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A pressure-driven column-based technique for the efficient extraction of DNA from respiratory samples

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ABSTRACT

Currently molecular techniques are a broadly accepted tool for diagnosis and are able to benefit patients in clinical practice. The polymerase chain reaction (PCR) has been especially incorporated into practical applications that are already in widespread use across the globe. With regard to the initial DNA extraction from clinically relevant samples, a number of commercially available kits are commonly used and are also designed to be easy to handle and less labor-intensive. In this study, the pressure system extracting DNA in column-based kit was developed, and its utility was compared with the centrifuge method using sputum from patients who were diagnosed with pneumonia. Also, due to the compact size and rapid processing time, the practical application of the pressure-based system incorporated into an automated pipetting machine was evaluated through clinical study. Our data suggests that DNA extraction by pressure was capable of serving as a substitute for the centrifuge method, and the compact and automatic nature of the pressure system device provided rapid and valuable information for clinical practice.

1. Introduction

Currently molecular techniques have been involved in clinical practice and are already in widespread use throughout the globe. Notably, real-time PCR using respiratory specimens for identification of pathogens has been one of the significant clinical examinations [1,2]. Although DNA extraction from respiratory samples, sputum in particular, is by no means easy due to its viscosity. There are several kit options available for DNA extraction depending on the sample type [3–6]; however only column-based extraction has been used most for respiratory specimens [2,7–10]. Extracting DNA using spin columns requires a centrifuge as part of the process; however, the centrifuge is not a device that can be readily downsized. The vacuum procedure is also available but it requires an adequate means of waste disposal and special equipment. Hence, a conceivable alternative for the extraction of DNA from respiratory samples could be conducted by applying pressure to the column instead of centrifuging.

Miniaturization and automation have been sought-after technologies in medical devices [11–13]. The more compact the instrument is the more acceptable and practical it becomes at the bedside. Likewise, automatic equipment is able to provide the product with less intricacy, time and error, while complying with regulatory requirements. A newly developed DNA extraction method should therefore carry both of these benefits unlike existing methods.

In this study, the pressure system extracting DNA in column-based kit was developed, and its utility was compared with the centrifuge method by using sputum from patients who were diagnosed with pneumonia. In addition, the system was incorporated into an automatic pipetting device, which was evaluated through clinical study. Our data suggests that DNA extraction by pressure was capable of serving as a substitute for the centrifuge, and the compact and automatic device installing the pressure system provided rapid and valuable information in clinical practice.

2. Materials and methods

2.1. Respiratory specimen (sputum)

Sputum was collected from patients with pneumonia at the Saitama Medical University Hospital. The sample was mixed well by pipetting and divided into 2 parts; one was submitted for a regular bacteriological test (smear or culture) and the other for DNA extraction and real-time PCR analysis. To select purulent sputum, samples with an M2–P3 gross appearance were analyzed [1]. From each sample, an aliquot was stored at −80 °C for laboratory analysis.

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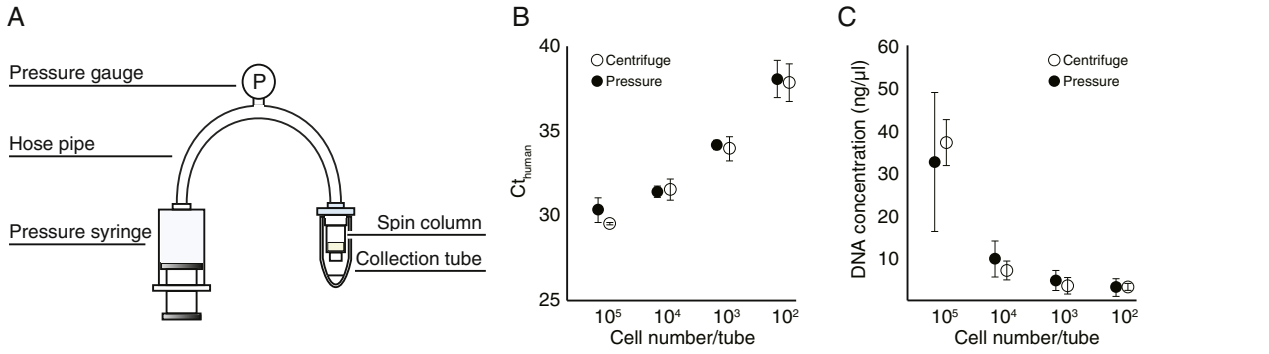

Fig. 1. Pilot experiment using the pressure-driven system. A. The scheme of manual pressurizer. The hand-made pressure was supplied from the pressure syringe to the column through a hosepipe and measured by pressure gauge. B. Relationship between cell number and Ct. Cultured A549 cells were suspended in DMEM and serially diluted. DNA was purified by the pressurizer and centrifuge methods, and a DNA sequence specific for human cell was amplified by real-time PCR. Experiments were performed at least three times. Error bars indicated mean ± SE. C. Relationship between cell number and DNA concentration. DNA was purified in the same manner as panel B. DNA concentration was measured based on DNA absorbance. Experiments were performed at least three times. Error bars indicated mean ± SE.

2.2. DNA preparation and quantification

The sample was diluted with an equal volume of phosphate-buffered saline (PBS) and homogenized by vortexing. Two hundred microliters of the homogenate was taken and mixed with 200 μl AL buffer (Qiagen, Tokyo, Japan) containing 20 μl proteinase K (Takara Bio Inc., Shiga, Japan), and the resultant mixture was incubated at 56 °C for 1 h. The protocols for pressure based or centrifuge based DNA extraction are described in Fig. 3. DNA was isolated into 100 or 500 μl of TE buffer with the QIAamp DNA Blood Mini Kit (Qiagen, Tokyo, Japan). Based on DNA absorbance, its concentration and purity were measured with a spectrophotometer GeneQuant Pro (GE healthcare, Tokyo, Japan). The ratio of nucleic acid to protein absorbance (260/280) was used as an indicator of the purity of DNA samples.

2.3. PCR reaction

The final solution of the PCR reaction contained 12.5 μl of the TAKARA Premix Ex Taq (Takara Bio Inc., Shiga, Japan), 300 nM of each primer, 100 nM–300 nM of the fluorescence-labeled TaqMan probe, 1.0 μl of purified DNA and deionized distilled water up to 25.0 μl. The PCR was performed by starting at 95 °C for 30 s followed by 40 cycles at 95 °C for 8 s, 61 °C for 25 s, and 72 °C for 20 s using the SmartCycler II (Cepheid, Sunnyvale, CA). The sequences of primer and probe or its concentration were described in [2].

2.4. HIRA-TAN system

We have adopted novel real-time PCR system named HIRA-TAN (Human cell-controlled Identification of the Respiratory Agent from "TAN" (which means sputum in Japanese)), which was able to discriminate therapeutic targets from commensal organisms (e.g. *Streptococcus pneumoniae* or *Pseudomonas aeruginosa*) and to detect foreign organisms (e.g. *Mycoplasma pneumoniae* or *Mycobacterium tuberculosis*) in the sputum. HIRA-TAN was capable of screening 23 target genes derived from respiratory pathogens in a lump and diagnosing the therapeutic target(s) among them. To determine the pathogenic role from commensal organisms detected by the real-time PCR, the HIRA-TAN system adopted $\Delta Ct_{pathogen}$ (difference between Ct_{human} and $Ct_{pathogen}$), implying the pathogen to human cell number ratio. Through the previous projects, the $\Delta Ct_{pathogen}$ cutoff for each commensal organism was setup and successfully able to distinguish the therapeutic target from among detected commensal organisms. The technical details in the real-time PCR reaction and the HIRA-TAN system were discussed in more detail in [1,2].

2.5. Patients

A diagnosis of pneumonia was clinically made when patients presented with both acutely produced/exacerbated illness, including cough, fever, sputum production, dyspnea, or chest discomfort/pain, and newly developed pulmonary infiltrates in a chest radiological examination without other alternative cause. Patients were eligible when they had pneumonia, were age 18 years or older and gave informed consent.

2.6. Ethical consideration

As to using patient's sample and the HIRA-TAN system for identification of respiratory pathogens, the research protocol was approved by the institutional review boards of the Saitama Medical University Hospital: IRB number 11-047 (February 8th, 2012).

3. Results

3.1. Applying pressure in place of a centrifuge to extract DNA

In order to test our hypothesis, first we have designed a small pressurizer, which was manually able to apply pressure onto a column through a hosepipe with a 1.2 mm inner diameter (Fig. 1A). DNA extraction from cultured human cells using the pressurizer showed a comparable copy number and quantity of DNA as compared to the centrifuge (Fig. 1B, C). Secondly, using the identical sputum stored at −80 °C, we measured the extracted DNA concentration and purity, Ct_{human} [the human cell-specific gene with Ct (threshold cycle) value by real-time PCR] and identification concordance for the respiratory pathogen diagnosed by the HIRA-TAN system (see Materials and methods) between the hand-pressured and centrifuged method. These two approaches demonstrated similar results (Table 1), strongly indicating the potential of the pressure method as being one of the tools for DNA extraction.

Table 1
Comparison of manually hand-pressured and centrifuged method.

	Centrifuged extraction	Hand-pressured extraction
DNA concentration (ng/μl)	102.8 ± 93.8	91.8 ± 53.8
DNA purity (260/280)	1.67 ± 0.09	1.60 ± 0.11
Ct_{human}	23.9 ± 2.2	23.0 ± 1.82
The number of pathogen detected by real-time PCR	12	12 (1.0)
The number of pathogen diagnosed as therapeutic target	8	8 (1.0)

Data for DNA concentration and purity and Ct_{human} (Ct value for human cell specific gene) were mean ± SD. The concordance ratios for pathogen detection and diagnosis are shown in parentheses respectively. Data were collected from 11 stored sputum samples.