

MEDICOLEGAL USE OF LACTOBACILLI MARKERS IN IDENTIFICATION OF HUMAN VAGINAL SECRETIONS

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ABSTRACT

The identification of body fluids at a crime scene is considered as a very important aspect of forensic science. Messenger RNA has recently been shown to be sufficiently stable to be considered suitable for use in forensic identification of body fluids.

Aim of the work: To assess the usefulness of using *Lactobacillus crispatus* and *Lactobacillus gasseri* genetic markers in forensic identification of human vaginal secretions found in crime scene.

Subjects and methods: All body fluid samples were taken from fifty volunteers: blood, saliva, semen, menstrual blood and vaginal secretions. Also different body fluid mixtures were prepared. Ribonucleic acid (RNA) was extracted from all these samples and then multiplex Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) method and gel electrophoresis were done.

Results: The *Lactobacilli* gene markers were proved to be present in vaginal fluid. They were present in both premenopausal and postmenopausal women. The *Lactobacilli* gene markers were present only in vaginal fluid and not in other body fluids. Also, they were present in body fluid mixtures only containing vaginal fluid.

Conclusion: The *Lactobacilli* markers are present in vaginal fluid without affection of the age on their presence. The *Lactobacilli* markers are specific to vaginal fluid and effective markers for its forensic identification even when they are mixed with other body fluids.

Recommendations: The *Lactobacilli* markers are useful markers for the vaginal fluid. The multiplex RT-PCR can be used as effective method for identification of body fluids at crime scene.

Key words: Forensic Identification, Lactobacilli bacteria, multiplex RT-PCR, Vaginal secretions.

1. INTRODUCTION AND AIM OF THE WORK:

The detection and identification of body fluids at a crime scene is considered as a very important aspect of forensic science (Ponce and Pascual, 1999; Tobe et al., 2007). Messenger RNA (mRNA), once thought to be unstable and to degrade rapidly, has recently been shown to be sufficiently stable to be considered suitable for use in forensic science (Zubakov et al., 2008; Setzer et al., 2008).

In order for mRNA profiling to be of use in forensic casework it was important to develop a multiplex reverse transcriptase polymerase chain reaction 'multiplex RT-PCR' method for the simultaneous analysis of body fluid-specific genes in a single sample extract (Juusola and Ballantyne, 2005).

In previous studies, human beta-defensin 1 (HBD-1) and mucin 4 (MUC4) have been used for the identification of vaginal secretions (Alvarez et al., 2004; Juusola and Ballantyne, 2005; Nussbaumer et al., 2006; Setzer et al., 2008).

However, MUC4 and HBD1 have also been reported to be expressed in saliva and salivary glands.

Lactobacilli species have been found to be the predominant bacteria in the vagina of women (Va' squez et al., 2002; Nam et al., 2007; Witkin et al., 2007).

Two *Lactobacilli* bacteria species have been reported to be specific to the vagina (Song et al., 2000; Witkin et al., 2007).

The 16S-23S rRNA intergenic spacer region (ISR) has been found to be a suitable marker to identify closely related species (Song et al., 2000).

The aim of this study is to assess the usefulness of using *Lactobacilli* markers in forensic identification of human vaginal secretions found in crime scene by incorporating *Lactobacilli* markers into mRNA multiplex assay to identify vaginal secretion and differentiate it from other body fluids.

2. SUBJECTS AND METHODS

2.1. Study population and sampling:

This study was done after taking acceptance of Institution Review Board (IRB) of faculty of medicine Zagazig University.

Inclusion criteria:

Fifty volunteers from Zagazig university hospitals were incorporated in this study after fully informed consent. These volunteers were 30 married females and 20 males and their age were between 20 and 55 years.

Exclusion criteria:

Women with genital infection, Women with systemic disease, pregnant women and Smokers were excluded from this study.

Sampling:

Vaginal swabs were collected from 12 females from different ages by rolling the sterile cotton swabs through the vagina.

Menstrual blood swabs were collected from 2.3. 8 females by rolling the sterile swabs in the menstrual blood.

Saliva (100-150µl) was collected from 5 males and 5 females (100-150µl) by asking participants to spit in the 1.5ml graduated RNase free centrifuge tubes in the morning before eating.

Blood samples (0.5-1ml) were collected by venipuncture from 5 males and 5 females. Freshly ejaculated Semen samples (0.5-1ml) were collected in plastic containers from 10 males.

2.2. Preparation of swabs:

All body fluids either individual or mixed were placed on swabs.

1- Preparation of individual body fluid swabs:

Vaginal swabs and menstrual swabs were ready.

Aliquots of saliva (about 50µl from each sample), blood (about 50µl from each sample) and

The amount taken from each primer (**Table 1**) was adjusted according to the final optimized concentration.

semen (about 50µl from each sample) were placed on sterile cotton swabs.

2- Preparing the mixed body fluid swabs:

Vaginal fluid containing mixtures: The vaginal swabs were placed on 50µl semen to form Semen and vaginal fluid mixture. Also the vaginal swabs were placed on blood (50µl) and semen (50µl) mixture to form Blood, semen and vaginal fluid mixture.

Mixtures not containing vaginal fluid: Blood (50µl) and semen (50µl) mixture was placed on sterile cotton swabs. Also Blood (50µl), semen (50µl) and saliva (50µl) mixture was placed on sterile cotton swabs.

RNA extraction:

RNA was extracted using GeneJET™ RNA Purification Kit (*Fermentas, EU*) according to the manufacturer's instructions. The purified RNA was stored at -20°C until use in the reverse transcriptase PCR.

2.4. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR):

This was done by using Thermo scientific Verso 1-step RT-PCR kit (*Thermo scientific, UK*).

The RT-PCR was performed in a single tube in a total reaction volume of 25µl containing: 0.5µl Verso Enzyme Mix, 12.5µl 1-Step PCR Master Mix, 1.25µl RT Enhancer 1.25µl from all primers (0.625µl from the forward primers and 0.625µl from the reverse primers), 3µl from the RNA extract and 6.5µl nuclease free water.

Table (1): Primers used in the multiplex reverse transcriptase PCR reaction:

Body fluid	Gene	Primer sequence (5'→3')	Size (bp)
Blood	GlycoA	Forward: CAG ACA AAT GAT ACG CAC AAA CG	188
		Reverse: CCA ATA ACA CCA GCC ATC ACC	
Mensrual blood	MMP11	Forward: CAA GAC TCA CCG AGA AGG GG	173
		Reverse: TAG CGA AAG GTG TAG AAG GCG	
Saliva	HIS	Forward: TGG GGC ATG ATT ATG GAG GTT Reverse: CAG AAA CAG CAG TGA AAA CAG CTT	233
	STATH	Forward: CTT GAG TAA AAG AGA ACC CAG CCA Reverse: TTC TGG AAC TGG CTG ATA AGG G	162
Semen	PRM2	Forward: CGT GAG GAG CCT GAG CGA Reverse: CGA TGC TGC CGC CTG T	201
	TGM4	Forward: TGA GAA AGG CCA GGG CG Reverse: AAT CGA AGC CTG TCA CAC TGC	215
Housekeeping	TEF	Forward: TGG GCC ATC AAC TGA GAA AGA	206

genes		Reverse: TCT CCC TAC ACT TCA ACT GCA CA	
	G6PDH	Forward: ATC ATC GTG GAG AAG CCC TTC Reverse: GTT CCA GAT GGG GCC GA	181
	UCE	Forward: AAT GAT CTG GCA CGG GAC C Reverse: ATC GTA GAA TAT CAA GAC AAA TGC TGC	241
Vaginal fluid	<i>L. crispatus</i>	Forward: CAG AGC AAG CGG AAG CAC A Reverse: CAT CTC TGC ATT GGG TTC CC	279
	<i>L. gasseri</i>	Forward: GAG AAA GCC AAG CGG AAG C Reverse: TTG CTT ACT TAC TGC TCC CCG	253

Reaction mixtures were subjected to the cycling program in a DNA heated lid thermal cycler. The PCR conditions were 50°C for 15 min, 95°C for 2 min, 30 cycles of (94°C for 30 s, 58°C for 30 s and 72°C for 90 s) a final step of 72°C for 45 min.

2.5. Agarose gel electrophoresis:

RT-PCR products were separated on 3% agarose gels. The gel was stained with ethidium bromide and photographed under UV transillumination.

3. RESULTS

3.1. The presence of *Lactobacilli* genes in vaginal fluid:

The *Lactobacilli* genes bands were detected in all vaginal samples by percentage 100% in conjunction with the housekeeping genes bands which were already found in these samples (Table 2).

3.2. The *Lactobacilli* bacterial genes in premenopausal and postmenopausal women:

The genes bands of *L. gasseri* and *L. crispatus* were detected in vaginal secretions of premenopausal group in all samples by percentage 100% and also postmenopausal group in all samples by percentage 100% (table 3).

3.3. The *Lactobacilli* bacterial genes in the separate body fluids:

The genes bands of *L. gasseri* and *L. crispatus* were detected in all samples of vaginal secretions by percentage 100% and in some samples of menstrual blood by percentage 66.7% but not in any sample of the other body fluid tested (blood, semen and saliva) (Table 4).

3.4. The *Lactobacilli* bacterial genes in the mixed body fluids:

The genes bands of *L. gasseri* and *L. crispatus* were detected in vaginal fluid containing mixtures but not in the mixtures not containing the vaginal fluid.

They appeared in Semen and Vaginal fluid mixture by percentage 100% and in Blood, Semen and Vaginal fluid mixture by percentage 100%. They didn't appear in any sample of Blood and Semen mixture or Blood, Semen and Saliva mixture (Table 5).

4. DISCUSSION

Analysis of cell-specific mRNA expression is a new technique for the identification of body fluids from biological stains (Haas et al., 2009).

The aim of this experimental study is to assess the usefulness of using *Lactobacilli* RNA markers for identification of vaginal fluid in crime scenes by incorporating them with other body fluid markers in the multiplex reverse transcription-polymerase chain reaction (RT-PCR) system used for body fluids identification

Regarding the presence of *Lactobacilli* genes in vaginal fluid, This study proved the presence of the 16S–23S ISR of *L. gasseri* and *L. crispatus* in vaginal secretions.

Lactobacilli species have been found to be the predominant bacteria in the vagina of women (Va'squez et al., 2002; Nam et al., 2007; Witkin et al., 2007).

Regarding the presence of the *Lactobacilli* bacterial genes in premenopausal and postmenopausal women, The genes bands of *L. gasseri* and *L. crispatus* were detected in vaginal secretions of premenopausal and that of postmenopausal women. This means that there is no effect of child bearing period on the detection of these genes and hence its effectiveness as a vaginal markers.

Also, the 16S–23S ISR of *L. gasseri* and *L. crispatus* were proved to be present in vaginal secretions in (Fleming and Harbison, 2010) study in both premenopausal and postmenopausal women.

Regarding the specificity of the *Lactobacilli* markers to the vaginal fluid, This study found that the *Lactobacilli* markers were present only in vaginal fluid (Figure 1) and some

cases of menstrual blood samples (Figure 2) and not in blood, saliva or semen.

The menstrual blood samples showing *Lactobacilli* markers can be differentiated by menstrual blood specific marker (MMP11) which can't be detected in vaginal fluid.

From this result, the specificity of *Lactobacilli* markers to vaginal fluid was proved and this will be of great importance especially in sexual crimes.

These two *Lactobacilli* bacteria species were reported to be specific to the vagina in previous studies (Song et al., 2000; Witkin et al., 2007).

Another study (Fleming and Harbison, 2010) found also that *Lactobacilli* ribosomal RNA markers were present only in vaginal fluid and not in blood, saliva, semen.

Also Akutsu et al. (2012) in his study reported that 16S ribosomal RNA genes of *L.*

crispatus could be effective specific markers for forensic identification of vaginal fluid.

Lactobacilli markers can be used instead of HBD1 and MUC4 markers which were used in previous studies for the identification of vaginal secretions Alvarez et al., 2004; Juusola and Ballantyne, 2005; Nussbaumer et al., 2006; Setzer et al., 2008).

These markers were proved to be non specific and reported to be expressed in saliva and salivary glands (Abiko et al., 2003 ; Liu et al., 2003)

Regarding the effectiveness of use of the *Lactobacilli* markers in the detection of vaginal fluid in mixed stains, The *Lactobacilli* markers were found in mixtures containing vaginal fluid (Semen and Vaginal fluid & Blood, Semen and Vaginal fluid) by 100% (Figures 3 and 4) and they weren't found in mixtures not containing vaginal fluids (Blood and Semen & Blood, Semen and Saliva) by 0% (Figures 5 and 6).

Table (2): The presence of *Lactobacilli* gene with the house keeping genes in the vaginal fluid:

Vaginal gene markers	Vaginal fluid samples						N	%
	1	2	3	4	5	6		
Vaginal <i>Lactobacilli</i> gene markers	+	+	+	+	+	+	6	100.0
Housekeeping genes	+	+	+	+	+	+	6	100.0

P = 1.0 → Non significant

Table (3): The presence of *Lactobacilli* gene in premenopausal and postmenopausal women:

Vaginal gene markers	Vaginal fluid samples						N	%
	1	2	3	4	5	6		
Premenopausal	+	+	+	+	+	+	6	100.0
Postmenopausal	+	+	+	+	+	+	6	100.0

P = 1.0 → Non significant

Table (4): *Lactobacilli* genes in the separate body fluids:

Body fluid	Presence of <i>Lactobacilli</i> bacterial gene markers						N	%
	1	2	3	4	5	6		
Vaginal fluid	+	+	+	+	+	+	6	100.0
Saliva	-	-	-	-	-	-	0	0.0
Semen	-	-	-	-	-	-	0	0.0
Blood	-	-	-	-	-	-	0	0.0
Menstrual blood	+	+	+	+	-	-	4	66.7

P < 0.001 → highly significant

Table (5): *Lactobacilli* genes in mixed body fluids:

Body fluid mixture		Presence of <i>Lactobacilli</i> bacterial gene markers						N	%
		1	2	3	4	5	6		
Mixtures containing vaginal fluids	Semen and Vaginal fluid	+	+	+	+	+	+	6	100.0
	Blood, Semen and Vaginal fluid	+	+	+	+	+	+	6	100.0
Mixtures without vaginal fluid	Blood and Semen	-	-	-	-	-	-	0	0.0
	Blood, Semen and Saliva	-	-	-	-	-	-	0	0.0

P < 0.001 → highly significant

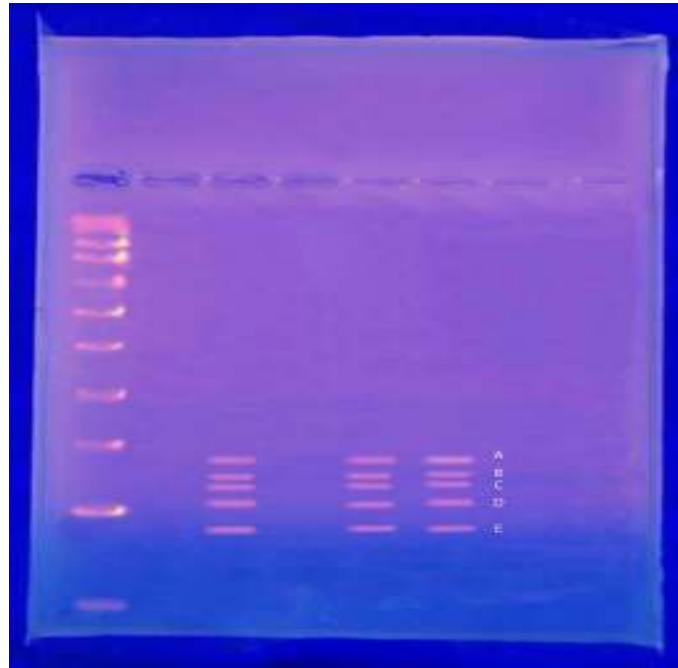


Figure (1): The presence of *Lactobacilli* genes (*L. crispatus*→ **A** and *L. gasseri*→ **B**) with the three housekeeping genes (UCE→ **C**, TEF→**D** and G6PDH→ **E**) in vaginal fluid samples.

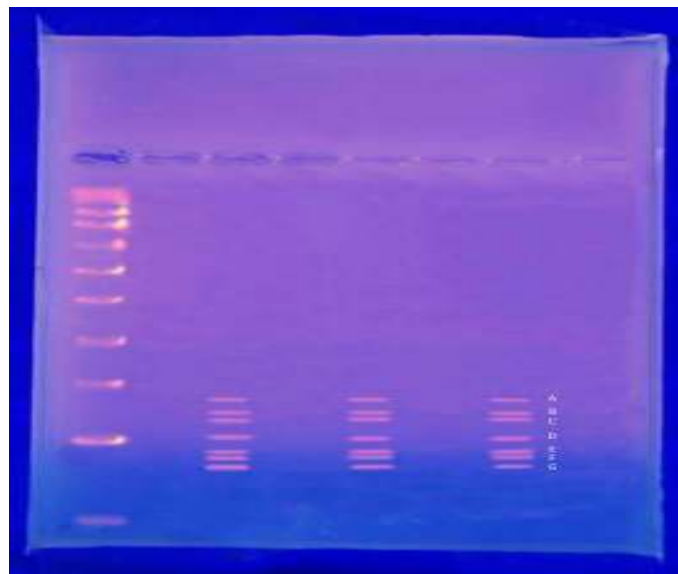


Figure (2): The presence of menstrual blood specific gene (matrix metalloproteinase11→ **G**) with the three housekeeping genes (UCE→ **C**, TEF→ **D** and G6PDH→ **F**) in the menstrual blood samples. There *Lactobacilli* genes are detected (*L. crispatus*→ **A** and *L. gasseri*→ **B**). The blood specific gene (glycophorinA → **E**) is also detected.

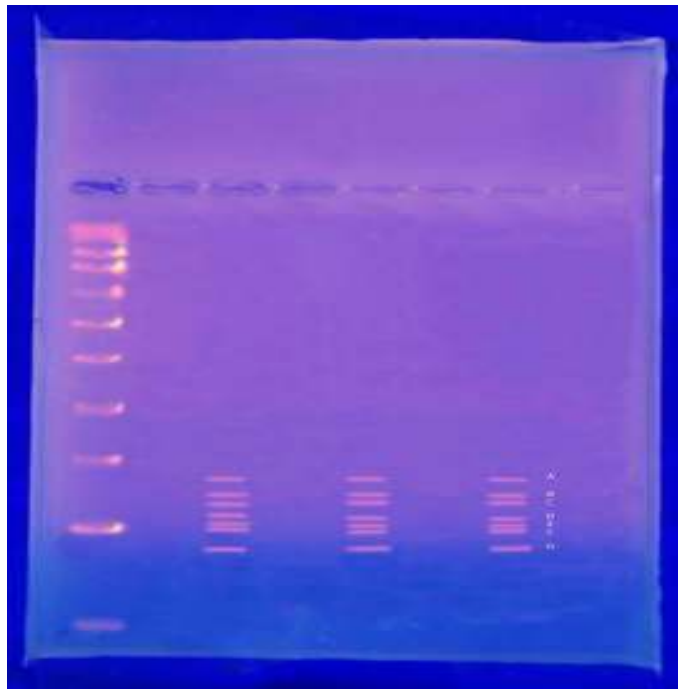


Figure (3): The presence of vaginal *Lactobacilli* genes (*L. crispatus*→ **A** and *L. gasseri*→ **B**) genes and semen specific genes (protamine 2→ **F** and transglutaminase 4 genes→ **D**) with the three housekeeping genes (UCE→ **C**, TEF→ **E** and G6PDH→ **G**) in the vaginal fluid – semen mixture.

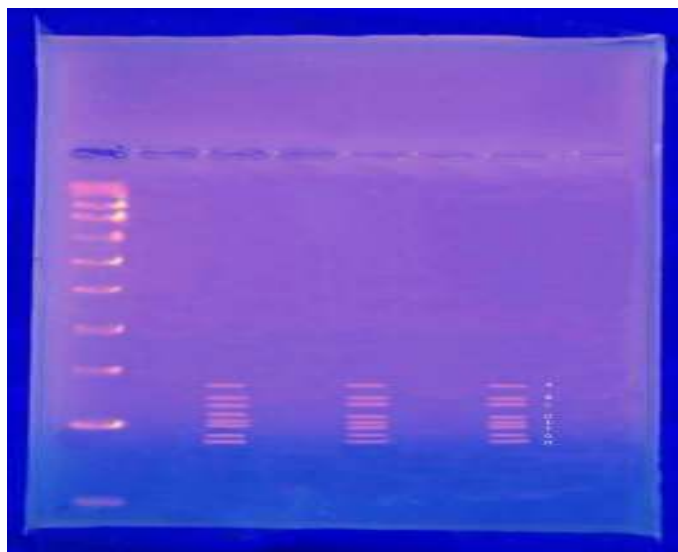


Figure (4): The presence of vaginal *Lactobacilli* genes (*L. crispatus*→ **A** and *L. gasseri*→ **B**) genes, semen specific genes (protamine 2→ **F** and transglutaminase 4 genes→ **D**) and blood specific gene (glycophorinA→ **G**) with the three housekeeping genes (UCE→ **C**, TEF→ **E** and G6PDH→ **H**) in the blood, semen and vaginal fluid mixture.

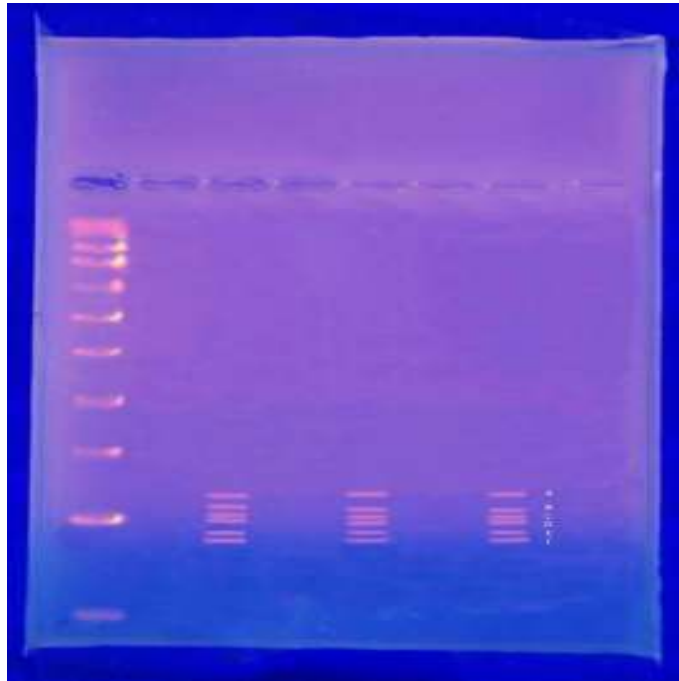


Figure (5): The presence of blood specific gene (glycophorinA→ E) and semen specific genes (protamine 2→ D and transglutaminase 4 → B) with the three housekeeping genes (UCE→ A, TEF→ C and G6PDH→ F) in the blood - semen mixture. There is no detection of *L. gasseri* and *L. crispatus* genes of the vaginal fluid.

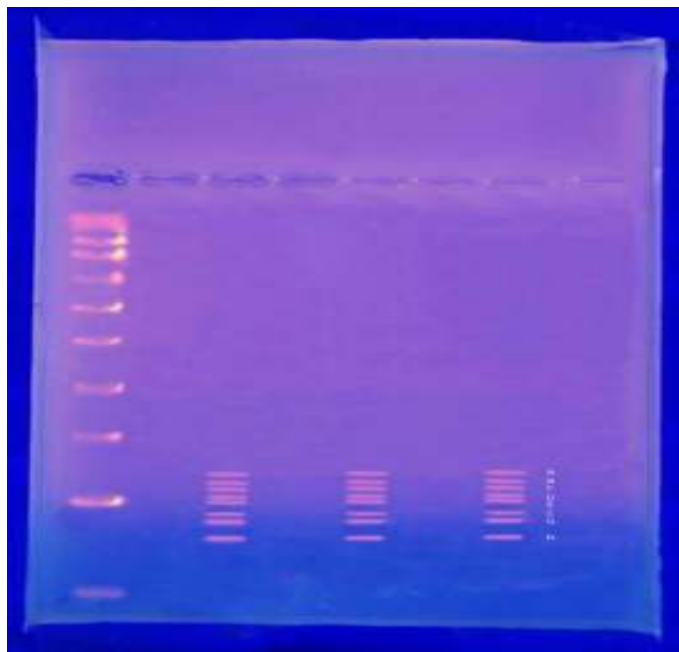


Figure (6): The presence of blood specific gene (glycophorinA→ F) and semen specific genes (protamine 2→ E and transglutaminase 4 → C) and saliva specific genes (histatin 3→ B and statherin→ H) with the three housekeeping genes (UCE→ A, TEF→ D and G6PDH→ G) in the blood, semen and salivary fluid mixture. There is no detection of *L. gasseri* and *L. crispatus* genes of the vaginal fluid.

5. CONCLUSION

The *Lactobacilli* markers are present in vaginal fluid. There is no affection of age on its presence. The *Lactobacilli* markers are specific to vaginal fluid and they aren't present in other body fluids. The *Lactobacilli* markers are effective in the detection of vaginal fluid in body fluid mixtures.

6. RECOMMENDATIONS

The use of the *Lactobacilli* markers as specific markers for the vaginal fluid is recommended instead of the older markers which were found to be non specific. Also the search for other specific markers for vaginal fluid is recommended.

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الاستخدام الطبى الشرعى لدلالات العصيات اللبنية فى الاستعراف على الافرازات المهبليه للانسان

ان استخدام تفاعل البلمرة المتسلسل العكسى المتعدد هو طريقة جديدة للاستعراف على سوائل الجسم المختلفة في مسرح الجريمة في خطوة واحدة حيث أنه تم اكتشاف دلالات من نوع الحمض الريبيى النووى خاصة بكل نوع من سوائل الجسم المختلفة (الدم واللعباب والسائل المنوى والافرازات المهبليه ودم الحيض) وتستطيع الاستعراف عليه دون غيره من سوائل الجسم الأخرى. وكان هدف هذه الدراسة هو:

استخدام دلالات جينية جديدة واثبات فائدتها فى الاستعراف على الافرازات المهبليه فى مسرح الجريمة وهى الدلالات الجينية للعصيات اللبنية.
الطريقة المستخدمة:

اشتملت هذه الدراسة على ٢٠ رجل و ٣٠ سيدة ما بين ٢٠ و ٥٥ عام وتم أخذ عينات مختلفة من جميع سوائل الجسم (الدم واللعباب والسائل المنوى والافرازات المهبليه ودم الحيض) بعد أخذ الموافقة من المتبرعين وقد تم عمل تفاعل البلمرة المتسلسل العكسى المتعدد لكل العينات السابقة بعد تحضيرها.

وقد اظهرت نتائج هذا البحث ما يلى:

١- أن الدلالات الجينية للعصيات اللبنية توجد في الافرازات المهبليه للانسان حيث أنها ظهرت في كل عينات الافرازات المهبليه مع جينات التدبير المنزلى.
٢- أن الدلالات الجينية للعصيات اللبنية لا تتأثر بالسن حيث أنها ظهرت في كل عينات الافرازات المهبليه من السيدات قبل وبعد بلوغ سن اليأس.

٣- أن الدلالات الجينية للعصيات اللبنية تستطيع تمييز الافرازات المهبليه بدقة عالية من بين كل سوائل الجسم المختلفة.

٤- ان الدلالات الجينية للعصيات اللبنية تستطيع تمييز الافرازات المهبليه الموجودة ضمن خليط من سوائل الجسم المختلفة يحتوى علي الافرازات المهبليه.

التوصيات:

استخدام الدلالات الجينية للعصيات اللبنية بدلا من الدلالات القديمة للاستعراف على الافرازات المهبليه والوارد وجودها في مسرح الجريمة وخاصة جرائم الاغتصاب