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Technical Note

Improvement and automation of a real-time PCR assay for vaginal fluids

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ABSTRACT

The identification of vaginal fluids is crucial in forensic science. Several molecular protocols based on PCR amplification of mfDNA (microflora DNA) specific for vaginal bacteria are now available. Unfortunately mfDNA extraction and PCR reactions require manual optimization of several steps. The aim of present study was the verification of a partial automatization of vaginal fluids identification through two instruments widely diffused in forensic laboratories: EZ1 Advanced robot and Rotor Gene Q 5Plex HRM. Moreover, taking advantage of 5-plex thermocycler technology, the ForFluid kit performances were improved by expanding the mfDNA characterization panel with a new bacterial target for vaginal fluids and with an internal positive control (IPC) to monitor PCR inhibition. Results underlined the feasibility of a semi-automated extraction of mfDNA using a BioRobot and demonstrated the analytical improvements of the kit.

1. Introduction

The identification of vaginal fluid at the crime scene can play a pivotal role during the analysis of a presumed sexual assault. At the moment, no standardized presumptive tests are available for this specific body fluid. At the end of last century the Lugol's iodine test was proposed as a sensitive assay for the identification of vaginal epithelial cells. The reaction involved the iodine and the glycogen present in those cells. Unfortunately the identification of Lugol-positive cells in the male urinary tract strongly reduced the application of this assay in forensic investigations [1–3]. With the development of nucleic acid techniques the forensic approach to the body fluids identification problem changed significantly [4]. In the last few years several authors described different protocols of molecular biology that can be useful during technical investigations. Transcriptomic analysis have been suggested as a possible application to many biological sample, but the degradation of RNA still remain a limitation point [5–7]. In addition, at the moment RNA is not routinely used in forensic institutes. On the other side, several protocols based on DNA characterization showed reproducible and robust results. Tissue-specific DNA methylation patterns have been investigated by Pyrosequencing, and the technique can be applied to a variety of biological fluids which may be present at crime scene [8,9]. In addition, considering that the vagina is characterized by specific bacteria, it has been speculated by several authors that the analysis of microflora DNA (mfDNA) can lead to the identification of vaginal fluids and their discrimination to other biological samples [10–13]. Starting from these considerations, a multiplex real time-PCR assay (ForFLUID kit, Molecular Digital Diagnostics, Viterbo, Italy) was developed for the identification of vaginal fluids by detection of genome DNA from six specific bacteria [10]: the assay was tested on 48 samples, including vaginal secretions, saliva, faeces and mixtures of body fluids. Recently, an interlaboratory evaluation was performed on vaginal fluids and eight forensic units confirmed the absence of false positives and a precision rate over 75% [14] confirming the appropriateness of the selected molecular tools for the identification of vaginal fluids. The absence of false positive is an important characteristic for a forensic product, but the reduction of the number of false negative it is also a desirable improvement. While part of these errors can be linked by the specific conditions of forensic samples, scientists can surely reduce interferences due to laboratory manipulations. In particular, the DNA extraction protocol is a crucial step that can dramatically affect genomic analysis. In order to reduce this variability, automated extraction

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procedures are becoming widely used in many forensic laboratories. The majority of DNA extraction protocols are forensic validated for human genomic analysis, but the hardness of bacterial wall (especially of Gram positives) introduces serious questions on reproducibility and sensibility. The present technical note reports the adaptation of previous described protocols for the ForFLUID kit to an automated DNA extraction procedure based on the usage of the BioRobot EZ1 Advanced XL (Qiagen, Hilden, Germany). In addition, the feasibility and utility of the extension of the bacteria panel considered by the kit was investigated.

2. Materials and methods

2.1. Samples

A total of 17 samples were considered for this study: twelve vaginal swabs, two mixtures prepared started from vaginal and oral/faecal sample freshly collected, one oral swab and two casework samples (Table 1). Swabs were collected according to standard clinical procedure, to reduce environmental contamination, from voluntary participants aged 30–67. The swab were stored in dry conditions and processed for DNA extraction the day after sampling. Casework samples were from forensic archives, where they were stored at −20 °C (CW1), at RT or in fridge (CW2).

2.2. DNA extraction

Bacterial DNA was purified by enzymatic pretreatment according to the isolation of genomic DNA of Gram positive bacteria protocol (Qiagen, Hilden, Germany). Each swab was washed in 500 μl of sterile PBS buffer (AppliChem GmbH, Darmstadt, Germany) at room temperature in agitation for 45 min. After the removal of the swab the buffer was centrifuged at 10,000 rmp and the bacterial pellet was resuspended in 180 μl of the appropriate enzyme solution according to the instructions of EZ1® Virus Mini Kit v. 2.0 (20 mg/ml lysozyme; 20 mM Tris–HCl, pH8.0; 2 mM EDTA; 1.2% Triton X-100) added with 564 units/ml lysostafin (Sigma–Aldrich, St. Louis, MO, USA) and 500 units/ml of mutanolysin (Sigma–Aldrich). After an incubation for 1 h at 37 °C in agitation, Proteinase K was added and the solution was incubated at 56° temperature in agitation for 30 min. The bacterial DNA was extracted by the BioRobot EZ1® Advanced XL Workstation using the EZ1® Virus Mini Kit v. 2.0 and the EZ1® Advanced Virus Card v2.0 (Qiagen, Hilden, Germany) by using a protocol based upon a silica covered magnetic particle technology: final elution was performed in 60 μl of TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA).

Negative controls, consisting of clean swabs not contaminated by bacteria, were processed in parallel.

2.3. Real-time PCR conditions and results interpretation

The real time PCR was performed using ForFLUID kit (Molecular Digital Diagnostic, Viterbo, Italy), containing three multiplex master mixes with the primers and probes described by Giampaoli et al. [10,14]. In addition, using Primer Express (v. 2.0.0; Thermo Fisher Scientific, Waltham, MA, USA) specific primers and probes for *Lactobacillus iners* has been designed and added to the mix containing those for *Lactobacillus gasseri* and *Lactobacillus crispatus*. An internal positive control (IPC) has been designed on the DNA sequence for the protein EGFP (Table 2). Reactions were performed in 25 μl: each primer was at a final concentration of 900 nM, while probes were at 250 nM. The IPC was a fragment of EGFP coding region of approximatively 150 nt: 0.5 ng of IPC were present in each reaction.

The amplification has been performed on a Rotor Gene Q 5Plex HRM (Qiagen, Hilden, Germany) according to the user's manual protocol. Data were analyzed in the Absolute Quantification mode in order to obtain C_T value. Results were interpreted according Giampaoli et al. [10,14] considering a clear detection of the target for $C_T \leq 30$, a clear exclusion of the target for $C_T \geq 35$ and an inconclusive result for $30 < C_T < 35$.

3. Results and discussion

To evaluate the feasibility of a semi-automated DNA extraction protocol and the subsequent real-time PCR amplification of

Table 1
Information on the donor of vaginal samples.

Sample	Age	Ethnicity	Menstrual day	Terapy
V1	36	Caucasian	p	
V2	30	Caucasian	p	
V3	35	Asian	p	
V4	37	Caucasian	p	
V5	30	Caucasian	p	
V6	39	Caucasian	19	o-p
V7	67	Caucasian	m	
V8	31	Caucasian	8	
V9	38	Caucasian	post p	
V10	46	Caucasian	a	
V11[a]	44	Caucasian	8	
V12	33	Caucasian	7	g
VS	–	–	–	
VF	–	–	–	
O	–	–	–	
CW1	–	–	–	
CW1	–	–	–	

p, pregnancy; post p, post partum; a, amenorrhea; o-p, oestrogen-progesterone pill; g, gonadotropins; m, menopause.
[a] Bleeding due to menometrorrhagia.

Table 2
Primers and probes used in the real time PCR.

Bacteria	Oligonucleotide name	Sequence (50–30)
L. crispatus	L_cri_f2	GCACTAACAGCCGAAGAAGG
	L_cri_r2	TTCGGATATCTCCGGATCAC
	L_cri_PR	FAM-CGAAAAGCTTCGGGGAGCGGT-BHQ1
L. gasseri	L_gas_f2	AGATTGAAGAGCGACCGAGA
	L_gas_r2	CCTTCATCGGCTTCTAGTGC
	L_gas_PR	JOE-AAGGGCGCATGGTGAATGCCT-BHQ1
S. salivarius	S_sal_f2	GATGCCAAGGGTGAAGTTGT
	S_sal_r2	GAGCCATCAGGATTCGTAGC
	S_sal_PR	JOE-TGGCGAACAGACGATCAACGG-BHQ1
S. mutans	S_mut_f2	CGGTTCTCAGCAAGACATGA
	S_mut_r2	ATGGTACCCAATCCGCAATA
	S_mut_PR	FAM-TGCAGTTAAAGCTCTGCATAAAAGCGG-BHQ1
S. aureus	SA1_f	ACACCTGAAACAAAGCATCC
	SA1_r	CGCTAAGCCACGTCCATATT
	SA1_PR	FAM-TGGTCCTGAAGCAAGTGCATTTACGA-BHQ1
Enterococcus spp. [29]	ECST784F	AGAAATTCCAAACGAACTTG
	ENC854R	CAGTGCTCTACCTCCATCATT
	GPL813TQ	JOE-TGGTTCTCTCCGAAATAGCTTTAGGGCTA-BHQ1
L. iners	Lin01F	GTCTGCCTTGAAGATCGGAGT
	Lin01R	CGATCTCTTGGGCAGGTTAC
	Lin01P	ROX-TACAGGCTAGCGGCGGACGG-BHQ2
IPC	f_egfp	AGAACGGCATCAAGGTGAAC
	r_egfp	TGCTCAGGTAGTGGTTGTCG
	pr_egfp	[cy5]CAACATCGAGGACGGCAGCG[BHQ2]