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(54) Title: METHOD AND REAGENT KIT FOR THE IDENTIFICATION OF BIOLOGICAL FLUIDS IN A SAMPLE

(57) Abstract: A method of determining the origin of biological fluids or tracks and a kit of reagents for their identification in a sample was invented by using molecular components as indicators and in particular microbial genomic sequences of the micro-floras present in the fluids and that are present in the respective mucous membranes of origin. A set of protocols and reagents are properly defined and optimized to provide a kit applicable for forensic purposes and other uses such as laboratory analysis and / or research studies in various sectors. The kit allows the identification of a biological fluid, or its traces on a matrix, starting from the identification of genomic sequences of bacterial DNA, and includes a component (component A) for the multiple amplification and a second component (component B) for hybridization. The component B comprises at least one probe specific for each bacterial species considered for the identification of biological fluid in a sample.

DESCRIPTION

The present invention relates to the field of biotechnology, and in particular concerns a method and a kit of reagents used in a simple way for the identification of biological fluids in a sample of any kind, for example consisting of forensic findings, clinical samples, swabs from outpatient or hospital, findings at a crime scene and / or traces of interest to forensic science investigations of police and private investigators.

It 's well known that in the forensic field, to trace the origin of a stain or trace left on finding laboratory analyses are used by departments in charge of the scientific investigations. The cases of kidnapping and sexual violence, as many other situations of interest in forensic medicine, have recently highlighted the importance of having scientific instruments to help reconstruct the crime scene and gather information on traces compatible with the presence of biological fluids on objects, clothing, artifacts found at the crime scene. In this context, it assumes specific relevance, for example, the possibility of being able to distinguish on a finding a possible trace of vaginal fluid from other biological fluids such as saliva, rectal mucus, sperm, or other organic materials such as animal blood, some beverages, or food.

Are already known some methods for the identification of biological fluids in a sample, however, such techniques are often unreliable, or generally require the application of complex and expensive instrumentation, as well as particularly high professional skills. Furthermore, no known method is able to ensure sufficient sensitivity and specificity appropriate to the analysis of complex and heterogeneous samples as well as with the possibility of distinguishing simultaneously a wide range of biological fluids. The known methods are based, for example on physical or biochemical-enzymatic properties of the biological fluid, which can be strongly influenced by the action of degradation, dehydration, and / or alteration as a result of exposure to various environmental, chemical, physical or biological agents -especially if prolonged in time.

A general investigation approach, which proved to be effective, technologically advanced, and currently in use in laboratories for scientific investigations is based on DNA analysis. However, molecular biology and analyses already available to identify and classify traces of human DNA, are not applicable to the determination of the type of a biological fluid, in that the DNA of an individual is identical in different tissues of the same subject, not allowing therefore, neither to differentiate the type nor the origin tissue of any biological fluid. An alternative method always based on molecular biology techniques, but still experimental, could be based on analysis of mRNA expression typical of the cells of mucosal tissue the biological fluid derives from, but this approach is still hypothetical and is in its turn strongly influenced by the action of environmental agents, as the RNA molecules are especially perishable also due to the ubiquitous and massive presence in the organic materials and the environment, indoor and outdoor, of agents able to degrade it (RNase), which would make any finding difficult to use. Therefore, at the moment, , the benefits of the acquisition of molecular biology, ie high speed, sensitivity, specificity, reproducibility, are not applied to answer these questions of compatibility in investigations of possible traces of body fluids through a diagnostic system in a broader spectrum .

The purpose of the present invention is therefore to provide a method and a kit for the identification of a range of biological fluids that can be used in a simple way and with the aid of instrumentation commonly present in small and medium molecular biology laboratories, disseminated in the territory. The method can however easily be adapted to other instruments and methods for the analysis of the DNA including the latest techniques for NGS (next generation sequencing). Said aim is achieved with a method and a kit whose main features are specified in the first claim and other features are specified in subsequent claims.

The kit according to the present invention is based on molecular biology techniques customary in forensic laboratories and applicable also to traces of biological fluids of forensic interest, and it is based on the technique of polymerase chain reaction and hybridization techniques, but the present invention differs from what is currently available primarily because it relies on the amplification

and identification not of human genomic or non-mitochondrial (mtDNA) sequences, but of different genome sequences belonging to various species that dwell the human body regions of origin of biological fluids under consideration and that constitute the microflora (mfDNA).
Complex bacterial populations colonize, in fact, the various body districts, such as the oral and nasal cavities, the genital and anal areas. Although the individual microbial species, in response to particular pathological or behavioral conditions, can be found in several districts, each region of the body tends to create a stable balance between the various bacteria, generating a well defined population. It is then possible to speak of "signature" of each microbial typical district. The vaginal mucosa, for example, is characterized by a complex microbial population that in non-pathological conditions, is made up by elements belonging to the genus Lactobacillus, such as L. iners, L. crispatus, and L. gasseri, but also several other species are present, such as indicated in Examples 4 to 7. These lactobacilli play an important protective function by acidification of the tissue, making it less colonizable by pathogenic species (Verstraelen H. Cutting edge: the vaginal microflora and bacterial vaginosis. Verh K Acad Geneeskd Belg., 2008; 70 (3) :147-74.). The use of molecular techniques has contributed in recent years to characterize with high accuracy the population of vaginal bacteria. It was possible to pinpoint that women belonging to different nationalities, can present a different microbial pattern. According to Dumonceaux and colleagues (TJ Dumonceaux, Schellenberg J, Goleski V, Hill JE, Jaoko W, Kimani J, Money D, Ball TB, Plummer FA, Severini A. Associated with Multiplex detection of bacteria with normal microbiota and bacterial vaginosis in vaginal swabs by use of oligonucleotide-coupled fluorescent Microspheres. J Clin Microbiol. Dec 2009, 47 (12) :4067-77. Epub 2009 Sep 30) African subjects show good levels of L. iners, but not of L. crispatus. Ravel and colleagues (J Ravel, Gajer P, Abdo Z, Schneider GM, Koenig SS, SL McCulle, Karlebach S, Gorle R, Russell J, Tacket CO, Brotman RM, Davis CC, Ault K, Peralta L, Forney LJ. Vaginal Microbiome of reproductive-age women. Proc Natl Acad Sci U.S. A. 2011 Mar 15; 108 Suppl 1:4680-7. Epub 2010 Jun 3) have reported differences in the microbial population correlated to ethnic specific values of vaginal pH. The vaginal flora is affected also by the clinical background of the subject. It is widely documented the reduction of Lactobacilli in favor of the proliferation of A. vaginae and G. vaginalis in cases of bacterial vaginosis (CS Bradshaw, SN Tabrizi, CK Fairley, AN Morton, and Rudland, SM Garland. The association of Atopobium vaginae and Gardnerella vaginalis with bacterial vaginosis and recurrence after oral metronidazole therapy. J Infect Dis. Sep 15 2006 , 194 (6) :828-36. Epub 2006 Aug 16). According Zozaya-Hinchliffe and colleagues (Zozaya-Hinchliffe M, Lillis R, Martin DH, MJ Ferris. Quantitative PCR assessments of bacterial species in women with and without bacterial vaginosis. J Clin Microbiol. May 2010, 48 (5) :1812-9 . Epub 2010 Mar 19) the population of L. iners is not particularly affected by situations of bacterial vaginosis. Since the different methods available for the identification of vaginal fluid are limited and inadequate, and as the identification of traces of vaginal fluid is a major need for the forensic field, different approaches have been tried and some commercial kits are available, though they possess a sensitivity and specificity too low. Generally they rely, in fact, on the identification of enzymatic and / or antigenic properties, and depend upon the integrity of protein products (for example, the salivary amylase). Therefore, they are greatly influenced also by the easy degradability of protein in the environment. The DNA is more resistant, but the techniques of analysis of the human genome currently used in forensic and biomedical laboratories, do not allow, to date, to distinguish the source of fluids, since the primary structure of the DNA of an individual is identical in different tissues. On the basis of these and other considerations several studies have been developed, including those of Frumkin (2010) based on the methylation of the human genome in different tissues and those of Fleming and Harbison (2010) who have suggested alternative approaches, including those based use of differences in gene expression in cells of the vaginal mucosa or into cells of the microflora, having also proposed a multiple amplification of bacterial mRNA.
However, it is not yet described an approach for the integrated and comparative identification of the different biological fluids on the basis of the genomic DNA of the microflora (mfDNA) usable as an

indicator for the simultaneous and comparative identification of two or more biological fluids. This approach allows not only to acquire a direct indication of compatibility between a sample and a fluid, but also indirectly via the comparison and the exclusion of compatibility with other biological fluids or with other matrices, in which microbial species may be present, for example environment (land, water, indoor and outdoor air, etc.) or food (milk, flour, dough, etc..). A series of micro-organisms found in the different mucous membranes, epithelium and in the relative biological fluids is shown in the examples no. 4, 5, 6, 7, 8.

Given the impossibility of defining a microbial species typical and unique for vaginal secretions, and certainly always absent in other body fluids, an integrated test of multi-target type assumes a crucial and significant value in identifying the microbial "signature" of a biological fluid. This approach differs from the simple association of one or more microorganisms to a fluid and regards instead the analysis of a fingerprint of the genomes of the microflora, ie the prevailing representation of the different species typical of that district, the mucosa, the biological fluid. a comparison between the genomes of different organisms, both bacteria, but also fungi, protozoa, viruses or bacteriophages, which constitute the microflora and that may be encountered in the different biological fluids. This analysis can be done with different techniques of molecular biology including the techniques of amplification or sequencing of DNA, including real-time PCR and Next Generation Sequencing. The application of this method allows analysis based on the characterization of the DNA of the microflora from specific sequences for one or more microbial species vaginal and at the same time also for one or more bacteria from other possible microflora, allowing to identify or exclude the vaginal origin of a fluid in a sample, and indicating the possible contamination with possible other biological fluids.

A method of determining the origin of fluids or biological specimens and kits of reagents for their identification in a sample has been developed by using as an indicator of molecular components and in particular microbial genomic sequences of the microflora present in the fluids and which dwell in the respective mucosae of origin. A set of protocols and reagents properly defined and optimized provide a kit applicable for forensic purposes and other applications such as laboratory analysis and / or research studies in various sectors. The kit allows the identification of a biological fluid, or its traces on a matrix, starting from the identification of genomic sequences of bacterial DNA, and includes a component (component A) for the multiplex amplification and a second component (component B) for hybridization. The component B includes at least one probe specific for each bacterial species considered for the identification of biological fluid in a sample.

Other elements that compose the kit are the usual reagents and instruments used for these types of analyses, as well as instructions for use.

The kit according to the present invention by identifying characteristic properties of the microflora of the biological fluid from the genome allows, in fact, to make a diagnosis of compatibility and / or exclusion compared to other biological fluids. This arrangement makes it possible to define a compatibility between a finding and a biological fluid, even if the finding was not immediately picked up and / or has been exposed to adverse environmental conditions, since the resistance of the bacterial DNA is high enough, as it is known . In addition, the kit can be applied in conventional molecular biology procedures, already used for analysis of traces of human DNA by applying already available technologies and skills and providing added value to the investigation. A considerable advantage with regard to the speed of amplification -which is in turn is directly proportional to the sensitivity of the method- is supported by the high-microbial load present in the fluid, which in the human microflora tends to exceed values of the order of 10^6-10^9 micro-organisms per milliliter, for bacteria, but also for viruses, bacteriophages, protozoa, fungi or any other microbial species.

A further advantage of the kit according to the present invention, compared to other methods based on polymerase chain reaction, consists in the fact that it does not require the use of restriction enzymes, of apparatus for electrophoresis or dying of the DNA, but it is based on the hybridization with a probe, detectable by a classic tool for real-time amplification, allowing to search for multiple

microbial indicators simultaneously in a single test -qualitative and quantitative-allowing also an automation of the analysis. However it is not precluded the possibility to make a preliminary assessment by electrophoresis and / or digestion with restriction enzymes according to the traditional procedures.

Another advantage of the kit according to the present invention consists in the fact that the amplification for one or more biological fluids can be realized in a single operation even with more indicators microbial parallel, adding to a mixture of standard reaction only the A and B components and DNA sample diluted in water.

Further advantages and features of the kit according to the present invention will become apparent to those skilled in the field from the following detailed description of an embodiment thereof with reference to the attached working examples.

The kit according to the present invention comprises two components for each biological fluid. The first of these components (component A) includes the reagents for the multiple amplification, and the second (component B) reagents for hybridization.

Component A consists of an amplification mixture that includes all the necessary reagents according to the known process for the polymerase chain reaction, commonly known as PCR and described inter alia in U.S. patents 4,683,195 and 4,683,202. Such reagents are: the reaction buffer that allows to create the conditions for the functionality of the enzyme (pH, salt concentration), the dNTP ie deoxynucleotide triphosphates consisting of nitrogenous bases (adenine, guanine, cytosine, thymidine) monomers required for the reaction polymerase; magnesium chloride (MgCl2) necessary to ensure specificity and efficiency of the reaction (annealing of primers and functionality of the polymerase), and the mixture of the primers that oligonucleotides of specific sequence, required to initiate the amplification of DNA complementary to each other according to the well-known principle of the polymerase chain reaction.

To identify the primers suitable to be used in the reaction mixtures, and that allow the amplification of the indicators used for the identification of the different biological fluids, it was necessary to first conduct research in the literature and in the data banks on microflora and on regions of genomic DNA that were compatible with the intended purposes, including the use of bioinformatics tools. Then, the individual sequences of the primers were chosen, which have been synthesized. Afterwards, further studies of bioinformatics and laboratory to detect and / or optimize the mutual relations between the different oligonucleotides in the reaction mixtures, as well as suitable conditions of magnesium and the temperature of annealing of primers to the DNA have been carried out. In this way, in the components A of the kit, according to the present invention, for each biological fluid, reagents are present and assayed in such a way as to enable the simultaneous amplification of segments of genes of different bacterial species. The research carried out has shown that such mixtures are sufficiently stable that they can be included in the kit as such. Therefore, they will allow the diagnostic laboratory not to dose the various reagents separately, as it is usually done.

According to a preferred embodiment of the invention, the mixture A includes the primers corresponding to different biological species, for example for the vaginal fluid sequences belonging to the genome of Lattobacillus gasseri and Lattobacillus crispatus or Lattobacillus iners; for saliva to Streptococcus salivarius and Streptococcus mutans ; for rectal mucus to Escherichia coli and Enterococcus faecalis. In this case, each mixture contains about 20 parts of a 100 micromolar solution of the primers corresponding to each species. These rates may of course vary within certain limits, which can also be of about 10%, without impairing the effectiveness of the mixture and its stability. The primers can be ordered from several companies including, for example, Sigma-Aldrich, MWG, PRIMM. Some 'primers sequences used from 5'-3, forward and reverse respectively, are specified below, and will then be resumed in the following examples:

L. crispatus:

(SEQ ID 1) GCACTAACAGCCGAAGAAGG

(SEQ ID 2) TTCGGATATCTCCGGATCAC

L. gasseri:
(SEQ ID 3) AGATTGAAGAGCGACCGAGA
(SEQ ID 4) CCTTCATCGGCTTCTAGTGC
L. INERS:
(SEQ ID 5) CGGTGAATTGATTGCTGATG
(SEQ ID 6) ATCGGCTTCCATCTTGTCAT
S. mutans:
(SEQ ID 7) CGGTTCTCAGCAAGACATGA
(SEQ ID 8) ATGGTACCCAATCCGCAATA
S. salivarius:
(SEQ ID 9) GATGCCAAGGGTGAAGTTGT
(SEQ ID 10) GAGCCATCAG GATTCGTAGC
E. coli:
(SEQ ID 11) CCCTTACGCT GAAGAGATGC
(SEQ ID 12) GAGGTTAAAG CCGACAGCAG
E. faecalis:
(SEQ ID 13) AGAAATTCCA AACGAACTTG
(SEQ ID 14) CAGTGCTCTA CCTCCATCATT
To increase the types of biological fluids identifiable with the kit according to the present invention, it is possible to add to the mixture the primers corresponding to other characteristics of the different species microflora human and / or animal and / or belonging to specific environmental matrices and / or foodstuffs interest in a differential diagnosis, and also in a method of diagnosis by exclusion. For example, to highlight the presence of traces of bovine blood is possible to add approximately 10 parts of a solution 100 micromolar of the following primers 5'-3 'corresponding to the species Bos taurus:
(SEQ ID 15) and CAGCCCCATC AAACATTTCATC (SEQ ID 16) CCCGTAATAA TATAAGCCTCGTCC, and providing for the component B, a probe sequence (SEQ ID 17) ATATCTGCCG AGACGTGAACTACG.
Considering now the B component of the kit according to the present invention, it consists of a solution containing the probes. Probe means an oligonucleotide of known sequence specific for the biological species sought, which can be done by synthesizing by outsourcing to appropriate companies or synthesized directly in the laboratory with the use of an instrument called oligonucleotide synthesizer. The important feature of the probe is that the sequence of bases A, C, G, T (adenine, cytosine, guanine, thymine) is defined.
According to a preferred embodiment of the invention, the kit includes probes corresponding to different human biological fluids, for example, vaginal fluid, saliva, mucus, rectal, and is thus allowed the identification of at least two different human biological fluids. The probes have the function of linking the DNA segments that are amplified using the mixtures of component A. These segments will undergo a hybridization reaction allowing to identify the biological fluids sought. For hybridization known methods and standard solutions can be used and for the detection any system can be used. In this case, at least two triads primer-probe for each biological fluid have been used simultaneously, corresponding respectively to at least two bacterial indicators. In particular, in this case, has been used an approach based on real-time amplification (Instrument ABI 7000, Applied Biosystems, similar instruments are available but also from other suppliers, for example, Biorad) and the oligonucleotide sequences were labeled (Sigma) with a system for detection by fluorescence, according to classical protocols for real-time PCR, known to the industry.
The sequences of oligonucleotide probes specific for the fluids mentioned above are shown below, indicated with also a type of dye used:
Vaginal fluid:
(SEQ ID 18) L. crispatus: [FAM] CGAAAAGCTTCGGGGAGCGGT [BLACK_QUENCER]
(SEQ ID 19) L. gasseri: [JOE] AAGGGCGCATGGTGAATGCCT [BLACK_QUENCER]

(SEQ ID 20) L. INERS: [FAM] TGGAACGAGTAGGACACGATGGTG [BLACK_QUENCER]
Saliva:
(SEQ ID 21) S. mutans: [FAM] TGCAGTTAAAGCTCTGCATAAAAGCGG [BLACK_QUENCER]
(SEQ ID 22) S. salivarius [JOE] TGGCGAACAGACGATCAACGG [BLACK_QUENCER]
Rectal mucus:
(SEQ ID 23) E. coli: [FAM] TGGGCAGATGAACATGGCATCG [BLACK_QUENCER]
(SEQ ID 24) E. faecalis [JOE] TGGTTCTCTCCGAAATAGCTTTAGGGCTA [BLACK_QUENCER]
Other probes corresponding to other components of microflora useful in the further characterization of the same fluid or other identifying matrices, can be added in order to obtain a reaction that can allow the kit according to the present invention to identify the same procedure with the same fluid with more probes and / or other possible biological contaminants of interest in a differential diagnosis of exclusion.
At the time of use, the components A and B will be added to the necessary reagents for the amplification reaction, according to known protocols. In particular, individual reagents are acquirable by different companies (SIGMA, Fermentas, Promega), and are typically used under the following final concentrations: 10mM Tris-HCl pH 8.3 (20 ° C), 50 mM KCl, 1.5 MgCl mM, 0.2 mM dNTP (Also, it is also possible to improve the yield and stability, adding stabilizers and / or a reference dye, acquired by different companies, and to be dosed according to protocols already known). However, for the real time amplification ready made solutions can be also used, which are obtainable from different companies (Sigma, Applied Biosystems).
The present invention will be illustrated by means of the following examples and of the accompanying figures, which should not be considered limitative but illustrative of the scope of the invention and wherein:
FIGURE I, referring to Example 1, relates to the identification of vaginal fluid. The sample of DNA extracted from vaginal swab was positive for the presence of L. gasseri and L. crispatus, while it does not appear significant signal for the indicators of other biological fluids such as saliva and / or rectal mucus.
FIGURE II, referring to Example 3, relates to the calibration curve (E. faecalis, serial dilutions) ...

EXAMPLE 1
Preparation of the components A and B.
For the preparation of component A, 20 parts of a solution 100 µM of each primer were diluted in ultrapure water. In particular, oligonucleotides were synthesized (SIGMA-Aldrich), received lyophilized and resuspended in sterile ultrapure water to a final concentration of 100 µM
The preparation of component A for the identification of vaginal fluid was carried out in the following way: in a test tube of 2 ml 100 µl of ultrapure sterile water, and respectively 100 µl of the primer with sequence GCACTAACAGCCGAAGAAGG (SEQ ID 1) , 100µl of the primer with sequence TTCGGATATCTCCGGATCAC (SEQ ID 2), 100 µl of the primer with sequence AGATTGAAGAGCGACCGAGA (SEQ ID 3), 100µl of the primer with sequence CCTTCATCGGCTTCTAGTGC (SEQ ID 4) were added.
The preparation of component A for the identification of saliva was carried out in the following way: in a test tube containing 2 ml 100 µl f ultrapure sterile water, and respectively 100 µl of the primer with sequence CGGTTCTCAGCAAGACATGA (SEQ ID 7), 100 µl of the primer with sequence ATGGTACCCAATCCGCAATA (SEQ ID 8), 100 µl of the primer with sequence GATGCCAAGGGTGAAGTTGT (SEQ ID 9), 100 µl of the primer with sequence GAGCCATCAGGATTCGTAGC (SEQ ID 10) were added.
The preparation of component A for the identification of rectal mucus was carried out in the following way: in a test tube containing 2 ml of ultrapure sterile water, and respectively 100 µl of

the primer with sequence CCCTTACGCTGAAGAGATGC (SEQ ID 11) 100 µl of the primer with sequence GAGGTTAAAGCCGACAGCAG (SEQ ID 12), 100 µl of the primer with sequence AGAAATTCCAAACGAACTTG (SEQ ID 13), 100 µl of the primer with sequence CAGTGCTCTACCTCCATCATT (SEQ ID 14) were added.
For the preparation of component B, 5 parts of a solution 100 µM of each probe were diluted in ultrapure water In particular, oligonucleotides labeled at 5 'with a fluorophore and the 3' end with a quencher were synthesized (SIGMA-Aldrich), received lyophilized and resuspended in ultrapure sterile water to a final 100 µM concentration
The preparation of component B for the identification of vaginal fluid was carried out in the following way: in a test tube containing 2 ml, 450 µl of ultrapure sterile water, and respectively 25 µl were added of each of the following labeled probes of sequence:
[FAM] CGAAAAGCTTCGGGGAGCGGT [BLACK_QUENCER] (SEQ ID 18)
[JOE] AAGGGCGCATGGTGAATGCCT [BLACK_QUENCER] (SEQ ID 19)
The preparation of component B for the identification of saliva was carried out in the following way: in a test tube containing 2 ml , 450 µl of ultrapure sterile water, and respectively 25 µl were added of each of the following labeled probes of sequence:
[FAM] TGCAGTTAAAGCTCTGCATAAAAGCGG [BLACK_QUENCER] (SEQ ID 21)
[JOE] TGGCGAACAGACGATCAACGG [BLACK_QUENCER] (SEQ ID 22)
For the preparation of component B for the identification of mucus rectal µwe proceeded as follows: in a test tube containing 2 ml , 450 µl of ultrapure sterile water, and respectively 25 µl were added of each of the following labeled probes of sequence:
[FAM] TGGGCAGATGAACATGGCATCG [BLACK_QUENCER] (SEQ ID 23)
[JOE] TGGTTCTCTCCGAAATAGCTTTAGGGCTA [BLACK_QUENCER] (SEQ ID 24)
All the solutions once prepared were aliquoted and stored at -20 ° C, ready for use.

EXAMPLE 2
Analysis of saliva, vaginal and fecal samples.
- DNA was isolated from oral (n = 5), vaginal (n = 5) and rectal (n = 2) swabs (stored at room temperature for at least 72 hours after collection, to allow dehydration and simulate environmental exposure, in analogy to what may happen in cases of forensic interest), and processed according to traditional methods of extraction that took account of good laboratory practice, general principles for the extraction of bacterial DNA and the general principles for the extraction of DNA from forensic and / or ancient samples. In particular GenElute Bacterial Genomic DNA kit (Sigma-Aldrich NA2100) has been used with some modifications to the protocol. Briefly, the swab was washed in 500 ul of sterile PBS for 45 minutes under stirring at room temperature. The eluate was centrifuged and the pellet frozen at -20 ° C for 20 minutes. Then, Glass Beads (Sigma-Aldrich G1145) were added together with 200 ul Lysozime solution. With a sterile pestle we disintegrated accurately the pellet and then incubated at 37 ° C for 30 minutes. Then it was proceeded by adding RNase and Proteinase K, reaching the elution up to a final volume of 50 l.
- To the samples above were added to the analysis also 3 negative controls, consisting of ultrapure sterile water, and 3 positive controls, consisting of bacterial DNA of the species sought, diluted in sterile water ultrapure to a final concentration of the order of 0.2 ng / l;
- The amplification reactions were conducted on an ABI 7000 instrument from Applied Biosystems, in a volume of 25 ul total, programmed as follows: denaturation 95 ° C for 10 minutes, followed by 40 cycles at 95 ° C for 15 seconds and 60 ° C for one minute. Each of the DNA samples described above and of the positive and negative controls, was analyzed in parallel for the search of three biological fluids: vaginal, salivary, rectal, using the respective components A and B prepared as described in Example 1. For each sample, were added 10 l of DNA in a test tube 0.2 ml thin wall in which they were aliquoted 1.25 µl of component A, 1.25 µl of component B and 12.5 µl of amplification reaction 2X of the company Applied Biosystems. Each DNA sample was amplified in parallel in three tubes using respectively the components A and B for the vaginal fluid, for saliva

and rectal mucus. The results observed (Figure 1) made it possible to correctly assign each sample to the fluid of origin.

EXAMPLE 3

Analysis of samples of vaginal fluid, calibration curves, mixture of components A and B in the solution AB.

Using the general conditions of DNA extraction and amplification previously described in Example 2, steps were taken to analyze additional samples of vaginal fluid, even for complex situations from the storage conditions (eg humidity, temperature, contamination with other fluids biological, conditions of superimposed infection, presence of blood, retention over 10 days at room temperature).

In addition, some samples were prepared of bacterial DNA serially diluted in order to use them in order to estimate a threshold of sensitivity and specificity of the method, measurable in CT (threshold cycle). In particular, for this aspect probes and primers for Lactobacillus gasseri, and Enterococcus faecalis have been used. The DNA samples were extracted and processed as described in example 2, except that has been used a 1:1 mixture of component A and component B, from which 2.5 µl in each tube were aliquoted.

Out of 15 analyzed samples of vaginal fluid 14 have provided a correct result. A sample showed no signal, but it has proved that id did not contain amplifiable bacterial DNA; The serial dilutions allowed to establish an acceptable threshold for the non-specificity, to be placed within the 35 cycle, and also made it possible to detect up to a concentration of 6 fg per reaction tube, which corresponds to little more than a bacterial genome (Dodd and Pemberton, 1998). In conclusion, the application has shown sufficient sensitivity and specificity, as well as rapid execution of the analysis of the DNA sample with a achievement of the result within a few hours (<3h), with high reproducibility in different types of samples.

EXAMPLE 4

Example of other microorganisms found in the vaginal microflora to be used alone or in admixture among them:

Actinomyces neuii, Aerococcus christensenii, Atopobium vaginae, Atopobium vaginae, Bacteroides uniformis, Bifidobacterium bifidum, Bifidobacterium longum subsp. longum, Chloroflexi spp., Clostridiales, Dialister spp., Enterococcus faecalis, Enterococcus faecium, Escherichia coli, Finegoldia magna, Gardnerella vaginalis, Lactobacillus acidophilus phage kc005, kc007 phage Lactobacillus acidophilus, Lactobacillus acidophilus phage kc012, kc021 phage Lactobacillus acidophilus, Lactobacillus casei, coleohominis Lactobacillus, Lactobacillus coleohominis, Lactobacillus crispatus, Lactobacillus delbrueckii phage kc023, kc031 Lactobacillus delbrueckii phage, phage kc039 Lactobacillus delbrueckii, Lactobacillus fermentum, Lactobacillus fermentum, Lactobacillus gasseri, Lactobacillus helveticus, Lactobacillus iners, Lactobacillus jensenii, Lactobacillus rhamnosus, Lactobacillus salivarius, Lactobacillus spp , Lactobacillus vaginalis, Leptotrichia spp., Megasphaera spp., Olsenella spp., Pediococcus acidilactici, Peptoniphilus indolicus, Peptostreptococcus anaerobius, Porphyromonas asaccharolytica, Prevotella bivia, Shuttleworthia spp., Staphylococcus capitis, Staphylococcus epidermidis, Staphylococcus haemolyticus, Staphylococcus hominis, Streptobacillus spp. , Streptococcus agalactiae, Streptococcus anginosus, Streptococcus mutans, Streptococcus salivarius, Tricomonas Faetus, Tricomonas vaginalis

EXAMPLE 5

Example of other microorganisms found in the oral microflora to be used alone or in admixture among them:

Acanthamoeba castellanii, Acanthamoeba culbertsoni, Acanthamoeba Acanthamoeba polyphaga

odontolyticus, Actinomyces naeslundii, Actinomyces spp., Bifidobacterium adolescentis, Bifidobacterium longum, Bifidobacterium dentium, Campylobacter gracilis, Dialister invisus, Eikenella corrodens, Eikenella corrodens, Enterococcus faecalis, nodatum Eubacterium, Fusobacterium nucleatum, Gemella Haemolysans , Gemella sanguinis, Giardia lamblia, Granulicatella adiacens, Granulicatella elegans, Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus crispatus Lactobacillus delbrueckii, Lactobacillus fermentum, Lactobacillus gallinarum, Lactobacillus gasseri, Lactobacillus paracasei, Lactobacillus salivarius, Lactobacillus ultunensis, Lactobacillus rhamnosus, Naegleria fowleri, Olsenella uli , Porphyromonas gingivalis, Porphyromonas micra, Porphyromonas endodontalis, Prevotella baroniae, Prevotella denticola, Prevotella spp., Pseudoramibacter spp., dentocariosa Rothia, Streptococcus gordonii, Streptococcus intermedius, Streptococcus oralis, Streptococcus salivarius, Streptococcus sanguinis, Streptococcus sobrinus, Scardovia wiggsiae, Selenomonas noxia , Selenomonas spp., Streptococcus mutans, Streptococcus-specific phages, Tannerella forsythia, Tetratricomonas buccalis, TM7 phylum, Treponema denticola, Treponema denticola, Tricomonas tenax, Veillonella spp.

EXAMPLE 6

Example of other microorganisms found in the intestinal microflora be used alone or in admixture among them:

Acinetobacter baumannii, Actinomyces meyeri, Actinomyces radingae, urogenitalis Actinomyces, Aerococcus christensenii, Aerococcus viridans, Agrobacterium radiobacter, Alloscardovia omnicolens, Anaerococcus tetradius, Anaerococcus vaginalis, Atopobium vaginae, Bacteriofagoides lambda, M13 Bacteriofagoides, Bacteroides uniformis, Balantidium coli, Balantidium coli, Bifidobacterium bifidum , Bifidobacterium breve, Bifidobacterium longum subsp. infantis, Bifidobacterium longum subsp. longum, Chilomastix mesnili, Corynebacterium accolens, Cryptosporidium parvum, Cryptosporidium parvum, Cyclospora cayetanensis, Dientamoeba fragilis, Embadomonas intestinalis, Encephalitozoon intestinalis, Endolimax nana, Entamoeba chattoni, Entamoeba coli, Entamoeba dispar, Entamoeba hartmanni, Entamoeba histolytica, Entamoeba moshkovskii, Enterobacteria phage, Enterobacteria phage P2, Enterococcus avium, Enterococcus faecalis, Enterococcus faecium, Enterocytozoon bieneusi, Enteromonas hominis, Escherichia coli, Finegoldia magna, Fusobacterium gonidioformans, Gardnerella vaginalis, Giardia lamblia, Iodamoeba butschlii, Isospora belli, Isospora belli, Klebsiella planticola, Lactobacillus casei, Lactobacillus crispatus, Lactobacillus gasseri, Lactobacillus helveticus, Lactobacillus INERS, Lactobacillus jensenii, Lactobacillus mucosae, Lactobacillus rhamnosus, Lactobacillus salivarius, Peptoniphilus indolicus, Peptostreptococcus anaerobius, Peptostreptococcus sp., Prevotella bivia, Pseudomonas stutzeri, Sarcocystis spp., Staphylococcus aureus, Staphylococcus capitis, Staphylococcus epidermidis, Staphylococcus haemolyticus, Staphylococcus hominis, Staphylococcus warneri, streptococcal phage C1 Streptococcus agalactiae, Streptococcus anginosus, Streptococcus bovis, Streptococcus intermedius, Streptococcus mitis group, Streptococcus parasanguinis, Streptococcus salivarius, Trichomonas hominis, Trichomonas vaginalis, Varibaculum cambriense, Weissella paramesenteroides.

EXAMPLE 7

Example of microorganisms found in the nasal microflora to be used alone or in admixture among them:

Accolens Corynebacterium, Corynebacterium mucifaciens, pseudiptheriticum Corynebacterium, Mycobacterium fallax, Orthomyxovirus, Propionibacterium acnes, Rinoviriadeae, Streptococcus mitis

EXAMPLE 7

Example of microorganisms found on the skin:

Aspergillus, Aureobasidium, Brevibacteriaceae, Candida, Corynebacteriaceae, Dermabacteraceae,

Epidermophyton, Leptosphaerulina, Micrococcaceae, Myrothecium, Moraxellaceae, Staphylococcus hominis, Staphylococcus capitis, Staphylococcus epidermidis, Streptococcus epidermidis, Streptococcus hominis, Trichophyton, Xanthomonadaceae,

In conclusion, as it appears evident from what has indeed shown, in the application of the kit to be used in a device for the realtime amplification, the components A and B for each biological fluid, may be mixed in a single solution (component AB). In addition, primers and probes can be increased in number within the limits of the equipment used and the number of fluorochromes available for the marking of the probes within a single tube. Also the amplification mixture can be prepared according to protocols known by the user, or purchased ready-made from companies such as Sigma or Applied Biosystems, having the user simply to add the DNA of the sample to be examined, and the mixture of primers and probes (component A and component B or component AB). In any case, to complete the use of the kit in its application in instruments for real-time PCR provided by different companies (Biorad, Applied Biosystems, WVR), as well as the equipment calibrated and suitably programmed for the amplification conditions, it will be necessary to employ the positive and negative controls tubes and appropriate criteria for the interpretation of results, but all this material and general procedures are already known in the general methodology and therefore need no special description. If appropriate, the completion of the kit can be carried out by the diagnostic laboratory itself, which can simply adapt the protocol to its instrument adapting to their needs the use of components A and B according to the present invention.
Any variants and / or additions may be made by those skilled in the field to the embodiment described here and depicted within the embodimvinvinent of the invention itself.

CLAIMS

1) Method for the identification and detection of the nature of biological fluids, that employs DNA sequences of microorganisms present in the microflora that resides in the mucosa and epithelia of humans or animals.

2) Method according to claim 1 comprising the steps of:
- Take a sample to be analyzed;
- Isolating the genomic DNA or a portion of it from the microflora present on the sample;
- Perform the detection of DNA sequences and
- Identify the nature of the sample.

3) Method according to any one of claims 1-2 in which the DNA belongs to the genomic or mitochondrial sequence of microorganisms chosen from bacteria, fungi, protozoa, viruses and bacteriophages that are present in the microflora of the districts of origin of biological fluids such as the oral and nasal cavity, skin, genital and anal areas, as an example.

4) The method according to any one of claims 1-3 wherein the detection is carried out with molecular biology techniques including techniques of amplification or sequencing of DNA, including real-time PCR and Next Generation Sequencing.

5) reagent kit for conducting the method according to any one of claims 1-4, comprising a first component (component A) for the amplification of multiple primers containing one or more genes of microorganisms belonging to the microflora present in a biological fluid and a second component (component B) for hybridization with the probe.

6) The kit according to claim 5, characterized by the mixing of component A of the primers and probes of the component B in a single solution that is to say in a single component AB.

7) The kit according to any of claims 5-6, characterized in that the amplification mixture component A comprises a pair of primers corresponding to one or more of the species selected from: Lattobacillus crispatus, Lactobacillus gasseri, Lactobacillus INERS, Streptococcus salivarius, Streptococcus mutans, E. coli, E faecalis, Bos Taurus and the component B comprises the corresponding probes.

8) The kit according to any of claims 5-6, characterized in that the amplification mixture component A comprises a pair of primers corresponding to the species Lattobacillus crispatus GCACTAACAGCCGAAGAAGG sequences (SEQ ID 1) and TTCGGATATCTCCGGATCAC (SEQ ID 2); and a pair of primers corresponding to the species Lactobacillus gasseri AGATTGAAGAGCGACCGAGA (SEQ ID 3) and CCTTCATCGGCTTCTAGTGC (SEQ ID 4).

9) A reagent mixture according to claim 4 characterized in that the amplification mixture component A comprises a pair of primers corresponding to the species Streptococcus salivarius GATGCCAAGGGTGAAGTTGT (SEQ ID 9) and GAGCCATCAGGATTCGTAGC (SEQ ID 10); and a pair of primers corresponding to the species Streptococcus mutans CGGTTCTCAGCAAGACATGA (SEQ ID 7) and ATGGTACCCAATCCGCAATA (SEQ ID 8).

10) A reagent mixture according to claim 4, characterized in that the mixture of component B comprises a hybridization probe corresponding to the species Lactobacillus crispatus CGAAAAGCTTCGGGGAGCGGT sequence (SEQ ID 18); and a probe corresponding to the species Lactobacillus gasseri AAGGGCGCATGGTGAATGCCT sequence (SEQ ID 19)

11) A reagent mixture according to claim 4, characterized in that the mixture of component B comprises a hybridization probe corresponding to the species Streptococcus salivarius TGGCGAACAGACGATCAACGG sequence (SEQ ID 22); and a probe corresponding to the species Streptococcus mutans TGCAGTTAAAGCTCTGCATAAAAGCGG sequence (SEQ ID 21).

12) A reagent mixture according to any one of the preceding claims 3-8 in which the component A contains at least one pair of primers for Enterococcus faecalis and the component B contains at least one probe for Enterococcus faecalis.

13) reagent kit according to any one of the preceding claims 3-9, characterized in that the support for the probes is made up of a microchip / microarray adapted to be introduced into an automated device for the recognition of biological fluids.

14) reagent kit according to any one of the preceding claims which uses oligonucleotides described by means of other methods of DNA amplification and / or hybridization and / or sequencing, for the purpose of identification of a biological fluid through the analysis of sequences of components of the microflora used as markers of the presence of a biological fluid in a sample, which includes but is not limited to, the identification of vaginal fluid from human or animal.

15) Use of genomic DNA or portions thereof belonging to the microflora of the human and animal biological fluids for the purpose of identification of said biological fluid through the analysis of said DNA sequences or portions thereof.

16) Use according to claim 12 wherein the genomic DNA or portions thereof belonging to a species chosen from: vaginal microflora: Lattobacillus crispatus, Lactobacillus gasseri, Lactobacillus INERS, Actinomyces neuii, Aerococcus christensenii, Atopobium vaginae, Atopobium vaginae, Bacteroides uniformis, Bifidobacterium bifidum, Bifidobacterium longum subsp. longum, Chloroflexi spp., Clostridiales, Dialister spp., Enterococcus faecalis, Enterococcus faecium, Escherichia coli, Finegoldia magna, Gardnerella vaginalis, Lactobacillus acidophilus phage kc005, kc007 phage Lactobacillus acidophilus, Lactobacillus acidophilus phage kc012, kc021 phage Lactobacillus acidophilus, Lactobacillus casei, coleohominis Lactobacillus, Lactobacillus coleohominis, Lactobacillus crispatus, Lactobacillus delbrueckii phage kc023, kc031 Lactobacillus delbrueckii phage, phage kc039 Lactobacillus delbrueckii, Lactobacillus fermentum, Lactobacillus fermentum, Lactobacillus gasseri, Lactobacillus helveticus, Lactobacillus INERS, Lactobacillus jensenii, Lactobacillus rhamnosus, Lactobacillus salivarius, Lactobacillus spp , Lactobacillus vaginalis, Leptotrichia spp., Megasphaera spp., Olsenella spp., Pediococcus acidilactici, Peptoniphilus indolicus, Peptostreptococcus anaerobius, Porphyromonas asaccharolytica, Prevotella bivia, Shuttleworthia spp., Staphylococcus capitis, Staphylococcus epidermidis, Staphylococcus haemolyticus, Staphylococcus hominis, Streptobacillus spp. , Streptococcus agalactiae, Streptococcus anginosus, Streptococcus mutans, Streptococcus salivarius, Tricomonas Faetus, Tricomonas vaginalis and mixtures thereof; oral microflora: Streptococcus salivarius, Streptococcus mutans, Acanthamoeba castellanii, Acanthamoeba culbertsoni, Acanthamoeba Acanthamoeba polyphaga odontolyticus, Actinomyces naeslundii, Actinomyces spp., Bifidobacterium adolescentis, Bifidobacterium longum, Bifidobacterium dentium, Campylobacter gracilis, Dialister invisus, Eikenella corrodens, Eikenella corrodens, Enterococcus faecalis, nodatum Eubacterium, Fusobacterium nucleatum, Gemella Haemolysans, Gemella sanguinis, Giardia lamblia, Granulicatella adiacens, Granulicatella elegans, Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus crispatus Lactobacillus delbrueckii, Lactobacillus fermentum, Lactobacillus gallinarum, Lactobacillus gasseri, Lactobacillus paracasei, Lactobacillus salivarius, Lactobacillus

ultunensis, Lactobacillus rhamnosus, Naegleria fowleri, Olsenella uli, Porphyromonas gingivalis, Porphyromonas micra, Porphyromonas endodontalis, Prevotella baroniae, Prevotella denticola, Prevotella spp. , Pseudoramibacter spp., dentocariosa Rothia, Streptococcus gordonii, Streptococcus intermedius, Streptococcus oralis, Streptococcus salivarius, Streptococcus sanguinis, Streptococcus sobrinus, Scardovia wiggsiae, Selenomonas noxia, Selenomonas spp., Streptococcus mutans, Streptococcus-specific phages, Tannerella forsythia, Tetratricomonas buccalis, TM7 phylum, Treponema denticola, Treponema denticola, Tricomonas tenax, Veillonella spp. and mixtures thereof; intestinal microflora: E. coli, E faecalis, Acinetobacter baumannii, Actinomyces meyeri, Actinomyces radingae, urogenitalis Actinomyces, Aerococcus christensenii, Aerococcus viridans, Agrobacterium radiobacter, Alloscardovia omnicolens, Anaerococcus tetradius, Anaerococcus vaginalis, Atopobium vaginae, Bacteriofagoides lambda, M13 Bacteriofagoides, Bacteroides uniformis, Balantidium coli, Balantidium coli, Bifidobacterium bifidum, Bifidobacterium breve, Bifidobacterium longum subsp. infantis, Bifidobacterium longum subsp. longum, Chilomastix mesnili, Corynebacterium accolens, Cryptosporidium parvum, Cryptosporidium parvum, Cyclospora cayetanensis, Dientamoeba fragilis, Embadomonas intestinalis, Encephalitozoon intestinalis, Endolimax nana, Entamoeba chattoni, Entamoeba coli, Entamoeba dispar, Entamoeba hartmanni, Entamoeba histolytica, Entamoeba moshkovskii, Enterobacteria phage, Enterobacteria phage P2, Enterococcus avium, Enterococcus faecalis, Enterococcus faecium, Enterocytozoon bieneusi, Enteromonas hominis, Escherichia coli, Finegoldia magna, Fusobacterium gonidioformans, Gardnerella vaginalis, Giardia lamblia, Iodamoeba butschlii, Isospora belli, Isospora belli, Klebsiella planticola, Lactobacillus casei, Lactobacillus crispatus, Lactobacillus gasseri, Lactobacillus helveticus, Lactobacillus INERS, Lactobacillus jensenii, Lactobacillus mucosae, Lactobacillus rhamnosus, Lactobacillus salivarius, Peptoniphilus indolicus, Peptostreptococcus anaerobius, Peptostreptococcus sp., Prevotella bivia, Pseudomonas stutzeri, Sarcocystis spp., Staphylococcus aureus, Staphylococcus capitis, Staphylococcus epidermidis, Staphylococcus haemolyticus, Staphylococcus hominis, Staphylococcus warneri, streptococcal phage C1 Streptococcus agalactiae, Streptococcus anginosus, Streptococcus bovis, Streptococcus intermedius, Streptococcus mitis group, Streptococcus parasanguinis, Streptococcus salivarius, Trichomonas hominis, Trichomonas vaginalis, Varibaculum cambriense, Weissella and related paramesenteroides mixtures; nasal microflora: accolens Corynebacterium, Corynebacterium mucifaciens, pseudiptheriticum Corynebacterium, Mycobacterium fallax, Orthomyxovirus, Propionibacterium acnes, Rinoviriadeae, Streptococcus mitis, and mixtures thereof; microorganisms found on the skin: Aspergillus, Aureobasidium, Brevibacteriaceae, Candida, Corynebacteriaceae, Dermabacteraceae, Epidermophyton, Leptosphaerulina , Micrococcaceae, Myrothecium, Moraxellaceae, Staphylococcus hominis, Staphylococcus capitis, Staphylococcus epidermidis, Streptococcus epidermidis, Streptococcus hominis, Trichophyton, Xanthomonadaceae and mixtures thereof; blood such as that of the Bos Taurus; and mixtures thereof.

FIG. I

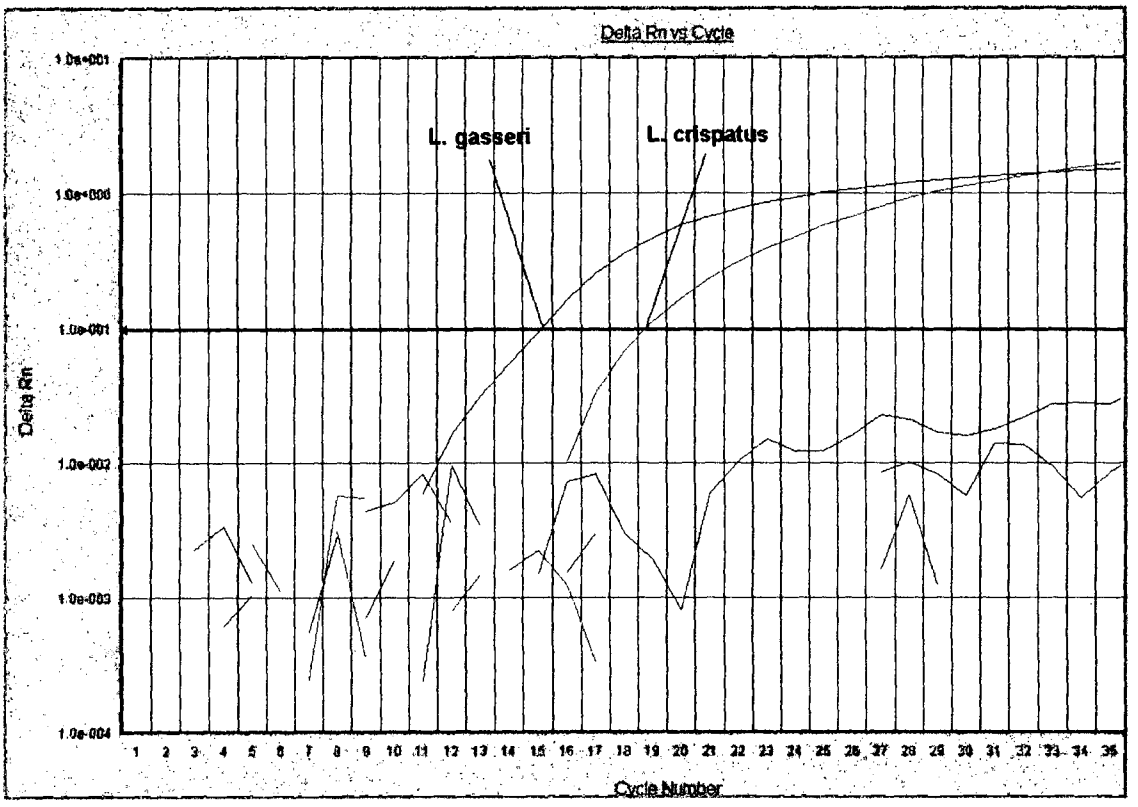
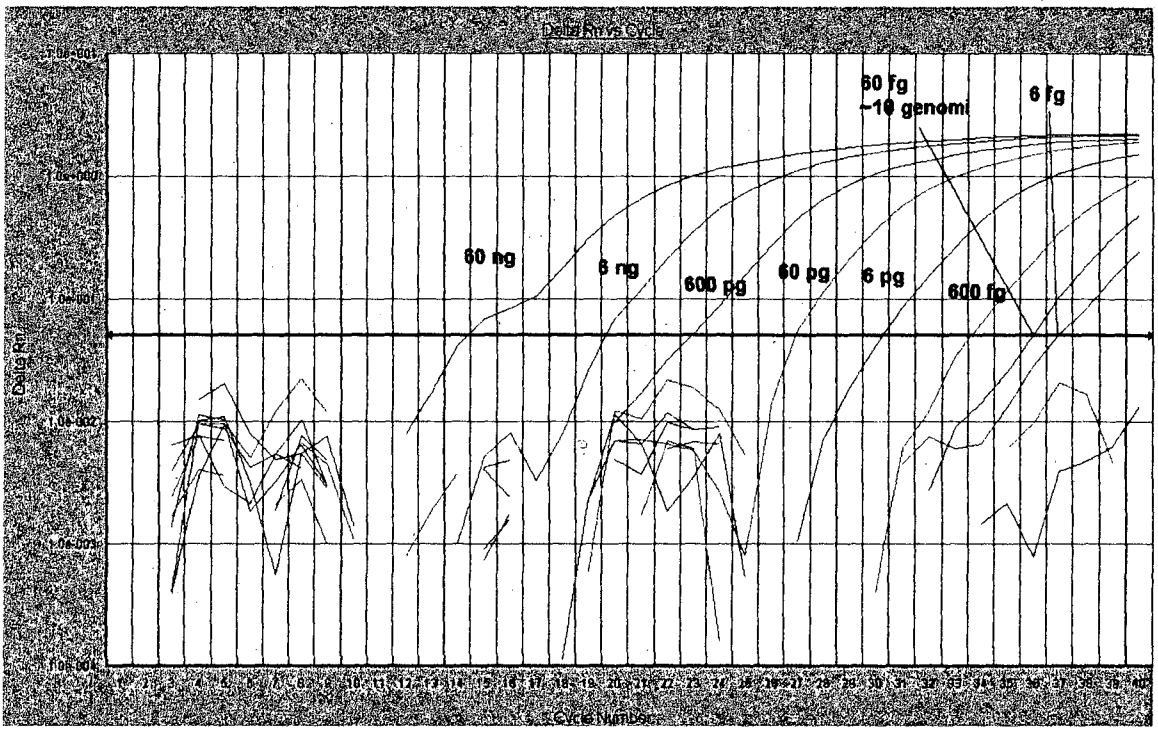

Delta Rn vs Cycle
L. gasseri
L. crispatus
Delta Rn
Cycle Number

FIG. II


Delta Rn vs Cycle
60 fg
~10 genom
6 fg
60 ng
6 ng
600 pg
60 pg
6 pg
600 fg
Delta Rn
Cycle Number

INTERNATIONAL SEARCH REPORT

International application No
PCT/IT2012/000061

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, Sequence Search, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NAKANISHI H ET AL: "A novel method for the identification of saliva by detecting oral streptococci using PCR", FORENSIC SCIENCE INTERNATIONAL, ELSEVIER SCIENTIFIC PUBLISHERS IRELAND LTD, IE, vol. 183, no. 1-3, 10 January 2009 (2009-01-10), pages 20-23, XP025868799, ISSN: 0379-0738, DOI: 10.1016/J.FORSCIINT.2008.10.003 [retrieved on 2008-11-04] the whole document ----- -/--	1-4,9, 11,15,16

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 June 2012	15/06/2012

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Santagati, Fabio

INTERNATIONAL SEARCH REPORT

International application No
PCT/IT2012/000061

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	ANDREA E DONALDSON ET AL: "Using oral microbial DNA analysis to identify expirated bloodspatter", INTERNATIONAL JOURNAL OF LEGAL MEDICINE, SPRINGER, BERLIN, DE, vol. 124, no. 6, 17 February 2010 (2010-02-17), pages 569-576, XP019847706, ISSN: 1437-1596 the whole document	1-4,15, 16
X	FLEMING R I ET AL: "The use of bacteria for the identification of vaginal secretions", FORENSIC SCIENCE INTERNATIONAL: GENETICS, ELSEVIER BV, NETHERLANDS, vol. 4, no. 5, 1 October 2010 (2010-10-01) , pages 311-315, XP027299830, ISSN: 1872-4973, DOI: 10.1016/J.FSIGEN.2009.11.008 [retrieved on 2010-09-18] the whole document	1-4,8, 15,16
X	WO 03/095677 A1 (MEDIGENES [KR]; LEE SANG-YUP [KR]; CHANG KYUNG-HEE [KR]; YOO SO-YOUNG) 20 November 2003 (2003-11-20) claims 107, 108	5-7,13, 14
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X	WO 2008/153893 A2 (UNIV NEW YORK SCHOOL MEDICINE [US]; LI YIHONG [US]; CAUFIELD PAGE W [U) 18 December 2008 (2008-12-18) paragraph [0096]; claim 10	5-7,13, 14
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INTERNATIONAL SEARCH REPORT

International application No
PCT/IT2012/000061

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IT2012/000061

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		JP 2006508670 A	16-03-2006
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		WO 2009149072 A2	10-12-2009