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SEX IDENTIFICATION IN HUMANS BY VARIABLE-STRINGENCY PCR OF Y CHROMOSOME SPECIFIC ALPHA SATELLITE

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Abstract. A method called variable-stringency PCR was designed to identify the sex of an unknown origin human sample. The reaction amplified a specific 281/285 bp product from male DNA, and some random DNA products from both male and female samples by using specific primers for the alphoid region of the Y chromosome. The amplification of Y chromosome alphoid DNA is a very robust reaction, not very sensitive to the quality and source of the DNA sample. The variable-stringency profile of the amplification reaction consisted in a succession of high-stringency / low-stringency / high-stringency stages, in all cases the stringency being determined by the annealing temperature in the PCR reaction. A blind test was performed, with samples of unknown sex, and the sex identification was successful in all cases.

Résumé. Une méthode appelée PCR en conditions de stringence variable a été développée, pour identifier le sexe d'un individu inconnu, dont on analyse un échantillon d'ADN. La méthode consiste en l'amplification d'une série de fragments de 281-285 paires de bases quand on utilise de l'ADN provenant d'un home, alors qu'une série de fragments aléatoires sont amplifies également chez les deux sexes. On a utilisé des amorces spécifiques pour la région alphoid du chromosome Y, cette réaction étant très robuste et peu sensible au qualité de l'ADN. La réaction d'amplification consiste en une succession de trois stages haute stringence/faible stringence/haute stringence, stringence déterminеé par la température d'alignement d'amorces.

Key words: sex identification, alphoid, variable stringency PCR, Y chromosome.

INTRODUCTION

Sex identification is a frequently encountered problem in various fields of medicine and anthropology. The most reliable methods nowadays use the polymerase chain reaction (PCR) technique for the sex diagnostic of a tissue sample. PCR is a method of amplifying small quantities of relatively short target sequences of DNA using sequence-specific oligonucleotide primers and thermostable Taq DNA polymerase.

Since the male and female genome differs by the presence of the Y chromosome in males, the basic idea would be to design a PCR reaction able to amplify a DNA fragment from the Y chromosome, when male DNA is used, together with the amplification of another sequence from a different region of the human genome. This fragment would serve as a positive internal control of the amplification reaction, allowing us to say that a sample which exhibits only the amplification of the non-Y chromosome fragment is a female sample. In the absence of this internal control, we cannot say whether the amplification failure occurred because we are dealing with a female-origin sample, or there was a technical problem with the amplification reaction. To achieve this goal, a multiplex (usually duplex) PCR reaction is carried out, with one pair of primers amplifying the sex-

linked locus, and the other pair(s) of primers amplifying the so called internal control. This method was used for the sex diagnostic in humans using the SRY gene.

Another technical solution for the sex identification assay would be to co-amplify in the same reaction, with a single pair of primers, two homologous DNA sequences, the first one from the Y chromosome, the other one from another chromosome, as is the case of amelogenin and ZFY/ZFX genes (homologous on both the X and Y chromosomes) and the alpha-satellite DNA (homologous on all chromosomes). Since the homology between the two DNA sequences is not perfect, it is possible to design primers which will yield amplification products of different sizes from male, respectively female DNA, allowing the sex discrimination based on the size of the amplification products, or on the use of a further restriction digestion reaction to discriminate the two sexes. Yet another technical variant is the use of the so-called low-stringency PCR, in which the cycling conditions allow the non specific annealing of primers at low annealing temperatures, thus generating random amplified DNA fragments, together with the specific Y chromosome DNA fragment. These random amplified DNA fragments would serve as an internal control of the amplification reaction, and the reaction would use just one pair of primers, avoiding the complications of designing a multiplex PCR. The method was used for sex diagnostic using the ZFY locus.

In this report we describe a method of sex identification in humans using Y chromosome specific primers for the centromeric alphoid region, in variable stringency conditions of the PCR amplification reaction. Alphoid DNA is a family of tandemely repeated simple sequences found mainly at the centromeres of all human chromosomes. The Y chromosome alphoid DNA is organized into tandemely repeated units, most of which are about 5.7-kb long, and some of them 6.0-kb long. The primers used for the PCR-based sex identification method presented here were chosen from within the 342-bp region that is present in 6.0-kb Y alphoid units, but absent from the 5.7-kb units. These primers co amplify two types of loci, one left side locus 281-bp long and one-to several 285-bp long loci in the right side of the alphoid array on the Y chromosome (Fig. 1). In a regular

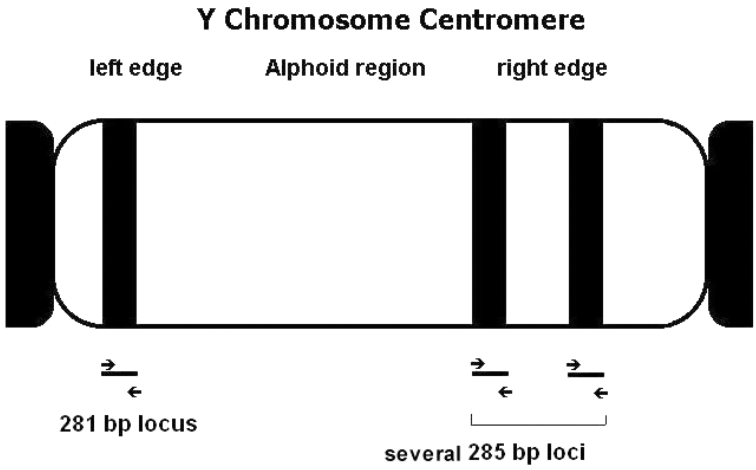

Fig. 1 – The strucuture of the alphoid array on the human Y chromosome.

agarose gel, both these amplification products are seen as a single band (designated 281/285 bp band, in this paper) when male DNA is used as template, and are absent when female DNA is used as a template. The variable stringency amplification reaction yields also some random amplified DNA products, which serve as an internal control for the amplification reaction.

MATERIAL AND METHODS

DNA extraction

Blood samples were collected in EDTA Vacutainer® tubes (Becton Dickinson, Plymouth, UK). Genomic DNA was extracted using the salting out method. In this method, the whole blood sample (2 ml) was mixed with 1X blood lysis buffer (3.1 M NH_4Cl, 0.2 M $KHCO_3$, 20 mM EDTA, pH 7.4) to a total volume of 9 ml, mixed gently by inversion (1 min), kept on ice for 30 minutes and centrifuged (3500 rpm, 10 min, 4°C). The supernatant was discarded, the pellet resuspended in 6 ml 1X blood lysis buffer and centrifuged as previously described. The supernatant was thoroughly discarded without disturbing the pellet. The pellet was then washed gently with 1 ml 1X blood lysis buffer and, after the removal of the supernatant, resuspended in 700 µl solution containing 100 µl 1X blood lysis buffer, 800 µl white lysis buffer (10mM TrisCl, 0.4 M NaCl, 2 mM EDTA, pH 8.0), 20 µl 20 mg/ml Proteinase K (Sigma, St. Louis, MO, USA) and 40 µl 20% SDS (Sigma, St. Louis, MO, USA). The mixture was incubated overnight at 37°C. After incubation, 240 µl of 6M NaCl (Sigma, St. Louis, MO, USA) was added and vortexed vigorously, then the mixture was centrifuged for 15 minutes at 3500 rpm without brake. The supernatant was transferred in a new tube and 40 µl 6M NaCl was added, then the mixture was centrifuged as previously. After centrifugation, the top aqueous layer was transferred into a clean tube containing 4 ml absolute ethanol (Sigma, St. Louis, MO, USA). The precipitated DNA was pelleted down and the supernatant discarded. The DNA pellet was washed gently with 70% ethanol, and the DNA pellet was air dried. The DNA was dissolved in 200 µl of ultra pure water and incubated for 30 minutes at 65°C. The DNA concentration and its relative purity were determined spectrophotometrically.

PCR conditions

The following primers were used for the amplification reaction: U972 (5'-TCT GAG ACA CTT CTT TGT GGT A-3') and L1214 (5'-CGC TCA AAA TAT CCA CTT TCA C-3'); the primer stock solution was prepared by dissolving the lyophilized primers in ultra pure water (18 $M\Omega cm^{-1}$ at 25°C) to a final concentration of 100 µM.

The final set-up of the PCR amplification included 40 ng DNA in a 15 µl reaction volume, containing 1X PCR buffer (Sigma, St. Louis, MO, USA), 1.5 mM $MgCl_2$ (Sigma, St. Louis, MO, USA), 200 µM of each dNTP (Sigma, St. Louis, MO, USA), 0.5 µM of each primer, and 0.5 units of Taq DNA Polymerase (Sigma, St. Louis, MO, USA). The DNA amplification was performed using a Techgene Thermal Cycler, with the following cycling conditions, defining the variable-stringency profile of the amplification reaction: a high-stringency stage (5 cycles of 1 min at 94°C, 1 min at 58°C, 1 min at 72°C), followed by a low-stringency stage (5 cycles of 1 min at 94°C, 1 min at 40°C, 1 min at 72°C), then a final high-

stringency stage (30 cycles of 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C), and a final extension for 5 min at 72°C.

Electrophoresis conditions

The PCR products were analyzed on 2% agarose gels. TBE (1X) (89 mmol/l Tris base, 89 mmol/l boric acid, 2 mmol/l EDTA, pH8.3) was used as electro-phoresis buffer. Gels were stained with 0.5 μg/ml ethidium bromide. The 100 bp ladder from Sigma (St. Louis, MO, USA) was used to assign the size of the PCR fragments. The Bio Rad Gel Doc 2000 system was used for the documentation of the gels.

RESULTS

When stringent conditions were used, the primers amplified the alphoid locus on the Y chromosome, resulting in a single 281/285 bp amplification product when male DNA is used and in the absence of any detectable amplification products with female DNA (data not shown). When the amplification program is altered to low stringency conditions (the low-stringency stage followed by the final high-stringency stage), both male and female DNA showed amplification of some random amplified DNA products, but the specific 281/285 bp product fails to amplify in male DNA (Fig. 2 a). Using the variable-stringency profile allowed us to obtain reliable amplification of the 281/285 bp product in male DNA, together with two other random amplified DNA products (aprox. 800 bp, and aprox. 210 bp), while the female DNA yielded only the two random amplified DNA products (Fig. 2 b).

DISCUSSIONS

We have developed a PCR method for sex diagnostic of human samples, using specific primers for the Y chromosome alphoid region, in a succession of

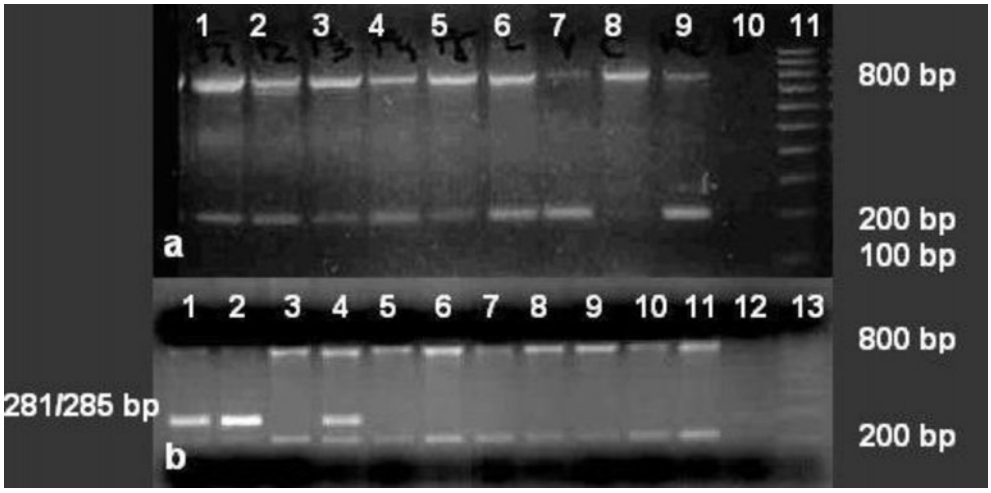

Fig. 2 – a) Low-stringency PCR. Lanes 1 to 5: female samples; lanes 6 to 9: male samples; lane 10: reaction negative control; lane 11: size marker 100 bp ladder (Sigma, St. Louis, MO, USA); b) Variable-stringency PCR. Lanes 1, 2 and 4: male samples; lanes 3, 5 to 11: female samples; lane 12: reaction negative control; lane 13: size marker 100 bp ladder (Sigma, St. Louis, MO, USA).

high-stringency/low-stringency/high-stringency conditions, which we designated as variable-stringency PCR. These variable stringency conditions allow the amplification of a specific 281/285 bp DNA fragment from male DNA, together with two others random amplified DNA fragments from both male and female DNA. These unspecific PCR products serve as an internal control demonstrating the efficiency of the amplification reaction.

Successful PCR depends on a number of factors, like the nature and concentration of the PCR buffer, the $MgCl_2$ and dNTP concentration, the annealing temperature, the extension time, the nature and concentration of various possible adjuvants, etc. Here we present some considerations regarding the PCR reaction we have set-up.

At DNA template quantities between 10 and 300 ng/15 μl reaction volume, the amplification efficiency showed no significant variation. The optimum $MgCl_2$ concentration we found was 1.5 mM. The concentration of the PCR primers in the final multiplex reaction was 0.5 μM, higher than the necessary primers concentration in a regular high-stringency PCR. This higher concentration is due to the low-stringency stage of the amplification profile, when a number of unspecific DNA sites compete for the primers.

The stringency profile of the PCR reaction was critical for the successful sex diagnostic. When low-stringency conditions were used (five low-stringency cycles, followed by 30 high-stringency conditions), the specific 281/285 bp product from male DNA failed to amplify, probably because of a too strong competition for primers during the low-stringency stage of the reaction. The problem was overcome by the variable-stringency profile of the reaction, by generating a higher number of 281/285 bp specific DNA products, before the low-stringency stage.

We used the sex diagnostic method presented here to assign the sex of some unknown samples, both males and females. The method provided the correct sex in all cases.

In conclusion, our study indicates that the co-amplification of both Y chromosome specific and random DNA sequences in a unique reaction mixture is a fast, sensitive and reliable method providing sex identification in humans, thus being very useful in forensic research.

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IDENTIFICAREA SEXULUI PRIN AMPLIFICAREA PRIN PCR ÎN CONDIȚII DE STRINGENȚĂ VARIABILĂ A REGIUNII ALFOID DE LA NIVELUL CROMOZOMULUI Y UMAN

REZUMAT

În aceasta lucrare prezentăm o variantă a tehnicii PCR, numită PCR în condiții de stringență variabilă, metodă utilizată pentru determinarea sexului la om, prin amplificarea unei regiuni specifice de la nivelul cromozomului Y uman. Reacția este foarte eficientă, fiind puțin sensibilă la calitatea și originea ADN utilizat. Metoda a fost testată prin determinarea sexului în cazul unor probe necunoscute, rezultatul fiind corect în toate cazurile.

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