>Rothia amarae</i>	
c)	Dialysate				<i>Klebsiella</i> sp.	1		
	<i>Staphylococcus epidermidis</i>	2	<i>Kocuria kristinae</i>					
	<i>Staphylococcus haemolyticus</i>	1	<i>Brevendimonas diminuta</i>	j)	Dialysate			
			<i>Arthrobacter aurescens</i>		<i>Staphylococcus pasteurii</i>	10	<i>Agrobacterium larrymoore</i>	10
	Saline				<i>Acinetobacter junii</i>	1	<i>Acinetobacter junii</i>	1
	<i>Staphylococcus haemolyticus</i>		<i>Pseudomonas oleovorans</i>	K)	Dialysate			
			<i>Brevendimonas diminuta</i>		<i>Kocuria rosea</i>	10	<i>Acinetobacter junii</i>	100
					<i>Staphylococcus pasteurii</i>	10	<i>Sphingomonas melonis</i>	10
d)	Dialysate				<i>Kocuria kristinae</i>	1	<i>Staphylococcus pasteurii</i>	1
	<i>Staphylococcus epidermidis</i>	1	<i>Brevendimonas nasdae</i>		<i>Staphylococcus epidermidis</i>	1		
	<i>Staphylococcus pasteurii</i>	1	<i>Pseudomonas oleovorans</i>		Dialysate (non-dominant)			
			<i>Massilia timonae</i>		<i>Staphylococcus pasteurii</i>	10	<i>Novosphingobium</i>	
			<i>Methylobacterium</i> sp.		<i>Kocuria rosea</i>	1	<i>subarcticum</i>	10
							<i>Staphylococcus epidermidis</i>	1
e)	Dialysate (non-dominant)							
	<i>Staphylococcus epidermidis</i>	1	<i>Brevendimonas</i> sp.					
	<i>Staphylococcus pasteurii</i>	1	<i>Bacteroidetes endosymbiont</i>					
	saline			i)	Dialysate			
	<i>Staphylococcus pasteurii</i>		<i>Brevendimonas nasdae</i>		<i>Staphylococcus pasteurii</i>	2	<i>Acinetobacter junii</i>	100
			<i>Methylobacterium</i> sp.		<i>Staphylococcus haemolyticus</i>	1	<i>Microbacterium imperiale</i>	1
	Saline				Dialysate (non-dominant)			
	<i>Staphylococcus capitis</i>		<i>Pantoea dispersa</i>		<i>Staphylococcus pasteurii</i>	3	<i>Nobosphingobium</i>	
			<i>Enterobacter</i> sp.		<i>Kocuria rosea</i>	2	<i>subarcticum</i>	100
					<i>Escherichia coli</i>	1	<i>Acinetobacter septicus</i>	100
							<i>Staphylococcus pasteurii</i>	1

Bacterial species and strains were identified by 16S rRNA gene homology. The numbers noted after the strains are based on colony counts that were recorded to reflect the ratios between bacterial species: differences within a dilution series are denoted as 1, 10, and 100 times higher, and differences within the same dilution plate as 1, 2, and 3 times higher.

samples. However, the “incubation” samples showed a decrease in Gram-positive cocci (16.7% in saline and 19.3% in dialysate), while Gram-negative bacilli increased to 66.7 % in saline and 66.1 % in dialysate (χ -square test, $P < 0.0001$). Gram-positive bacilli also increased from 2.3% to 16.7% in saline, and from 7.6 to 14.6% in dialysate. These changes in composition, resulting from the increased bacilli and decreased Gram-positive cocci were shown to be significant by Bonferroni’s post hoc test.

By 16S rRNA genome analysis, the genotypes of 70 strains (excluding overlapping strains in the same sample) were identified. The predominant phylotype of the “at GJ” samples was the phylum Firmicutes (Figure 2b). Coagulase-negative staphylococci, such as *Staphylococcus epidermidis* and *S. pasteurii*, were isolated from all 17 “at GJ” samples (Table 1). Both Proteobacteria and Actinobacteria were isolated in five samples, although they were the minority of microbes. The “incubation” samples showed a marked change in

microbe distribution, specifically, the bacteria of the phylum Firmicutes were predominant in only three samples (17.6%). Actinobacteria, alpha-Proteobacteria and gamma-Proteobacteria were predominant in 14 samples (82.4%, Fisher's exact probability, $P = 0.011$, Figure 2b). These results suggested that contamination by Gram-negative bacilli, a cause of endotoxin activity, may be derived from the hands (Figure 2). While coagulase-negative staphylococci were still isolated from five "incubation" samples (case a, g, k dominant, k non-dominant, and l in table), these were outnumbered by the increase in other microbes both in saline and dialysate. Similar results were observed when observing both the dominant and non-dominant hands of the same person (case d, k, and l), in confirmation with an early report (Fierer et al. 2008). After incubation, neither the bacterial growth rates nor the microbe composition were different between saline and dialysate (case a, c, and d in Figures 1 and table). The "at GJ" samples showed a higher diversity and harvested cell count in the dialysate, which may have been due to the presence of sugar or Ca^{2+} (average 2.45 stains in all dialysate and 1.33 in all saline. $P = 0.017$ by Students' unpaired t -test).

In conclusion, it is shown that bacteria of the phylum Proteobacteria, are the causative bacteria of aqueous contamination, because they were isolated from the hands of working staffs. Our modification in the GJ method was simple and significant in revealing the bacteria attached to the hands as a source of aqueous contamination. This method is reproducible with the specified medium, formulation of interest, and Gram stain, which may be used as an effective training protocol without need of any specialized equipment for hand hygiene procedures.

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