2.2 Methodology

THE USE OF A CHEMILUMINESCENT DETECTION SYSTEM FOR PATERNITY AND FORENSIC TESTING. VERIFICATION OF THE RELIABILITY OF THE OLIGONUCLEOTIDE-PROBES USED FOR GENETIC ANALYSIS

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SUMMARY

Several VNTR loci, that had been characterized by the use of P^{32}-labeled recombinant DNA fragments or oligonucleotides to the consensus sequence, were analyzed using oligonucleotide-Alkaline Phosphatase conjugates and a chemiluminescent detection method. The loci tested were D2S44, D4S163, D12S11, D14S13, D17S79, D18S27, DXYS14 and DNF24. Individuals, already typed using P^{32}-labeled probes, were used in this study. Pst I or Hae III-digested DNA from approximately 200 to 400 individuals, covering the full size range of alleles, were examined for each locus. The results obtained for these loci show that, with the exception of D2S44, all the alleles were recognized with the AP-oligos. D2S44 contained a subset of alleles that could only be detected by hybridization to recombinant DNA fragments. In forensic applications, results obtained using DNA recovered from evidentiary material show that most probes detect 10 ng of genomic DNA after an overnight exposure. This indicates that the sensitivity of this detection system is equal or better to that obtained with P^{32}-labeled probes.

INTRODUCTION

The use of oligonucleotides, conjugated with alkaline phosphatase (AP-oligo), and a chemiluminescent detection system provides a convenient and sensitive assay for the detection of DNA polymorphisms routinely used for identity testing (Baum et al. 1990). The detection of a polymorphism relies on the hybridization of oligonucleotides homologous to the variable number of repeats present at these loci. The size of an AP-oligo, used as a hybridization probe, varies up to 30 bases, while the size of the consensus sequences can vary from 14 to 72 bp. Thus, while some of the AP-oligos include up to 2 full copies of the consensus sequence others contain only a subset. Therefore, the purpose of this study was to test whether an AP-oligo can identify all the different size DNA fragments (alleles) of a locus.

MATERIALS AND METHODS

Isolation of DNA from samples of peripheral blood or blood stains was performed as described by Balazs et al. 1989 or Grimberg et al. 1989. DNA from semen stains was

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prepared by the procedure described by Giusti et al. 1986. DNA samples were digested with 10 fold excess of Hae III, Hinf I or Pst I according to manufacture recommendations. Fractionation of DNA fragments and transfer to nylon membrane has been described (Balazs et al. 1988).

Hybridization to AP-oligos to DNA-membranes was performed using the Quick-Light™ hybridization kit (Lifecodes Corp.). Briefly, membranes were placed in a plastic container, pre-hybridized for 10 min, hybridized for 10 min to the AP-oligo and washed 4 times for 10 min. each. All of these incubations were done at 55°C with agitation. Membranes were treated with a blocking solution, rinsed with buffer at room temperature, sprayed with Lumi-Phos 480™ (Lumigen Inc.), sealed in a plastic folder and exposed to XAR5 (Kodak) X-ray film for 1-6 hours at 37°C or overnight, at room temperature.

RESULTS AND DISCUSSION

Specificity and sensitivity of AP-oligos. DNA samples covering the full size range of alleles for a locus were selected from databases that had been developed using P^{32}-labeled probes. These samples were hybridized to AP-oligos and the patterns generated by these 2 types of probes were compared. For most of the loci examined, there was complete concordance between the patterns generated by both type of probes. The only exception was the oligonucleotide homologous to the consensus sequence for D2S44. This sequence does not anneal to a subset of alleles in approximately 4% of North American Black and 1% of North American Caucasian population. Although this does not pose a problem for establishing sample identity, it can result in false parental exclusion.

The temperature of the hybridization and washing steps (55°C) were found, in general, to produce minimal or undetectable cross-hybridization to DNA fragments from other loci. The AP-oligos used to detect D14S13 in Pst I and Hae III digested DNA identified, in addition to the main locus, several weaker polymorphic and monomorphic bands that did not permit easy interpretation of results. Increased stringency of hybridization or washing resulted in a loss in sensitivity without significant gain in specificity.

The sensitivity of the AP-probes was measured by hybridizing each probe to a membrane containing a serial dilution of Pst I or Hae III-digested genomic DNA, ranging from 500 to 5 ng/lane. The sensitivity of some of the AP-oligos was sufficient to detect as little as 10ng of genomic DNA after an overnight exposure.

Analysis of paternity cases. Each of the AP-oligos used in paternity testing, using Pst I-digested DNA, were hybridized to 400 - 500 paternity cases that had previously been typed using P^{32}-labeled probes. The most striking effect seen from the results was the increased sharpness of the bands with AP-oligos (Fig.1) as compared with P^{32}-probes (Fig.2). This resulted in easier visualization of closely spaced DNA fragments with concomitant increase in resolution.

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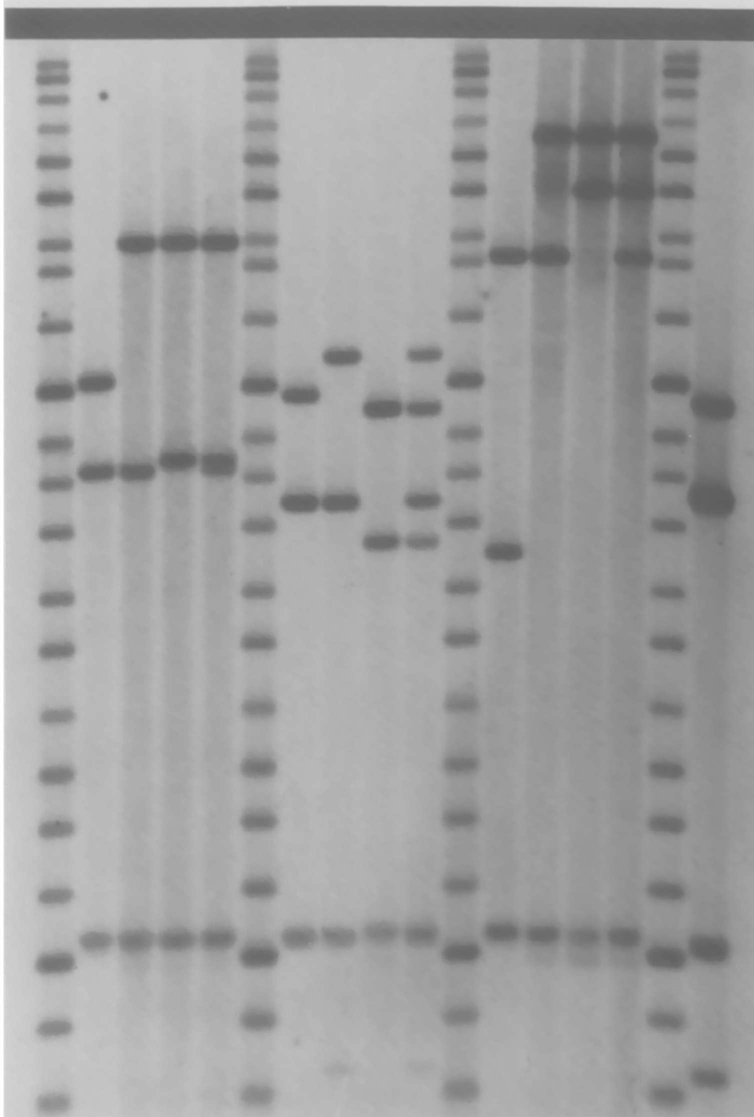
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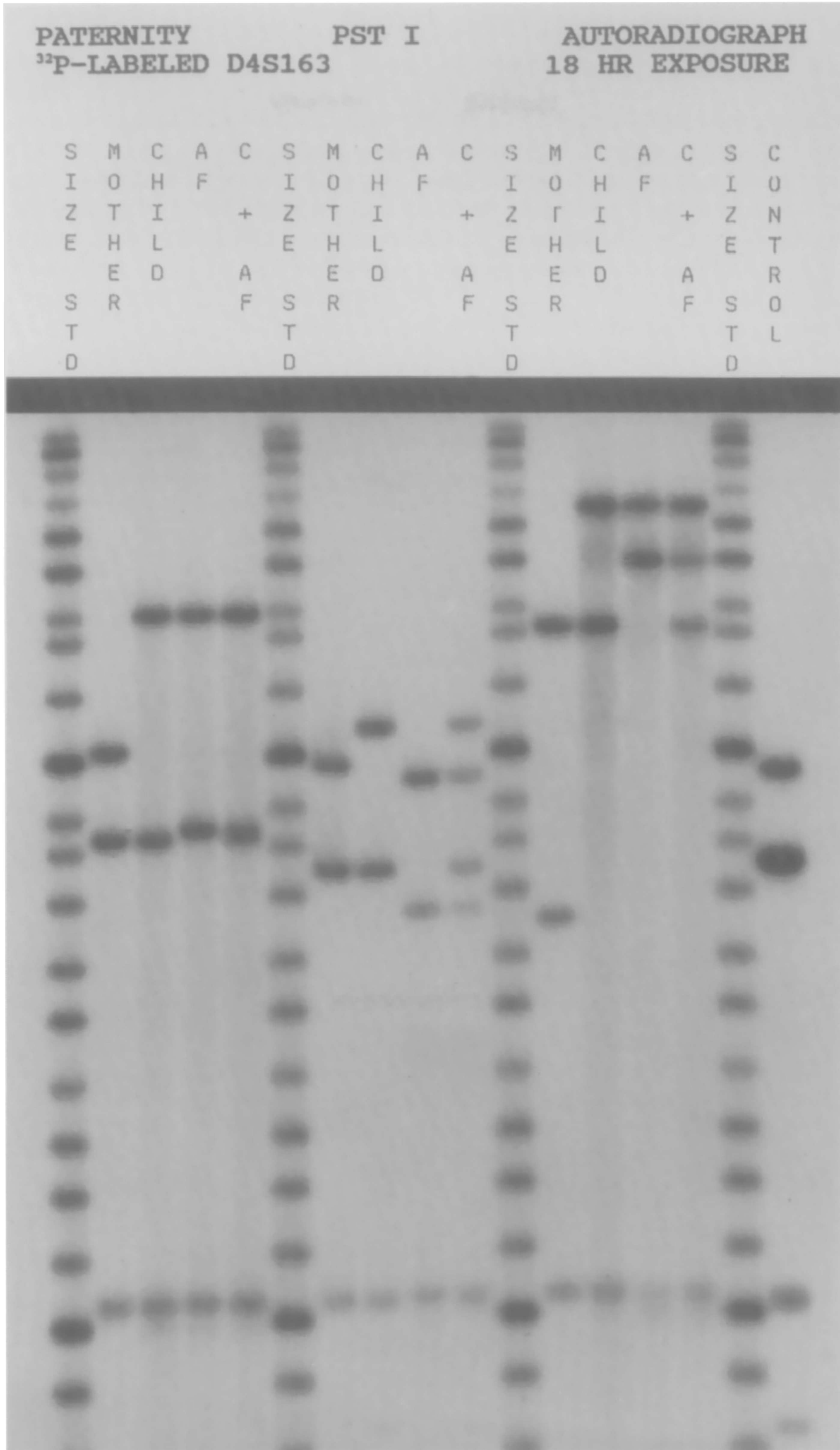
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The main advantages of the AP-probes are their convenience of use, speed, and safety. This methodology, when used in conjunction with fast and simple non-organic procedures for the isolation of DNA from blood and restriction enzyme digestion (Grimberg et al.1989), can produce samples that can be loaded into gels in under 8 hrs. By using the appropriate gel concentration, electrophoresis can be performed for 16 to 20 hrs, without sacrificing resolution. After DNA transfer to a nylon membrane (3 to 6 hrs), samples are ready for hybridization. As described in Materials and Methods, the entire process, from pre-hybridization to the point of exposing to film, takes 2 to 3 hrs. Paternity cases, prepared with 0.5 to 1 μg of DNA, produced sizable results in about 2 hrs of exposure at 37 °C. Therefore, the total time required to perform an entire paternity test can be less than 2 days.

Analysis of forensic cases. DNA samples were prepared from a variety of blood and semen stains. DNA was digested with Pst I or Hae III, fractionated by gel electrophoresis, transferred to nylon membrane (neutral or charged) and hybridized to AP-oligos. Membranes were repeatedly stripped and rehybridized up to 8 times with only small decrease in sensitivity. In addition, membranes that had been previously hybridized to P^{32}-labeled probes, and stripped, produced results comparable to new membranes. Finally, the results obtained by this non-isotopic detection system were the same as those obtained with P^{32}-labeled probes in terms of sensitivity and the final conclusions that could be drawn from them.

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