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A simple Preservation Method of Bird Feces for DNA Analysis: A Case Study on Chicken and Quail

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ABSTRACT

Standardized methods for fecal sample collection and safe long-distance transportation for DNA extraction are yet to be identified. Chickens and quails' samples were collected at 2weeks of age of birds housed in single cages. The samples were randomly divided into four groups and subjected to three treatments: storage in 75%(Group I) and 100% ethanol(Group III), freezing at −20°C(Group II), and immersing in 100% ethanol for 3 weeks followed by drying the samples for more than 60 days and transporting them to another country (Group IV). Our objectives were to quantify the DNA concentration and amplify a fragment of the gene from each sample successfully using the primers mcb398 and mcb869 through DNA barcoding. All samples were successfully amplified and PCR products were measured. The DNA relative density of Group I samples ranged from 97.9 to 293 and averaged 155.0 ± 92.5. In Group III with a mean value of 359.4 ±242.1. For chicken samples preserved at −20°C, the relative density ranged from 80.4 to 560 and averaged 220.6 ± 227.1. The mean values for the DNA relative density showed an increasing order from Group I to Group II and Group III. Statistics showed no significant differences between Groups I and II ($P \leq 0.05$) and between Groups III and II. Therefore, our data showed that the method of preserving samples in absolute ethanol and then drying them at room temperature or up to 45^{O}C achieves the best results. This method is inexpensive and safe for long-distance transportation and at airports.

طريقة بسيطة للحفاظ على DNA في براز الطيور: دراسة حالة عن الدجاج والسمان

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الملخص

لم يتم بعد تحديد الطرق الموحدة لجمع عينات البراز والنقل الآمن لمسافات طويلة لاستخراج الحمض النووي. تم جمع 24 عينة براز من الدجاج (العدد = 12) والسمان (العدد = 12). وجمعت عينات الدجاج من مجموعة دجاج مختارة عشوائيًا تتكون من 100 دجاجة و100 طائر السمان عند عمر أسبوعين وموجودة في أقفاص فردية. وقسمت العينات عشوائيا إلى أربع مجموعات وأخضعت لأربع معاملات. قمنا بمقارنة أربع طرق مختلفة لحفظ عينات براز الطيور: التخزين في 75% و100% من الإيثانول، والتجميد عند -20 درجة مئوية، والغمر في 100% من الإيثانول لمدة 3 أسابيع يليها تجفيف العينات لأكثر من 60 يومًا ونقلها إلى دولة اخرى. كانت أهدافنا هي قياس تركيز الحمض النووي وتضخيم جزء من الجين من كل عينة بنجاح باستخدام الاشعال mcb398 وmcb869 من خلال ترميز الحمض النووي .تم تضخيم جميع العينات بنجاح وتم قياس منتجات PCR. تراوحت الكثافة النسبية للحمض النووي لعينات المجموعة الأولى (المحفوظة في 75٪ من الإيثانول + التجفيف) من 97.9 إلى 293 وبلغ متوسطها 155.0 ± 92.5. في المجموعة الثالثة (المحفوظة في 100% إيثانول + تجفيف) بمتوسط قيمة 359.4 ±242.1. بالنسبة لعينات الدجاج المحفوظة عند -20 درجة مئوية (المجموعة الثانية)، تراوحت الكثافة النسبية من 80.4 إلى 560 وبلغ متوسطها 220.6 ± 227.1 .أظهرت القيم المتوسطة للكثافة النسبية للحمض النووي ترتيبًا متزايدًا من المجموعة الأولى إلى المجموعة الثانية والمجموعة الثالثة. أظهرت الإحصائيات عدم وجود فروق ذات دلالة إحصائية بين المجموعتين الأولى والثانية ($P<0.05$)وبين المجموعتين الثالثة والثانية ($P<0.05$) .لذلك، أظهرت بياناتنا أن طريقة حفظ العينات في الإيثانول المطلق ثم تجفيفها في درجة حرارة الغرفة أو حتى 45 درجة مئوية تحقق أفضل النتائج. هذه الطريقة غير مكلفة وآمنة للنقل لمسافات طويلة وفي المطارات.

الكلمات المفتاحية: البراز، الحفظ، الإيثانول، التجميد، التجفيف، الحمض النوويDNA .

INTRODUCTION

Feces contain exfoliated gut epithelial cells with the host's genomic and mitochondrial DNA (Qiao *et al*., 2018). Such DNA contains identical genetic information as tissue DNA, offering opportunities to mine the genetic information of animals through polymerase chain reaction (PCR) amplification, restriction fragment length polymorphism analysis, and sequencing technology for applications such as species identification (Kadri, 2020) gender determination, paternity testing, individualization, phylogenetic relationship inference, and even genomic studies (Syakalima *et al*., 2019). Feces also contain a large quantity of microbes that provide microbial genomic and plasmid DNA important for studies about the structure, function, and dynamics of gut microbial communities, as well as linking them to physiological homeostasis, infection, diseases, immunity, metabolism, behavior, development, evolution, etc. (Qiao *et al*., 2018). The collection of feces is non-invasive and easier than collecting other genetic materials from wild or captive animals. This makes feces the most desirable materials for relevant studies given the DNA can be well preserved.

The quality of isolated DNA is the most important factor influencing the success and accuracy of DNA analysis. However, fecal microbial communities tend to change quickly after defecation and exposure to the ambient environment. Host cells and DNA are also prone to degradation. Therefore, appropriate handling techniques and storage of feces when collected are needed (Zamil *et al*., 2021). To date, several categories of preservation methods have been developed and widely applied, including 1) cold preservation, i.e., using -20 to -80°C (Santos *et al*., 2019) and even liquid nitrogen to reduce the activity of nuclease; 2) chemical preservation, i.e., using chemical fixative

buffers to kill nuclease; (Liu *et al*., 2019) and 3) drying preservation, i.e., removing water from the sample to reduce the activity of nuclease (Vargas-pellicer *et al*., 2019).

Freezing at -80°C or in liquid nitrogen (-196°C) is the most frequently used method for long-term safe storage of biomaterials (Li *et al*., 2003). For short-term storage, freezing at -20 to -28°C is preferable (Michaud & Foran, 2011). However, it is often difficult to freeze samples immediately in the field, and there is often the risk of thawing during transportation, especially over long distances, even if the samples are frozen in time. Alternatively, chemical preservation is often used for the temporary storage of fecal samples during fieldwork. Ethanol is frequently used for this purpose because of the convenience of access and cost-effectiveness. Various concentrations of ethanol, viz. 75%, 90%, 95%, and 100%, have been used in experiments (Seutin *et al*., 1991) for PCR amplification, genotyping, and sequence analyses (Li *et al*., 2003). However, DNA tends to degrade dramatically when samples are stored for a long time in 75% ethanol (Seutin *et al*., 1991), and degradation is much less when stored in 100% ethanol (Ramón‐Laca *et al*., 2018). However, ethanol is a flammable liquid classified as "dangerous goods" and requires special packaging and transport, with the risk increasing with the concentration. A dried fecal sample is safe for transportation. However, the effectiveness of DNA preservation by drying is contradictory (Bubb *et al*., 2011). No general conclusion has been reached on the stability of and damage to DNA in dried samples (Wasser *et al*., 1997).

The ideal preservation method should be highly effective for DNA preservation, easy to perform *in situ* during fieldwork, safe for transportation, and cost-effective. When tissue or fecal sample is immersed in ethanol, ethanol penetrates into the cells rapidly, removes and replaces free water in the cells, and causes a change in the tertiary structure of proteins including enzymes involved in DNA degradation, facilitating the preservation of DNA and other cell components (Anchordoquy & Molina, 2007). We speculated that fecal samples could be safely preserved and shipped in water-proof packages at room temperature if they are treated with ethanol and subsequently dried. If this becomes possible, the collection and application of feces would be greatly supported. In this study, we tested the effectiveness of two schemes, 75% ethanol + drying and 100% ethanol + drying, using chicken and quail feces as examples.

MATERIAL AND METHODS

Feces collection

Chicken samples were collected from an arbitrarily selected chicken group consisting of 100 chickens and 100 quails at 2 weeks of age housed in single cages. A total of 24 fecal samples were collected from chicken (n=12) and quail (n=12). A clean plastic film was placed under the cage to collect fecal droppings. Fresh feces were collected using a trowel and a spatula without contamination from feathers and other debris. Each fecal sample was placed in a 10 ml tube. The samples were randomly divided into four groups and subjected to four treatments. The treatments I and III were stored in 75% and 100% ethanol at room temperature with a volume ratio of 4:1 (ethanol to samples), respectively. All samples were dried in a room temperature after a week and stored in airtight 10 ml centrifuge tubes at room temperature for a week until DNA extraction. Group II samples were stored for the same period in a refrigerator at -20°C until DNA extraction. In light of the results obtained from the chicken experiments, an additional 12 fecal samples from Japanese quails (6 samples for each sex), referred to as Group IV, were collected and preserved in 100% ethanol for a week with the same volume ratio. All samples (Group IV) were then dried under the same conditions and stored in airtight centrifuge tubes at 45°C temperature for 3 weeks

followed by drying the samples for more than 60 days and transporting them to another country, until DNA extraction.

DNA extraction and quantification

Each dried sample was homogenized by shaking the tube up and down, and 200 mg was weighed accurately on an electronic analytical balance (Analytical Balance ME104TE/00, Mettler Toledo, Germany) and transferred to a 1.5 ml tube. Samples preserved in a -20°C refrigerator were also dried at 45°C and 200 mg was collected from each sample after homogenization as above. The samples were refreshed by adding 100 µl TNE buffer (containing 10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, pH=8.0) to each tube and incubated at room temperature for 15 min. DNA extraction was then performed using the QIAamp DNA Stool Minikit (QIAGEN, Netherlands) following the manufacturer's instructions. The final volume of DNA extract was 30 µl.

PCR was performed to amplify a 472 bp fragment of the Cyt b gene for each sample using the primer mcb398: F 5'- ACCATGAGGACAAATATCATTCTG-3' and mcb869: R 5'-CCTCC TAGTTTGTTAGGGATTGATCG-3'. The reaction was set up in a 10 µl system containing 5 µl of 2× Easy Taq® PCR SuperMix (TransGen Biotech Co., Ltd. China), 0.2 µl each of the forward and reverse primers, 2.6 µl of ddH2O, and 2 µl of the DNA extract. Cycling was initiated with incubation at 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, 52°C for 30s, and 72°C for 1 min, with a final extension at 72°C for 5 min. PCR products were isolated on a 1.5% agarose gel and visualized under UV after fluorescent staining (6× DNA loading buffer, TransGen Biotech Co., Ltd. China). Images of each gel were captured using the GenoSens 2000 Touch system (Clinx Science Instruments Co. Ltd. China) and Image Studio Lite ver 5.2 (LI_COR Inc.) was used to quantify PCR products on the gel images. DNA quantity was expressed as the relative signal density normalized against the negative control on each gel.

Statistical Analysis

Experimental data are presented as the mean ± standard deviation (*SD)*. Statistical analyses were conducted using CoStat software (CoHort Software, Monterey, CA, USA). Differences between group means were tested using one-way analysis of variance (ANOVA), least significance difference (LSD) means comparison, and the Student's t test. Unless otherwise noted, statistical comparisons were performed at a P value of 0.05.

RESULTS AND DISCISSION

All samples were successfully amplified and PCR products were measured (Fig. 1a). The DNA relative density of Group I samples (preserved in 75% ethanol + drying) ranged from 97.9 to 293 and averaged 155.0 ± 92.5. This figure ranged between 99.4 and 664) in Group III (preserved in 100% ethanol + drying) with a mean value of 359.4 ±242.1. For chicken samples preserved at -20ºC (Group II), the relative density ranged from 80.4 to 560 and averaged 220.6 ± 227.1.

The mean values for the DNA relative density showed an increasing order from Group I to Group II and Group III. Statistics showed no significant differences between Groups I and II ($P \leqslant 0.05$) and between Groups III and II ($P \leqslant 0.05$). However, Group I was significantly lower than Group III ($P \leqslant 0.05$) (Fig. 2). For Group IV, the DNA relative density of female Japanese quail samples ranged from 146 to 1280 with a mean value of 515.7±662.1, while that of male samples ranged between 92.3 and 215, with an average of 152.8±61.4. No significant difference was detected between females and males ($P \leqslant 0.05$) (Fig. 1b). The average DNA density of all Group IV samples was 334.2±465.1. Compared to the chicken samples, the mean DNA density of Group

IV was not significantly different from that of Group III ($P \leqslant 0.05$) but was slightly greater than that of Group I ($P \leqslant 0.05$) and Group II ($P \leqslant 0.05$) (Fig. 2).

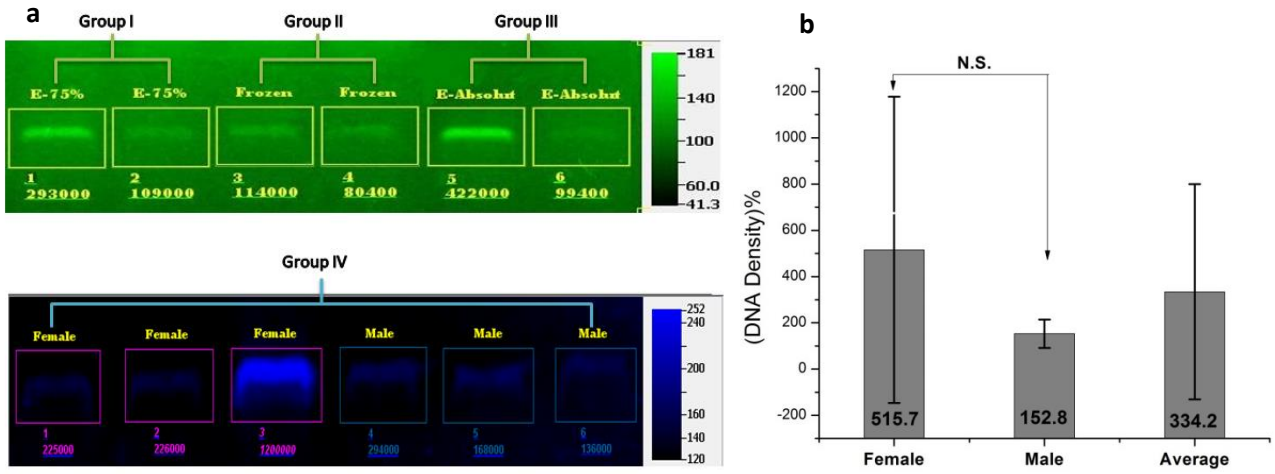

Fig.1 Fluorescently visualized PCR products and photodensity measurement of partial samples of four groups. **a** the frames are ranges defined to measure photodensity of the target bands. The groups are categorized into Group I (75% EtOH+drying), Group II (-20°C) to Group III (pure EtOH+drying). Group IV shows the Japanese quail samples that were preserved for two months after treatment using 100% ethanol and drying. **b** DNA was successfully extracted from Group IV after 2 months, and the density of DNA in female was higher than the male with nonsignificant differences ($P \leqslant 0.05$).

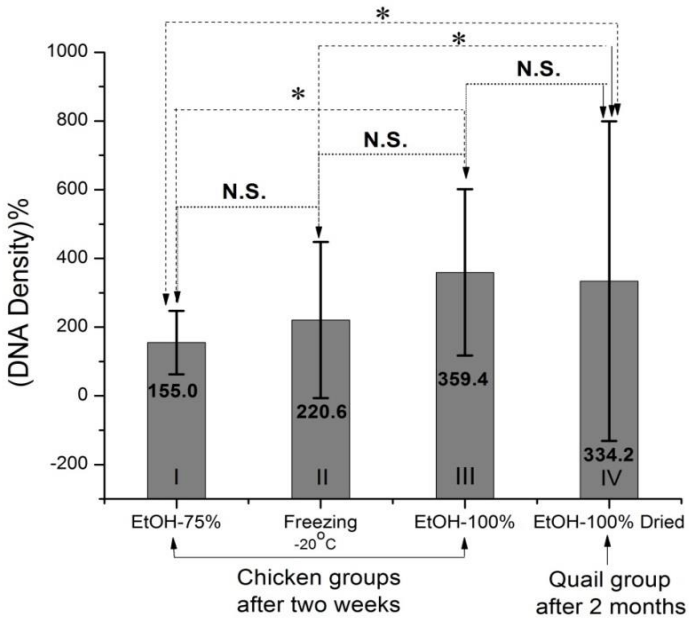

Fig. 2 Comparison of DNA relative density values of four experimental groups for chicken samples the means of DNA relative density showed an increasing order from Group I (75% EtOH+drying), Group II (-20° C) to Group III (pure EtOH+drying). Statistics did not detect significant difference between Group I and Group II ($P \leqslant 0.05$), and between Group III and Group II ($P \leqslant 0.05$). However, DNA relative density in Group I was significantly lower than Group III ($P \leqslant 0.05$). For Japanese quail samples preserved for two months after treatment using 100% ethanol and drying (Group IV), DNA relative density was greater than Group I ($P \leqslant 0.05$) and Group II ($P \leqslant 0.05$), and close to level of Group III of chicken samples ($P \leqslant 0.05$). * The asterisk indicates significant differences. N.S: Non-significant

DNA degradation of biological samples is an enzymatic digestion process that occurs in water solutions. The water shell around DNA is essential for it to maintain the correct conformation and charge, allowing enzymes to bind and cut DNA strands (Doughty *et al*., 2011). Changes in the bound water shell such as the removal of water may change the strength of hydrogen bonds and conformation of DNA (Khesbak *et al*., 2011), thus obstructing enzymatic cutting. Therefore, the removal of water from the ambient environment of DNA is safer than immobilizing degrading reactions by freezing the sample.

Drying by heating is a direct method to remove water from biomaterials. However, the process often requires temperature-controlled tools to avoid over-drying and thermal degradation of DNA (Alongi *et al*., 2015). Such tools are often unavailable for field sampling.

Chemical extraction is a safe method to remove water from bio-samples. Ethanol can form hydrogen bonds with water molecules and reduce the number of water molecules available to hydrate the DNA. Additionally, ethanol has a lower dielectric constant than water, which causes the DNA to aggregate with positive ions in the solution and precipitate from the solution (Fang *et al*., 1999). For similar reasons, ethanol may induce secondary structural changes favoring the precipitation of proteins (Yoshikawa *et al*., 2012). Therefore, ethanol is an ideal chemical to preserve DNA in bio-samples.

However, our results showed that preserving fecal samples in 75% ethanol for a week could not effectively prevent degradation and was even slightly worse than refrigerator preservation (Fig. 2). This is largely due to insufficient ethanol concentration to reduce the solubility of DNA because feces often contain 65% to 80% water, which further dilutes the ethanol. Studies have shown that DNA preservation can be improved when a high concentration (>90%) of ethanol is used (Reddy *et al*., 2012). Also, new findings on avian faecal samples relating to the two tested preservation methods, 95% ethanol and RNAlater, were deliver DNA of high quality and quantity (Edwards *et al*., 2023). This is in line with our study as both chicken and Japanese quail feces preserved in 100% ethanol yielded a greater quantity of PCR products than those preserved in 75% ethanol and by freezing at -20^{o}C (Fig. 2).

However, ethanol is flammable and often prohibited for transport. Removal of ethanol after fecal samples are treated may largely reduce the risk of sample handling. The feasibility of drying for DNA preservation after ethanol treatment was tested in the present study. The results demonstrated that drying at 45^{o}C followed by preservation in an airtight package at room temperature for a week to 2 months did not significantly reduce the DNA quality (Fig. 2). This suggests that treatment using 100% ethanol coupled with drying is an effective and safe approach for preserving and shipping avian fecal samples. This method can be used widely for sample collection in the field and in captivity.

CONCLUSION

The results of our experiments confirmed that fecal samples can be used as a reliable source of DNA, with appropriate collection and storage protocols. Therefore, we recommend storing samples in absolute ethanol and then drying them at 45°C and transporting them to the laboratory for DNA collection. This method of preservation is considered for the first time to our knowledge, and it is an inexpensive and safe method for transportation over long distances and at airports. This method is also a simplified and effective method for preserving genetic material from bird waste, which can be used by researchers in the agricultural, veterinary and zoological fields.

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AUTHORS' CONTRIBUTIONS

Ahmed conceived and designed the study. Ahmed and Nagam performed the experiments. Ahmed wrote the first draft of the manuscript. Ahmed, Nagam, Jacob, Zhang, and Xu revised the manuscript. All authors read and approved the final manuscript.

Conflict of interest: All authors declare there are no conflicts of interest.

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