An Application of Salivary DNA in Twin Research of Chinese Children

Yue Zhang and Chengye Ji

Institute of Child and Adolescent Health, Peking University, Beijing, China

Since saliva collection is noninvasive, painless and inexpensive, it may become an alternative to obtain genomic DNA, which is critical to evaluate zygosity and the role of genetic factors in twin research. This study provided a rough description of salivary DNA in Chinese twin children, and presented the DNA yield and quality extracted from saliva in a large-scale children sample, which supplied an example for saliva sample using in genetic epidemiology. Three milliliters of saliva was collected from 356 twin children aged 6 to 15, and DNA was extracted by a commercial DNA isolation kit. The DNA yield and purity was determined by spectrophotometry at 260nm and 280nm. The zygosity determination of the same-sex twins and the assay of Polymerase Chain Reaction-Restriction Fragment Length Polymorphism indicated the quality of salivary DNA. The amount of extracted DNA from three milliliters of saliva was about 34.91μg (2.20 ~ 122.04μg), average $OD_{260/280}$ values was 1.84. Saliva DNA is a reliable sample for the determination of twins' zygosity. We conclude that saliva may be a feasible and reliable source of DNA for genetic epidemiology studies, especially for twin research.

Keywords: saliva, DNA, twins, children, zygosity

Twins are an important biological sample for genetic epidemiology, especially for human gene and environment interaction. About 52 countries had established twin registers till 2006 (Busjahn & Hur, 2006). Classical twin studies depend on the comparison of concordance in monozygotic (MZ) and dizygotic (DZ) twins, and DNA is the most accurate method for zygosity determination. Moreover, twin research applies to evaluate the role of specific genetic factors in many fields (Chorbov et al., 2007; Hojlund et al., 2006; van den Ord et al., 2004). Thus, obtaining high quality genomic DNA is critical for twin research, in both zygosity determination (Hannelius et al., 2007) and genetic effects. Blood is still the primary method of obtaining samples for genetic epidemiology studies, but has disadvantages such as invasion, pain, special instruments and personnel, and the limited participation of subjects, especially children. It is necessary to research and consider efficient and acceptable methods for obtaining genomic DNA.

Saliva DNA is an important source for forensic casework and crime scenes (von Wurmb-Schwark et al., 2006), which has not yet been pervasively adopted in large-scale genetic epidemiology research. A recent study reports that salivary DNA in adult is comparable with blood in purity, genotyping, and PCR amplification (Hansen et al., 2007). Rogers et al. (2007) found that saliva provided a more sufficient quantity and better quality of DNA than the normally used buccal swab and brush techniques. Furthermore, saliva supplies a wide range of biomarkers including heavy metals, hormones, enzymes, immunoglobulins, toxin and their metabolites (Koh & Koh, 2007), and can be used in many medicine fields as a diagnostic fluid (Streckfus & Bigler, 2002). The studies based on salivary DNA have been reported (e.g., Herman et al., 2003; Khatib, 2005; van Schie & Wilson, 2000), and some researchers have given attention to the impact of storage conditions for salivary DNA during recent years (Ng et al., 2006; Ng et al., 2004; Quinque et al., 2006). Nevertheless, according to our knowledge, there are only a few large-sample studies on salivary DNA (Etter et al., 2005; Rylander-Rudqvist et al., 2006), especially in children, since previous studies were done by cytobrush, mouthwash, and swab techniques (e.g., Chen et al., 1999; Freeman et al., 1997; London et al., 2001).

The objectives of this study were to provide rough description of salivary DNA in Chinese twin children, and give a potential reliable source of DNA for genetic epidemiology studies, especially for twin research.

Materials and Methods

Subjects

As a pilot study being conducted in Beijing, China, the aim is to test the data collection and laboratory methods for studying the association between

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Address for correspondence: Chengye Ji, Institute of Child and Adolescent Health, Peking University Health Science Center, 38 Xueyuan Road, Haidian District, Beijing 100083, China. E-mail: jichengye@263.net

genetics and intelligence in twin children. Twins from elementary and junior high schools were registered by school doctors in Dongcheng District of Beijing City, and participated in the study after their parents were informed about the study and gave their consent. This study was approved by the Board of Medical Research and Human Ethics Committee of the Peking University Health Science Center.

Saliva Collection and DNA Extraction

The saliva samples were collected during school time, after informing the children or their parents 1 day prior. The children refrained from eating, chewing gum, or any oral hygiene procedures prior to the collection in the morning. Each child was asked to rinse his or her mouth with tap water and rest for about 5 minutes, then provide three milliliters of whole saliva in a 15-ml collecting tube noted with their name and collection time. Since the saliva samples would be used for hormone tests, we added nothing during transport and storage as previous description (Etter et al., 2005; Ng et al., 2004; Terasaki et al., 1998; van Schie & Wilson, 1997). Saliva samples collected at schools were transferred as soon as possible, and kept cold with ice by vacuum flask during transport, then frozen below –20 ºC and stored at –80 ºC until DNA extraction. All the sample collecting was done by our team members to ensure the saliva was supplied correctly.

The extraction of salivary DNA from the entire sample was completed within 3 months after collection. Saliva samples were centrifuged at 3000 rpm for 15 minutes after it had thawed. DNA was extracted with the NucleoSpin® Tissue kit (Macherey-Nagel Inc., Germany) according to the manufacturer's instruction. Total concentration and purity of DNA samples was determined by spectrophotometry at 260nm and 280nm using a UV-1601 spectrophotometer (Shimadzu Inc., Japan).

PCR and Genotyping

Determination of zygosity was made by Polymerase Chain Reaction – Short Tandem Repeat (PCR-STR) analysis with GenePrint® Fluorescent STR Systems Kit (Promega Inc., U.S.A.), comprising of 4 loci: D16S539, D7S820, D13S317 and D5S818. The accuracy of zygosity determination based on the four loci in Beijing population is 99.2%, comparable with another report of these four loci in Chinese population (99.6%) (Chen et al., 2007). Methods of PCR amplification, sample preparation and electrophoresis for the GenePrint® Fluorescent STR Systems Kit were performed according to the manufacturer's instruction. A four-marker multiplex PCR reaction was finished by 10 µl. The reaction contained dNTP (2 mM) 1.0 µl, 10 × buffer 1.0 µl, Taq DNA polymerase 0.55 U (Blend Taq-Plus-TOYOBO, Japan) , $MgCl_2$ (2 mM) 1.0 µl and Primer 0.8 µl. The amount of DNA was adjusted according to the referenced protocol, and also based on the measurement of the spectrophotometer. The PCR was carried out by the cycling program: initial denaturation 94 ºC for 11 minutes, then denaturation at 94 ºC for 60 seconds, annealing at 59 ºC for 60 seconds, and extension at 72 ºC for 60 seconds for 28 cycles, with a final extension of 60 ºC for 45 minutes. One microliter PCR product was added to 10 µl deionized formamideand, 0.5 µl ILS600 standard 95 ºC 4 min and then put at ice at once. The electrophoresis finished on an ABI 3130XL through ABI POP4. The analysis of size fragment was processed by GeneMaper software (version 3.5).

The first six salivary DNA samples from these subjects and three blood DNA samples of our previous subjects (Zhang et al., 2007) were performed for brain-derived neurotrophic factor (BDNF) val66met polymorphism by PCR-RFLP as Neves-Pereira et al. (2002) described. We used about 150 ng of total DNA and observed the 113 bp products through agarose electrophoresis gels. Then enzymatic digestion was made with 3 U Eco721 (MBI Fermentas) overnight. The fragments were separated on a 3.5% agarose gel at 100 V, and visualized with ethidium bromide (EB). The three possible genotypes (A/A, A/G, G/G) were defined by banding patterns of 113 bp, 113 / 78 bp and 78 bp, respectively.

Data Analysis

Data calculation and statistical analysis were performed using the SPSS for Windows program (version 11.5). The correlation of DNA yields in twin pair was 0.377 ($p < .01$), so one of each twin-pair or triplet were selected randomly for analysis. Two individual twin subjects were also included for analysis. Data were presented as mean ± standard deviation (SD). Mean DNA yields between genders or in age groups were compared using the unpaired t test after log transformation and nonparametric test, respectively.

Results

We registered 430 twin and triplet children (209 twin pairs and four sets of triplets) by school doctors. Of those, 356 children (181 boys and 175 girls) supplied saliva, including 174 twin pairs, two individual twin subjects and two sets of triplets. The mean age of subjects was 9.92 ± 2.86 years old, with the range from six to fifteen. The participation rate was 82.79%.

Overall average DNA yield from 3 ml of saliva was 34.91 ± 25.73 µg, with considerable variability from 2.20 to 122.04 µg, as shown in Table 1. The average yields of saliva DNA in different genders or age groups had no statistical significance (Table 1).

Average $OD_{260/280}$ ratio was 1.84 ± 0.17 (range = 1.26–2.20), which showed that the DNA purity was generally acceptable. The zygosity of 140 pairs same-sex twins was determined by salivary DNA. MZ twins were determined when all these loci were identical. As a result, 111 MZ twins and 29 same-sex dizygotic (DZS) twins were identified by PCR-STR. The successful rate of PCR-STR was about 99.30%, since two

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Table 1

Yields of Saliva DNA for Genders and Age Groups (μg / 3 ml)

	N	Mean	*SD*	Minimum	Maximal	*p* value
Gender						
boys	94	36.15	±26.56	2.94	120.25	0.64
girls	84	33.52	±24.84	2.20	122.04	
Age						
6–8	68	38.84	±26.29	2.20	121.38	0.11
9–11	55	34.00	±29.79	2.94	122.04	
12–15	55	30.95	±19.70	5.70	120.25	
Total	178	34.91	±25.73	2.20	122.04	

samples failed in the PCR. The ratio of MZ to DZ was approximately 1.82: 1, close to other reports of Chinese twins (Gao et al., 2006; Li et al., 2006). Figure 1 shows the result of zygosity determination.

In the assay of PCR-RFLP, all samples were successfully used to amplify the BDNF polymorphism and yield interpretable results by gel electrophoresis after PCR and digestion, as shown in Figure 2.

Discussion

The sociodemographic characteristics such as race/ethnicity and age are associated with participation of DNA collected that has been reported (Crider et al., 2006). Therefore, we tested the participation rate in our sample. The high participation rate (82.79%), consistent with other reports of adults (Hansen et al.,

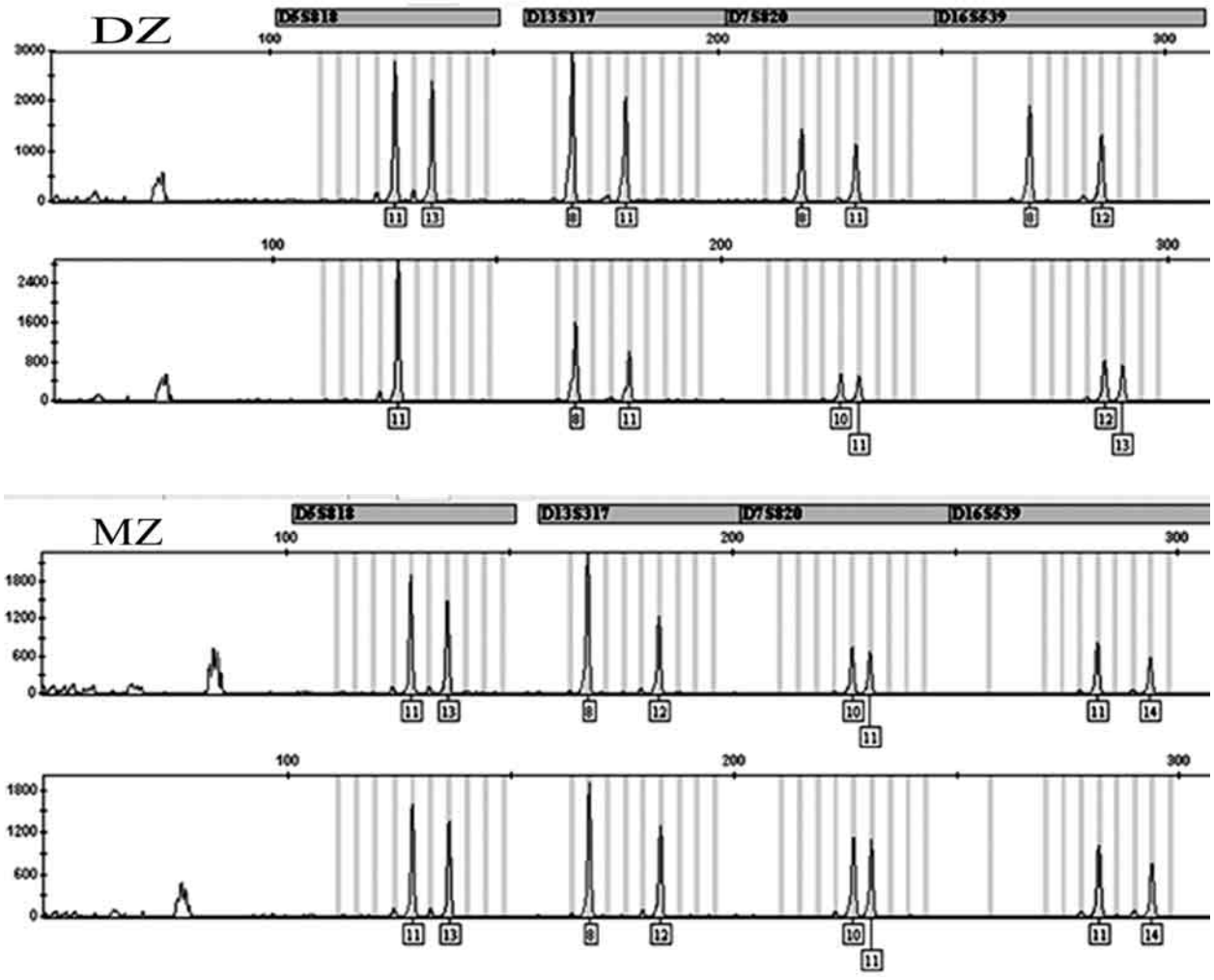

Figure 1

Zygosity determination for four microsatellite loci: D5S818, D7S820, D13S317, and D16S539. MZ was determined when all these loci were identical.

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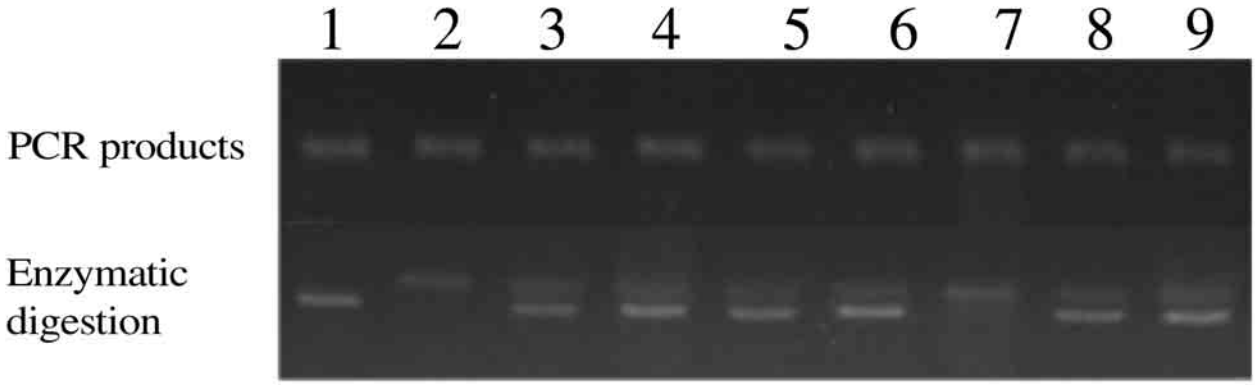

Figure 2

Result of PCR-RFLP. No. 1-3: blood DNA; No. 4-9: saliva DNA. The three possible genotypes were defined by banding patterns of 113bp, 113/ 78bp and 78bp, respectively.

2007; Rylander-Rudqvist et al., 2006), indicates that saliva collection for research is acceptable for children in China. We also believe that school doctors can play a useful role in explaining the purpose of this research and increase the participation rate, because the children and their parents do have more trust in the school teachers.

The overall average DNA yield of 3 ml saliva was 34.91 μg, which is less than that typically obtained from blood (20–80 μg / 2 ml), but higher than that using swab or cytobrush (1–2 μg / 1 swab or cytobrush) (Ng et al., 2006). The mean yield of DNA extracted in our salivary samples is lower than that in other reports, which collected saliva from adult samples by the Oragene kit and stored them at room temperature (Hansen et al., 2007; Rylander-Rudqvist et al., 2006). The difference of DNA yield may be affected mostly by collection and extraction methods or individual differences. In addition, storage conditions are also important for DNA yield and quality, such as temperature and storage and transfer time. To avoid high temperature and long transfer times, the samples were refrigerated and frozen more carefully during transport and storage, which can prevent DNA degradation and bacteria growth (Dixon et al., 2006, Rogers et al., 2007). As reported, high-quality DNA was obtained after transport at room temperature for 1 week and storage for 13–14 months at –70 °C (London et al., 2001). Ng et al. (2004) showed DNA was extracted successfully from saliva and gave an acceptable PCR result after storage at –70 °C for 1 month. As shown in our study, the saliva samples were stored –80 °C and DNA was extracted within 3 months, which gives important implications for planning a large-sample salivary biomarkers research project.

As the first research of salivary DNA in Chinese twin children, we found no statistical significance of salivary DNA in both genders and age groups, which consists with those reports in the adults or between adults and infants (Freeman et al., 1997; Le Marchand et al., 2001; van Schie & Wilson, 1997). The decrease of salivary DNA yield with age groups may be affected by some reasons such as exfoliated velocity of oral epithelium and need to be explored in the future. However, our result shows that sufficient DNA can be extracted from saliva in children, which is superior to that obtaining from children's mouthwash (0.7 μg/10 ml) (Chen et al., 1999).

One limitation of this study is that we couldn't distinguish human DNA and unhuman DNA by measurement. It has been reported that the proportion of human DNA in total saliva is about 40% and 68%, respectively (Quinque et al., 2006; Rylander-Rudqvist et al. 2006). According to this, the yield of human DNA in 3 ml saliva should be enough to test a large number of genetic markers for each PCR reaction which usually only requires 20–100 ng of template DNA.

The DNA quality of these saliva samples is acceptable by the average $OD_{260/280}$ rate, which indicates low protein and organic contamination. The DNA quality highly affects genotyping and PCR amplification (Hansen et al., 2007), therefore the DNA quality is essential for the success rate in genotype analyses. The high success rate (99.30%) of multiplex PCR-STR reaction demonstrates that DNA quality of saliva is satisfied for zygosity determination in twin research. In the PCR-RFLP assays, the visible bands of PCR and enzymatic digestion were observed at the correct size without a smear band, which implied that the salivary DNA quality is suitable for this assay and comparable to blood DNA for our ongoing research. The results of PCR-RFLP correspond well with other reports which showed that the success rates of 10 specific SNP assays were 96% (range = 91–100%) in Oragene saliva (Rylander-Rudqvist et al., 2006) and that of PCR-RFLP for DNA collected by mouthwash and toothbrush in children was 98.8% (London et al., 2001). Other papers showed that saliva samples were superior over swab and FTA cards samples in DNA quality (Hansen

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et al., 2007) and could identify different genetic markers (Quinque et al. 2006). According to the tests of zygosity determination and PCR-RFLP that were used for our salivary DNA samples, the results indicated the salivary DNA could be used for these assays. However, further work is needed to investigate the quality of salivary DNA for other genetic assays in large-sample.

In conclusion, we demonstrate that collecting saliva for large-scale twin research in Chinese children was feasible. The salivary samples appear to offer genomic DNA with adequate quantity and high quality. Saliva can be an alternative means of obtaining DNA for genetic epidemiology studies, especially for twin research.

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