TECHNICAL NOTE

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PCR-Based Forensic Testing of DNA from Stained Cytological Smears*

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ABSTRACT: Experiments were performed to evaluate the efficiency of PCR-STR (Short Tandem Repeats) and PCR-sequence polymorphisms for the identification of stained pap smears and postcoital slides stained with cytological and forensic techniques. HLA-DQA1, PolyMarker, Amelogenin, HUMTH01, HUMVWFA31, HUMF13B, and HUMFES/FPS were determined. With the exception of the forensic Baecchi stain, all the PCR-systems gave consistent results in comparison with the reference blood from the donors. Cytological stained smears can be important evidence for identification in sexual assault cases and in missing person cases.

KEYWORDS: forensic science, DNA typing, polymerase chain reaction, short tandem repeat, sexual assault evidence, HLA-DQA1, LDLR, HBGG, GYPA, D758, GC, TH01, VWFA31, F13B, FES/FPS, amelogenin, Pap smears, cytology slides, histological stains

In forensic sexual assault cases, a stained cytological smear is sometimes the only biological material submitted as evidence. Furthermore, it is possible that a cytological or a forensic stained slide could be used as reference material for the identification of remains or biological stains from a missing person.

During recent years, PCR based DNA systems have gained increasing popularity in the forensic laboratory because of their great sensitivity and their applicability to analysis of low quantity and degraded biological materials.

The aim of this study was to evaluate if DNA extracted from stained vaginal and postcoital smear slides could be amplified and provide reliable PCR-typing results with HLA-DQA1, PolyMarker, Amelogenin, HUMTH01, HUMVWFA31, HUM13B, and HUMFES/FPS (1–7). Three of the most commonly used cytological staining Papanicolaou (Pap), May-Grünwald-Giemsa (MGG), Hematoxylin-Eosin (HE) (8) and the forensic staining Baecchi (Bae) (9) were tested with the above mentioned PCR-systems.

Materials and Methods

Eighteen saliva and 84 vaginal or postcoital slides were collected from volunteers. One half of the slides were fixed by flame and the other half with a chemical fixing agent (spray Merckofix 3970, Merck Darmstadt). The fixed slides were stained with one of the following techniques (22 for each stain): Papanicolaou (Pap), May-Grünwald-Giemsa (MGG), Hemaoxylin-Eosin (HE), and Baecchi (Bae). The stained slides were mounted in "Canada" balsam, covered with a glass coverslip and left for two weeks at room temperature. Then the coverslip was removed by immersion in xylene overnight and the smeared region of each slide was swabbed with sterile cotton wetted with autoclaved distilled water.

The DNA from these swabs and the corresponding saliva samples from the male and female donors were extracted with Chelex (10). The Chelex procedure included an overnight incubation at 56°C with Proteinase K and DTT followed by an overnight ethanol precipitation at −20°C. The material from the postcoital slides was extracted by differential lysis and only the male fraction was tested. The quantity of human DNA was estimated by slot-blot analysis with the Human DNA Quantitation System (Life Technologies, Gaithersburg, MD) and 0.5 to 2 ng were amplified.

Amplitype HLA-DQA1 and PM typing were performed according to the recommended protocols in the kits (1,2). The PCR primers and the amplification conditions for the STR systems have been described previously: HUMTH01 (183 bp–207 bp) (4,11) and FES/FPS (211 bp–239 bp) (7,11), HUMVWFA31 (130 bp–166 bp) (5,12), HUMF13B (169 bp–189 bp) (6,13). All the systems were amplified adding 0.08 mg/mL bovine albumine fraction V (Sigma Chemicals, St-Louis, MO). With the exception of HLA-DQA1 and Amelogenin which were amplified in duplex (14), the other systems were amplified in singleplex. STR-detection was done by native Hydrolink or Polyacrylamide vertical gel electrophoresis in discontinuous buffer followed by silver staining (11).

Results and Discussion

After a legal request concerning a sexual assault case with only one cytological smear stained with Papanicolaou available as material for identification (Fig. 1), it was necessary to evaluate the possibility to extract, amplify, and type some DNA-PCR systems from stained vaginal and postcoital slides.

Papanicolaou, May-Grünwald-Giemsa, and Haematoxylin-Eosin are the most commonly used techniques in clinical and cytological laboratories. The Baecchi protocol is still often used in European forensic laboratories for detecting sperm.

Heat or chemical fixation of the slides did not significantly affect the DNA yield from the Chelex extraction or the results from the PCR amplification of the sequence polymorphism and STRs systems.

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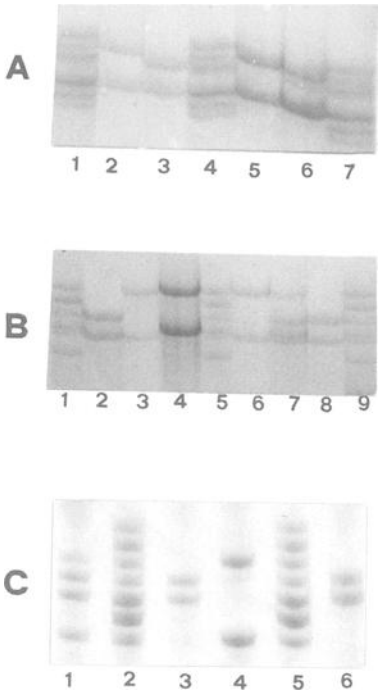

FIG. 1—*HUMTH01 (A), HUMFES/FPS (B), and HUMVWFA31 (C) typing results from blood, sperm and nonsperm fractions from a papanicolaou stained postcoital smear slide. (A) Allelic ladder (1,4,7), female blood (2), male blood (3), nonsperm fraction (5), sperm fraction (6). (B) Allelic ladder (1,5,9), female blood (2), control blood (3) male blood (4) control blood (6), sperm fraction (7), nonsperm fraction (8). (C) Allelic ladder (2,5), sperm fraction (1) female blood (3), male blood (4), nonsperm fraction (6).*

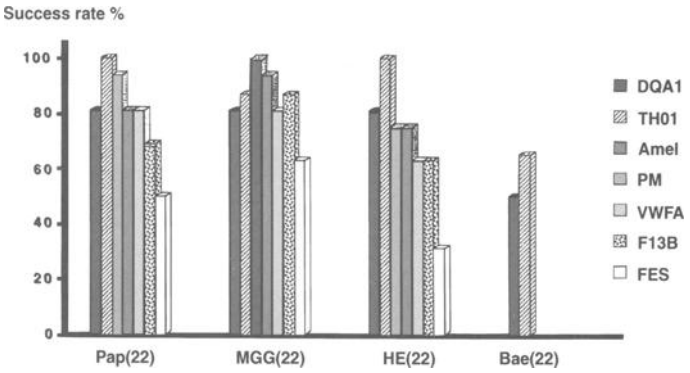

FIG. 2—*Success rate of PCR-systems from stained smear slides.*

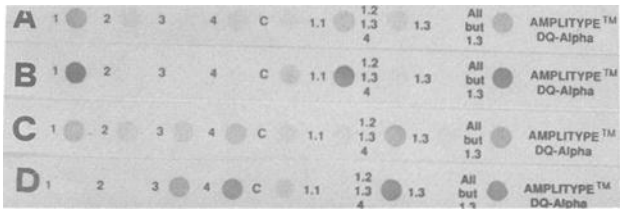

FIG. 3—*Amplitype HLADQA1 typing results from sperm fraction from two Baecchi and two HE stained postcoital smear slides. (A) Baecchi stained postcoital slide, (B) HE stained postcoital slide, (C) Baecchi stained postcoital slide, (D) HE stained postcoital slide.*

On the contrary, the staining techniques did have an effect on the recovery of DNA following Chelex extraction as reported in Table 1: DNA was recovered from all the cytological stained smears in sufficient quantities (1 to >10 ng) but the extraction from the Baecchi stained slides yielded only <1 to 1 ng of DNA.

For the cytological staining (Pap, MGG, HE) with the exception of HUMFES/FPS which is less sensitive with our protocol, the success rate of the PCR-systems fluctuated between 63% and 100% with the highest average rate of 95% obtained with the HUMTH01 (Fig. 2). For the Baecchi stained slides, only HLA-DQA1 and HUMTH01 were tested, because the DNA was degraded and in low quantity (<1 ng to 1 ng). HUMTH01 gave the best success rate at 65% whereas HLA-DQA1 dropped to 50% (Fig. 2). The inconclusive HUMTH01 results were often negative and sometimes resulted in nonspecific bands. The inconclusive HLA-DQA1 typization resulted in the presence of extraneous or unexpected dots which are greater than or equal in intensity to the internal control or "C" dot (Fig. 3). These unexpected alleles correspond neither to the male nor to the female alleles.

Although we have estimated rather than counted the number of spermatozoa and other cells present on each slide, the success rate of the typization correlated well with the microscopic estimation, except for the Baecchi stained slides which yielded less extracted DNA than expected.

TABLE 1—*DNA yield from stained slides: chelex extraction.*

Staining	Quantity
Papanicolaou	1 ng to > 10 ng
May-Grünwald-Giemsa	1 ng to > 10 ng
Hematoxylin-Eosin	<1 ng to > 10 ng
Baecchi	<1 ng to 1 ng

In agreement with some authors (15–18), the results show that the reagents used for the three cytological stains Pap, MGG, and HE do not interfere with the extraction, amplification, and typing of the stained cells. Furthermore, heat or chemical fixation of the cells did not play an important role.

On the other hand, the reagent used for the forensic Baecchi stain interfered with the extraction and the DNA was recovered in low yield. In order to explain this lower yield, we extracted smear slides stained with HE or Baecchi protocol using the phenol-chloroform procedure and compared the results on agarose gel with ethidium bromide (Fig. 4). The cells stained with HE contained high molecular weight DNA while DNA extracted from Baecchi stained cells was clearly degraded and lacked high molecular weight fraction. The two staining reagents used for the Baecchi stain, Fuchsin acid (Rubin S), and Methylene blue, are dissolved with 1% HCl to obtain a good contrast that allows easy detection of very rare spermatozoa heads (red) in a nonbiological support (blue). We have investigated the effect of the staining Fuchsin acid and methylene blue and the effect of 1% HCl on a phenol-chloroform extraction. As shown in Fig. 5, the acid fuchsin and methylene blue diluted in HCl 1% degraded fresh DNA whereas those two reagents diluted in water did not degrade the DNA and the STRs typing were conclusive (Fig. 6). This result is not surprising because strong acid induces the depurination and strand scission or base modification of the DNA structure.

Concerning the inconclusive PCR typing for the Baecchi stained slides, nonspecific or contaminated PCR products were the major problems when amplifying very low amounts of DNA or DNA of poor quality. Although the level of nucleotide misincorporation is weak, the nonspecific product of the Taq Polymerase as well

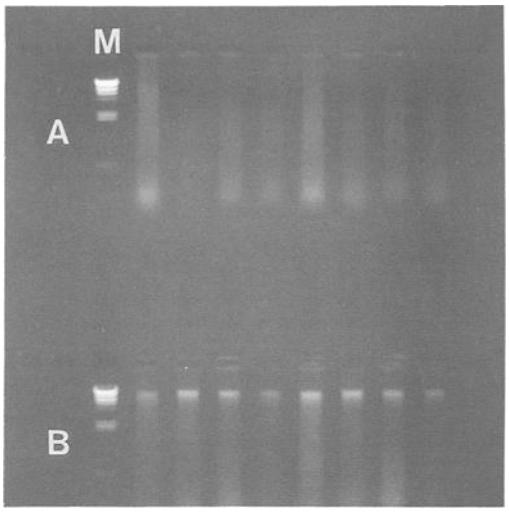

FIG. 4—*Phenol-chloroform extracted DNA from eight stained smear slides tested by agarose gel electrophoresis with ethidium bromide after Baecchi stain (A), and Hematoxylin-Eosin (B). The lambda DNA/Hind III digested molecular weight marker is in the first position.*

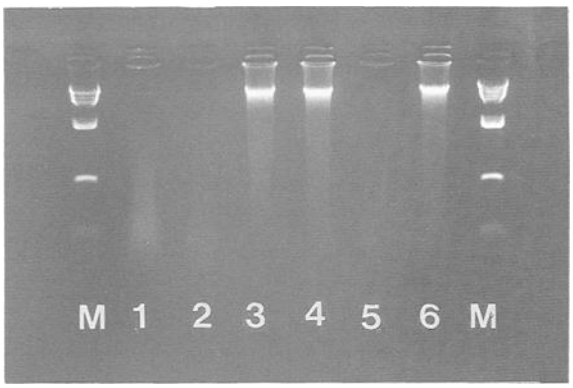

FIG. 5—*DNA from fresh sperm was extracted with phenol-chloroform after 30 min incubation at room temperature with acid fuschin diluted in 1% HCl (1), with methylene blue diluted in 1% HCl (2), with acid fuschin diluted in water (3), with methylene blue diluted in water (4), with HCl 1% (5), with TEN (6). Five microliters of each sample was electrophoresed on agarose and visualized with ethidium bromide. The lambda DNA/Hind III digested molecular weight marker is in the first and last position.*

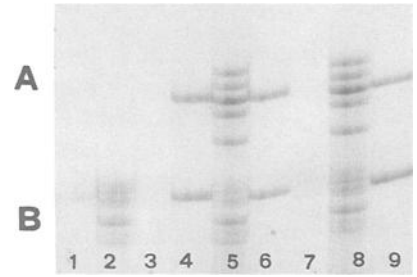

FIG. 6—*HUMFES/FPS (A) typing results from phenol-chloroform extracted sperm DNA after 30 min incubation at room temperature with acid fuschin diluted in water (4), with methylene blue diluted in water (6) and with TEN (9). The allelic ladder was in lanes 5 and 8. HUMTH01 (B) typing results from phenol-chloroform extracted sperm DNA after 30 min incubation at room temperature with acid fuschin diluted in 1% HCl (1), with methylene blue diluted in 1% HCl (3), with acid fuschin diluted in water (4), with methylene blue diluted in water (6), with HCl 1% (7), with TEN (9). The allelic ladder was in lanes 2, 5, and 8.*

as the contaminated products become detectable if the DNA template is limiting (19–22).

In conclusion, HLA-DQA1, PolyMarker, Amelogenin, HUMTH01, HUMVWFA31, HUMF13B, and HUMFES/FPS typing can be used for the identification of cytological stained Pap smears and postcoital slides. On the other hand, the forensic Baecchi technique is not suitable for the DNA analysis with forensic PCR systems because of the poor quality and the low quantity of DNA extracted from the stained cells.

Consequently, it is advised to adopt another sperm detection technique in the forensic laboratory, for instance, the Christmas Tree protocol (23), which is amenable to PCR typing as already reported for the Amplitype PM and HLA-DQA1 systems (15).

The successful results obtained from the stained cytological slides could be used as evidence in sexual assault cases and as reference material for the identification of remains or biological stains from a missing person.

Acknowledgments

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