An Improved Method for High Quality Metagenomics DNA Extraction from Human and Environmental Samples

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To explore the natural microbial community of any ecosystems by high-resolution molecular approaches including next generation sequencing, it is extremely important to develop a sensitive and reproducible DNA extraction method that facilitate isolation of microbial DNA of sufficient purity and quantity from culturable and uncultured microbial species living in that environment. Proper lysis of heterogeneous community microbial cells without damaging their genomes is a major challenge. In this study, we have developed an improved method for extraction of community DNA from different environmental and human origin samples. We introduced a combination of physical, chemical and mechanical lysis methods for proper lysis of microbial inhabitants. The community microbial DNA was precipitated by using salt and organic solvent. Both the quality and quantity of isolated DNA was compared with the existing methodologies and the supremacy of our method was confirmed. Maximum recovery of genomic DNA in the absence of substantial amount of impurities made the method convenient for nucleic acid extraction. The nucleic acids obtained using this method are suitable for different downstream applications. This improved method has been named as the THSTI method to depict the Institute where the method was developed.

Efficient extraction of high-quality, high molecular weight (HMW) community genomic DNA from limited amount of human origin or environmental samples carrying diverse microbial species is the key challenge for cutting edge downstream applications like next generation DNA sequencing (NGS). The NGS technology is often used to explore the identity and abundance of culturable and uncultured microbial species in its natural community and to decode the microbial genomes to investigate its functional repertoires. For such different applications including shotgun metagenomics it is very important to extract HMW community genomic DNA. Different microbes present in diverse ecosystems have different types of cell wall and cell membranes, which enclose their cytoplasm and genomic contents (Fig. 1). Harsh sample treatment could affect DNA quality, while mild process may cause partial lysis particularly for the classes of bacteria carrying thick layers of peptidoglycan. Therefore, it is important to optimize the cell lysis methods to obtain genomic DNA from abundant as well as...
rare representatives of each taxonomic groups possessing different thickness of cell wall and different layer of cell membranes with different embedded components casing their genomic contents.

Lyses of microbial cells expose their genomic DNA to different cellular and extracellular molecules including different type of nucleases. Despite its inert nature, double stranded DNA is physically fragile and highly susceptible to exo- and endonucleases, active forms of which are widely present in the matrix of most of the environmental and human samples analyzed in this study. Therefore, it is important to inactivate all the nucleases in lysis solution by incorporating strong denaturing agents or chemicals that chelate residual metallic ions from the suspension. Although, several commercial kits are now available to extract DNA from human and environmental samples, most of which uses silica-based column where DNA adsorb selectively to a stationary solid phase at high pH and high salt concentration. The major disadvantage for most of the commercial kits is insufficient recovery of genomic DNA from marginal amount of clinical or environmental samples. Furthermore, different DNA extraction kits have different biases, which can produce dramatically different results for the same sample. Several laboratories working on metagenomics reported different methods of community DNA extraction depending on the type of samples they used for analysis. Recently, International Human Microbiome Standards (IHMS) launched a guideline for standard operating procedures to optimize community DNA extraction methods from human fecal samples (http://www.microbiome-standards.org). So far, no attempt has been taken to develop a gold standard for community DNA extraction from both human and environmental origin samples.

In this study, we developed a highly sensitive method, by combining physical, mechanical and chemical lysis approaches, to isolate community bacterial DNA from different human and environmental samples (Fig. 2). All the selected samples harbor culturable and uncultured bacteria belonging to closely or distantly related taxonomic groups and having different thickness of cell wall and different layer of cell membranes (Fig. 1). We compared both the quality and quantity of isolated DNA with existing methodologies and observed that this approach worked best compared to currently available approaches. The isolated DNAs are suitable for all types of high-resolution downstream applications including shotgun metagenomics sequencing where HMW genomic DNA is preferable.

Results and Discussion

Spheroplast formation and DNA isolation. Both, environmental and human samples contain large numbers of microbial cells belonging to different phyla and they are reasonably heterogeneous in terms of their genomic contents, morphology and architecture of their cell wall (Fig. 1). To obtain sufficient amount of quality community DNA from Gram-positive and Gram-negative bacterial cells, it is important to preprocess the samples before adding lysis reagents. In this study, we used three different enzymes lysozyme, lysostaphin and mutanolysin that target either 1,4-beta glycoside-linkages or transpeptide bond in Gram-positive and Gram-negative bacterial cell wall and help in spheroplast formation. Spheroplast is highly susceptible to lysis reagents and labile to mechanical and physical forces.

For lysis, first we treated the spheroplast with Guanidinium thiocyanate (GITC) to disrupt the bacterial cell membrane and inactivate nucleases and other enzymes. Combining mechanical (bead beating) and thermal (heat) forces enabled final lysis. The recovery and quality of the isolated DNAs were confirmed by running the samples on agarose gel (Fig. 3). We used both environmental and human samples (Fig. 2), containing diverse
range of bacterial species including Gram-positive and Gram-negative bacteria possessing different types of cell wall, to confirm the suitability of the same method in wide range of samples. We successfully isolated reasonably good amount of quality DNA from all the tested samples (Fig. 3 and Table 1). DNA yield was typically ~1–109 μg, depending on the initial sample size and the way the sample was stored (Table 1). Total yield of DNA irrespective of the sample types was always higher in THSTI method compare to Kit and ALHS methods (Table 1). Average size of the DNA fragments recovered by THSTI method was ~20 kb (Fig. 3).
Several methods have been described for community microbial DNA extraction from several methods have been described for community microbial DNA extraction from various sources. Most of these methods, however, either require complex extraction procedures or are limited in their ability to recover sufficient amounts of DNA from low-abundance samples. In this study, we developed a new method for the isolation of high-quality DNA from various sources, including human and environmental samples.

### Table 1: Average concentration and total recovery of nucleic acids isolated from different environmental and human origin samples

| Sample          | Method   | Nucleic acid concn. (ng/μl) | Total recovery (ng) | 260/280     |
|-----------------|----------|-----------------------------|---------------------|-------------|
| Stool           | THSTI    | 543.3 ± 187.26 (DNA)        | 108660 ± 37520 (DNA)| 1.85 ± 0.06|
| Stool           | Kit      | 202.29 ± 105.63 (DNA)       | 20229.23 ± 10563 (DNA)| 1.94 ± 0.23|
| Stool           | ALHS     | 113.38 ± 62.26 (DNA + RNA)  | 11338.46 ± 6226 (DNA + RNA)| 1.67 ± 0.07|
| Vaginal Swab    | THSTI    | 104.77 ± 39.61 (DNA)        | 20955.38 ± 7923.13 (DNA)| 1.69 ± 0.12|
| Vaginal Swab    | Kit      | 8.37 ± 5.66 (DNA)           | 836.15 ± 566.7 (DNA)| 1.43 ± 0.58|
| Vaginal Swab    | ALHS     | 22.79 ± 9.5 (DNA + RNA)     | 2279.23 ± 906.02 (DNA + RNA)| 2.47 ± 1.01|
| Soil            | THSTI    | 53.16 ± 36.77 (DNA)         | 10633.84 ± 10317.18 (DNA)| 1.48 ± 0.041|
| Soil            | Kit      | 66.02 ± 70.13 (DNA)         | 6602.30 ± 7014 (DNA)| 1.16 ± 0.05|
| Soil            | ALHS     | 93.91 ± 103.17 (DNA + RNA)  | 9391.53 ± 7355.84 (DNA + RNA)| 1.44 ± 0.07|
| Sewage water    | THSTI    | 79.24 ± 80.71 (DNA)         | 15849.23 ± 12190 (DNA)| 1.71 ± 0.041|
| Sewage water    | Kit      | 14.47 ± 5.72 (DNA)          | 1447.69 ± 572 (DNA)| 1.68 ± 0.05|
| Sewage water    | ALHS     | 98.74 ± 60.95 (DNA + RNA)   | 9874.61 ± 8071 (DNA + RNA)| 2.14 ± 0.07|
| Gastric Tissue Biopsy | THSTI  | 53.9 (DNA)                  | 10780 (DNA)| 1.85 |
| Gastric Tissue Biopsy | Kit    | 126.5 (DNA+RNA)             | 12650 (DNA+RNA)| 1.4 |

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### Table 2: Minimum number of bacterial cells needed to isolate detectable amount of nucleic acids by using genomic DNA isolation kit or THSTI methods.

| CFU     | 10^6 | 10^7 | 10^8 | 10^9 | 10^10 | 10^11 |
|---------|------|------|------|------|-------|-------|
| Kit     | 6.27 | 0.28 | 0.03 | ND   | ND    | ND    |
| THSTI   | 10.3 | 1.25 | 0.1  | 0.03 | ND    | ND    |

### Assessment of the quality of isolated DNA.

Both the quality and quantity of isolated DNA were assessed by measuring the absorbance at 260 and 280 nm wavelengths (Table 1) and by visualizing extracted community DNA on agarose gel (Fig. 3). Most of the isolated DNA samples had OD260/OD280 ratio in between ~1.6 and ~1.9 except the genomic DNA isolated from soil sample (Table 1). We further confirmed the quality of isolated DNA by visualizing all the samples on 0.8% agarose gel containing DNA-intercalating agent ethidium bromide. Although, the gel electrophoresis is not very sensitive to measure the quantity of DNA but this is useful to analyze the stable RNA contamination, short fragment DNA contamination, and also shown the average size of isolated DNA. It is important to note that in THSTI and kit methods nucleic acids were treated with RNase to remove stable RNA while in automated liquid handling system the RNAse treatment step was absent. Thus, in terms of quality of DNA, the present method is free of from other nucleic acid impurities.

### Comparison of current method with available DNA isolation kits and automated nucleic acid extraction system.

Several methods have been described for community microbial DNA extraction from human and environmental origin samples. We compared the quality and quantity of DNA obtained from equal amount of same samples for all, except gastric tissue biopsy, using DNA isolation kit (Qiagen, Germany), and automated nucleic acid extraction system (MagNA pure, Roche Diagnostics, Swizerland). We observed that, when the tested samples, like stool specimen, contained large numbers of bacterial species, both automated nucleic acid extraction system and kit method could recover adequate amount of quality DNA for downstream applications. However, both the methods are not efficient to recover sufficient amount of DNA from low amount of microbial cells including vaginal swabs, where bacterial number was limited (Fig. 3 and Table 1). In contrast, the method developed in this study efficiently recover sufficient amount of genomic DNA even in samples with limited amount of bacterial cells (Fig. 3 and Table 2).

### Suitability of isolated DNA in different downstream applications.

To assure the quality of isolated nucleic acid, the samples were used for different downstream applications including PCR amplification (Fig. 4), restriction digestion (Fig. 5), cloning and sequencing of PCR products (Fig. 6). The PCR amplification of complete and partial 16S rRNA gene of bacterial DNA was done by using set of primer tagging with or without NGS.
specific adaptor and barcode sequences. The adaptor was selected based on the recommendation of 454 GS FLX + pyrosequencing platform (Table 3). We used different NGS primers specific for C1, C3 and C5 and C9 regions of
16S rRNA gene (Fig. 4 and Table 3). Sufficient amount of desired amplicon from each set of amplification reaction confirmed the suitability of isolated DNA for NGS application (Fig. 4). The complete 16S rRNA genes were amplified from the sewage water, soil, stool, GB and vaginal swabs genomic DNA and subset of them were used for cloning and sequencing reactions. Among thousands of clones obtained during cloning of 16S rRNA gene, few of them were randomly picked up for plasmid isolation. Eight representative recombinant clones of 16S rRNA gene amplified from sewage water DNA are shown (Fig. 6). Insert of subset of plasmids were sequenced in a capillary sequencer using universal M13F and/or M13R primers. Identity of 16S rRNA genes amplified from DNA sample of sewage water, soil, stool, GB and vaginal swabs were examined by using NCBI BLASTN program (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch) database. Although the sample size was small (n = 36), still we have identified multiple Gram-positive and Gram-negative bacterial species in different samples belonging to different bacterial classes (Table 4). Restriction digestions of subset of DNA samples were done using type II restriction endonuclease EcoRI (Fig. 5). Complete digestion of genomic DNA indicates absence of inhibitory compounds, possibly, in the isolated DNA samples.

### Conclusion

The method reported in this study is very efficient and economic to isolate community bacterial DNA from minimal amount of human and environmental samples. The quality and quantity of extracted DNA are suitable for various downstream applications including restriction enzyme digestion, PCR amplification using sequencing adaptor and barcode tagged primers used for NGS reactions. Compared to testified two methods, kit and automated nucleic acid extraction system, the recovery of community DNA in THSTI method is substantially higher. A limitation of the present method is the duration for extraction of DNA from the sample. This can be afforded, considering the quality, quantity and suitability of the isolated DNA for subsequent downstream applications.

### Methods and Materials

#### Samples

Sewage water and soil, two environmental samples used for this study, were collected from the National Capital Region, India. Stool samples were obtained from healthy adult volunteers. Gastric biopsy samples were obtained from Hinduja Hospital and Medical Research Centre, Mumbai, India. Vaginal swab samples were obtained from Department of Obstetrics and Gynecology, Maulana Azad Medical College, New Delhi, India and Pediatric Biology Center, Translational Health Science and Technology Institute, NCR Biotech Science Cluster, Faridabad, India. The human origin samples were collected after receiving approval from THSTI ethics committee and informed consent from the study subjects. Recombinant DNA works were carried out in “accordance” with the approved guidelines of THSTI biosafety committee. All other experimental protocols used in this study were carried out in “accordance” with the relevant guidelines and standard operating procedure (SOP) of Centre for human microbial ecology (CHME).

#### Enzymes

Lysozyme (10 mg/ml), mutanolysin (25 KU/ml) and lysostaphin (4 KU/ml) were used for removal of cell wall from Gram-positive and Gram-negative bacterial cells. All three enzymes were purchased from Sigma-Aldrich, USA. Both mRNA and stable RNA species were removed from the pool of nucleic acids by treating the samples with RNase (10 mg/ml).

#### Buffers

Tris-HCl (1M, pH 8.0 and pH 7.5) and Phosphate buffer (0.1 M, pH 8.0) were used to re-suspend the nucleic acids.

#### Other reagents

Following reagents were used at different stages of sample processing and DNA isolation: EDTA (0.5 M, pH 8.0), NaCl (5 M), PVPP (Mol wt 40,000), Guanidine thiocyanate (4 M), Sodium-acetate (3 M, pH 5.2), Potassium acetate (5 M, pH 5.2), N-Laurylsarcosine (10%), Glass beads (2.5 mm), Zirconia beads (0.1 mm), Ethanol (96%), Hydrochloric acid (HCl), Sterile deionized water (H2O). All the chemicals used in this study were purchased from Sigma-Aldrich, USA.

#### Glass Beads processing

The glass beads are very useful to detach microbes from the matrix of collected samples. 2.5 mm glass beads are suitable for bacterial cells. First, the glass beads (Biospec USA) were kept in 1.0% Triton-X solution for 30 minutes at room temperature and then washed 6-7 times with water. The washed beads were kept in an incubator over night at 55°C. Beads were autoclaved before use.

### Table 3. Primers used in this study to amplify partial or complete 16S rRNA gene.

| Name | Sequence (5'-3') |
|------|-----------------|
| 130  | GGCGGATCCAGAGGAGTGTTCCAGCCGC |
| 139  | GGCCCTCGAGAGTTGTAGCCTGGCTACG |
| 27F  | CCACTACCCCTGGTGCTTGGCAGCTCAGAGAGTTTGATCCTGGCTCAG |
| 534R | CCATCCTACCCCTGGTGCTCAGACCTCGAGCAGCAGTCAGATTACGCGGAGGGCAGG |
| 926R | CCATCTCATCCCCTGGTGCTCAGACCTCGAGCAGCCCAGTCAATTCMTTTRAGT |

Letter code: Bold font, Restriction enzyme binding sequence; Regular font, 16S rRNA gene specific sequence; Regular underline font, adaptor sequence for 454 GS FLX+ pyrosequencer; Italic font, Key sequence for 454 GS FLX+ pyrosequencer; Bold underline font, barcode (MID) sequence.
Gilbert, Michael S., et al. (2016). “Preparation of 0.1 mm Zirconia beads.” Scientific Reports 6:26775. DOI: 10.1038/srep26775.

**Preparation of 0.1 mm Zirconia beads.** First, the 0.1 mm Zirconia beads (Biospec USA) were washed with 1% Triton-X solution. All the detergent was removed by vigorous shaking and washing the beads 7–8 times in milliQ water until it does not foam anymore. The beads were resuspended in milliQ water and sterilized by autoclaving at 121°C for 15 min.

**Pre-processing and cell lysis.** First, fresh or freeze stored environmental (1 gm soil, 35 ml SW) or human samples (200 mg stool, 1 HVS, 1–5 mg GTB) carrying sufficient numbers of bacterial cells were transferred into a pre-chilled 2 ml microcentrifuge tube (MCT) and resuspended in 200 μl 50 mM Tris-1 mM EDTA buffer (pH 8.0). Since, all the samples contain both microbial cells and extracellular matrix like, mucin or undigested food particles, it is important to detach the microbes for adequate access of buffering agents and enzymes, used for spheroplast formation. With this aim, we added 4 glass beads (2.5 mm) and vortexed continuously for 1 min or until the sample was thoroughly homogenized. Then the glass beads were removed from the suspension by transferring supernatant into a fresh tube and enzyme cocktail containing 50 μl lysozyme (10 mg/ml); 6 μl mutanolysin (25 KU/ml), and 3 μl lysostaphin (4 KU/ml) was added. The cell suspension was incubated for 1 hour at 37°C to remove cell wall from bacterial cells.

Lysis of microbial cells was done by combining chemical, physical and mechanical approaches. First, 250 μl Guanidine thiocyanate (4 M) was added and mixed gently for 45 seconds. Then, 300 μl 10% N-Lauryl sarcosine was added and incubated for 10 minutes at 37°C in a vortex mixer (Thermomixer, Eppendorf, Germany) with mild shaking (300 rpm). After short incubation, the tubes were transferred into a pre-warmed water bath and incubated at 70°C for 1 hour. Mechanical lysis was done in a bead beater using 0.1 mm zirconia beads (BioSpec,

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### Table 4. Dominant bacterial species identified in the sewage water (SW) samples, soil samples (SS), stool samples (GM), vaginal swabs (HVS) and gastric tissue biopsy samples (GTB).

| Clone | Bacterial species | Systemic position | GenBank accession no. |
|-------|------------------|-------------------|------------------------|
| GM01-Pc | *Prevotella copri* | Class-Bacteroidetes, Phylum-Bacteroidetes | KX057366 |
| GM02-Pc | *Prevotella copri* | Class-Bacteroidetes, Phylum-Bacteroidetes | KX057367 |
| GM03-Pc | *Prevotella copri* | Class-Bacteroidetes, Phylum-Bacteroidetes | KX057368 |
| GM04-Pb | *Prevotellaaceae bacterium* | Class-Bacteroidetes, Phylum-Bacteroidetes | KX057369 |
| GM05-Pc | *Prevotella copri* | Class-Bacteroidetes, Phylum-Bacteroidetes | KX057370 |
| GM06-Pc | *Prevotella copri* | Class-Bacteroidetes, Phylum-Bacteroidetes | KX057371 |
| GM07-Pc | *Prevotella copri* | Class-Bacteroidetes, Phylum-Bacteroidetes | KX057372 |
| HVS01-LCr | *Lactobacillus crispatus* | Class-Bacilli, Phylum-Firmicutes | KX057346 |
| HVS02-LCr | *Lactobacillus crispatus* | Class-Bacilli, Phylum-Firmicutes | KX057347 |
| HVS03-LCr | *Lactobacillus crispatus* | Class-Bacilli, Phylum-Firmicutes | KX057348 |
| HVS04-LCr | *Lactobacillus crispatus* | Class-Bacilli, Phylum-Firmicutes | KX057349 |
| HVS05-LCr | *Lactobacillus crispatus* | Class-Bacilli, Phylum-Firmicutes | KX057350 |
| HVS06-LCr | *Lactobacillus crispatus* | Class-Bacilli, Phylum-Firmicutes | KX057351 |
| HVS07-Ljn | *Lactobacillus jensenii* | Class-Bacilli, Phylum-Firmicutes | KX057352 |
| HVS08-Lco | *Lactobacillus coleohominis* | Class-Bacilli, Phylum-Firmicutes | KX057353 |
| HVS09-Lco | *Lactobacillus coleohominis* | Class-Bacilli, Phylum-Firmicutes | KX057354 |
| HVS10-Lco | *Lactobacillus coleohominis* | Class-Bacilli, Phylum-Firmicutes | KX057355 |
| HVS11-Lco | *Lactobacillus coleohominis* | Class-Bacilli, Phylum-Firmicutes | KX057356 |
| HVS12-Lco | *Lactobacillus coleohominis* | Class-Bacilli, Phylum-Firmicutes | KX057357 |
| GTB01-Gh | Gemella haemolytica | Class-Bacilli, Phylum-Firmicutes | KX057343 |
| GTB02-Hp | Helicobacter pylori | Class-Alpha-proteobacteria, Phylum- Proteobacteria | KX057344 |
| GTB03-Hp | Helicobacter pylori | Class-Alpha-proteobacteria, Phylum- Proteobacteria | KX057345 |
| SW01-BP | Unculture betaproteobacterium | Class-Betaproteobacteria, Phylum-Proteobacteria | KX057358 |
| SW02-RB | Rhodobacteraceae bacterium | Class-Alphaproteobacteria, Phylum-Proteobacteria | KX057359 |
| SW03-Ac | Actinobacterium sp. | Class-Actinobacteria, Phylum- Actinobacteria | KX057360 |
| SW04-Ar | Arcobacter sp. | Class-Epsilonproteobacteria, Phylum-Proteobacteria | KX057361 |
| SW05-Mb | Macromonas bipunctata | Class-Betaproteobacteria, Phylum-Proteobacteria | KX057362 |
| SW06-Ab | Alcaligenaceae bacterium | Class-Betaproteobacteria, Phylum-Proteobacteria | KX057363 |
| SW07-Bs | Bordetella sp. | Class-Betaproteobacteria, Phylum-Proteobacteria | KX057364 |
| SW08-Pa | Pseudomonas aeruginosa | Class-gamma-proteobacteria, Phylum-Proteobacteria | KX057365 |
| SS01-Br | Bacillus infantis | Class-Bacilli, Phylum-Firmicutes | KX129724 |
| SS02-Rs | Rhodococcus sp. | Class-Alphaproteobacteria, Phylum-Proteobacteria | KX129725 |
| SS03-Br | Bacillus sp. | Class-Bacilli, Phylum-Firmicutes | KX129726 |
| SS04-Pt | Psychrobacter sp. | Class-Flavobacteria, Phylum-Bacteroidetes | KX129727 |
| SS05-Fc | Flavobacterium sp. | Class-Flavobacteria, Phylum-Bacteroidetes | KX129728 |
| SS06-Zp | Gramella sp. | Class-Flavobacteria, Phylum-Bacteroidetes | KX129729 |
USA). Around 300 mg of zirconia beads was added to the suspension and cell lysis was done by mechanical disruption using SpeedMill PLUS bead beater (Analytical Jena, Germany). Beating was done in two cycles (30 seconds each). Total program time for bacteria was 2 minutes. After completion of bead beating, 15 mg Polynvinylpolypyrrolidone (PVPP) was added to the suspension and mixed well by gentle vortexing of the sample. To remove the added beads, PVPP and all other cell debris, the suspension was spun down at 14000 rcf for 5 minutes in a microcentrifuge (5427R, Eppendorf, Germany).

**Organic extraction and precipitation of nucleic acids.** The supernatant was transferred into a fresh MCT. The pellet was washed with 500 μl Tris (50 mM)-EDTA (20 mM)-NaCl (100 mM)-PVPP (1%) and the supernatants were pooled. The genomic DNA was precipitated from the supernatant by adding two volumes of 96% ethanol. The organic solvent was mixed gently for one minute and kept five minutes at room temperature and the nucleic acids were recovered by centrifugation at maximum speed, 14000 rcf, for 10 minutes at 4 °C in a microcentrifuge. The supernatant was removed by mild aspiration and keeping the tube in an inverted position on adsorbent paper to let the fluid drain away. The pelleted nucleic acids were dried for 10–15 minutes at room temperature.

**Removal of RNA and purification of genomic DNA.** To remove all the RNA species that are present in the nucleic acid preparation, the pellet was dissolved in 450 μl phosphate buffer supplemented with 50 μl 3 M-potassium acetate. The pellet was dissolvis by pipetting and incubated on ice for 90 minutes. The tube was removed from ice and 2 μl RNase (10 mg/ml) was added and placed in a heating block (37 °C) for 30 minutes. The suspension was supplemented with 50 μl sodium-acetate (3 M) and 1 ml of ice-cold 96% ethanol. The DNA was precipitated by centrifugation at 14000 rcf for 10 minutes at 4 °C. To remove the excess salts, the pellet was washed with 70% ice-cold ethanol. The pellet was dried at room temperature and re-suspended in 200 μl Tris (10 mM)-EDTA (1 mM) buffer (pH 8.0) and dissolved DNA was stored at 4 °C.

**PCR amplification and cloning of community 16S rRNA gene.** PCR amplification of 16S rRNA gene for targeted metagenomics study was done using adaptor and barcode labeled conserved region specific primers and DNA free Q5 High-fidelity DNA polymerase (NEB, USA). Amplification was done in 50 μl reaction volume with 1–10 ng of template DNA and following the reaction conditions: 98 °C-2 minute (1 cycle), 98 °C-20 seconds, 50 °C-30 seconds, 72 °C-45 seconds (30 cycle), 72 °C-7 minute (1 cycle). The PCR products were electrophoresed on a 1% agarose gel, stained with ethidium bromide and photographed using a gel imaging system (Alphaimager, USA). PCR amplified 16S rRNA gene products from sewage water, soil, stool, GTB and vaginal swab samples were purified and cloned into pCR2.1 cloning vector and subsets of samples were sequenced in a capillary sequencer using vector specific M13F and/or M13R primers.

**Highlights**

- Sensitive method to isolate community bacterial DNA from different human origin and environmental samples.
- Efficient recovery and high purity of isolated DNA made this method attractive for high-resolution molecular applications.
- Would be gold standard for wide range of studies including environmental and clinical samples.
- Very economic compared to kits and automated DNA extraction methods.

**Box 1**

*Lysostaphin* is a known antimicrobial peptide, is a lytic enzyme that disrupts bacterial cell walls by catalyzing hydrolysis of 1,4-beta glycoside-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues present in the peptidoglycan layer.

*Lysostaphin*, a 27 KDa glycylglycine endopeptidase, used as antimicrobial agent against Gram-positive bacteria14. The endopeptidase works on the transpeptide bond of bacterial cell wall and removes the crosslinking peptide bridges.

*Mutanolysin* is a an N-acetylmuramidase that catalyzes the cleavage of β-N-acetylmuramyl-(1 → 4)-N-acetylg glucosamine linkage of the Gram-positive bacterial cell wall15. Its N-terminal end carries enzymatic domain where the C-terminal moieties are involved in substrate recognition and binding to the unique cell wall polymers. The enzyme is preferably used in the formation of spheroplasts and isolation of DNA from bacterial culture.

*Guanidinium thiocyanate* (GITC) is a chaotropic agent, used as strong denaturant to isolate nucleic acids from viral particles and bacterial cells16. GITC is used to lyse cells and inactivate RNase and DNase, the enzymes that is present in all bacterial cells and degrade RNA and DNA, respectively.

*Sodium lauroyl sarcosinate*, an amphiphilic amino acid anionic surfactant comprising hydrophobic 12-carbon aliphatic chain and the hydrophilic carboxylate, most often used in nucleic acid isolation from bacterial cells17. It helps in lysis of host cells and removing protein and broken cell walls from the suspension.

*Polyvinylpyrrolidone* (PVPP) is an insoluble, cross-linked form of polyvinylpyrrolidone. PVPP helps to remove the host secondary metabolites and other phenolic impurities from aqueous solution.

**Isopropanol and Ethanol.** Since, isopropanol is less volatile than ethanol and it co-precipitates simple sugars and salts with nucleic acids, precipitation of DNA with ice cold, 96% ethanol is preferable. DNA is a highly
polar molecule, because of its negatively charged phosphate residues in the nucleotide backbone. The repulsive forces that arise because of the exposed phosphate group between the polynucleotide chains need to be neutralized for effective precipitation of DNA. In the presence of 70% ethanol and 300 mM Na\(^{+}\) ions, the negative charges of the polynucleotide chains are reduced to the point where the DNA precipitates. It is important to note that ethanol precipitation of DNA can only be done if the cations are available in sufficient amount.

References

1. Brooks, J. P. et al. The truth about metagenomics: quantifying and counteracting bias in 16S rRNA studies. BMC Microbiol. 15, 66, doi:10.1186/s12866-015-0351-6 (2015).
2. Virgin, H. W. & Todd, J. A. Metagenomics and personalized medicine. Cell 147, 44–56, doi:10.1016/j.cell.2011.09.009 (2011).
3. Claesson, M. J. et al. Gut microbiota composition correlates with diet and health in the elderly. Nature 488, 178–184, doi:10.1038/nature11319 (2012).
4. Human Microbiome Project, C. Structure, function and diversity of the healthy human microbiome. Nature 486, 207–214, doi:10.1038/nature11234 (2012).
5. Human Microbiome Project, C. A framework for human microbiome research. Nature 486, 215–221, doi:10.1038/nature11209 (2012).
6. Tatsunenko, T. et al. Human gut microbiome viewed across age and geography. Nature 486, 222–227, doi:10.1038/nature11053 (2012).
7. Ghosh, T. S. et al. Gut microbiomes of Indian children of varying nutritional status. Plos one 9, e95547, doi:10.1371/journal.pone.095547 (2014).
8. Ravul, J. et al. Vaginal microbiome of reproductive-age women. Proceedings of the National Academy of Sciences of the United States of America 108 Suppl 1, 4680–4687, doi:10.1073/pnas.1002611107 (2011).
9. Mason, O. U. et al. Metagenomics reveals sediment microbial community response to Deepwater Horizon oil spill. The ISME journal, doi:10.1038/ismej.2013.254 (2014).
10. Hstao, A. et al. Members of the human gut microbiota involved in recovery from Vibrio cholerae infection. Nature, doi:10.1038/nature13738 (2014).
11. Gevers, D. et al. The treatment-naive microbiome in new-onset Crohn’s disease. Cell Host Microbe 15, 382–392, doi:10.1016/j.chom.2014.02.005 (2014).
12. Aagaard, K. et al. The placenta harbors a unique microbiome. Science translational medicine 6, 237ra265, doi:10.1126/scitranslmed.3008599 (2014).
13. Smith, M. I. et al. Gut microbiomes of Malawian twin pairs discordant for kwashikor. Science 339, 548–554, doi:10.1126/science.1229000 (2013).
14. Schindler, C. A. & Schuhardt, V. T. Purification and Properties of Lysostaphin—a Lytic Agent for Staphylococcus Aureus. Biochim. Biophys. Acta 97, 242–250 (1965).
15. Yokogawa, K., Kawata, S., Nishimura, S., Ikeda, Y. & Yoshimura, Y. Mutanolysin, bacteriolytic agent for cariogenic Streptococci: partial purification and properties. Antimicrob. Agents Chemother. 6, 156–165 (1974).
16. Chomczynski, P. & Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162, 156–159, doi:10.1006/abio.1987.9999 (1987).
17. Filip, C., Fletcher, G., Wulff, J. L. & Earhart, C. F. Solubilization of the cytoplasmic membrane of Escherichia coli by the ionic detergent sodium-lauryl sarcosinate. J. Bacteriol. 115, 717–722 (1973).

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Author Contributions

Conceived and designed the experiments: B.D. Identification and collection of samples: B.D., S.B., B.S., O.M., N.K., R.T., S.B., N.W., V.P., P.A., V.G.P., R.V., A.R.M., M.V. and G.B.N. Performed the experiments: B.D., S.B., B.S., O.M., D.A., N.K., M.D., A.F., P.K., S.S. and V.P. Analyzed the data: B.D., S.B., B.S., O.M., D.A., N.K., M.D., A.F., P.K., S.S., K.H.A., T.H., M.A., H.V., O.P., V.P., A.G.P., R.V., A.R.M., M.V., K.T., T.K. and G.B.N. Wrote the manuscript: B.D. All authors read and approved the manuscript.

Additional Information

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