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*Research Paper*

# Investigation of factors affecting the presence of *Lactobacillus spp*. used in identification of vaginal secretion

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**ABSTRACT**

The detection and identification of human body fluids at a crime scene is necessary in forensic anlysis, being a source for investigation and contributing to case evidence as regards the occurrence of an event. In addition to serological tests, methods based on human RNA analysis were developed for identification of body fluids and stains. The use of bacteria for the identification of body fluids was also being investigated and studies showed promising use of vaginal specific bacteria as a means of identifying vaginal secretions. In this study, the presence of the various *Lactobacillus spp*. including *Lactobacillus gasseri, Lactobacillus crispatus* and *Lactobacillus jensenii* were investigated in the vaginal swab samples taken from ninety-one (91) women cases with vaginal infections and postmenopausal. RNA isolation, cDNA synthesis and PCR were done respectively to 16S-23S rRNA intergenic spacer regions of the *Lactobacillus spp.* investigated. PCR products were run in capillary electrophoresis. It is believed that the negative results obtained cannot be used to exclude the presence of vaginal secretion as a result of undetected *Lactobacillus spp* in 12.9% of infection diagnosed samples and 46.6% of postmenopausal period cases. However, positive results may be valuable especially because no serological methods have been found in identification of vaginal secretion until now. In describing vaginal secretion, it is advisable to perform co-analysis of all three *Lactobacillus* species, and the presentation of any two *Lactobacillus* strains may support the possibility that the stain or biological material can be vaginal.

**Key words:** Sexual assault, *Lactobacillus*, RNA, vaginal secretion, body fluid identification.

## INTRODUCTION

In addition to revealing the genetic profile of the body fluids obtained from the site through DNA analysis, the origin of body fluids must also be determined (Hanson and Ballantyne, 2010; Virkler and Lednev, 2009). The identification of the origin is important in determining how the event occurred and contributes to case evidence (Hanson and Ballantyne, 2013).

In addition to murder or suicide, the resolution of sexual assault event is facilitated by the crime scene investigation. How and where the vaginal secretions are obtained from the scene on an object (apparel, furniture); looking at the distribution of biological fluids in the enviroment can support the victims or suspect's statements, if sexual activity took place voluntarily or not (Hanson and Ballantyne, 2013; Williams et al., 2013). For example, if a woman is sexually assaulted during her mensturation period, the solicitation from the victims lawyer to distinguish the menstural blood found on the accused from nose bleeding, could help in resolving the case in a healthy manner (Meissner and Ritz-Timme, 2010; Joanna et al., 2014). Likewise, the presence of a smeared spot on the victim or identification of the vaginal secretion on a penile

**Table 1:** Primers used in the study (F: Forward; R: Reverse).

| Variable | mRNA marker | Primer row (fluorescent dye) | References |
|---|---|---|---|
| Vaginal secretion | *L. jensen*ii (16S ribosomal RNA) | F:PET-AAGTCGAGCGAGCTTGCCTATTGAAAT<br>R:CGCCTTTTAAACTTCTTTCATGCGAAAGTAGC | (Roeder, 2013) |
| | *L. gasseri* (16S-23S intergenic Spacer region) | F: FAM- CATCGAGAAAGCCAAGCGGAAGC<br>R: GATCATTGCTTACTTACTGCTCCCCG | |
| | *L. crispatus* (16S-23S intergenic spacer region) | F:NED-GTATCCAGAGCAAGCGGAAGCACAC<br>R:GCATCTCTGCATTGGGTTCCCCC | |
| Control gene 1 | GAPDH | F: VIC-TCTTCACCACCACGGAGAA<br>R: AGGGGGCAGAGATGATGAC | (Haas, 2009) |
| Control gene 2 | UCE | F:VIC-AATGATCTGGCACGGGACC<br>R:ATCGTAGAATATCAAGACAAATGCTGC | (Fleming, 2010) |

swab can be used to confirm the presence of sexual activity (Hanson and Ballantyne, 2013; Hadzic et al., 2011).

Identification of vaginal secretion is a bit more complicated when compared to other body fluids. There have been many previous studies on this subject. Recently, studies supporting the use of bacterial RNAs of *Lactobacillus gasseri* (*L. gasseri*), *Lactobacillus crispatus (L. crispatus) and Lactobacillus jensenii* (*L. jensenii*) in the identification of vaginal secretion have been undertaken. In this study, the presence of these three bacterial species in the normal population, in cases with vaginal infection and in the postmenopausal age group; and the possibility of routine use of these strains in the identification of forensic vaginal secretion were investigated.

## MATERIALS AND METHODS

Vaginal swabs taken from the volunteers in Çukurova University, Medical Faculty Balcalı Hospital Obstetrics and Gynecology Clinic between 04.04.2016 to 20.06.2016 were included in this study. The samples were also investigated whether the 3 *Lactobacillus* type (*L. gasseri*, *L. crispatus* and *L. jensenii*) were affected due to vaginal infection and postmenopausal period. Required permissions were obtained from the Ethics Committee of the Faculty of Medicine of Çukurova University. Written and verbal consent was obtained from volunteers after being informed about the study. Financial support was provided from the Scientific Research Projects Unit of Cukurova University in order to be able to carry out this work with project code TTU-2016-5499.

Thirty (30) samples were chosen as control group from volunteers who were not suffering from any disease and were physically and hormonally normal as a result of physical examination and laboratory tests and were not on any medication from women who came for fertilization treatment to the Obstetrics and Gynecology Infertility Polyclinic. On the other hand, 30 samples were chosen as postmenopausal group from volunteers who came to gynecology polyclinic for annual menopause control examination and were not suffering from any disease and were physically and hormonally normal as a result of physical examination and laboratory tests and were not on any medication. Finally, 31 samples were chosen as infection group from voluntary patients just before the initiation of treatment from those who came to gynecological polyclinic because of stinking vaginal discharge and itching complaints and had vaginal infection diagnosis.

Samples were taken from the volunteers who gave verbal and written consent before the examination. Single-use, sterile packed and non-lubricated speculum were used for vaginal sampling. Speculum was inserted into vagina by giving lithotomy position. Samples were taken by gentle spinning 360° through the posterior fornix and vaginal wall with sterile cotton vaginal swabs. The samples were stored at -20°C until RNA isolation.

RNA isolation, cDNA synthesis and PCR were performed in the 16S-23S rRNA intergenic spacer region of *Lactobacillus* species, respectively (Table 1). After PCR, the samples were run on capillary electrophoresis.

## RESULTS

Thirty (30) samples were taken from each group for a successful statistical comparison. At this point, 30 samples for control group, 30 samples for infection group and 31 samples for postmenopausal group were collected from volunteers participating in this study. Groups were analyzed by means of age and the presence of *Lactobacillus* species. IBM SPSS Statistics Version 20.0 package program was used for statistical analysis of the data. Categorical measurements were summarized as number and percentage, while numerical measurements were summarized as mean and standard deviation. Chi-square test was performed to compare categorical measurements

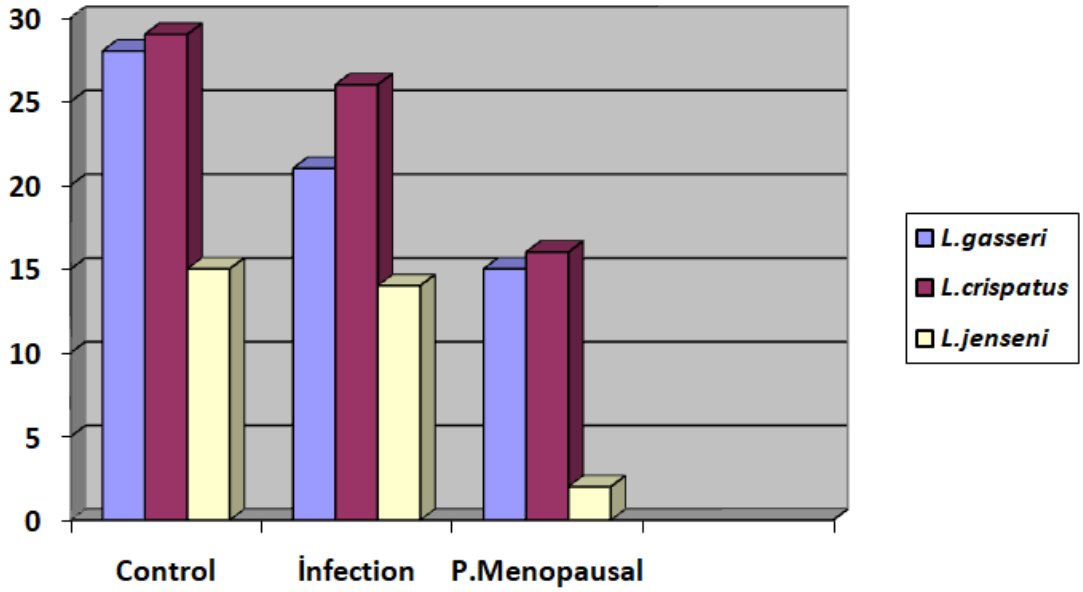


**Figure 1:** Detection ratio of *Lactobacillus* species according to groups.

between groups.

The age range of the control group was between 21 to 45 years, and the average age was 31.8. The age range of the infection group was between 19 to 47 years and the average age was 37.2. The age range of the postmenopausal group was between 44 to 70 years and the mean age was 56.8. The *Lactobacillus* species *L. gasseri*, *L. crispatus* and *L. jensenii* searched were examined sequentially according to the 3 groups.

### *Lactobacillus gasseri*

In the control group, 28 of the 30 cases were found to have *L. gasseri* and only 2 cases did not have the *L. gasseri* strain. When we compared infection and postmenopausal age group with control group; *L. gasseri* positivity was found in 21 of the 31 cases in the infection group, while bacteria was not detected in 10 cases. Similarly, *L. gasseri* was detected in 15 of the postmenopausal cases, but not in the remaining 15 patients. The statistical analysis showed that there was a significant reduction in the detection rate of bacterial strain in both the infection group and the postmenopausal group ($p<0.001$).

### *Lactobacillus crispatus*

In the control group, 29 of the 30 cases were found to have Lcris and only 1 case did not have the Lcris strain. When we compared infection and postmenopausal age group with control group; *L. crispatus* positivity was found in 26 of the 31 cases in the infection group, while bacteria was not detected in 5 cases. On the other hand, *L. crispatus* was detected in 16 of the postmenopausal cases, but not in the remaining 14 patients. The statistical analysis showed that there was a significant reduction in the detection rate of bacterial strain in both the infection group and the postmenopausal group ($p<0.001$).

### *Lactobacillus jensenii*

In contrast to the other two bacteria strains, it was also found to be detected with a low ratio in the control group. *L. jensenii* was detected only in 15 of 30 cases and the remaining 15 patients did not have *L. jensenii* strain. Similar to *L. crispatus*, no significant difference was found between control and infection groups in which *L. jensenii* was positive in 14 of 31 cases and was not detected in the remaining 17 cases. In the postmenopausal period, there was a serious decrease and only 2 of 30 cases were found to have *L. jensenii* (Figure 1). According to the results of statistical analysis, it was seen that there was a significant decrease in the detection rate of bacterial strain in the postmenopausal group when compared with control and infection groups ($p<0.001$).

Examining the the double and triple combination of all three bacterial strains in our study, *L. gasseri* and *L. crispatus* were found to be more common in all three groups. There was a decrease in the triple combination of *L. gasseri*, *L. crispatus* and *L. jenseni* and the combination of *L. gasseri* and *L. jensenii* due to the decrease in the detection rates of *L. jensenii* (Table 2). When there are no *Lactobacillus* strains or at least one or two bacterial strains in the groups, no bacteria were detected in 3.3% of the cases in the control group, 12.9% of the cases of infection and 46.6% of cases after the menopausal period (Table 3).

**Table 2:** Coexistence of *Lactobacillus* strains.

| Group | Control | Infection | Post menopausal |
|---|---|---|---|
| *L. gasseri* + *L. crispatus* + *L. jensenii* | 15 | 10 | 2 |
| *L. gasseri* + *L. crispatus* | 28 | 20 | 15 |
| *L. gasseri* + *L. jensenii* | 15 | 10 | 2 |

**Table 3:** Presence of *Lactobacillus* strains.

| Group | Control | Infection | Post menopausal |
|---|---|---|---|
| Presence of any of the two strains | 28 | 24 | 15 |
| Presence of at least one strain | 29 | 27 | 16 |
| Presence of no strain | 1 | 4 | 14 |

## DISCUSSION

Currently, immunological and enzymatic kits are utilized for the identification of a wide variety of biological fluids. Semegolin antigen / prostate-specific antigen in semen, amylase in the saliva fluid, glycophorin / hemoglobin in the stains, and specific antibodies proving Tamm-Horsfall protein in the urine were produced.

The methods used to demonstrate the presence of vaginal fluid are much more complex (Taylor, 1983). Firstly, the identification of the glycogen present in the vaginal epithelial cell by the Lugol-iodine reaction was first used to demonstrate the increased vaginal secretion on the person's penis that was alleged to be guilty. However, the demonstration of the presence of Lugol-positive epithelial cells in the urinary system of man has reduced the use of this method in forensic applications (Pothof et al., 2009). RT-PCR-mediated studies of MUC-4, HBD-1, ESR-1 mRNAs have been used to identify vaginal secretion.

Recently, results of studies supporting the use of *L. gasseri*, *L. crispatus* and *L. Jensenii* bacteria RNAs in the identification of vaginal fluid have been taken. This study routinely explored the use of these three bacterial strains in the identification of forensic vaginal fluids in the normal population, cases with vaginal infection and in postmenopausal age groups.

*Lactobacillus* species dominant in the vaginal environment may differ due to ethnic groups. In the studies on whites, blacks, asian and spanish speaking races in America; *L. iners, L. crispatusun, L. gasseri* and *L. jensenii* were found to be in varying amounts between the groups (Jacques et al., 2015). One study described *L. acidophilus L. crispatusun* and *L. jensenii* as the most common vaginal *Lactobacillus* species in the vaginal flora of healthy women (Famularo et al., 2001). In another study, to determine predominant *Lactobacillus* species in the vaginal flora, *L. gasseri* and *L. crispatusun* were reported to be the most dominant combination in Turkey and America (Kılıç et al., 2001; Aslım and Kılıç, 2006). *L. crispatus* has been shown to be present in all populations, although the predominant *Lactobacillus* species can be altered, as can be seen in studies.

In the control group of our study, *L. gasseri and L. crispatus* co-detection rates was found to be 93.3%, and the presence of all three bacterias together with *L. jensenii* remained at 50%. The other two bacterias other than *L. jensenii* were found to be the ideal combination for the identification of vaginal secretion. Previously, in a 41-sample study in which all three bacterial mRNAs for vaginal fluid identification were investigated, the samples were taken at different stages of the menstrual cycle and found to be 73.2% of *L. gasseri* and 68.3% of *L. crispatus* (Jakubowska et al., 2013). In another study, *L. gasseri* and *L. crispatus* were found together in all of the 14 vaginal swabs and in another study, *L. gasseri* and *L. crispatusun* were found together in all of the 10 vaginal swabs (Rachel, 2010; Vüqar, 2015). *L. gasseri* and *L. crispatus* can be observed to be very high together as seen in our study.

Examining cases of vaginal infection in our study, *L. gasseri*, *L. crispatus* and *L. jensenii* were found to be 68, 84 and 45%, respectively. It was observed that *L. gasseri* decreased significantly in the presence of infection as compared to the other two *Lactobacillus* strains, the sensitivity was higher, and there was a slight decrease in *L. crispatus* and *L. jensenii*, although not significant. The reasons for the change in the vaginal flora include: antibiotic, cytostatic, corticosteroid, antiviral, antifungal drugs and radiotherapy, vaginal shower, malformation after surgery and radiotherapy, anatomical deformities, cysts, hives, polyps, immunosuppression (AIDS etc) treatment, aging or hormonal changes due to oral contraceptive use, uncontrolled diabetes, foreign bodies (RIA, Diaphragm, Buffer etc) and spermicidal agents (Mardh, 1991).

Depending on these factors, predominance of *Lactobacillus* in the vaginal flora is decreasing. Lactobacillus prevent other pathogenic microorganisms from clinging to epithelial cell receptors, make the microenvironment unviable by producing antimicrobial substances such as hydrogen peroxide, bacteriocin, bacteriocin-like substances and biosurfactants, and finally prevents vaginal infection

(Boris and Barbes, 2000; Ocana and Nader-Macias, 2002). However, vaginal infections such as BV, candida vaginitis and trichomoniasis can occur with decreasing *Lactobacillus*. As observed in our work, there has been a decrease in the detection rates of all three *Lactobacillus* species, with more in *L. gasseri*.

Another group studied was the postmenopausal period. When the detection status of the *Lactobacillus* species in the postmenopausal period was examined, it was found that the presence of *Lactobacillus* decreased significantly during menopause compared to the control and infection group. Detection ratios of *L. gasseri*, *L. crispatus* and *L. jensenii* were found to be 50, 53 and 7%, respectively. In healthy women, the physiological conditions of the vagina can also change in different phases of life. The change in hormonal levels before and after puberty and postmenopausal period, and consequently the increase or decrease in the amount of estrogen, also affects *Lactobacillus*. While there was an increase in *Lactobacillus* in newborn and postpubertal period, there was a decrease in prepubertal and postmenopausal period (McGroarty, 1996; Reid and Burton, 2002). Similarly, in this study, there was a decrease in the detection rate due to hormonal effects in all three *Lactobacillus* species.

Recently, vaginal secretion has been identified using MUC-4, the major component of vaginal mucus, and HBD-1, a vaginal antimicrobial polypeptide (Lindenbergh et al., 2012; Haas et al., 2009). Especially when we look at some studies with MUC-4, it was seen that all of the vaginal swab specimens taken from the women in the reproductive period had visible MUC-4, while none of the women in the menopausal period could be detected. MUC-4 was detected in 33 of the vaginal swab samples taken from women aged between 25 and 50 years; and on the other hand, in vaginal swab samples of 9 women, MUC-4 was not detected. The 9 cases in which MUC-4 could not be detected belong to postmenopausal period (Hadzic et al., 2011). This is explained by vaginal atrophy and vaginal dryness due to hormonal effects. However, in our study, it was found that the combination of *L. gasseri* and *L. crispatus* was found to be up to 50%, despite the decrease in postmenopausal period.

Apart from this, MUC-4 and also HBD-1 can be seen in the saliva outside the target tissue, therefore specific markers for saliva, like STATH and HTN3 are used, after it is shown that the existing material is not saliva, the fluid may be stated as vaginal fluid (Cossu et al., 2009; Nussbaumer et al., 2006). The absence *Lactobacillus* species in saliva and semen is an advantage over MUC-4 and HBD-1; the use of an extra marker is not required. In cases where vaginal smear is taken before puberty between the ages of 4 to 12 and after antibiotic use where *Lactobacilus* count is diminished, MUC-4 still can be accounted and is said to be more effective in such cases.

As a result, detection of no bacteria in 12.9% of patients with infection diagnosis and 46.6% of postmenopausal
cases, suggests that negative results in identifying vaginal fluid or stain can not be used to exclude vaginal secretion, but positive results may be very important, especially since there has been no serologic method to identify vaginal secretion.

Considering that the age group of cases exposed to sexual assault is mostly post puberty period, it is thought that in a large part of the events this can provide a prediction about the detection of vaginal fluid.

In describing vaginal secretion, it is advisable to perform co-analysis of all three *Lactobacillus* species, and the presentation of any two *Lactobacillus* strains may support the possibility that the stain or biological material can be vaginal.

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