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SIMPLE NON-RADIOISOTOPIC DNA FINGERPRINTING WITH DIGOXIGENIN LABELLED M13 PHAGE USING CHROMOGENIC AND CHEMILUMINESCENT DETECTION SYSTEMS

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ABSTRACT

Improved conditions for non-radioisotopic DNA fingerprinting analysis using digoxigenin M13 probe were developed. Highly resolved banding patterns were obtained with specific-primed labelling of M13 DNA and Photogene membrane as a blotting medium under low stringency of hybridization conditions and post-hybridization washes. DNA fingerprinting of one family with 4 children and a pair of identical twins were performed and the signals were detected by both colourimetric and chemiluminescent detection systems. Readable pattern was generated with as little as 0.5 μg of human genomic DNA. This non-radioisotopic DNA fingerprinting was simple, reliable and should be applicable as an alternative to the isotopic method.

INTRODUCTION

DNA fingerprinting technique which was first described by Jeffreys *et al.*[1] has proven useful for individual identification [2,3], paternity testing [4,5] and forensic medicine [6]. The M13 bacteriophage, one of the most commonly available vector in the laboratory, has been widely employed to detect the hypervariable polymorphic patterns [7-9]. Generally, M13 DNA is labelled with the radioisotope phosphorus 32 which poses a health hazard, and the probe is unstable due to the short half-life. Therefore, alternative methods for non-radioactive labelling of probe have been developed for DNA fingerprinting analysis [10-12].

Recently, we have described a procedure for performing DNA fingerprints by specific-primed labelling of M13 probe with both ^{32}P and digoxigenin [3]. Here, improved conditions for DNA fingerprinting analysis using digoxigenin-labelled probe were developed. The procedure utilized both colourimetric and chemiluminescent methods to detect antidigoxigenin conjugated alkaline phosphatase. From our study, the optimal conditions for non-radioisotopic DNA fingerprinting analysis were obtained using the specific-primed labelling of M13 DNA with digoxigenin and the following conditions: 16 h of electrophoresis at 2.5 V/cm, vacuum blot on Photogene membrane, low stringency hybridization conditions and low stringency post-hybridization washes. The DNA fingerprints obtained under these conditions showed clear, intense banding patterns.

MATERIALS AND METHODS

DNA Preparation

Human DNA was extracted from white blood cells by a "salting out" method [13] and digested to completion with restriction endonuclease *Hae* III (BRL) at 5 U/μg under standard conditions [14].

Digested DNA was then size-fractionated by electrophoresis in a 20 cm long, 0.7% agarose gel with a Tris-borate buffer at 50 V for 16 h. DNA was then transferred to a Photogene membrane (BRL) using a vacuum blotting apparatus (2016 Vacugene LKB Bromma). The blotting steps were as described by Olszewska & Ken [15] except that the gel was denatured in 1.5 M NaCl, 1.5 M NaOH for 15 min and the vacuum blotting was continued for 2 h in 1M ammonium acetate to ensure maximum transfer of high molecular weight fragments. Afterwards the nylon membrane was removed and immersed in an excess of 1.5 M NaCl-1.5 M NaOH solution for 1 min to ensure complete denaturation of immobilized DNA. The membrane was then neutralized for 5 min in an excess of 1M ammonium acetate, then air dried for 10 min.

NON-RADIOISOTOPIC LABELLING OF M13 DNA WITH DIGOXIGENIN-11-dUTP

The labelling was done by primer extension using the specific 13-base oligonucleotide 5' AGCGTTTGCCATC 3' as a primer according to procedures previously described [3].

HYBRIDIZATION AND POST-HYBRIDIZATION WASHES

The Photogene membrane with newly transferred DNA was prehybridized and hybridized as described by Westneat *et al.*[16] Hybridization was carried out at 60 °C overnight or at least 3 h with the addition of digoxigenin-labelled probe (200-250 ng of probe /ml of hybridization buffer). After hybridization the membrane was washed twice with 2 x SSC containing 0.1% SDS at room temperature for 30 min and rinsed briefly with 1 x SSC.

DETECTION OF HYBRIDIZED PROBE

After the post-hybridization washes, the membrane was briefly washed in a washing buffer containing 100 mM Tris-HCl, pH 7.5, and 150 mM NaCl and blocked with the blocking reagent (provided by Boehringer Mannhiem) for 30 min at room temperature. After being washed briefly in washing buffer, the membrane was incubated at room temperature for 30 min with antidigoxigenin-conjugated alkaline phosphatase which was diluted to 150 mU/ml in washing buffer. The membrane was then washed 2 times for 15 min in washing buffer and equilibrated for 2 min in an equilibration buffer containing 100 mM Tris-HCl, pH 9.5,100 mM NaCl, and 50 mM $MgCl_2$. After equilibration the membrane was treated with alkaline phosphatase substrate, the NBT/BCIP, for colourimetric detection or the substituted dioxetane, 4-methoxy-4-(3-phosphatephenyl) spiro [1,2-dioxetane-3,2'-adamantane] (PPD) for chemiluminescent detection.

COLOURIMETRIC DETECTION

The membrane was incubated in the dark with a solution containing 45 µl of NBT solution (75 mg/ml of NBT in 70% dimethylformamide), 35 µl of X-phosphate solution (75 mg/ml of BCIP in 70% dimethyl-formamide) and 10 ml of equilibration buffer. The colour development was allowed to proceed for 3-16 h at room temperature. The membrane was washed for 5 min with 10 mM Tris-HCl, pH 7.4, and 1 mM EDTA, air dried and photographed.

CHEMILUMINESCENT DETECTION

The dioxetane reagent supplied by Lumigen, Inc. (Detroit, MI) was diluted tenfold and pipetted over the membrane. The membrane was then wrapped with plastic wrap and stored in the dark at room temperature for 45 min. The signals were recorded on the XAR film (Kodak, Rochester, New York) by 15 min exposure at room temperature.

RESULTS AND DISCUSSION

The conditions described in this paper were developed to provide a simple, rapid and reliable procedure to perform non-radioisotopic DNA fingerprints with digoxigenin-labelled M13 phage. Our previous study [3] had shown improvement of DNA fingerprinting pattern by specific-primed labelling of M13 DNA in which two 15-bp repeated core sequences of M13 DNA were labelled. We have now further investigated other conditions in order to enhance the banding patterns. For blotting medium, the Photogene membrane was found to be a good blotting medium due to its consistent ability to produce clear and distinct DNA fingerprints. Lower background signal was observed compared to the Genescreen Plus membrane reported previously [3]. Moreover, in our hand, the Genescreen Plus membrane was found to vary from lot to lot. The stringency of post-hybridization washes was modified from that of the radioisotopic method. Post-hybridization washes at lower stringency (2 x SSC, 0.1% SDS at room temperature for 60 min) yielded a superior banding pattern with a clean background. With these conditions DNA fingerprinting patterns were consistently obtained and the representative results are shown in Fig.1. The DNA from a 4-child family and a pair of twins from an unrelated family were hybridized with digoxigenin-labelled M13 probe and the signals were detected by colourimetric method using substrate NBT/BCIP. Strong intense bands were developed after 3 h.

The duration of colour development varied upon the amount of target DNA. When amounts of the target DNA were 5-10 µg, strong bands were visible in 2 to 3 min and the complete banding pattern was obtained after 3 h. Amounts of target DNA of less than 1 µg required at least 16 h for complete signal generation. Longer incubation time with the substrate caused a higher background signal (data not shown).

The sensitivity of detection was studied in order to compare with the radioisotopic and the other non-radioisotopic methods. Decreasing amounts of genomic DNA were digested, electrophoresed, blotted and hybridized; a readable pattern was obtained even when as little as 0.5 µg of sample was applied (Fig.2). Well resolved pattern was obtained

when 10-2 μg of samples were applied. Increasing the time for colour development to 16 h was needed to see the complete banding pattern. The sensitivity obtained under our conditions was comparable to other non-radioisotopic methods reported by other workers[10,11]. However, less sensitivity was found when compared with the radioisotopic method where only 0.25 μg of target DNA is required for a readable pattern[3].

The conditions reported in this paper were also applied to the chemiluminescent detection system with PPD substrate. This is the first time that the use of chemiluminescent method has been described for the detection of the hypervariable polymorphic DNA fingerprints. The chemiluminescent detection of a 4-child family and a pair of twins (see Fig.1) is shown in Fig. 3. A tenfold dilution of the dioxetane reagent was needed to decrease the background signals. Light output was recorded by 15 min exposure to X-ray film after 45 min of incubation with the PPD substrate. Thus, the results can be obtained within 1 h after adding PPD which was more rapid compared to 24-48 h required for autoradiography of radioactive method. Longer incubation with the substrate did not significantly increase the signal intensity. Increased exposure time however caused high background signal and should be avoided. The chemiluminescent system has been reported by other investigators to have higher sensitivity than the colourimetric method for the detection of viral and plasmid DNA[17]. However under our conditions, no significant difference in sensitivity was observed. Furthermore, detection using a chemiluminescent substrate created a higher background signal. Therefore, sensitivity was lost due to low signal to noise ratio. Another chemiluminescent detection system using horseradish peroxidase, or the ECL detection system, was also applied to DNA fingerprinting analysis. The banding patterns showed a high background signal (data not shown). Nevertheless, the chemiluminescent detection has the advantages of rapid detection time and simple manipulative steps. In addition, the filter can be reused, unlike the colourimetric method where the insoluble coloured product is very difficult to remove from the filter.

The DNA fingerprinting analysis using a non-radioisotopic labeled M13 probe described here should provide an alternative to the radioisotopic method despite its less sensitivity. It should replace the radioisotopic method for applications where the amount of the target DNA is not a limiting factor. These applications include paternity testing, monitoring reconstitution in bone marrow transplantation, and certain medical diagnosis.

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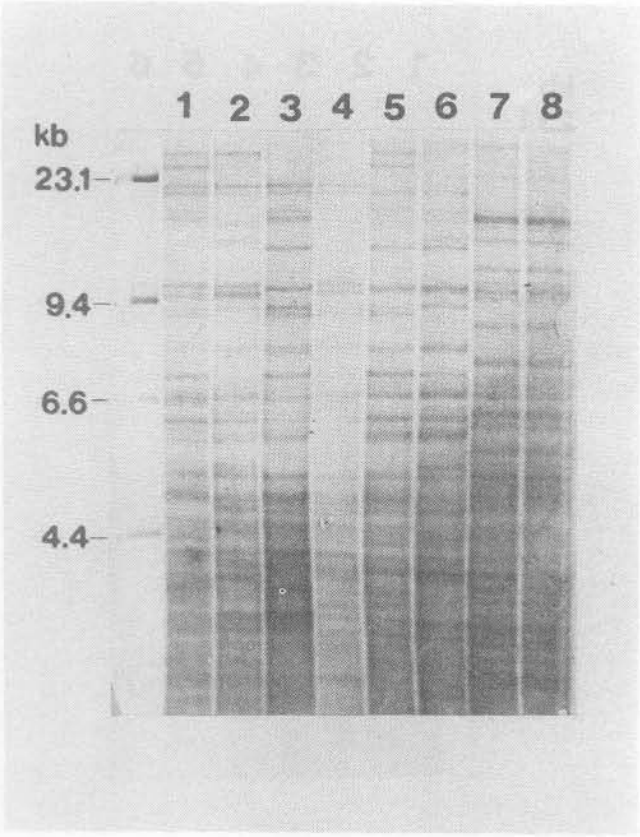

Fig 1. Colourimetric detection of DNA fingerprints from a 4-child family and a pair of identical twins hybridized with digoxigenated M13 probe. Ten micrograms of DNA were digested with *Hae* III, electrophoresed on 0.7% agarose gel at 50 V for 20 h and blotted on to a Photogene membrane. The hybridization, washing condition, and detection conditions were as described in materials and methods. Colour reaction with substrate BCIP/NBT was performed for 3 h.

lane 1	DNA fingerprint of father
lane 2	DNA fingerprint of elder sister
lane 3	DNA fingerprint of elder brother
lane 4	DNA fingerprint of younger sister
lane 5	DNA fingerprint of younger brother
lane 6	DNA fingerprint of mother
lanes 7-8	DNA fingerprints of identical twins

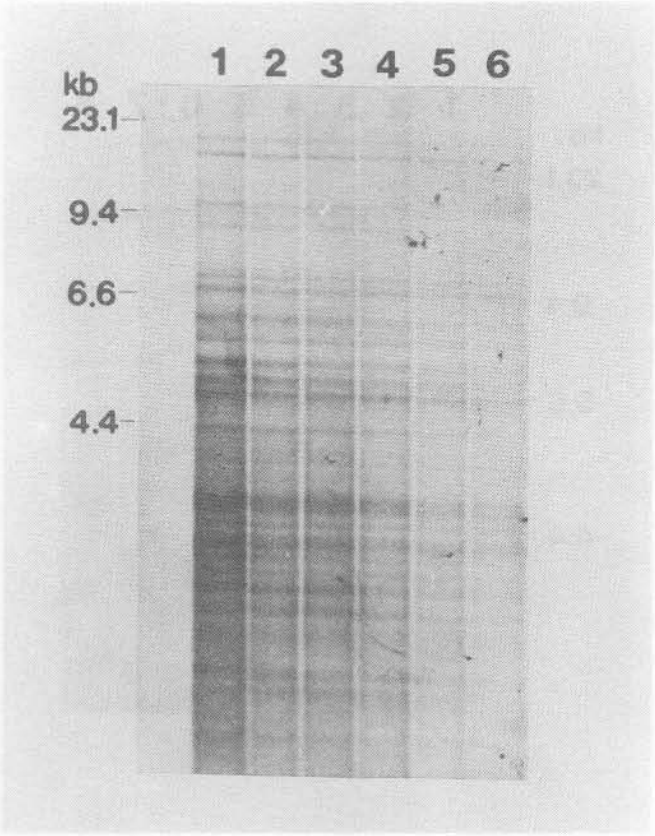

Fig 2. Colourimetric detection of DNA fingerprints of the same individual obtained by using specific-primed digoxigenated M13 probe hybridized to the electrophoresed human DNA after digestion with restriction enzyme *Hae* III. The amounts of DNA in lanes 1, 2, 3, 4, 5, and 6 were 10, 5, 3, 2, 1, and 0.5 μg respectively. The hybridization, washing condition, and detection conditions were as described in materials and methods. Colour reaction with substrate BCIP/NBT was performed for 16 h.

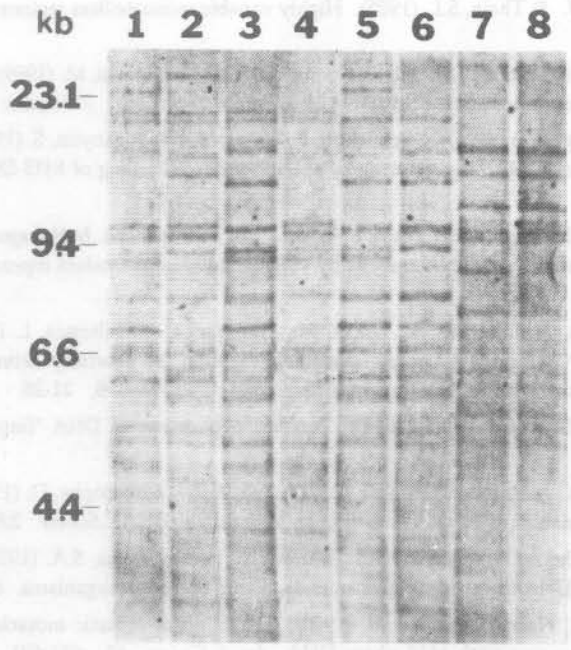

Fig 3. Chemiluminescent detection of DNA fingerprints from a 4-child family and a pair of identical twins hybridized with digoxigenated M13 probe. Ten micrograms of DNA were digested with *Hae* III, electrophoresed on 0.7% agarose gel at 50 V for 20 h and blotted onto a Photogene membrane. The hybridization, washing condition, and detection conditions were as described in materials and methods. The film exposure was for 15 min after 1 h incubation with substrate PPD.
Lanes 1-8 are the same as in Fig. 1.

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บทคัดย่อ

ได้ทำการพัฒนาการสร้างลายพิมพ์ดีเอ็นเอด้วยวิธีที่ไม่ต้องอาศัยสารกัมมันตภาพรังสีโดยเลือกใช้ไวรัสM13ที่เตรียมขึ้นเอง แล้วนำมาติดฉลากด้วย digoxigenin ตามด้วยการแสดงสัญญานแบบสี (chromogenic detection) และแบบแสง (chemiluminescent detection) พบว่าสามารถพัฒนาจนได้ลายพิมพ์ดีเอ็นเอที่ชัดเจน เมื่อทำการติดฉลาก M13 โดยวิธี specific-prime labelling ในการศึกษานี้ได้เลือกใช้ Photogene membrane เป็น blotting medium ภายใต้สภาวะในการทำ hybridization และ post-hybridization ที่ stringency ต่ำ

วิธีที่พัฒนาได้ถูกนำไปใช้ตรวจสอบลายพิมพ์ดีเอ็นเอของครอบครัวที่มีลูก 4 คน และตรวจการเป็นฝาแฝดที่เกิดจากไข่ใบเดียวกัน นอกจากนี้ยังสามารถทำได้โดยใช้ดีเอ็นเอเป้าหมายเพียง 0.5 ไมโครกรัมเท่านั้น การสร้างลายพิมพ์ดีเอ็นเอโดยใช้เทคนิคที่ไม่ใช้สารกัมมันตภาพรังสีนี้มีข้อดี คือ ง่าย เชื่อถือได้ สะดวกและปลอดภัย ซึ่งสามารถนำมาใช้แทนสารกัมมันตภาพรังสีได้