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A rapid method for detection of Y-chromosomal DNA from dried blood specimens by the polymerase chain reaction

Michal Witt* and Robert P. Erickson

Department of Human Genetics and Department of Pediatrics and Communicable Diseases, University of Michigan Medical School, Ann Arbor, MI 48109, USA

Summary. The alphoid satellite family is the only repetitive DNA family showing chromosome specificity. We have developed a simple, rapid, and reliable test for sex diagnosis based on detection of these sequences in undigested genomic DNA using the polymerase chain reaction. In our test, dried blood specimens were the source of DNA. When female DNA was used as a template for the reaction, only the expected 130-bp X-chromosome-specific fragment was detected, while with male DNA both the expected 170-bp Y-chromosome-specific and X-chromosome-specific fragments were detected. The Y-chromosome-specific fragment was further characterized by restriction enzyme analysis. The Y fragment was detectable when DNA obtained from an equivalent of 10 µl of spotted blood was used in the reaction, whereas detection of the X fragment was possible with DNA from an equivalent of 5 µl of blood. Our test may find various applications in newborn screening and in forensic science.

Introduction

The precise determination of the sex of fetuses or individuals, using molecular biology technology is a matter of interest to many laboratories. Several rapid and accurate methods of "sexing" human DNA have been developed, focusing on the detection of Y-specific chromosomal sequences (Lau et al. 1984; Lau and Schonberg 1984; Lau 1985; Disteche et al. 1984; Stalvey and Erickson 1987). These methods are extremely useful in clinical practice for prenatal diagnosis of sex-linked disorders where fetal sex needs to be determined first. These techniques can also be used in following host versus graft cells in patients with bone marrow transplants from the opposite sex (Yam et al. 1987) or in searching for XO/XY mosaicism in patients with Turner syndrome (Stalvey et al. 1988). Some of these methods may be of use in detecting structural aberrations of Y chromosomes (Stalvey et al. 1988) or in sexing DNA for forensic purposes when incompletely degraded DNA is available (Witt and Erickson 1989). Probes specific for repeat sequences derived from the heterochromatic region of the long arm of the Y chromosome (Cooke 1976) were used to develop the first simple screening test for sex diagnosis through dot blot hybridization in small samples of unrestricted genomic DNA (Lau et al. 1984). These probes have also been used for in situ hybridizations (Lau 1985; Lau and Schonberg 1984). However, the use of a probe specific to the heterochromatic region of the Y chromosome may be of limited reliability because of its inability to detect Y sequences in individuals lacking this region of the Y chromosome, as demonstrated in some normal, fertile males (McKay et al. 1978; Bobrow et al. 1971). Conversely, this region of the Y may be carried as a translocation in 1 of 3,000 apparently normal females (Cooke and Noel 1979). Fragments of Yq may also be absent in Turner syndrome mosaics whose marker chromosome is a Y derivative (Magenis and Donlon 1982). Thus, probes for the heterochromatic region of the long arm of the Y chromosome have an increased risk of an incorrect diagnosis of sex. These reservations can also be applied to the method presented by Kogan et al. (1987) in which the polymerase chain reaction (PCR) was used to detect the presence of sequences of this region in chorionic villi for prenatal sex diagnosis.

The only repetitive DNA family likely to exhibit the properties of significant chromosome specificity is the alphoid (alpha) satellite family located in pericentromeric regions of all human chromosomes (Willard and Waye 1987). Alphoid satellite sequences on the Y have a higher repeat organization detected with restriction enzymes such as *Eco*RI (Wolfe et al. 1985): a 5.5-kb *Eco*RI fragment was found to be characteristic for these Y alphoid repeats. Dosage experiments have demonstrated that there are about 100 copies of this fragment on the Y chromosome (Wolfe et al. 1985). A sex diagnosis procedure for blotted DNA using probe Y97 specific for Y-chromosomal alphoid repeats has previously been reported (Stalvey and Erickson 1987).

We have developed a simple, rapid, highly sensitive, and accurate method of determination of sex through the PCR (Scharf et al. 1986) in which dried blood spots are used as a source of DNA template. This method may be useful in neonatal screening, since such dried specimens are already routinely used in screening for some genetic diseases (Guthrie 1980) and in forensic research for the analysis of biological evidence (Witt and Erickson 1989).

* *Permanent address:* Institute of Human Genetics, Polish Academy of Sciences, Poznan, Poland

Offprint requests to: R. P. Erickson, Division of Pediatric Genetics, Department of Human Genetics, Medical Science II M4708, 1500 East Medical Center Drive, Ann Arbor, MI 48109–0618, USA

Materials and methods

Blood was drawn into standard EDTA-containing tubes and 50-µl aliquots were spotted on Whatman 3MM filter paper to form standard spots. Spots were dried and stored at room temperature. For DNA isolation dried specimens were cut into pieces and rehydrated in 3 ml 0.85% NaCl for 2 h during which they were occasionally shaked (McCabe et al. 1987). Cells were spun down in Eppendorf tubes in a "Microfuge" for 2 min. The remaining filter pieces were washed with 1.5 ml 0.85% NaCl and the elutant was spun down in the same Eppendorf tubes as above. The supernatant was discarded and the cellular pellet was resuspended in 50 µl of sterile water and boiled for 10 min. This 50-µl DNA solution was used as a template for the polymerase chain reaction.

Two sets of oligonucleotide primers were used: Y1, Y2 flanking the 170-bp fragment of the alphoid repeats of the human Y chromosome (Y1: ATGATAGAACGGAAATATG; Y2: AGTAGAATGCAAAGGGCTCC, Wolfe et al. 1985) and X1, X2 flanking the 130-bp fragment of alphoid repeats of the human X chromosome (X1: AATCATCAAATGGAGATTTG; X2: GTTCAGCTCTGTGAGTGAAA, Waye and Willard 1985). Oligonucleotides were synthesized on an Applied Biosystem 380A DNA Synthesizer and purified by HPLC. For PCR, a slightly modified protocol from that provided by the Cetus Corp. was applied utilizing 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min in a reaction buffer containing 50 m*M* KCl, 10 m*M* TRIS-HCl pH 8.3, 1.5 m*M* $MgCl_2$, 0.9% gelatin, 2.5 units of Taq polymerase (New England BioLabs), 200 µmole of each dNTP and 1 µmole of each primer per 100 µl reaction mixture. A 50-µl sample of reaction mixture was analyzed on 4% composite agarose gel (3% NuSieve GTG and 1% regular low electroendoosmosis agarose) in tris-borate buffer in the presence of 0.0002% ethidium bromide. For restriction analysis, the product in the remaining 50 µl of reaction mixture was precipitated with ethanol, redissolved in restriction enzyme buffer, and then digested with an appropriate restriction enzyme.

When dried specimens of blood collected by heel- or finger-stick and not previously treated with an anticoagulant were used, elution of cells from the filter was performed for 5 h in the presence of 100 µg Proteinase K. All other technical details were as described above.

Restriction sites of the 170-bp Y-chromosomal fragment were identified using DNA/protein sequence analysis software no. 01030 (International Biotechnologies, Inc.).

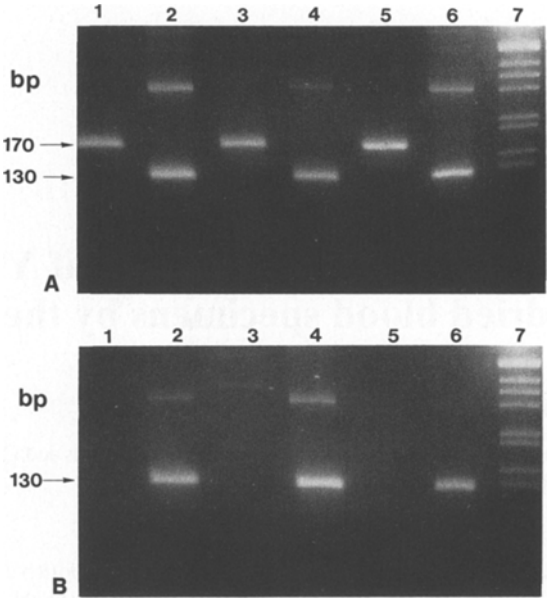

Fig. 1A, B. Sexing of DNA eluted from blood spots applying the polymerase chain reaction. **A** DNA from three different males (*lanes 1* and *2, 3* and *4,* and *5* and *6*) used as a template; *lanes 1, 3, 5* with primers Y1, Y2; *lanes 2, 4, 6* with primers X1, X2; *lane 7* size marker (from the bottom to the top: 142 bp, 154 bp, 200 bp, 220 bp, 298 bp, 344 bp, etc.). **B** DNA from three different females (*lanes 1* and *2, 3* and *4,* and *5* and *6*) used as a template; *lanes 1, 3, 5* with primers Y1, Y2; *lanes 2, 4, 6* with primers X1, X2; *lane 7* size marker (as in **A**)

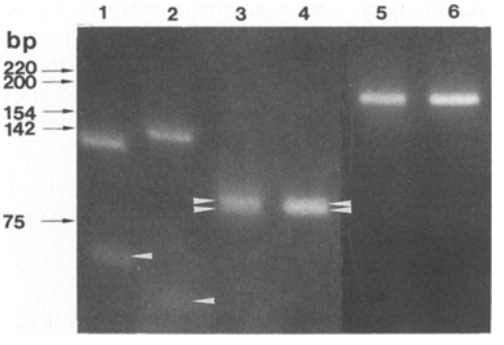

Fig. 2. Restriction analysis of the 170-bp Y-chromosome-specific fragment. *Lane 1 Dde*I digestion, *lane 2 Hin*fI digestion, *lane 3 Hpa*II digestion, *lane 4 Nci*I digestion, *lane 5 Eco*RI digestion, *lane 6* undigested 170-bp fragment. Positions of size markers shown on the left

Results

When DNA from dried specimens of male blood was used, the 170-bp fragment was effectively synthesized with Y1,Y2, and the 130-bp fragment was synthesized with X1,X2 primers (Fig. 1A). The 170-bp amplification product was not detected in female DNA from dried spots after PCR using Y-specific primers, whereas a control reaction utilizing X1,X2 primers resulted in the 130-bp X-specific fragment (Fig. 1B). The same patterns of PCR products were obtained for different males and for different females (Fig. 1). The conditions of the reaction (incubation time and temperature, amount of enzyme, number of cycles, reaction buffer) were optimized to minimize the background of unspecific amplification products (Witt and Erickson 1988). Although the polymerase chain reaction is very Mg^{2+} sensitive, the male-female difference was independent of Mg^{2+} concentration within the range of 1–10 m*M* (data not shown) and unequivocal in the cases studied.

To characterize further the Y-chromosomal PCR product, restriction sites of the 170-bp fragment were identified by computer pogram, and restriction enzyme digestion of the ethanol-precipitated fragment was performed. *Dde*I digestion resulted in 122-bp and 48-bp fragments (Fig. 2, lane 1); *Hin*fI digestion showed 132-bp and 38-bp fragments (Fig. 2, lane 2); *Hpa*II digestion gave 88-bp and 82-bp fragments (Fig. 2, lane 3); and *Nci*I digestion resulted in 87-bp and 83-bp fragments (Fig. 2, lane 4). *Eco*RI, which does not have any cutting sites within the analyzed sequence, did not cut the 170-bp fragment at all, and the product of *Eco*RI treatment was identical with

the uncut 170-bp Y-specific fragment (Fig. 2, lanes 5 and 6, respectively). These results were as anticipated from computer analysis.

To determine the limit of sensitivity of this method smaller amounts of whole blood were blotted on the filter paper, the DNA was eluted, and PCR was run under standard conditions. We found that a visible Y-chromosomal fragment could be synthesized when as little as 10 µl of male blood was blotted while the X-chromosomal fragment could be synthesized when DNA was eluted from a spot equivalent to 5 µl of male or female whole blood (not shown). The only difference between the products of PCR obtained from extremely small amounts of DNA (5–10 µl of blood spotted) and those obtained from larger spots was that the former ones were slightly fainter on an agarose gel than the latter.

An attempt was made to synthesize both fragments in one tube containing male DNA. The synthesis of both, using a high temperature of annealing (55°C) to enhance the specificity of the reaction, was impossible even when ratios of Y- and X-specific primers were changed to favor the Y (1:1–100:1). In all cases only the 130-bp band was obtained (data not shown). When annealing was performed at 37°C, synthesis of both the 170-bp and the 130-bp fragments in one tube was possible, but these mild conditions also permitted the synthesis of numerous unspecific products of higher molecular weight (not shown).

Identical results were obtained when spots of non-anticoagulated blood were used as a source of DNA for PCR sexing. In order to release white blood cells from such spots, simultaneous rehydration and proteolysis with Proteinase K were necessary. In our study we were able to diagnose sex using spots up to 8 years of age (data not shown).

Discussion

We have demonstrated a very simple sex diagnosis test in which dried blood spots on filter paper were used as a source of DNA template for PCR. We believe that this method represents a significant improvement over previous tests as far as ease, minimal time requirement, and reliability is concerned. Dried blood spots have already been employed in newborn screening programs for phenylketonuria (Guthrie 1961), other inborn errors of metabolism (Guthrie 1980), and genetic diseases (Orfanos and Naylor 1984). The advantages of using these spots are ease of collection, transport, storage, and the amenability to automation of the diagnostic procedures. Previously dried specimens are an invaluable source of DNA for different kinds of retrospective analyses, while fresh blood spots may be used for prospective studies and current diagnoses. A rapid sex diagnostic test may be of use in cases in ambiguous genitalia, true hermaphroditism, suspected 21-hydroxylase deficiency, etc.

The band patterns unique to males and females were identically reproduced when either pure DNA (not shown) or DNA eluted from dried blood spots was used as a template for PCR (Fig. 1A, B), illustrating that purification of DNA for this technique is unnecessary. Very faint bands of higher molecular weight nonspecific products were sometimes present (e.g., Fig. 1B, lane 3) and varied from experiment to experiment and from batch to batch of primers. The extremely high sensitivity of this method allowed us to use amounts of dried blood as small as 5–10 µl to detect target sequences. This means that even fragments of standard spots, equivalent to 50 µl of the whole blood, can be used for the diagnosis of sex by the polymerase chain reaction. The enormous sensitivity of PCR is a well-known fact based on the many experiments performed using DNA isolted from very small amounts of cells such as buccal epidermal cells obtained from a mouth wash (Lench et al. 1988), a single hair root (Higuchi et al. 1988), an unfertilized oocyte (Rappolee et al. 1988), or single sperm (Li et al. 1988). However, the advantage of this technique is also its disadvantage – the sensitivity of this method may easily result in detection of trace amounts of contamination (Lo et al. 1988). Special care has to be taken for handling blood spots; even epidermal cells of the person preparing DNA samples may bias the result of the analysis.

The restriction enzyme analysis is consistent with the presumed identity of the 170-bp fragment as the Y-specific sequence. Furthermore, blind studies performed on DNA samples from 15 different individuals also have confirmed this assumption. Comparison of the 340-bp consensus sequence of the alphoid repeat element from the X chromosome with the sequence of several repeat elements of the Y chromosome revealed about 70% homology between them (Wolfe et al. 1985). However, our primers, Y1 and Y2, were chosen to be in a region of least homology to the X, and a Y-specific signal was never detected when the female template was used.

The fact that we were unable to synthesize both fragments in one tube containing male DNA cannot be explained by a dimer formation of primers since the synthesis of the 130-bp fragment has never been inhibited. This problem may be explained by the relative availability of both template sequences and the competition for enzyme molecules. Alphoid blocks on the Y chromosome are repeated only about 100 times (Wolfe et al. 1985), whereas alphoid blocks on the X chromosome are present in about 5,000 copies (Waye and Willard 1985). At a lower temperature of hybridization this competition does not seem to be as strong.

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