DETERMINING RISK OF VAGINAL INFECTION

FIELD OF THE INVENTION

The present invention relates to means, uses, and methods for determining the risk of a vaginal infection in a subject.

BACKGROUND OF THE INVENTION

Vaginitis i.e., vaginal infection is the most common gynecological problem in adult women. Infectious vaginitis presents itself in three primary forms: bacterial vaginosis, *Candida* vaginitis, and *Trichomonas* vaginitis. Bacterial vaginosis is caused by an overgrowth of facultative anaerobic bacteria in the vaginal microflora. Typically, the symptoms of bacterial vaginosis are non-specific and differential diagnosis is problematic.

The microbial composition of the vagina (i.e., the vaginal microflora, vaginal microbiota or vaginal microbiome) has a significant role in women's health e.g., for the propagation of STDs (sexual transmitted diseases) or for the risk of preterm birth during pregnancy. Typical bacterial species in the vagina are benign and beneficial and the dominance of *Lactobacillus* spp. protect the body from the signs of clinical infection of facultative pathogenic microbes. Under normal circumstances, beneficial microbial species maintain a healthy balance of growth with the dominance of *Lactobacillus* spp.. However, a disturbance of this balance (i.e., dysbiosis) can negatively impact women's health, such as causing bacterial vaginosis, increase the risk of acquiring STDs, or cause reproductive complications such as spontaneous abortions, premature births, or low fetal birth weight. As such, assessment of the vaginal ecosystem is important for preventing and treating such conditions.

The Amsel Criteria for bacterial vaginosis include pH-value, evaluating the presence of clue cells, white discharge, and an odor of amines after mixing with KOH.

Microscopic evaluation for bacterial vaginosis commonly uses the Nugent scoring system which is a scoring system for gram-stained vaginal swabs to diagnose e.g. bacterial vaginosis. Thereby, the score is calculated by assessing the presence or absence of large gram-positive rods (*Lactobacillus* spp. morphotypes, decrease in *Lactobacillus* spp. morphotypes scored as 0 to 4), small gram-variable rods (*Gardnerella vaginalis* morphotypes, scored as 4 to 0), and curved gram-variable rods (*Mobiluncus* spp. Morphotypes, specifically *Mobiluncus curtisii subsp. curtisii*, scored as 2 to 0). A

score of 7 to 10 is consistent with bacterial vaginosis. Performing the Nugent score requires a trained and experienced microscopist. Furthermore, this is a purely microscopic method which does not allow the clear identification of a specific microorganism and thus, does not allow the specific determination of specific microorganisms such as e.g., specific bacteria. Thus, the use of cultural methods and other methods is limited, as these methods do not differentiate between colonization, contamination and infection. In addition, these methods have a time-latency in common that prolongs the patient's symptoms and disease.

US2020/248239 A1 discloses DNA-based methods and reagents for the detection of various bacterial species such as *Candida albicans*, *Gardnerella vaginalis*, *Prevotella bivia*, *Lactobacillus iners*, *Lactobacillus crispatus*, *Lactobacillus gasseri*, and *Lactobacillus jensenii* for the detection of vaginal infection.

Basavaprabhu H. N. et al. discuss the influence of probiotics on bacterial vaginosis and shows, in Table 1, various microbiome compositions in combination with different levels of vaginal health (Microbial Pathogenesis, 2020, vol. 141, pp: 104029)

Jespers V. et al., discusses the positive influence of *Lactobacillus crispatus* and *Lactobacillus vaginalis* on vaginal health with regard to maintaining the vaginal microbiome and HIV infection. Figure 1 shows the presence of specific bacterial species and the status of bacterial vaginosis. (BMC Infectious Diseases, 2015, vol. 15, no. 1, pp: 115)

US2016/0040216 A1 discloses systems and methods for determining the vaginal microbiome and its health. The methods are based on a modified PCR method that blocks the amplification of the dominant species, thus improving the detection of less dominant species. The resulting results can be used to determine the health of the vaginal microbiome and, for example, the ratio of dominant species to non-dominant species can be determined. All of these methods require specialized laboratories and experts and may take several days to provide results. Currently, there is no comprehensive on-site test available which allows clear determination of the vaginal microbiota. Although laboratory tests for the detection of individual infectious organisms exist e.g., for *Candida albicans*, and although there are next generation sequencing (NGS), qPCR, and probe hybridization (DNA based methods known, there is no system such as rapid test or point-of-care test available which provides a comprehensive and reliable means for determining the overall risk of vaginal infections based on the specific detection of different microorganisms at the genus or species level.

Thus, there is a need for reliable, rapid, inexpensive, and simple means and methods for determining the risk of vaginal infection. Specifically, there is a need for such means and methods which allow the self-collection and self-testing by a subject.

SUMMARY OF THE INVENTION

It is the object of the present invention to provide a new system and method for determining the risk of vaginal infection.

The objective is solved by the subject matter of the present invention.

The inventors of the present invention have found that detecting the herein described microorganisms allows determining the risk of vaginal infection.

The present invention provides a system for *in vitro* determining the risk of vaginal infection in a subject, said system comprising

a) means for specifically detecting in a vaginal sample of said subject:

i. *Candida albicans*;

ii. *Gardnerella spp,* specifically *Gardnerella vaginalis*;

iii. *Prevotella bivia*;

iv. *Lactobacillus iners*; and

v. at least one of *Lactobacillus* species belonging to the *Lactobacillus acidophilus* complex, and *Lacticaseibacillus species* belonging to the *Lacticaseibacillus casei* complex;

and;

b) means for assigning a status of either positive or negative for each of i., ii., iii., iv., and v. based on a);

wherein the risk of vaginal infection in the subject is:

I. low if the status of i., ii., and iii., is negative and the status of v. is positive;

II. intermediate if the status of i. is negative, the status of at least one of ii., and iii. is positive, and the status of v. is positive;

III. intermediate if the status of i., ii., iii., iv., and v. is negative;

IV. high if the status of i. is positive; and

V. high if the status of at least one of ii., iii., and iv. is positive and the status of v. is negative.

Specifically, wherein the *Lactobacillus acidophilus* complex comprises *Lactobacillus crispatus*, *Lactobacillus gasseri*, and *Lactobacillus jensenii*.

Specifically, wherein the *Lacticaseibacilli casei* complex comprises *Lacticaseibacillus rhamnosus*, *Lacticaseibacillus casei*, and *Lacticaseibacillus paracasei.*

Specifically, further comprising means for detecting vi. sialidase and means for assigning a status of either positive or negative for vi..

Specifically, wherein the status of vi. is positive if the sample has a sialidase activity of 5 +/- 2 U/mL or more.

Specifically, wherein the risk of the subject is:

VI. intermediate if the status of vi. is positive, the status of i. is negative, and the status of v. is positive; and

VII. high if the status of vi. is positive, and the status of v. is negative.

Specifically, wherein the means for detecting are selected from the group consisting of antibodies, primers, probes, and chromogenic substrates.

Specifically, wherein the status of i. is positive if *Candida albicans* is detected at a level corresponding to a cfu/mL of 10^4 or copies/ml of 10^4 or higher.

Specifically, wherein the status of ii. is positive if *Gardnerella sp.,* specifically *Gardnerella vaginalis* is detected at a level corresponding to a cfu/mL of 10^4 or copies/ml of 10^4 or higher.

Specifically, wherein the status of iii. is positive if *Prevotella bivia* is detected at a level corresponding to a cfu/mL of 10^4 or copies/ml of 10^4 or higher.

Specifically, wherein the status of iv. is positive if *Lactobacillus iners* is detected at a level corresponding to a cfu/mL of 10^4 or copies/ml of 10^4 or higher.

Specifically, wherein the status of v. is positive if at least one of lactobacilli belonging to the *Lactobacillus acidophilus* complex, and lacticaseibacilli belonging to the *Lacticaseibacillus casei* complex is detected at a level corresponding to a cfu/mL of 10^5 or copies/ml of 10^5 or higher, or if the sum lactobacilli belonging to the *Lactobacillus acidophilus* complex, and lacticaseibacilli belonging to the *Lacticaseibacillus casei* complex is a level corresponding to cfu/mL of 10^5 or copies/ml of 10^5 or higher .

Specifically, wherein the system is a point-of-care test.

Specifically, further comprising means for detecting *Trichomonas* sp.

Specifically, wherein the risk of vaginal infection in the subject is high if the status of *Trichomonas* sp. is positive.

According to the invention there is further provided a kit comprising the system described herein, further comprising an instruction manual.

According to the invention there is further provided a use of the system described herein or the kit described herein for determining the risk of a vaginal infection in a subject.

According to the invention there is further provided an *in vitro* method of determining the risk of vaginal infection in a subject, said method comprising

a) specifically detecting in a vaginal sample from the subject:

i. *Candida albicans*;

ii. *Gardnerella sp,* specifically *Gardnerella vaginalis*;

iii. *Prevotella bivia*;

iv. *Lactobacillus iners*; and

v. at least one of lactobacilli belonging to the *Lactobacillus acidophilus* complex, and lacticaseibacilli belonging to the *Lacticaseibacillus casei* complex;

and;

b) assigning a status of either positive or negative for each of i., ii., iii., iv., and v. based on a);

wherein the risk of vaginal infection in the subject is:

I. low if the status of i., ii., and iii., is negative and the status of v. is positive;

II. intermediate if the status of i. is negative, the status of at least one of ii., and iii. is positive, and the status of v. is positive;

III. intermediate if the status of i., ii., iii., iv., and v. is negative;

IV. high if the status of i. is positive; and

V. high if the status of at least one of ii., iii., and iv. is positive and the status of v. is negative.

Specifically, wherein the *Lactobacillus acidophilus* complex comprises *Lactobacillus crispatus*, *Lactobacillus gasseri*, and *Lactobacillus jensenii.*

Specifically, wherein the *Lacticaseibacilli casei* complex comprises *Lacticaseibacillus rhamnosus*, *Lacticaseibacillus casei*, and *Lacticaseibacillus paracasei.*

Specifically, further comprising detecting vi. sialidase and assigning a status of either positive of negative for vi..

Specifically, wherein the status of vi. is positive if the sample has a sialidase activity of 5 +/- 2 U/mL or more.

Specifically, wherein the risk of the subject is:

VI. intermediate if the status of vi. is positive, the status of i. is negative, and the status of v. is positive; and

VII. high if the status of vi. is positive, and the status of v. is negative.

Specifically, wherein detecting is performed by a method selected from the group consisting of PCR, RNA sequencing-based approaches, DNA sequencing based approaches, cfu determination, ELISA, enzyme test, DNA-hybridisation, RNA-hybridisation, Loop-mediated Isothermal Amplification, probes, reporter systems, and combinations thereof.

Specifically, wherein the status of i. is positive if *Candida albicans* is detected at a level corresponding to a cfu/mL of 10^4 or copies/ml of 10^4 or higher.

Specifically, wherein the status of ii. is positive if *Gardnerella spp.* specifically *Gardnerella vaginalis* is detected at a level corresponding to a cfu/mL of 10^4 or copies/ml of 10^4 or higher.

Specifically, wherein the status of iii. is positive if *Prevotella bivia* is detected at a level corresponding to a cfu/mL of 10^4 or copies/ml of 10^4 or higher.

Specifically, wherein the status of iv. is positive if *Lactobacillus iners* is detected at a level corresponding to a cfu/mL of 10^4 or copies/ml of 10^4 or higher.

Specifically, wherein the status of v. is positive if at least one of lactobacilli belonging to the *Lactobacillus acidophilus* complex, and lacticaseibacilli belonging to the *Lacticaseibacillus casei* complex is detected at a level corresponding to a cfu/mL of 10^5 or copies/ml of 10^5 or higher, or if the sum lactobacilli belonging to the *Lactobacillus acidophilus* complex, and lacticaseibacilli belonging to the *Lacticaseibacillus casei* complex is detected at a level corresponding to a cfu/mL of $10^{5,}$ or copies/ml of 10^5 or higher.

Specifically, further comprising detecting *Trichomonas* sp.

Specifically, wherein the risk of vaginal infection in the subject is high if the status of *Trichomonas* sp. is positive.

FIGURES

Figure 1: Graphical representation of exemplary layouts of the test system. Fig. 1A shows an exemplary layout of a system described herein comprising determination of sialidase. Fig. 1B shows an exemplary layout of a system described herein. Fig. 1C and Fig. 1 D shows an exemplary layout of the system described herein, but divided in two units.

Figure 2: Overview regarding test procedure and central statements of the test system.

Figure 3: Summed bacterial count of *Lb. crispatus, Lb. jensenii* and *Lb. gasseri* from vaginal swabs on MRS agar

Figure 4: *Lb. crispatus, Lb. gasseri* and *Lb. jensenii* bacterial counts from vaginal swabs on MRS agar

Figure 5: Bacterial count of *Gardnerella* spp., *Prevotella* spp. and *Lactobacillus iners* from vaginal swabs on Columbia blood agar

Figure 6: Copy number of *Lb. crispatus, Lb. gasseri,* and *Lb. jensenii* determined in vaginal bast streaks by qPCR

Figure 7: Summed-up copy numbers of *Lb. crispatus*, *Lb. gasseri* and *Lb. jensenii* determined in vaginal bast streaks by qPCR

Figure 8: Copy number of *Gardnerella vaginalis*, *Prevotella* spp. and *Lactobacillus iners* determined in in vaginal swabs determined by qPCR

DETAILED DESCRIPTION

Unless indicated or defined otherwise, all terms used herein have their usual meaning in the art, which will be clear to the skilled person. Reference is for example made to the standard handbooks, such as Sambrook et al, "Molecular Cloning: A Laboratory Manual" (4th Ed.), Vols. 1 -3, Cold Spring Harbor Laboratory Press (2012); Krebs et al., "Lewin´s Genes XI", Jones & Bartlett Learning, (2017), Brock Mikrobiologie: Michael T. Madigan, Kelly S. Bender, et al. Pearson Studium – Biologie 2020; The ELISA Guidebook (2nd ED.) from Methods in Molecular Biology – Book Series, Walker J.M. (Springer), The Immunoassay Handbook (4th Ed.), 2013 Wild D. (Elsevier); Lactic Acid Bacteria – Biodiversity and Taxonomy (2014) Holzapfel W.H. (Hrsg.) & Wood B.J.B. (Hrsg.) Wiley-Blackwell; Microbiology: A Laboratory Manual, 12th edition (2021) Cappuccino J.G. & Welsh C.T., Pearson; Galask R P and Larsen B, Clinical Perspective in Obstetrics and Gynecology, 1986, “Infectious Diseases in the Female Patient”; Schwierzt A, Advances in Experimental Medicine and Biology, 2016, vol. 902, “Microbiota of the Human Body”; Friese K et al., Infektionskrankheiten in Gynäkologie und Geburtshilfe, 2003; Petersen Eiko E, Infektionen in Gynäkologie und Geburtshilfe, 2011; and Neumann G., Schäfer A., Mendling W. “Phasenkontrast-Mikroskopie in der Frauenarztpraxis”, Springer-Verlag (2014).

The subject matter of the claims specifically refers to artificial products or methods employing or producing such artificial products, which may be variants of native (wild type) products, or products isolated by technical means. Though there can be a certain degree of sequence identity to the native structure, it is well understood that the materials, methods and uses of the invention, e.g., specifically referring to isolated nucleic acid sequences, amino acid sequences, fusion constructs, expression constructs, transformed host cells and modified proteins, are "man-made" or synthetic, and are therefore not considered as a result of "laws of nature".

The terms "comprise", "contain", "have", and "include" as used herein can be used synonymously and shall be understood as an open definition, allowing further members or parts or elements. "Consisting" is considered as a closest definition without further elements of the consisting definition feature. Thus "comprising" is broader and contains the "consisting" definition.

The term "about" as used herein refers to the same value or a value differing by +/-5 % of the given value.

As used herein and in the claims, the singular form, for example "a", "an" and "the" includes the plural, unless the context clearly dictates otherwise.

The term "**vaginal infection**" or vaginitis as used herein refers to an infection-related inflammatory state of the vagina. Symptoms of either a vaginal infection or vaginitis may include vulvar and vaginal redness, swelling, edema, vaginal discharge, itching, burning, pain, and a strong odor. A final diagnosis may need a clinical check by a physician.

The term "**risk**" as used herein in the context of a vaginal infection refers to the likelihood that a subject has or will develop a vaginal infection. The vaginal infection can ultimately be diagnosed by a physician.

According to one embodiment, determining the risk of vaginal infection is determining a vaginal infection.

According to one embodiment, the invention described herein provides assessment of healthy vaginal microbiota and a deviation therefrom.

According to a specific embodiment, the healthy vaginal microbiota is a physiologic vaginal microbiota.

According to one embodiment, the risk is either low, intermediate, or high.

According to one embodiment, the risk may be shown as colors, e.g., the risk is either low (green), intermediate (yellow), or high (red).

According to one embodiment, a low risk means that the subject has physiologically healthy vaginal microbiota.

According to one embodiment, a low risk means that the subject does not have a vaginal infection and that the likelihood of developing a vaginal infection is low.

Low risk of vaginal infection may mean that the subject does not need further observation or treatment by a physician.

According to one embodiment, an intermediate or high risk means that the subject has a deviation from physiologically healthy vaginal microbiota.

According to one embodiment, an intermediate risk of vaginal infection means that the subject does not have a vaginal infection which requires immediate treatment but that there is a risk of developing a vaginal infection.

Intermediate risk may mean that the subject should monitor the risk of vaginal infection and may possibly receive treatment to reduce the risk of vaginal infection.

According to a specific embodiment, a high risk of vaginal infection means that the subject has a vaginal infection or that the risk of developing a vaginal infection is high. High risk of vaginal infection may require immediate treatment by a physician.

According to one embodiment, the invention described herein comprises detection of specific microorganisms. Specifically, the specific microorganisms are selected from microorganisms characteristic for non-healthy vaginal microbiota and microorganisms characteristic for healthy vaginal microbiota.

According to one embodiment, examples of microorganisms characteristic for healthy vaginal microbiota are of lactobacilli belonging to the *Lactobacillus acidophilus* complex, and lacticaseibacilli belonging to the *Lacticaseibacillus casei* complex.

According to a specific embodiment, the *Lactobacillus acidophilus* complex comprises *Lactobacillus crispatus*, *Lactobacillus gasseri*, and *Lactobacillus jensenii*.

According to a specific embodiment, the *Lacticaseibacilli casei* complex comprises *Lacticaseibacillus rhamnosus*, *Lacticaseibacillus casei*, and *Lacticaseibacillus paracasei.*

According to one embodiment, at least one, at least two, at least three, at least four, at least five, or all of *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus jensenii*, *Lactocaseibacillus rhamnosus, Lacticaseibacillus casei,* and *Lacticaseibacillus paracasei* are detected.

According to a specific embodiment, *Lactobacillus crispatus* is detected.

According to a specific embodiment, *Lactobacillus gasseri* is detected.

According to a specific embodiment, *Lactobacillus jensenii* is detected.

According to a specific embodiment, *Lactocaseibacillus rhamnosus* is detected.

According to a specific embodiment, *Lacticaseibacillus casei* is detected.

According to a specific embodiment, *Lacticaseibacillus paracasei* is detected.

According to a specific embodiment, described herein is detecting of *Lacticaseibacillus casei* and/or *Lacticaseibacillus paracasei.* Specifically, *Lacticaseibacillus casei* and/or *Lacticaseibacillus paracasei* are detected if the sample is obtained from a woman undergoing menopause or is postmenopausal. More specifically, *Lacticaseibacillus casei* and/or *Lacticaseibacillus paracasei* may be detected in addition to at least one, at least two, at least three, or all of *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus jensenii*, and *Lactocaseibacillus rhamnosus* if the sample is obtained from a woman undergoing menopause or is postmenopausal.

According to an alternative embodiment, described herein is detecting of at least one, at least two, at least three, or all of *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus jensenii*, and *Lactocaseibacillus rhamnosus.* Specifically, at least one, at least two, at least three, or all of *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus jensenii*, and *Lactocaseibacillus rhamnosus* are detected if the sample is obtained from a woman which is not undergoing menopause or is postmenopausal.

According to a specific embodiment, the selection of specific microorganisms to be detected from the group consisting of *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus jensenii*, *Lactocaseibacillus rhamnosus, Lacticaseibacillus casei, Lacticaseibacillus paracasei,* and the combination thereof for detecting may be adapted based on the specific use of the invention described herein.

According to one embodiment, examples of microorganisms characteristic for unhealthy vaginal microbiota are *Candida albicans, Gardnerella vaginalis, Prevotella bivia, and Lactobacillus iners.*

The term "**specifically detecting**" as used herein refers to the detection of the specific microorganism, the specific enzyme, or a further specific marker.

In general, microorganisms such as bacteria and yeasts might be detected by different methods. However, methods such as Gram-staining methods do not allow the specific detection of a specific bacterium but allow only to classify bacterial species into two large groups: Gram-positive bacteria and gram-negative bacteria. Alternatively, classification can also be according to cell shape, such as, but not limited to spherical

(cocci), rod (long and short rods, bacilli), spiral (spirilla), comma (vibrios) or corkscrew (spirochaetes). According to one embodiment, specifically detecting may mean identifying the specific microorganism genus or species, identifying a specific complex of microorganisms, identifying an enzyme, or identifying a further specific marker.

According to a specific embodiment, the following microorganisms, enzymes, and further markers are specifically detected:

i. *Candida albicans*;
ii. *Gardnerella spp.,* specifically *Gardnerella vaginalis*;
iii. *Prevotella bivia*;
iv. *Lactobacillus iners*;
v. at least one of lactobacilli belonging to the *Lactobacillus acidophilus* complex, and lacticaseibacilli belonging to the *Lacticaseibacillus casei* complex*;*
vi. optionally sialidase enzyme; and
vii. optionally further markers.

According to a specific embodiment, the following microorganisms, enzymes, and further markers are specifically detected:

i. *Candida albicans*;
ii. *Gardnerella spp.,* specifically *Gardnerella vaginalis*;
iii. *Prevotella bivia*;
iv. *Lactobacillus iners*;
v. at least one of *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus jensenii*, *Lactocaseibacillus rhamnosus, Lacticaseibacillus casei,* and *Lacticaseibacillus paracasei;*
vi. optionally sialidase enzyme; and
vii. optionally further markers.

Candida albicans is generally known in the field. For example, a specific strain of "***Candida albicans***" is also known under SC531 according to the NCBI taxonomy system.

Gardnerella spp. is generally known in the field. Non limiting examples are *Gardnerella greenwoodii*, *G. leopoldii*, *G. pickettii*, *G. piotii*, *G. swidsinskii* and *G. vaginalis.* For example, a specific strain of *"**Gardnerella vaginalis**"* is also known under type strain DSM No. 4944.

Prevotella bivia is generally known in the field. For example, a specific strain of "***Prevotella bivia***" is also known under type strain: NCTC10287, having accession numbers ATCC 14018; DUKES 594.

Lactobacillus iners is generally known in the field. For example, a specific strain of ***Lactobacillus iners*** is also known under type strain: CCUG 28746 = CIP 105923 = DSM 13335 = JCM 12513 = LMG 18914. *Lactobacillus iners* has accession numbers: Genbank 16S rRNA gene accession number: Y16329; Genbank genome accession number: AZET00000000. *Lactobacillus iners* has a year of valid or effective publication: 1999 DOI: 10.1099/00207713-49-1-217.

Lactobacillus crispatus is generally known in the field. For example, a specific strain of ***Lactobacillus crispatus*** is also known under type strain: ATCC 33820= CCUG 30722 = CIP 102990 = CIPP II = DSM 20584 = JCM 1185 = LMG 9479 = VPI 3199. *Lactobacillus crispatus* has accession numbers: Genbank 16S rRNA gene accession number: AF257097; Genbank genome accession numbers: AZCW00000000. *Lactobacillus crispatus* has a year of valid or effective publication: 1953, DOI: 10.1099/00207713-33-2-426.

Lactobacillus jensenii is generally known in the field. For example, a specific strain of ***Lactobacillus jensenii*** is also known under type strain: 62GT = ATCC 25258T = CCUG 21961T = CCUG 35572T = CIP 69.17T = DSM 20557 = JCM 1146T = JCM 15953T = LMG 6414T = NRRL B-4550T. *Lactobacillus jensenii* has accession numbers: Genbank 16S rRNA gene accession number: AF243176; Genbank genome accession number: AYYU00000000. *Lactobacillus jensenii* has a year of valid or effective publication: 1970, DOI: 10.1099/00221287-62-2-219.

Lactobacillus gasseri is generally known in the field. For example, a specific strain of ***Lactobacillus gasseri*** is also known under type strain: 63 AM of Gasser = ATCC 33323= CCUG 31451 = CIP 102991 = DSM 20243 = JCM 1131 = LMG 9203 = NRRL B-14168 = NRRL B-4240. *Lactobacillus gasseri* has accession numbers: Genbank 16S rRNA gene accession number: AF519171; Genbank genome accession number: NC_008530. *Lactobacillus gasseri* has a year of valid or effective publication: 1980, DOI: 10.1016/S0172-5564(80)80019-4.

Lactocaseibacillus rhamnosus is generally known in the field. For example, a specific strain of ***Lactocaseibacillus rhamnosus*** is also known under type strain: ATCC 7469= CCUG 21452 = CIP A157 = DSM 20021 = IFO (now NBRC) 3425 = JCM 1136 = LMG 6400 = NCAIM B.01147 = NCCB 46033 = NCIMB 6375 (formerly

NCDO 243) = NCTC 12953 = NRRL B-442 = VKM B-574. *Lactocaseibacillus rhamnosus* has the following basonyms (old species names): *Lactobacillus rhamnosus, Lactobacillus casei subsp. rhamnosus.* The name rejection was published in DOI: 10.1099/00207713-39-2-105. *Lactocaseibacillus rhamnosus* has accession numbers: Genbank 16S rRNA gene accession number: D16552; Genbank genome accession number: AZCQ00000000. *Lactocaseibacillus rhamnosus* has a year of valid or effective publication: 1968 DOI: 10.1099/00207713-39-2-105.

Lacticaseibacillus casei is generally known in the field. For example, a specific strain of ***Lacticaseibacillus casei*** is also known under type strain: Orla-Jensen 7 ("Streptobacterium casei") = Hucker03 = OrlandL-323 = Tittsler303 = ATCC 393= BCRC (formerly CCRC) 10697 = CECT 475 = CIP 103137 = DSM 20011 = IAM 12473 = JCM 1134 = KCTC 3109 = LMG 6904 = NBRC (formerly IFO) 15883 = NCIMB 11970 (NCDO 161) = NRRL B-1922. *Lacticaseibacillus casei* has the following basonym (old species name): *Lactobacillus casei. Lacticaseibacillus casei* has accession numbers: Genbank 16S rRNA gene accession number: AF469172; Genbank genome accession number: BALS00000000. *Lacticaseibacillus casei* has a year of valid or effective publication: 1916 DOI: 10.1099/00207713-21-1-69.

Lacticaseibacillus paracasei is generally known in the field. For example, a specific strain of ***Lacticaseibacillus paracasei*** is also known under *Lacticaseibacillus paracasei subsp. paracasei* and to type strain: DSM 5622 = ATCC 25302T = A24-2-1T = AS 1.2826T = JCM 8130T = LMG 13087T. *Lacticaseibacillus paracasei* has the following basonyms (old species names): *Lactobacillus paracasei subsp. paracasei, Lactobacillus casei subsp. alactosus, Lactobacillus casei subsp. pseudoplantarum.* The name rejection was published in DOI: 10.1099/00207713-39-2-105. *Lacticaseibacillus paracasei* has accession numbers: Genbank 16S rRNA gene accession number: D79212; Genbank genome accession number: AZGH00000000. *Lacticaseibacillus paracasei* has a year of valid or effective publication: 1989 DOI: 10.1099/00207713-39-2-105.

According to one embodiment, the system described herein is configured to assign a status of positive or negative for a detected microorganism described herein. According to a specific embodiment, the system is further configured to assign a status of positive of negative for sialidase. According to a specific embodiment, the system is further configured to assign a status of positive of negative for a further marker.

According to a specific embodiment, the system described herein comprises means for assigning a status of either positive or negative for each of i., ii., iii., iv., and v. based on a). According to a specific embodiment, the status may also be assigned for any further detected sialidase or any further marker described herein.

According to one embodiment, assigning a status of positive or negative is based on specifically detecting.

According to a specific embodiment, if the detection shows that a microorganism, sialidase, or a further marker is present then the status of is positive.

According to a specific embodiment, if the detection shows that a microorganism, sialidase, or a further marker is not present then the status is negative.

According to a specific embodiment, a status of positive or negative may be obtained by comparison of the result of specifically detecting with a predetermined reference value and assigning a status of positive if said result is above the reference value or negative if the result is below the reference value.

According to a specific embodiment, said reference value may be a threshold.

According to one embodiment, thresholds for assigning a positive or negative status may be adapted for specific purposes or use cases.

According to a specific embodiment, thresholds for assigning a positive or negative status may be the detection limit of the respective microorganism, enzyme, or further marker detected.

According to one embodiment, thresholds for assigning a positive or negative status may be determined by considering the bacterial count or cfu. Specifically, for the specific detecting principle implemented, e.g., ELISA, the threshold may be determined using cfu determination principles as a reference or calibration system.

According to one embodiment, thresholds for assigning a positive or negative status may be determined by considering the Nugent scores. Specifically, for the specific detecting principle implemented, e.g., ELISA, the threshold may be determined using Nugent score principles as a reference or calibration system.

According to a specific embodiment, the status of i. is positive if *Candida albicans* is detected at a level corresponding to a cfu/mL of 10^4, 10^5, 10^6, 10^7, 10^8, or higher.

According to a specific embodiment, the status of ii. is positive if *Gardnerella spp.,* specifically *Gardnerella vaginalis* is detected at a level corresponding to a cfu/mL of 10^4, 10^5, 10^6, 10^7, 10^8, 10^9, or higher.

According to a specific embodiment, the status of iii. is positive if *Prevotella sp.* is detected at a level corresponding to a cfu/mL of 10^4, 10^5, 10^6, 10^7, 10^8, 10^9, or higher.

According to a specific embodiment, the status of iv. is positive if *Lactobacillus iners* is detected at a level corresponding to a cfu/mL of 10^4, 10^5, 10^6, 10^7, 10^8, or higher.

According to a specific embodiment, the status of v. is positive if at least one of *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus jensenii*, *Lacticaseibacillus rhamnosus, Lacticaseibacillus casei,* and *Lacticaseibacillus paracasei* is detected at a level corresponding to a cfu/mL of 10^5, 10^6, 10^7, 10^8, or higher.

According to a specific embodiment, the status of v. is positive if the sum of at least two of *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus jensenii*, *Lactocaseibacillus rhamnosus, Lacticaseibacillus casei,* and *Lacticaseibacillus paracasei* is a level corresponding to a cfu/mL of 10^5, 10^6, 10^7, 10^8, 10^9, $10^{10,}$ or higher.

According to a specific embodiment, the status of v. is positive if the sum of at least three of *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus jensenii*, *Lactocaseibacillus rhamnosus, Lacticaseibacillus casei,* and *Lacticaseibacillus paracasei* is a level corresponding to a cfu/mL of 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10}, or higher.

According to a specific embodiment, the status of v. is positive if the sum of at least four of *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus jensenii*, *Lactocaseibacillus rhamnosus, Lacticaseibacillus casei,* and *Lacticaseibacillus paracasei* is a level corresponding to a cfu/mL of 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10}, or higher.

According to a specific embodiment, the status of v. is positive if the sum of at least five of *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus jensenii*, *Lactocaseibacillus rhamnosus, Lacticaseibacillus casei,* and *Lacticaseibacillus paracasei* is a level corresponding to a cfu/mL of 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10}, or higher.

According to a specific embodiment, the status of v. is positive if the sum of *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus jensenii*, *Lactocaseibacillus rhamnosus, Lacticaseibacillus casei,* and *Lacticaseibacillus paracasei* is a level corresponding to a cfu/mL of 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10}, or higher.

According to a specific embodiment, assigning the status of positive or negative for iv. *Lactobacillus iners*; and v. at least one of lactobacilli belonging to the *Lactobacillus acidophilus* complex, and lacticaseibacilli belonging to the *Lacticaseibacillus casei* complex may be based on the comparison of detection result of iv. and v.. Specifically, iv. may be assigned a positive status and v. may be assigned a negative status if the level of iv. is higher than the level of v.

According to one embodiment, means for specifically detecting are means for PCR, such as, but not limited real time-PCR. Specifically, primers for the respective microorganisms described herein are used for PCR. Such primers are well known to the skilled person and are selected based on the respective organism to be detected in the sample.

According to one embodiment, means for specifically detecting are means for performing a microbiological method, specifically by a cultivation-based microbiological method. More specifically, the samples are cultivated on plates and the total bacterial count per mL of vaginal secretion is calculated by counting the plates. Alternatively, the bacterial count of individual microorganisms can be determined by counting the colonies of uniform colony morphology. Identification at species level can carried out using the matrix assisted laser desorption ionization (MALDI)-TOF mass spectrometry methods and instruments.

According to a specific embodiment, the status of i. is positive if *Candida albicans* is detected at a level corresponding to 10^4 copies/ml or higher, specifically 10^5,10^6, 10^7, 10^8, 10^9, 10^{10} copies/ml, or higher.

According to a specific embodiment, the status of ii. is positive if *Gardnerella spp.,* specifically *Gardnerella vaginalis,* is detected at a level corresponding to 10^4 copies/ml and higher, specifically 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10} copies/ml, or higher.

According to a specific embodiment, the status of iii. is positive if *Prevotella sp*. is detected at a level corresponding to 10^4 copies/ml and higher, specifically 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10} copies/ml, or higher.

According to a specific embodiment, the status of iv. is positive if *Lactobacillus iners* is detected at a level corresponding to 10^4 copies/ml and higher, specifically 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10} copies/ml, or higher.

According to a specific embodiment, the status of v. is positive if at least one of *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus jensenii*, *Lacticaseibacillus rhamnosus, Lacticaseibacillus casei,* and *Lacticaseibacillus paracasei* is detected at a level corresponding to 10^5 copies/ml and higher, specifically 10^6, 10^7, 10^8, 10^9, 10^{10} copies/ml, or higher.

According to a specific embodiment, the status of v. is positive if the sum of at least two of *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus jensenii*, *Lactocaseibacillus rhamnosus, Lacticaseibacillus casei,* and *Lacticaseibacillus*

paracasei is a level corresponding to 10^5 copies/ml and higher, specifically 10^6, 10^7, 10^8, 10^9, 10^{10} copies/ml, or higher.

According to a specific embodiment, the status of v. is positive if the sum of at least three of *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus jensenii*, *Lactocaseibacillus rhamnosus, Lacticaseibacillus casei,* and *Lacticaseibacillus paracasei* is a level corresponding to 10^5 copies/ml and higher, specifically 10^6, 10^7, 10^8, 10^9, 10^{10} copies/ml, or higher.

According to a specific embodiment, the status of v. is positive if the sum of at least four of *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus jensenii*, *Lactocaseibacillus rhamnosus, Lacticaseibacillus casei,* and *Lacticaseibacillus paracasei* is a level corresponding to 10^5 copies/ml and higher, specifically 10^6, 10^7, 10^8, 10^9, 10^{10} copies/ml, or higher.

According to a specific embodiment, the status of v. is positive if the sum of at least five of *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus jensenii*, *Lactocaseibacillus rhamnosus, Lacticaseibacillus casei,* and *Lacticaseibacillus paracasei* is a level corresponding to 10^5 copies/ml and higher, specifically 10^6, 10^7, 10^8, 10^9, 10^{10} copies/ml, or higher.

According to a specific embodiment, the status of v. is positive if the sum of *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus jensenii*, *Lactocaseibacillus rhamnosus, Lacticaseibacillus casei,* and *Lacticaseibacillus paracasei* is a level corresponding to 10^5 copies/ml and higher, specifically 10^6, 10^7, 10^8, 10^9, 10^{10} copies/ml, or higher.

According to a specific embodiment, assigning the status of positive or negative for iv. *Lactobacillus iners*; and v. at least one of lactobacilli belonging to the *Lactobacillus acidophilus* complex, and lacticaseibacilli belonging to the *Lacticaseibacillus casei* complex may be based on the comparison of detection result of iv. and v.. Specifically, iv. may be assigned a positive status and v. may be assigned a negative status if the level of iv. is higher than the level of v.

According to one embodiment, the system described herein further comprises detecting vi. sialidase and means for assigning a status of either positive or negative for vi..

According to one embodiment, the system described herein further comprises detecting vi. sialidase and assigning a status of either positive or negative for vi..

The term "**sialidase**" (EC 3.2.1.18) as used herein refers to an enzyme catalyzing the hydrolysis of glycosidic linkages of terminal sialic acid residues in oligosaccharides, glycoproteins, glycolipids, colominic acid and synthetic substrates. Sialidase is also known as exo-α-sialidase, neuraminidase, α-neuraminidase, or acetylneuraminidase. The systematic name is acetylneuraminyl hydrolase.

According to one embodiment, the status of vi. or sialidase is positive if the sample has a sialidase activity of 5 +/- 2 U/mL or more. Specifically, the status is positive if the sample has a sialidase activity of at least 3, 4, 5, 6, 7 U/ml, or more U/ml.

According to one embodiment, the risk of the subject is:

VI. intermediate if the status of vi. is positive, the status of i. is negative, and the status of v. is positive; and

VII. high if the status of vi. is positive, and the status of v. is negative

According to one embodiment, *Trichomonas* sp. is a further marker.

According to a specific embodiment, *Trichomonas* sp. is *Trichomonas vaginalis*.

According to one embodiment, the system described herein further comprises means for detecting *Trichomonas* sp.. According to a specific embodiment, the risk of vaginal infection in the subject is high if the status of *Trichomonas* sp. is positive.

According to one embodiment, the system and method described herein further comprises detecting *Trichomonas* sp.. According to a specific embodiment, the risk of vaginal infection in the subject is high if the status of *Trichomonas* sp. is positive.

According to one embodiment, *Candida glabrata* is a further marker.

According to one embodiment, the system and method described herein further comprises means for detecting *Candida glabrata*. According to a specific embodiment, the risk of vaginal infection in the subject is high if the status of *Candida glabrata* is positive.

According to one embodiment, the system and method described herein further comprises detecting *Candida glabrata*. According to a specific embodiment, the risk of vaginal infection in the subject is high if the status of *Candida glabrata* is positive.

According to an alternative embodiment of the invention, alternatively or additionally to detection of *Prevotella bivia,* any one of *Prevotella amnii, Prevotella melaninogenica,* or *Prevotella timonensis* may be detected.

According to one embodiment, the system described herein further comprises means for detecting any one of *Prevotella amnii, Prevotella melaninogenica,* or

Prevotella timonensis. According to a specific embodiment, the risk of vaginal infection in the subject is high if the status of any one of *Prevotella amnii, Prevotella melaninogenica,* or *Prevotella timonensis* is positive.

According to one embodiment, the system and method described herein further comprises detecting any one of *Prevotella amnii, Prevotella melaninogenica,* or *Prevotella timonensis*. According to a specific embodiment, the risk of vaginal infection in the subject is high if the status of any one of *Prevotella amnii, Prevotella melaninogenica,* or *Prevotella timonensis* is positive.

According to one embodiment, the methods and means described herein may further comprise one or more of determination of further pathogenic species, determination of the pH value, staining of smears, and determination of the Nugent score.

According to one embodiment, detecting may be qualitatively, semi-quantitatively, or quantitatively.

According to one embodiment, means for specifically detecting are means for PCR, RNA sequencing based approaches, DNA sequencing based approaches, cfu determination, ELISA, enzyme test, DNA-hybridisation, RNA-hybridisation, Loop-mediated Isothermal Amplification, probes, reporter systems, and combinations thereof.

According to one embodiment, such means for specifically detecting may be antibodies, primers, probes, chromogenic substrates, and combinations thereof.

According to a specific embodiment, antibodies used in an ELISA test system are means for specifically detecting microorganisms.

According to a specific embodiment, chromogenic substrates used in an enzymatic assay are means for specifically detecting sialidase.

According to one embodiment, methods for specifically detecting are PCR, RNA sequencing based approaches, DNA sequencing based approaches, cfu determination, ELISA, enzyme test, DNA-hybridisation, RNA-hybridisation, Loop-mediated Isothermal Amplification, probes, reporter systems, and combinations thereof.

According to a specific embodiment, RNA-hybridisation is sRNA hybridisation.

Non-limiting examples of PCR are qPCR and digital PCR.

According to a specific embodiment, cfu determination may comprise the use of selective media, elective media, and/or specific incubation conditions. Non-limiting examples of specific incubation conditions are specific temperature, anaerobic incubation, and aerobic incubation.

Non-limiting examples of DNA sequencing-based approaches are next-generation sequencing (e.g. Illumina Inc. San Diego, CA, USA), and/or third-generation sequencing (e.g. Pacific Biosciences, Menlo Park CA, USA; Oxford Nanopore Technologies, Oxford Science Park, UK) techniques that can be used alone or in combination to obtain high-quality genomes of isolated bacteria and sequence amplicons or metagenomes of vaginal swab samples.

Non-limiting examples of RNA sequencing-based approaches are next-generation sequencing (e.g. Illumina Inc. San Diego, CA, USA), and/or third-generation sequencing (e.g. Pacific Biosciences, Menlo Park CA, USA; Oxford Nanopore Technologies, Oxford Science Park, UK) techniques that can be used alone or in combination to obtain high-quality metatranscriptomes of vaginal swab samples.

According to one embodiment, detecting sialidase is performed by ELISA or a chromogenic enzyme assay.

In general, chromogenic or fluorogenic substrates and assays for determining sialidase activity or presence in a sample are known in the field.

According to a specific embodiment, a chromogenic assay for sialidase detection comprises 5-bromo-4-chloro-3-indolyl-alpha-DN-acetylneuraminic acid which is hydrolyzed to 5-bromo-4-chloro-3-hydroxyindole by sialidase and leads to the formation of an indigo pigment upon oxidation (red to bluish/purple color).

According to one embodiment, the vaginal sample is obtained from a subject.

According to one embodiment, the vaginal sample may be vaginal fluid, also known as cervico-vaginal fluid. According to an alternative embodiment, the fluid collected may be cervical fluid, cervical mucous and/or material from the cervical mucous plug.

According to one embodiment, the invention described herein provides determination of the risk of vaginal infections based on a vaginal swab of the subject. According to a specific embodiment, the invention described herein provides determination of the risk of vaginal infections based on a single vaginal swab of the subject.

According to one embodiment, the sample is self-collected vaginal fluid. For example, the sample may be self-collected using a vaginal swab. Alternatively, the vaginal fluid may be collected in a surgical, hospital or clinical setting. For example, the vaginal fluid, cervical mucous and/or material from the cervical mucous plug could be

collected by a healthcare provider, using a vaginal swab with or without a speculum. The sample may further be a sample of douche fluid collected following vaginal douching.

According to a specific embodiment, the vaginal swabs may be dry swabs.

According to a specific embodiment, the vaginal swabs may be dry swabs and are immediately placed into a liquid medium after sampling. Specifically, said liquid medium may be water, a buffer, or a physiological sodium chloride solution.

According to a specific embodiment, the vaginal sample may be pretreated before analysis.

According to a specific embodiment, the vaginal sample may be pretreated by one or more of DNA extraction, RNA extraction, rRNA extraction, 16S rRNA extraction, and pH adjustment.

The term "**subject**" as used herein refers to any being that can get a vaginal infection.

According to one embodiment, the subject is a human, specifically a woman.

According to a specific embodiment, the woman is pregnant, non-pregnant, is undergoing menopause, or is postmenopausal.

According to a specific embodiment, the subject has symptoms of vaginal infection or does not have symptoms of vaginal infection.

According to a specific embodiment, the subject is a pregnant woman not having symptoms of vaginal infection.

According to one embodiment, the subject is a pregnant woman and determining the risk of vaginal infection is performed for determining the risk of preterm birth, specifically infection-associated preterm birth.

According to a specific embodiment, a low risk of vaginal infection of a pregnant woman may indicate a low risk of preterm birth.

The term "**preterm birth**" as used herein refers to birth before 37 weeks' of pregnancy are completed. There are sub-categories of preterm birth: extremely preterm (<28 weeks), very preterm (28 to <32 weeks), and moderate to late preterm (32 weeks +).

According to one embodiment, the invention described herein provides methods and means of determining if a pregnant woman would benefit from treatment to prevent infection-associated preterm birth.

According to one embodiment, determining the risk of preterm birth is carried out during pregnancy. According to a specific embodiment, determining the risk of preterm birth is carried out in the late first trimester, and/or in the early second trimester.

According to a specific embodiment, the risk of vaginal infection is presented as an easy-to-read result such as by a color scoring system.

According to one embodiment, a scoring system is implemented for evaluation of the risk of vaginal infection which provides consequences of action for the subject. According to a specific embodiment, the risk of vaginal infection is scored for low, intermediate, or high risk of vaginal infection.

According to a specific embodiment, the scoring system provides the risk of vaginal infection in the form of a traffic light color code.

According to a specific embodiment, a template may be used for evaluation or scoring of the risk of vaginal infection.

According to one embodiment, the scoring system described herein provides an interpretation of the results obtained by the methods and means described herein which allows drawing of an action by the subject.

According to one embodiment, a scoring system is described herein which is implemented in a software application. According to a specific embodiment, the software application is an app for mobile electronic devices. Specifically, the mobile electronic device may be connectable to a smartphone. Alternatively, the mobile device is a smartphone or a comparable device.

According to a specific embodiment, the results of the invention described herein may be easily shared with a physician or healthcare provider.

According to one embodiment, the invention described herein provides a computer-implemented evaluation of the data obtained by the methods and means described herein. According to a specific embodiment, a software application uses an algorithm for data interpretation. According to another specific embodiment, artificial intelligence is used.

According to one embodiment, the methods and means described herein may be implemented in a semi-automated or automated system.

According to a specific embodiment, the system may comprise a computer which is configured to assign a status of positive or negative based on the detection of the marker described herein.

According to one embodiment, detecting may provide a visible result for a positive or negative status. Therefore, the system described herein may be configured to assign a status of positive or negative based on the detection of the marker described herein.

According to one embodiment, the risk of vaginal infection is low, intermediate, or high according to the following Table 1.

Table 1: i. *Candida albicans;* ii. *Gardnerella vaginalis;* iii. *Prevotella bivia;* iv. *Lactobacillus iners;* v. at least one of lactobacilli belonging to the *Lactobacillus acidophilus* complex, and lacticaseibacilli belonging to the *Lacticaseibacillus casei* complex*;* **+**: positive status; **-**: negative status; **+/-**: negative or positive status, inter. (intermediate)

	Risk							
	low	inter.	inter.	inter.	high	high	high	high
i.	**-**	**-**	**-**	**-**	**+**	**+/-**	**+/-**	**+/-**
ii.	**-**	**+**	**+/-**	**-**	**+/-**	**+**	**+/-**	**+/-**
iii.	**-**	**+/-**	**+**	**-**	**+/-**	**+/-**	**+**	**+/-**
iv.	**+/-**	**+/-**	**+/-**	**-**	**+/-**	**+/-**	**+/-**	**+**
v.	**+**	**+**	**+**	**-**	**+/-**	**-**	**-**	**-**

According to one embodiment, the risk of vaginal infection is low, intermediate, or high according to the following Table 2.

Table 2: i. *Candida albicans;* ii. *Gardnerella vaginalis;* iii. *Prevotella bivia;* iv. *Lactobacillus iners;* v. at least one of lactobacilli belonging to the *Lactobacillus acidophilus* complex, and lacticaseibacilli belonging to the *Lacticaseibacillus casei* complex*;* vi. Neuraminidase / Sialidase (enzyme); **+**: positive status; **-**: negative status; **+/-**: negative or positive status; inter. (intermediate)

	Risk									
	low	inter.	inter.	inter.	inter.	high	high	high	high	high
i.	**-**	**-**	**-**	**-**	**-**	**+**	**+/-**	**+/-**	**+/-**	**+/-**
ii.	**-**	**+**	**+/-**	**-**	**+/-**	**+/-**	**+**	**+/-**	**+/-**	**+/-**
iii.	**-**	**+/-**	**+**	**-**	**+/-**	**+/-**	**+/-**	**+**	**+/-**	**+/-**
iv.	**+/-**	**+/-**	**+/-**	**-**	**+/-**	**+/-**	**+/-**	**+/-**	**+**	**+/-**
v.	**+**	**+**	**+**	**-**	**+**	**+/-**	**-**	**-**	**-**	**-**
vi.	**-**	**+/-**	**+/-**	**+/-**	**+**	**+/-**	**+/-**	**+/-**	**+/-**	**+**

According to one embodiment, means for detecting and assigning a status of either positive or negative may be performed by one device or may be performed by separate devices.

According to one embodiment, the system described herein may be a single device or may be split into several devices.

According to a specific embodiment, means for detecting and assigning may be included in a point-of-care test device.

For example, the system described herein may be a lateral-flow assay (LFA) in which detection of a marker provides a visible sign that the marker is above a pre-defined threshold i.e., assigning a positive status, or below a pre-defined threshold i.e., assigning a negative status.

In general, lateral-flow assays are known in the field for detecting several different target analytes. Typical construction of an LFA is a paper-based test strip with the general operation principle as follows: The sample containing the target analyte in liquid form is applied to a sample pad. The liquid then moves via capillary action through the different regions on the test strip. The sample with target analyte first migrates through a conjugate pad that comprises a variety of detection reagents including e.g., antibodies specific to the target analyte. After interacting with the detection reagents, the sample which contains the target analyte bound to a detection reagent e.g., an antibody continues on to a detection zone where the target analyte bound to a detection reagent e.g., an antibody is immobilized via capture reagents. Finally, an absorbent pad at the end of the test strip absorbs any excess liquid remaining after the sample has passed through the test strip.

According to one embodiment, the system is configured for providing “one-step” or “lateral flow” detection of the markers described herein from a sample collected from a subject.

According to one embodiment, the system described herein is a lateral-flow device for detecting. According to a specific embodiment, the lateral-flow device comprises detection reagents for detecting i. *Candida albicans;* ii. *Gardnerella vaginalis;* iii. *Prevotella bivia;* iv. *Lactobacillus iners;* v. at least one of lactobacilli belonging to the *Lactobacillus acidophilus* complex, and lacticaseibacilli belonging to the *Lacticaseibacillus casei* complex. According to a specific embodiment, the lateral-flow device may further comprise means for detecting sialidase/neuraminidase.

According to one embodiment, the system described herein comprises a lateral-flow device for detecting i. *Candida albicans;* ii. *Gardnerella vaginalis;* iii. *Prevotella bivia;* iv. *Lactobacillus iners;* v. at least one of lactobacilli belonging to the *Lactobacillus acidophilus* complex, and lacticaseibacilli belonging to the *Lacticaseibacillus casei* complex, wherein said lateral-flow device further comprises sialidase detection.

According to one embodiment, the present invention relates to a rapid screening tool for determining the risk of vaginal infection in a subject sample.

According to one embodiment, described herein is a point-of-care assay device comprising

a. a sample application zone, and

b. a detection zone comprising:

a first detection reagent with binding affinity to i. *Candida albicans;*

a second detection reagent with binding affinity to ii. *Gardnerella spp.,* specifically *Gardnerella vaginalis*;

a third detection reagent with binding affinity to iii. *Prevotella bivia*;

a fourth detection reagent with binding affinity to iv. *Lactobacillus iners*; and

a fifth detection reagent with binding affinity to v. at least one of lactobacilli belonging to the *Lactobacillus acidophilus* complex, and lacticaseibacilli belonging to the *Lacticaseibacillus casei* complex*;*

wherein the device is configured to determine the risk of vaginal infection in a subject.

According to a specific embodiment, the device is configured to detect i. *Candida albicans;* ii. *Gardnerella spp.,* specifically *Gardnerella vaginalis;* iii. *Prevotella bivia*; iv. *Lactobacillus iners;* and v at least one of lactobacilli belonging to the *Lactobacillus acidophilus* complex, and lacticaseibacilli belonging to the *Lacticaseibacillus casei* complex in a sample from a subject to discriminate between low, intermediate, and high risk of vaginal infection.

According to a specific embodiment, the device further comprises a sixth detection reagent for specifically detecting vi. sialidase/neuraminidase.

According to a specific embodiment, for the point-of-care assay different devices may be applicable e.g., lateral flow immunoassay device.

According to a specific embodiment, the device comprises a lateral flow immunoassay device.

According to one embodiment, the system or device comprises a lateral flow technology with nucleic acid hybridization technique. Specifically, ribosomal RNA sequences are detected by hybridization with a capture probe on the membrane integrated inside a lateral flow test housing and a detection probe conjugated to e.g., colloidal gold. Specifically, after cell lysis and equilibration in hybridization buffer the lysed cell supernatant is applied to the sample pad of the lateral flow device. The specific target sequence in case of a positive sample as well as the inbuilt reaction control will than hybridize to the specific capture probe and be visualized on the reaction zone without additional user interference.

According to a further specific embodiment, in the case sialidase is also to be detected, the device further comprises an enzymatic assay device.

According to another specific embodiment, in the case sialidase is also to be detected, the device comprises a lateral flow immunoassay device and an enzymatic assay device.

According to a specific embodiment, the device comprises a detection system which is designed to interact with a mobile computer device to generate an output.

According to one embodiment, the lateral flow immunoassay device is a test strip. Specifically, the test strip is a paper-based test strip.

According to a specific embodiment, the device includes a sample application zone and a detection zone.

According to a specific embodiment, the device includes at least one detection reagent specific to i. *Candida albicans;* ii. *Gardnerella spp.,* specifically *Gardnerella vaginalis;* iii. *Prevotella bivia*; iv. *Lactobacillus iners;* and v. at least one of lactobacilli belonging to the *Lactobacillus acidophilus* complex, and lacticaseibacilli belonging to the *Lacticaseibacillus casei* complex. Optionally, the detection zone includes also a reagent specific to sialidase.

According to a specific embodiment, the detection zone may be functionalized by immobilizing various receptor molecules which specifically bind to the respective markers. The receptor molecules may be selected from natural origin, e.g., antibodies, antibody fragments or the like. The receptor molecules may be synthetically produced molecules, e.g., aptamers. The receptor molecules may also be molecules which are formed due to the activity of an enzyme e.g., due to the reaction of sialidase. The detection zone may comprise at least one sensor region, on which, as receptors for sensing the respective markers, antibodies, or other specificity imparting receptors, such

as aptamers, which specifically bind to the respective markers to be detected, are arranged. Thus, an effective, easy to apply functionalization of the sensor region surface is achieved.

According to a specific embodiment, the antibodies or other specificity imparting receptors such as e.g., aptamers have a high selectivity for detecting specific markers. In relation to possible receptors, the antibodies arranged on the sensor surface or other specificity imparting receptors such as aptamers e, g. bind the marker to be detected and, in the process, lead to a change in the properties.

According to a further embodiment, the device described herein further includes a reagent zone such as a conjugate pad that comprises a variety of detection reagents. The reagent zone comprises at least one detection reagent specific to i. *Candida albicans;* ii. *Gardnerella spp.,* specifically *Gardnerella vaginalis;* iii. *Prevotella bivia*; iv. *Lactobacillus iners;* and v. at least one of lactobacilli belonging to the *Lactobacillus acidophilus* complex, and lacticaseibacilli belonging to the *Lacticaseibacillus casei* complex. Optionally, the reagent zone includes also a reagent specific to sialidase. Thereby, when the marker to be detected is present in the sample and contacts the detection reagent, a labeled complex is formed.

According to a further embodiment, the detection zone on the device described herein includes a marker binding partner which binds to the labeled reagent complex.

According to one embodiment, the device may be a chromatography test strip.

According to a specific embodiment, the presence of a marker described herein is indicated by a test line visible to the naked eye. The presence of a marker may be indicated by a test line for each marker to be detected.

According to a specific embodiment, the test lines are on the same device but spatially separate from each other.

In one embodiment of the invention, the sample to be analyzed is applied to a carrier. The carrier can be made of one single chromatographic material, or preferably several capillary active materials made of the same or different materials and fixed on a carrier backing. These materials are in close contact with each other so as to form a transport path along which a liquid driven by capillary forces flows from an application zone, passing the reagent zone, towards one or more detection zones.

Depending on the type of detection method, different detection reagents are present in the reagent zone, which is located between the application zone and the detection zone. In a sandwich immunoassay, it is preferred to have a labeled, non-

immobilized detection reagent in the reagent zone that is specific to the marker to be detected. Thus, when a marker present in the sample contacts the corresponding labeled detection reagent present in the reagent zone, a labeled complex is formed between the marker and the corresponding labeled detection reagent. The labeled complex in turn is capable of forming a further complex with an immobilized marker binding partner in the detection zone. In a competitive immunoassay, the reagent zone preferably contains a labeled, non-immobilized marker analogue which competes with the marker for the immobilized marker binding partner in the detection zone. The marker binding partners in the reagent zone and in the detection zone are preferably monoclonal, polyclonal or recombinant antibodies or fragments of antibodies capable of specific binding to the corresponding marker.

Detection of the marker may be achieved in the detection zone. The immobilized molecule binds the labeled complex or the labeled marker-analogue by immune reaction or other reaction in the detection zone, thus building up a visible test line in the detection zone during the process. Preferably, the label is an optically detectable label. Forming a complex at the detection zone immobilizes the label and the test line becomes visible for the naked eye, indicating a positive test result. Suitable are direct labels e.g., particularly gold labels which can be best recognized by the naked eye. Additionally, an electronically read out device (e.g. on the basis of a photometrical, acoustic, impedimetric, potentiometric and/or amperometric transducer) can be used to obtain more precise results and a semi-quantification of the analyte. Other suitable labels may be latex, fluorophores or phosphorophores.

According to a specific embodiment, the detection reagents are selected from antibodies, synthetic molecules, nucleotides, nucleic acids, aptamers, peptides, proteins, enzymes, probes, and sensors.

According to a specific embodiment, the detection reagents are labelled with a detectable marker.

According to a specific embodiment, the detectable marker is selected from an enzyme label, fluorescent label, radiolabel, particulate label, colored latex particle, colored plastic particle, a colored phosphor particle, and a fluorescent particle.

According to a specific embodiment, the device further comprises a test window configured to allow observation of the test results.

According to one embodiment, the detection reagents are chemically conjugated to the detectable marker to form a permanent, irreversible reagent-marker complex.

According to a specific embodiment, the detection reagents or the detectable marker are configured to be visible to a user when the sample is positive for i. *Candida albicans;* ii. *Gardnerella spp.,* specifically *Gardnerella vaginalis;* iii. *Prevotella bivia*; iv. *Lactobacillus iners;* and v. at least one of lactobacilli belonging to the *Lactobacillus acidophilus* complex, and lacticaseibacilli belonging to the *Lacticaseibacillus casei* complex.

According to a specific embodiment, the detection reagents or the detectable marker are configured to be visible to a user when the sample is positive for i. *Candida albicans;* ii. *Gardnerella spp.,* specifically *Gardnerella vaginalis;* iii. *Prevotella bivia*; iv. *Lactobacillus iners;* v. at least one of lactobacilli belonging to the *Lactobacillus acidophilus* complex, and lacticaseibacilli belonging to the *Lacticaseibacillus casei* complex; and vi. sialidase.

According to a specific embodiment, the device is configured to qualitatively, semi-quantitatively, or quantitatively detect i. *Candida albicans;* ii. *Gardnerella spp.,* specifically *Gardnerella vaginalis;* iii. *Prevotella bivia*; iv. *Lactobacillus iners;* v. at least one of lactobacilli belonging to the *Lactobacillus acidophilus* complex, and lacticaseibacilli belonging to the *Lacticaseibacillus casei* complex; and optionally vi. sialidase.

According to one embodiment, described herein is also a method of in vitro determining the risk of vaginal infection in a subject, comprising the steps of

a. providing a sample from said subject;

b. providing the point-of-care device described herein;

c. applying the sample to the device;

d. observing the detection such as the absence or presence of the detectable reagent-marker complex to determine whether the sample has a positive or negative status for i. *Candida albicans;* ii. *Gardnerella spp.,* specifically *Gardnerella vaginalis;* iii. *Prevotella bivia*; iv. *Lactobacillus iners;* and v. at least one of lactobacilli belonging to the *Lactobacillus acidophilus* complex, and lacticaseibacilli belonging to the *Lacticaseibacillus casei* complex; wherein

e. negative status of i., ii., and iii., and the positive status of v. is indicative for a low risk of vaginal infection;

f. negative status of i., positive status of at least one of ii. and iii., and positive status of v. is indicative for an intermediate risk of vaginal infection;

g. negative status of i., ii., iii., iv., and v. is indicative for an intermediate;

h. positive status of i. is indicative of a high risk of vaginal infection;

i. positive status of at least one of ii., iii., and iv., and negative status of v. is indicative for a high risk of vaginal infection; and

j. thereby determining the risk of vaginal infection of the subject.

According to one embodiment, a kit is provided comprising the system or device described herein and an instruction manual. According to a specific embodiment, the kit may further comprise means for collecting a sample such as a swab.

According to one embodiment, the herein described kit may be used by a subject for self-colleting a sample, delivering the kit to a laboratory for determining the risk of vaginal infection, and receiving the results from the laboratory. According to an alternative embodiment, the herein described kit may be used by a subject for self-collecting a sample and self-determining the risk of vaginal infection using the means provided in the kit.

According to one embodiment, the invention described herein for determining the risk of vaginal infections further provides determining if the subject would benefit from treatment based on the risk determined by the methods and means described herein.

According to one embodiment, the invention described herein further provides a method to treat a subject at intermediate or high risk of vaginal infection.

According to a specific embodiment, treatment of high risk of vaginal infection includes antibiotic therapy.

According to a specific embodiment, treatment of high risk of vaginal infection includes antibiotic therapy and probiotic therapy.

According to a specific embodiment, treatment of intermediate risk of vaginal infection includes probiotic therapy.

According to a specific embodiment, low risk of vaginal infection does not require treatment.

According to one embodiment, the following items are described herein:

1. A system for *in vitro* determining the risk of vaginal infection in a subject, said system comprising

a) means for specifically detecting in a vaginal sample of said subject:

i. *Candida albicans*;

ii. *Gardnerella spp.,* specifically *Gardnerella vaginalis*;

iii. *Prevotella bivia*;

iv. *Lactobacillus iners*; and

v. at least one of lactobacilli belonging to the *Lactobacillus acidophilus* complex, and lacticaseibacilli belonging to the *Lacticaseibacillus casei* complex;

and;

b) means for assigning a status of either positive or negative for each of i., ii., iii., iv., and v. based on a);

wherein the risk of vaginal infection in the subject is:

I. low if the status of i., ii., and iii., is negative and the status of v. is positive;

II. intermediate if the status of i. is negative, the status of at least one of ii., and iii. is positive, and the status of v. is positive;

III. intermediate if the status of i., ii., iii., iv., and v. is negative;

IV. high if the status of i. is positive; and

V. high if the status of at least one of ii., iii., and iv. is positive and the status of v. is negative.

2. The system of item 1, wherein the *Lactobacillus acidophilus* complex comprises *Lactobacillus crispatus*, *Lactobacillus gasseri*, and *Lactobacillus jensenii*.

3. The system of item 1 or 2, wherein the *Lacticaseibacilli casei* complex comprises *Lacticaseibacillus rhamnosus*, *Lacticaseibacillus casei*, and *Lacticaseibacillus paracasei.*

4. The system of any one of items 1 to 3, further comprising means for detecting vi. sialidase and means for assigning a status of either positive or negative for vi..

5. The system of item 4, wherein the status of vi. is positive if the sample has a sialidase activity of 5 +/- 2 U/mL or more.

6. The system of item 4 or 5, wherein the risk of the subject is:

VI. intermediate if the status of vi. is positive, the status of i. is negative, and the status of v. is positive; and

VII. high if the status of vi. is positive, and the status of v. is negative.

7. The system of any one of items 1 to 6, wherein the means for detecting are selected from the group consisting of antibodies, primers, probes, and chromogenic substrates.

8. The system of any one of items 1 to 7, wherein the status of i. is positive if *Candida albicans* is detected at a level corresponding to a cfu/mL of 10^4 or higher.

9. The system of any one of items 1 to 8, wherein the status of ii. is positive if *Gardnerella spp.,* specifically *Gardnerella vaginalis,* is detected at a level corresponding to a cfu/mL of 10^5 or higher.

10. The system of any one of items 1 to 9, wherein the status of iii. is positive if *Prevotella bivia* is detected at a level corresponding to a cfu/mL of 10^5 or higher.

11. The system of any one of items 1 to 10, wherein the status of iv. is positive if *Lactobacillus iners* is detected at a level corresponding to a cfu/mL of 10^4 or higher.

12. The system of any one of items 1 to 11, wherein the status of v. is positive if at least one of lactobacilli belonging to the *Lactobacillus acidophilus* complex, and lacticaseibacilli belonging to the *Lacticaseibacillus casei* complex is detected at a level corresponding to a cfu/mL of 10^4 or higher, or if the sum lactobacilli belonging to the *Lactobacillus acidophilus* complex, and lacticaseibacilli belonging to the *Lacticaseibacillus casei* complex is a level corresponding to a cfu/mL of 10^6 or higher.

13. The system of any one of items 1 to 12, wherein the system is a point-of-care test.

14. The system of any one of items 1 to 13, further comprising means for detecting *Trichomonas* sp.

15. The system of item 14, wherein the risk of vaginal infection in the subject is high if the status of *Trichomonas* sp. is positive.

16. A kit comprising the system of any one items 1 to 15, further comprising an instruction manual.

17. Use of the system of any one of items 1 to 15 or the kit of item 16 for determining the risk of a vaginal infection in a subject.

18. An *in vitro* method of determining the risk of vaginal infection in a subject, said method comprising

a) specifically detecting in a vaginal sample from the subject:

i. *Candida albicans*;

ii. *Gardnerella spp.,* specifically *Gardnerella vaginalis*;

iii. *Prevotella bivia*;

iv. *Lactobacillus iners*; and

v. at least one of lactobacilli belonging to the *Lactobacillus acidophilus* complex, and lacticaseibacilli belonging to the *Lacticaseibacillus casei* complex;

and;

b) assigning a status of either positive or negative for each of i., ii., iii., iv., and v. based on a);

wherein the risk of vaginal infection in the subject is:

I. low if the status of i., ii., and iii., is negative and the status of v. is positive;

II. intermediate if the status of i. is negative, the status of at least one of ii., and iii. is positive, and the status of v. is positive;

III. intermediate if the status of i., ii., iii., iv., and v. is negative;

IV. high if the status of i. is positive; and

V. high if the status of at least one of ii., iii., and iv. is positive and the status of v. is negative.

19. The method of item 18, wherein the *Lactobacillus acidophilus* complex comprises *Lactobacillus* crispatus, *Lactobacillus* gasseri, and *Lactobacillus* jensenii.

20. The method of item 18 or 19, wherein the *Lacticaseibacilli casei* complex comprises *Lacticaseibacillus rhamnosus*, *Lacticaseibacillus casei*, and *Lacticaseibacillus paracasei.*

21. The method of any one of items 18 to 20, further comprising detecting vi. sialidase and assigning a status of either positive of negative for vi..

22. The method of item 21, wherein the status of vi. is positive if the sample has a sialidase activity of 5 +/- 2 U/mL or more.

23. The method of item 21 or 22, wherein the risk of the subject is:

VI. intermediate if the status of vi. is positive, the status of i. is negative, and the status of v. is positive; and

VII. high if the status of vi. is positive, and the status of v. is negative.

24. The method of any one of items 18 to 23, wherein detecting is performed by a method selected from the group consisting of PCR, RNA sequencing-based approaches, DNA sequencing based approaches, cfu determination, ELISA, enzyme test, DNA-hybridisation, RNA-hybridisation, Loop-mediated Isothermal Amplification, probes, reporter systems, and combinations thereof.

25. The method of any one of items 18 to 24, wherein the status of i. is positive if *Candida albicans* is detected at a level corresponding to a cfu/mL of 10^4 or higher.

26. The method of any one of items 18 to 25, wherein the status of ii. is positive if *Gardnerella spp.,* specifically *Gardnerella vaginalis,* is detected at a level corresponding to a cfu/mL of 10^5 or higher.

27. The method of any one of items 18 to 26, wherein the status of iii. is positive if *Prevotella bivia* is detected at a level corresponding to a cfu/mL of 10^5 or higher.

28. The method of any one of items 18 to 27, wherein the status of iv. is positive if *Lactobacillus iners* is detected at a level corresponding to a cfu/mL of 10^4 or higher.

29. The method of any one of items 18 to 28, wherein the status of v. is positive if at least one of lactobacilli belonging to the *Lactobacillus acidophilus* complex, and lacticaseibacilli belonging to the *Lacticaseibacillus casei* complex is detected at a level corresponding to a cfu/mL of 10^4 or higher, or if the sum lactobacilli belonging to the *Lactobacillus acidophilus* complex, and *lacticaseibacilli* belonging to the *Lacticaseibacillus casei* complex is detected at a level corresponding to a cfu/mL of 10^6 or higher.

30. The method of any one of items 18 to 29, further comprising detecting Trichomonas sp.

31. The system of item 30, wherein the risk of vaginal infection in the subject is high if the status of Trichomonas sp. is positive.

EXAMPLES

The examples described herein are illustrative of the present invention and are not intended to be limitations thereon. Many modifications and variations may be made to the techniques described and illustrated herein without departing from scope of the invention.

Example 1: Sialidase quantification in the vaginal smear

A color reaction test for testing specific enzymes such as neuraminidase/sialidase may also be integrated. The point-of-care assay (layout variant Fig. 1b) contains a reaction well for the use of 5-bromo-4-chloro-3-indolyl-α-DN-acetylneuraminic acid as reagent. This constitutes a chromogenic assay for sialidase detection that leads to hydrolysis reaction and the formation of an indigo pigment upon oxidation that results in a red to bluish/purple color. The development of the color indicates the presence of sialidase in the sample, and the intensity of the color is in relation to the amount of sialidase in the vaginal smear.

Vaginal secretion is obtained from the vagina using a sterile cotton swab; for the self-test, the swab should be inserted 10-15 cm vaginally. Alternatively, the swab can be taken by a physician during a gynecological examination. The swab, after being saturated with vaginal secretion, is rinsed in an appropriate buffer solution in a small

plastic eprouvette. The buffer solution extracts the neuraminidase/sialidase. The swab must be rinsed in the solution for about 30 seconds and squeezed out at the edge of the eprouvette with a rotating movement. The eprouvette is then closed with a valve stopper to apply the solution to the neuraminidase/sialidase reaction well of the test plate (Fig. 1b). A drop of approx. 50 µl of the sample solution is added to the neuraminidase/sialidase reaction well of the test plate (Fig. 1b). Then a drop of approx. 50 µl of 5-bromo-4-chloro-3-indolyl-α-DN-acetylneuraminic acid as reagent is added to the reaction well. After an incubation time of 25 min at room temperature the result is evaluated. A positive result (presence of sialidase/neuraminidase) is indicated by a red to bluish/purple color of the reaction zone. A negative result is displayed by no or a yellowish color of the reaction zone.

Example 2: Point-of-care assay - Lateral-flow device

The point-of-care assay is performed with a lateral-flow device. The reagents needed for each marker to be detected are included in the lateral-flow device.

Vaginal secretion is obtained from the vagina using a sterile cotton swab; for the self-test, the swab should be inserted 10-15 cm vaginally. Alternatively, the swab can be taken by a physician during a gynecological examination. The swab, after being saturated with vaginal secretion, is rinsed in an appropriate buffer solution in a small plastic eprouvette. The buffer solution extracts the antigens to be tested of the microorganisms and/or the enzymes (neuraminidase / sialidase) and/or DNA/RNA.

The swab is rinsed in the solution for about 30 seconds and squeezed out at the edge of the eprouvette with a rotating movement. The eprouvette is then closed with a valve stopper to apply the solution to the test plate of the system.

The carrier plate used for this purpose consists of an arrangement of several lateral flow tests, each divided into a sample introduction area and an analysis area. In the analysis area there are markings for the control band "C" (a corresponding band becoming visible indicates the functionality of the test) and at least one test line "T" (a band becoming visible indicates the presence of the sought bacterium/yeast/molecules).

The measurement principles used correspond to an enzyme-linked immunosorbent assay (ELISA) for the detection of the antigens of the sought bacteria/yeasts/enzyme (neuraminidase/sialidase). The sialidase may be detected in the filling well as described in example 1.

The principle of the test is the following: The sample is applied to the sample application zone of the test. The sample application zone comprises a sponge which holds an excess of sample fluid. Once soaked, the fluid flows to the conjugate pad in which the particles, called conjugates, are stored. The conjugate pad contains all the reagents required for an optimized chemical reaction between the target markers and its binding partner (antibody) that has been immobilized on the particle's surface. This marks target particles as they pass through the pad and continue across to the test and control lines. The test line shows a signal. The control line contains affinity ligands which show whether the sample has flowed through and the biomolecules in the conjugate pad are active. After passing these reaction zones, the fluid enters the final porous material, the wick, that simply acts as a waste container.

Table 3: Tabular representation of the test system and the detection zone

	Filling well	**Test line (T)**	**Control line (C)**
Lb. crispatus		Detection zone	Detection zone
Lb. gasseri		Detection zone	Detection zone
Lb. jensenii		Detection zone	Detection zone
Lc. rhamnosus		Detection zone	Detection zone
Lc. casei		Detection zone	Detection zone
Lc. paracasei		Detection zone	Detection zone
Lb. iners		Detection zone	Detection zone
Gardnerella vaginalis		Detection zone	Detection zone
Prevotella bivia		Detection zone	Detection zone
Candida albicans		Detection zone	Detection zone
Sialidase		Detection zone	Detection zone
Sialidase	Detection zone	n/a	n/a
Further markers		Detection zone	Detection zone

The graphical representation of a possible layout of the test plate is shown in Figure 1.

<u>Overall process/interpretation of results and possible basic statements of the system.</u>

Figure 2 shows the overall scheme of the test system. Starting with the triggering moment up to the interpretation scheme in traffic light colors and the options for action derived from it.

Based on the appearance of a test line or a color development in the filling well, for each organism and enzyme, the status of each organism and enzyme is determined. For example, if a test line appears for *Candida albicans*, the status for *Candida albicans*

is positive. Based on the status of each tested organism and enzyme, a low, intermediate, or high status is assigned based on the following Table 4. A color scheme for analyzing the result may be implemented, e.g., for low risk a green color, for intermediate risk a yellow color, and for high risk a red color.

Table 4: i. *Candida albicans;* ii. *Gardnerella vaginalis;* iii. *Prevotella bivia;* iv. *Lactobacillus iners;* v. at least one of lactobacilli belonging to the *Lactobacillus acidophilus* complex, and lacticaseibacilli belonging to the *Lacticaseibacillus casei* complex*;* vi. Neuraminidase / Sialidase (enzyme); **+**: positive status; **-**: negative status; **+/-**: negative or positive status; inter. (intermediate)

	Risk									
	low	inter.	inter.	inter.	inter.	high	high	high	high	high
i.	**-**	**-**	**-**	**-**	**-**	**+**	**+/-**	**+/-**	**+/-**	**+/-**
ii.	**-**	**+**	**+/-**	**-**	**+/-**	**+/-**	**+**	**+/-**	**+/-**	**+/-**
iii.	**-**	**+/-**	**+**	**-**	**+/-**	**+/-**	**+/-**	**+**	**+/-**	**+/-**
iv.	**+/-**	**+/-**	**+/-**	**-**	**+/-**	**+/-**	**+/-**	**+/-**	**+**	**+/-**
v.	**+**	**+**	**+**	**-**	**+**	**+/-**	**-**	**-**	**-**	**-**
vi.	**-**	**+/-**	**+/-**	**+/-**	**+**	**+/-**	**+/-**	**+/-**	**+/-**	**+**

The assessment "Green" follows physiologically normal vaginal flora, no evidence of infection. No consequences are needed.

The assessment "red" reflects a bacterial infection that requires treatment.

The assessment „yellow" reflects an intermediate vaginal microbiota, which requires further observation or, depending on the symptoms, medical clarification.

Example 3: <u>Quantification of microorganisms by microbiological and PCR methods</u>

<u>Materials and Methods:</u>

The following vaginal swabs were analysed according to the system and method described herein.

Table 5: Vaginal swabs and associated purity grades determined by the Medical University of Vienna, which were used for the preliminary evaluation

Degree of purity	**Patient ID**
1 (Nugent Score 0 Normal Flora)	3
	4
	13
	14

	21
2 (Nugent Score 4-6 Intermediate Flora)	6
	34
	35
3 (Nugent Score 7-10 Bacterial vaginosis)	12
	19

The eluted vaginal secretions from two swabs were vortexed for several seconds and then pipetted together. To obtain the residual fluid from the swabs, these were centrifuged with the swab side up in centrifugation tubes at 8000 x *g* for 10 minutes at 4°C. The liquid was then pipetted up and down several times to obtain the pooled vaginal fluid. After pipetting up and down several times, the liquid was pipetted into the pooled vaginal fluid.

The smear fluid was diluted decimally in Dilucups with Maximal Recovery Diluent (LabRobot, catalogue number 40DEMRD6) and 100 µL of the smears or their dilution levels (based on final concentration on the plate) were spread on Candida Ident Agar (Merck, catalogue number: 94382) for quantification of yeasts, GranuCult® Prime MRS agar (Merck, 1.10660) for quantification of Lactobacilli from the acidophilus complex and MRS agar supplemented with 50 mg/L vancomycin hydrochloride (MRS-V) (Sigma-Aldrich, V8138) for quantification of *Lacticaseibacillus* species. For the identification of *Lactobacillus iners*, *Prevotella bivia* and *Gardnerella vaginalis* Columbia agar supplemented with 5% sheep blood (Biomerieux, 43049) was used.

To the MRS and MRS-V agar, 0.5 g/L L-cysteine hydrochloride monohydrate (Sigma-Aldrich, catalogue number C7880) was added to improve anaerobiosis, and 40 mg/L cycloheximide (Sigma-Aldrich, catalogue number C7698) was added to inhibit the growth of yeasts/fungi. The plates were incubated at 37°C for 5 days under anaerobic conditions. Only the Candida Ident Agar was incubated under aerobic conditions. At the end of the incubation period, the total bacterial count per mL of vaginal secretion was calculated by counting the plates with a colony count between 10 and 300 colonies. In addition, the bacterial count of individual species was determined by counting the colonies of uniform colony morphology. Identification at species level was carried out using the MALDI Biotyper® from Bruker.

DNA was extracted from 1 mL of swab fluid using the DNeasy PowerSoil Pro Kit (Qiagen, catalogue number 47014). The liquid was centrifuged under the above conditions and the pellet was resuspended in 250 µL of the liquid Amies transport buffer

provided in the Copan ESwab system after removal of the supernatant. DNA was then extracted according to the manufacturer's protocol. The real-time PCR was carried out using the qTower^3 from Analytik Jena.

Quantification of microorganisms by cultivation-based microbiological methods

1. Quantification of yeasts

Determination of the cultural bacterial count of yeasts by cultivation on Candida Ident Agar

In none of the 10 analysed samples *Candia albicans* species could be detected.

2. Quantification of *Lacticaseibacillus* species

The species *Lacticaseibacillus rhamnosus*, *Lacticaseibacillus casei* and *Lacticaseibacillus paracsei* couldn't be detected in the analysed samples.

3. Quantification of *Lactobacillus* species

Figure 3 shows the total count of *Lactobacillus* species (consisting of *Lb. crispatus*, *Lb. gasseri* and *Lb. jensenii*) on MRS agar with a threshold set at $1.0*10^5$ log CFU/mL.

Lb. crispatus is the most common and dominating species identified on MRS agar. The bacterial count was highest in patients with healthy vaginal flora and was detected in all patients from this group. The bacterial counts were between 5.0 and 7.3 log cfu/mL. Among the patients with intermediate flora, the species was identified in all three swabs, with bacterial counts of 5.2 and 6.5 log cfu/mL. Among the swabs from patients with bacterial vaginosis no *Lb. crispatus* was detected.

Lb. gasseri was detected in three patients with healthy vaginal flora and two patients with intermediate vaginal flora. *Lb. jensenii* was only detected in one patient with healthy vaginal flora and two patients with intermediate vaginal flora.

Figure 4 shows the bacterial counts of *Lb. crispatus, Lb. gasseri* and *Lb. jensenii* from vaginal swabs on MRS agar with a threshold set at $1.0*10^4$ log cfu/mL. In some samples, the bacterial count of each species could not be determined due to the

similar colony morphology of those species. In those cases, the bacterial counts of the two relevant species are summed up.

Conclusion: The bacterial count determined on MRS agar tended to be higher in patients with healthy vaginal flora. *Lb. crispatus* was the most common species identified on MRS agar. The lactic acid bacterial count decreased in patients with intermediate vaginal flora and especially with bacterial vaginosis (below threshold).

4. Quantification of *Lactobacillus iners, Gardnerella vaginalis.* and *Prevotella spp.*

Determination of the bacterial count on Columbia blood agar

Figure 5 shows the *Lactobacillus iners*, *Gardnerella spp.* and *Prevotella* spp. results of the bacterial count on Columbia blood agar with a threshold set at $1.0*10^4$ log cfu/mL.

Lactobacillus iners was detected in patient 3 with healthy vaginal flora according to the Nugent score. Potentially pathogenic species were predominantly detected in swabs from patients with bacterial vaginosis. *Prevotella* species were identified in both patients with bacterial vaginosis. *Gardnerella species* were detected in two swabs from patients with intermediate flora (34, 35) and in both patients with bacterial vaginosis. Figure 5 shows the bacterial count of *Lactobacillus iners*, *Gardnerella* spp. and *Prevotella* spp. from vaginal swabs on Columbia blood agar.

Table 6: displays the respective assignment to low, medium and high risk of vaginal infection according to the microbiological quantification methods summarized above:

Patient ID	*Candida albicans* (i)	*Gardnerella* spp. (ii)	Prevotella spp. (iii)	*Lb. iners* (iv)	*Lactobacillus acidophilus* complex (v)	*Lb. crispatus* (v)	*Lb. gasseri* (v)	*Lb. jensenii* (v)	*Lacticase ibacillus casei* complex (v)	*Lc. rhamnosus* (v)	*Lc. casei* (v)	*Lc. paracasei* (v)	Result
3	-	-	-	+	+	+	+	-	-	-	---	--	low (I) iv+v positive
4	-	-	-	-	+	+	+	-	-	-	---	--	low (I) v positive
13	-	-	-	-	+	+	+	+	-	-	---	--	low (I) v positive
14	-	-	-	-	+	+	-	-	-	-	---	--	low (I) v positive
21	-	-	-	-	+	+	-	-	-	-	---	--	low (I) v positive
6	-	-	-	-	+	+	+	+	-	-	---	--	low (I) v positive
34	-	+	-	-	+	+	-	-	-	-	---	--	intermediate (II) ii+v positive
35	-	+	-	-	+	+	+	+	-	-	---	--	intermediate (II) ii+v positive
12	-	+	+	-	-	-	-	-	-	-	---	--	high (V) ii+v positive
19	-	+	+	-	-	-	-	-	-	-	---	--	high (V) ii+v positive

Quantification of microorganisms by real-time PCR

1. Determination of the *Candida albicans* copy number using real-time PCR

The primers designed by Ogata et al. (2015) , which amplify a 53 bp fragment of the 26S rRNA gene, were used for the molecular biological detection of *C. albicans*. Quantification was performed with a standard curve of a pure culture of the *C. albicans* type strain ATCC 18804.

Conclusion: Molecular biology revealed a *C. albicans* copy number of less than 2 log levels in all patients and thus determined to be negative.

2. Determination of the *Lacticaseibacillus rhamnosus, Lactocaseibacillus casei* and *Lactocaseibacillus paracasei* copy number using qPCR techniques

For the molecular biological detection of *Lc. rhamnosus*, the primers designed by Song et al. (2000) were used, which amplify a 113 bp fragment of the 16S-23S rRNA intergenic spacer region. The standard curve was prepared from a pure culture of the *Lc. rhamnosus type strain* DSM 20021. In the swabs from the patients, the species could not be detected either microbiologically or molecularly.

For the detection of *Lc. casei* and *Lc. paracasei*, the primers designed by Kim et al., 2020) were used. *Lc.casei* DNA is detected by amplifying a 139 bp fragment of the Putative glycoside hydrolase gene. The *Lc. paracasei* primers bind a 135 bp fragment of the Quinone reductase related Zn-dependent oxidoreductase gene. The standard curves were constructed using serial dilutions of genomic DNA of the type strains (*Lc. casei* DSM 20011, *Lc. paracasei subsp. paracasei* DSM 5622). By application of the qPCR techniques in all smears, a *Lc.* casei *copy number* of less than 3.5 log copies/mL was determined. The *Lc. paracasei* copy number was also less than 3.5 log copies/mL in all smears and was thus determined negative.

3. Determination of *Lb. crispatus, Lb. gasseri* and *Lb. jensenii* copy number by real-time PCR

The primer and probes for a multiplex real-time PCR detection of *Lb. crispatus*, *Lb. jensenii*, *Lb. gasseri* and *Lb. iners* were designed by Balashov et al. (2014), amplifying a 136 bp fragment of the *tuf* gene. For quantification, standard curves were generated by dilution of the genomic DNA of the type strains (*Lb. crispatus* DSM 20584, *Lb. gasseri* DSM 20243, *Lb. jensenii* DSM 20557, *Lb. iners* LMG 18914). To achieve lower detection limits, the species were detected in singleplex assays.

Figure 6 shows the copy number of *Lb. crispatus, Lb. gasseri,* and *Lb. jensenii* determined in vaginal bast streaks by qPCR.

With exception of sample 19, using real-time PCR, *Lb. crispatus* was detected in all smears in which the species was also identified by cultural methods. In swabs with ID 3 and 34, the copy numbers were lower than 5 log copies/mL and almost one log level lower than the bacterial counts. In swab 19, *Lb. crispatus* was detected with *a* copy number of 5.4, while the species could not be detected by microbiological methods.

Lb. gasseri was detected in two patients (3 & 4) with normal vaginal flora at similar levels as the bacterial plate counts. Using real-time PCR, the species was detected in all patients with intermediate flora (6, 34 and 35) but only in sample 35 a copy number higher than 5 log copies/mL was quantified. A copy number of 4 log copies/mL was detected in one of the patients with BV (ID 19), while the species could not be detected by microbiological methods.

Lb. jensenii was detected in one patient with normal flora and two patients with intermediate flora with a copy number higher than 5 log copies /mL. The copy numbers were between 0.1 to 0.8 log levels higher than the bacterial counts on MRS agar. The species was detected in another patient with intermediate flora (ID34) with a copy number of only 2.7 log copies/mL. In that sample, the species was not identified on MRS agar, likely due to the overgrowth with *Lb. crispatus*.

According to the qPCR results, *Lb. crispatus* appears to be the predominant species in patients with normal vaginal flora, while the species *Lb. gasseri* and *Lb. jensenii* might be dominating in patients with intermediate flora.

Figure 7 displays the summed-up copy numbers of *Lb. crispatus*, *Lb. gasseri* and *Lb. jensenii.* With exception of swab with ID 3, the highest copy numbers were detected in patients with a healthy vaginal flora. Lower copy numbers were quantified in swabs from patients with intermediate flora or BV.

4. Determination of the copy number of *Lactobacillus iners, Gardnerella vaginalis* and *Prevotella* species using real-time PCR

The primers designed by Zozaya-Hinchliffe et al. (2010) were used for the molecular biological detection of *Gardnerella vaginalis* and *Prevotella species*. The standard curves were created with a dilution series of the genomic DNA of the *Gardnerella vaginalis* type strain ATCC 14018 and *Prevotella bivia* DSMZ 20514.

Using real-time PCR, *Lb. iners* was detected in smears with ID 3, and 13. Microbiologically, the species could only be detected in smear 3. *Gardnerella vaginalis* was quantified in two swabs of patients with intermediate flora and the two swabs of patients with bacterial vaginosis with a copy number of more than 5 log copies/mL. Similar bacterial counts of *Gardnerella species* were identified in the same swabs by growth of Columbia blood agar. Using real-time PCR, *Prevotella* species were detected in smears 6 and 35 with intermediate flora and smears 12 and 19 with bacterial vaginosis with a copy number higher than 5 log copies/mL. Microbiologically, *Prevotella* species were only detected in the patients with bacterial vaginosis.

Figure 8 shows the copy number of *Lactobacillus iners, Gardnerella vaginalis* and *Prevotella* species in vaginal swabs determined by qPCR.

Conclusion: Pathogenic species such as *Gardnerella vaginalis* or *Prevotella species* were present in higher numbers in patients with intermediate vaginal flora and bacterial vaginosis.

Table 7: displays the respective assignment to low, medium and high risk of vaginal infection according to the PCR quantification methods summarized above:

Patient ID	*Candida albicans* (i)	*Gardnerella vaginalis* (ii)	Prevotella spp. (iii)	*Lb. iners* (iv)	*Lactobacillus acidophilus* complex (v)	*Lb. crispatus* (v)	*Lb. gasseri* (v)	*Lb. Jensenii* (v)	*Lactica seibacillus casei* complex (v)	*Lc. rhamnosus* (v)	*Lc. casei* (v)	*Lc. paracasei* (v)	Result
3	-	-	-	+	+	-	+	-	-	-	-	-	low (I) iv + v positive
4	-	-	-	-	+	+	+	-	-	-	-	-	low (I) v positive
13	-	-	-	+	+	+	-	+	-	-	-	-	low (II) v positive
14	-	-	-	-	+	+	-	-	-	-	-	-	low (I) v positive
21	-	-	-	-	+	+	-	-	-	-	-	-	low (I) v positive
6	-	-	+	-	+	+	-	+	-	-	-	-	intermediate (II) iii+v positive
34	-	+	-	-	+	-	-	-	-	-	-	-	intermediate (II) ii+v positive
35	-	+	+	-	+	+	+	+	-	-	-	-	intermediate (II) ii+iii+v positive
12	-	+	+	-	-	-	-	-	-	-	-	-	high (v is negative)
19	-	+	+	-	+	+	-	-	-	-	-	-	high (v negative)

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CLAIMS

1. A system for *in vitro* determining the risk of vaginal infection in a subject, said system comprising

a) means for specifically detecting in a vaginal sample of said subject:

i. *Candida albicans*;

ii. *Gardnerella spp.,* specifically *Gardnerella vaginalis*;

iii. *Prevotella bivia*;

iv. *Lactobacillus iners*; and

v. at least one of lactobacilli belonging to the *Lactobacillus acidophilus* complex, and lacticaseibacilli belonging to the *Lacticaseibacillus casei* complex;

and;

b) means for assigning a status of either positive or negative for each of i., ii., iii., iv., and v. based on a);

wherein the risk of vaginal infection in the subject is:

I. low if the status of i., ii., and iii., is negative and the status of v. is positive;

II. intermediate if the status of i. is negative, the status of at least one of ii., and iii. is positive, and the status of v. is positive;

III. intermediate if the status of i., ii., iii., iv., and v. is negative;

IV. high if the status of i. is positive; and

V. high if the status of at least one of ii., iii., and iv. is positive and the status of v. is negative.

2. The system of claim 1, wherein the Lactobacillus acidophilus complex comprises *Lactobacillus crispatus*, *Lactobacillus gasseri*, and *Lactobacillus jensenii.*

3. The system of claim 1 or 2, wherein the *Lacticaseibacilli casei* complex comprises *Lacticaseibacillus rhamnosus*, *Lacticaseibacillus casei*, and *Lacticaseibacillus paracasei.*

4. The system of any one of claims 1 to 3, further comprising means for detecting vi. sialidase and means for assigning a status of either positive or negative for vi..

5. The system of claim 4, wherein the status of vi. is positive if the sample has a sialidase activity of 5 +/- 2 U/mL or more.

6. The system of claim 4 or 5, wherein the risk of the subject is:

VI. intermediate if the status of vi. is positive, the status of i. is negative, and the status of v. is positive; and

VII. high if the status of vi. is positive, and the status of v. is negative.

7. The system of any one of claims 1 to 6, wherein the means for detecting are selected from the group consisting of antibodies, primers, probes, and chromogenic substrates.

8. The system of any one of claims 1 to 7, wherein the status of i. is positive if *Candida albicans* is detected at a level corresponding to a cfu/mL of 10^4 or copies/ml of 10^4, or higher.

9. The system of any one of claims 1 to 8, wherein the status of ii. is positive if *Gardnerella spp.,* specifically *Gardnerella vaginalis,* is detected at a level corresponding to a cfu/mL of 10^4 or copies/ml of 10^4, or higher.

10. The system of any one of claims 1 to 9, wherein the status of iii. is positive if *Prevotella bivia* is detected at a level corresponding to a cfu/mL of 10^4 or copies/ml of 10^4, or higher.

11. The system of any one of claims 1 to 10, wherein the status of iv. is positive if *Lactobacillus iners* is detected at a level corresponding to a cfu/mL of 10^4 or copies/ml of 10^4, or higher.

12. The system of any one of claims 1 to 11, wherein the status of v. is positive if at least one of lactobacilli belonging to the *Lactobacillus acidophilus* complex, and lacticaseibacilli belonging to the *Lacticaseibacillus casei* complex is detected at a level corresponding to a cfu/mL of 10^5 or copies/ml of 10^5, or higher, or if the sum lactobacilli belonging to the *Lactobacillus acidophilus* complex, and lacticaseibacilli belonging to the *Lacticaseibacillus casei* complex is a level corresponding to a cfu/mL of 10^5 or copies/ml of 10^5, or higher.

13. The system of any one of claims 1 to 12, wherein the system is a point-of-care test.

14. The system of any one of claims 1 to 13, further comprising means for detecting *Trichomonas* sp.

15. The system of claim 14, wherein the risk of vaginal infection in the subject is high if the status of *Trichomonas* sp. is positive.

16. A kit comprising the system of any one claims 1 to 15, further comprising an instruction manual.

17. Use of the system of any one of claims 1 to 15 or the kit of claim 16 for determining the risk of a vaginal infection in a subject.

18. An *in vitro* method of determining the risk of vaginal infection in a subject, said method comprising

a) specifically detecting in a vaginal sample from the subject:

i. *Candida albicans*;

ii. *Gardnerella spp.,* specifically *Gardnerella vaginalis*;

iii. *Prevotella bivia*;

iv. *Lactobacillus iners*; and

v. at least one of lactobacilli belonging to the *Lactobacillus acidophilus* complex, and lacticaseibacilli belonging to the *Lacticaseibacillus casei* complex;

and;

b) assigning a status of either positive or negative for each of i., ii., iii., iv., and v. based on a);

wherein the risk of vaginal infection in the subject is:

I. low if the status of i., ii., and iii., is negative and the status of v. is positive;

II. intermediate if the status of i. is negative, the status of at least one of ii., and iii. is positive, and the status of v. is positive;

III. intermediate if the status of i., ii., iii., iv., and v. is negative;

IV. high if the status of i. is positive; and

V. high if the status of at least one of ii., iii., and iv. is positive and the status of v. is negative.

19. The method of claim 18, wherein the *Lactobacillus acidophilus* complex comprises *Lactobacillus* crispatus, *Lactobacillus* gasseri, and *Lactobacillus* jensenii.

20. The method of claim 18 or 19, wherein the *Lacticaseibacilli casei* complex comprises *Lacticaseibacillus rhamnosus*, *Lacticaseibacillus casei*, and *Lacticaseibacillus paracasei.*

21. The method of any one of claims 18 to 20, further comprising detecting vi. sialidase and assigning a status of either positive of negative for vi..

22. The method of claim 21, wherein the status of vi. is positive if the sample has a sialidase activity of 5 +/- 2 U/mL or more.

23. The method of claim 21 or 22, wherein the risk of the subject is:

VI. intermediate if the status of vi. is positive, the status of i. is negative, and the status of v. is positive; and

VII. high if the status of vi. is positive, and the status of v. is negative.

24. The method of any one of claims 18 to 23, wherein detecting is performed by a method selected from the group consisting of PCR, real-time PCR, RNA sequencing-based approaches, DNA sequencing based approaches, cfu determination, ELISA, enzyme test, DNA-hybridisation, RNA-hybridisation, Loop-mediated Isothermal Amplification, probes, reporter systems, and combinations thereof.

25. The method of any one of claims 18 to 24, wherein the status of i. is positive if *Candida albicans* is detected at a level corresponding to a cfu/mL of 10^4 or copies/ml of 10^4 or higher.

26. The method of any one of claims 18 to 25, wherein the status of ii. is positive if *Gardnerella spp.,* specifically *Gardnerella vaginalis,* is detected at a level corresponding to a cfu/mL of 10^4 or copies/ml of 10^4 or higher.

27. The method of any one of claims 18 to 26, wherein the status of iii. is positive if *Prevotella bivia* is detected at a level corresponding to a cfu/mL of 10^4 or copies/ml of 10^4 or higher.

28. The method of any one of claims 18 to 27, wherein the status of iv. is positive if *Lactobacillus iners* is detected at a level corresponding to a cfu/mL of 10^4 or copies/ml of 10^4 or higher.

29. The method of any one of claims 18 to 28, wherein the status of v. is positive if at least one of lactobacilli belonging to the *Lactobacillus acidophilus* complex, and lacticaseibacilli belonging to the *Lacticaseibacillus casei* complex is detected at a level corresponding to a cfu/mL of 10^5 or copies/ml of 10^5 or higher, or if the sum lactobacilli belonging to the *Lactobacillus acidophilus* complex, and *lacticaseibacilli* belonging to the *Lacticaseibacillus casei* complex is detected at a level corresponding to a cfu/mL of 10^5 or copies/ml of 10^5 or higher.

30. The method of any one of claims 18 to 29, further comprising detecting Trichomonas sp.

31. The system of claim 30, wherein the risk of vaginal infection in the subject is high if the status of Trichomonas sp. is positive.

32. A kit comprising the system of any one claims 1 to 15, further comprising an instruction manual.

33. Use of the system of any one of claims 1 to 15 or the kit of claim 32 for determining the risk of a vaginal infection in a subject.