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Sex determination and species exclusion in forensic samples with probe cY97

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Summary. A total of 120 human samples of blood, saliva and semen stains, hair roots, bone and skin fragments, obtained from 30 males and 16 females were analyzed in Southern blots with probe cY97. Only the male samples gave a specific band of 5.7 kb. In dot blot, under high stringency conditions, male DNA gave signals equivalent to a quantity of female DNA eight times higher. Probe cY97 did not react with 9 different vertebrate species but gave a signal for monkey DNA when used at low stringency. The advantage of using a probe specific for the centromeric region for sex determination and species exclusion is discussed.

Key words: Sex identification – Species exclusion – Southern blot – Dot blot – cY97

Zusammenfassung. Insgesamt wurden 120 menschliche Blutproben, Speichelproben und Spermaspuren, Haarwurzeln, Knochen und Hautpartikel, welche von 30 männlichen und 16 weiblichen Personen erhalten wurden, in der Southern-Blot-Technik analysiert mit Hilfe der Probe cY97. Nur die männlichen Proben gaben eine spezifische Bande von 5,7 Kb. Im Dot-Blot, unter hochstringenten Bedingungen, ergab die männliche DNA Signale, welche einer 8-fach höheren Menge von weiblicher DNA entsprachen. Die Proben cY97 reagierte nicht mit 9 verschiedenen Wirbeltierspezies, aber sie ergab ein Signal für Affen-DNA, wenn unter geringen Stringenzbedingungen hybridisiert wurde. Der Vorteil des Gebrauchs einer Probe zur Geschlechtsbestimmung und zum Speziesausschluß, welche spezifisch für die Centromer-Region ist, wird diskutiert.

Schlüsselwörter: Geschlechtsbestimmung – Speziesausschluß – Southern-Blot – Dot-Blot – cY97

Introduction

In criminal cases, it is relevant to determine the species and the sex of the donor of biological samples found at the crime scene. Usually morphological, histological, cytological and immunological methods are used [1]. In recent years recombinant DNA techniques have been adopted to resolve forensic casework such as paternity determination, personal identification and sex determination.

Positive identification of males is generally based on DNA probes recognizing repeated sequences of the heterochromatic region of the Y chromosome long arm [2–7]. Sometimes, these probes can give false negatives because the long arm of the Y chromosome can be lost without affecting the normal male phenotype [8, 9].

In this paper we report a method for species exclusion and positive human Y chromosome identification using the probe cY97 on samples typically investigated in forensic medicine. The probe recognizes the centromeric alphoid repeats and gives a 5.7 Kb band specific for the human Y chromosome [10–14].

Materials and methods

DNA extraction and purification. Known quantities (1, 2, 5, 10, 20 µl) of blood of human male and female, monkey (*M. rhesus*, female), dog, cat, horse, cow, pig, rabbit, mouse, chicken and sardine were spotted onto cloth and air dried. DNA was extracted and purified as reported by Gill [3]. DNA was also extracted from human semen and saliva stains, hair roots, skin and bone fragments. When extracting small amounts of DNA, glycogen was added before the ethanol precipitation [15]. DNA was quantitated with the fluorescent dye Hoechst 33258 and the fluorometer Hoefer TKO 100.

Controls were carried out on the human Y chromosome bearing somatic hybrid 3E7 and on the revertant 7/2 which has lost the Y chromosome [16].

Digestion and Southern blot hybridization. The samples were digested with *Eco*RI, *Pst*I, *Rsa*I, *Hin*fI, *Msp*I and *Hin*dIII according to the manufacturer's recommendations. Electrophoresis was carried out for 18 h at 50 V in 0.7% agarose gel in TBE (90 m*M* Tris, 9 m*M* boric acid, 2 m*M* Na_2EDTA). Standard procedure was adopted for the Southern blots [17]. The 2.6 and 2.9 kb *Eco*RI Y-specific fragments were purified from cosmid cY97 [11] and ^{32}P dCTP labelled by random primed DNA synthesis [18] to obtain a specific activity of 3×10^9 cpm/µg. Prehybridization (60 min in 7% SDS, 0.5 *M* phosphate buffer pH 7.2, 1 m*M* Na_2EDTA, 0.1% BSA) and hybridization (overnight in 1 × SSC. 6% PEG MW 6,000) were performed at 65°C [19]. The filters were then washed in 0.5 × SSC, 0.1% SDS at 65°C.

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Dot blots. DNA was denatured and spotted onto Hybond-N (Amersham) according to the manufacturer's recommendations. The probe was labelled and hybridized as reported above. The filters were washed in 1 × SSC, 0.1% SDS at 65°C and exposed for 6–24 h without drying at −70°C with intensifying screens. After the first exposure the filters were washed again at higher stringency (0.1 × SSC) and exposed for 1–7 days. To check for the presence of DNA on the filters they were stripped and reprobed with probe lambda 33.6 [20] which detects the minisatellite DNA in all vertebrates so far tested.

Some experiments were also carried out using the digoxigenin-11-UTP DNA labelling and detection kit (Boehringer).

Results

The probe cY97 detected a 5.7 kb fragment in human male DNA and in the hybrid 3E7 DNA digested with *Eco*RI. This fragment was never observed in human female DNA and the revertant 7/2 gave no signal (Fig. 1). The 5.7 kb Y specific fragment could also be detected in *Pst*I, *Rsa*I, *Msp*I, *Hin*dIII digests and in the widely used *Hin*fI digest. All Southern blots were hybridized under low stringency conditions to obtain a typical α-satellite ladder as positive signal even from female DNA.

Scalar quantities of DNA were loaded onto a gel to evaluate the resolution limit of the system. An unambiguous signal of 5.7 kb was detectable in male DNA samples of 50 ng and less (Fig. 2).

A total of 120 human samples of blood, saliva and semen stains, hair roots, bone and skin fragments were analyzed (Fig. 3). The results were always in agreement with the known sex of the donors (30 males and 16 females).

Under high stringency conditions the dot blots gave strong signals for human males only and weak or no signal for females (Fig. 4A). Interpretable results were obtained from a total of 143 samples (blood stains, hair roots, saliva stains, etc.). The same difference between male and female DNA was obtained when some of these samples were tested with the nonradioactive labelling system reported in materials and methods (Fig. 5). In this case the sensitivity of the test was slightly reduced.

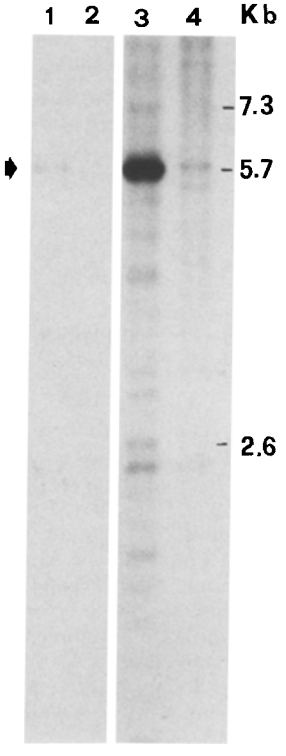

Fig. 1. Southern blot of DNA extracted from: cell hybrid 3E7 *(lane 1)*, cell hybrid revertant 7/2 *(lane 2)*, human male *(lane 3)* and human female *(lane 4)*. The samples were digested with *Eco*RI and probed with cY97 under low stringency conditions (0.5 × SSC, 65°C). The *arrow* indicates the 5.7 Kb band

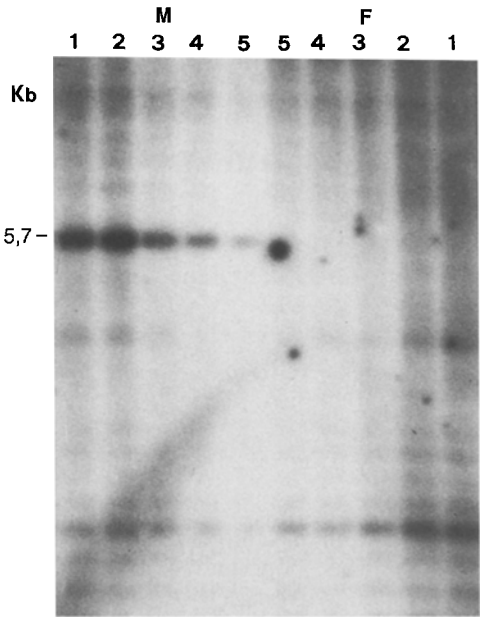

Fig. 2. Southern blot of scalar quantities (800; 400; 200; 100; 50 ng; from *lane 1 to lane 5* respectively) of human male *(M)* and female *(F)* DNA. The samples were digested with *Eco*RI and probed with cY97 under low stringency conditions (0.5 × SSC, 65°C)

Strong signals, irrespective of the sex, were obtained in dot blots of human and monkey samples under low stringency conditions whereas the other vertebrates tested (dog, cat, horse, cow, pig, rabbit, mouse, chicken and sardine) gave no signal (Fig. 4B). The same filters, stripped and reprobed with the minisatellite probe lambda 33.6, gave a strong hybridization signal for all the samples tested (Fig. 4C).

The discrimination limits of cY97 in dot blot was tested comparing scalar quantities of female and male DNA. Equivalent signal intensities were obtained when the amount of female DNA was roughly 8 times the amount of male DNA (Fig. 6B). The same filter was reprobed at low stringency (Fig. 6A) to check that the amount of female and male DNA loaded was the same.

Discussion

As previously reported [10–14], probe cY97 showed a male specific band of 5.7 kb in Southern blots which allows positive identification of human males. Females can be positively identified by the typical ladder of bands of the alpha satellite which is obtained at low stringency conditions. The sensitivity of the test is high and a signal can be obtained from less than 50 ng of undegraded DNA corresponding to less than 1 µl of blood or to a

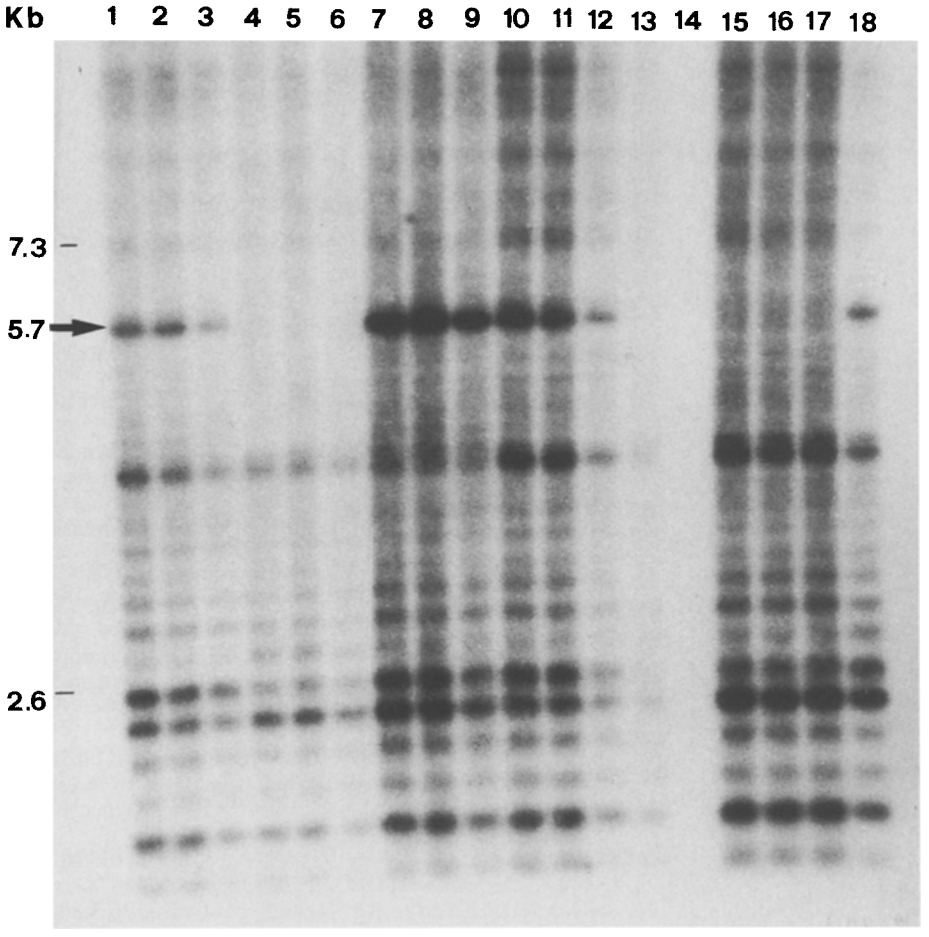

Fig. 3. Southern blot of DNA obtained from: male skin fragment, 15 µg *(lane 1);* 2 and 1 male hair roots (*lanes 2 and 3* respectively); 2 and 4 female hair roots (*lanes 4 and 5* respectively); female saliva stain of 10 µl (*lane 6*); male bone fragment (*lane 7*); male bloodstains (20, 10, 5, 2, 1 µl, *lanes 8–12* respectively); female bloodstains (1, 2, 5, 10, 20 µl, *lanes 13–17* respectively); semen stain 2 µl (*lane 18*). The samples were digested with *Eco*RI and probed with cY97 under low stringency conditions (0.5 × SSC, 65°C), the 5.7 Kb band is indicated by an *arrow*

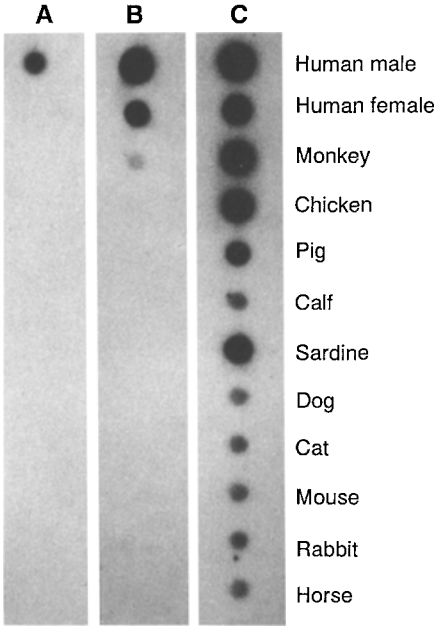

Fig. 4A–C. Dot blot of human male, human female and DNA from 10 other vertebrate tested with probe cY97 (**A** and **B**) and probe lambda 33.6 (**C**). The same filter was initially tested under low stringency conditions **B**, then under high stringency **A** and finally stripped and reprobed with the minisatellite lambda 33.6 **C**. The DNA was extracted from 5 µl bloodstains

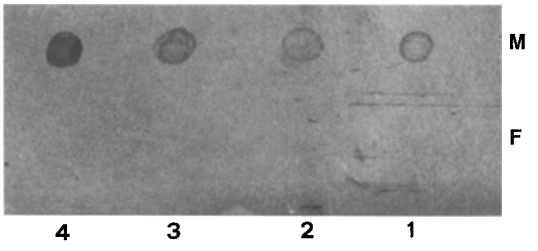

Fig. 5. Dot blot (high stringency) detected with a non-radioactive method with probe cY97. *M:* male samples. *F:* female samples. The DNAs were extracted from 1–4 hair roots as reported in the figure

single hair root [21]. The same sensitivity was reported by Tho et al. [10].

Another advantage offered by the probe cY97, even when used at low stringency, is the possibility of exclusion of the vertebrate species tested here. Most probably all vertebrate species except higher primates, can be excluded, but an extensive screening of all vertebrates would be beyond the limits of this research.

The identification of sex in dot blots at high stringency requires some precautions because the signal given by a male DNA can be mistaken for the signal given by an 8 times excess of female DNA. In this case the minisatel-

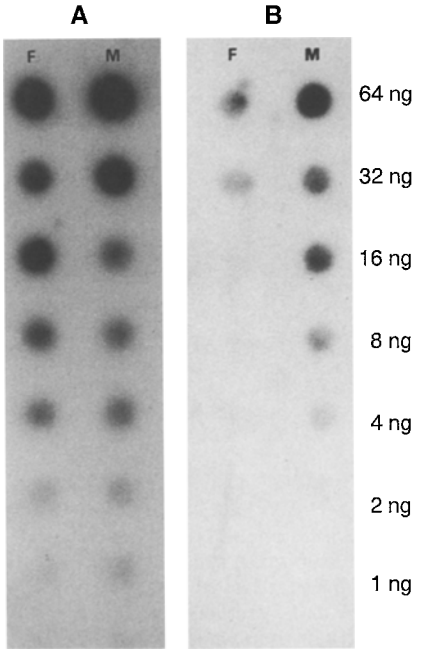

Fig. 6A, B. Quantitative comparison of human male *(M)* and female *(F)* DNA. The samples were tested with probe cY97 under low stringency **A** to check that the amounts of male and female DNA loaded on the filter were roughly the same. When the same filter was reprobed under high stringecy **B** with probe cY97 it showed a stronger signal for male DNA

lite lambda 33.6 was used to quantitate the DNA and to positively confirm the presence of DNA on the filter, even if non-human DNA is present [22]. Because of the complexity of reference controls, we think that the dot blot method is more useful for species exclusion rather than for sex determination.

Until now sex determination by DNA analysis has been carried out mainly with probes recognizing repeated sequences of the Yq arm [2–7]. This arm can be reduced and even lost without affecting a normal male phenotype [8, 9] and in 1 out of 3,000 normal females it is carried as a translocation [23]. This will cause at least a corresponding probability of false positives and of false negatives. Since probe cY97 recognizes the centromeric sequences of the Y chromosome, false positives and negatives should be limited only to XX males and XY females whose incidence is rather low. Nevertheless all these genetic anomalies could be covered by the combined use of arm specific probes and of a centromeric specific probe such as cY97.

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