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Influence of swabbing solution and swab type on DNA recovery from rigid environmental surfaces

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ABSTRACT

Determination of the metagenome has become an important component of forensic identification, which requires efficient environmental sampling techniques. Therefore, in this study, we compared the efficiency of sample collection using swabbing with cotton swabs and three types of medical swabs (S7, S22, S24) along with three different solutions: phosphate-buffered saline (PBS), 1% Tween 20 + 1% glycerol in PBS (TG), and GS commercial solution (Noble Bio, Hwaseong, Republic of Korea). Combinations of the three solutions with the three types of swabs were tested at different volumes (cotton swab, S7: 0, 30, 50, 70 μL; S22, S24: 0, 70, 100, 130 μL). *Escherichia coli* and *Staphylococcus aureus* were selected as representative environmental microbial samples, and the number of colony-forming units (CFUs), DNA concentration, and DNA copy numbers were compared across groups. The sampling process had a clear effect on the efficiency of extraction, which allowed for determination of a more efficient sample sampling method. In particular, cotton swabs showed 2–10-fold greater CFUs of both species than the medical swabs, and resulted in significantly greater amounts of extracted DNA. TG was found to be the most efficient solution for bacterial DNA extraction, with higher CFUs and DNA obtained than with the other three solutions at all volumes tested. This study highlights the need for a standardized sampling method that can be applied to all environmental samples, especially for microbial quantification, and provides valuable reference data for the efficient collection of environmental samples for metagenomic analyses in microbial-based forensic assessments.

1. Introduction

Owing to recent advances in sequencing technology, including the development of next-generation sequencing that yields large amounts of results in a short time, forensics analyses have expanded to include metagenomics evaluations, by assessing the microorganisms that are abundant in the human body as well as in the environment (Schmedes et al., 2016). However, it is still a difficult task to study microorganisms by sampling and culturing in a natural environment (Qin et al., 2010). Microorganisms exist in diverse environments, including on human surfaces as well as in soil, water, and other life forms. With regards to forensic research, there has been a shift in recent years from a focus on analyses of human DNA to identifying the specific microbial signatures of individuals, including their resident microbes (Lee et al., 2016; Woerner et al., 2019), using a metagenomics approach. It is now also possible to use saliva DNA to distinguish individuals more efficiently based on the representative bacteria of the oral cavity, which can confirm that the stain found at the crime scene is indeed saliva (Gevers et al., 2012). In addition, many researchers are actively studying the relationship of microorganisms with their environments, with a particular focus on implications for human health and welfare (Qin et al., 2010). For example, the NIH Common Fund has constructed the HMP (http://commonfund.nih.gov/hmp/) to facilitate research on interactions of human health and microbial communities in five main body regions: airway, skin, oral, gastrointestinal, and vaginal samples. The main benefit of the HMP is to provide a set of standards for the microbiome of healthy individuals as a reference, and to make this resource available to all researchers (Gervers et al., 2012).

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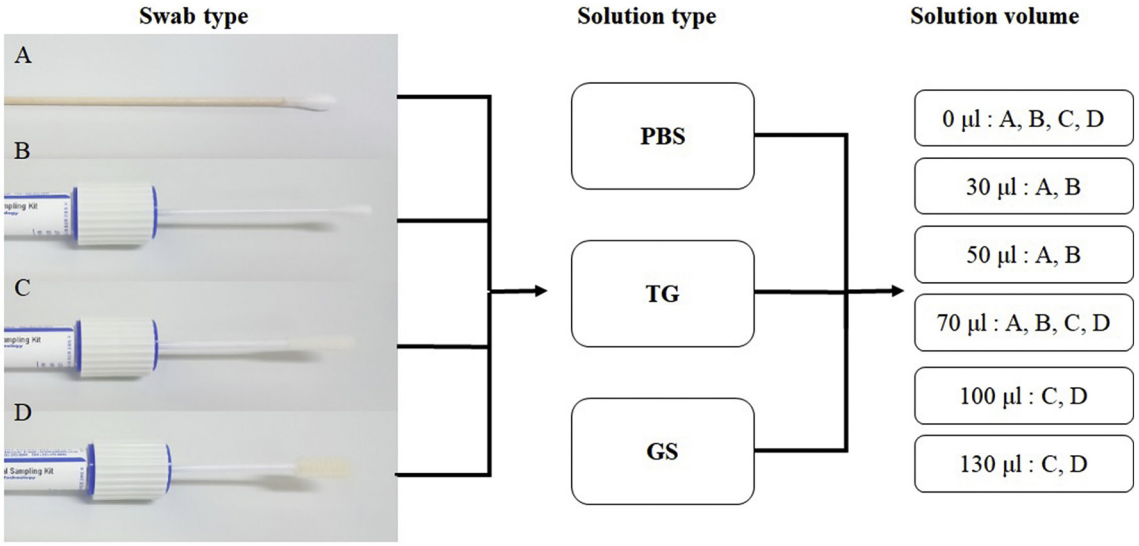

Fig. 1. Schematic of experiment according to swab type and solution type. Three kinds of solutions (1× PBS, TG; 1% Tween 20 + 1% Glycerol in PBS, GS; Noble Bio, Hwaseong, Republic of Korea) and four kinds of swabs (A: cotton swabs, B, C, D: S7, S22, S24 medical swabs) were used. The amount of solution wetted on the swab was selected by measuring the weight of the swab. Through each scheme, the efficiency of collection of environmental microorganisms by swab type and solution type and volume was tested.

Initial metagenomics studies, including those applied to forensics, mainly used sampling methods with tools such as adhesive tape with non-tacky surfaces treated with 95% ethanol (Hanssen et al., 2017; Verdon et al., 2010). Thereafter, a sample method using a swab gradually became standard practice. In particular, for the application of metagenomics to forensics, a swab is typically used for sampling from the human body as well as the environment (Woerner et al., 2019; Maestre et al., 2018), which are then applied to NGS and downstream processes for genetic analyses. Swab sampling is also commonly applied for metagenomics in a wide variety of fields besides forensics, including for microbiome and ecological analyses, medical diagnosis, biotechnology, and biological systems; thus, metagenomics is also known as environmental community genomics. Once the sample is obtained, DNA is directly extracted from microbial aggregates followed by analysis of microbial genomes through sequencing (Handelsman, 2004). Along with the emergence of new techniques, the importance of sampling methods has been emphasized.

Swabbing has been shown to be an effective method of sampling from the skin for investigating bacterial diversity, which is a common goal of metagenomics research (Grice et al., 2008; Pang and Cheung, 2007; Anzai-Kanto et al., 2005). Swabs have also long been used for forensic human sample collection, such as for STR analysis with blood, saliva, semen, and fingerprint samples collected from outpatients (Liu, 2015; Williamson, 2012; Van Oorschot and Jones, 1997). In general, cotton swabs are the most common sampling tool for metagenomics research that are coated with specific solutions (Pechal et al., 2014). In particular, a mixed solution of 0.15 M NaCl and 0.1% Tween 20 has been used for sampling to assess the influence of internal and external individual factors on the resident bacterial community composition (Ying et al., 2015; Fierer et al., 2008). In addition, a mixed solution of NaCl and Tween 20 was used to confirm the level of agreement in bacterial composition between samples taken from cell phones and personal footprints (Lax et al., 2015). In another study, the efficiency of human cell sampling was compared using six kinds of solutions, which revealed that Triton X-100 was the most efficient for sampling human-contacted objects (Fierer et al., 2010). However, this has only been confirmed for analyses of human cells, and it is therefore necessary to confirm the most efficient solution and sampling method for a mixture of different kinds of samples.

Accordingly, in this study, we compared the efficiencies of different types of swabs (a cotton swab and three types of medical swabs) and solutions of different volumes [phosphate-buffered saline (PBS), 1% Tween20 + 1% glycerol in PBS (TG), and GS commercial solution (Noble Bio, Hwaseong, Republic of Korea)] on the efficiency of bacterial quantification. We focused on *Escherichia coli* and *Staphylococcus aureus* as representative gram-negative and gram-positive bacteria, respectively, in environments, including humans. We compared the quantitative values with three assays [colony forming units (CFU) count, gDNA concentration measurements, and gDNA copy number] for the combinations of different solutions and swabs to identify the most relatively efficient sampling method for metagenomics and downstream analyses. These results can serve as a reference for standardizing the sampling method according to swab type (size, weight, solution volume, and the kind of solution) to obtain reliable results that are comparable across studies in forensics applications, and can further contribute to the application in metagenomic for other research fields.

2. Material and methods

2.1. Bacteria culture and sample preparation

S. aureus ATCC 25923 and *E. coli* ATCC 8739 obtained from Korea Centers for Disease Control and Prevention were cultured in nutrient agar medium at 36.5 °C for 24 h. One of the colonies was selected and further cultured in Luria-Bertani (LB) broth medium at 36.5 °C for 24 h. The cultured bacterial medium was placed on a desk surface using a sterilized cotton swab, and the medium was completely dried for 1 h.

To compare the bacterial collection efficiency with different sampling methods, GS commercial solution (Noble Bio, Hwaseong, Republic of Korea) (Fierer et al., 2008; Si et al., 2015), PBS solution (20× PBS Buffer; LPS solution, Daejeon, Republic of Korea) diluted 1× with sterilized distilled water, and TG solution prepared by mixing 1% Tween 20 + 1% glycerol in PBS were used for sample collection. In addition, cotton swabs and three types of medical swabs (S7, S22, S24; Noble Bio, Hwaseong, Republic of Korea) were used with each type of solution to compare the sampling efficiency according to the type and of swab and solution. In the experiment, the cotton, S7, S22, and S24 cotton swab types were labeled as swabs A, B, C, and D, respectively (Fig. 1). The various swabs tested were of similar weights and sizes to avoid effects due to swab size. The weights of 40 swabs were

determined on an electronic scale (Ohaus Adventurer, USA).

To compare the efficiency of bacterial collection, the solution volume was adjusted according to the type and weight of the swab (A and B swab: 0, 30, 50, 70 μL; C and D swab: 0, 70, 100, 130 μL). The volume of the solution was determined so that the entire swab could be covered in the solution according to its weight, which was determined to be 70 μL for A and B swab, and 100 μL for C and D swab. To compare the collection efficiency according to the volume of the solution, swabs A and B were further tested with 50 and 100 μL of solution, while swabs C and D were tested with 70 and 130 μL of solution (Fig. 1).

2.2. Sample preparation for CFU determination

The artificial sample prepared on the table was collected using different combinations of the prepared swabs and solutions and placed in 5 mL of LB medium. Each of the swabs was treated with Branson 8200 Ultrasonic Cleaner (Branson Ultrasonics Corp., Danbury, USA) and mixed on MS1 shakers (IKA®, Germany) for approximately 1 min. The LB medium containing each swab was serially diluted in PBS (1:10), and then 100 μL of the diluted solution was plated on mannitol salt agar (BD Diagnostic Systems, Sparks, MD, USA) medium and MacConkey (BD Diagnostic Systems, Sparks, MD, USA) medium for *E. coli* and *S. aureus*, respectively, and incubated at 36.5 °C for 24 h. The cultured colonies were counted and converted to CFU for analysis.

2.3. DNA extraction

The samples from the table were combined with each type of swab and solution, and only the head of the swab was cut and placed in a 1.5 mL tube. The collected samples were treated with 1 mL of PBS for 2 h and then centrifuged at 7500 rpm for 10 min. The swab was removed and mixed with 180 μL ATL buffer, and the tube was stimulated for 10 min with a water-bath type ultrasonic cleaner (Jeiotech, Daejeon, Republic of Korea) and treated with 20 μL Proteinase K (Bioneer, Daejeon, Republic of Korea). DNA extraction was performed using QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany), which is suitable for DNA extraction of bacteria (Wade et al., 2005). DNA extraction was performed according to the manufacturer's instructions with certain modifications as previously described (Sarah and Foram, 2013).

The experiment was performed three times for each combination of the swab and each solution type and volume. The DNA was eluted with 100 μL of elution solution. The DNA concentration was measured three times using a Qubit 4 fluorometer (Invitrogen™, Thermo Fisher Scientific, Waltham, USA). The extracted DNA solution was stored at −70 °C until analyzed.

2.4. Number of DNA copies determined by quantitative polymerase chain reaction (qPCR)

qPCR was used to compare bacterial collection efficiencies by type of swab, and type and volume of solution to measure the number of bacterial DNA copies in the sample. In the quantitative analysis, one of the genes of *E. coli* and *S. aureus* was selected as a standard gene for comparison (Table 1). To generate the standard curve, bacterial DNA was treated with TA cloning kit (Enzynomics, Daejeon, Republic of Korea) according to the manufacturer's instructions. The number of DNA copies (ng/*μL*) was calculated using the "dsDNA copy number calculator" (https://cels.uri.edu/gsc/cndna.html) from cycle threshold (Ct) values. The data of all samples are expressed as an average of log10 values. The standard curve was constructed by serial dilutions of the copy numbers of constructed genes to 10^8–10^4. The copies number of the samples was calculated using standard curve and Ct values.

Bacterial gDNA was amplified using the QuantStudio 3 Real-Time PCR system (Applied Biosystems, USA) and processed with SYBR green (TOPreal ™ qPCR 2× PreMIX, Enzynomics, Daejeon, Republic of Korea) to measure amplification products in 96-well plates. PCR was performed with 2 μL of template DNA, 10 μL of SYBR green, 2 μL of 12.5 pM forward and reverse primer, and 4 μL of distilled water in a total volume of 20 μL. The primers are listed in Table 1. PCR amplification proceeded through the following steps: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s for *E. coli;* and 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 15 s, and 72 °C for 30 s for *S. aureus*. Melting temperature analysis was also performed to confirm the primer efficiency. The blank sample was not amplified by PCR using primers for *E. coli* or *S. aureus*.

2.5. Statistical analyses

Statistical analysis was performed using Statistical Package for Social Sciences (SPSS, Chicago, IL, USA). Comparison of weight according to swab type and comparison of DNA extraction efficiency according to type and volume of the solution was performed by a one-sample *t*-test (Box et al., 1978). Comparisons of the bacterial collection efficiencies from the PBS, TG, and GS by volume were analyzed using the non-parametric Kruskal-Wallis test, which determines whether a sample occurred in the same distribution and is suitable for comparisons of two or more independent samples of the same or different size. The Mann-Whitney *U* test is used in the same context when there are only two groups. Thus, the sampling efficiencies in pair-wise comparisons between PBS, TG, and GS were compared using the Mann-Whitney U test (Mann and Whitney, 1947).

3. Results

3.1. Bacterial recovery with PBS, TG, and GS

As shown in Table 2, there was no significant difference in the mean weights of the four swab types (Supplementary Table 1, one-sample *t*-test, $p > .05$). Therefore, each swab had a similar weight and similar harvesting potential. Based on the CFU count, A swabs showed the highest recovery efficiency among the four swabs tested for both *E. coli* and *S. aureus,* with 2–10-times higher CFUs than B and D swabs; bacteria colonies were scarcely detected from C swabs. The A swab showed a significantly higher average CFU, depending on the type of solution, than the three types of swabs (Supplement Figs. 1–2, Kruskal-Wallis, $p < .05$). The TG had greater efficiency at bacterial recovery than PBS and GS overall, although TG did not always result in the greatest CFU count. In particular, high recoveries of 2869 and 2084 CFUs/plate were obtained with 50 μL and 70 μL TG on A swabs, respectively (Fig. 2).

Table 1
PCR primers used for quantitative real-time PCR.

Bacteria	Gene names	Primer sequences (5′ → 3′)		NCBI references
E. coli	*OmpA*	Forward	GC CAG CTG AGC AAC CTG GAT CC	NC_000913.3
		Reverse	GT GCG GAG ATT TTG TCT GCC GG	
S. aureus	*Fgbps*	Forward	GC GTC AAC AGC AGA TGC GAG CG	NC_007795.1
		Reverse	TA TTA AAT TGT GGA CGT GCA CC	

Table 2
Weight of swabs by type.

Swab	A	B	C	D
1	0.1026	0.1666	0.4782	0.3574
2	0.1040	0.1670	0.4786	0.3579
3	0.1000	0.1666	0.4783	0.3574
4	0.1019	0.1668	0.4786	0.3578
5	0.1027	0.1665	0.4782	0.3575
6	0.1023	0.1668	0.4785	0.3577
7	0.1006	0.1665	0.4781	0.3574
8	0.1002	0.1667	0.4784	0.3576
9	0.1043	0.1665	0.4782	0.3574
10	0.1037	0.1667	0.4783	0.3577
Mean ± S.D	0.10223 ± 0.00148	0.16667 ± 0.00016	0.47834 ± 0.00017	0.35758 ± 0.00018

3.2. DNA concentration according to the volume and type of collection solution

Similar to the CFU analysis, the greatest amount of DNA was obtained from swab A compared to the other three swabs for all three solutions. In addition, the TG resulted in a DNA concentration above the median for all swabs. Different DNA concentrations were obtained for the three solution types, and that obtained from the TG sample was approximately 2–4 times higher than that obtained from the PBS sample (Fig. 3), regardless of volume. In particular, 50 μL and 70 μL of TG resulted in the highest concentrations of DNA obtained at 0.347 and 0.324 ng/μL, respectively (Fig. 3). Swabs A, B, and D were significantly different depending on the type of solution (Supplementary Fig. 3, Kruskal-Wallis, A, B, D swabs: $p < .05$). The results of swab C showed no statistical significance (Supplementary Fig. 3, Kruskal-Wallis, C swab: $p > .05$). Therefore, at the tested DNA concentration, swab C is unsuitable as a sample swab.

3.3. DNA copy numbers according to the volume and type of collection solution

Finally, we assessed the bacterial collection efficiency with different swabs and solutions according to qPCR. Similar to the results described above, the TG recovered DNA of both bacteria with high efficiency from several of the swabs. In particular, the A swabs resulted in higher copy numbers than the other swabs; for example, A swabs with 50 μL and 70 μL TG resulted in 5.51E + 06 and 4.57E + 06 DNA copies, respectively (Fig. 4). Swabs A, B, C, and D showed significant differences depending on the type (Supplementary Fig. 5, Kruskal-Wallis, $p < .05$) and volume (Supplementary Fig. 6, Kruskal-Wallis, $p < .05$) of the solution.

4. Discussion

Given the ubiquity of microorganisms, they serve as important biological tools in a variety of research applications and fields. Despite numerous studies focused on the efficient harvesting of environmental samples, including microorganisms, there is still no standard method (Hanssen et al., 2017; Verdon et al., 2010). This may be due to differences in sample collection methods, samplers, environmental conditions, and bacterial composition. Among the various sampling methods, swab-based collection is a non-invasive and simple approach (Quaak et al., 2018). In addition, microbial populations present in cotton swab samples from skin samples have shown similar DNA yields to samples

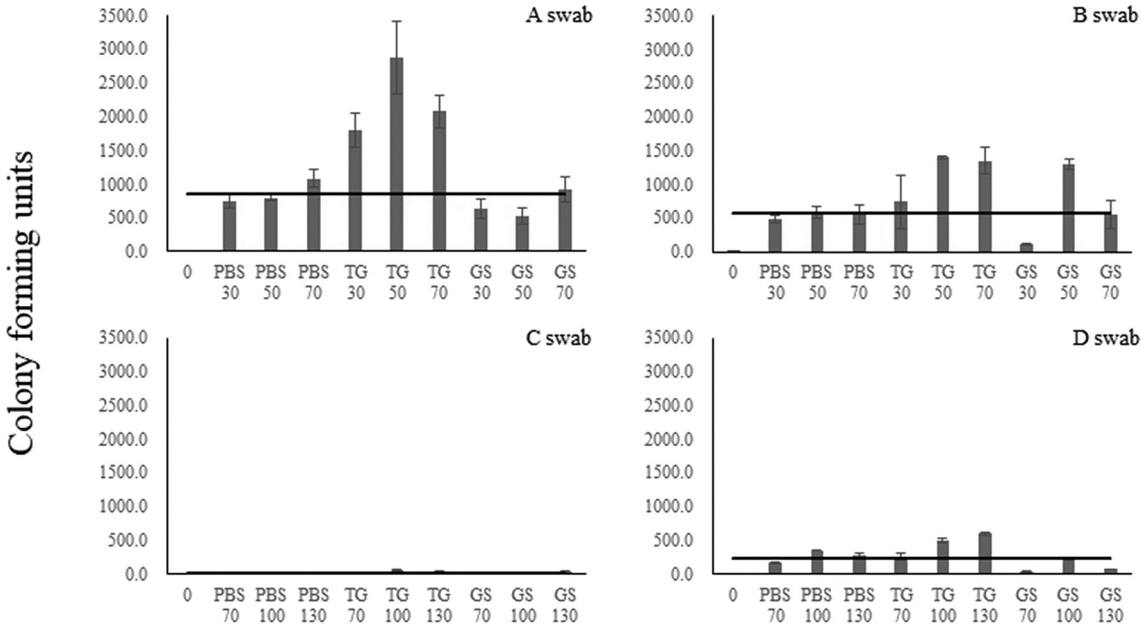

Fig. 2. Number of colonies about environmental samples collected by swabs. The cultured *E. coli* and *S. aureus* mixed LB solution was dried on the experimental table and collected with three solutions and four kinds of cotton swabs. The swab samples were diluted 10-fold in PBS, and then cultured in MSA and Mac medium for 24 h at 36.5 °C. Cotton swab tended to have the highest number of colony counts and the highest observed value was 2.87×10^3 CFUs/mL in 50 μL of TG. In addition, it was confirmed that the environmental samples were efficiently collected in the order of TG, PBS and GS. The experiment was repeated 3 times, and the mean value, a median and standard error of the number of colonies were shown in the graph.

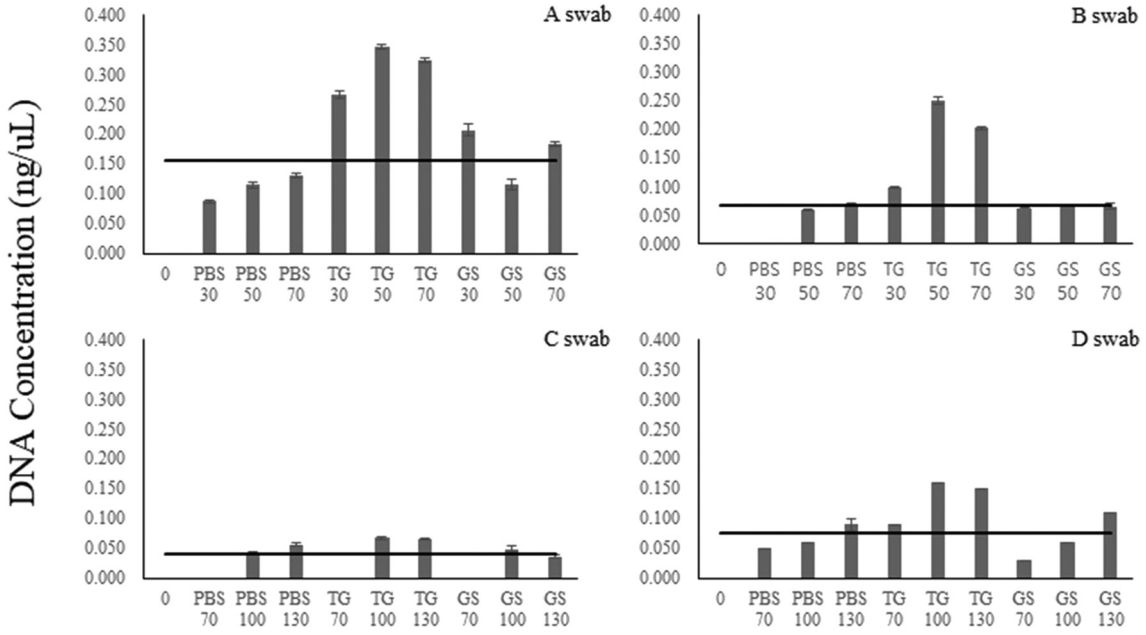

Fig. 3. gDNA concentration about environmental samples collected by swabs. The cultured *E. coli* and *S. aureus* mixed LB solution was dried on the experimental table and collected with three solutions and four kinds of cotton swabs. Cotton swab tended to be best tools for extracted to DNA from environmental samples, and cotton swab - 50 μL TG solution was measured the highest concentration (0.347 ng / μL). In addition, it has been confirmed that the TG solution is most efficient for DNA extraction. The experiment was repeated 3 times, and the mean value, a median and standard error of the number of colonies were shown in the graph.

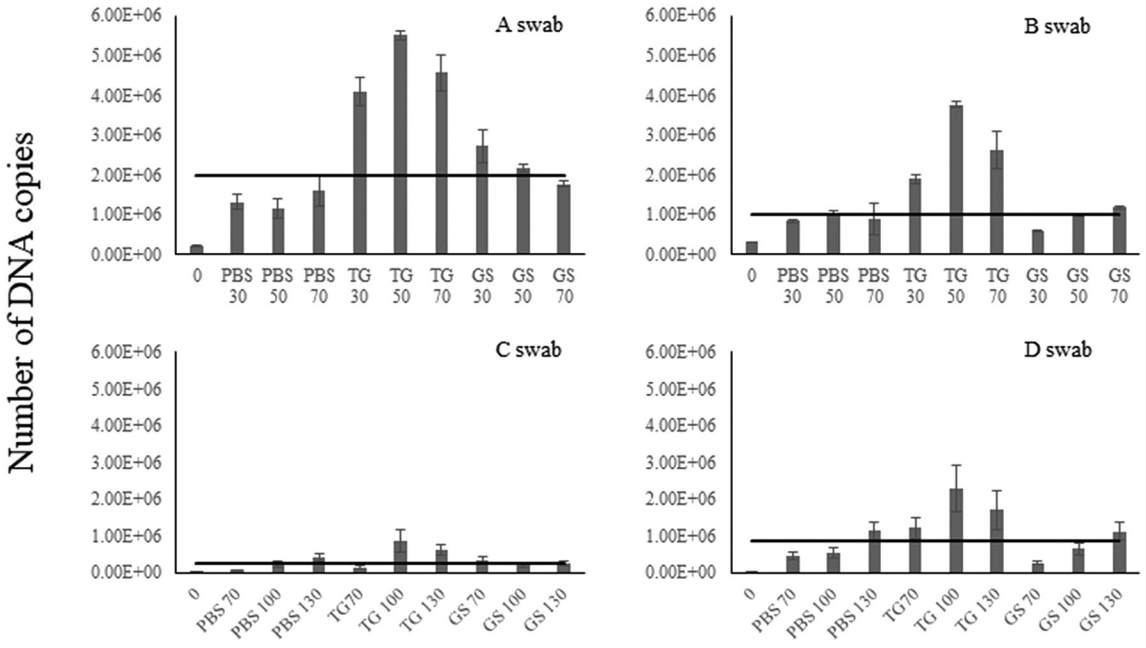

Fig. 4. Number of DNA copies by quantitative PCR about environmental samples collected by swabs. The cultured *E. coli* and *S. aureus* mixed LB solution was dried on the experimental table and collected with three solutions and four kinds of cotton swabs. The number of copies of environmental sample DNA collected by cotton swabs was higher than that of other medical swabs. The highest value was confirmed 5.51E + 06 copies of the cotton swab - 50 μL TG solution. Similar trends were also observed with DNA concentration measurements. The experiment was repeated 3 times, and the mean value, a median and standard error of the number of colonies were shown in the graph.

collected from scraping and biopsies (Grice et al., 2008). For forensic applications, individual identification is possible even with a very small amount of extracted DNA using STR analysis from samples collected from swab sampling at the contact area of the object and the finger (Liu, 2015). Thus, swabs are commonly used for the collection of many medical and forensic research samples. Our recent work in metagenomics showed that cotton swabs were effective in identifying various types of bacteria in a sample (Grice et al., 2008). However, this procedure requires more standardized sampling methods. Accordingly, we compared the efficiencies of bacterial DNA extraction and amplification with different types of swabs and solutions. Overall, we found that the TG solution, the combination of Tween 20 and glycerol, had the greatest efficiency compared to GS and PBS, and the standard cotton swab showed better efficiencies than three types of medical swabs for all three assessments (CFU count, DNA concentration, and DNA copy number); thus, the best efficiency was found with the combination of

TG and cotton swab. Swabs A (cotton), B (S7), and C (S22) showed statistically significant effects with respect to colony, DNA concentration, and DNA copy number, while C (S22) swabs showed significant or unfavorable results. Therefore, the C (S22) swab is the most unsuitable tool for sampling, and A (cotton) swabs are the most suitable.

The TG solution is amphiphilic, meaning that it is soluble in both water and non-polar solvents with a detergent basis, and organic molecules in cells such as lipids and proteins are recovered in the solution. PBS lacks these properties and thus may be less effective in harvesting complete cellular components. Moreover, the inclusion of 0.1% Tween 20 aids in cell lysis during DNA extraction (Anzai-Kanto et al., 2005; Williamson, 2012; Van Oorschot and Jones, 1997), allowing for the urea of cells to float in aqueous solution, thereby promoting cell recovery during swab collection. Tween 20 is more efficient than PBS for cell/DNA recovery and has thus long been used in forensic laboratories for DNA extraction. Therefore, we assessed whether concentrations of the solution higher than 0.1% would lead to higher DNA yields. Higher Triton X-100 concentrations did not provide significantly more DNA than lower concentrations within the optimal range of 1% to 5%, but showed a general tendency to produce more DNA. However, the higher the Triton X-100 concentration, the higher the probability of precipitation. Thus, a 1% solution is best suited for sample collection purposes. Although the use of Triton X-100 may affect DNA recovery when using certain commercial DNA extraction kits, we did not observe any of the negative effects of Triton X-100 swabbing even after prolonged storage. Therefore, Tween 20, as an alternative detergent solution to Triton X-100, showed better efficiency at a concentration of 1% compared with 0.1%, and also improved sample storage and DNA separation by mixing with glycerol.

To minimize the number of variables, each sample was cultured under the same conditions, smeared on the same desk, dried, and sampled with swab A (cotton). Based on the colony recovery rate of bacteria, DNA concentration, and copy number, the efficiency of the detergent-based solution was better than that of PBS, in line with previous studies (Daniel et al., 1995). Interestingly, the collection efficiency was also found to vary according to the type of swab. Swab A (cotton) showed the best recovery rate overall. Thus, our results confirmed that not only the selection of the solution but also the combination of the solution and the swab is an important consideration for obtaining high recovery in environmental samples for metagenomic forensic applications.

Overall, we found that sampling of 50 μL of TG using a conventional cotton swab is the most suitable condition for standardization of environmental sample collection. However, there are still some questions about standardization. Environmental samples are not only diverse in their nature and components but are also collected by a variety of people, and thus do not represent a single collection. Therefore, further research is needed to establish a more efficient sampling method for collecting various materials by various individuals. Nonetheless, this study identified the most efficient way to recover high CFUs and DNA, and highlights the importance of considering the combination of solutions, swabs, and the solution volume for each collection.

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Declarations of conflicting interests

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2019.04.011.

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