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Validation Studies on the Analysis of the HLA DQα Locus Using the Polymerase Chain Reaction

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ABSTRACT: A series of experiments has been performed to evaluate typing of the HLA DQα gene by polymerase chain reaction (PCR) amplification of the gene and subsequent hybridization with sequence-specific oligonucleotide probes. These experiments were designed to evaluate DQα typing for analysis of evidentiary specimens. Bloodstains were exposed to a variety of conditions and environmental insults. These conditions included exposure to many different types of substrates, various microorganisms that could be encountered in evidentiary stains, sunlight, and a variety of chemical contaminants. Varying amounts of genomic deoxyribonucleic acid (DNA) were amplified to test the sensitivity of DQα typing. The sensitivity of the PCR technique raises the concern that DNA from sources other than the evidentiary material could be detected. A series of experiments was done to evaluate the question of DNA contamination. Purified DNA samples with different DQα types were mixed in different ratios to determine the ratio at which it could not be determined whether an allele was from the sample or the contaminant. Samples were exposed to a variety of situations that could lead to contamination, such as extensive handling and exposure to coughing or sweaty clothing, to other wet bloodstains, and to saliva. The DQα types were determined from 469 individuals from three sample populations (Caucasian, black, and Hispanic), and the genotype frequencies were compared with frequencies previously reported by others. DNA samples from old cases [which had previously been analyzed by restriction fragment length polymorphism (RFLP) typing of variable number of tandem repeat sequences] were typed. All samples that were excluded by DQα typing were also excluded by RFLP analysis, and all samples that were included by RFLP analysis were included by DQα typing. Finally, the problem of allele dropout, or the failure to detect particular alleles, was noted and alleviated by performing the typing under appropriate conditions. The results of these validation experiments indicate that typing of the DQα gene by PCR and detection of specific alleles can be accomplished, when the typing is done using proper protocols, without producing false positive or false negative results.

KEYWORDS: pathology and biology, genetic typing, polymerase chain reaction (PCR), deoxyribonucleic acid (DNA)

The detection of variable number of tandem repeat (VNTR) sequences by restriction fragment length polymorphism (RFLP) analysis is a powerful technique for characterizing forensic science samples [*1,2*]. Analysis of samples with several polymorphic VNTR probes requires a minimum of 10 to 50 ng of relatively undegraded deoxyribonucleic acid

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(DNA). However, some evidentiary samples do not contain this much DNA, or the DNA is degraded to an extent that it is not suitable for RFLP analysis. Genetic information can be obtained from such samples, as well as from samples with DNA of sufficient quality and quantity for RFLP analysis, by amplification of the DNA through the polymerase chain reaction (PCR) [3], followed by detection of polymorphic alleles. Furthermore, genetic typing via PCR amplification can be accomplished in a few days, in contrast to the several weeks required for RFLP typing.

Target DNA molecules are amplified in PCR by a series of denaturation, primer annealing, and primer extension steps. During each cycle, the target DNA is replicated. The choice of primers and reaction conditions allows amplification of specific regions of the target DNA, generating millions of copies of that region. The use of the thermally stable enzyme *Taq* polymerase for primer extension [4] facilitates the automation of the PCR process by the use of a thermal cycling machine.

A kit is commercially available (AmpliType®, Cetus Corp., Emeryville, California) which contains reagents to amplify and type a region of one of the major histocompatibility complex genes, DQα [5]. This Class II antigen gene is polymorphic, exhibiting six common alleles: four nominal alleles, Alleles 1, 2, 3, and 4, and the 1 allele subtypes 1.1, 1.2, and 1.3. Thus, 21 genotypes can be detected in the population. The variations that determine these alleles are located in a 242 base pairs (bp) region (239 bp for Alleles 2 and 4) in the second exon of the DQα gene. Primers that flank this region are used in the amplification, and allele-specific oligonucleotide (ASO) probes have been developed to detect each of the DQα alleles [5,6]. These probes are fixed to a nylon membrane (typing strips), and DQα alleles are identified by hybridization with the amplified DQα DNA.

Before a technique can be used to analyze evidentiary samples, it must be properly validated [7]. To this end, a series of experiments has been carried out using the PCR/DQα typing system. These experiments included (1) evaluation of various DNA extraction methods for their effectiveness at yielding DNA of the quality needed for amplification; (2) amplification and typing of DNA exposed to a variety of contaminants, adventitious substances, and environmental insults; (3) amplification and typing of old case samples previously analyzed by RFLP technology; and (4) determination of population frequency data for the DQα alleles for several ethnic groups.

Materials and Methods

DNA Extraction

DNA for PCR amplification was extracted from liquid blood samples as has been previously described [1]. A variety of methods for DNA extraction from bloodstains were used for the validation experiments described below and are outlined here. Some bloodstains were extracted using an organic extraction procedure, previously described [2]. This procedure included sodium dodecyl sulfate (SDS)/proteinase K digestion, phenol/chloroform/isoamyl alcohol (24:24:1) extraction, and ethanol precipitation. Other stains were extracted using a modified organic extraction protocol as follows: The stains were soaked in 500 μl of water at 56°C for 2 h prior to SDS/proteinase K digestion. After the water soak, the stain cuttings were placed in the lid of the microcentrifuge tube (Sarstedt, previously punctured), and the tubes were spun in a microfuge for 1 min. The cuttings were placed in a clean tube and extracted as described by Budowle et al. [2]. For six substrates (oilcloth, vinyl upholstery, and scrapings from plastic, drywall, wood, and leaves) the pellet resulting from the centrifugation was also extracted using the organic extraction procedure. Some stains were extracted using a Centricon extraction procedure. These stains were extracted using the organic extraction protocol, except that three

organic extractions (that is, phenol, chloroform, and isoamyl alcohol) were done. Following the organic extractions, the samples were extracted one time with 500 μl of butanol and then placed in a Centricon 100® microconcentrator (Amicon), to which 1.5 mL of TE buffer [consisting of 10m*M* tris(hydroxymethyl)aminomethane (Tris)/chlorine (Cl) and 0.1m*M* ethylenediaminetetraacetic acid (EDTA), at pH 8.0] had been added. The tubes were subjected to centrifugation at 1000 × *g* for 20 min in a Sorvall RC-5 centrifuge (DuPont) using the GSA rotor. Two millilitres of TE buffer were added, and the tubes were subjected to centrifugation again. This wash step was repeated once more (for a total of three washes), then the sample was collected by centrifugation at 500 × *g* for 5 min. In addition, some bloodstains were extracted using a nonorganic extraction procedure described by Grimberg et al. [*8*], which included lysis of cells with Triton X-100, followed by nuclear lysis with proteinase K and lithium chloride precipitation. The extraction method used for each set of experiments is indicated in the sections below.

DNA Amplification

DNA samples were amplified in 50 or 100-μL reactions containing 50m*M* potassium chloride (KCl), 10m*M* Tris-Cl, at pH 8.3, 1.5m*M* magnesium chloride ($MgCl_2$), 0.01% gelatin, 0.2m*M* dATP, dCTP, dGTP, and TTP, 0.2m*M* DQα primers (GH26 and GH27 [*5*], and 1.25 units of *Taq* polymerase per 50 μL of reaction volume. Some samples were amplified in 100-μL reactions using prepackaged 2 × premixes (Cetus Corp., Emeryville, California). All the samples were overlaid with 50 μL of mineral oil, then amplified in Perkin-Elmer/Cetus thermal cyclers (Model TC-1). The PCR profiles used for each set of experiments are listed in the sections below. The profiles used were those recommended by Cetus Corp. at the time a particular experiment was performed; these profiles changed several times during the development of the kit by Cetus. An evaluation of the PCR product was done by subjecting 5 or 10% of each sample to electrophoresis on 2% agarose gels made in TBE (consisting of 100m*M* Tris, 100m*M* boric acid, and 2m*M* EDTA, at pH 8.3), containing 0.5 μg/mL of ethidium bromide, and visualization of the 239 or 242 bp DQα product using an ultraviolet light.

DQα Typing

Amplified DQα DNA samples were typed with allele-specific oligonucleotide (ASO) probes using either the dot blot procedure [*6*] or the reverse dot blot procedure [*5*] [AmpliType® DQα typing kit (Cetus Corp., Emeryville, California)]. In the dot blot procedure, the horseradish-peroxidase-labeled probes were hybridized with nylon membranes to which amplified DQα DNA had been fixed. This was accomplished by denaturing 20 μL of amplified DNA in 1600 μL of 0.4*N* sodium hydroxide (NaOH) and applying 200 μL to a nylon membrane through each of eight wells in a dot blot apparatus. The membrane was then cut so that each sample could be hybridized with one of the eight probes. The concentration of each probe during hybridization was as follows: DQA1, 0.75 pmol/mL; DQA2, 0.5 pmol/mL; DQA3, 0.25 pmol/mL; DQA4, 0.5 pmol/mL; DQA1.1, DQA(1.2, 1.3, 4), DQA1.3, and "all but DQA1.3" were all 0.5 pmol/mL. Hybridization was done as described by Higuchi et al. [*6*]. Duplex formation was detected by the horseradish peroxidase catalyzed reaction, which oxidizes the colorless soluble substrate 3,3′,5,5′-tetramethylbenzidine (TMB) and forms a blue precipitate.

The reverse dot blot procedure utilized biotin-labeled primers in the amplification. During hybridization of amplified DQα DNA with the typing strips, a streptavidin/horseradish peroxidase complex was added. The streptavidin binds to biotin (present on the 5′ end of every amplified DNA molecule), and hybridization was detected by oxidation of TMB. Thirty-five microlitres of each 100-μL amplification reaction was used for typing. The hybridizations and stringency washes were done at 55°C.

Repetitive Analysis of Identical Samples

DNA was isolated from a sample of liquid blood (DQα type 1.1, 4 [*1*]). One hundred aliquots of 100 ng each were amplified (at 94°C for 60 s, 60°C for 30 s, 72°C for 30 s for 34 cycles, followed by a final 7-min extension at 72°C) and typed using the reverse dot blot format.

Effect of Various Hybridization/Wash Temperatures

DNA was isolated from three 50-μL bloodstains (DQα types 1.1, 4; 1.2, 3; and 1.3, 4) using the Centricon extraction method. Twelve replicate amplifications of each sample were done, each using 5% of the extract. The amplification conditions were at 94°C for 60 s, 60°C for 30 s, 72° for 30 s for 34 cycles, followed by a final 7-min extension at 72°C. Two replicates of each sample were typed using the reverse dot blot format at each of six different hybridization/wash temperatures (52, 53, 54, 56, 57, and 58°C). Thus, six samples were typed at each of the six different temperatures.

Environmental Insult Studies

Substrates—Fifty-microlitre bloodstains were made on a variety of substrates (Table 1), dried, and then stored at −20°C from several days to several months. The substrates included a wide variety of fabric types, vinyl, plastic, drywall, wood, linoleum, metal, and leaves. DNA was extracted from replicate 50-μL stains using each of the four methods described above (organic, modified organic, Centricon, and nonorganic). For the organic, modified organic, and nonorganic methods, 2.5% of each extract was amplified (93°C for 30 s, 55°C for 30 s, 72°C for 30 s for 35 cycles, followed by a final 7-min extension at 72°C) in 50-μL reactions. The samples were typed using the dot blot format. For the Centricon method, 25% of each extract was amplified, and the samples were typed using the reverse dot blot format.

Chemicals—Bloodstains contaminated with a variety of substances were prepared as previously described [*9,10*]. Washed cotton sheeting was spotted with 50 μL of each contaminant, allowed to dry briefly, and spotted with 50 μL of whole blood. The contaminants used included unleaded gasoline, 10W40 motor oil, 10% SDS, 0.1*M* potassium phosphate (KPO_4) buffer (pH 6.8), household bleach, 1*N* NaOH, and 1*M* acetic acid. In addition, 50 μL of blood was spotted on 0.1 g of soil. Stains consisting of only the contaminants were made to serve as negative controls. All the experimental and control stains were made in duplicate. After storage at room temperature for 5 days (shielded from light), DNA was extracted from all stains using the Centricon purification procedure. Approximately 4% of each sample was amplified (93°C for 30 s, 55°C for 30 s, 72°C for 30 s for 35 cycles, followed by a final 7-min extension at 72°C), and the samples were typed using the reverse dot blot format.

Microorganisms—Four microorganisms commonly expected to be associated with evidentiary stains were cultured overnight [*10*]. These included the bacteria *Escherichia coli*, *Bacillus subtilis*, and *Staphylococcus epidermidis* and the yeast *Candida albicans*. Aliquots of 50 μL of the overnight cultures were spotted on washed cotton sheeting. The stains were allowed to dry briefly, and 50-μL bloodstains were made on top of the microbial stains. All the stains were made in duplicate. Duplicate microbial stains were made and not stained with blood to serve as negative controls. After 5 days at room temperature, DNA was extracted from all stains using the Centricon purification procedure. Approximately 4% of each sample was amplified (94°C for 60 s, 60°C for 30 s, 72°C for 30 s for 32 cycles, followed by a final 7-min extension at 72°C), and the samples were typed using the reverse dot blot format.

TABLE 1—*Amplification and typing of DNA from bloodstains on various substrates.*[a]

Sample	Extraction Method				
	O	NO	MO	MO(P)	CE
Scrapings from glass	–	+	+	ND	+
Cotton sheeting	–	+	+	ND	+
Tee shirt	–	+	+	ND	+
Sweatshirt	+	+	+	ND	+
Lightweight cotton weave	–	+	+	ND	–
White denim	–	+	+	ND	–
Blue denim	–	–	–	ND	–
Corduroy	–	+	+	ND	–
Terry cloth	–	+	+	ND	+
Sweater	–	+	+	ND	+
Lightweight wool blend	–	+	+	ND	+
Heavy-weight wool blend	+	+	+	ND	+
Polyester	–	+	+	ND	+
Nylon stocking	–	+	+	ND	+
Nylon lace	–	+	+	ND	+
Acetate lining	–	+	+	ND	+
Peau de soie	–	+	+	ND	–
Filter paper	+	+	+	ND	+
Brown paper bag	–	+	+	ND	–
Suede fabric	–	+	–	ND	–
Suede leather	–	+	+	ND	+
Cotton upholstery	–	–	–	ND	–
Oilcloth	+	+	+	+	+
Cotton backing of oilcloth	–	+	+	ND	+
Vinyl upholstery	+	+	+	+	+
Knit back of vinyl upholstery	–	+	+	ND	+
Scrapings from plastic	–	+	ND	+	ND
Scrapings from drywall	–	–	–	+	–
Scrapings from wood	–	–	–	+	–
Scrapings from linoleum	+	+	+	ND	+
Scrapings from metal	–	+	+	ND	ND
Scrapings from leaves	–	–	–	–	–
Carpet	+	+	+	ND	+
Nylon panties	–	+	+	ND	ND
Total (n = 34)	7 (21%)	29 (85%)	30 (91%)		20 (67%)

[a] Key to abbreviations:
O = organic extraction method.
NO = nonorganic extraction method.
MO = modified organic extraction method.
MO(P) = pellet from modified organic extraction method.
CE = Centricon extraction method.
\+ = typing results obtained.
– = no typing results.
ND = amplification/typing not done.

Sunlight exposure—Fifty-microlitre bloodstains were made on washed cotton sheeting, and the stains were placed in a greenhouse. Half of the stains were protected from light, while half were exposed to daylight through the greenhouse glass. The stains were collected at 1, 2, 3, 4, 7, 8, 9, 10, and 11 days, and at 2, 4, 6, 8, 12, 16, and 20 weeks (that is, a total of 16 time periods). The stains were placed in the greenhouse in July; therefore, the 20-week samples were collected at the end of November. Some stains were placed outdoors in clear plastic petri dishes. Half of these stains were protected from light. The

samples were collected daily for 20 days (except that no 19-day collection was made) and stored at −70°C until it was convenient to perform the DNA extractions, which were done using the Centricon purification procedure. Approximately 4% (greenhouse) or 8% (petri dish) of each sample was amplified as follows: (*a*) 93°C for 30 s, 55°C for 30 s, 72°C for 30 s for 35 cycles, followed by a 7-min extension at 72°C for the greenhouse stains exposed for up to two weeks; (*b*) 94°C for 60 s, 60°C for 30 s, 72°C for 30 s for 34 cycles, followed by a 7-min extension at 72°C for the greenhouse samples exposed for more than two weeks; and (*c*) 94°C for 60 s, 60°C for 30 s, 72°C for 30 s for 32 cycles, followed by a 7-min extension at 72°C for samples in the petri dishes. The samples were then typed using the reverse dot blot format. Duplicate stains were made and analyzed.

Sensitivity of Detection and Detection of Mixed Samples

Genomic DNA extracted from whole blood [*1*] from samples with three different DQα genotypes (1.2, 3; 1.2, 4; and 1.1, 3) was quantified by spectrophotometry and serially diluted. The following quantities of DNA of each sample were amplified: 500, 250, 125, 63, 31, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, and 0.063 ng. The amplification conditions were 94°C for 60 s, 60°C for 30 s, 72°C for 30 s for 32 cycles, followed by a final 7-min extension at 72°C. The samples were typed using the reverse dot blot format.

Serial dilutions of spectrophotometrically quantified DNA samples with DQα types 1.2, 4, and 1.1, 3 were mixed in the amounts and ratios listed in Table 2. Mixtures of DNA were made with 1.2, 4 as the majority type and 1.1, 3 as the minority type, as well as with 1.1, 3 as the majority type and 1.2, 4 as the minority type. The mixtures were amplified (94°C for 60 s, 60°C for 30 s, 72°C for 30 s for 32 cycles, followed by a final 7-min extension at 72°C) and typed using the reverse dot blot format.

Induced Contamination and Effects of Sample Handling

The effects of exposure to a variety of possible sources of DNA contamination on the amplification and typing of samples were evaluated. Bloodstains were exposed to a variety

TABLE 2—*Composition of mixed DNA samples.*[a]

Amount of Majority Type, ng	Amount of Minority Type, ng	Ratio
200	200	1:1
20	20	1:1
2	2	1:1
0.2	0.2	1:1
15	10	3:2
10	5	2:1
20	5	4:1
25	5	5:1
35	5	7:1
200	20	10:1
200	2	100:1
200	0.2	1000:1
20	2	10:1
20	0.2	100:1
2	0.2	10:1

[a]The mixtures were made with DNA with DQα type 1.2, 4 as the majority type and 1.1, 3 as the minority type, as well as with 1.1, 3 as the majority type and 1.2, 4 as the minority type. The DNA quantity was determined spectrophotometrically.

of conditions which potentially induce DNA contamination. Table 3 lists the various contamination scenarios set up. In addition, the stain extraction buffer and PCR premix were exposed to shed scalp skin, introduced by a laboratory worker scratching her head over open tubes. Stains composed of blood (of one DQα type) and saliva (of another DQα type) were also made. DNA was extracted from all the stains, and 4% of each sample was amplified (94°C for 60 s, 60°C for 30 s, 72°C for 30 s for 32 cycles, followed by a final 7-min extension at 72°C). The samples were typed using the reverse dot-blot method.

"Evidence" from a mock crime scene class was kindly provided by R. E. Gaensslen (University of New Haven). These items were evaluated for potential contamination introduced by handlers of evidence or by wearers of the clothing. The evidence consisted of a shirt and a pair of slacks which were purchased new and laundered; and a shirt and pair of shorts which had been worn extensively by one person and which were laundered prior to use in the crime scene class. The evidentiary items were stained with pig blood. Twenty cuttings were taken from these four items and extracted using the Centricon method. Approximately 25% of each sample was amplified (94°C for 60 s, 60°C for 30 s, 72°C for 30 s for 32 cycles, followed by a 7-min extension at 72°C), and the samples were typed using the reverse dot blot format.

TABLE 3—*Induced contamination and mixed stains.*

Contamination Source	Primary Type	Contaminant Type	Alleles Detected
Induced Contamination			
Dried stains stored together	1.1, 4	3, 3	1.1, 3, 4[a]
	3, 3	1.1, 4	3
Wet stains stored together	1.1, 4	3, 3	1.1, 3, 4[a,b]
	3, 3	1.1, 4	1.1, 3, 4[a,b]
1 μL blood/shirt with heavy perspiration	1.1, 4	1.1, 2	1.1, 4[b]
10 μL blood/shirt with light perspiration	1.1, 4	1.1, 2	1.1, 4[b]
Cutting from shirt with heavy perspiration	NA	1.1, 2	–[b]
Cutting from shirt with light perspiration	NA	1.1, 2	–[b]
1 μL bloodstains/5 min handling	1.1, 4	3, 3	1.1, 4[b]
10 μL bloodstains/5 min handling	1.1, 4	3, 3	1.1, 4
	1.1, 4	2, 2	1.1, 4
1 μL bloodstain/1 min coughing	1.1, 4	3, 3	1.1, 4[b]
10 μL bloodstain/1 min coughing	1.1, 4	3, 3	1.1, 4
	1.1, 4	2, 2	1.1, 4
1 μL bloodstain/30 s shed scalp cells	1.1, 4	3, 3	1.1, 4[b]
10 μL bloodstain/30 s shed scalp cells	1.1, 4	3, 3	1.1, 4
	1.1, 4	2, 2	1.1, 4
Extraction buffer/30 s shed scalp cells	NA	3, 3	–[b]
PCR premix/30 s shed scalp cells	NA	3, 3	–
10 μL bloodstain/contaminated scissors	1.1, 4	3, 3	1.1, 4
Mixed Stains			
10 μL bloodstain/10 μL saliva	1.1, 4	3, 3	1.1, 3, 4[c]
1 μL bloodstain/10 μL saliva	1.1, 4	3, 3	1.1, 3, 4[d]
10 μL bloodstain/1 μL saliva	1.1, 4	3, 3	1.1, 3, 4[e]
1 μL bloodstain/1 μL saliva	1.1, 4	3, 3	1.1, 3, 4[d]
10 μL bloodstain/1 cm^2 saliva-soaked cloth	1.1, 4	3, 3	1.1, 3, 4[d]
1 μL bloodstain/1 cm^2 saliva-soaked cloth	1.1, 4	3, 3	1.1, 3, 4[d]

[a]The 3 allele was less intense than the C dot.
[b]An amount of 10 μL (20% of extract) was amplified in addition to the usual 2 μL (4%).
[c]The 3 allele was more intense than the 1.1 and 4 alleles; the 1.1 and 4 alleles were approximately equal to the C dot.
[d]The 1.1 and 4 alleles were less intense than the C dot.
[e]The 1.1, 3, and 4 alleles were of approximately equal intensity and more intense than the C dot.

Analysis of Old Case Samples

DNA samples extracted from old casework samples at the Federal Bureau of Investigation (FBI) Laboratory were obtained (243 samples from 44 cases). The samples had been extracted as previously described [*2*]. Approximately 10% of the extracted DNA from case stains or vaginal swabs was amplified (93°C for 30 s, 55°C for 30 s, 72°C for 30 s for 35 cycles, followed by a 7-min extension at 72°C) and typed using either the dot blot or reverse dot blot procedure. The results were compared with RFLP results obtained in the FBI Laboratory.

Population Frequency Data

Blood samples from 150 Caucasian volunteer donors at the FBI Academy and from 193 blacks and 128 Hispanics from Baylor University School of Medicine were analyzed. The DNA was extracted from liquid whole blood samples according to the method described by Budowle and Baechtel [*1*]. Approximately 8 ng of DNA was used for PCR. The amplification conditions were (*a*) for the Caucasian samples, 93°C for 30 s, 55°C for 30 s, 72°C for 30 s for 35 cycles, followed by a 7-min extension at 72°C; (*b*) for the black and Hispanic samples, 94°C for 60 s, 60°C for 30 s, 72°C for 30 s for 34 cycles, followed by a 7-min extension at 72°C. The samples were typed using the reverse dot blot format.

Results

Comparison of DNA Extraction Methods and Effect of Various Substrates on Amplification and Typing of Bloodstain DNA

To evaluate various extraction methods for their effectiveness in yielding amplifiable DNA from bloodstains made on a variety of substrates, bloodstains (DQα type 1.1, 4) were made on approximately 30 different fabric types (both synthetic and natural), as well as on drywall, linoleum, metal, paper, wood, and leaves. Table 1 lists the substrates used and typing results obtained from DNA extracted from bloodstains on these substrates. The organic extraction procedure for DNA from bloodstains for RFLP analysis [*2*] was found often to be unsuccessful in yielding DNA of the purity needed for PCR amplification. The extracted DNA apparently contained an inhibitor of PCR. The organic extraction procedure described by Budowle et al. [*2*] yielded interpretable typing results from 21% of these stains (all were conclusive calls and correctly typed). Using the water presoak, Centricon purification, or nonorganic extraction methods, typing results (all correct) were obtained from 91, 67, and 85% of the stains, respectively. Only DNA samples extracted from bloodstains on leaves, denim, and cotton upholstery failed to amplify using any of the four methods described. No DNA was detected in extracts of bloodstains on leaves, while the DNA from denim and upholstery appeared intact and of sufficient quantity, as judged by appearance on ethidium-bromide-stained agarose gels. These latter two substrates probably contained an inhibitor of PCR which was not removed by any of the extraction procedures.

Repetitive Analysis of Identical Samples

One hundred aliquots of 100 ng of DNA isolated from a liquid whole blood sample were amplified and typed (DQα type 1.1, 4): 98 typed were correctly; 2 samples, which would have been judged too faint to call (that is, there was no visible hybridization to the C probe), did not show the 1.1 allele at all. This problem of allele dropout appears to be alleviated by avoiding amplification in the front two rows of the thermal cycler (see the section on Allele Dropout below).

Effect of Various Hybridization/Wash Temperatures

The Cetus protocol for hybridization of amplified DNA with allele-specific probes using the reverse dot-blot format specifies that hybridization and washing must be carried out in a shaking water bath at 55°C (±1°C). Hybridizations and washings were done for a set of replicate samples at 52, 53, 54, 56, 57, and 58°C. Below 54°C, cross-hybridization was seen, especially between the DQA1 allele subtyping probes. Above 56°C, loss of hybridization occurred, especially from the DQA1 subtyping and DQA3 probes. Thus, care must be taken to ensure that the water bath used for these steps is at the proper temperature. Samples of known DQα type should be used as positive controls to indicate that proper hybridization conditions have been achieved.

Effect of Sunlight on Bloodstain DNA

Replicate bloodstains (50 μL) were exposed to summer sunlight in a greenhouse or outdoors protected by a clear plastic petri dish. Control samples were placed in the greenhouse but protected from sunlight. Stains in the greenhouse were collected daily for 2 weeks, and at 3, 4, 6, 8, 12, 16, and 20 weeks. Stains placed outdoors were collected daily for 3 weeks. DNA from stains exposed to light in the greenhouse could be amplified and correctly typed through 6 weeks. DNA extracted from stains exposed longer than 6 weeks appeared to be highly degraded on ethidium-bromide-stained agarose gels and failed to amplify or type. DNA from control stains stored until 20 weeks in the greenhouse typed correctly. DNA extracted from stains placed outdoors through 3 weeks amplified and typed correctly, although by 3 weeks the spots were faint (but darker than the C dot and therefore interpretable), which results were approximately equivalent to those obtained from bloodstains stored in the greenhouse for 6 weeks.

Chemical Contamination of Bloodstains

Bloodstains were contaminated by a variety of substances [*9,10*], including gasoline, motor oil, acid, base, bleach, soap, salt, and soil. All the samples typed correctly except those contaminated with soil. No DNA was apparent on ethidium-bromide-stained agarose gels when the extracts from the bloodstained soil were analyzed. Possible explanations for the amplification failure of DNA from soil-contaminated blood are that components of the soil bind the DNA, making it impossible to extract, or that the bloodstain DNA was completely degraded, or that the samples may have contained inhibitors of PCR.

Microorganism Contamination of Bloodstains

DNA extracted from replicate bloodstains contaminated with one of three species of bacteria (*Escherichia coli*, *Bacillus subtilis*, and *Staphylococcus epidermidis*) and the fungus *Candida albicans* [*10*], as well as DNA from duplicate control stains (consisting of the microorganisms only), was analyzed. DNA from the microorganism stains failed to amplify or type, while the human DNA extracted from all bloodstains contaminated with microorganisms typed correctly.

Sensitivity of Detection and Detection of Mixed Samples

The sensitivity of the test and its ability to detect contaminating DNA in mixed samples was evaluated by amplifying varying amounts of genomic DNA from one source or from samples containing two different sources of DNA with different DQα types. Typing results were obtained from 2 ng of the 1.2,3 allele sample and from as little as 500 pg of

the 1.2, 4 and 1.1, 3 samples. Hybridization to the allelic typing probes could be detected from even less DNA (down to 65 pg), but the hybridization to the control probe was not detectable. These latter strips were judged uninterpretable. DNA samples with two different DQα types were mixed in different amounts and different ratios (Table 2). If the majority type was 5, 7, or 10 times greater than the minority, the minority type was quite visible but less intense than the control dot. If the majority type was 100 times greater, the minority type was visible but quite faint and less intense than the C dot. If the majority type was 1000 times greater, the minority type was not detected. The ratios, rather than the overall amount of DNA, appeared to be more important in determining the relative intensities of the dots, because the patterns were very similar for 200/20 ng, 20/2 ng, and 2/0.2 ng. For 1:1, 3:2, 2:1, and 4:1 ratios, the contaminant type was of equal or greater intensity than the C dot. Thus, some mixed samples, if they were both homozygotes, or if one was a heterozygote and one a homozygote and shared its allele with the heterozygote, would not be detected as mixes, even if present in unequal amounts. The possibility of mixed samples would have to be taken into account in the interpretation of certain cases, particularly if only one genetic locus is typed.

Induced Contamination and Effects of Sample Handling

Because PCR allows the analysis of very small amounts of DNA, it is of concern whether DNA from sources other than the evidentiary material can be introduced prior to amplification and detected. This type of contamination could possibly interfere with interpretation of DQα dot blot patterns. A variety of samples was set up to test whether various potential sources of human DNA could be detected (Table 3). No detectable contamination was introduced by handling or coughing or from the dirty, sweaty clothing. However, as expected, two wet stains, when dried in contact with one another, could cross-contaminate each other. DNA from a 10-μL bloodstain made on top of a 10-μL saliva stain showed DQα alleles from both sources, with the saliva type being significantly stronger. DNA from a 10-μL bloodstain on a 1-μL saliva stain showed alleles from both sources in approximately equal proportions. This is of significance since in forensic science cases the presence of saliva may be undetectable or unknown. This observation of the contribution of DNA from saliva has significance for the interpretation of forensic science cases.

As another way of testing for contamination introduced by "routine" evidence handling, "evidence" from a mock crime scene class was processed. The blood present was pig blood, thus, any DQα types obtained would have arisen from contamination introduced by the personnel collecting the evidence or by the person who had worn the clothing. Twenty cuttings were taken from four items of clothing. No typing results were obtained from any of the cuttings, nor was any DNA present on a slot-blot hybridization test for human DNA (sensitive to less than 100 pg DNA) [*11*].

Population Studies

Population samples were analyzed to determine the frequencies of DQα genotypes. A total of 150 Caucasian samples, 193 black samples, and 126 Hispanic samples were typed. The allele and genotype frequencies are given in Tables 4 and 5, respectively. The expected genotype frequencies were calculated from the allele frequencies and compared with the observed values using chi-square analysis. All classes (that is, genotypes) with less than 3 observations were pooled. For the Caucasian population, the chi-square value is 12.14, $0.500 < P < 0.750$, df = 13; for the black population, 11.52, $0.250 < P < 0.500$, df = 10; and for the Hispanic population, 11.36, $0.250 < P < 0.500$, df = 10. Thus, the three population samples satisfy Hardy-Weinberg expectations. A computer program developed by G. Carmody (Carleton University, Ottawa, Ontario, Canada),

TABLE 4—*DQα allele frequencies.*[a]

Allele	Caucasian, *N*	Black, *N*	Hispanic, *N*
1.1	37 (0.123)	53 (0.137)	32 (0.127)
1.2	54 (0.180)	99 (0.256)	34 (0.135)
1.3	26 (0.087)	17 (0.044)	10 (0.040)
2	44 (0.147)	45 (0.117)	22 (0.087)
3	45 (0.150)	39 (0.101)	56 (0.222)
4	94 (0.313)	133 (0.345)	98 (0.389)
Total[b]	300	386	252

[a]The values are the numbers of observations; the values in parentheses are the allele frequencies.
[b]The total numbers indicate the numbers of chromosomes typed.

TABLE 5—*DQα genotype frequencies.*[a]

DQα Genotype	Caucasian, *N*	Black, *N*	Hispanic, *N*
1.1	3 (0.020)	4 (0.021)	4 (0.032)
1.1, 1.2	9 (0.060)	15 (0.078)	4 (0.032)
1.1, 1.3	3 (0.020)	2 (0.010)	1 (0.008)
1.1, 2	8 (0.053)	6 (0.031)	3 (0.024)
1.1, 3	2 (0.013)	5 (0.026)	6 (0.048)
1.1, 4	9 (0.060)	17 (0.088)	10 (0.079)
1.2	5 (0.033)	9 (0.047)	2 (0.016)
1.2, 1.3	3 (0.020)	3 (0.016)	4 (0.032)
1.2, 2	9 (0.060)	11 (0.057)	4 (0.032)
1.2, 3	6 (0.040)	12 (0.062)	8 (0.064)
1.2, 4	17 (0.113)	40 (0.207)	10 (0.079)
1.3	1 (0.007)	1 (0.005)	0 (0.000)
1.3, 2	3 (0.020)	5 (0.026)	0 (0.000)
1.3, 3	4 (0.027)	2 (0.010)	3 (0.024)
1.3, 4	11 (0.073)	3 (0.016)	2 (0.016)
2	2 (0.013)	2 (0.010)	1 (0.008)
2, 3	7 (0.047)	2 (0.010)	4 (0.032)
2, 4	13 (0.087)	17 (0.088)	9 (0.071)
3	7 (0.047)	1 (0.005)	5 (0.040)
3, 4	12 (0.080)	16 (0.083)	25 (0.198)
4	16 (0.098)	20 (0.119)	21 (0.167)
Total[b]	150	193	126

[a]The values are the numbers of individuals carrying a particular DQα type; the values in parentheses are the frequencies of the DQα types.
[b]The total numbers indicate the numbers of persons typed.

using an algorithm devised by Roff and Bentzen [*12*], was used to test the homogeneity of genotype frequencies for each population sample determined in this study with population samples reported by Helmuth et al. [*13*]. The chi-square value for three Caucasian population samples (two analyzed by Helmuth et al. [*13*] and one analyzed here) is 64.722, $P = 0.3370$ (± 0.0149), and for three black populations (two analyzed by Helmuth et al. [*13*] and one analyzed here) is 38.3059, $P = 0.5580$ (± 0.0157). Therefore, the samples can be pooled to form one combined Caucasian data base ($N = 737$) and one combined black data base ($N = 589$). When the two Hispanic populations described in Helmuth et al. [*13*] and the one described here were compared, the chi-square value was 83.6547, $P = 0.0000$. Pairwise comparisons were then done on the three populations.

The "RBL Hispanic" population [13] was similar to the Hispanic population described here (chi-square, 19.4379, P = 0.4210, ±0.0156), but the Mexican population analyzed by Helmuth et al. [13] was dissimilar to both of the other Hispanic populations (P = 0.0000 for both comparisons). The power of discrimination is calculated to be 0.93 for Caucasians, 0.90 for the South Carolina black population, 0.94 for the Baylor black population, and 0.90 for the Hispanic population. These values are similar to those previously published [13].

Allele Dropout

During the course of the validation experiments, it was noted that in some samples, previously determined to be DQA 1.1, 4, only the 4 allele was detected (which would have been interpreted as a 4 homozygote) [14]. This mistyping was not caused by any particular contaminant introduced in the validation experiments but, rather, appeared to occur sporadically. It was determined that the dropout was due to failure of amplification of the 1.1 allele [14] (unpublished observations, Russell Higuchi and P. Sean Walsh, Cetus Corp., Emeryville, California), and that the dropout could occur with any of the 1 alleles (1.1, 1.2, or 1.3) [14] and was probably due to insufficient denaturation of the 1 alleles during PCR. The 1 alleles are more G/C rich than are alleles 2, 3, and 4 [15], and therefore the duplex DNA of the 1 alleles could be more difficult to denature. Dropout occurred more frequently when using an older thermal cycler, as well as when using the front row of newer machines. Denaturation temperatures within tubes were monitored by placing a thermocouple into a tube containing 100 μL of PCR reaction mix. The PCR run parameters were 93°C for 30 s, 55°C for 30 s, and 72°C for 30 s. One, two, or three measurements were made for each well of two different thermal cyclers. Table 6 lists the peak temperatures measured in each well of each thermal cycler. As much as a 4°C difference in peak temperatures was noted as being reached in different

TABLE 6—*Peak sample temperatures for denaturing in two thermal cyclers.*[a]

				Thermal Cycler "A"				
Back	88.8	89.5	89.8	89.6	89.5	89.6	89.1	89.4
	89.6	90.5	89.6	90.3	90.4	90.5	88.9	89.8
					90.1		89.7	
							89.9	
	89.7	91.6	90.0	90.4	90.7	91.2	90.7	90.3
	88.8	89.3	91.2	91.0	91.4	90.2	90.8	90.1
							90.6	
	88.7	89.4	89.7	90.1	89.9	90.3	90.0	89.9
Front	88.4	88.4	88.7	88.7	88.7	89.0	89.0	89.7
				Thermal Cycler "B"				
Back	91.2	91.7	91.9	92.0	92.0	91.5	91.6	90.1
	91.1	91.5	91.7	92.0	92.0	91.5	91.5	90.1
	91.7	91.9	92.2	92.2	92.4	92.5	92.2	91.2
								91.2
	91.5	91.6	91.8	92.2	92.4	92.1	91.8	91.6
								91.6
	90.5	91.0	91.1	91.2	91.2	91.2	91.0	90.7
								90.7
	89.6	89.9	89.7	90.2	90.0	89.8	90.1	89.9
	89.7							89.7
Front	89.0	88.9	88.9	88.9	89.3	89.0	88.7	89.0
					88.9	88.5	89.2	89.0

[a]The thermal cyclers were programed for 93°C, 30 s denaturation; the values are in degrees Celsius.

TABLE 7—*Analysis of differentially extracted samples from 74 case samples.*

PCR Clean, RFLP Clean,[a]	PCR Mixed, RFLP Clean,[a]	PCR Mixed, RFLP Mixed
	Female DNA Fractions	
37	3	4
	Male DNA Fractions	
22	4	4

[a]In clean samples, no male alleles were detected in the female fraction or vice versa; in mixed samples, male alleles were detected in the female fraction or vice versa.

wells during the same PCR run. New software installed in the older machine (thermal cycler A), denaturing at 94°C for 1 min, and avoidance of the use of the first two rows of the thermal cycler have alleviated the problem of allele dropout.

Analysis of Old Case Samples

Samples from old cases (243 samples from 44 cases) were obtained from the FBI Laboratory, and the DNA was extracted using the organic extraction method, amplified, and typed. Typing results were obtained from 63% of all samples (58% of known bloodstains, 67% of questioned bloodstains, 68% of female fractions of differentially extracted vaginal swabs or stains, and 62% of male DNA fractions). The failure of amplification of DNA from known bloodstains may have been due to the presence of an inhibitor not eliminated by the organic extraction protocol. Failure of amplification of DNA samples from the evidentiary samples could be due to the presence of inhibitors of PCR in the DNA extracts (41% of the samples that gave no PCR result did yield RFLP results) or to insufficient quality or quantity of the DNA (59% of the samples that gave no PCR result also gave no RFLP result). All the samples that matched by RFLP analysis also matched by DQα analysis (8 cases had sufficient data to make this comparison). Of 3 cases that excluded suspects based on RFLP results and for which there was sufficient data to compare DQα typing results, 2 were also excluded by DQα analysis and 1 was included. Occasional DQα inclusions would be expected in cases that excluded on the basis of RFLP analysis because of the relative informativeness of the two methods. The female and male DNA fractions from two differentially extracted vaginal swabs from each of two cases (of 44) that were inconclusive by RFLP analysis were typeable for DQα. Generally, contamination of samples by other DNA did not appear to be a problem except in some differentially extracted vaginal swabs. Table 7 shows these results. If RFLP results showed mixed types, DQα results did also (4 out of 4 female DNA fractions and 4 out of 4 male DNA fractions from differentially extracted samples). If RFLP results appeared clean (that is, no detectable male profile in the female fraction and vice versa), then usually the DQα typings did not appear mixed (37 of 40 female DNA fractions and 22 of 26 male DNA fractions). Three of 40 female DNA fractions showed the presence of the suspect's DQα alleles, and 4 of 26 male DNA fractions showed the presence of the victim's DQα alleles.

Discussion

The validation studies of HLA DQα typing described here demonstrate that the HLA DQα gene can be successfully typed using the AmpliType DQα DNA typing kit (Cetus).

A variety of conditions that may be encountered in forensic casework do not produce false positives or negatives when the typing is done under proper protocols.

The choice of DNA extraction procedures for PCR is important. A method that yields DNA of the purity necessary for PCR by removing potential inhibitors of PCR should be used. The method described by Budowle et al. [2], while effective for RFLP analysis, is not the most desirable approach for obtaining DNA that can be amplified. The other methods described, the modified organic extraction method (which includes soaking the stain in water prior to organic extraction), the nonorganic extraction method, and the Centricon dialysis/concentration method, are all more effective than the organic extraction method for preparing DNA of the purity needed for amplification.

The DQα amplification and typing system was shown to be relatively unaffected by various environmental insults to bloodstains. Chemical and microbial contaminants that may be encountered in connection with evidentiary stains do not prevent obtaining interpretable typing results. Bloodstains deposited on a wide variety of substrates yielded correct typing results; however, bloodstains deposited on soil, leaves, and certain fabric types (such as blue denim and cotton upholstery) did not yield DNA that could be amplified and typed. No DNA was detected from extracts of bloodstains on soil and leaves (when analyzed on agarose gels) and thus was either completely degraded or unextractable from those substrates. DNA obtained from stains on blue denim and cotton upholstery was of high molecular weight and of sufficient quantity but failed to amplify. This failure is probably due to the presence of inhibitors of PCR which co-purify with the DNA. Another extraction method using Chelex 100® ion-exchange resin [*16*] has been reported to be effective in yielding amplifiable DNA from these substrates [*17*]. Exposure to sunlight eventually causes degradation of DNA in stains to the point that no typing results can be obtained. Because DQα typing requires only the presence of a minimal amount of DNA over 242 base pairs in length, typing results can be obtained after a longer period of exposure when using PCR and DQα typing than when using RFLP analysis [*10*] (6 weeks versus 10 to 12 days when exposed to sunlight in a greenhouse in the summer). Most important, however, no adventitious substances led to mistyping: either the correct type or no type was obtained.

The sensitivity experiments described demonstrated that it was possible to type 500 pg of genomic DNA. Hybridization to typing probes was visible when even lesser amounts of DNA were amplified, but hybridization to the C probe was not detectable, rendering the strips uninterpretable. Quantitation of human DNA by slot blot hybridization of genomic DNA with a human alphoid DNA probe (D17Z1) serves as an independent means of quantifying sample DNA [*11*]; however, there may not always be a relationship between the amount of DNA in a sample and the dot intensity. The extent of amplification, and dot intensity, may be decreased by other factors, such as inhibitors of PCR. Thus, estimates of DNA quantity from slot blots should not be a predictor of the intensity of dot blots.

The sensitivity of PCR raises the concern that contamination of evidentiary samples from sources of DNA other than the stain itself could interfere with the typing results. The results of the sample handling experiments demonstrate that routine handling of evidence did not introduce contaminating DNA from the handler to the sample. Although there has been a report of shed scalp skin introducing DNA contamination [*18*], the results reported here indicate that none of the following introduced detectable DNA contamination: extensive handling of a sample, coughing on a sample, shed scalp skin, scissors previously used to cut other bloodstains, or clothing subjected to heavy perspiration. The number of cycles used for amplification of the DQα gene (that is, 32) is such that amplification and detection of minute amounts of DNA, such as might be imparted by handling or shed skin cells would be unlikely. Some of the validation studies described here were done using 34 or 35 cycles, which was according to protocols recommended

by Cetus Corp. at earlier stages of development of the AmpliType kit. The results of the contamination studies only apply to typing done using 32 cycles; the use of more cycles may, in fact, allow detection of smaller amounts of contaminating DNA. Measures must be taken by laboratories using PCR analysis to limit laboratory induced contamination by amplified DNA.

The typing results obtained from the mixed blood and saliva stains demonstrate that the possibility of a body fluid stain actually being a mixed stain must be considered when interpreting dot blots. It may be difficult in some cases to discount the possibility of mixed body fluids when using only one genetic test. However, when results from multiple loci are available, the likelihood of a result being attributable to a mixed stain as opposed to being attributable to a heterozygote will be more readily assessed.

The problem of allele dropout, encountered during the course of the validation experiments, is one of critical importance to forensic scientists, as it could lead to mistyping of samples. Dropout of the DQα 1 alleles was originally detected using denaturing conditions of 93°C for 30 s. The possible explanation for the cause of dropout of the 1 alleles is failure of denaturation of these alleles during PCR because of their higher GC content relative to that of alleles 2, 3, and 4 [*15*]. This phenomenon occurred more frequently when the samples were in the front two wells of the thermal cycler. Measurements of sample temperatures indicated that samples in these rows did not heat up as quickly as samples in other rows of the thermal cycler. The avoidance of the front two rows of the thermal cycler, combined with more stringent denaturation conditions (94°C for 60 s), alleviate this problem.

Analysis of old case sample DNAs using the AmpliType kit demonstrated that DQα typing could be done on the range of samples normally encountered in casework. Many of the samples that failed to amplify or type were known samples from victims or suspects. All of these were bloodstains. It is anticipated that use of a different extraction protocol will improve the success rate of typing. Greater efficiency of typing is expected with the use of the Chelex extraction protocol, which is effective at yielding amplifiable DNA from many substrates, including those which did not yield amplifiable DNA when using the four methods described here [*17*]. Typing results from differentially extracted vaginal swabs demonstrated that cross-contamination of the sperm and nonsperm DNA fractions can occur and must be considered in interpretation of results.

In conclusion, PCR amplification of DNA from samples exposed to a variety of insults yields correct DQα typing results. The DQα alleles present in a sample at the level of sensitivity of the test were reliably detected, and no false results were produced as long as the test was carried out under conditions that prevented allele dropout. The typing system appears relatively resistant to a variety of environmental insults, and factors that do influence the test serve to give no results rather than false results. The use of an extraction protocol that is effective in removing inhibitors of PCR is a most important factor in yielding positive test results. Interpretation of typing results must always be done with caution, taking into account the possibility of mixed samples. Studies are under way currently evaluating a large number of case samples (previously analyzed by RFLP typing) to determine the relative efficiency of DQα typing via reverse dot blot hybridization to allele-specific probes (and the Chelex protocol for DNA extraction) for providing results from DNA from evidentiary material.

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