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Effect of different fixatives on yield of DNA from human fecal samples

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Abstract

Fixation and transportation of human fecal samples is often difficult in geographically remote locations due to unavailability of options for immediate freezing. In this study effectiveness of five different chemical fixatives were evaluated on human fecal samples including for supernatant using RNAProtect® Bacteria Reagent (Qiagen), 95% ethanol, acetone, TRIZol® and a mixture of all these fixatives, in addition to immediate freezing. DNA was extracted from the fecal samples using QIAamp® Fast DNA Stool Mini Kit as well as quality and yield of extracted DNA was monitored for a period of 30 days. It was found that except TRIZol®, all other preservatives showed good DNA quality and yield for a period of one month based on agarose gel electrophoresis, Nanodrop and Qubit fluorometric measurements. It was also found that supernatant of fecal sample fixed with RNAProtect Bacteria Reagent gave reliable DNA yield in comparison to other various fixatives. The study also revealed that quality and yield of DNA from fecal samples fixed in acetone were very promising since it is a cost-effective fixative. Overall, the study shows future applicability for downstream DNA analyses of the RNAProtect® Bacteria Reagent, 95% ethanol, acetone, and a mixture of all these fixatives for fixing human fecal samples to be collected from geographically remote locations or in regions where available resources are largely limited.

Introduction

Over the last few decades, human gut microbiome has garnered particular attention globally to understand implications for human health (Turnbaugh et al 2007, Clemente et al 2012, HMP Consortium 2012, Fan and Pedersen 2020). Changes in gut microbiota has been linked to various diseases including obesity (Ley et al 2006, Turnbaugh et al 2006), diabetes (Frank et al 2007, Lewis et al 2015, Fan and Pedersen 2020) and cardiovascular diseases (Yamashita et al 2016, Jie et al 2017). Majority of the human gut microbiome studies are performed by undertaking analyses of human fecal samples (Gorzelak et al 2015). Apart from being routinely used for diagnosis of infection, or gastrointestinal (GI) problems, human fecal samples are used as part of non-invasive methods for detection of colorectal cancer or other nucleic acid-based GI diseases (He et al 2019). In studies involving human fecal samples, collection and preservation remains very challenging; especially from areas which are geographically remote and therefore it is not feasible to store the samples immediately at −20 °C or similar temperatures. Various types of fixatives such as ethanol (Nagy 2010, Song et al 2016), acetone (Fukatsu 1999), fecal occult blood test (FOBT) cards, fecal immunochemical test (FIT) tubes (Vogtmann et al 2017), OMNigen® GUT (Choo et al 2015, Penington et al 2018, Chen et al 2019), RNAlater®, and Norgen Stool Nucleic Acid Collection and Preservation System (Chen et al 2019) have been investigated previously for preserving different types of samples across ambient temperatures.

The use of high concentration of ethanol as a tissue fixative is well known for a long time and over the years this solvent is being increasingly used for undertaking downstream molecular work (Giannella et al 1997). Ethanol is considered as an excellent fixative due to its ability to slow down enzymatic processes and denaturation of DNA as well as protecting it from DNA degrading enzymes (Kilpatrick 2002, Srinivasan et al 2002, Marquina et al 2021). Though the concentration of ethanol as a fixative varies from 70% to 96% or even
higher but higher ethanol concentration is recommended for optimal DNA preservation (Nagy 2010, Flournoy et al 1996, Marquina et al 2021). Absolute ethanol is an excellent fixative for preserving both high molecular weight DNA and RNA (Giannella et al 1997, Noguchi et al 1997, Srinivasan et al 2002). Acetone can be also used as a fixative for subsequent extraction of DNA; Fukatsu (1999) demonstrated that for insects and their endosymbions, acetone was a better fixative than ethanol particularly when high level of water is present in the material. It has been also shown that RNA and proteins were also better preserved in acetone for several years and that since most biological materials have high water content, acetone may be a better fixative than ethanol (Fukatsu 1999). Goetze and Jungbluth (2013) however found that DNA preservation in acetone was comparable to that with ethanol based on short- (30 days) and long-term (4 months) laboratory and field experiments. TRIZol® is generally used for extraction of RNA and proteins; however only limited number of studies have described its role in DNA extraction due to low DNA purity and the need for additional purification steps (Braakman et al 2015, Campbell et al 2015, Bo et al 2021). RNAprotect® Bacteria Reagent (RNAprotect B.R.) is generally used for stabilizing RNA before bacterial cells are lysed and is suitable for use in both Gram-positive and Gram-negative bacteria (RNAprotect® Bacteria Reagent Handbook, 2020). To date, RNAprotect® Bacteria Reagent has not been evaluated as a potential fixative for human fecal samples and towards undertaking subsequent molecular analyses. Since no single fixative is perfect for preserving all types of tissues or samples, a mixture of various fixatives has also been tried in order to balance shortcomings (Sato et al 1990, Foss et al 1994, Shibutani et al 2000; Olert et al 2001, Srinivasan et al 2002).

The choice of fixation method should depend on research questions and scope of the study along with practicality for collection, storage and transport of samples. For human gut microbiome, various studies have recommended 95% ethanol among other fixatives when the requirement for immediate freezing of the fecal samples at −20°C or lower are not met (Flores et al 2015, Song et al 2016, Vogtmann et al 2017, Wang et al 2018, Tang et al 2020, Marotz et al 2021). For analysis of colorectal cancer or other GI diseases, human feces containing human DNA needs to be preserved which is in very small ratio in comparison to the total DNA due to large bacterial contribution (Whitney et al 2004). For such studies different fixation methods needs to be utilized. The selection of fixative is particularly crucial when collection of human fecal samples is to be undertaken from geographical areas considered to be remote, does not have access to resources, cost-effectiveness of resources and transportation of samples within a set time frame may not be possible.

This study aims to investigate which fixative is most suitable for effectively fixing human fecal samples and can be subsequently used to extract high quality and high yield of DNA for up to a month while keeping in mind the cost-effectiveness of the fixative. In this study, we have evaluated the effectiveness of 5 different fixatives for human fecal samples, namely, RNAprotect® Bacteria Reagent, 95% molecular grade ethanol, acetone, TRIZol® and mixture of all the four fixatives in addition to a control which was stored at −20 °C without any fixative. We also checked if the supernatant of the fixative-added homogenized fecal sample can give sufficient DNA yield and quality. The samples were extracted ten times within a period of one month to see if DNA quality and yield varies in different fixatives in comparison to control.

Material and methods

Fecal sample collection and preservation

Fecal sample from a healthy individual aged 26 years was collected with informed consent in a sterile container and 2 g wet mass was immediately aliquoted in 6 sterile containers. RNAprotect® Bacteria Reagent (Qiagen), 95% molecular grade ethanol (Merck) (referred to as 95% ethanol henceforth), acetone (Merck) and TRIZol® (Invitrogen) were added in equal ratio (2.5: 1) in 4 containers containing human fecal sample. In the fifth container containing fecal sample, all the fixatives mixed in equal volume were added in the same volume to mass ratio. The sixth container had fecal sample without any added fixative and was immediately stored within 30 min of collection at −20 °C. All other containers containing fecal sample and fixatives were stored at ambient temperature of 20 °C. The feces to fixative ratio was same for all the five containers and they were homogenized by vigorous vortexing for 10 min.

DNA extraction

DNA was extracted manually using QIAamp® Fast DNA Stool Mini Kit as per manufacturer’s instructions (Qiagen) with one modification. For the fixed samples, 200 mg or 200 μl of the homogenized fecal sample or the supernatant was used for DNA extraction. For the frozen sample, 200 mg of the sample was weighed immediately and used for DNA extraction. DNA was extracted on Day 1, Day 3, Day 5, Day 7, Day 9, Day 12, Day 15, Day 20, Day 25 and Day 30 from all the six containers. On Day 1 and Day 3, DNA was initially extracted from the supernatant generated after vigorously vortexing fecal samples fixed earlier with respective fixatives; from
Day 5 onwards DNA extraction was carried out from the remaining slurry. DNA was finally eluted in 100 μl ATE buffer instead of 200 μl as stated in the manual.

**DNA yield and quality analysis**

The yield of DNA and quality were analyzed by agarose gel electrophoresis and compared with the DNA ladder. The extracted DNA which exhibited band intensity lesser than 40 ng with respect to DNA ladder were categorized as low yield and those which were equal or brighter than 100 ng were categorized as high yield. The DNA concentration and purity (260:280) were also measured on NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific). Subsequently, concentration of dsDNA was also measured by Qubit 3.0 Fluorometer (Thermo Fisher Scientific) using dsDNA BR Assay Kit (Thermo Fisher Scientific). In this study, 2–10 ng μl⁻¹ was considered as low yield, 10–50 ng μl⁻¹ as medium yield and >50 ng μl⁻¹ as high yield based on the NanoDrop measurement.

**Result and discussion**

In figure 1, agarose gel images reflected that the yield of DNA on Day 1, Day 3 and Day 5 was very low for fecal samples preserved in 95% ethanol, acetone, TRIzol and mixed fixative. The observed low yield of DNA could be due to the supernatant left after homogenization which was used for extraction across the respective days. From the supernatant, only RNAprotect showed high yield in DNA in Day 1 and Day 3 along with that of control as evident in figure 1. On Day 5, DNA yield was clearly reflected in the gel for sample fixed with mixed fixative. From Day 7 onwards with the exception of the TRIzol® fixed sample, high DNA yield was observed in the other samples (figure 1) which may be due to the presence of left-over slurry of fecal sample subsequently used for DNA extraction. DNA of the fecal sample preserved in RNAprotect® B.R. showed highest DNA yield up to Day 7 of the experiment. Samples stored in RNAprotect® B.R., 95% ethanol and acetone exhibited high DNA yield up to Day 30 (figure 1). The fecal sample stored in mixed fixative gave high DNA yield with the exception on Day 30, in which the yield was medium based on gel electrophoresis. Fecal sample frozen at −20 °C gave medium DNA yield from Day 12 to Day 30. TRIzol® gave the lowest yield among all the fixatives compared to control (figure 2). All the above DNA yield representations were qualitatively assessed based on agarose gel electrophoresis and compared against DNA ladder of known concentration.

The total DNA concentration determined by NanoDrop also revealed low concentration or no DNA on Day 1 and Day 3 for fecal samples preserved in 95% ethanol, acetone, TRIzol® and mixed fixative. In case of RNAprotect® B.R., the Day 1 and Day 3 DNA concentrations were 15.2 ng μl⁻¹ and 123.7 ng μl⁻¹ respectively whereas that of control the concentrations were 31.1 ng μl⁻¹ and 54.3 ng μl⁻¹ respectively (figure 2). It also
established TRIzol® as the weakest fixative amongst all and the DNA concentration ranged between 7.1 ng μl⁻¹ to 91.4 ng μl⁻¹ throughout the study period. The present study also showed that DNA concentration is highest in frozen fecal sample for up to Day 30 (figure 2) whereas the gel electrophoresis exhibited medium DNA yield from Day 12 to Day 30 (figure 1). Fecal sample fixed with 95% ethanol on Day 5, Day 7 and Day 12 exhibited DNA concentration of 10.5 ng μl⁻¹, 24.0 ng μl⁻¹ and 25.5 ng μl⁻¹ respectively. Overall yield of DNA was found to be consistent in fecal sample fixed with RNAprotect® B.R. and the DNA concentration ranged between 15.2 ng μl⁻¹ on Day 1 and 384.5 ng μl⁻¹ on Day 30; highest DNA concentration of 679.0 ng μl⁻¹ was found on Day 25. The study has also revealed that with the exception of Day 1 and Day 3, DNA from acetone and TRIzol® fixed fecal samples and for Day 1 of fecal sample fixed with mixed fixatives, most of the extracted DNA were of good quality (figure 3).

DNA concentration was also determined by Qubit fluorometer which can accurately and selectively measure dsDNA concentrations. Similar to Nanodrop result, the supernatant which was used to extract DNA for Day 1 and Day 3 yielded sufficient amount of DNA only in fecal samples fixed using RNAprotect® B.R. (figure 4). The TRIzol® fixed fecal sample gave lowest yield compared to other fixatives throughout the study period. On Day 5, fecal samples fixed in RNAprotect B.R. and mixed reagent gave better yield than other fixatives. From Day 7

Figure 2. Total DNA yield of fecal sample preserved in different fixatives and extracted at different time periods based on NanoDrop measurement.

Figure 3. Nanodrop measurement of A₂₆₀/A₂₈₀ ratio of fecal sample preserved in different fixatives and extracted at different time periods.
onwards DNA yield for all the samples, with the exception of TRIzol® fixed fecal sample, was good when compared to that of the control (figure 4). DNA yield of fecal samples fixed with acetone was more consistent from Day 7 till Day 30. Total DNA yield was higher than dsDNA for fecal samples fixed in different fixatives during the entire span of the experiment (figure 5).

The result from this study indicates that all fixatives with the exception of TRIzol® gave sufficient DNA yield for a period of one month as confirmed by agarose gel electrophoresis, Nanodrop and Qubit measurements. This study has also shown that only fecal samples preserved in RNAprotect® B.R. showed consistently good yield of DNA when supernatant of the same is to be used for downstream molecular analyses. This suggests that RNAprotect® B.R. can act as a stabilizer and enhance lysis which helps to release DNA from the lysed cells and subsequent extraction from the supernatant. In contrast, rest of the fixatives tested do not lyse the cells which renders the supernatant unusable for DNA extraction. The cell lysis leading to DNA release however, needs to be checked using additional experiments. The use of RNAprotect in assessing transcriptome of stool microbiome has been undertaken (Reck et al 2015); this reagent has not been used in any study linked to human gut microbiome particularly from South Asia. It should be also noted that while frozen fecal sample resulted in higher DNA yield on average, this sample is undiluted and does not necessarily lead to higher yields when dilution is taken into account for other samples. In the present study it has been shown that among all fixatives
with the exception of TRIzol® there is no inconsistency in terms of quantity and quality of DNA when extracted from homogenized slurry. The 95% ethanol has been already established as a good fixative for fixing fecal samples and for undertaking subsequent human gut microbiome analysis (Song et al 2016); in our study we found that yield and concentration of DNA improved with the use of this fixative. The use of acetone as a fixative showed promising result with consistent yield of DNA along with quality. Given, the cost of acetone is fractional, there is huge potential of this fixative for application in fixing human fecal samples following collection from geographically remote locations or in resource challenged regions, followed by subsequent transportation to undertake downstream molecular analyses. The other fixatives used in this study which showed good DNA yield and quality from human fecal samples should be further investigated for potential use in human gut microbiome studies. In particular, RNAProtect® B.R. holds potential for further evaluation but the cost effectiveness of this reagent can be a challenge when undertaking human microbiome studies in developing countries.

The current study design was focused on collection and fixation of fecal samples in only one tube followed by one extraction per time point to show the temporal trend in variation of DNA yield. In-depth conclusions would require analyses from multiple collections and storage in different types of vials to incorporate the influence of collection and storage methods on total DNA yield. The microbial population load is not homogeneous within the same fecal sample. It has been shown that within a single bowel movement, there could be variations in the microbial composition between different collection points of the fecal samples depending upon the consistency of the feces (Wu et al 2019). Therefore, multi-tube collections and extraction could show variations in total DNA yield owing to differential microbial load in the fecal sample and was therefore avoided in the present study.

To conclude, the overall findings from this study are particularly relevant when collection of fecal samples is to be undertaken from geographically remote locations which have no access to immediate freezing and also in regions where access to available resources for storage are largely limited or cost is an issue.

**Data availability statement**

All data that support the findings of this study are included within the article (and any supplementary files).

**Conflict of interests**

The authors declare no conflict of interests.

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