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Development of a New Application for Comprehensive Viability Analysis Based on Microbiome Analysis by Next-Generation Sequencing: Insights into Staphylococcal Carriage in Human Nasal Cavities

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ABSTRACT The nasal carriage rate of *Staphylococcus aureus* in human is 25 to 30%, and *S. aureus* sporadically causes severe infections. However, the mechanisms underlying staphylococcal carriage remain largely unknown. In the present study, we constructed an *rpoB*-based microbiome method for staphylococcal species discrimination. Based on a microbiome scheme targeting viable cell DNA using propidium monoazide (PMA) dye (PMA microbiome method), we also developed a new method to allow the comprehensive viability analysis of any bacterial taxon. To clarify the ecological distribution of staphylococci in the nasal microbiota, we applied these methods in 46 nasal specimens from healthy adults. PMA microbiome results showed that *Staphylococcaceae* and *Corynebacteriaceae* were the most predominant viable taxa (average relative abundance: 0.435262 and 0.375195, respectively), and *Staphylococcus epidermidis* exhibited the highest viability in the nasal microbiota. *Staphylococcus aureus* detection rates from nasal specimens by *rpoB*-based conventional and PMA microbiome methods were 84.8% (39 of 46) and 69.5% (32 of 46), respectively, which substantially exceeded the values obtained by a culture method using identical specimens (36.9%). Our results suggest that *Staphylococcaceae* species, especially *S. epidermidis*, adapted most successfully to human nasal cavity. High detection of *S. aureus* DNA by microbiome methods suggests that almost all healthy adults are consistently exposed to *S. aureus* in everyday life. Furthermore, the large difference in *S. aureus* detection rates between culture and microbiome methods suggests that *S. aureus* cells frequently exist in a viable but nonculturable state in nasal cavities. Our method and findings will contribute to a better understanding of the mechanisms underlying carriage of indigenous bacteria.

IMPORTANCE Metagenomic analyses, such as 16S rRNA microbiome methods, have provided new insights in various research fields. However, conventional 16S rRNA microbiome methods do not permit taxonomic analysis of only the viable bacteria in a sample and have poor resolving power below the genus level. Our new schemes allowed for viable cell-specific analysis and species discrimination, and nasal microbiome data using these methods provided some interesting findings regarding staphylococcal nasal carriage. According to our comprehensive viability analysis, the high viability of *Staphylococcus* species, especially *Staphylococcus epidermidis*, in human nasal carriage suggests that this taxon has adapted most successfully to human nasal tissue. Also, a higher detection rate of *S. aureus* DNA by microbiome methods (84.8%) than by a culture method (36.9%) suggests that almost all healthy adults are consistently exposed to *Staphylococcus aureus* in the medium and long term. Our findings will contribute to a better understanding of the mechanisms underlying the carriage of indigenous bacteria.

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The genus *Staphylococcus* is a member of the skin and nasal microbiotas in humans and various animals. *Staphylococcus aureus* is a major opportunistic pathogen that can cause various infections, and methicillin-resistant *S. aureus* (MRSA) remains a significant clinical concern in both hospital-acquired and community-acquired infections worldwide (1–3).

Bacteria living in the nasal cavities of hosts can serve as a reservoir for the spread of the pathogen and can predispose the host to subsequent infections (4, 5). The predominant site of *S. aureus* colonization in human beings is the anterior nares, and nasal carriage is a significant risk factor for infection (6, 7). Therefore, cultivation of nasal swabs has been performed for MRSA screening as a part of infection control measures (8–10). However, the nares are not necessarily the most prevalent sites for carriage of *Staphylococcus* colonization in some animal species, and perineal or rectal swabs have been used for culture of staphylococcal strains in dogs and cats (11). Thus, it is likely that staphylococcal species exhibit host- and tissue-tropism (12, 13). Although recent advances have furthered our understanding of *Staphylococcus*-host interaction during colonization, staphylococcal carriage results from complex relationships with the host immune system and other unknown factors (7, 14–19). Further verification by various research techniques is desirable in order to understand the underlying mechanisms involved in staphylococcal carriage.

Recently, next-generation sequencing (NGS) technologies have become a valuable tool for whole-genome sequencing of bacterial isolates, molecular typing, and microbiome analysis. Studies using microbiome schemes with NGS have focused on the relationships between microbiota and diseases such as atopic dermatitis, inflammatory bowel disease, diabetes, rheumatoid arthritis, multiple sclerosis, ophthalmic disease, Parkinson's disease, and *Clostridium difficile* infection (7, 20–28). Thus, metagenomic analyses, such as 16S rRNA amplicon sequencing, have provided new insight into the correlation between microbial ecology and diseases. However, the 16S rRNA microbiome method has poor resolving power below the genus level (29, 30), whereas it exhibits high taxonomic universality. Furthermore, a conventional microbiome scheme using NGS that targets DNA molecules does not permit taxonomic analysis of only the viable bacteria localized in biological samples, which contain not only viable cells but also dead cells and cell-free DNA. Thus, further development of metagenomic application is desirable to meet various research needs.

In the present study, we created a microbiome method based on the RNA polymerase beta-subunit (*rpoB*) gene, whose partial sequences have been widely used in phylogenetic analysis and species identification using Sanger sequencing, due to higher diversity than that of 16S rRNA (31–33). Our method allowed microbiome analysis at the species level within the family *Staphylococcaceae*. Using a microbiome scheme targeting viable cell DNA using propidium monoazide (PMA) dye (PMA microbiome method), which has been conventionally used for viable cell DNA-specific PCR techniques as a photoreactive reagent (34), we developed a new application for a comprehensive viability analysis based on the combination of conventional and PMA microbiome methods, using identical specimens. Here, we analyzed the human nasal microbiota in healthy individuals, using these methods to clarify the ecological aspects of staphylococci in human nasal tissues.

RESULTS

16S rRNA-based conventional and PMA microbiome methods in nasal specimens from 46 healthy human individuals. 16S rRNA PCR amplification was successful in all nasal specimens, regardless of PMA pretreatment. From DNA libraries of 16S rRNA PCR products, we obtained averages of 68,654 and 87,086 reads per sample for PMA and conventional microbiome methods, derived from viable cell DNA and total DNA, respectively (see Table S2 in the supplemental material). In the 16S rRNA-based method, average numbers of operational taxonomic units (OTUs) of 30.84 and 15.65

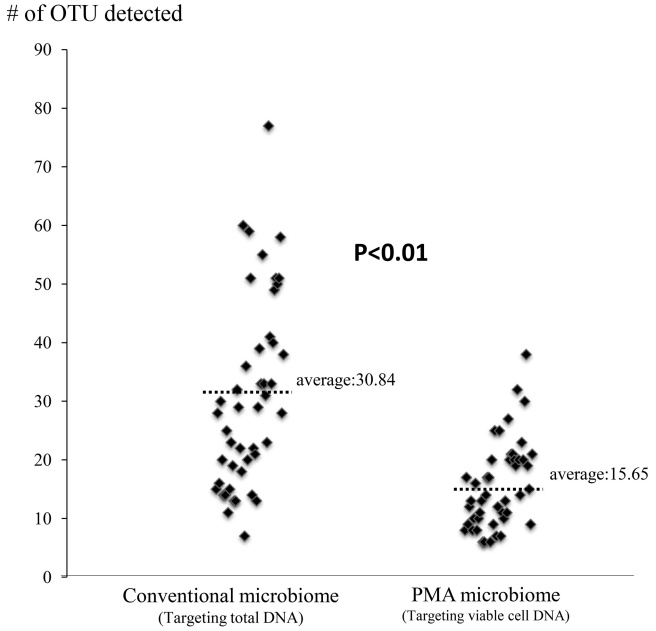

FIG 1 Comparisons using conventional and PMA microbiome methods between numbers of detected operational taxonomic units (OTUs) in 46 nasal specimens.

were detected by conventional microbiome and PMA microbiome methods, respectively. PMAxx pretreatment of nasal samples significantly decreased the number of OTUs, by about 50% at the family level ($P < 0.01$) (Fig. 1). Thus, a great difference between conventional and PMA microbiome methods was observed, which may be explained by dead cell DNA and cell-free DNA contamination in nasal samples.

In the 16S rRNA-based conventional microbiome method targeting total DNA, *Corynebacteriaceae* (average relative abundance, 0.432636) was the most frequently identified taxon, followed by *Staphylococcaceae* (0.332492), *Peptoniphilaceae* (0.078314), and *Carnobacteriaceae* (0.057320). These four families accounted for about 90% of the nasal microbiota. In the 16Sr RNA-based PMA microbiome method targeting viable cell DNA, *Staphylococcaceae* was the most predominant family (0.435262), followed by *Corynebacteriaceae* (0.375195), *Peptoniphilaceae* (0.051435), and *Carnobacteriaceae* (0.049584) (Table 1). As shown in Fig. 2, these four taxa showed low abundance rates as dead cell DNA; these rates were calculated in the comprehensive viability analysis.

Relative abundances of *Neisseriaceae*, *Streptococcaceae*, and *Prevotellaceae* decreased considerably from 0.013481, 0.006436, and 0.003313 in the conventional microbiome method to 0.000875, 0.000281, and 0.000118 in the PMA microbiome method, respectively. A similar tendency was observed in carriage rates (Table S2 in the supplemental material). In other words, high occupancy as dead cell DNA in these taxa was observed in the comprehensive viability analysis. These results indicated that *Neisseriaceae*, *Streptococcaceae*, and *Prevotellaceae* species exhibited lower viability in the nasal cavity, compared with those of the top four families. In contrast, although the family *Bacillaceae* was a minor taxon in the nasal microbiota, it exhibited higher viability. However, the family *Propionibacteriaceae*, which contains *Propionibacterium acnes*, known as one of the major members in skin microbiota, was frequently detected (73.9%, 34 of 46), it exhibited lower viability than the top four families in nasal microbiota (Fig. 2). *Pseudomonadaceae* and *Comamonadaceae* taxa also exhibited low abundance rates on average. However, we speculated that this result was not reliable, because almost half of all individual samples had negative values for the difference of base 10 logarithm (log10) values for *Pseudomonadaceae* and *Comamonadaceae* in the comprehensive viability analysis, which theoretically should yield positive values. This tendency might result from high physiological polymorphism among members of this taxon or from low compatibility with the PMA assay.

TABLE 1 Abundances of the top 23 families in 46 nasal specimens, analyzed by 16S rRNA-based conventional and PMA microbiome methods

Detection method	PMA pretreatment	Target	Taxon (at family level)	Avg relative abundance	SD	Range	No. (%) of carrying individuals
Conventional microbiome	No	Total DNA	*Corynebacteriaceae*	0.432636	0.224033	0.03542–0.94036	46 (100)
			Staphylococcaceae	0.332492	0.211770	0.00609–0.70232	46 (100)
			Peptoniphilaceae	0.078314	0.128043	0–0.53797	45 (97.8)
			Carnobacteriaceae	0.057320	0.142349	0–0.50016	35 (76.1)
			Neisseriaceae	0.013481	0.030994	0–0.17038	35 (76.1)
			Streptococcaceae	0.006436	0.015632	0–0.09634	42 (91.3)
			Prevotellaceae	0.003313	0.014259	0–0.09575	25 (54.3)
			Veillonellaceae	0.002820	0.005593	0–0.02469	32 (69.6)
			Moraxellaceae	0.002342	0.006233	0–0.03139	31 (67.4)
			Micrococcaceae	0.002046	0.004192	0.00009–0.02416	46 (100)
			Actinomycetaceae	0.001871	0.005384	0–0.03158	31 (67.4)
			Rhodobacteraceae	0.001860	0.009140	0–0.06212	35 (76.1)
			Bacillaceae	0.001764	0.003830	0–0.02457	44 (95.7)
			Nocardioidaceae	0.001641	0.010470	0–0.07110	16 (34.8)
			Comamonadaceae	0.001542	0.005149	0–0.02664	20 (43.5)
			Campylobacteraceae	0.001489	0.008156	0–0.05517	12 (26.1)
			Pasteurellaceae	0.001479	0.006021	0–0.04039	19 (41.3)
			Nocardiaceae	0.001199	0.003816	0–0.02550	32 (69.6)
			Pseudomonadaceae	0.001053	0.002712	0–0.01532	24 (52.2)
			Flavobacteriaceae	0.001012	0.004295	0–0.02832	21 (45.7)
			Fusobacteriaceae	0.000794	0.003083	0–0.01533	13 (28.3)
			Porphyromonadaceae	0.000785	0.003707	0–0.02470	13 (28.3)
			Propionibacteriaceae	0.000784	0.002567	0–0.01756	34 (73.9)
PMA microbiome	Yes	DNA from viable cells	*Staphylococcaceae*	0.435262	0.267030	0.00353–0.88256	46 (100)
			Corynebacteriaceae	0.375195	0.270469	0.02003–0.93683	46 (100)
			Peptoniphilaceae	0.051435	0.120054	0–0.48423	40 (87.0)
			Carnobacteriaceae	0.049584	0.162910	0–0.81601	22 (47.8)
			Comamonadaceae	0.004733	0.011920	0–0.04991	24 (52.2)
			Pseudomonadaceae	0.004095	0.012675	0–0.07054	29 (63.0)
			Methylobacteriaceae	0.003856	0.010689	0–0.05960	20 (43.5)
			Rhizobiaceae	0.003689	0.012118	0–0.07630	22 (47.8)
			Moraxellaceae	0.003413	0.012987	0–0.07695	17 (37.0)
			Spirulinaceae	0.002952	0.018891	0–0.12822	6 (13.0)
			Bacillaceae	0.002644	0.007264	0–0.04269	39 (84.8)
			Flavobacteriaceae	0.001685	0.005480	0–0.03279	12 (26.1)
			Bradyrhizobiaceae	0.001100	0.002961	0–0.01748	19 (41.3)
			Micrococcaceae	0.000959	0.003140	0–0.02023	27 (58.7)
			Neisseriaceae	0.000875	0.002374	0–0.01326	15 (32.6)
			Campylobacteraceae	0.000872	0.005914	0–0.04011	1 (2.2)
			Rhodobacteraceae	0.000839	0.003142	0–0.02005	12 (26.1)
			Veillonellaceae	0.000722	0.002429	0–0.01264	8 (17.4)
			Caulobacteraceae	0.000573	0.002545	0–0.01635	9 (19.6)
			Cytophagaceae	0.000563	0.003722	0–0.02525	2 (4.3)
			Propionibacteriaceae	0.000471	0.002388	0–0.01622	19 (41.3)
			Hymenobacteraceae	0.000463	0.003033	0–0.02058	3 (6.5)
			Enterobacteriaceae	0.000441	0.002295	0–0.01504	6 (13.0)

***rpoB*-based conventional and PMA microbiome methods in nasal specimens from 46 healthy human individuals.** *rpoB* PCR amplification was successful in all nasal specimens, regardless of PMAxx pretreatment. From DNA libraries of *rpoB* PCR products, we obtained on average 393,996 and 338,391 reads per sample for PMA and conventional microbiome methods, respectively (see Table S3 in the supplemental material). In the *rpoB*-based method, the average number of OTUs was 10.13 and 6.54 by conventional and PMA microbiome methods, respectively; PMAxx pretreatment of nasal samples significantly decreased the number of OTUs by about 65% at the species level ($P < 0.01$). Thus, a great difference between conventional and PMA microbiome methods was also observed in the *rpoB*-based microbiome method.

In the *rpoB*-based conventional microbiome method targeting total DNA, *S. epidermidis* (average relative abundance, 0.714484) was the most predominant species,

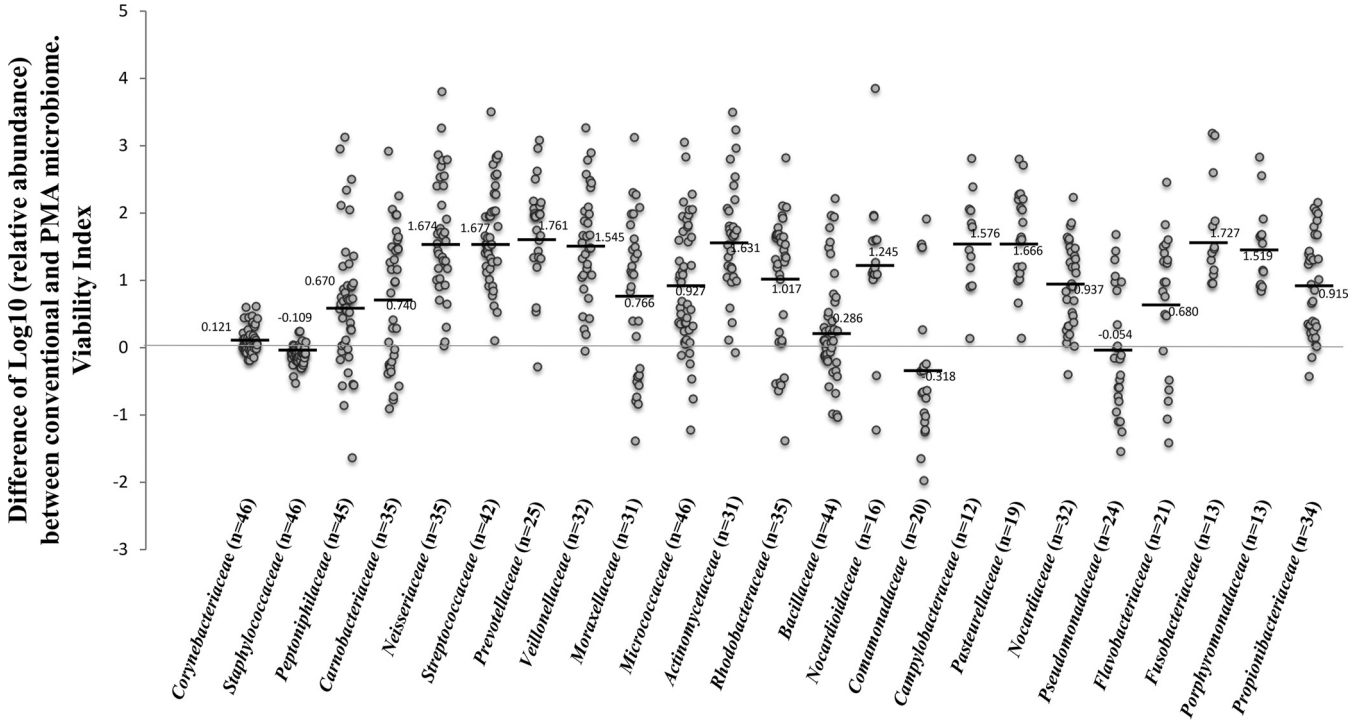

FIG 2 Interfamily comparison in the comprehensive viability analysis based on the 16S rRNA-based microbiome method in 46 nasal specimens from healthy adults. Gray circles represent the differences in relative abundances (log10 values) measured by conventional microbiome analysis (targeting total DNA) and PMA microbiome analysis (targeting viable cell DNA), which approximately indicates the abundance ratios of dead cell DNA to viable cell DNA (viability index). Black lines indicate average values. Larger numeric values indicates lower viability.

followed by *S. aureus* (0.122471), *Staphylococcus capitis* (0.032634), and *Staphylococcus hominis* (0.015187). These four species accounted for about 90% of the *Staphylococcaceae* nasal microbiota (Table 2).

In the *rpoB*-based PMA microbiome method targeting viable cell DNA, *S. epidermidis* (0.746362) was predominant, followed by *S. aureus* (0.114128), *S. capitis* (0.032272), and *S. hominis* (0.014198) (Table 2). As shown in Fig. 3, these species, other than *S. hominis*, showed low abundance rates as dead cell DNA, i.e., high viability in the comprehensive viability analysis. The viability of *S. aureus* was lower than that of *S. epidermidis*.

Although *S. capitis* and *Staphylococcus warneri* exhibited minor abundances in the nasal microbiota compared with *S. epidermidis* and *S. aureus*, the former two species exhibited high detection rates among staphylococcal species. Although, the *S. hominis* was frequently detected (93.4%, 43 of 46) (Table 2), it exhibited the lowest viability in nasal microbiota among top four species (Fig. 2).

Comparison of *S. aureus* detection rates in 46 nasal specimens by the microbiome method using NGS, PCR, and culture methods. Surprisingly, the *S. aureus* detection rates in nasal specimens from 46 healthy adults by the *rpoB*-based microbiome method targeting total and viable cell DNAs using NGS were 84.8% (39 of 46) and 69.5% (32 of 46), respectively (Table 3). These values substantially exceeded the widely reported nasal carriage rate of 25 to 30% (6).

In order to verify the high *S. aureus* detection rates from nasal specimens by NGS methods, we compared the detection rates between NGS, conventional *nuc*-PCR, and culture methods targeting *S. aureus*, using identical test specimens. As shown in Table 3, the *S. aureus* detection rate by both culture and *nuc*-PCR methods was 36.9% (17 of 46). These detection rates were comparable to those of previous reports (6), indicating that the study population consisting of 46 healthy adults was representative of the general population in terms of *S. aureus* nasal carriage rate. In previous studies, the diversity of *S. aureus* detection rates using conventional PCR methods may have resulted from multiple factors such as sampling methods, target genes, and PCR

TABLE 2 Abundances of *Staphylococcaceae* species in 46 human nasal specimens, analyzed by *rpoB*-based conventional and PMA microbiome methods

Detection method	PMA pretreatment	Target	Taxon (at species level)	Avg relative abundance	SD	Range	No. (%) of carrying individuals[a]
Conventional microbiome	No	Total DNA	*Staphylococcus epidermidis*	0.714484	0.254244	0.078705–0.992350	46 (100)
			Staphylococcus capitis	0.032634	0.076620	0.000007–0.479128	45 (97.8)
			Staphylococcus hominis	0.015187	0.033089	0.000002–0.189123	43 (93.4)
			Staphylococcus aureus	0.122471	0.196776	0–0.658542	39 (84.8)
			Staphylococcus warneri	0.001562	0.003929	0–0.020466	34 (73.9)
			Staphylococcus lugdunensis	0.015154	0.045635	0–0.228220	25 (54.3)
			Staphylococcus haemolyticus	0.001613	0.007908	0–0.053760	22 (47.8)
			Staphylococcus pasteuri	0.000786	0.002383	0–0.014888	22 (47.8)
			Staphylococcus caprae	0.002001	0.008156	0–0.050478	19 (41.3)
			Staphylococcus cohnii	0.000344	0.001092	0–0.006115	18 (39.1)
			Staphylococcus saprophyticus	0.000184	0.000583	0–0.003516	11 (23.9)
			Staphylococcus pettenkoferi	0.000169	0.000732	0–0.003712	8 (17.4)
PMA microbiome	Yes	DNA from viable cells	*Staphylococcus epidermidis*	0.746362	0.246951	0.114632–0.995250	46 (100)
			Staphylococcus capitis	0.032272	0.078119	0–0.443570	40 (86.9)
			Staphylococcus hominis	0.014198	0.049433	0–0.294620	24 (52.1)
			Staphylococcus aureus	0.114128	0.193522	0–0.740368	32 (69.5)
			Staphylococcus warneri	0.000580	0.001921	0–0.012093	18 (39.1)
			Staphylococcus lugdunensis	0.013273	0.045749	0–0.256855	16 (34.7)
			Staphylococcus haemolyticus	0.001809	0.011967	0–0.081190	6 (13.0)
			Staphylococcus pasteuri	0.000901	0.005444	0–0.036879	5 (10.8)
			Staphylococcus caprae	0.000848	0.004944	0–0.033304	3 (6.5)
			Staphylococcus cohnii	0.000373	0.002385	0–0.016173	3 (6.5)
			Staphylococcus saprophyticus	0.000059	0.000350	0–0.002354	2 (4.3)
			Staphylococcus pettenkoferi	0.000153	0.000874	0–0.005834	2 (4.3)

[a] $>1e^{-5}$ of relative abundance was defined as the threshold for a positive decision.

conditions (35–37). In the present study, culture-positive and -negative samples for *S. aureus* were independently applied to Illumina MiSeq runs, and the high *S. aureus* detection rate obtained by NGS methods was free from the influence of barcode sorting error in the MiSeq system. Therefore, it is likely that the *S. aureus* detection rates obtained by NGS methods were reliable.

The average relative abundances of 17 *S. aureus* culture-positive specimens using *rpoB*-based conventional and PMA microbiome methods were 0.277602 (range, 0.079416 to 0.569537) and 0.288543 (range, 0.031136 to 0.723341), respectively, which were higher than those of 29 *S. aureus* culture-negative specimens (average, 0.004587; range, 0 to 0.088988 for *rpoB*; and average, 0.006253; range, 0 to 0.164649 for PMA microbiome) (see Table S1 in the supplemental material). Consequently, NGS (threshold level of $>1e^{-05}$ relative abundances of *S. aureus*) and culture methods could detect the existence of an *S. aureus* cell in the sample population at sensitivity levels of 10^{-5} and 10^{-2}, respectively. The sensitivity of the PCR-based method was comparable to that of the culture method. Thus, it was likely that the microbiome method exhibited about 1,000 times greater sensitivity than the culture and conventional PCR methods in the detection of *S. aureus*.

DISCUSSION

An increasing number of studies on the relationship between microbiota and diseases have been reported, following the widespread use of the 16S rRNA metagenomic method (20–28). Additionally, there have been reports on analytical applications in microbiome methods, such as species-level and strain-level analytical methods (38–40). Checinska et al. and Venkateswaran et al. previously reported that differentiating between viable and yet-to-be-cultivable microbial populations requires an appropriate sample processing technology, such as the PMA assay, and conducted a microbiome analysis with PMA treatment, using samples from devices in the International Space Station (41, 42). However, there have been few reports on microbiome analyses targeting viable bacterial populations. Our results from viable cell DNA-specific

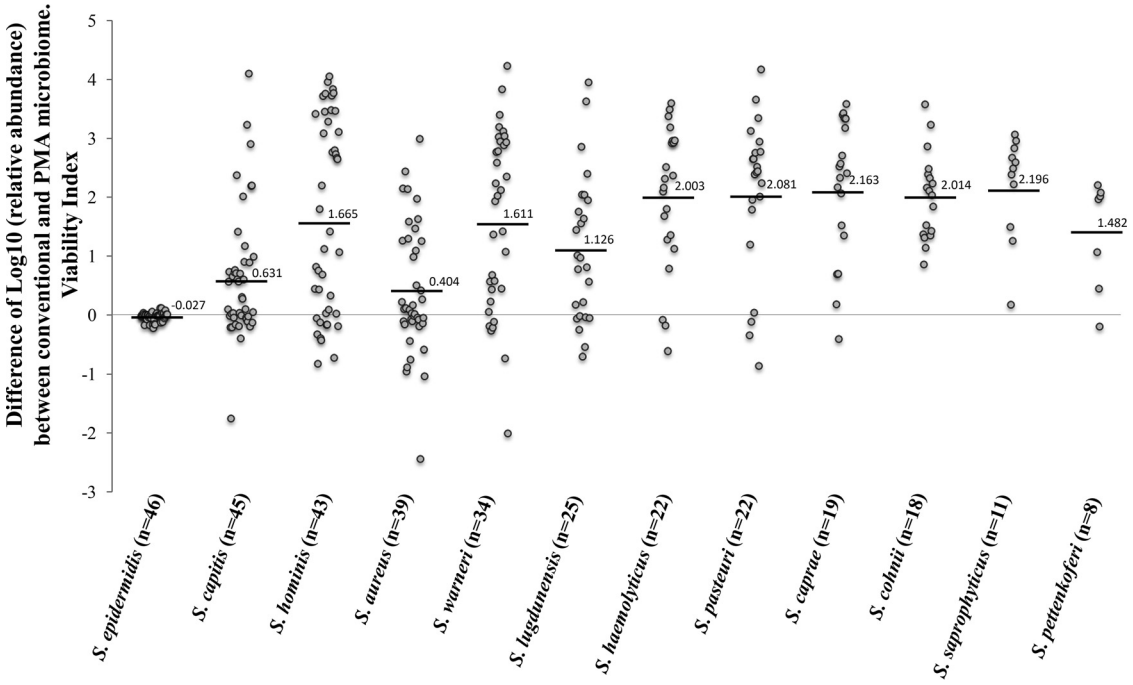

FIG 3 Interspecies comparison in the comprehensive viability analysis based on the *rpoB*-based microbiome method in 46 nasal specimens. Gray circles represent the differences in relative abundances (log10 values) measured by conventional microbiome analysis (targeting total DNA) and PMA microbiome analysis (targeting viable cell DNA), which approximately indicates the abundance ratios of dead cell DNA to viable cell DNA (viability index). Black lines indicate average values. Larger numeric values indicate lower viability.

microbiome analysis using a PMA dye indicated that biological samples, such as nasal specimens, contained not only viable cells but also dead cells and cell-free DNA derived from various bacterial taxa, which were not negligible. Consequently, our scheme, a combination of conventional and PMA microbiome methods using identical test specimens, allowed for viability analysis of any bacterial taxon in the anterior nares.

16S rRNA and shotgun metagenomic methods are universally applicable for any target organism and are useful as primary screening methods (43). However, these schemes exhibited low sensitivity for an especially minor taxon in the community (44) and had the critical limitation of poor resolving power at the species level (29, 30). Our *rpoB*-based microbiome method was validated for *Staphylococcaceae*-specific deep sequencing and species level analysis and was found to be functional as a secondary screening analysis targeting *Staphylococcaceae* following a 16S rRNA-based microbiome method. Further development of secondary screening methods targeting specific organisms is desirable for microbiome research.

In the current study, *Neisseriaceae* and *Streptococcaceae* species exhibited low viability in the nasal microbiota. *Neisseria meningitidis* and *Streptococcus pyogenes*, which occasionally cause life-threatening diseases, frequently colonize nasopharyngeal mucosa rather than nasal mucosa (45, 46). In contrast, high viability of the family *Staphylococcaceae*, especially the genus *Staphylococcus*, was found in the current study, suggesting that this taxon had evolved to colonize nasal tissues. Consequently, the

TABLE 3 Comparison of *Staphylococcus aureus* detection rates in 46 nasal specimens by various methods

Detection method	PMAxx pretreatment	Target	No. (%) of *S. aureus*-positive samples
Microbiome based on *rpoB* gene by NGS	No	Total DNA	39 (84.8)
	Yes	DNA from viable cells	32 (69.5)
nuc-PCR	No	Total DNA	17 (36.9)
Culture using a mannitol salt agar		Culturable viable cells	17 (36.9)

difference in tissue tropism between *Neisseriaceae*, *Streptococcaceae*, and *Staphylococcaceae* families was successfully quantified by our comprehensive viability analysis method, although it was undeterminable by the conventional microbiome method targeting total DNA.

The results of the comprehensive viability analysis suggested that *Staphylococcaceae* species adapted most successfully to the nasal cavity. In particular, high compatibility of *S. epidermidis* in human nasal tissue was observed compared with that of other species within the genus *Staphylococcus*. To date, more than 40 species have been taxonomically described in the genus *Staphylococcus* (47). Staphylococcal species exhibit host specificity at the species and clone levels (12, 13, 48). Joseph et al. speculated that a high frequency of the livestock-associated *S. aureus* clonal complex 398 (CC398) from human tongue dorsum samples, following those of human-related CC30, CC8, and CC45, likely resulted from ingestion of food (40). The combination of our comprehensive viability analysis with microbiome method used in this study may allow the differentiation of whether detected CC398 *S. aureus* DNA was derived from endogenous *S. aureus* living cells or from exogenous dead cells contained in pork meats. Our analysis scheme will contribute to a better understanding of host specificity and mechanisms underlying staphylococcal carriage.

Interestingly, *S. aureus* DNA was detected from almost all nasal specimens by the *rpoB*-based microbiome method targeting total DNA using NGS in the current study. Joseph et al. also reported previously that the nasal carriage rate of *S. aureus* was as high as 57%, using a shotgun metagenomic analysis (40). Our results (84.8%) exceeded the detection rate, suggesting that this difference resulted from a higher specificity and depth against *Staphylococcaceae* species of our *rpoB*-based method than those of the shotgun metagenomic method. Nonetheless, high nasal carriage rates of *S. aureus* observed in studies using NGS methods contrast with the widely reported carriage rate of 25 to 30% in healthy individuals by the conventional culture method. This discrepancy could be explained by the high *in vivo* stability of DNA molecules as a target for measurement. In previous reports, longitudinal studies using a culture method showed that persistent and intermittent *S. aureus* carriers had carriage rates of 12 to 30% and 16 to 70%, respectively (6, 49–53). Although long-term observation of *S. aureus* carriage has not been performed, a highly sensitive NGS method might be able to detect rudimentary *S. aureus* DNA molecules derived from *S. aureus* cells previously carried in the nares. Consequently, our results suggested that all healthy adults were consistently exposed to *S. aureus* in everyday life. Therefore, a negative culture result in screening for *S. aureus* or MRSA at a specific point in time does not completely eliminate the risk of infections with them. The low detection rate in *nuc*-PCR (36.9%) suggests that a conventional PCR is insensitive compared to real-time PCR and NGS methods, which exhibit about 100 to 1,000 times higher sensitivity than that of conventional PCR (54, 55).

In the present study, the detection rate of *S. aureus* from nasal specimens by the *rpoB*-based PMA microbiome method targeting viable cell DNA was 69.5%, which substantially exceeded the values obtained by a culture method (36.9%). This large difference in results in the identical test specimens between culture and PMA microbiome methods targeting viable cells suggested that *S. aureus* cells frequently exist in the viable but nonculturable (VBNC) state in the nasal microbiota. The VBNC state is considered a survival strategy adopted by many bacteria in response to stress and can be induced by limiting environmental conditions (56). Starvation, low temperature, antibiotic pressure, *Cinnamomum verum* essential oil, drying, and mammalian abscesses have been reported as factors that induce the VBNC state in *S. aureus* (56–61). Therefore, further studies are needed to elucidate the role of the VBNC state in staphylococcal carriage.

To our knowledge, this is the first report on the methodological development of a culture-independent, comprehensive viability analysis based on a microbiome method by NGS. We anticipate that this comprehensive viability analysis can be widely applied to various experimental systems, such as comparative analysis of tissue tropism among

various bacterial taxa existing in the human body, *in vivo* efficacy testing of antibiotics or disinfectants, assessment of the impact of antimicrobial agents on indigenous microbiota, and monitoring of spoilage bacteria contained in food samples, which have traditionally used culture-dependent methods. In addition, our study suggested high compatibility of *S. epidermidis* with respect to human nasal tissue and involvement of the VBNC state of *S. aureus* cells in human nasal carriage. Our findings will contribute to a better understanding of the mechanisms underlying the carriage of indigenous bacteria.

MATERIALS AND METHODS

Study design and participants. Nasal swabs were obtained from 46 healthy volunteers (16 males and 30 females), who were individuals with ($n = 3$) and without ($n = 43$) a history of antibiotic use within 1 month. The mean age of the volunteers was 26.1 years (range, 19 to 71 years) (Table S1 in the supplemental material).

The study protocol was reviewed and approved by the ethics committee of the Juntendo Institutional Review Board, Juntendo University School of Medicine, and the clinical study committee of Juntendo University Hospital (no. 17-021). All participants provided their informed consent prior to sampling.

Sample collection and processing. Nasal samples were carefully collected from nasal vestibules of both nostrils using a seed swab γ1 (Eiken Chemical Co., Ltd., Tokyo, Japan) and transported to our laboratory immediately after collection. Swab specimens from participants were suspended in 1.0 ml of sterile saline. Each sample solution was divided into two separate 500-μl aliquots. One aliquot was used for the culture-based method and analyses targeting total DNA such as PCR and conventional microbiome methods; the other aliquot was used for the PMA microbiome method targeting viable cell DNA using PMAxx dye (Biotium, Inc., CA) (62–64).

Sample preparation for the PMA microbiome method based on selective detection of viable cell DNA using PMAxx dye. We performed the viability-PCR assay using the PMAxx dye according to the manufacturer's protocol (Biotium, Inc., CA). This dye is cell membrane impermeable, and thus can be selectively used to modify only exposed DNA from dead cells and cell-free DNA in extracellular fluid, the DNA of which has been PMAxx-modified. The PMAxx-modified, exposed DNA cannot be amplified by PCR. We applied this assay via PCR to an Illumina NGS platform. PMAxx-treated cells were washed twice in 200 μl of Tris-EDTA (TE) buffer. The metagenomic analysis using samples processed by this method is referred to here as the PMA microbiome method. Sample processing by PMAxx pretreatment was not performed for the conventional microbiome method targeting total DNA.

DNA extraction. We used each diluted nasal swab sample, with or without PMAxx pretreatment, for DNA extraction. Samples were pretreated in TE buffer with 50 U of achromopeptidase overnight at 37°C, and then digested with 100 U of proteinase K overnight at 50°C. We next isolated DNA by the phenol-chloroform isoamyl alcohol (PCI) method. Finally, DNA samples were eluted in 50 μl of TE buffer, following ethanol precipitation.

Preparation of PCR amplicons and DNA libraries for microbiome analyses based on 16S rRNA gene and *rpoB* amplicon sequencing. We performed an Illumina 16S metagenomic sequencing protocol, which targeted the V3 to V4 region of bacterial and archaeal 16S rRNA genes, for comprehensive analysis of the nasal microbiota, following the manufacturer's workflow.

For species-level analysis within the family *Staphylococcaceae*, we constructed a method of microbiome analysis based on partial sequences of *rpoB*, which encodes the highly conserved β subunit of the bacterial RNA polymerase and has been widely used as a suitable target for staphylococcal species identification (31). In order to amplify variable regions of *rpoB* genes in the family *Staphylococcaceae*, degenerate primers were designed by multiple alignments of amino acid and nucleotide sequences in multiple species belonging to the family *Staphylococcaceae* (see figures in the supplemental material), which are available from NCBI databases (see Table S4 in the supplemental material). Finally, the degenerate primers rpoB-F (5′-GCVAACATGCAACGWCAAGC-3′) and rpoB-R (5′-CCTTTWGGHGTNACTTT ACC-3′) were designed as a set of universal primers for amplification of a 629-bp region within the *rpoB* coding region. The closest related species were *Staphylococcus pseudintermedius* and *Staphylococcus delphini*, which exhibited 99.3% similarity (a difference of 4 bp of 629 bp).

We used the KOD Multi and Epi enzyme (Toyobo Co., Ltd., Osaka, Japan), which has acceptable high fidelity and low amplification bias, as a PCR polymerase for microbiome analysis using PCR. The reaction mixture for the PCR consisted of 70 ng of DNA extract in a total volume of 25 μl, composed of 0.5 μl of KOD polymerase, 50 pmol of each primer, and 12.5 μl of 2× KOD buffer. The following PCR conditions were used: 94°C for 2 min; 25 to 30 cycles at 98°C for 20 s, 54°C for 30 s, and 68°C for 1 min; and 68°C for 2 min. DNA fragments were analyzed by electrophoresis in 1× Tris-acetate-EDTA on a 1% agarose gel stained with ethidium bromide. The PCR products were then purified using AMPure beads (Beckman Coulter, Inc., CA), according to the manufacturer's protocol.

DNA libraries for sequencing were prepared by transposon-based fragmentation of the 629-bp *rpoB* PCR products using a Nextera XT DNA sample prep kit (Illumina, San Diego, CA). DNA libraries obtained in this manner were highly heterogeneous, and sequencing reads with sufficient quality were generated; we thus treated multiple samples collectively.

NucleoMag NGS Cleanup and Size Select technology (Macherey-Nagel, Düren, Germany) was used twice for cleanup and size selection of NGS libraries, according to a protocol for removing adapter dimers.

NGS, sequencing read processing, and taxonomic analysis. Sequencing was performed using a MiSeq reagent kit v3 (600 cycles) and a paired-end 2 × 300-bp cycle run on an Illumina MiSeq sequencing system. After sequencing, MiSeq-read 1 and 2 reads were stitched by FLASh (http://ccb.jhu.edu/software/FLASH/) (65), resulting in significant improvement of taxonomic clustering because of longer reads. The merged reads were filtered and trimmed by removing bases with quality value (QV) scores of 20 or less and read lengths shorter than 250 bases, and then converted from FASTQ to FASTA format using FASTX toolkit ver. 0.0.14 (http://hannonlab.cshl.edu/fastx_toolkit).

Taxonomic assignments of 16S rRNA and *rpoB* genes were performed using the BLASTN program of BLAST+ software, with E value cutoffs of e^{-10} and e^{-20}, respectively. The reference sequence file of the bacterial 16S rRNA genes was obtained from the NCBI database, and that of *rpoB* was edited in the present study (Table S4 in the supplemental material). Taxonomic classification of 16S rRNA genes was performed using MEGAN6 software (66). *rpoB* reads were manually processed; those with less than 95% nucleotide identity compared with any species in the family *Staphylococcaceae* were omitted from species-level identification.

An operational taxonomic unit (OTU) is a cluster of similar sequence variants, which is dependent on sequence diversity in the target gene. OTUs in 16S rRNA genes and *rpoB* were represented at the family and species levels in the present study, respectively.

Detection of *S. aureus* by conventional culture, PCR, and *rpoB*-based microbiome methods. Ten microliters of suspended solution from the nasal swab was inoculated onto mannitol salt agar (Nissui Co., Ltd., Tokyo, Japan) and incubated at 37°C for 48 h for selective isolation of staphylococci. Among colonies exhibiting catalase-positive and Gram-positive cocci that were presumptively identified as staphylococci by colony morphology, representatives of each colony type were subcultured on Trypticase soy agar II with 5% sheep blood (TSAB; BD Japan, Co., Ltd., Tokyo, Japan). A single colony was suspended in 50 μl of TE buffer (10 mM Tris and 1 mM EDTA [pH 8.0]) with 10 U of achromopeptidase (Wako Chemical Co., Ltd., Osaka, Japan) to a 1.0 McFarland standard, and the suspension was incubated at 50°C for 30 min. The crude DNA solution was then used for species identification by the *nuc*-PCR method, as reported previously (67, 68).

Using DNA extracts from nasal specimens, we performed *S. aureus* detection by a *nuc*-PCR and *rpoB*-based microbiome. Seventy nanograms of DNA extract was applied for each method. In a *nuc*-PCR, presence or absence of DNA fragments was visually inspected by electrophoresis in 1× Tris-acetate-EDTA on a 1% agarose gel stained with ethidium bromide. In the *rpoB*-based microbiome method of *S. aureus* detection, at least 100,000 valid bacterial *rpoB* sequencing reads were obtained per sample. We determined whether the sample was *S. aureus* positive or negative given a threshold level of $>1e^{-05}$ relative abundance of *S. aureus*.

Development of a new application for comprehensive viability analysis by the combination of conventional and PMA microbiome methods. In order to compare the viabilities among any bacterial taxa in human nasal microbiota in 46 healthy individuals, we developed a new method for comprehensive viability analysis based on the combination of conventional microbiome and PMA microbiome methods.

First, the base 10 logarithm (log10) of relative abundance was calculated from the results of the conventional microbiome method targeting total DNA and the PMA microbiome method targeting viable cell DNA. Second, the differences between their values were calculated. Thus, the differences of log10 values of relative abundances between total DNA and viable cell DNA approximately represented the occupancy of dead cell DNA and cell-free DNA. The ranges and averages calculated according to bacterial taxa were then used for intertaxon comparisons. Larger numeric values indicate less viability.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.00517-18.

SUPPLEMENTAL FILE 1, PDF file, 1.0 MB.
SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.
SUPPLEMENTAL FILE 3, XLSX file, 0.3 MB.
SUPPLEMENTAL FILE 4, XLSX file, 0.2 MB.
SUPPLEMENTAL FILE 5, XLSX file, 0.3 MB.

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