Assessment of bacterial contamination of toothbrushes using Illumina MiSeq

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Toothbrushes are commonly used to remove dental plaque, and the presence of bacteria on toothbrushes has been previously reported. Toothbrushes are contaminated by many bacteria after brushing, and contaminated toothbrushes can cause oral and systemic diseases. Toothbrush contamination was studied previously, but the study was limited because it only identified specific bacteria using a general bacterial culture method. To overcome this limitation, we used Illumina sequencing to identify microorganisms present on toothbrushes. Toothbrush samples were divided into two groups according to the storage location: a toothbrush stored in the office or a toothbrush in the bathroom. Samples were sequenced using Illumina sequencing. *Enterococcus* (30.76%), *Pseudomonas* (21.85%), *Streptococcus* (14.94%), and *Lactobacillus* (5.15%) were the predominant bacteria found on the toothbrushes stored in the office. *Streptococcus* (19.73%), *Pseudomonas* (16.08%), *Enterococcus* (8.16%), and *Neisseria* (7.04%) were the predominant species on the toothbrushes stored in the bathroom. In addition, 36.29% of the bacteria on the toothbrushes stored in the office and 33.77% of the bacteria on the toothbrushes stored in the bathroom were identified as potentially pathogenic bacteria. Both groups included microorganisms such as *Streptococcus*, *Actinomyces*, *Porphyromonas*, and *Fusobacterium* that are related to oral disease. This study confirmed the high contamination rate of used toothbrushes and demonstrated that repeated use of toothbrushes could lead to contamination by pathogenic bacteria.

Key Words: Bacteria, Bathroom, Dental plaque, Toothbrush

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INTRODUCTION

Toothbrushes are generally used to remove dental plaque and some studies demonstrate the presence of bacteria on toothbrushes [1-3]. Many bacteria are found on toothbrushes after brushing [4], and these bacteria can survive for one day to one week after brushing [5]. Toothbrushes can be contaminated by the oral environment, hands, and contaminated containers [6]. Contaminated toothbrushes

can cause oral disease, sepsis, and many systemic diseases of the cardiovascular system, respiratory organs, and the kidneys [7].

Various studies reported the number and types microorganisms present on toothbrushes and reported contamination by pathogenic microorganisms after using toothbrushes [8-13]. An average of 10^3 to 10^5 colony-forming unit (CFU) of bacteria were found per toothbrush. Malmberg et al. [10] examined the distribution of microorganisms in 44

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toothbrushes collected from four nurseries. *Streptococcus* like *Streptococcus salivarius*, *Streptococcus sanguinis*, and *Streptococcus mitis* were most prevalent in these studies, and *Haemophilus* was found in 82% of the samples. Researchers also found oral microorganisms associated with periodontal disease and fungi. Another study found that 70% of toothbrushes were contaminated with pathogenic microorganisms after use [14].

Most of these studies used a method of culturing bacteria in a specific medium. The identified microorganisms were identified using Gram staining and biochemical characteristics. However, this identification method is limited because it cannot detect microorganism species that are not cultured. Sometimes, identification using biochemical properties may not match the characteristics of any known species or genus.

To overcome these limitations, this study used Illumina sequencing to identify microorganisms present on toothbrushes.

MATERIALS AND METHODS

Sampling

A total of 10 toothbrush samples were obtained by collecting 2 toothbrushes (group A; toothbrushes stored in the office, group B; toothbrushes stored in the bathroom) from 5 healthy adults (aged 20–50). The toothbrush sample was transferred to a test tube containing 10 mL of sterile distilled water and vortexed for 5 minutes to remove the bacteria from the head of toothbrush.

Genomic DNA extraction

The PureLink® Genomic DNA Mini Kit (Invitrogen, Waltham, MA, USA) was used to extract DNA from the 10 toothbrushes and DNA was extracted according to the manufacturer's instructions. For Illumina sequencing, individual DNA samples belonging to each group were mixed in equal proportions.

Fig. 1. Rarefaction curve of the bacterial communities in toothbrush samples. Group A, toothbrushes stored in the office; group B, toothbrushes stored in the bathroom; OTU, operational taxonomic units.

Polymerase chain reaction amplification and Illumina sequencing

For the metagenome analysis of extracted DNA samples, we used previously reported experimental methods [15,16].

Rarefaction curve was shown in Fig. 1.

Pathogenic bacteria analysis

The bacterial genus was confirmed using Illumina sequencing analysis. The bacterial genera with evidence of pathogenicity in the last 10 years were identified using the PubMed database [17]. A species was deemed pathogenic if one of the bacteria belonging to the discovered bacterium genus was a species known to be pathogenic.

RESULTS

Bacterial diversity of toothbrush samples

Table 1 shows the microbial diversity of the two toothbrush groups with different storage locations (group A, group B). In group A, 78,171 reads and 126 genera were obtained, while 83,021 reads and 151 genera were obtained in group B. In group A, *Enterococcus* (30.76%), *Pseudomonas* (21.85%), *Streptococcus* (14.94%) and *Lactobacillus* (5.15%) were most abundant. In group B, *Strep-*

Table 1. List of genera identified on toothbrushes using Illumina MiSeq

Phylum	Class	Genus	Count	
			Group A	Group B
Bacteria				
Actinobacteria	Actinobacteria_c	<i>Actinobaculum</i>	13	7
		<i>Actinomyces</i>	401	786
		<i>Antricoccus</i>	-	11
		<i>Arsenicicoccus</i>	-	11
		<i>Bifidobacterium</i>	282	403
		<i>Brachybacterium</i>	-	15
		<i>Brevibacterium</i>	131	218
		<i>Corynebacterium</i>	69	99
		<i>Dietzia</i>	-	4
		<i>Gardnerella</i>	-	7
		<i>Kocuria</i>	32	583
		<i>Microbacterium</i>	3	-
		<i>Phycococcus</i>	-	2
		<i>Propionibacterium</i>	4	6
		<i>Rhodococcus</i>	3	70
		<i>Rothia</i>	1,557	2,908
	Coriobacteriia	<i>Atopobium</i>	29	130
		<i>Collinsella</i>	9	14
		<i>Senegalimassilia</i>	-	8
Bacteroidetes	Bacteroidia	<i>Alistipes</i>	58	890
		<i>Alloprevotella</i>	125	959
		<i>Bacteroides</i>	-	221
		<i>Dysgonomonas</i>	2	-
		<i>Marinifilum</i>	1	-
		<i>Parabacteroides</i>	-	20
		<i>Porphyromonas</i>	152	937
		<i>Prevotella</i>	861	3,813
		<i>Tannerella</i>	3	72
	Flavobacteria	<i>Bergeyella</i>	17	88
		<i>Capnocytophaga</i>	81	162
		<i>Chryseobacterium</i>	-	14
		<i>Maritimimonas</i>	-	2
		<i>Sphingobacterium</i>	5	140
		<i>Deinococcus</i>	-	102
Firmicutes	Bacilli	<i>Aerococcus</i>	-	1
		<i>Alkalibacterium</i>	1	-
		<i>Bacillus</i>	55	-
		<i>Enterococcus</i>	24,088	6,790
		<i>Gemella</i>	232	1,994
		<i>Granulicatella</i>	205	309
		<i>Lactobacillus</i>	4,032	647
		<i>Lactococcus</i>	98	3
		<i>Staphylococcus</i>	64	-
		<i>Streptococcus</i>	11,703	16,410
		<i>Vagococcus</i>	1	-
		<i>Weissella</i>	7	-
	Clostridia	<i>Acetatifactor</i>	-	7
		<i>Acidaminobacter</i>	81	3
		<i>Agathobacter</i>	-	4
		<i>Alkalibacter</i>	37	-

Table 1. Continued

Phylum	Class	Genus	Count	
			Group A	Group B
Fusobacteria	Fusobacteria_c	<i>Alkaliphilus</i>	31	-
		<i>Anaerovorax</i>	3	-
		<i>Blautia</i>	5	32
		<i>Butyricicoccus</i>	-	5
		<i>Caproiciproducens</i>	-	2
		<i>Catonella</i>	8	70
		<i>Clostridium</i>	12	129
		<i>Dorea</i>	7	13
		<i>Eisenbergiella</i>	23	597
		<i>Eubacterium</i>	42	210
		<i>Faecalibacterium</i>	6	64
		<i>Filifactor</i>	-	4
		<i>Fusicatenibacter</i>	6	10
		<i>Intestinibacter</i>	4	-
		<i>Lachnoanaerobaculum</i>	45	74
		<i>Lachnospira</i>	-	14
		<i>Marvinbryantia</i>	-	1
		<i>Mogibacterium</i>	1	-
		<i>Moryella</i>	45	69
		<i>Oribacterium</i>	57	73
		<i>Oscillibacter</i>	5	107
		<i>Peptostreptococcus</i>	25	190
		<i>Pseudoflavonifractor</i>	3	126
		<i>Romboutsia</i>	3	5
		<i>Roseburia</i>	-	6
		<i>Ruminococcus</i>	14	31
		<i>Sporobacter</i>	-	5
		<i>Subdoligranulum</i>	-	11
		<i>Tindallia</i>	11	-
	Erysipelotrichi	<i>Bulleidia</i>	32	36
	Negativicutes	<i>Catenibacterium</i>	-	14
		<i>Holdemanella</i>	2	6
		<i>Dialister</i>	2	12
		<i>Megamonas</i>	-	5
		<i>Megasphaera</i>	5	14
		<i>Pelosinus</i>	10	10
		<i>Phascolarctobacterium</i>	-	9
		<i>Selenomonas</i>	21	137
		<i>Veillonella</i>	1,261	3,757
	Tissierellia	<i>Dethiosulfatibacter</i>	114	-
Fusobacteria	Fusobacteria_c	<i>Parvimonas</i>	24	141
		<i>Tissierella</i>	157	-
		<i>Fusobacterium</i>	400	1,923
Proteobacteria	Alphaproteobacteria	<i>Leptotrichia</i>	111	893
		<i>Streptobacillus</i>	-	16
		<i>Altererythrobacter</i>	-	7
		<i>Aureimonas</i>	13	15
		<i>Brevundimonas</i>	56	7
		<i>Novosphingobium</i>	5	-
		<i>Rhizobium</i>	467	18
		<i>Rhodobacter</i>	-	5

Table 1. Continued

Phylum	Class	Genus	Count	
			Group A	Group B
	Betaproteobacteria	<i>Roseomonas</i>	18	2
		<i>Sphingobium</i>	8	5
		<i>Sphingomonas</i>	17	2
		<i>Acidovorax</i>	13	13
		<i>Comamonas</i>	3	7
		<i>Curvibacter</i>	6	6
		<i>Dechloromonas</i>	29	18
		<i>Delftia</i>	61	35
		<i>Duganella</i>	3	-
		<i>Eikenella</i>	-	1
		<i>Herbaspirillum</i>	3	-
		<i>Janthinobacterium</i>	3,393	2,110
		<i>Kingella</i>	11	65
		<i>Lautropia</i>	23	198
		<i>Neisseria</i>	1,067	5,854
		<i>Paucibacter</i>	53	43
		<i>Simonsiella</i>	-	7
		<i>Sutterella</i>	-	4
		<i>Thauera</i>	2	1
		<i>Undibacterium</i>	6	11
	Deltaproteobacteria	<i>Desulfonatronum</i>	194	10
	Epsilonproteobacteria	<i>Desulfovibrio</i>	-	7
		<i>Arcobacter</i>	-	3
		<i>Campylobacter</i>	15	114
		<i>Helicobacter</i>	-	1
	Gammaproteobacteria	<i>Sulfurovum</i>	2	3
		<i>Acinetobacter</i>	2,937	840
		<i>Actinobacillus</i>	3	101
		<i>Aeromonas</i>	389	264
		<i>Aggregatibacter</i>	7	122
		<i>Buttiauxella</i>	10	18
		<i>Cardiobacterium</i>	12	37
		<i>Cedecea</i>	68	36
		<i>Citrobacter</i>	27	12
		<i>Enhydrobacter</i>	195	332
		<i>Enterobacter</i>	458	129
		<i>Erwinia</i>	1,707	173
		<i>Escherichia</i>	43	11
		<i>Haemophilus</i>	556	2,047
		<i>Halomonas</i>	23	16
		<i>Halotalea</i>	3	-
		<i>Klebsiella</i>	5	156
		<i>Moraxella</i>	-	3
		<i>Morganella</i>	143	132
		<i>Pantoea</i>	133	4,109
		<i>Pseudoalteromonas</i>	-	1
		<i>Pseudomonas</i>	17,110	13,374
		<i>Rahnella</i>	15	5
		<i>Serratia</i>	-	8
		<i>Stenotrophomonas</i>	1,081	761
		<i>Vibrio</i>	2	8

Table 1. Continued

Phylum	Class	Genus	Count	
			Group A	Group B
		<i>Woeseia</i>	2	-
		<i>Xanthomonas</i>	-	349
		<i>Yersinia</i>	54	15
Saccharibacteria_TM7	Saccharimonas_c	<i>Saccharimonas</i>	31	101
Spirochaetes	Spirochaetes_c	<i>Treponema</i>	7	11
Synergistetes	Synergistia	<i>Aminivibrio</i>	2	-
		<i>Fretibacterium</i>	-	6
Tenericutes	Mollicutes	<i>Acholeplasma_g2</i>	7	13
Verrucomicrobia	Verrucomicrobiae	<i>Akkermansia</i>	4	95
Unknown			732	3,473
Total			78,171	83,021

Group A, toothbrushes stored in the office; group B, toothbrushes stored in the bathroom; -, not available.

Fig. 2. Relative abundance of bacterial genera in toothbrush samples. Group A, toothbrushes stored in the office; group B, toothbrushes stored in the bathroom.

Streptococcus (19.73%), *Pseudomonas* (16.08%), *Enterococcus* (8.16%) and *Neisseria* (7.04%) were most abundant (Fig. 2-4).

Pathogenic bacteria

In group A, 45 out of 124 genera (36.29%) belonged to pathogenic species. *Enterococcus* was the most common genus, and we also found pathogenic species in the genera *Pseudomonas*, *Acinetobacter*, *Neisseria*, *Haemophilus*, *Corynebacterium*, *Staphylococcus*, *Bacillus*, and *Escherichia*. We also detected bacteria associated with oral diseases like *Streptococcus*, *Actinomyces*, *Fusobacterium*, *Porphyromonas*, *Treponema*, *Aggregatibacter*, and *Tannerella*. In group

B, 51 genera out of 151 genera (33.77%) contained pathogenic species. *Streptococcus* was the most common, and we also found pathogenic species such as *Pseudomonas*, *Enterococcus*, *Neisseria*, *Haemophilus*, *Corynebacterium*, *Escherichia*, and *Helicobacter*. We also found bacteria associated with oral diseases like *Streptococcus*, *Fusobacterium*, *Porphyromonas*, *Actinomyces*, *Aggregatibacter*, *Tannerella*, and *Treponema*.

DISCUSSION

The toothbrush is contaminated from the point of use, and the contamination increases when the toothbrush is used repeatedly [18,19]. In addition, 80% of toothbrushes are known to be contaminated before use [8]. The number of bacteria found on each toothbrush is highly variable and was reported to average from 10^3 to 10^5 CFU per toothbrush [8,10,12,13]. Glass and Lare [19] examined the microbial distribution on toothbrushes used by adults and found various genera, including pathogenic species like *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas*. In addition, Malmberg et al. [10] investigated children's toothbrushes, finding mostly aerobic microorganisms and bacteria like *Staphylococci* and *Pseudomonas*, and fungi. In a study by Mehta et al. [14], 70% of toothbrushes used in the experiment were found to be severely contaminated by pathogenic microorganisms. Studies on microbial contamination of toothbrushes have been done before, but most

Fig. 3. Bacterial community diversity in toothbrush samples (group A, toothbrushes stored in the office).

of them have identified bacteria based on general bacterial culture methods. Therefore, the risk of contaminated toothbrushes may be underestimated.

In our study, the *Enterococcus* genus known to have multi-drug resistance was found most frequently. We also found the genus *Pseudomonas*, an opportunistic infectious bacteria, and *Streptococcus*, a bacterium related to oral diseases. These results were similar to most other studies, but there were differences in the abundances found for each group. Among the toothbrushes stored in office, *Enterococcus* was observed at the highest abundance, followed by *Pseudomonas*, *Streptococcus*, and *Lactobacillus*. The *Streptococcus* genus was most frequently observed in toothbrushes stored in the bathroom, followed by *Pseudomonas*, *Enterococcus*, and *Neisseria*. The proportion of genera containing pathogenic species was observed to be 36.29% in group A and 33.77% in group B. In both groups,

we found *Haemophilus*, which can cause a deadly respiratory infection, *Corynebacterium*, which is known to be the causative agent of diphtheria, and *Neisseria*, which can cause septicemia and gonorrhea. In group A, we found *Bacillus* and *Staphylococcus*. In group B, we found *Helicobacter*, which can cause stomach inflammation, gastric cancer, and peptic ulcer disease. In addition, we found several oral bacteria that are related to oral diseases. In both groups, we also found *Streptococcus* and *Actinomyces*, causative bacteria of dental caries, and *Porphyromonas*, *Fusobacterium*, *Aggregatibacter*, *Tannerella*, and *Treponema*, which are associated with periodontal disease.

Repeated use of contaminated toothbrushes may increase the possibility of bacterial infection [18]. We also found pathogenic bacteria that do not directly cause infection in healthy individuals but could be fatal to patients with weak immune systems. Thus, there is a need to reduce microbial

Fig. 4. Bacterial community diversity in toothbrush samples (group B, toothbrushes stored in the bathroom).

contamination of toothbrushes. The contamination of the toothbrush can be influenced by factors like the shape of the toothbrush, the toothpaste, the storage of the toothbrush, the frequency of brushing, and the period of use. We obtained toothbrush samples in two groups according to storage location (group A, group B). Because toothbrushes are often stored in bathrooms, they can be exposed to enteric bacteria that are aerosolized [12]. We expected enteric bacteria to be found only in toothbrush samples stored in the bathroom, but enteric bacteria were isolated in both groups of toothbrushes. The toothbrush stored in the office seems to be contaminated with enteric bacteria via the human hand or other infection routes.

Based on previous studies, patients with systemic diseases are advised to frequently replace their toothbrush [4,9,20]. The American Dental Association recommends re-

placing the toothbrush every three months [4], and patients with oral disease also reported reduced symptoms after replacing toothbrushes [9]. Many previous studies demonstrated that brushing is effective in improving oral hygiene [21,22]. There is also a risk of pathogenic microbial transfer due to toothbrush contamination and the positive role of such a toothbrush. Therefore, further research is needed to propose a method to reduce toothbrush contamination and establish better hygiene standards.

CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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