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ORIGINAL ARTICLE

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DNA extraction from the most difficult microorganisms including *Mycobacterium, Staphylococcus,* and *Malassezia* in five minutes using sand

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ABSTRACT • *Staphylococcus, Mycobacterium,* and yeasts have complex cell walls that impede cell lysis and the recovery of DNA using conventional extraction methods. Previously, we showed that sand particles effectively disrupt the cell walls of *Staphylococcus* and *Mycobacterium* for conducting DNA and RNA extraction. In this study, we aimed to test whether sand treatment of *Staphylococcus, Mycobacterium,* and *Malassezia* enables the extraction of usable DNA for polymerase chain reaction directly, without proteinase K, phenol-chloroform, and ethanol precipitation treatments. In our protocol, one or two colonies of each microorganism were mixed with sand particles in 100 µl ddH_2O and vortexed for 3 minutes and the supernatant was used in the polymerase chain reaction protocol. The results showed that sand treatment of *Staphylococcus, Mycobacterium* and *Malassezia* allowed sufficient DNA to be extracted while the obtained DNA was pure enough to conduct polymerase chain reaction and restriction enzyme digestion. We conclude that using sand for DNA extraction has important cost advantages while enabling DNA extraction to be completed in only five minutes. The method's simplicity also reduces the risk of contamination in studies involving many examples.

INTRODUCTION

Obtaining sufficient quantities of pure DNA is crucial for polymerase chain reaction (PCR). Different DNA extraction protocols, such as phenol-chloroform, proteinase K, glass beads, thermal shock and boiling, have been used successfully for DNA isolation from gram-negative bacteria (1,2). However, *Staphylococcus, Mycobacterium,* and yeasts have complex cell walls that impede cell lysis and the recovery of DNA using conventional extraction methods so the complex cell wall structure must be broken down to isolate DNA or RNA from these microorganisms (3-6). For the *Staphylococcus* genus, such as gram-positive bacteria, this can be achieved by forming spheroplasts using lysostaphin, and for the *Mycobacterium* genus by using chemicals like cetyltrimethyl ammonium bromide (CTAB) (4,7).

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In a previous study, we used sand particles to mechanically remove the cell wall of *Staphylococcus* and *Mycobacterium* for DNA or RNA extraction (8). In that study, after being vortexed for 3 minutes, the bacteria-sand mixture was treated using the proteinase K and phenol-chloroform, and ethanol precipitation protocols to obtain DNA while the guanidinium thiocyanate-phenol-chloroform protocol was followed for RNA extraction. Our sand method enabled sufficient amounts of pure DNA and RNA, which are usually difficult to obtain, to be extracted from *Staphylococcus, Mycobacterium.* In addition, when we compared we found that far more DNA was obtained using sand than glass beads and the same amount of DNA was obtained as the lysostaphin-treated microorganism.

In this study, we investigated whether the protease K, phenol-chloroform, and ethanol precipitation protocols are needed to obtain sufficient amounts of pure DNA for PCR using sand treatment alone. To test this, *Staphylococcus, Mycobacterium* and *Malassezia* were vortexed with sand particles for 3 minutes before the supernatant was checked for DNA quantity and usability for PCR. The quality of the DNA template was also tested using the 2kbp PCR protocol.

MATERIALS and METHODS

Sand Preparation

Sand was obtained from a natural stream (chosen because the edge structure of stream sand particles is sharper than that of sea sand) and sieved for 0.5-3 mm size (Figure 1). The resulting fine-grained sand was thoroughly cleaned with ddH_2O to eliminate all dirt and dust without losing small particles autoclaving to sterilize it (9).

Microorganisms and Growth Conditions

Previously described methicillin-resistant *Staphylococcus aureus* (MRSA) containing the exfoliative toxin A gene-encoding phage (9), *Escherichia coli* DH5α *(E. coli)* and, *Mycobacterium tuberculosis* H37Rv ATCC 25618 (10), *Malesseiza furfur* (CBS 7,019) (11), strains were used in the study. LB (Luria-Bertani) Agar (Difco, USA) was used for to culture of the *Staphylococcus* and *E. coli.* Lowenstein-Jensen (LJ) (Becton, Dickinson, USA) agar media was used for the growth of the *M. tuberculosis* (10). The p63 (TP63), transcript variant 1 (ACCESSION NM_003722) cloned pcDNA-3 vector (previously constructed in our laboratory) was transformed into the *E. coli* DH5α and grown on the LB agar containing ampicillin. *Malassezia* was inoculated in modified Leeming–Notman agar (MLNA) (1% w/v peptone, 1% w/v glucose, 0.2% w/v yeast extract, 0.8% desiccated ox bile, 0.1% v/v glycerol, 0.05% w/v glycerol monostearate, 0.5% v/v Tween 60, 2% v/v oleic acid, and 1% w/v agar in distilled water) supplemented with cycloheximide (0.5%) and chloramphenicol (0.05 %). The culture was incubated at 32°C for 2 weeks (11).

Figure 1 Prepared sand as described in the Materials and Methods section was shown.

DNA Extraction Using Sand

One or two colonies of each microorganism were mixed in 100 µl ddH_2O with 100 mg sand. The bacteria-sand mixture was then vortexed at maximum speed for 3 minutes before another 100 µl ddH_2O was added to the tube, mixed and centrifuged for

20 seconds at maximum speed. The supernatant was collected for PCR. Ten µl of DNA from each supernatant sample was run and analyzed by ethidium bromide (EtBr) (AppliChem, Germany) treated agarose gel electrophoresis.

Primers and PCR Protocol

The primers used in the study are presented in the table.

PCR

Amplification was performed on a total of 50 µl containing 2 µl of the DNA template, 20 pmol of each primer, 2.5 mmol/L of the four deoxynucleotides and 2.5 U of Taq polymerase (Thermo Scintific, Lithuania). The reaction mixtures were subjected to 38 cycles of amplification in an S1000 Thermal cycler (BioRad, CA USA). The PCR machine was programmed as follows: 94°C for 2 minutes to denature the template; 94°C for 45 seconds for denaturation; 60°C for 45 seconds for annealing; and 68°C for 45 or 90 seconds for extension (depending on PCR product length). After amplification, the PCR products were analyzed by EtBr treated agarose gel electrophoresis.

Restriction Enzyme Digestion

The DNA obtained from the microorganisms using the sand protocol were cut with the rare cutter enzyme *HindIII* (NEB, UK) and the frequent cutter enzyme *HinfI* (NEB, UK), according to the supplier's recommended protocol before analysis by EtBr treated agarose gel electrophoresis.

RESULTS

Two colonies from the MRSA and *M. tuberculosis H37Rv ATCC 25618, E. coli* DH5α and one colony from *Malassezia* were selected from the medium. After vortexing the individual sample with sand in 100 µl sterile ddH_2O, another 100 µl H_2O was added and briefly centrifuged. The supernatant was collected, and 10-15 µl of the supernatant was run on agarose gel. Figure 2 shows the individual DNAs run in the agarose gel.

To investigate the usefulness of the obtained DNA, PCR was performed using individual supernatants obtained from the sand extraction protocol with specific primers for genes identified in the genome of the chosen MRSA and *M. tuberculosis, Malassezia furfur* from our previous studies (see Table 1). The figures 3, 4, and 5 show the PCR products from specific microorganisms.

To test DNA integrity, the *E. coli* colonies transformed with pcDNA3-p63cDNA were treated with sand to obtain the supernatant. The PCR was performed using the two primer set available in our laboratory. One primer set covered the whole p63

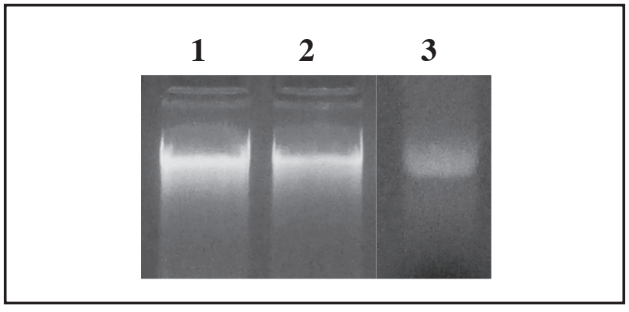

Figure 2 DNAs obtained from MRSA (lane 1);, *Mycobacterium* (lane 2);, *Malassezia* (lane 3) using the sand method.

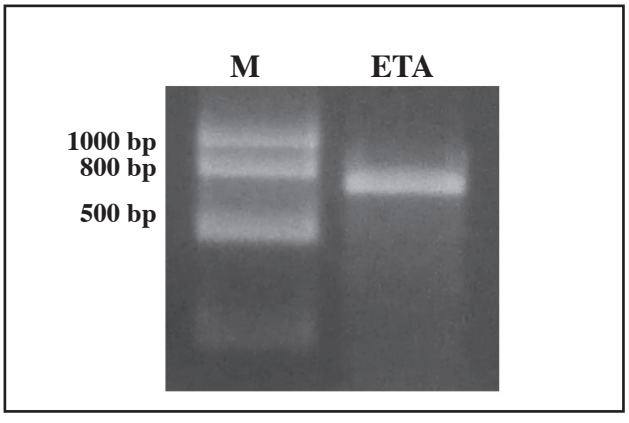

Figure 3 PCR product of the DNA obtained from ETA phage containing MRSA using ETA specific primers. M, marker containing 1000, 800, 500 and 200 bp bands.

Table 1 Primer sequences, length, and specificity

Name of primer	Sequence	Used for	Length (bp)	Used in
drrA-F	ATGGTGGACATCTTGTCGAC	*Mycobacterium* drrA efflux pump	158	(10)
drrA-R	AGGTTCTGCTCACCGGAAAG			
Eta-F	CTATTTACTGTAGGAGCTAG	MRSA containing ETA phage genome	742	(9)
Eta-R	ATTTATTTGATGCTCTCTAT			
M.fur-F	CAAATGACGTATCATGCCATGC	*Malassezia furfur* 5.8S rRNA	252	(11)
M.fur-R	TCCCTTTCAGAGCGGTTTGC			
P63var1-F	TTTGAAACTTCACGGTGTG	P63 var1 cDNA cloning	2042	this experiement
P63var1-R	TCACTCCCCCTCCTCTTTGAT			
pcDNA-F	CTAGAGAACCCACTGCTTACT	pcDNA sequence primers	2321	this experiement
pcDNA-R	TTCCAGGGTCAAGGAAGGCA			

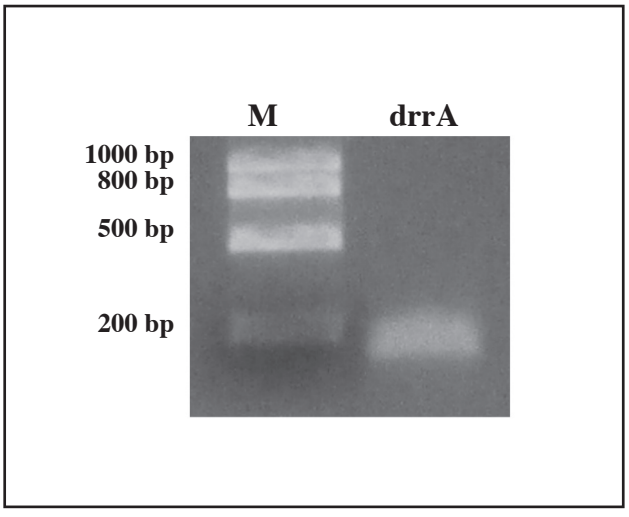

Figure 4 PCR product of the DNA obtained from *Mycobacterium* using drrA specific primers. M, marker containing 1000, 800, 500 and 200 bp bands.

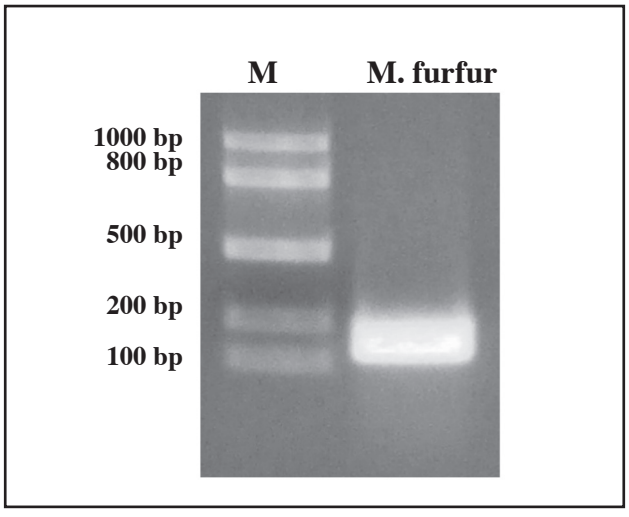

Figure 5 PCR product of the DNA obtained from *M. furfur* using *M. furfur* specific primers. M, marker containing 1000, 800, 500, 200, and 100 bp bands.

cDNA, which is 2042 bp, while the second set was chosen from the forward and reverse side of the cloning site of the pcDNA-3-p63 vector plasmid, which produced 2321 bp PCR product. Figure 6 shows the results for the PCR products obtained from the first primer set covering the whole p63 cDNA and the results for the PCR products obtained from the second primer set which covers the p63 and parts of the pcDNA vector in addition to the whole p63 cDNA.

The quality of the extracted DNA was also tested by restriction enzyme digestion. Figure 7 shows that the DNA obtained from the sand protocol was successfully digested by both the rare cutter enzyme *HindIII* and the frequent cutter enzyme *HinfI*.

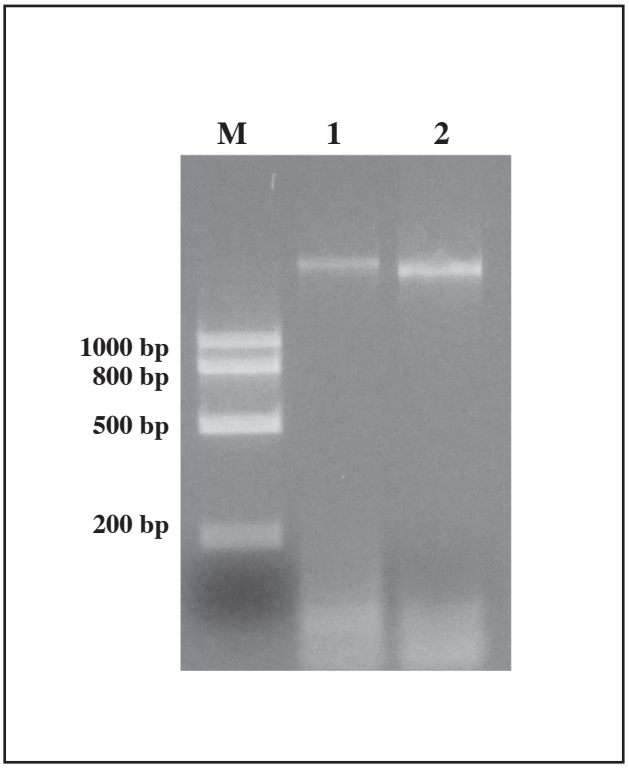

Figure 6 PCR product of the DNA obtained from E.coli DH5α transformed with pcDNA-p63 vector plasmid using primers chosen on pcDNA vector (lane 1), primers chosen on p63 cDNA (lane 2). M, marker containing 1000, 800, 500 and 200 bp bands.

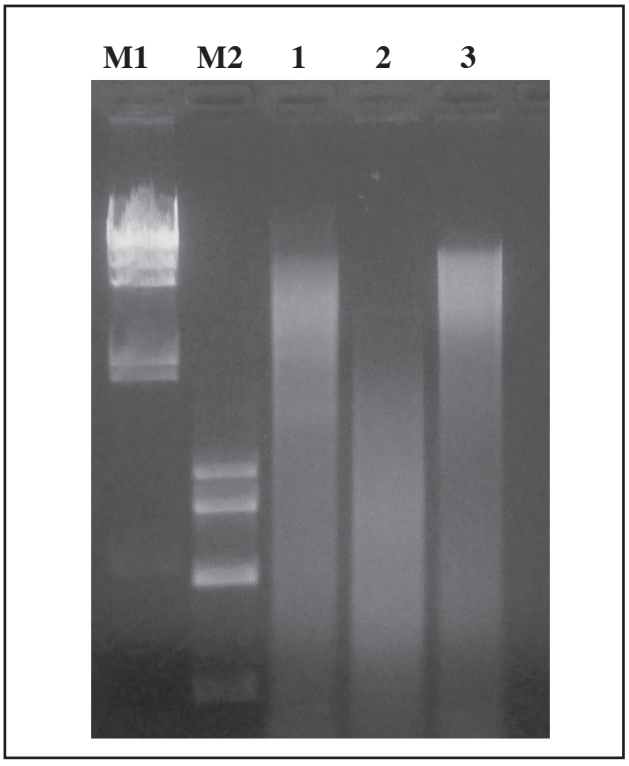

Figure 7 DNA obtained from MRSA using the sand protocol digested with HindIII (lane1), HinfI (lane 2), and not digested (lane 3). M1, Lamda HindIII marker, M2, marker containing 1000, 800, 500 and 200 bp bands.

DISCUSSION

Because of their thick peptidoglycan layer, Gram-positive bacteria are resistant to the lysis required for DNA extraction while isolation of DNA from *Mycobacterium*, which also incorporate many complex lipids in their cell walls, is also difficult. Without disruption of the cell, standard extraction methods are ineffective so for these bacteria it is necessary to eliminate the cell wall for the cell to form a spheroplast. To transform *Staphylococcus* into its spheroplast form, lysostaphin is used while for *Mycobacterium*, CTAB is used to remove the cell wall. Similar to *Staphylococcus* and *Mycobacterium* simple lysis procedures, such as the use of sequential freeze-thaw cycles or incubation with hot detergent and proteases, have not produced high yields of DNA from many fungal species (12).

In this report, we described an extremely easy method for DNA extraction from the most difficult microorganisms. In our previous study, we showed that a sand protocol effectively disrupts cell walls of *Staphylococcus* and *Mycobacterium* without the need for any chemicals. However, in the previous study, after the sand treatment, we treated the bacteria with proteinase K and phenol-chloroform, and ethanol precipitation protocols, which require considerable time to complete DNA extraction. Therefore, the present study investigated, firstly, whether cell wall disruption alone is enough to release DNA into the supernatant without proteinase K

treatment, and secondly whether there is a PCR inhibitor effect of the sand treatment. Thirdly, we tested whether DNA integrity is affected by the sand treatment. Our results showed that using sand for DNA extraction enabled sufficient amounts of DNA to be extracted. The obtained DNA was also sufficiently pure to successfully conduct both PCR and restriction enzyme digestion. Finally, PCR products of over 2000bp length were obtained using Taq polymerase enzyme.

The sand method uses the same physical mechanism as glass beads to disrupt bacteria cell walls mechanically. Because sand is a naturally occurring granular material composed of finely graded particles, we predicted that sand would be more effective than glass since the former's surface is sharper and stronger than that of glass beads. Indeed in the previous experiment, we found that far more DNA was obtained using sand than glass beads (9).

Some studies have described using silica and zirconium particles in addition to glass beads to disrupt bacteria cell walls as both particles, silica particularly, bind the DNA with high affinity (13). Although we didn't investigate this in the present study, we would predict that using these materials together would yield less DNA than using the sand treatment. In addition, silica and zirconium need to be pre-treated with other chemicals before applying to DNA extraction.

The sand method described in this study enabled sufficient DNA to be extracted from bacteria with rigid cell walls that normally make this process difficult. The obtained DNA is also sufficiently pure to successfully conduct PCR and restriction enzyme digestion. We, therefore conclude that using the sand method has important advantages, particularly decreasing costs and reducing the time need to extract DNA. Pre-prepared sand can be either used or stored almost indefinitely in the laboratory while our new method's simplicity reduces the risk of contamination in epidemiological studies involving multiple samples.

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Conflict of interest: None.

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