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(56) Fremdragne publikationer:
US-A- 5 573 927
US-A1- 2007 109 535
US-A1- 2011 143 332
US-A1- 2012 149 599
US-A1- 2014 377 795
US-A1- 2016 053 295
Anonymous: "Combined Dielectrophoresis-Raman Setup for the Classification of Pathogens Recovered from the Urinary Tract - Analytical Chemistry (ACS Publications)", , 14. Oktober 2013 (2013-10-14), XP055425245, Gefunden im Internet: URL:http://pubs.acs.org/doi/10.1021/ac4021 616 [gefunden am 2017-11-15]
K. MAQUELIN ET AL: "Prospective Study of the Performance of Vibrational Spectroscopies for Rapid Identification of Bacterial and Fungal Pathogens Recovered from Blood Cultures", JOURNAL OF CLINICAL MICROBIOLOGY, Bd. 41, Nr. 1, 1. Januar 2003 (2003-01-01) , Seiten 324-329, XP055345994, US ISSN: 0095-1137, DOI: 10.1128/JCM.41.1.324-329.2003

Fortsættes ...

COMBINED OPTICAL-SPECTROSCOPIC METHOD FOR IDENTIFYING MICROBIAL PATHOGENS

The invention relates to methods for determining a microbial pathogen. In addition, the invention is directed to methods for determining a microbial pathogen and its anti-infective resistance. In particular, the invention relates to a method for determining a bacterium and its antibiotic resistance.

Technological Background

The currently established techniques for phenotypic resistance testing from patient materials can be based on at least two culturing steps, and thus, the analysis result can be available at the earliest 48 hours after sampling. The first culturing step in this process can be to obtain a pure bacterial culture, the second involves resistance testing itself. Common tests for resistance testing can include the agar diffusion test, the E-test and the microdilution test. The identification of antibiotic resistance by Raman spectroscopy was shown for vancomycin-resistant enterococci (see, e.g., Schroeder et al., Scientific Reports, 2015). Further, certain algorithms exist that record the morphology of bacteria under the influence of antibiotics. However, these algorithms require the prior identification of the bacteria and in tests with several clinical isolates showed an error rate of almost 10% (see, e.g., Choi et al., Science Translational Medicine, 2015).

Automated assays likely require larger amounts of biological material to be obtained by overnight culturing. In addition, the diagnosis often takes 8 to 10 h, or in some cases even longer. Newer approaches based on genotypic identification and characterization of antibiotic resistance can be significantly faster and can often be performed directly from patient material, although they require precise knowledge of characteristic nucleic acid sequences and therefore often fail to identify rapidly mutating multi-resistant Gram-negative pathogens.

US 2011/143332 A1 discloses a method and a device for producing a profile that identifies a microorganism based on surface-enhanced Raman scattering (SERS).

Therefore, it is not possible to perform a fast and reliable antibiotic resistance analysis of unidentified microbial pathogens such as bacteria with previous techniques. In order to optimally tailor anti-infective therapy, in particular antibiotic therapy, to the

patient and his microbial pathogen, shorter determination times, in particular of antibiotic resistance, would be desirable. In addition, the use of broad-spectrum antibiotics could be reduced and wrong therapies for unknown resistances avoided.

Abstract of the invention

It can therefore be seen as the object of the invention to provide an improved method for determining a microbial pathogen. In particular, it is preferable to provide a method for a simultaneous determination of a microbial pathogen and its anti-infective resistance. In particular, it can be considered as an object of the invention to provide a method to simultaneously determine a bacterium and its antibiotic resistance.

The object of the invention is achieved by means of the method of independent claim 1. Further advantages and developments are given in the subclaims.

In general, the present invention relates to a method for determining a microbial pathogen comprising the steps of Raman spectroscopy of a sample comprising at least one microbial pathogen and optical detection of the sample.

The present invention relates to a method according to the appended claim 1 for determining a microbial pathogen and its anti-infective resistance in a sample comprising the microbial pathogen and an anti-infective agent.

The optical detection can be done by microscopy, holographic detection or dynamic light scattering. In preferred embodiments, the optical detection takes place by microscopy. The microscopic method can be, for example, transmitted light microscopy or fluorescence microscopy. Transmitted light microscopy is used as the preferred microscopic method. When determining the morphology of the microbial pathogen by optical detection, quantitative morphology parameters are determined. Accordingly, the determination of the microbial pathogen is based on the Raman spectrum and the quantitative morphology parameters of the microbial pathogen.

By combining of the optical-morphological determination and the Raman spectroscopic determination, a fast and reliable determination of the microbial pathogen can be facilitated. The identification of the microbial pathogen and, if necessary, its anti-infective resistance can be performed using an automated analysis, e.g., using multivariable statistical methods and comparison with databases that are available locally or centrally.

This can facilitate the determination of the microbial pathogens and their antibiotic resistance in a short time using a small number of microbial pathogens. Within a very short time and with a small number of pathogens, the resistance level and/or the minimum inhibitory concentration of an anti-infective agent for the (to be determined) microbial pathogen can be determined. On the basis of the information obtained, the therapy can be specifically adapted to the specific pathogen.

The minimum inhibition concentration can be the concentration at which a threshold value (e.g., band ratio in the Raman spectra or certain extent of morphological change or certain reduction of bacterial growth compared to the untreated sample) is not reached or not exceeded. By comparing the minimum inhibitory concentration determined in this manner with the sensitivity limit values (e.g., according to the EUCAST guideline), the unknown microbial pathogens to be characterized can be classified in the resistance levels, e.g., sensitive, intermediate or resistant.

The terms “determining”, “determination” and “identification” may be used interchangeably in the present application.

A preferred embodiment relates to a method for determining the microbial pathogen and its anti-infective resistance comprising the steps of Raman spectroscopy of a sample comprising at least one microbial pathogen and an anti-infective and optical detection of the sample, and determination of the anti-infective resistance based on the Raman spectrum and the morphology of the microbial pathogen.

In a specific embodiment, the method further comprises the following step:

- determining the cell growth of the microbial pathogen by optical detection,

wherein the determination of anti-infective resistance can be based on the determined Raman spectrum, the determined morphology and the determined cell growth of the microbial pathogen.

When determining the anti-infective resistance of the microbial pathogen, a first indicator of the resistance of the microbial pathogen to the anti-infective agent can be used, wherein the first indicator of the resistance of the microbial pathogen to the anti-infective agent can be or include an unchanged cell morphology of the microbial pathogen compared to negative control data. Further, when determining the anti-infective resistance of the microbial pathogen, a second indicator of the resistance of the microbial pathogen to the anti-infective agent can be used, wherein the second indicator for the

resistance of the microbial pathogen to the anti-infective agent can be or include an unchanged Raman spectrum of the microbial pathogen or a Raman spectrum of the microbial pathogen characteristically altered for a particular resistance mechanism, as compared to negative control data. In addition, a third indicator of resistance of the microbial pathogen to the anti-infective agent can be used, each determining the anti-infective resistance of the microbial pathogen, wherein the third indicator of resistance of the microbial pathogen to the anti-infective agent is an unchanged or slightly unchanged cell growth of the microbial pathogen compared to negative control data.

The negative control data may be data from a sample containing the microbial pathogen without anti-infective agent. The data of this sample may already be available, e.g., as data of a sample which has already been analyzed at an earlier stage according to the exemplary method. Alternatively, the sample can be analyzed with the microbial pathogen without anti-infective agent in parallel to the sample with the anti-infective agent. In another embodiment, the negative control data can originate from the same sample before it has come into contact with the anti-infective.

In particular, the present application relates to a method for determining a microbial pathogen and its anti-infective resistance in a sample comprising the microbial pathogen and an anti-infective agent, comprising the following steps: cultivating the sample comprising at least one microbial pathogen and an anti-infective, determining the Raman spectrum of the microbial pathogen by Raman spectroscopy of the sample under at least two different anti-infective conditions, and determining the morphology of the microbial pathogen by optical detection of the sample under at least two different anti-infective conditions.

In particular, the present application relates to a method for determining a microbial pathogen and its anti-infective resistance, comprising the following steps: cultivating a sample comprising at least one microbial pathogen and one anti-infective; using Raman spectroscopy of the sample at at least two time points during cultivating the sample; and utilizing an optical detection of the sample at at least two time points during cultivating the sample.

"Under at least two different anti-infective agents conditions" in context of the invention means that the sample is exposed to either a different anti-infective concentration and/or a different duration of action of the anti-infective. In one exemplary embodiment, the sample can be divided into two or more aliquots (parts), with a different

concentration of anti-infective added to each aliquot. This can indicate that no anti-infective is added to one unit of the sample while a certain concentration of anti-infective is added to another unit. After the duration of action of the anti-infective, the measurement can take place at any time point after the anti-infective has taken effect. The anti-infective's duration of action or persistency can be, e.g., about 5 min, 10 min, 15 min, 20 min, 30 min, 40 min, 45 min, 50 min, 60 min, 70 min, 80 min, 90 min, 120 min or longer. The person skilled in the art can certainly understand and determine the duration of action of anti-infectives.

In another embodiment, the Raman spectrum and the morphology of the sample can be determined at at least two time points. Wherein at least one earlier time at which the anti-infective has not yet been added or at which the anti-infective has not yet been able to take effect, and at least one later time at which the anti-infective has taken effect is measured.

In one embodiment, the anti-infective is not yet added to the earlier time point. Alternatively, the anti-infective can be added at the earlier time point, and can have a shorter duration of action to the sample at the earlier time point than at the later time point. The earlier time for determining the Raman spectrum and morphology of the sample can be, for example, about 5 min after anti-infective addition or earlier, preferably about 3 min after anti-infective addition or earlier, more preferably about 2 min after anti-infective addition or earlier, particularly preferably about 1 min after anti-infective addition or earlier or before anti-infective addition. The subsequent time point in time can be, for example, after approx. 10 min, 15 min, 20 min, 30 min, 40 min, 45 min, 50 min, 60 min, 70 min, 80 min, 90 min, 120 min or longer after anti-infective addition.

The combination of optical-morphological and Raman spectroscopic analyses can facilitate the simultaneous identification of the microbial pathogen, as well as the determination of anti-infective resistance in one test. In addition, the combined exemplary method can facilitate a faster and at the same time more sensitive resistance testing. The method can be relatively easy to parallelize, and can therefore be used in routine diagnostics for testing several anti-infectives (resistogram), in particular antibiotics. For this method, small amounts of microbial pathogens, such as, e.g., 100-1000 bacteria per test, can be sufficient. Therefore, time-consuming cultivation and isolation steps can be avoided.

The combination of optical detection and Raman spectroscopy may serve to locate

the microorganisms, and both techniques can also provide important information for the determination of the microbial pathogen and/or its antibiotic resistance. This can mean that not only data from Raman spectroscopy, but also morphological parameters determined by optical detection are used to determine the microbial pathogen and/or its antibiotic resistance. Optical detection can therefore not only used to determine the local position of the microbial pathogen.

In particular, the application relates to methods for determining a microbial pathogen and its anti-infective resistance in a sample comprising the microbial pathogen and an anti-infective, comprising the following steps: cultivating the sample comprising at least one microbial pathogen and one anti-infective, determining the Raman spectrum of the microbial pathogen by Raman spectroscopy of the sample under at least two different anti-infective conditions, determining the morphology of the microbial pathogen by optical detection of the sample under at least two different anti-infective conditions, determining the microbial pathogen based on the Raman spectrum and the morphology of the microbial pathogen, and determining the anti-infective resistance of the microbial pathogen based on the Raman spectrum and the morphology of the microbial pathogen.

In particular, the application relates to methods for determining a bacterium and its antibiotic resistance, comprising the following steps: culturing a sample comprising at least one bacterium and an antibiotic; using Raman spectroscopy of the sample at at least two time points during cultivating the sample; and utilizing the optical detection of the sample at at least two time points during cultivating the sample. In particular, using such exemplary embodiments, it is possible to identify a multi-resistant Gram-negative bacterium and its antibiotic resistance. Therefore, the exemplary methods of the present disclosure can be more beneficial than molecular biological methods based on the determination of certain resistance genes. The prior methods have not yet been able to reliably detect the multi-resistant Gram-negative pathogens (MRGN) with their high genetic variability that are currently on the increase. Further, using the exemplary embodiments of the present disclosure, it is possible to identify a fungus and of determining its antifungal resistance.

Typically, Raman spectroscopy takes place at single cell level. This can mean that Raman spectroscopy can be carried out on individual microbial pathogens after a wide field scan in which the individual microbial pathogens can be detected by image recognition algorithms. Alternatively or in addition, Raman spectroscopy can be

performed on several bacteria in the image field simultaneously. For the identification of mixed infections, segregation algorithms can be used.

Optical detection can be used both in the wide field to determine the number of microbial pathogens in the sample and at the single cell level to determine the cell morphology of the microbial pathogen. In order to be able to reliably detect the morphology of the microbial pathogen in the fluid, inhomogeneous electric fields can be used to align the dielectric micro-organisms in the E-field. This exemplary orientation can reduce the random arrangement of micro-organisms. In particular, for non-round pathogens (e.g., rods) this can be of interest by simplifying the data analysis, and reducing the number of microorganisms at least optically detected for a certain accuracy. In one embodiment, the Raman spectrum can be measured at different time points during cultivation, and thus information about the biochemical fingerprint of the microbial pathogen is obtained. This can facilitate the determination of the effect of the anti-infective to be determined, i.e. a successful inhibition/killing by the anti-infective or induction of various resistance mechanisms. Thus, the exemplary combination of the Raman spectroscopy procedure and an optical-morphological analysis can facilitate the effective determination of the microorganism (identification), the determination of the number of microbial pathogens in the sample, the determination of whether resistance is present and the mechanism of action of antibiotic resistance.

In the current state of the art, there is no procedure that can provide this information within a short time (a few hours) from the smallest sample material (few bacteria in suspension).

In specific embodiments, the method may additionally be performed with a control sample containing at least the one microbial pathogen but no anti-infective, e.g., an antibiotic. For example, the procedure can also be performed with several samples containing at least one microbial pathogen and a different anti-infective such as an antibiotic. Alternatively or additionally, the exemplary method can be performed in several samples, each containing at least one microbial pathogen and one anti-infective in a different concentration, in order to obtain quantitative information on the minimum inhibitory concentration (MIC). In addition, an antibiotic-sensitive test strain can be used as an additional control sample. The same antibiotic compounds can be added to the samples with the antibiotic-sensitive test strain in the same concentrations as in the samples with the microbial pathogen to be tested.

The optical detection can take place at the beginning of cultivation and at intervals of not more than about 60 minutes, preferably not more than about 30 minutes, more preferably not more than about 15 minutes, and particularly preferably not more than about 5 minutes.

Raman spectroscopy can be performed at the beginning of cultivation and at intervals of maximum 60 minutes, preferably maximum 30 minutes.

By means of the method of the invention, the determination of the microbial pathogen and its anti-infective resistance can be completed within about 180 minutes, preferably about 150 minutes, more preferably less than about 120 minutes, and most preferably less than about 60 minutes.

Typically, the method relates to the identification of pathogenic microbial pathogens. Therefore, the sample can be obtained from an individual, preferably a mammal, particularly preferably a human. The sample can be a body fluid such as a urine sample or a microcolony transferred into a medium.

For the methods according to the invention, only 50 to 1000, preferably 100 to 1000, microbial pathogens are required in one sample. Typically, a sample contains $5x10^1$ to $1x10^5$ preferably $1x10^2$ to $1x10^5$, more preferably $5x10^2$ to $1x10^5$ microbial pathogens. In further exemplary embodiments, the sample can contain $1x10^3$ to $1x10^4$ microbial pathogens.

In one embodiment, an electric inhomogeneous field can be generated in the sample chamber.

Another aspect of the application relates to a system for determining a microbial pathogen, comprising

- a Raman spectrometer to provide a Raman spectrum of the microbial pathogen,
- a device for optical detection of the microbial pathogen to provide optical data of the microbial pathogen.

wherein the system can be arranged to determine the morphology of the microbial pathogen by analysing the optical data and the microbial pathogen based on a combination of the Raman spectrum and the morphological data of the microbial pathogen.

In a specific embodiment, the system can also be configured to determine anti-

infective resistance on a combination of the specific Raman spectrum and the specific morphology of the microbial pathogen. By using different anti-infective concentrations, the minimum inhibitory concentration of the pathogen can also be determined. For this exemplary purpose, anti-infective concentrations around the clinical breakpoint (e.g., from EUCAST guidelines) can be used.

In one embodiment, the system can additionally comprise a device which can generate an inhomogeneous electric field in a sample to be determined. Such device can include electrodes, for example. The field strength can be individually adapted to the sample, the chamber and the electrode geometry, and can be between about 2 and 60 V. If there is no active movement for heat dissipation and the sample chamber material is poorly heat conductive, about 16 V should not be exceeded to avoid heating effects.

Another aspect of the application relates to a program element for determining a pathogen which, when executed on a processor, instructs the processor to perform the following steps:

- determining a morphology of the microbial pathogen based on the data of a device for optical detection of the pathogen, and
- determining the microbial pathogen based on a Raman spectrum of the microbial pathogen and the morphology of the microbial pathogen.

In one embodiment, the program element directs the processor to further perform the following step:

- determining the anti-infective resistance of the microbial pathogen based on the Raman spectrum and the morphology of the microbial pathogen.

Another aspect of the invention relates to a computer-accessible medium on which a program element is stored which, when executed on a processor, instructs the processor to perform the following steps:

- determining a Raman spectrum of the microbial pathogen based on spectroscopic data from a Raman spectrometer,
- determining a morphology of the microbial pathogen based on the data of a device for optical detection of the pathogen, and
- determining the microbial pathogen based on the Raman spectrum and the morphology of the microbial pathogen.

In one embodiment, the computer-readable medium can further instruct the processor to perform the following step: determining the anti-infective resistance of the microbial pathogen based on the Raman spectrum and the morphology of the microbial pathogen.

In order to facilitate the optical recording of morphology, the sample chamber may contain electrodes, which can generate an inhomogeneous electric field and thus exert a force on the microorganisms contained in the sample. By this force microbial pathogens are driven into a certain orientation and/or enriched on certain regions of the measuring chip. The anti-infective can have an influence on the polarizability of the microorganism. The effect of an electric field on the sample can thus contribute to an enrichment in the detection field, as well as to a differentiation of the microorganism.

Description of Preferred Embodiments

It should be noted that "comprising" does not exclude other elements or steps, and "a/an" or "one" does not exclude a plurality. Furthermore, it should be pointed out that features or steps that are described with reference to one of the exemplary embodiments can also be used in combination with other features or steps of other described exemplary embodiments.

In particular, it should be noted that the methods for determining a microbial pathogen also relate to the determination of several microbial pathogens, i.e. that, for example, several different pathogens are contained in one sample or several samples with different pathogens are examined in parallel. Likewise, more than one anti-infective can be added to a sample or several samples with anti-infectives can be examined in parallel. Combinations of several pathogens and anti-infectives are of course also intended.

The present application relates to a method for determining the microbial pathogen comprising the steps of Raman spectroscopy of a sample comprising at least one microbial pathogen and optical detection of the sample.

A preferred embodiment relates to a method for determining the microbial pathogen and its anti-infective resistance comprising the steps of Raman spectroscopy of a sample comprising at least one microbial pathogen and an anti-infective and optical detection of the sample.

A specific embodiment relates to a method for determining the microbial pathogen and its anti-infective resistance, comprising the following steps:

- cultivating a sample comprising at least one microbial pathogen and an anti-infective agent;
- Raman spectroscopy of the sample at at least two times during cultivating the sample;
- optical detection of the sample at at least two times during cultivating the sample;

Particularly preferred embodiments relate to methods for determining a microbial pathogen and its anti-infective resistance, comprising the following steps:

- cultivating a sample comprising at least one microbial pathogen and an anti-infective agent;
- determining the Raman spectrum of the microbial pathogen by Raman spectroscopy at at least two times during cultivating the sample;
- determining the morphology of the microbial pathogen by optical detection at at least two times during cultivating the sample;
- determining the microbial pathogen and its anti-infective resistance based on the Raman spectrum and the morphology of the microbial pathogen.

Another embodiment relates to a method for determining a microbial pathogen and its anti-infective resistance, comprising the following steps:

- dividing the sample comprising at least one microbial pathogen into at least two aliquots;
- adding different concentrations of the anti-infective to the individual aliquots;
- cultivating the aliquots;
- determining the Raman spectrum of the microbial pathogen by Raman spectroscopy;
- determining the morphology of the microbial pathogen by optical detection;
- determining the microbial pathogen and its anti-infective resistance based on the Raman spectrum and the morphology of the microbial pathogen.

Typically, the determination of the Raman spectrum and the determination of the morphology typically take place after the application time anti-infective agent has acted for a period of time.

The concentrations used are preferably in the range of the limit value for sensitivity and resistance. For this purpose, the person skilled in the art can, for example, use the breakpoints in the breakpoint table of the *European Committee on Antimicrobial Susceptibility Testing* (EUCAST) as a guide (http://eucast.org/clinical_breakpoints/). For example, the concentration used can be 0.1 times, 0.2 times, 0.3 times, 0.4 times, 0.5 times, 0.6 times, 0.7 times, 0.8 times, 0.9 times, 1 time, 2 times, 4 times, 8 times or more than 8 times the EUCAST breaking point. In one embodiment, the used concentration is 4 times the EUCAST breaking point.

As an internal quality control, the samples can also be examined at the time before the addition of the anti-infective or directly after the addition of the anti-infective, also with the aid of Raman spectroscopy and optical analysis. The term "microbial pathogens" includes microorganisms such as bacteria, archaea and fungi. In particular, the term relates to microorganisms that are pathogenic for animals, preferably mammals, particularly preferably humans.

In preferred embodiments, the bacterium can be a multi-resistant gram-negative bacterium. Examples of multi-resistant Gram-negative bacteria are Enterobacteriaceae, e.g. *Escherichia coli, Klebsiella pneumoniae, Klebsiella oxytoca, Proteus spp., Enterobacter spp., Pseudomonas aeruginosa and Acinetobacter baumannii*.

The term "anti-infective" can cover compounds that act against microorganisms, i.e., kill the microorganisms or inhibit their growth. The term includes, for example, antibiotics (against bacteria), antivirals (against viruses), antifungals (against fungal) and anthelmintics (against worms). In particular, the term "anti-infective" covers antibiotics and antifungals. Anti-infectives and antibiotics include β-lactams, glycopeptides, polyketides, macrolide antibiotics, aminoglycoside antibiotics, polypeptide antibiotics, quinolones (and fluoroquinolones) and sulfonamides. Antibiotics work for example by inhibiting cell wall synthesis (e.g. penicillin), inhibiting protein biosynthesis (e.g., kanamycin, neomycin and chloramphenicol), inhibiting correct nucleic acid polymerization (e.g., rifampicin and ciprofloxacin).

Therefore, the methods of the invention can be suitable for the determination of bacteria and their antibiotic resistance. Thus, a sample comprising at least one bacterium

and one antibiotic can be subjected to Raman spectroscopy and optical detection. In addition, the methods according to the exemplary embodiments of the present disclosure can be suitable for the determination of fungal and their antifungal resistance, wherein a sample comprises at least one fungus and one antifungal is subjected to Raman spectroscopy and optical detection.

An anti-infective resistant microorganism can be used for attenuating or completely neutralizing the growth-inhibiting or killing effect. It is possible to use different strategies for this purpose, e.g., modify the target structure of the anti-infective so that the drug can no longer bind, synthesize enzymes that degrade the anti-infective and render it ineffective, synthesize alternative proteins that take over the function of the protein inhibited by the anti-infective or open up alternative metabolic pathways, the inhibited molecule in such a large excess that the anti-infective concentrations present can only block a part of the target structures and the rest is still present, changes in the membrane structure so that the substances do not enter the cell in the first place (reduced uptake) or can be actively pumped out again (e.g., Efflux pumps). Such exemplary changes lead to the fact that the microorganism can continue to grow despite the presence of the anti-infective (resistance), but there are also some morphological and biochemical changes that can be detected optically as well as by Raman spectroscopy. The above-described resistance mechanisms can occur either individually or in combination. By using electrical fields, the changes may be made more easily visible.

Preferred embodiments relate to methods for determining a bacterium and its antibiotic resistance, comprising the following steps:

- cultivating a sample comprising at least one bacterium and one antibiotic;
- Raman spectroscopy of the sample at at least two times during cultivating the sample;
- optical detection of the sample at at least two times during cultivating the sample.

Particularly preferred embodiments relate to methods for determining a bacterium and its antibiotic resistance, comprising the following steps:

- cultivating a sample comprising at least one bacterium and one antibiotic;
- determining the Raman spectrum of the bacterium by Raman spectroscopy at at least two times during cultivating the sample;

- determining the morphology of the bacterium by optical detection at at least two times during cultivating the sample;
- determining the bacterium and its antibiotic resistance based on the Raman spectrum and the morphology of the bacterium.

Alternative embodiments relate to methods for determining a fungus and its antifungal resistance, comprising the following steps:

- cultivating a sample comprising at least one fungus and an antifungal agent;
- Raman spectroscopy of the sample at at least two times during cultivating the sample;
- optical detection of the sample at at least two times during cultivating the sample.

Particularly preferred embodiments relate to methods for determining a fungus and its antimycotic resistance, comprising the following steps:

- cultivating of a sample comprising at least one fungus and an antifungal agent;
- determining the Raman spectrum of the fungus by Raman spectroscopy at at least two times during cultivating the sample;
- determining the morphology of the fungus by optical detection at at least two times during cultivating the sample;
- determining the fungus and its antifungal resistance based on the Raman spectrum and the morphology of the fungus.

The time intervals between the at least two time points can be defined elsewhere. In certain exemplary embodiments, at least at the first time point (when optical detection and/or Raman spectroscopy takes place) the sample is not yet mixed with the anti-infective. The anti-infective is added before the next time point, for example the second time point (at which optical detection and/or Raman spectroscopy takes place).

An unchanged cell morphology of the microbial pathogen compared to negative control data can be an indicator of resistance of the microbial pathogen to the anti-infective. In addition, unchanged cell growth of the microbial pathogen compared to negative control data may be an indicator of resistance of the microbial pathogen to the

anti-infective. In addition, an unchanged Raman spectrum of the microbial pathogen or a Raman spectrum characteristically altered for a particular resistance mechanism compared to negative control data may be an indicator of the resistance of the microbial pathogen to the anti-infective. In other words, in determination of the anti-infective resistance of the microbial pathogen, a first indicator of the resistance of the microbial pathogen to the anti-infective agent may be used, wherein the first indicator of the resistance of the microbial pathogen to the anti-infective agent is an unchanged or resistance mechanism characteristic change in the cell morphology of the microbial pathogen compared to negative control data. In addition, in determining the anti-infective resistance of the microbial pathogen, a second indicator of the resistance of the microbial pathogen to the anti-infective agent may be used, wherein the second indicator of the resistance of the microbial pathogen to the anti-infective agent is an unchanged Raman spectrum of the microbial pathogen or a Raman spectrum of the microbial pathogen characteristically altered for a particular resistance mechanism compared to negative control data. The term "a Raman spectrum characteristically altered for a particular resistance mechanism" can include characteristic spectral changes known to the person skilled in the art. For certain resistance mechanisms a changed amino acid composition of the bacterium can be detected (e.g. VanA, VanB, VanC, VanD, VanE, VanG, especially VanA, VanB and in particular of vancomycin resistance), in the case of other resistance mechanisms an increased production of certain enzymes or the substances cleaved by these enzymes can be detected, e.g. in the case of β-lactam resistance an increased production of β-lactamases or an increased hydrolysis of the β-lactam ring of the β-lactam antibiotics used.

In addition, in determining the anti-infective resistance of the microbial pathogen, a third indicator of the resistance of the microbial pathogen to the anti-infective agent can be used, wherein the third indicator of the resistance of the microbial pathogen to the anti-infective agent is an unchanged cell growth of the microbial pathogen compared to negative control data.

The term "unaltered growth", as used herein, can also include slightly reduced cell growth. This means that cell growth is reduced by less than 10%, less than 5%, less than 3% or less than 1% compared to negative control data. Cell growth is usually determined by observing the cell number over time, i.e. the growth rate. Cell growth can also be detected morphologically, e.g. by counting cells with a morphology typical for cell division.

Conversely, an altered cell morphology of the microbial pathogen compared to negative control data may be an indicator of sensitivity of the microbial pathogen to the anti-infective. In particular, changes in cell morphology that are characteristic of the sensitivity of the microbial pathogen must be taken into account. In certain exemplary embodiments, minor initial shape changes can therefore be neglected. The morphological changes are often characteristic for certain classes of antibiotics. In addition, altered cell growth of the microbial pathogen compared to negative control data may be an indicator of sensitivity of the microbial pathogen to the anti-infective. In addition, a Raman spectrum of the microbial pathogen characteristically altered for sensitivity to the anti-infective agent compared to negative control data may be an indicator for the sensitivity of the microbial pathogen to the anti-infective agent.

The negative control data can originate from a sample containing the microbial pathogen without anti-infective agent. The data of this sample may already be available, e.g. as data of a sample which has already been analysed at an earlier stage according to the exemplary embodiment of the present disclosure. Alternatively, the sample can be analysed with the microbial pathogen without anti-infective agent in parallel to the sample with the anti-infective agent. In another exemplary embodiments, the negative control data may originate from the same sample before it comes into contact with the anti-infective. In addition, the negative control data could come from a sample of an anti-infective resistant strain with or without anti-infective agent, in particular from a sample of an anti-infective resistant strain.

A sample of an anti-infective with a microbial pathogen that is sensitive to this anti-infective (comparison pathogen) could serve as a positive control.

The reference pathogen is preferred from the same species, particularly preferably from the same type of pathogen to be examined. The genus and type of the unknown microbial pathogen can, for example, be determined directly in the method described here. An unchanged cell morphology of the microbial pathogen in comparison to the positive control data can then be an indicator of a sensitivity of the microbial pathogen to the anti-infective agent. Furthermore, unchanged cell growth of the microbial pathogen compared to the positive control data can be an indicator of the sensitivity of the microbial pathogen to the anti-infective agent. In addition, an unchanged Raman spectrum of the microbial pathogen compared to the positive control data can be an indicator of the sensitivity of the microbial pathogen to the anti-infective agent.

Optical detection can be performed by microscopy, holographic detection or dynamic light scattering, preferably microscopy. Typically, microscopy is light microscopy. The term "light microscopy" is familiar to person skilled in the art and includes, among others, transmitted light microscopy, reflected light microscopy, fluorescence microscopy, holography, interference contrast microscopy, polarization microscopy and confocal microscopy or combinations thereof. In preferred embodiments, transmitted light microscopy is used for optical detection. In an exemplary embodiment, a confocal microscope can be used in a transmitted light mode.

Therefore, Raman spectrometers with integrated optical detection devices, in particular microscopes such as transmitted light microscopes or confocal microscopes, can be suitable for carrying out the exemplary methods of the present disclosure. In particular, Raman spectrometers with integrated transmitted light and/or fluorescence microscopes (e.g. alpha300 from WiTEC, Ulm, Germany, inVia confocal Raman microscope from RENISHAW, United Kingdom or XploRA™PLUS from HORIBA) are particularly suitable. Such transmitted light and/or fluorescence microscope can be or include a confocal microscope. It can also be advantageous if a white light source is integrated for optical detection. A laser source is required in particular for Raman spectroscopy. The Raman spectrometer can have a detector, a spectrometer and usually at least one filter.

A lens with a high numerical aperture, i.e., with a numerical aperture greater than or equal to 0.8 and preferably greater than or equal to 0.9, is used for single cell analysis. Alternatively, individual microorganisms can be locally enriched by the use of inhomogeneous electric fields (e.g. by negative dielectrophoresis accumulation of bacteria in the middle of the sample vessel). Subsequently, Raman spectroscopy can be used to obtain mean value spectra of all microbial pathogens in focus.

Raman spectroscopic methods for the determination of anti-infective resistances are known to persons skilled in the art (see, e.g. Schroeder et al.). In Raman spectroscopy, a sample can be excited by monochromatic light (e.g. laser). This also produces inelastically scattered light on the sample, the frequency of which differs from that of the stimulating light. These frequency differences, the so-called Raman shift, contain information about the oscillation states of the molecules and thus about the chemical composition of the microorganism.

Typically, Raman spectroscopy determination is performed at single cell level. For

this purpose, for example, a wide-field image of the microhole chamber is taken in white light. The individual bacteria are detected by an image recognition algorithm which is coupled to the hardware of the device. These can be approached automatically, for example by moving the sample stage or by moving the optics. The Raman measurement can then be performed on a single bacterium. The signal is optimized in the Z-focus plane. In the pure medium, the effect of optical traps can also be exploited to achieve an optimal focus. With optimal focusing, the background fluorescence of the medium hardly plays a role. Wavelength-modulated excitation can also be used for robust data analysis. If the signal of a single microorganism is too weak, these can also be enriched at certain points in the sample vessel, e.g. over the measuring range (e.g. by using inhomogeneous electrical fields). Accumulation can then take place at previously defined points in the sample vessel, which can be approached automatically. For the optical analysis of the number and morphology, all microorganisms visible in the image field of the microscope are recorded and used for the evaluation. For Raman spectroscopic analysis, the microorganisms accumulated by the effect of the electric field are used. The electrode structure must be adapted to the size of the microorganisms to be examined.

The sample vessel can be the well of a multiwell plate, a perforated chamber, a microcontainer, or the sample area of a measuring chip without restriction.

Optical detection can be used to determine the cell count, growth rate and/or cell morphology of the microbial pathogen in the sample. This makes it possible, for example, to detect morphological changes caused by the influence of the anti-infective. In addition, the optional use of inhomogeneous electric fields makes it easier to visualize changes in polarizability and can thus be used for faster detection of resistances. The directional orientation, which can be achieved by applying an electric field, can also reduce the scattering of the determined optical parameters. This may shorten the duration of the determination of anti-infective resistance.

The term “morphology” refers to parameters that describe the shape of the microbial pathogen, e.g. size, roundness, area, ratio of area to perimeter, ratio of longest extension (length) to shortest extension (width). Preferred parameters are size, roundness and area. These parameters or parameters derived therefrom (e.g. parameters of Zernike polynomials) can be extracted from the data of the optical detection by means of automatic image recognition algorithms.

To determine the quantitative information on the morphological change,

microscopy is performed at single-cell level. For this purpose, a wide-field image in white light is first taken at each time interval and the various bacteria are automatically recognised and characterised by appropriate image recognition algorithms (see above).

Anti-infective resistance is determined by optical detection and Raman spectroscopy. This can mean that the optical detection data, in particular the microscopy image data and the Raman spectra, are used for the evaluation.

Using holographic approaches or dynamic light scattering, averaged data can be captured on the image section. Particularly with holographic approaches, the characteristics of the individual microbes can also be determined by data evaluation.

In addition, the growth rate of the microbial pathogens can be determined. For this purpose, wide-field images are taken at different time points during the cultivation of the sample. Automatic image recognition algorithms determine the number of microorganisms in the sample. The growth rate is determined by the change in the number of micro-organisms over time in the sample.

The determination of the microbial pathogen and, if necessary, its anti-infective resistance is carried out by automated analysis using multivariate statistical methods, such as, e.g., main component analysis, neural networks or support vector machines and various correlation algorithms. These automated analysis methods can be trained with image data and Raman spectra of different microorganisms (especially bacteria) with different anti-infectives (especially antibiotics). The optical and spectral data are independent, but reflect the same changes in the microorganisms. This increases the accuracy by combining the information. This allows shorter analysis times to be achieved with high accuracy. For the combined evaluation of the spectral information and the image data, two approaches are possible: a quantitative parameters (e.g. number of microbial pathogens, roundness, size, form factor (aspect ratio), etc.) are determined from the image data and added to the Raman data (intensity over wave numbers) for multivariate statistical evaluation; and b. From the Raman data, marker bands are identified by statistical evaluation (specific to the mechanism of action of the anti-infective used) and from the relative intensities of the marker bands individual variables are generated, which can be evaluated together with the quantitative parameters from the optical analysis in a mixed statistical model.

Typically the microorganisms (e.g., bacteria) are used as pure suspension in the exemplary methods of the present disclosure. For this purpose, the microorganisms (e.g.,

bacteria) are introduced into the chambers of a microwell plate intended for analysis as an aliquot of the suspension. Since the same volume and the same concentration are always required for all chambers, filling can be carried out automatically by appropriate devices. Gravity causes the microorganisms (e.g. bacteria) to sink to the ground. Alternatively, the microorganisms could also be embedded in a suitable embedding medium, e.g., agarose. The corresponding diffusion times of the anti-infective agent must be took into consideration. Alternatively, the pathogen suspension (e.g. bacterial suspension) can be passed through a suitable microfluidic plate.

The terms “microhole plate” and “microwell plate” can be used interchangeably and refer to titer plates with a filling volume of 0.1 µl to 2000 µl, preferably 0.5 µl to 1000 µl, more preferably 1 µl to 500 µl, even more preferably 2 µl to 200 µl, most preferably 5 µl to 100 µl, for example 10 µl. Microhole plates are usually made of plastic. Preferred are micro hole plates with a glass bottom. The bottom may have a thin coating of agarose or poly-L-lysine. This can favour the accumulation of bacteria near the glass bottom. If electrodes are to be used (to generate an inhomogeneous electric field), they are also applied to the bottom plate. An arrangement in the walls of the perforated chambers is also conceivable for the application described here. In preferred designs, perforated chamber plates are used which do not influence the growth of the microbial pathogens. By the use of micro hole plates only little bacterial material is needed. Micro hole plates are inexpensive and therefore suitable for low-cost routine diagnostic applications. The bottom of the chamber of the hole plate should be wetted by the pathogen suspension. The size of the chamber can be adapted to the image field of the microscope used.

For cultivating the microorganisms, media are suitable which are suitable for culturing a broad spectrum of microorganisms, for example heart-brain broth (typical composition: calf s brain infusion 12.5 g/l; bovine heart infusion 5.0 g/l; proteose peptone 10.0 g/l; glucose 2.0 g/l; sodium chloride 5.0 g/l; disodium hydrogen phosphate 2.5 g/l; pH 7.4 ± 0.2; e.g. from Carl Roth, Germany).

The microbial pathogens are cultivated at a temperature that is as optimal as possible for the cell division of the pathogen. As a rule, cultivation takes place between 25°C and 39°C, preferably between 30°C and 38°C, particularly at 37°C For continuous temperature maintenance, a sample chamber can be used which is set to the desired temperature, e.g. 37°C Since Raman spectroscopy and optical detection are performed parallel to cultivation, the sample chamber on or around the Raman spectrometer with

integrated optical detection device is arranged in the device so that the sample can be kept at the desired temperature during Raman spectroscopy and optical detection. The temperature fuse can be either local (e.g. by heating foil) or global (includes microhole plate and parts of the optical structure). The light cable can be conducted through optical fibres.

In a particular embodiment, the device has two sample chambers at different temperatures. For example, one sample chamber can be set to 30 °C and the second sample chamber to 37 °C. The sample is first introduced into one of the two chambers, for example the chamber at 37 °C, and the microbial pathogens are identified by optical detection and/or Raman spectroscopy. If the identified pathogen is known to grow better at 30 °C, the micro-perforated plate is inserted into the second chamber. The introduction of the sample into the chamber can take place automatically. Alternatively, a sample chamber could be used and the temperature of the sample chamber could be adjusted to the optimal growth temperature of the pathogen after the identification of the microbial pathogen. In a further embodiment, two samples, which are identical in terms of the microbial pathogen and (if applicable) the antibiotic, are cultivated in parallel in two chambers with different temperatures, e.g. in one chamber heated to 37 °C and one to 30 °C.

The described method can basically be used for all anti-infectives (especially antibiotics). For example, a pure sample without anti-infective additive or a pure sample without anti-infective resistance is used as control. Alternatively, comparative data records can be used that are already available for the corresponding pathogen (e.g., comparable pathogen of the same species) in combination with the corresponding anti-infectives. In one embodiment, comparative data sets are used in which the same anti-infective agent is added to the sample to be tested, but the microbial pathogen differs from the sample to be tested. The anti-infective may already be lyophilized in the chambers of the hole plate. Depending on the origin of the sample material (blood culture, respiratory tract, urinary tract etc.) an appropriate cartridge can be selected and filled.

The anti-infective is dosed into the chambers after Raman spectroscopic identification of the germs. For this purpose, storage vessels with injection connections, for example, can be built into the device. In this version, it can be recommended to run a test strain with known sensitivity in a row of chambers in order to simultaneously control

the effectiveness of the anti-infective.

In a specific embodiment, a large amount of the bacterial suspension (sufficient for the required antibiotic testing plus control) is poured into a central chamber. After Raman spectroscopic identification of the pathogens in the device, a micro hole plate is selected, which contains the relevant antibiotics in the relevant concentrations, and the bacterial suspension is passed microfluidically into the corresponding chambers.

In one embodiment, the method can be performed with at least one control sample. This control sample may contain at least one microbial pathogen without anti-infective agent. Thereby the growth rate and cell morphology of the sample(s) comprising the microbial pathogen and an anti-infective can be compared with the growth rate and cell morphology of the control sample(s) comprising the microbial pathogen without the anti-infective.

In addition, the exemplary method may be performed in a sample comprising an anti-infective-sensitive test strain.

In a further exemplary embodiment, the method can be performed on several samples, each containing a microbial pathogen and a different anti-infective. Since the exemplary procedure of the present disclosure can be parallelized, it is thus possible to simultaneously test the resistance of the microbial pathogen against several anti-infectives. This is due to the increasing incidence of multi-resistant microbial pathogens, especially multi-resistant bacteria (e.g., methicillin-resistant *Staphylococcus aureus* (MRSA), multi-resistant *Clostridium difficile* but also multi-resistant Gram-negative bacteria (MRGN) such as multiresistant *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, or Enterobacteria such as *Escherichia coli* and *Klebsiella pneumoniae*) of enormous importance.

In another embodiment, the method can be carried out on several samples, each containing a microbial pathogen and an anti-infective agent in different concentrations. This makes it easy to determine the minimum inhibitory concentration.

It is clear, that the present invention can also be suitable for the parallel analysis of different anti-infectives in different concentrations.

In specific embodiments, the optical detection takes place at the beginning of cultivation and at intervals not exceeding 120 minutes, preferably 60 minutes, particularly preferably not more than 30 minutes, more preferably not more than 15 minutes, even more preferably not more than 5 minutes. Raman spectroscopy shall be carried out at the

beginning of cultivation and at intervals not exceeding 120 minutes, preferably not exceeding 60 minutes, particularly preferably not exceeding 30 minutes.

By means of the method of the invention, the determination of the microbial pathogen and its anti-infective resistance can be completed within 180 minutes, preferably 150 minutes, more preferably 120 minutes, especially preferably 60 minutes. Applied technologies for determining the genus and the type of the microbial pathogen can be Raman spectroscopy and for determining the senility of anti-infective a combination of Raman spectroscopy and optical analysis can be used. Identification and resistance determination can be carried out in the same device. No further sample pretreatment steps are necessary. The required sample material is minimal.

Typically the method relates to the identification of pathogenic microbial pathogens. Therefore, the sample is taken from an individual, preferably a mammal, particularly preferably a human. The sample may be a body fluid, e.g. a urine sample, or a microcolony transferred into a medium. The urine sample is typically filtered using a filter that separates impurities but allows microbial pathogens to pass through. Complex samples, which usually contain many components, such as blood samples, or also colonizing flora, such as aspirating material, are, as established in microbiological laboratories and known by person skilled in the art, spread out and cultivated for a short time, e.g. less than 6 h, preferably less than 4 h, more preferably less than 3 h, especially preferably less than 2 h. Since only little bacterial material is required, short incubation times of approx. 2-3 h are sufficient to obtain enough sample material. Alternatively, microfluidic purification steps are also conceivable, which make the microbial pathogens directly accessible from the complex patient material for further analysis with the exemplary embodiments described herein. Such a pre-purification can also be directly coupled to the titer plate system described here.

For the methods according to the exemplary embodiments, only 50 to 1000, preferably 100 to 1000, microbial pathogens are required in one sample. Typically a sample contains $5x10^1$ to $1x10^5$ preferably $1x10^2$ to $1x10^5$, more preferably $5x10^2$ to $1x10^5$ microbial pathogens. In particularly preferred designs, the sample contains $1x10^3$ to $1x10^4$ microbial pathogens. Since only a small number of pathogens is necessary both for the determination of the pathogens and for the determination of their anti-infective resistance, one or more preculture steps, which are necessary in the state of the art methods, can be resigned. As these pre-culture steps usually take between 6 and 24

hours, the result can be achieved much faster in the present method after sampling. The duration of the exemplary procedure for determining the pathogens and/or their anti-infective resistance may be reduced accordingly. Thus, the determination of the microbial pathogen and its anti-infective resistance is possible within 180 minutes, preferably 150 minutes, especially preferred 120 minutes after sampling.

A specific embodiment relates to a method for determining the microbial pathogen and its anti-infective resistance, comprising the following steps:

- cultivating a sample comprising at least one microbial pathogen and an anti-infective agent;
- a cultivation of a control sample comprising at least one microbial pathogenwithout anti-infective agent;
- a Raman spectroscopy of the samples at at least two time points during cultivating the sample; and
- an optical detection of the samples at at least two time points during cultivating the sample.

Another aspect relates to a system for determining a microbial pathogen, comprising:

- Raman spectrometer to provide spectroscopic data of the microbial pathogen; and
- device for optical detection of the microbial pathogen to provide optical data of the microbial pathogen,

wherein the system is arranged to determine the morphology of the microbial pathogen by analysing the optical data and to determine the microbial pathogen based on a combination of the Raman spectrum and the morphological data of the microbial pathogen.

A embodiment relates to a system for determining a microbial pathogen, comprising:

- Raman spectrometer to provide spectroscopic data of the microbial pathogen; and
- device for optical detection of the microbial pathogen to provide optical data of the microbial pathogen,

wherein the system is arranged to process a Raman spectrum of the microbial pathogen, to determine the morphology of the microbial pathogen by analysis of the optical data, and to determine the microbial pathogen based on a combination of the Raman spectrum and the morphological data of the microbial pathogen.

In a specific embodiment, the system can also be configured to determine anti-infective resistance based on a combination of the specific Raman spectrum and the specific morphology of the microbial pathogen.

Another aspect relates to a program element for determining a pathogen that, when executed on a processor, configures the processor to perform the following steps:

- determining a morphology of the microbial pathogen based on the data of a device for optical detection of the microbial pathogen, and
- determining the microbial pathogen based on a Raman spectrum and the morphology of the microbial pathogen.

Another embodiment relates to a program element for determining a pathogen that, when executed on a processor, configures the processor to perform the following steps:

- processing of a Raman spectrum of the microbial pathogen,
- determining a morphology of the microbial pathogen based on the data of a device for optical detection of the microbial pathogen, and
- determining the microbial pathogen based on the processed Raman spectrum and the morphology of the microbial pathogen.

The term “processing of a Raman spectrum” covers common methods known to person skilled in the art for signal optimization of the Raman spectrum, such as background correction, normalization or wave number correction.

In one embodiment, the program element instructs the processor to also perform the following step:

- determining the anti-infective resistance of the microbial pathogen based on the Raman spectrum and the morphology of the microbial pathogen after interaction with the anti-infective agent.

The program element can be part of a computer program, but it can also be independent. For example, the program element can be used to update an already existing program element.

Another aspect relates to a computer-readable medium on which a program element is stored which, when executed on a processor, instructs the processor to perform the following steps:

- determining a morphology of the microbial pathogen based on the data of a device for optical detection of the pathogen, and
- determining the microbial pathogen based on the Raman spectrum and the morphology of the microbial pathogen.

Another embodiment relates to a computer-readable medium on which a program element is stored which, when executed on a processor, instructs the processor to perform the following steps:

- processing of a Raman spectrum of the microbial pathogen,
- determining a morphology of the microbial pathogen based on the data of a device for optical detection of the pathogen, and
- determining the microbial pathogen based on the processed Raman spectrum and the morphology of the microbial pathogen.

In one embodiment, the computer-readable medium can further instruct the processor to perform the following step: determining the anti-infective resistance of the microbial pathogen based on the Raman spectrum and the morphology of the microbial pathogen.

The computer-readable medium can be viewed as a storage medium, e.g. a USB stick, CD or DVD, data storage device, hard disk or any other medium on which a program element as described above can be stored.

Exemplary Embodiment 1

- Preparation of a bacterial suspension and introduction into chambers of a microhole chamber plate (filling volume of chamber ≤ 100 µl, aliquot 10 µl): One microhole chamber contains no antibiotic, the other microhole chambers contain one or

different antibiotics.

- Raman spectroscopic identification of the pathogens by recording individual spectra of several individual pathogens in the measuring chamber and comparing the spectra with a database for determining the type of bacteria: Using appropriate image recognition algorithms, which are coupled to the hardware of the device, different bacteria can be automatically approached. Incubation of the bacteria at 37°C

- Tracking the growth of bacteria with the aid of white light microscopy and subsequent image evaluation: A wide-field image of each hole chamber is taken every approx. 15 minutes. Image recognition algorithms provide quantitative information about morphological changes. In the evaluation, this information is evaluated relative to the control without antibiotic.

- Raman spectroscopic characterization: After 60 and 120 minutes, Raman spectroscopic characterization is performed on approximately 10-20 bacteria in the individual chambers. By analysing the Raman spectra, quantitative information about the biochemical fingerprint, i.e. information about the biochemical composition of the bacteria, is obtained. In the evaluation, this information is determined relative to the control without antibiotic. Based on the image information, the bacteria are automatically approached by the automated procedure of the sample stage.

- Automated analysis of antibiotic resistance by integrating data on growth curves, quantitative information on morphological changes, and Raman spectroscopic characterization: Spectral changes of bacterial Raman spectra in the antibiotic-added chambers are compared with those in the control chamber without antibiotic addition. If the changes characteristic of the mechanism of action of the antibiotic occur, this is an indicator of sensitivity. If no differences are detectable or the differences characteristic of a particular resistance mechanism are detectable, this is an indicator of resistance. The comparison is made by statistical evaluation of the (pretreated) Raman spectra using multivariate methods (e.g. main component analysis, linear discriminant analysis, support vector analysis or others). Similarly, indicators of resistance and sensitivity are obtained from the analysis of growth characteristics and morphological changes. These three indicators are used with the aid of a weighted statistical procedure to make statements about the resistance or sensitivity of the examined bacterium to the various antibiotics.

Optical detection is performed on a Raman spectrometer with integrated confocal

microscope with white light illumination (e.g. inVia confocal Raman microscope from RENISHAW, United Kingdom or XploRA™PLUS from HORIBA).

Exemplary Embodiment 2

- Introduction of aliquots of a bacterial suspension into chambers of a microhole chamber plate (filling volume of the chamber ≤ 100 µl, aliquot 10 µl). Each chamber contains a different concentration of an antibiotic whose resistance is to be tested (concentration range: 0 µg/ml to 4-fold breakpoint according to EUCAST). The chambers are equipped with electrodes that enable the generation of an electric field.
- Switching on the electric field. The bacteria are concentrated at a defined point in the perforated chamber. The Raman spectroscopic identification of the pathogens is carried out by recording Raman spectra at the defined position in the measuring chip and comparing the spectra with a database to determine the type of bacteria. The following incubation of the bacteria at 37 degrees centigrade can take place without the generation of an electric field.
- Tracking the growth of bacteria with the aid of white light microscopy and subsequent image evaluation: A wide-field image is taken in each perforated chamber every approx. 5-15 minutes. Image recognition algorithms acquire quantitative information about morphological changes. For enrichment and alignment of the bacteria (especially interesting for rod-shaped bacteria) the electric field can be switched on immediately before the microscope image is taken. The evaluation includes the dynamic change of the optical parameters (and the polarizability, if applicable). As a control both time 0 min and the aliquot without antibiotic are used.
- Raman spectroscopic characterization: After 30, 60 and 90 minutes, Raman spectroscopic characterization takes place within the bacterial cloud enriched by the electric field. Several spectra are recorded in each measuring chamber. By analysing the Raman spectra, quantitative information about the biochemical fingerprint, i.e. information about the biochemical composition of the bacteria, is obtained. Depending on the antibiotic, the evaluation may include the formation of band ratios or multivariate statistical methods compared to the control without antibiotic.
- Automated analysis of antibiotic resistance and determination of the minimum inhibitor concentration: For this purpose, both image information and Raman

data are evaluated together for each antibiotic concentration. This includes information on bacterial growth, quantitative parameters of morphology as well as characteristic Raman bands. For each concentration of antibiotics, it is determined whether the concentration of antibiotics added was sufficient to inhibit bacterial growth or not. The minimum inhibition concentration is the concentration at which a threshold value (e.g. band ratio in the Raman spectra or certain extent of morphological change or certain reduction of bacterial growth compared to the untreated sample) is not reached or not exceeded. The threshold values are characteristic for certain antibiotic classes and duration of action. By comparing the minimum inhibitory concentration determined in this way with the sensitivity limit values (e.g. according to the EUCAST guideline), the unknown bacteria to be characterised are classified in the resistant levels sensitive, intermediate (defined for some bacterial/antibiotic combinations) or resistant.

In the following, an exemplary embodiment of the invention will be discussed in more detail with reference to the accompanying drawing:

FIG. 1: Overview of a system for determining a microbial pathogen

FIG. 1 shows a system for determining a microbial pathogen (1) which has a camera for optical detection. The exciters (5) to be determined are located in a perforated chamber of a hole chamber plate (3) which has a plurality of hole chambers (4). The optical-morphological detection is performed by a camera (2). A white light source (9) illuminates the sample through the objective (8). Laser (6) and dispersive element with detector (7) forming the Raman spectrometer. The excitation light path and the detection light path pass through the objective (8). The detector (7) is used for Raman spectroscopic detection. The calculation unit (10) serves to determine the pathogens and, if applicable, their anti-infective resistance by determining a morphology of the pathogens based on the data of the camera and determining the pathogens (and, if applicable, their anti-infective resistance) based on the Raman spectrum and the morphology of the microbial pathogen.

FIG. 2: 2A. Raman spectra of an E. coli strain after 90 minutes of exposure to different concentrations of Ciprofloxacin. In accordance with classical microbiological methods, the MHK of this strain was determined to 0.032 µg Ciprofloxacin/ml. Thus this strain is sensitive to Ciprofloxacin. 2B. Enlargement of a particular section of FIG. 2A (the order of the spectra for the band ~1485 cm^{-1} follows the concentrations given on the right,

for the Raman band at 1450 cm^{-1} the order is exactly reversed).

FIG. 3: 3A. Raman spectra of another *E. coli* strain after 90 minutes of duration of action to different concentrations of Ciprofloxacin. In accordance with classical microbiological methods, the MHK of this strain was determined to 1 µg Ciprofloxacin/ml. This makes this strain ciprofloxacin-resistant. 3B. Enlargement of a particular section of FIG. 3A (the order of the spectra for the band ~1485 cm^{-1} follows the concentrations given on the right, for the Raman band at 1450 cm^{-1} the order is exactly reversed).

FIG. 4: Morphology of *E. coli* strains without antibiotic treatment (4A and 4C) and 180 min after treatment with piperacillin/tazobactam (4B: sensitive strain; 4D: resistant strain).

Patentkrav

1. Fremgangsmåde til bestemmelse af et mikrobielt patogen og en anti-infektionsmiddelresistens deraf i en prøve omfattende det mikrobielle patogen og et anti-infektionsmiddel, omfattende følgende trin:

- dyrkning af prøven omfattende mindst ét mikrobielt patogen og et anti-infektionsmiddel;
- bestemmelse af det mikrobielle patogens Raman-spektre ved hjælp af Raman-spektroskopi af prøven under mindst to forskellige anti-infektionsmiddel-betingelser,
- bestemmelse af kvantitative morfologiske parametre af det mikrobielle patogen ved optisk detektering af prøven under mindst to forskellige anti-infektionsmiddel-betingelser
- bestemmelse af det mikrobielle patogen baseret på Raman-spektrene og de kvantitative morfologiske parametre af det mikrobielle patogen,
- bestemmelse af det mikrobielle patogens anti-infektionsmiddelresistens baseret på Raman-spektrene og de kvantitative morfologiske parametre af det mikrobielle patogen.

2. Fremgangsmåde ifølge krav 1, hvor de mindst to forskellige anti-infektionsmiddel-betingelser er to forskellige anti-infektionsmiddel-koncentrationer og/eller to forskellige virkningsvarigheder af anti-infektionsmidlet.

3. Fremgangsmåde ifølge et af kravene 1 eller 2, omfattende følgende trin:

- bestemmelse af det mikrobielle patogens Raman-spektre ved hjælp af Raman-spektroskopi af prøven på mindst to tidspunkter under dyrkning af prøven,
- bestemmelse af de kvantitative morfologiske parametre af det mikrobielle patogen ved hjælp af optisk detektering af prøven på mindst to tidspunkter under dyrkning af prøven,
- bestemmelse af det mikrobielle patogen baseret på Raman-spektrene og de kvantitative morfologiske parametre af det mikrobielle patogen,
- bestemmelse af det mikrobielle patogens anti-infektionsmiddelresistens baseret på Raman-spektrene og de kvantitative morfologiske parametre af det mikrobielle patogen.

4. Fremgangsmåde ifølge et af de foregående krav, hvor den optiske detektering sker ved mikroskopi, holografisk detektering eller dynamisk lysspredning, hvor mikroskopien er transmitteret lysmikroskopi eller fluorescensmikroskopi.

5. Fremgangsmåde ifølge et af de foregående krav, yderligere omfattende trinnet:

- bestemmelse af cellevæksten af det mikrobielle patogen ved optisk detektering;

hvor bestemmelsen af anti-infektionsmiddelresistensen er baseret på de bestemte Raman-spektre, de bestemte kvantitative morfologiske parametre og den bestemte cellevækst af det mikrobielle patogen.

6. Fremgangsmåde ifølge et af de foregående krav, hvor det mindst ene mikrobielle patogen er en bakterie, og anti-infektionsmidlet er et antibiotikum.

7. Fremgangsmåde ifølge et af de foregående krav, hvor der ved bestemmelse af det mikrobielle patogens anti-infektionsmiddelresistens anvendes en første indikator for det mikrobielle patogens resistens over for anti-infektionsmidlet, hvor den første indikator for det mikrobielle patogens resistens over for anti-infektionsmidlet er en uændret cellemorfologi af det mikrobielle patogen sammenlignet med negative kontroldata.

8. Fremgangsmåde ifølge krav 7, hvor der ved bestemmelse af det mikrobielle patogens anti-infektionsmiddelresistens anvendes en anden indikator for det mikrobielle patogens resistens over for anti-infektionsmidlet,
hvor den anden indikator for det mikrobielle patogens resistens over for anti-infektionsmidlet er et Raman-spektrum af det mikrobielle patogen, som er uændret, eller som ændres på en måde, som er karakteristisk for en bestemt resistensmekanisme sammenlignet med negative kontroldata, og/eller
hvor der ved bestemmelse af det mikrobielle patogens anti-infektionsmiddelresistens anvendes en tredje indikator for det mikrobielle patogens resistens over for anti-infektionsmidlet,
hvor den tredje indikator for det mikrobielle patogens resistens over for anti-infektionsmidlet er en uændret cellevækst af det mikrobielle patogen

sammenlignet med negative kontroldata.

9. Fremgangsmåde ifølge et af kravene 7 eller 8, hvor de negative kontroldata stammer fra en prøve, som omfatter det mikrobielle patogen uden anti-infektionsmiddel.

10. Fremgangsmåde ifølge et af de foregående krav, hvor fremgangsmåden yderligere udføres med en kontrolprøve indeholdende det mindst ene mikrobielle patogen uden anti-infektionsmiddel, og/eller hvor fremgangsmåden varer mindre end 180 minutter, fortrinsvis mindre end 150 minutter, mere fortrinsvis mindre end 120 minutter, mest fortrinsvis mindre end 60 minutter.

11. Fremgangsmåde ifølge et af de foregående krav, hvor prøven er taget fra et individ, fortrinsvis fra et pattedyr, især fortrinsvis fra et menneske, og/eller indeholder $1*10^2$ til $1*10^5$, fortrinsvis $5*10^3$ til $1*10^4$ mikrobielle patogener.

12. Fremgangsmåde ifølge et af de foregående krav, hvor der i prøven genereres et elektrisk uhomogent felt.

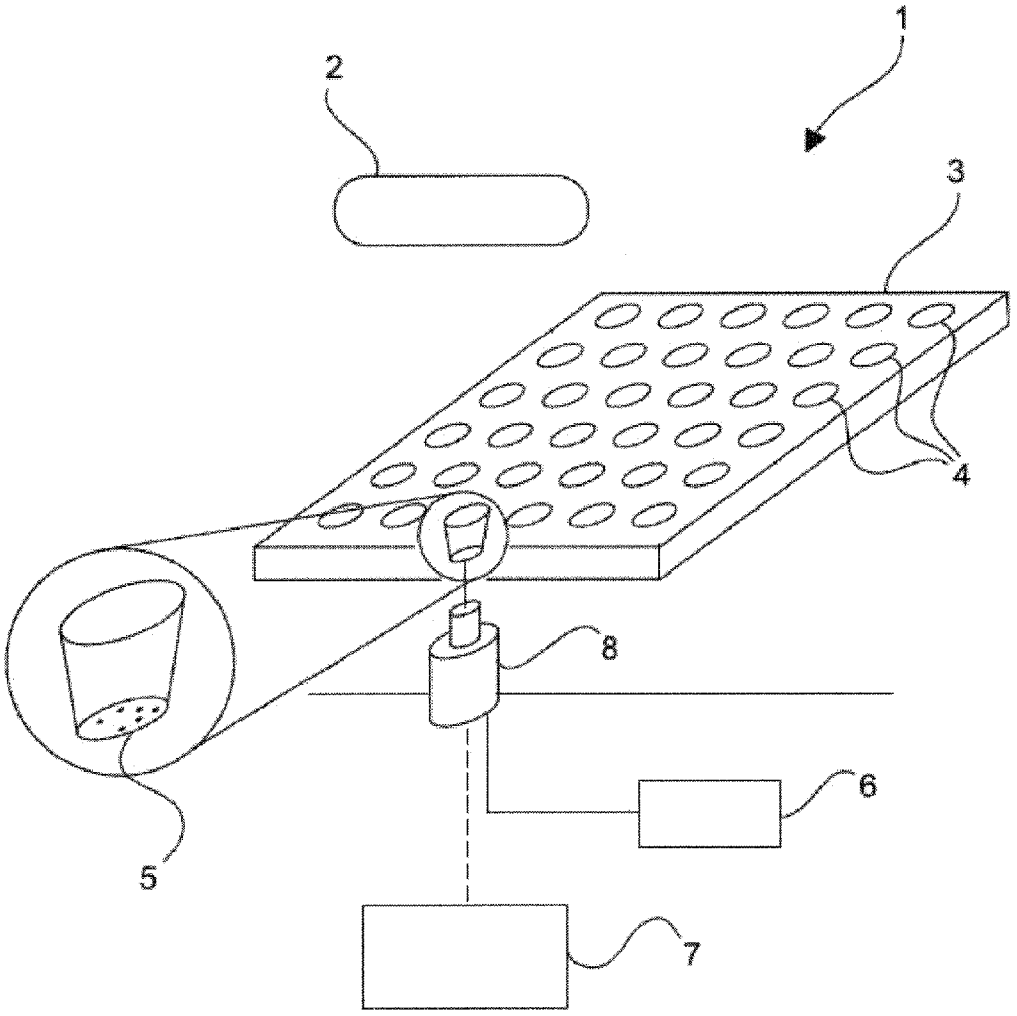
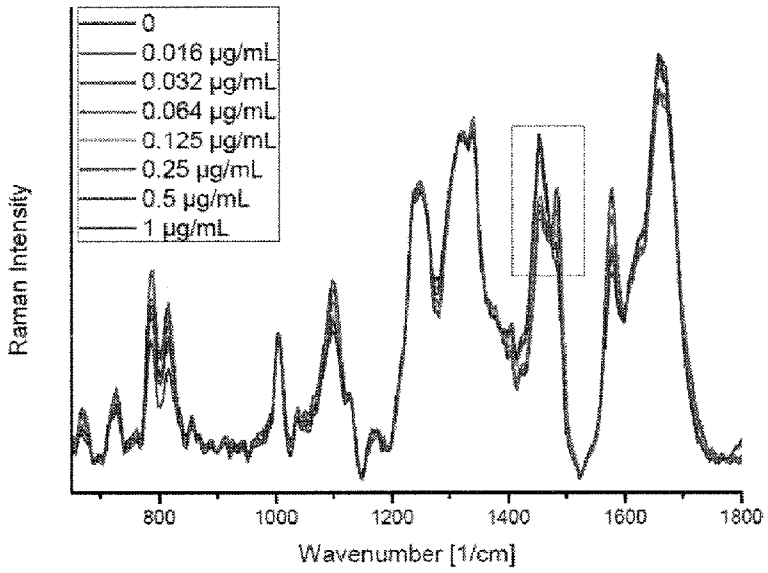


Fig. 1

2A


0
0.016 µg/mL
0.032 µg/mL
0.064 µg/mL
0.125 µg/mL
0.25 µg/mL
0.5 µg/mL
1 µg/mL
Raman Intensity
800
1000
1200
1400
1600
1800
Wavenumber [1/cm]

2B

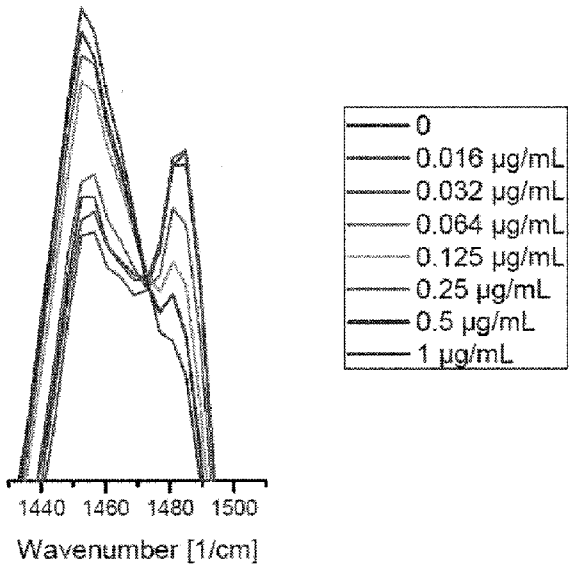
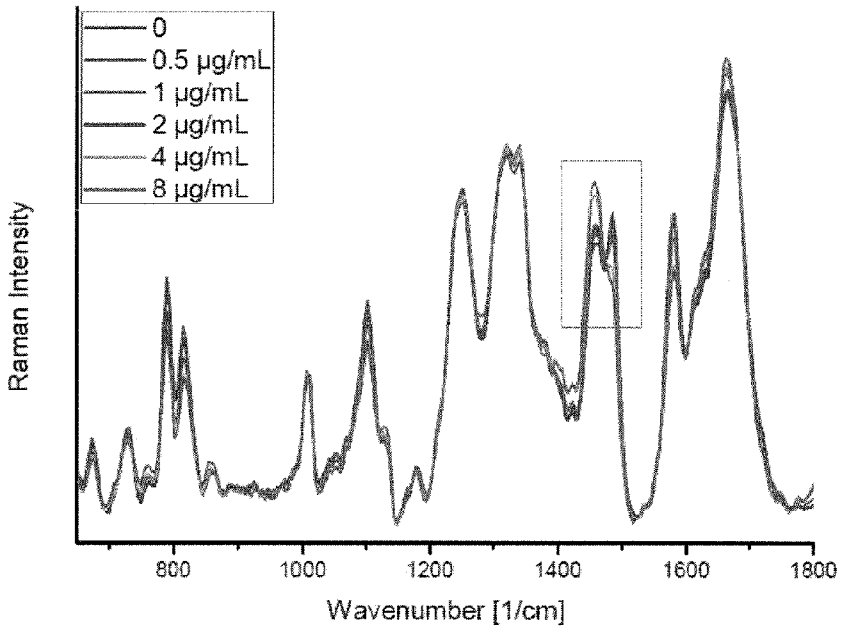

0
0.016 µg/mL
0.032 µg/mL
0.064 µg/mL
0.125 µg/mL
0.25 µg/mL
0.5 µg/mL
1 µg/mL
1440
1460
1480
1500
Wavenumber [1/cm]

Fig. 2

3A


0
0.5 µg/mL
1 µg/mL
2 µg/mL
4 µg/mL
8 µg/mL
Raman Intensity
800
1000
1200
1400
1600
1800
Wavenumber [1/cm]

3B

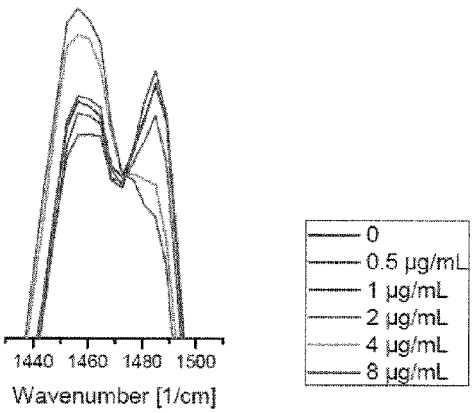
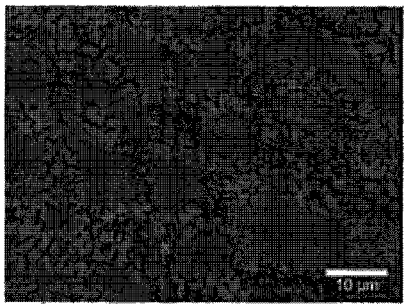
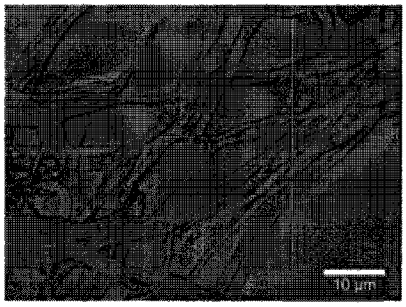

1440
1460
1480
1500
Wavenumber [1/cm]
0
0.5 µg/mL
1 µg/mL
2 µg/mL
4 µg/mL
8 µg/mL

Fig. 3



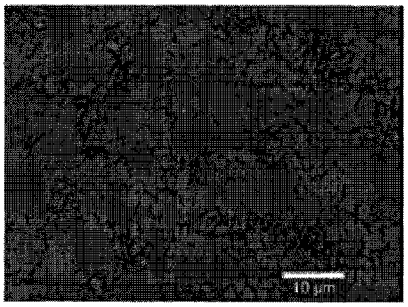
4A

E. coli strain untreated, after 180 min



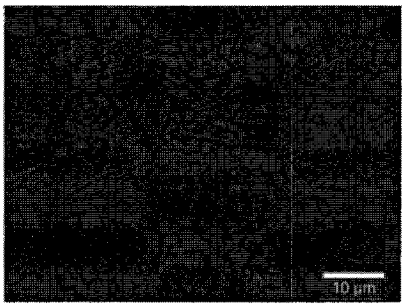
4B

E. coli strain, 180 min treated with piperazillin/ tazobactam



4C

E. coli 180 min, without antibiotics



4D

e. coli with piperazillin/ tazobactam after 180 min --> resistent

Fig. 4