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Mixture interpretation: Experimental and simulated reevaluation of qualitative analysis

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ABSTRACT

We present here analytical data using the 15 STR typing (Identifiler) kit regarding heterozygote balance in experimental DNA samples including one or two persons. Surprisingly, the allelic imbalance was observed even in samples consisting of only one person but adequate DNA for the standard protocol. The variance of heterozygote balance was more expanded in two-person mixtures than in one-person samples. Therefore, it is not suitable to use allelic peak heights/areas for estimating the genotypes of the contributors such as the quantitative analysis. We also reevaluated the effectiveness of qualitative analysis by simulation, i.e. consideration of the probability of all possible genotype combinations from the typing results of a mixed DNA sample. As demonstrated, the qualitative analysis using 15 STR loci is still extremely effective even in a mixture from two or three individuals.

1. Introduction

In Japanese forensic casework, DNA typing for 15 autosomal microsatellite or short tandem repeat (STR) loci using AmpFℓSTR® Identifiler® PCR Amplification Kit (Applied Biosystems, Foster City, CA) is currently used for individual identification. As the kit is highly sensitive, the correct genotype is usually identified with less than 1 ng of human genomic DNA.

High sensitivity has also enabled analysis of mixed DNA samples in current forensic cases. In addition, forensic samples often contain a low amount of DNA, or the DNA is degraded by environmental factors including temperature, humidity, ultraviolet radiation, pH, and microorganisms [1]. As a result, short allele peaks are often observed in the electropherogram chart, and they are prone to misinterpretation due to stochastic effects such as allelic imbalance, drop-out, drop-in, and laboratory-based contamination [2–5]. An allele from the minor contributor may not be distinguished from a stutter peak of the major contributor [6]. Due to the complexity of mixture interpretation, mixed stains are rarely analyzed in current Japanese forensic casework. However, forensic demand of mixture analysis has increased in crime cases, especially sex-related offenses.

In several countries, guidelines for mixture interpretation have been developed, and mixture analysis has been applied for forensic practices [7–10]. In particular, considering the ratio of two allele peak heights of a heterozygote, i.e. heterozygote balance (H_b or H'_b) [7,8,11–13] or peak height ratio [14] seems to be essential to estimate genotypes of the contributors. The general definition for heterozygote balance is $H'_b = \varphi_a/\varphi_b$, where φ_a is the peak height (or areas) of the shorter allele, and φ_b is the taller one [7]. The International Society of Forensic Genetics (ISFG) DNA commission noted that if two independent alleles of a heterozygote are derived from the same contributor, the H'_b of the two alleles is more than 0.6 [7]. In the United Kingdom (UK) DNA working group's report, the H'_b is $\geqslant$0.5 [8]. The recommendation of the threshold value of H'_b by ISFG was determined using the data from an original multiplex system of six STR loci [15] and the AmpFℓSTR® SGM Plus® PCR Amplification Kit [16], not the AmpFℓSTR® Identifiler® PCR Amplification Kit (Identifiler). It does not seem that the ISFG guideline considers the difference among the multiplex kits. In the UK report, experimental data was not shown. Besides, investigation of heterozygote balance in a mixture is insufficient, although heterozygote balance is used in practical analysis of mixed stains.

Another definition is $H_b = \varphi_{HMW}/\varphi_{LMW}$, where φ_{HMW} is the height of the high molecular weight (HMW) allele, and φ_{LMW} is the height of the low molecular weight (LMW) allele. Recently, Bright et al. experimentally recommended that the H_b from a

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heterozygote using Identifiler should be within the range of $0.6 \leqslant H_b \leqslant 1.66$ [11,12]. However, it is difficult to evaluate the range of H_b since the ratio of DNA amounts in two contributors was not obviously shown.

We experimentally investigated the distribution for H_b using pristine DNA samples of a one person and mixtures containing two persons in Identifiler testing. Furthermore, usefulness of quantitative analysis, (i.e. taking account of peak heights/areas and restricted allelic combinations in each contributor), was compared to that of qualitative analysis, (i.e. not taking account of peak heights/areas and considering all possible sets of genotypes about in each contributor). In this comparison, we generated computer-based mixtures of 2–5 persons and estimated the likelihood ratios (LRs) in each mixture by qualitative analysis supposing that the genotypes of only the suspect (S) or both the suspect and the victim (V) are known.

2. Materials and methods

2.1. Investigation of the variability in heterozygote balance

Genomic DNA from pristine blood samples donated by 32 individuals was extracted using the DNA Isolation Kit for Mammalian Blood (Roche Applied Science, Mannheim, Germany) according to the manufacturer's protocol. Extracted DNA was quantified using NanoDrop 1000 (NanoDrop Technologies, Inc.). Each sample was then diluted in TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA) to 10 ng/μl. In order to raise the precision of concentration of the DNA solution, the DNA concentrations in the diluted samples were measured again in duplicate using the Quantifiler® Human DNA Quantification Kit (Applied Biosystems), and the mean values were used for preparation of DNA samples with one or two person(s).

DNA samples from 20 individuals were diluted in water to two concentrations, i.e., 0.1 ng/μl ($n = 20$) and 0.05 ng/μl ($n = 20$). In two-person mixed samples, we made 20 pairs of major contributor (MA) and minor contributor (MI). In each MA and MI pair, DNA mixture was prepared at the four ratios of 9:1, 4:1, 3:1, and 1:1, and the total DNA concentration was adjusted to 0.1 ng/μl. The total DNA concentrations in all samples and the DNA concentrations of MA in two-person mixed samples were within the recommended concentration of the protocol of the Identifiler kit (0.05–0.125 ng/μl).

All DNA samples were amplified in triplicate. The standard 28 cycle amplification protocol was used according to the manufacturer's protocol for the following 15 STR loci: D8S1179 (D8), D21S11 (D21), D7S820 (D7), CSF1PO (CSF), D3S1358 (D3), TH01 (TH), D13S317 (D13), D16S539 (D16), D2S1338 (D2), D19S433 (D19), vWA (vWA), TPOX (TPO), D18S51 (D18), D5S818 (D5), FGA (FGA) and an Amelogenin gender marker (Am). PCR products were then analyzed by a genetic analyzer (ABI 310xl, Applied Biosystems), and the genotypes were determined by computer software (GeneMapper v 4.0, Applied Biosystems). Using the value of relative fluorescent unit (RFU) obtained from an electropherogram (EPG), H_b was calculated using non-overlapped heterozygote alleles derived from a contributor. The analytical threshold was determined 50 RFU and peaks below the threshold were not considered in this study.

For the evaluation of the heterozygote balance, we adopted $H_b = \varphi_{HMW}/\varphi_{LMW}$. The normal range of H_b equivalent to ISFG and UK guidelines are $0.6 \leqslant H_b \leqslant 1.66$ and $0.5 \leqslant H_b \leqslant 2.0$, respectively. The difference of H_b variances in samples of various DNA concentration was investigated by F-test of equality of variances using the R statistical software (version 2.13.1) [17]. Computed p-values were corrected by Bonferroni method, and the level of significance was 0.05.

2.2. Simulated likelihood ratios in suspect identification

The likelihood ratio (LR) compares the probabilities of the evidence under alternative hypotheses. In mixed DNA investigation of forensic cases, the two hypotheses of LR are the prosecution hypothesis (H_p) and the defense hypothesis (H_d). Given in a two-person mixed stain (M) and that all observed alleles can be explained by the genotype of the victim (V) and the suspect (S), H_p means that M contains DNA of V and S, whereas H_d means that M contains V and one unknown contributor (U). The LR of this case is defined as follows:

$$LR = \frac{Pr(M|H_p)}{Pr(M|H_d)} = \frac{Pr(M|V,S)}{Pr(M|V,U)}$$

As for practical LR calculation, there are two approaches, quantitative analysis [18–21] and qualitative analysis [22,23]. Quantitative analysis is generally expected to obtain the larger LR values than qualitative analysis as long as the peak heights/areas have fidelity to the amount of template DNA, while the latter approach is free from prejudice and more conservative than the former one.

We adopted LR values by qualitative analysis in the present study. First, based on previously reported Japanese allele frequencies investigated by Identifiler kit [24], we generated genotypes of 50,000 simulated individuals and selected 10,000 individuals as S, and another 10,000 ones as V. We then synthesized computer-based 10,000 'mixtures' including 2–5 persons. LR values were evaluated in two scenarios, i.e., whether the victim's DNA inclusion in mixture is already known or not. In the former scenario, a suspect and a victim, (and unknown person(s)) contribute to a mixed stain (designated as "SV"). In the latter scenario, "S" pattern, we masked the victim's contribution to the mixture, and consider only a suspect and unknown contributor(s) in H_p.

We used a formula for the LR calculation of a DNA mixture in qualitative analysis by Weir et al. [23]. The formula needs four variables, i.e., the number of contributors in the mixture, the number of known contributors (SV or S), the profile of the mixture, and the genotypes of known contributors. Using these variables, we calculated the LR values in every case. The density of LR values was estimated by Kernel Density Estimation (KDE) to draw graphs. This simulation and density estimate were written in R language (version 2.13.1).

As far as we know, there are few publications about the strength of the LR value supporting the evidence. In this study, we temporarily used LR threshold = 1000 by Evett and Weir's recommendation [25] and compared LR values of the minimum, 99% and 95% lower limit (LL).

3. Results

3.1. Reproducibility of peak height values

In this study, experimental samples were amplified and genotyped in triplicate. The peak heights and H_b values varied even from the aliquoted samples of a DNA solution amplified simultaneously (Fig. 1A and B). Allelic imbalance is the phenomenon in which a calculated H_b value is not satisfied with the recommended range. In general, low-template DNA has a high possibility to cause allelic imbalance [2,4,5]. Surprisingly, allelic imbalance was observed one time out of three in samples containing adequate DNA to detect allelic balance i.e., 0.1 ng/μl one-person DNA ($H_b = 0.55$ in Fig. 1A) and 0.09 ng/μl MA's DNA ($H_b = 0.43$ in Fig. 1B). Therefore, peak height values are sometimes poorly reproducible and provide unreliable information even in standard DNA analysis.