MICROFLUIDIC DROPLET-BASED DETECTION OF HETERORESISTANCE

TECHNICAL FIELD

The present invention generally relates to detection of heteroresistance in a microbial population, and in particular to a method and system for droplet-based detection of heteroresistance.

BACKGROUND

Antibiotic resistance is emerging as a severe threat to human health. To tackle this, innovative diagnostic tools are required for a better understanding of this phenomenon. A single bacterial cell in a population can demonstrate heterogeneity in terms of response to the antibiotic resulting in difficulty in the classification of the bacteria either as susceptible or resistant and thereby difficulty in finalizing the treatment. Heteroresistance is the phenomenon wherein subpopulations of presumed isogenic bacteria show varied antibiotic susceptibilities.

The current gold standard for the determination of heteroresistance is population analysis profiling (PAP). PAP is usually performed by inoculation of a bacterial population onto culture plates, flasks, or tubes containing increasing concentrations of antibiotic, quantifying the bacterial growth at each antibiotic concentration, and, finally, graphical analysis of the results.

Current PAP methods require quite large volumes of fluids, typically in the range of mL up to L, comparatively large bacterial samples and reagents. A further drawback is that the results from PAP methods are generally available first after at least 24 hours of incubation.

There is therefore a need for techniques allowing detection of heteroresistance in a microbial population, such as in a bacterial population, which is not marred by the shortcomings of current PAP methods.

Scheler et al., Droplet-based digital antibiotic susceptibility screen reveals single-cell clonal heteroresistance in an isogenic bacterial population, *Scientific Reports* 10: 32828 (2020) developed a droplet-based digital minimum inhibitory concentration (MIC) screen for quantifying the single-cell distribution of phenotypic responses to antibiotics. Lyu et al., Phenotyping antibiotic resistance with single-cell resolution for the detection of heteroresistance, *Sensors and Actuators B: Chemical* 270: 396-404 (2018) describes a droplet microfluidics method to encapsulate single cells from a population consisting of a mixture of antibiotic-sensitive and antibiotic-resistant bacteria. Co-encapsulating viability probe alamarBlue with the cells allows the use of fluorescent droplets as a read-out for droplets that

contain live cells after their exposure to antibiotics. Enumerating the fluorescent droplets thus gives the number of resistant cells in the population. The method enables the quantitative phenotyping of heterogeneous resistance, or heteroresistance, with single-cell resolution.

SUMMARY

It is a general objective to detect heteroresistance in a microbial population.

It is a particular objective to enable detection of heteroresistance in a microbial population using smaller sample volume and shorter incubation times than PAP-based methods.

These and other objectives are met by embodiments of the present invention.

The present invention is defined in the independent claims. Further embodiments of the invention are defined by the dependent claims.

An aspect of the invention relates to a method of detecting heteroresistance in a microbial population of microbes. The method comprises generating droplets comprising at least one microbe and an antimicrobial agent. The method also comprises incubating the droplets in conditions supporting growth of the microbes in the droplets. The method further comprises monitoring the size of the droplets during incubation and detecting heteroresistance to the antimicrobial agent in the microbial population based on detection of a shrinkage in size of at least one droplet comprising at least one microbe but no shrinkage in size of at least one other droplet comprising at least one microbe.

Another aspect of the invention relates to a system for detecting heteroresistance in a microbial population of microbes. The system comprises a microfluidic device, a camera, a memory and a processor. The microfluidic device comprises a sample inlet configured to receive a sample comprising microbes and an antimicrobial agent in a culture medium, an oil inlet configured to receive an oil sample, a droplet generator in fluid connection with the sample inlet and the oil inlet and configured to generate droplets comprising at least one microbe and the antimicrobial agent in the culture medium, and an incubation chamber in fluid connection with the droplet generator and configured to house a plurality of droplets in conditions supporting growth of the microbes in the droplets. The camera is arranged to take images of the droplets in the incubation chamber. The memory is configured to store program instructions and the processor is configured, when executing the program instructions, to process the at least one image to determine sizes of droplets in the incubation chamber, and detect heteroresistance to the

antimicrobial agent in the microbial population based on detection of a shrinkage in size of at least one droplet comprising at least one microbe but no shrinkage in size of at least one other droplet comprising at least one microbe.

The present invention provides a droplet-based detection of heteroresistance in a microbial population. Accordingly, smaller sample volumes as compared to prior art PAP-based techniques are needed and the detection results can be obtained with much shorter incubation periods as compared to such PAP-based techniques.

BRIEF DESCRIPTION OF THE DRAWINGS

The embodiments, together with further objects and advantages thereof, may best be understood by making reference to the following description taken together with the accompanying drawings, in which:

Fig. 1 is a flow chart illustrating a method of detecting heteroresistance in a microbial population according to an embodiment.

Fig. 2 is a flow chart illustrating an embodiment of the droplet generating step in Fig. 1.

Fig. 3 is a flow chart illustrating an embodiment of the monitoring step in Fig. 1.

Fig. 4 is a flow chart illustrating additional, optional steps of the method shown in Fig. 1 according to an embodiment.

Fig. 5 is a flow chart illustrating additional, optional steps of the method shown in Fig. 1 according to another embodiment.

Fig. 6 is a flow chart illustrating additional, optional steps of the method shown in Fig. 1 according to a further embodiment.

Fig. 7 illustrates *Escherichia coli* MG1655 in MH broth with different colony forming units (CFU) per mL to have droplets with bacterial encapsulation and growth, (7a) 10^4 CFU/mL (7b) 10^5 CFU/mL (7c) 5×10^5 CFU/mL.

Fig. 8 illustrates the effects of susceptible *E. coli* MG1655 against two antibiotics with 10^5 CFU/mL, (8a)

2 mg/L CTX (8b) 0.5 mg/L GEN.

Fig. 9 illustrates the results of spiking susceptible *E. coli* with resistant *E. coli* in the presence of antibiotics to detect the subpopulation of resistant *E. coli.* (9a) *E. coli* MG1655+CTX resistant *E. coli* strain DA62594 with 10^{-3} frequency in the presence of 2 mg/L of CTX, 2.5×10^{7} CFU/mL. (9b) *E. coli* MG1655+CTX resistant *E. coli* strain DA62594 with 10^{-5} frequency in the presence of 2 mg/L of CTX 5×10^{8} CFU/mL. (9c) *E. coli* MG1655+CTX resistant *E. coli* strain DA62594 with 10^{-6} frequency in the presence of 2 mg/L of CTX, 5×10^{8} CFU/mL. (9d) *E. coli* MG1655+GEN resistant *E. coli* strain DA63654 with 10^{-5} frequency in the presence of 4 mg/L of GEN, 5×10^{8} CFU/mL. (9e) *E. coli* MG1655+GEN resistant *E. coli* strain DA63654 with 10^{-6} frequency in the presence of 4 mg/L of GEN, 5×10^{8} CFU/mL. (9f) *E. coli* MG1655+TET resistant *E. coli* strain DA34827 with 10^{-5} frequency in the presence of 5 mg/L of TET, 5×10^{8} CFU/mL. (9g) *E. coli* MG1655+TET resistant *E. coli* strain DA34827 with 10^{-6} frequency in the presence of 5 mg/L of TET, 5×10^{8} CFU/mL.

Fig. 10 illustrates results with clinically heteroresistant strains against CTX and GEN to detect subpopulation of these strains. (10a) *E. coli* DA63082 clinically hetero-resistant strain against CTX. *E. coli* DA63082 was tested experimentally two times (n_1 and n_2) both are plotted side by side. (10b) *E. coli* DA63744 clinically hetero-resistant strain against CTX. *E. coli* DA63744 was tested experimentally two times (n_1 and n_2) both are plotted side by side. (10c) *E. coli* DA63082 clinically hetero-resistant strain against GEN. *E. coli* DA63082 was tested experimentally two times (n_1 and n_2) both are plotted side by side. (10d) *E. coli* DA63340 clinically hetero-resistant strain against GEN. *E. coli* DA63340 was tested experimentally two times (n_1 and n_2) both are plotted side by side.

Fig. 11 is a schematic block diagram of a system for detecting heteroresistance in a microbial population according to an embodiment.

Fig. 12 is a flow chart illustrating a change in size distribution of droplets over time.

Fig. 13 are photographs showing droplet shrinkage associated with bacterial growth with droplet diameter shown at 0 hours, 12 hours and 24 hours for the case of 30 % empty droplets and 70 % droplets with bacteria.

Fig. 14 is a schematic block diagram of a multiplex microfluidic chip according to an embodiment.

Fig. 15 illustrates detection of resistant subpopulations using clinical isolates of *E. coli* against multiple antibiotics using a multiplex microfluidic chip. (15a) DA63333 was non-HR against both CTX and GEN. DA63333 was tested using a multiplex microfluidic chip, the normalized droplet size was plotted against time and only the control experiment showed shrinkage. At the same time, there was no shrinkage and bacterial growth observed in the CTX and GEN droplets. (15b) DA63660 showed HR against CTX and non-HR against GEN. DA63660 was tested using a multiplex microfluidic chip. The normalized droplet size was plotted against time and both the control and CTX experiments showed shrinkage while there was no shrinkage and bacterial growth observed in the GEN droplets. (15c) DA62906 showed HR against GEN and non-HR against CTX. DA62906 was tested using a multiplex microfluidic chip. The normalized droplet size was plotted against time and both the control and GEN experiments showed shrinkage while there was no shrinkage and bacterial growth observed in the CTX droplets. Each strain was tested in two independent experiments (N_1 and N_2) with data plotted side by side demonstrating droplet size reduction caused by the bacterial growth.

Fig. 16 is a schematic showing steps implemented to find out the effect of droplet size on MG1655 growth. (16a) Schematic showing flow-focusing device used for droplet generation, where light mineral oil with 3% PGPR was used as a continuous phase and MG1655 in MH broth was used as a dispersed phase. (16b) Droplets generated were transferred to Eppendorf® tubes for incubation. (16c) Protocols to be implemented after incubation time. (16d) The resulting CFU was determined by growing MG1655 on agar plates incubated at 37°C for 24 hours.

Fig. 17 illustrates the effect of droplet size on MG1655 growth. (17a) CFU of MG1655 plotted against time for various droplet diameters. (17b) Normalized CFU of MG1655 plotted against time for various droplet diameters.

Fig. 18 illustrates detection of resistant subpopulations using clinical isolates of *A. baumannii* (DA33098) with heteroresistance against a single antibiotic Amikacin (8 mg/L) using (18a) image texture parameter homogeneity and (18b) Image texture parameter correlation. In both plots, time in hours is plotted on the X-axis and the texture parameter on the Y-axis.

DETAILED DESCRIPTION

The present invention generally relates to detection of heteroresistance in a microbial population, and in particular to a method and system for droplet-based detection of heteroresistance.

There is an ever-increasing problem of antimicrobial resistance of microorganisms to antimicrobial agents, for instance the emergence of antibiotic-resistant bacteria. Generally, microbial populations can develop heterogeneity in their response to antimicrobial agents. For instance, microorganisms derived from a susceptible population can develop mutations in genes that affect the activity of antimicrobial agents, resulting in preserved survival in the presence of the antimicrobial agent. Alternatively, foreign genetic material can be acquired through horizontal gene transfer to microorganisms resulting in an antimicrobial resistant subpopulation. Heteroresistance occurs when a subpopulation of the microorganisms shows an increased level of resistance compared to the main microbial population.

The present invention enables detection of such heteroresistance in a microbial population using a droplet-based approach. Such droplet-based detection of heteroresistance has significant advantages as compared to the traditional PAP-based techniques used in the art to detect heteroresistance. Firstly, the invention can detect heteroresistance in a microbial population even from very small sample volumes. A drawback of PAP-based techniques is that they require quite large sample volumes, typically in the range of mL up to L. The present invention, however, can use sub-mL samples, such as using sample volumes in the µL to mL range or indeed even smaller volumes. In fact, the droplets generated in the present invention typically have a respective volume in the fL to nL range so the present invention can be used with smaller sample volumes than PAP-based techniques.

Secondly, PAP-based techniques require quite long incubation periods, typically at least 24 hours, before any heteroresistance can be detected. Experimental results as presented herein indicate that heteroresistance can be detected already with merely 12-18 hours of incubation thereby significantly speeding up the heteroresistance detection process as compared to prior art PAP-based techniques.

The sensitivity of the heteroresistance detection of the invention is further very high and is capable of detecting heteroresistance at a frequency of at least 10^{-6} in a microbial population.

The prior art droplet-based microfluidic assays to detect heteroresistance as recited in the background section employ single-cell encapsulation per droplet resulting in a lot of empty droplets, and to detect low subpopulation frequency of $10^{-6,}$ these methods will require around 5 to 6 million droplets along with transfer in between chips. In addition, the methods require labelling of viable cells with fluorescent or colored dyes. Hence, there is a need for a method and system that will reduce the number of droplets required along with no in-between chip transfer steps. As shown herein, the invention could improve the detection limit with just 200 to 300 droplets and have detection on the same chip as droplet generation.

By tracking size changes in hundreds of such droplets, it was possible to identify rare subpopulations (frequency of 10^{-6}) that continued to grow even at antibiotic concentrations inhibiting most cells. This growth was quantified through osmotically induced size changes (5-10 % reduction in droplet size compared to droplet without any growth) in the droplets, driven by the metabolic activity of the growing cells.

Accordingly, an aspect of the invention relates to a method of detecting heteroresistance in a microbial population of microbes, see Fig. 1. The method comprises generating, in step S1, droplets comprising at least one microbe and an antimicrobial agent. The droplets are incubated in step S2 in conditions supporting growth of the microbes in the droplets. The size of the droplets is monitored during incubation in step S3. The method then allows detection, in step S4, of heteroresistance to the antimicrobial agent in the microbial population based on detection of a shrinkage in size of at least one droplet comprising at least one microbe but no shrinkage in size of at least one other droplet comprising at least one microbe.

The present invention thereby utilizes the phenomenon that the size or volume of microbial-containing droplets change, such as decrease, if the microbes are growing inside the droplets. However, the size of droplets containing non-growing microbes or empty droplets, i.e., droplets not containing any microbes, do not change in size or volume, or may indeed, if adjacent to droplets containing growing microbes, increase in size or volume. This decrease in size or volume of droplets containing growing microbes is believed to be due to a change in the chemical composition of the droplets as the growing microbes consume nutrients. This change in chemical composition may result in the mass transfer of water from the droplet with growing microbes to the surroundings, such as into adjacent droplets in contact with the droplet as shown in Fig. 13. Accordingly, droplets with growing microbes will typically shrink in size while adjacent droplets with non-growing microbes or empty droplets can, due to the mass transfer of water, increase in size. This means that the distribution in droplet size will change over time if the microbial population comprises a heteroresistant subpopulation as schematically illustrated in Fig. 12. Thus, the droplets generated in step S1 initially have an initial or starting size distribution as represented by the droplet diameter at 0 hours in Fig. 12. If there are both resistant and susceptible microbes in the microbial population, the droplet size distribution will change over time as the droplets are incubated in step S2. In more detail, droplets comprising resistant microbes will typically shrink in size as the microbes are growing inside the droplets even in the presence of the antimicrobial agent. However, droplets comprising susceptible microbes or empty droplets will not shrink in size or even increase in size. The droplet size distribution will thereby change during incubation as schematically shown in Fig. 12 with one smaller droplet size distribution corresponding to droplets with growing microbes and one larger droplet size

distribution corresponding to droplets with non-growing microbes and empty droplets. In addition, the peak corresponding to smaller droplet size (growing microbes) is generally smaller than the peak corresponding to larger droplet size (non-growing microbes).

"Microbe" or "microorganism" as used herein refers to an organism of microscopic dimensions, which may exist in a single-celled form or as a colony of cells. The microbes have a sufficient small cell size to fit within droplets generated as described herein and are further capable of growing inside the droplets in the absence of any antimicrobial agents. Typical examples of microbes that can be investigated according to the invention include bacteria and unicellular eukaryotes, such as fungi and unicellular protists. In an embodiment, the microbes are bacteria or fungi, preferably bacteria. In a preferred embodiment, the bacteria are Gram negative bacteria.

"Antimicrobial agent" as used herein refers to an agent that kills microbes, also referred to as microbicide, or stops the growth of microbes, also referred to as microbiostatic agent or microbiostat. For instance, antibacterial agents or antibiotics could be used as antimicrobial agent for bacteria, whereas antifungal agents or antifungals could be used as antimicrobial agent for fungi.

In an embodiment, step S1 of Fig. 1 comprises generating droplets comprising at least one microbe and the antimicrobial agent in a culture medium. In this embodiment, the antimicrobial agent is thereby dissolved or dispersed in a culture medium that supports growth of the microbes. The particular culture medium used depends on the type of microbes to be tested. For instance, the culture medium could be a bacterial growth medium if the microbes are bacteria and a fungi growth medium if the microbes are fungi. The culture medium is in liquid form in room temperature (20-25°C) or at the temperature, at which the method is run, such as about 37°C, to enable generation of droplets in step S1.

Illustrative, but non-limiting, examples of culture medium that could be used to generate droplets in step S1 include lysogeny broth (LB), also referred to as Luria-Bertani broth, and Mueller Hinton (MH) broth for bacteria and Sabouraud dextrose media for fungi.

The droplets as generated in step S1 are typically a mixture of droplets of culture medium comprising the antimicrobial agent but no microbes (denoted "empty" droplets herein) and droplets of culture medium comprising the antimicrobial agent and comprising one or multiple, i.e., at least two, microbes. The ratio between so-called empty droplets, i.e., not containing any microbes, and microbe-containing droplets is

at least partly dependent on the number of microbes in the original sample, such as the number of colony forming units (CFUs) per unit volume, such as CFU/mL in the original sample. The higher concentration (CFU/mL) of microbes the higher number of droplets generated in step S1 will contain one or more microbes and the higher the average number of microbes per droplet.

In an embodiment, the microbe-containing droplets, i.e., droplets comprising at least one microbe, constitute less than 75 %, preferably less than 50 % of the droplets generated in step S1. In a preferred embodiment, the majority of the droplets generated in step S1 are empty droplets and the minority of the generated droplets are microbe-containing droplets. As an example, the microbe-containing droplets constitute less than 25 %, preferably less than 10 %, and more preferably less than 5 % of the droplets generated in step S1. Experimental data as presented herein demonstrate that droplet shrinkage (indicating microbe growth) and swelling (indicating no growth) depend at least partly on the number of empty droplets. When only a few droplets contain growing microbes, they are mainly in contact with droplets without microbial growth resulting in maximum shrinkage due to surrounding droplets supporting mass transfer. In contrast, when most droplets contain growing microbes, the size difference between microbe-containing droplets and empty droplets is less noticeable as there are fewer surrounding droplets for the mass transfer process. This sensitivity at lower frequency is a particular strength of the invention. In fact, even when only about 1 % of the droplets were microbe-containing droplets, growth of microbes could be detected.

Thus, in an embodiment, step S1 in Fig. 1 comprises generating microbe-containing droplets comprising at least one microbe per microbe-containing droplet and empty droplets that do not contain any microbe. In this embodiment, the microbe-containing droplets constitute less than 75 %, preferably less than 50 %, more preferably less than 25 %, and most preferably less than 10 %, of the droplets generated in step S1.

It is only droplets containing one or more microbes that are resistant against the antimicrobial agent that will change, such as shrink, in size during the incubation in step S2, whereas the size of empty droplets or droplets containing susceptible microbes will not change, such as shrink, in size but rather maintain their size or even increase in size as mentioned above, see Fig. 13.

This also means that if the sample to be tested only contains susceptible microbes, then there will be no change, such as shrinkage, in size of droplets during incubation and the droplet sizes should remain fairly the same as at the start of the incubation. Correspondingly, if the sample to be tested only contains

resistant microbes, then all droplets containing microbes will shrink so that there will not be any microbial-containing droplets with the original size or an increase in size. Thus, heteroresistance in a microbial population can be detected by a differentiation in droplet size between different microbe-containing droplets, i.e., droplets containing resistant and growing microbes will shrink in size whereas droplets containing susceptible and non-growing microbes will not shrink in size, such as no change or an increase in droplet size. A microbial population with only resistant microbes or only susceptible microbes will instead not result in the differentiation in droplet size.

In an embodiment, step S4 in Fig. 1 comprises detecting heteroresistance to the antimicrobial agent in the microbial population based on detection of a change in size of at least one droplet comprising at least one microbe but no change in size of at least one other droplet comprising at least one microbe and/or an opposite change in size of the least one other droplet comprising at least one microbe. Opposite change as used herein indicate a change in size in an opposite direction as compared to change in size, i.e., an increase in size is an opposite change to a decrease in size and a decrease in size is an opposite change to an increase in size.

Hence, in an embodiment step S4 of Fig. 1 comprises detecting heteroresistance to the antimicrobial agent in the microbial population based on detection of a reduction in diameter of at least one droplet comprising at least one microbe but no reduction in diameter of at least one other droplet comprising at least one microbe. For instance, step S4 comprises detecting heteroresistance to the antimicrobial agent in the microbial population based on detection of a reduction in diameter of at least one droplet comprising at least one microbe but no change in diameter or an increase in diameter of at least one other droplet comprising at least one microbe.

The method as shown in Fig. 1 can be used to test a single antimicrobial agent and whether the microbes show any heteroresistance to the antimicrobial agent. Alternatively, the microbes could be exposed to a mixture of multiple antimicrobial agents or one antimicrobial agent and another therapeutic agent to detect any heteroresistance in the microbial population with regard to the mixture of agents. For instance, sometimes combination antibiotics are used, in which two active ingredients are added together for additional therapeutic effect. As illustrative examples, amoxicillin is often used together with clavulanic acid, and trimethoprim can be combined with sulfamethoxazole. In such a case, step S1 in Fig. 1 comprises generating droplets comprising at least one microbe and multiple antimicrobial agents or an antimicrobial agent and another therapeutic agent.

The generation of droplets in step S1 and the incubation of droplets in step S2 are preferably performed in a microfluidic device 10, see Fig. 11. In such a case, step S1 of Fig. 1 can be performed as shown in Fig. 2. In this embodiment, the method comprises flowing a continuous phase comprising an oil in a microfluidic channel 14 in step S10 and flowing a dispersed phase comprising the microbes and the antimicrobial agent in the culture medium in a perpendicular microfluidic channel 12 in step S11. Steps S10 and S11 are typically performed in parallel by simultaneously flowing the continuous phase and the dispersed phase. A next step S12 comprises generating the droplets at a T-junction 15 between the microfluidic channel 14 and the perpendicular microfluidic channel 12.

Hence, in an embodiment, the invention utilizes two-phase flow microfluidics to enable generation or formation of droplets inside an immiscible carrier fluid. In a typical T-junction configuration, as shown in Fig. 11, the two phases flow through orthogonal channels 12, 14 and form droplets where they meet. In such a configuration, the size of the droplets generally depends on the flow rates of the two liquids, the dimensions of the channels 12, 14, the relative viscosity between the two liquids, and presence of any surfactants and their concentrations.

In such an embodiment, an oil is used as the carrier fluid, which is commonly referred to as continuous phase. The culture medium with the microbes and antimicrobial agent is then dispersed into the oil in step S12 at the T-junction 15 to form droplets of the culture medium carried by the oil. The continuous phase flown in the microfluidic channel 14 could be any oil, in which the culture medium is immiscible. An illustrative, but non-limiting, example of such an oil is a mineral oil (paraffinum liquidum), and in particular a light mineral oil (paraffinum perliquidum). A light mineral oil typically has a kinematic viscosity within 14 to 17 cSt at 40°C or 3 to 11 cSt at 100°C, dynamic viscosity within 2 to 400 cP at room temperature.

In an embodiment, the continuous phase is an oil, such as a mineral oil, further comprising at least one surfactant. Illustrative, but non-limiting, examples of such surfactants include polyglycerol polyricinoleate (PGPR), sorbitan monooleate (Span 80), cetyl polyethylene glycol/polypropylene glycol-10/1 dimethicone (ABIL® EM 90), and fluorinated surfactants, such as Krytox® or PicoSurf®.

The present invention is, however, not limited to generating droplets using a T-junction configuration as discussed above in connection with Fig. 2. Also, other methods of generating droplets of culture medium comprising microbes and antimicrobial agent could be used. For instance, a flow focusing device (FFD) could be used to generate such droplets. A FFD generally comprises three microfluidic inlet channels

converging into a microfluidic channel via a narrow orifice. The dispersed phase is then contained in the middle inlet channel and is squeezed by the continuous phase flows from two opposing side channels. In such a case, both phases pass through the narrow orifice that is located downstream of the three inlet channels. The stream of the dispersed phase becomes narrow and finally breaks into droplets. Generally, the droplet size in an FFD is determined by the flow rates of the two phases, by the flow rate ratio in addition to the microfluidic channel geometries and the viscosities of the two phases.

Other examples of droplet generating structures that could be used to generate droplets include co-flowing devices, and cross-flow devices.

In an embodiment, step S1 in Fig. 1 comprises generating the droplets comprising at least one microbe and the antimicrobial agent at a droplet generator 19 of a microfluidic device 10. In this embodiment, step S2 comprises incubating the droplets in conditions supporting growth of the microbes in the droplets in an incubation chamber 17 of the microfluidic device 10 in fluid connection with the droplet generator 19. Step S3 comprises, in this embodiment, monitoring the size of the droplets during incubation in the incubation chamber 17.

Thus, the method as shown in Fig. 1 is preferably performed on-chip, i.e., with steps S1-S3 performed in the microfluidic device 10. This is a significant advantage as compared to the prior art techniques mentioned in the background section, which uses a droplet generation chip and a droplet counting chip. Thus, the droplet generation chip is used to generate droplets, which are then collected from the droplet generation chip and incubated off-chip in a test tube and then added to a droplet counting chip, where the actual analysis is taking place. The present invention therefore provides a more user-friendly method allowing the method steps S1 to S3 to be performed on-chip in a single microfluidic device 10.

In an embodiment, step S1 comprises microbe-containing droplets comprising, on average, multiple microbes per microbe-containing droplet, preferably at least 10 microbes per microbe-containing droplet, more preferably at least 25 microbes per microbe-containing droplet, even more preferably at least 50 microbes per microbe-containing droplet, and most preferably from 100 up to 3000 microbes per microbe-containing droplet.

Having a plurality of microbes in the microbe-containing droplets means that fewer droplets are needed in order to detect heteroresistance. As is shown herein, generating microbe-containing droplets containing, on average, from 100 up to 3000 microbes per microbe-containing droplet enabled

heteroresistance detection even at a frequency as low as 10^{-6} using merely 200 to 300 droplets. This should be compared to the prior art techniques mentioned in the background section, which require about 5 to 6 million droplets in order to detect a heteroresistant subpopulation frequency of 10^{-6}.

In an embodiment, step S1 comprises generating droplets comprising the at least one microbe and the antimicrobial agent and having an average diameter selected within an interval of from 150 up to 300 µm. Average diameter as used herein indicate that there will be a distribution in the sizes of the droplets generated in step S1 around this average diameter as schematically shown in Fig. 12. Accordingly, some droplets generated in step S1 will have a diameter larger than the average diameter, whereas other droplets generated in step S1 will have a diameter smaller than the average diameter. In other words, the droplets generated in step S1 do not necessarily have to have the same diameter but the average diameter of the generated droplets is preferably within the interval of from 150 up to 300 µm. Accordingly, individual droplets generated in step S1 may have a diameter smaller than 150 µm or larger than 300 µm.

In a preferred embodiment, the average diameter of the droplets generated in step S1 is selected within an interval of from 175 up to 275 µm and more preferably selected within an interval of from 200 up to 250 µm.

In an embodiment, step S1 comprises generating droplets comprising the at least one microbe and the antimicrobial agent and having an average volume selected within an interval of from 1 up to 15 nL. Average volume as used herein indicate that there will be a distribution in the volumes of the droplets generated in step S1 around this average volume. Accordingly, some droplets generated in step S1 will have a volume larger than the average volume, whereas other droplets generated in step S1 will have a volume smaller than the average volume. In other words, the droplets generated in step S1 do not necessarily have to have the same volume but the average volume of the generated droplets is preferably within the interval of from 1 up to 15 nL. Accordingly, individual droplets generated in step S1 may have a volume smaller than 1 nL or larger than 15 nL.

In a preferred embodiment, the average volume of the droplets generated in step S1 is selected within an interval of from 2 up to 10 nL, and more preferably selected within an interval of from 4 up to 8.5 nL.

As mentioned in the foregoing, the size of the droplets, such as in terms of average diameter or average volume, can be tailored based on the flow rates of the continuous phase and the dispersed phase, the

relative viscosity between the phases, and the microfluidic channel geometries used, for instance, in the T-junction configuration or the FFD.

The above-mentioned preferred average diameters and average volumes are, though, highly suitable to enable generation of droplets comprising one or multiple microbes per droplet also for small sample volumes.

The number of microbes captured in droplets generated in step S1 depends at least partly on the size (diameter or volume) of the droplets, the concentration of microbes (CFU per unit volume) and the type of microbes. As an illustrative example, droplets with above-exemplified preferred droplet diameters and volumes could contain from one up to several thousand bacteria per droplet. For instance, a bacteria-containing droplet could contain from 1 up to about 5,000 or 6,000 bacteria.

Step S2 of Fig. 1 comprises incubating the droplets in conditions supporting growth of the microbes in the droplets. Hence, the incubation in step S2 is taking place in conditions that allow resistant microbes to grow inside the droplets. For instance, the temperature could be maintained at a target temperature or temperature range, such as at or close to 37°C, to support such microbial growth.

In an embodiment, the incubation in step S2 is performed while the droplets maintain physical contact with neighboring droplets. For instance, the droplets generated in a microfluidic device 10 in Fig. 11 at the T-junction 15 could be transported into an incubation chamber 17 designed to house a plurality of the droplets generated in step S1 at the T-junction 15. The droplets are then preferably incubated in the incubation chamber 17 while maintaining droplets in physical contact with neighboring droplets.

In other words, step S2 in Fig. 2 preferably comprises incubating the droplets in conditions supporting growth of the microbes in the droplets while maintaining the droplets in proximity of, preferably contact with, each other to enable mass transfer of water between neighboring droplets.

This is schematically shown in Fig. 13 illustrating photographs taken of some droplets in the incubation chamber 17 of a microfluidic device 10. As is shown in the photographs, the droplets are preferably maintained in physical contact with neighboring droplets to enable mass transfer of water between droplets as microbes are growing inside some of the droplets. In the first photograph taken prior to the start of incubation (0 hours), two neighboring droplets have fairly the same diameter 218 vs. 219 μm. One of the droplets contains resistant microbe(s), whereas the neighboring droplet contains susceptible

microbe(s). At 12 hours of incubation, see middle photograph, the growth of the microbe(s) in one of the droplets has resulted in a droplet shrinkage from a diameter of 219 µm down to 213 µm, whereas the neighboring droplet has increased in size from a diameter of 218 µm up to 229 µm. The droplet shrinkage and growth are even further evident following 24 hours of incubation where the droplet with growing microbe(s) have shrunk from 219 µm down to 206 µm, whereas the droplet lacking microbial growth has increased in size from 218 µm up to 235 µm. This change in droplet size during incubation is probably due to mass transfer of water from droplets with microbial growth into neighboring or adjacent droplets lacking microbial growth.

The monitoring of the size of the droplets in step S3 of Fig. 1 may be performed as shown in Fig. 3. In this embodiment, step S3 comprises taking at least one image of the droplets during incubation in step S20.

For instance, an initial or first image is preferably taken prior to incubation or at a first time point shortly following start of the incubation of the droplets in step S2. This corresponds to the photograph to the left in Fig. 13. One or more subsequent images are then taken at one or more time points during incubation, such as represented by the photographs at 12 hours and 24 hours of incubation, respectively, in Fig. 13. In such a case, any change, such as shrinkage, in size of droplets can be determined based on a comparison of the initial or first image with the one or more subsequent images. Thus, in such an embodiment, the detection of heteroresistance in step S4 is performed at least partly based on the comparison of the initial or first image with the one or more subsequent images.

A single subsequent image could be taken in step S20, such as at a defined time period following start of incubation at step S2. This defined time period is preferably selected within an interval of from 6 hours up to 24 hours, more preferably selected within an interval of from 9 hours up to 24 hours, such as selected within an interval of from 12 up to 18 hours. The defined time point could, for instance, be about 12 hours or 18 hours following start of incubation.

Alternatively, multiple subsequent images could be taken in step S20 at different time periods following start of incubation at step S2. For instance, images could be taken at 6, 12, 18 and optionally also at 24 hours following start of incubation as an illustrative, but non-limiting, examples. As is shown in Fig. 7, the differentiation in droplet size start to appear already at 6 hours of incubation and become evident at 12 hours of incubation in the case 30% or 70% of the droplets contained growing microbes, see Figs. 7b and 7c.

This means that it could be advantageous to take multiple images in step S20 at different time periods following the start of the incubation to monitor the progress in droplet size development over time until a clear difference in droplet size can be detected, if the sample contained a heteroresistant population.

Fig. 4 is a flow chart illustrating additional steps according to an embodiment of the method in Fig. 1. In this embodiment, an initial distribution of sizes of the droplets comprising at least one microbe prior to incubating the droplets or at a first time point during incubating the droplets is determined in step S30. A next step S31 comprises determining a subsequent distribution of sizes of the droplets comprising at least one microbe at a later time point during incubating the droplets. The method then continues to step S4 in Fig. 1, which comprises, in this embodiment, detecting heteroresistance to the antimicrobial agent in the microbial population based on a comparison of the subsequent distribution of sizes and the initial distribution of sizes.

The initial and subsequent size distributions could be determined in steps S30 and S31 by analysis of images taken of the droplets in step S20 as mentioned in the foregoing. In such a case, a graph or histogram representing the initial and subsequent size distributions could be generated by counting the number of microbial-containing droplets having a given droplet size or size range. Fig. 12 illustrates an example of a graph showing such initial (0 h) and subsequent (12 h) distributions of sizes. The initial distribution of sizes indicates that the droplets have a uniform size distribution around an average diameter whereas, following 12 hours of incubation, droplets containing growing microbes will shrink in size whereas droplets containing non-growing microbes and empty droplets will rather increase in size giving a clear differentiation in these two populations of droplets.

Fig. 5 is a flow chart illustrating additional steps according to another embodiment of the method in Fig. 1. In this embodiment, a plurality of droplets comprising at least one microbe and the antimicrobial agent is identified in step S40. The following steps S41 and S42 are then performed for each such identified droplet, which is schematically indicated by the loop L1. Step S41 comprises determining an initial size of the identified droplet prior to incubating the droplet or at a first time point during incubating the droplets, whereas the following step S42 comprises determining a subsequent size of the identified droplet at a later time point during incubating the droplets. The method then continues to step S4 in Fig. 1, which comprises, in this embodiment, detecting heteroresistance to the antimicrobial agent in the microbial population based on pairwise comparisons of the subsequent sizes and the initial sizes for the identified droplets.

The embodiment shown in Fig. 5, thus, monitors individual droplets, such as individual droplets in the incubation chamber 17 of the microfluidic device 10 in Fig. 11. The sizes of such individual droplets prior to and following incubation are then compared to determine if any of the droplets have shrunk in size and whether any other droplets have not significantly changed in size or increased in size. This approach is schematically shown in Fig. 13 where two individual droplets are monitored at 0, 12 and 24 hours of incubation and where the diameters of the droplets are indicated at each monitoring occasion.

Fig. 6 is a flow chart illustrating additional steps according to another embodiment of the method in Fig. 1. In this embodiment, an average diameter of the droplets comprising at least one microbe is determined in step S50 prior to incubating the droplets or at a first time point during incubating the droplets. A next step S51 comprises calculating, for each droplet of a plurality of droplets comprising at least one microbe, a normalized droplet size representing a ratio between a diameter of the droplet and the average diameter. The method then continues to step S4 in Fig. 1, which comprises, in this embodiment, detecting heteroresistance to the antimicrobial agent in the microbial population based on a distribution of the normalized droplet sizes.

Figs. 7 to 10 illustrate such an approach by plotting normalized droplet sizes prior to incubation (0 hours) and at different time points (6, 12, 18, 24 hours) during incubation. As is shown in, for instance, Figs. 9-10 the distribution of the normalized droplet sizes changes over time if the microbial population show heteroresistance to the antimicrobial agent. However, no such change in the distribution of the normalized droplet sizes is seen for a purely susceptible microbial population as shown in Fig. 8.

Thus, various embodiments could be used to detect heteroresistance by monitoring distribution of droplet sizes or by monitoring individual droplets. The latter approach is suitable when the droplets remain in place in the incubation chamber 17 during incubation as this simplifies monitoring the same droplets at the different time occasions. For instance, an initial image of the droplets can be taken prior to incubating the droplets or at the first time point during incubating the droplets. The image is processed to enable detection of the image coordinates (pixels) of selected microbial-containing droplets. As an example, the two droplets shown in Fig. 13 could be identified as having droplet center at pixels (X_1, Y_1) and (X_2, Y_2) and having initial diameter of $D_{0,1}$=218 µm and $D_{0,2}$=219 µm, respectively. A subsequent image is then taken on the droplets in the incubation chamber 17, such as following 12 hours of incubation, and the diameters of the droplets at the same positions (X_1, Y_1) and (X_2, Y_2) in the subsequent image are then determined $D_{12,1}$=229 µm and $D_{12,2}$=213 µm, respectively.

It will generally be harder to track individual droplets if the droplets are allowed to move slightly within the incubation chamber 17 during incubation. In such a case, it may be more appropriate to determine distributions of sizes or distributions of normalized droplet sizes and use such distributions to detect any heteroresistance to the antimicrobial agent in the microbial population in step S4 of Fig. 1.

The heteroresistance detection of the embodiments can be further improved by using the detection not only based on droplet sizes but also based on droplet texture features. Thus, in such an approach texture features are determined for the monitored droplets and used together with droplet size in the heteroresistance detection. Texture feature of a droplet as used herein is a feature representative of the texture of the droplet.

As an illustrative, but non-limiting example, the texture feature could be a gray level co-occurrence matrix (GLCM) texture feature. GLCM is a statistical method used in image processing to analyze texture features. There are various GLCM texture features including contrast, dissimilarity (DIS), homogeneity (HOM), angular second moment (ASM), maximum probability (MAX), entropy (ENT), energy, shade, prominence, mean, variance, and correlation. As an example, correlation assesses how correlated a pixel is to its neighbor pixel, whereas homogeneity evaluates the closeness of the distribution of elements in the GLCM to the GLCM diagonal and thereby indicates how uniform the texture is.

The correlation feature is calculated as:

$$Correlation = \sum_{i,j=0}^{N-1} P_{ij} \frac{(i-\mu)(j-\mu)}{\sigma^2}$$

and the homogeneity feature is calculated as:

$$Homogeneity = \sum_{i,j=0}^{N-1} \frac{P_{ij}}{1+(i-j)^2}$$

wherein P_{ij} represents element i, j of the normalized symmetrical GLCM, N represents the number of gray levels in the image, μ is the GLCM mean calculated as

$$\mu = \sum_{i,j=0}^{N-1} iP_{ij}$$

and σ^2 is the variance of the intensities of all reference pixels int eh relationships that contributed to the GLCM calculated as

$$\sigma^2 = \sum_{i,j=0}^{N-1} P_{ij}(i-\mu)^2$$

In an embodiment, the method therefore also comprises determining, for at least a subset of the monitored droplets, at least one texture feature. In such a case, step S4 in Fig. 1 comprises detecting heteroresistance to the antimicrobial agent in the microbial population based on detection of the shrinkage in size of at least one droplet comprising at least one microbe but no shrinkage in size of at least one other droplet comprising at least one microbe and based on the at least one texture feature.

In an embodiment, the at least one texture feature is at least one GLCM texture feature. In a particular embodiment, the at least one GLCM texture feature is GLCM correlation or homogeneity. Thus, in a particular embodiment, the detection of heteroresistance in step S4 is based on droplet sizes and GLCM correlation, based on droplet sizes and GLCM homogeneity, or based on droplet sizes, GLCM correlation and GLCM homogeneity.

The present invention also relates to a system 1 for detecting heteroresistance in a microbial population as schematically shown in Fig. 11. The system 1 comprises a microfluidic device 10, a camera 30, a memory 22 and a processor 21.

The microfluidic device 10 comprises a sample inlet 11 configured to receive a sample comprising microbes and an antimicrobial agent in a culture medium and an oil inlet 13 configured to receive an oil sample. The microfluidic device 10 also comprises a droplet generator 19 in fluid connection with the sample inlet 11 and the oil inlet 13 and configured to generate droplets comprising at least one microbe and the antimicrobial agent in the culture medium. The microfluidic device 10 also comprises an incubation chamber 17 in fluid connection with the droplet generator 19 and configured to house a plurality of droplets in conditions supporting growth of the microbes in the droplets.

The camera 30 of the system 1 is configured to take images of the droplets in the incubation chamber 17. The memory 22 is configured to store program instructions and the processor 21 is configured, when executing the program instructions, to process the at least one image to determine sizes of droplets in the incubation chamber 17 and detect heteroresistance to the antimicrobial agent in the microbial population based on detection of a shrinkage in size of at least one droplet comprising at least one microbe but no shrinkage in size of at least one other droplet comprising at least one microbe.

In an embodiment, the microfluidic device 10 comprises a microfluidic channel 14 in fluid connection with the oil inlet 13 and a perpendicular microfluidic channel 12 in fluid connection with the sample inlet 11. In such an embodiment, the droplet generator 19 comprises a T-junction 15 between the microfluidic channel 14 and the perpendicular microfluidic channel 12. This means that the perpendicular microfluidic channel 12 has a first end connected to or in fluid connection with the sample inlet 11 and a second, opposite end connected to the microfluidic channel 14, wherein the perpendicular microfluidic channel 12 and the microfluidic channel 14 interconnect at an angle of or at least close to 90° in the T-junction 15.

In another embodiment, the droplet generator is in the form of an FFD. For instance, the microfluidic device comprises a first microfluidic channel having a first end in fluid connection with the oil inlet, a second microfluidic channel having a first end in fluid connection with the oil inlet and a perpendicular microfluidic channel having a first end in fluid connection with the sample inlet. In such an embodiment, the droplet generator comprises a 4-way junction between a respective second, opposite end of the first and second microfluidic channels and of the perpendicular microfluidic channel and an orifice channel having a diameter that is smaller than the diameters of the first and second microfluidic channel and of the perpendicular microfluidic channel. The channels are arranged, clockwise or counter-clockwise, at the 4-way junction, first microfluidic channel, orifice channel, second microfluidic channel and perpendicular microfluidic channel.

Other examples of droplet generators 19 include co-flowing devices, and cross-flow devices.

In an embodiment, the microfluidic device 10 comprises a microfluidic meander channel 16 interconnecting the droplet generator 19 and the incubation chamber 17. Such a microfluidic meander channel 16 provides a prolonged flow path for the droplets from the droplet generator 19 up to the incubation chamber 17. This may be advantageous for the stability of the droplets by avoiding sudden changes in its velocity just after the droplet generation, which promotes formation of droplets with fairly

uniform initial droplet sizes and also facilitates entry and distribution of the droplets inside the incubation chamber 17.

The droplets enter the incubation chamber 17, such as from the microfluidic meander channel 16, at a first end of the incubation chamber 17. The incubation chamber 17 then preferably comprises an outlet 18, such as at a second, opposite end of the incubation chamber 17. In such a case, surplus carrier fluid, i.e., oil, is preferably allowed to leave the incubation chamber 17 through the outlet 18 while the droplets are restricted from leaving the outlet 18. This restriction could be implemented in the form of a filter or obstruction arranged to enable the oil to flow through the filter or past the obstruction and into the outlet 18 but restrict the droplets from leaving the incubation chamber 17.

In an embodiment, the droplet generator 19 is configured to generate microbe-containing droplets comprising, on average, at least 10 microbes per microbe-containing droplet, preferably at least 25 microbes per microbe-containing droplet, more preferably at least 50 microbes per microbe-containing droplet, and most preferably from 100 up to 3000 microbes per microbe-containing droplet.

In an embodiment, the droplet generator 19 is configured to generate microbe-containing droplets comprising at least one microbe per microbe-containing droplet and empty droplets that do not contain any microbe. The microbe-containing droplets constitute less than 75 %, preferably less than 50 %, more preferably less than 25 %, and most preferably less than 10 %, of the droplets.

In an embodiment, the droplet generator 19 is configured to generate droplets comprising the at least one microbe and the antimicrobial agent in the culture medium and having an average diameter selected within an interval of from 125 up to 300 µm, preferably selected within an interval of from 150 up to 275 µm, and more preferably selected within an interval of from 175 up to 225 µm, such as from 180 up to 220 µm.

In an embodiment, the droplet generator 19 is configured to generate droplets comprising the at least one microbe and the antimicrobial agent in the culture medium and having an average volume selected within an interval of from 1 up to 15 nL, preferably selected within an interval of from 2 up to 10 nL, and more preferably selected within an interval of from 4 up to 8.5 nL.

The incubation chamber 17 is preferably configured to house the plurality of droplets in conditions supporting growth of the microbes in the droplets while maintaining droplets in physical contact with

neighboring droplets. For instance, the incubation chamber 17 could be configured to house the plurality of droplets in conditions supporting growth of the microbes in the droplets while maintaining droplets in proximity of each other to enable mass transfer of water between neighboring droplets.

In an embodiment, the processor 21 is, when executing the program instructions, configured to process the at least one image to detect heteroresistance to the antimicrobial agent in the microbial population based on detection of a change in size of at least one droplet comprising at least one microbe but no change and/or an opposite change in size of at least one other droplet comprising at least one microbe.

In an embodiment, the processor 21 is, when executing the program instructions, configured to process the at least one image to determine diameters of droplets in the incubation chamber 17 and detect heteroresistance to the antimicrobial agent in the microbial population based on detection of a reduction in diameter of at least one droplet comprising at least one microbe but no reduction in diameter of at least one other droplet comprising at least one microbe.

The processor 21 is, in a particular embodiment and when executing the program instructions, configured to detect heteroresistance to the antimicrobial agent in the microbial population based on detection of a reduction in diameter of at least one droplet comprising at least one microbe but no change in diameter or an increase in diameter of at least one other droplet comprising at least one microbe.

In an embodiment, the processor 21 is, when executing the program instructions, configured to determine an initial distribution of sizes of the droplets comprising at least one microbe prior to incubating the droplets or at a first time point during incubating the droplets and determine a subsequent distribution of sizes of the droplets comprising at least one microbe at a later time point during incubating the droplets. The processor is 21, in this embodiment, also configured to detect heteroresistance to the antimicrobial agent in the microbial population based on a comparison of the subsequent distribution of sizes and the initial distribution of sizes.

In another embodiment, the processor 21 is, when executing the program instructions, configured to identify a plurality of droplets comprising at least one microbe and an antimicrobial agent in the at least one image. The processor 21 is, in this embodiment, also configured to determine, for each identified droplet of the plurality of droplets, an initial size of the identified droplet prior to incubating the droplets or at a first time point during incubating the droplets and determine, for each identified droplet of the plurality of droplets, a subsequent size of the identified droplet at a later time point during incubating the droplets.

The processor 21 is further configured to detect heteroresistance to the antimicrobial agent in the microbial population based on pairwise comparisons of the subsequent sizes and the initial sizes for identified droplets.

In a further embodiment, the processor 21 is, when executing the program instructions, configured to determine, based on the at least one image, an average diameter of the droplets comprising at least one microbe prior to incubating the droplets or at a first time point during incubating the droplets. The processor 21 is also configured to calculate, for each droplet of a plurality of droplets comprising at least one microbe, a normalized droplet size representing a ratio between a diameter of the droplet and the average diameter. In this embodiment, the processor 21 is configured to detect heteroresistance to the antimicrobial agent in the microbial population based on a distribution of the normalized droplet sizes.

The camera 30 of the system 1 could be any camera or image capturing equipment capable of taking images of the droplets in the incubation chamber 17 and wherein the images can be processed by the processor 21 to determine or at least estimate sizes of droplets. The camera 30 may optionally be connected to or arranged in relation to a microscope in order to take images of the droplets in the incubation chamber 17. Various types of cameras 30 could be used in the system 1 including, but not limited to, charged coupled device (CCD) cameras, complementary metal oxide semiconductor (CMOS) cameras, digital high-speed cameras, universal serial bus (USB) digital microscope cameras and mobile phone cameras.

The processor 21 and the memory 22 are interconnected to each other to enable normal software execution. The processor 21 and the memory 22 may be implemented in a computer 20 as shown in Fig. 11. The computer 20 may also include an input and output (I/O) unit (not shown) connected to the processor 21 and/or the memory 22 to enable reception of image data from a camera 30. The I/O unit could then be implemented to enable wireless communication with external devices, such as the camera 30, for instance in the form of a receiver and optionally a transmitter, or a transceiver, or as a general I/O port for wired communication with external devices, such as the camera 30.

The term processor 21 as used herein should be interpreted in a general sense as any circuitry, system or device capable of executing program code or computer program instructions to perform a particular processing, determining or computing task. The processor 21 does not have to be dedicated to only execute the above-described steps, functions, procedure and/or blocks, but may also execute other tasks.

The microfluidic device 10 can be manufactured using various manufacturing processes including, but not limited, to three-dimensional (3D) printing, molding, machining, casting and sculpting.

In an embodiment, the microfluidic device 10 is made of an optically clear or transparent material to facilitate imaging of the droplets in the incubation chamber 17. Alternatively, at least a portion of the microfluidic device 10 aligned with the incubation chamber 17 is transparent whereas any remaining portion of the microfluidic device 10 does not necessarily have to be made of a transparent material. A further alternative is to have a microfluidic device 10 comprising a substrate comprising the above-mentioned microfluidic channels 12, 14, 16 and the incubation chamber 17 in the form of open channels and an open chamber. In such a case, a transparent lid or cover could be attached to the substrate to close the open channels and the open chamber to form the microfluidic channels 12, 14, 16 and the incubation chamber 17. The above-mentioned inlets 11, 13 could then be in the form of openings in the lid or cover or openings in a first main surface of the substrate opposite to a second main surface, to which the lid or cover is attached.

The substrate could be made of various plastics including, but not limited to, polydimethylsiloxane (PDMS), poly(methyl methacrylate) (PMMA), polylactic acid (PLA), acrylonitrile butadiene styrene (ABS), polystyrene (PS), thermoplastic elastomer (TPE), polyamide (PA), polyaryletherketone (PAEK), polycaprolactone (PCL), poly(lactic-co-glycolc acid) (PLGA), poly(ethylene glycol) (PEG), or other materials, such as silicon or glass.

The above-mentioned optional lid or cover could be made of a transparent plastic, such as selected from the above-mentioned list.

The microfluidic device 10 of the system 1 could be used to analyze one microbial population and one antimicrobial agent. It is, however, possible to design a microfluidic device 10 that enables multiplexing, i.e., analyzing multiple microbial populations and/or multiple antimicrobial agents in parallel. Such a microfluidic device 10 is schematically shown in Fig. 14. The microfluidic device 10 comprises multiple sample inlets 11A, 11B, 11C, multiple droplet generators 19A, 19B, 19C and multiple incubation chambers 17A, 17B, 17C. In such an embodiment, each sample inlet 11A, 11B, 11C of the multiple sample inlets 11A, 11B, 11C is configured to receive a respective sample comprising microbes and an antimicrobial agent in a culture medium. Each droplet generator 19A, 19B, 19C of the multiple droplet generators 19A, 19B, 19C is in fluid connection with the oil inlet 13 and a respective sample inlet 11A,

11B, 11C of the multiple sample inlets 11A, 11B, 11C and configured to generate droplets comprising at least one microbe and the antimicrobial agent in the culture medium. Each incubation chamber 17A, 17B, 17C of the multiple incubation chambers 17A, 17B, 17C is in fluid connection with a respective droplet generator 19A, 19B, 19C of the multiple droplet generator 19A, 19B, 19C and configured to house a plurality of droplets in conditions supporting growth of the microbes in the droplets.

In the multiplexing embodiment, the microfluidic device 10 thereby comprises $N \geq 2$ sample inlets 11A, 11B, 11C, N droplet generators 19A, 19B, 19C and N incubation chambers 17A, 17B, 17C to enable running N analysis of microbes in parallel. In Fig. 14, a single oil inlet 13 is in fluid connection with each of the droplet generators 19A, 19B, 19C. In an alternative embodiment, the microfluidic device 10 comprises N oil inlets. In such a case, each oil inlet is in fluid connection with a respective droplet generator 19A, 19B, 19C.

The microfluidic device 10 of Fig. 14 enables testing different microbial populations in parallel. Alternatively, or in addition, one and the same microbial population could be analyzed but with different antimicrobial agents and/or different concentrations of a given antimicrobial agent.

The different droplet generators 19A, 19B, 19C and incubation chambers 17A, 17B, 17C are preferably implemented as described in the foregoing. For instance, each incubation chamber 17A, 17B, 17C is preferably in fluid connection with a respective outlet 18A, 18B, 18C as shown in Fig. 14.

The following Examples show the development of a microfluidic device that can be used in a droplet-based microfluidic assay to assess heteroresistance (HR) in bacterial populations. This assay involved encapsulating approximately 100 to 3,000 bacteria per droplet, depending on the type of antibiotic used, droplet size, and the frequency of the resistant subpopulation. When the bacterial population inside the droplet was exposed to antibiotics the resistant subpopulation grew, resulting in a 5-10% shrinkage of the droplet compared to droplets with only susceptible bacteria where growth was inhibited. The shrinkage of droplet size was a reliable indicator of the growth of resistant bacteria and could be precisely quantified by microscopy.

The experiments demonstrated that droplet shrinkage (indicating bacterial growth) and swelling (indicating no growth) depended on the number of inert droplets, i.e., droplets not containing any bacterial cells, in the incubation chamber. When only a few droplets contained growing bacteria, they were mainly in contact with droplets without bacterial growth resulting in maximum shrinkage due to surrounding

droplets supporting mass transfer. In contrast, when most droplets contained growing bacteria, the size difference was less noticeable as there were fewer surrounding droplets for the mass transfer process. This sensitivity at lower frequencies is a particular strength for HR detection.

The method outpaced the current PAP test in terms of detection time and automation capabilities. Results could be obtained within 12 to 24 hours (depending on the antibiotic), which compared to the PAP test took at least one additional day. This invention can thereby be used in identifying HR bacteria and subsequently adjusting treatment to reduce complications, failure, and death. This invention also significantly improved the detection limit for HR as compared to single encapsulation droplet-based standard AST methods since such single encapsulation droplet-based standard AST methods would require approximately 5.5×10^{6} and 5.5×10^{7} droplets to detect lower frequencies, such as 10^{-6} or 10^{-7}. In contrast, the invention can detect subpopulations at a frequency of 10^{-6} using only 200 to 300 droplets, representing a 20,000-fold reduction in the number of droplets needed.

The microfluidic chip could be designed to support multiplexing, allowing the detection of resistant subpopulations across multiple antibiotics simultaneously, making it a more efficient approach. Beyond its time-saving benefits, the invention offers complete automation potential via integration with a flow control system and microscope. It can also facilitate the isolation of rare antibiotic-resistant phenotypes for further genotypic and phenotypic analysis when combined with active sorting methods like acoustic and electrical sorting based on droplet size disparity Additionally, unlike the standard PAP test, which requires approximately 25 mL of MH broth media and antibiotics per agar plate, the invention can use significantly less volumes, such as about 5 µL, resulting in a 1/5000 reduction in the consumption of antibiotics.

EXAMPLES

EXAMPLE 1

Microfluidic device design, fabrication, and setup

A microfluidic device 10 was constructed as two parts: a droplet generation part and a droplet storage and incubation part. The droplet generation part comprises a standard T-junction 15, see Fig. 11, with an oil inlet 13 configured to receive light mineral oil with 3 % polyglycerol polyricinoleate (PGPR) as surfactant and in fluid connection with a microfluidic channel 14 having an average channel width of 150 µm. A sample inlet 11 is configured to receive a biological sample with microbes + antimicrobial agent and is in fluid connection with the T-junction 15 with a perpendicular microfluidic channel 12 having an average channel width of 80 µm. The droplet generator 19 generates droplets with diameters in the 180

to 220 µm range. The droplet storage and incubation part comprise an incubation chamber 17 designed to hold around 2000 to 3500 droplets.

In the case of the multiplex microfluidic chip, similar dimensions were used along with multiple droplet generators and incubation chambers. The microfluidic device mold was 3D printed using an ASIGA printer. The master mold was then used for PDMS (Polydimethylsiloxane) casting with a 10:1 ratio of base to curing agent from Sylgard 184. Then the PDMS replica was punched at inlets and outlets with a 1 mm punch (Welltec) and bonded to a glass slide (Epredia) with a plasma oven (Diener Electronic, Germany). Syringe pumps (Cetoni, Nemesys pumps, Germany) were used to drive all fluids and the whole microfluidic setup was kept inside a stage-top incubator (OKOlab) maintained at 37°C and 80% humidity for the experiments.

A microfluidic device mold was 3D printed using an ASIGA printer. The master mold was then used for polydimethylsiloxane (PDMS) casting with a 10:1 ratio of base to Sylgard™ 184 curing agent. The PDMS replica was punched at the inlets 11, 13 and outlet 18 with a 1 mm punch (Welltec) and bonded to a Epredia™ glass slide with a plasma oven (Diener Electronic, German) to close the microfluidic channels 12, 14, 16 and the incubation chamber 17. Syringe pumps (Cetoni, Nemesys pumps, Germany) were used to push the fluids into the microfluidic device 10 and the whole microfluidic setup was kept inside a stage-top incubator (Okolab) maintained at 37°C and 80% humidity.

Microscopy

An Olympus inverted microscope (IX73, Olympus) with a 10× objective, a motorized stage for X-Y-Z direction, and a camera 30 (C11440, Hamamatsu) was used for image acquisition. The microfluidic device 10 was placed in a stage-top incubator (Okolab), which was kept on the microscope stage holder for imaging. Once droplets were stable in the incubation chamber 17 (stopped moving after droplet generation was stopped), each location was marked with a motorized stage in X-Y direction. Each location was then imaged every hour for the next 24 or 30 hours. Each experiment was run for 24 to 30 hours based on the antimicrobial agent and microbial strain used.

Image processing

The images that were obtained every hour were processed with a custom-made computational pipeline implemented in Phyton to find out the droplet diameter every hour and shrinkage in droplet diameter due to microbial growth. Briefly, raw grayscale images were smoothened (Gaussian 31×31 kernel, standard deviation: 5), opened (21×21 kernel), min-max-normalized, and converted to unsigned 8-bit integer format. Droplet boundaries were identified by thresholding pre-processed images using Otsu's method

(three classes: “droplet boundary”, “other droplet content”, “background”). The binary “droplet boundaries” masks were subjected to a circle Hough transform, droplet centroids were located by identifying peaks in accumulator-normalized Hough space, and maximum droplet radii were computed using a Euclidean distance transform of the “droplet boundaries” masks. Preliminary binary droplet masks were generated by labeling both “droplet boundaries” and “droplet disks” (given by droplet centroids and maximum radii) as regions corresponding to droplets. Droplets were then segmented based on a Euclidean distance transform of the preliminary droplet masks using a droplet centroid-seeded Watershed algorithm. Droplets touching the border of an image were excluded. Droplet label masks were exported as TIFF files, and tabular droplet information (accumulator values, centroids, radii) was saved in CSV format. For downstream analysis, droplet sizes were normalized by the average droplet diameter at 0 hours and plotted against time every 6 hours for 24 to 30 hours.

Bacterial strains and culturing conditions

Mueller-Hinton (MH) (Difco) medium was used for growth in broth and agar plates. Antibiotics (Gentamicin, Cefotaxime, and Tetracycline) were purchased from Sigma-Aldrich. For every experiment, bacteria frozen in 10 % dimethyl sulfoxide (DMSO) stocks were streaked on MH agar plates and incubated at 37°C overnight. Single colonies from the platers were inoculated in 1 mL MH broth, incubated at 37°C, 199 rpm agitation, overnight, and subsequently used for each experiment. Antibiotic stocks were prepared fresh from powder stock (Sigma Aldrich) in sterile phosphate-buffered saline (PBS) (Sigma-Aldrich) and used within 16 h of preparation.

When the clinical isolates were incubated in the droplet system in the presence of 4 mg/L GEN, they exhibited filamentous growth. This growth pattern interfered with the system's capacity to identify empty droplets. Therefore, we adjusted the protocol to use a lower concentration of 0.5 mg/L for this specific combination, which allowed for effective segmentation of droplets containing growth.

EXAMPLE 2

Example 2 evaluated optimal ratio of filled (bacteria-containing) to empty droplets by varying the concentration of bacteria in the initial droplet-forming suspension.

In this Example 2, Mueller Hinton (MH) broth media was used as an aqueous phase with different colony-forming units (CFU) of susceptible *Escherichia coli* (MG1655) in the microfluidic device described in Example 1. Fig. 7a shows the normalized droplet size at intervals of 0, 6, 12, 18, and 24 hours with *E. coli* MG1655 in MH broth as a continuous phase with 10^4 CFU/mL. Normalized droplet size represents

the ratio of droplet diameter at any given time and average droplet diameter at 0 hours. In Fig. 7a, N_d represents the number of droplets imaged and analyzed every hour for 24 hours, while N_{GS} represents droplets that showed bacterial growth and shrinkage of the droplet. In Fig. 7a only 1% of the droplets showed growth and shrinkage since 10^4 CFU/mL resulted in 1% of droplets with single or double encapsulation of bacteria and the rest of the droplets (99 %) were empty (without encapsulation of bacteria). In this Example 2, 927 droplets were analyzed and after 24 hours only 8 droplets showed bacterial growth and droplet shrinkage. This Example 2 showed that, with shrinkage-based detection, we can even detect a 1% subpopulation of *E. coli* and there was no overlap in size distribution between droplets with and without growth.

The above-presented example was repeated but with an initial CFU at 10^5, which resulted in around 70% empty droplets and around 30% droplets with single or multiple *E. coli* MG1655 encapsulations. In this set of experiments, an increase in the size of the droplets without bacterial growth due to mass transfer from droplets with bacterial growth was observed. In this experiment, N_d = 2270 and N_{GS} = 588, see Fig. 7b.

The example was further repeated with the initial CFU at 5×10^5, which resulted in around 30% empty droplets and around 70% droplets with single or multiple encapsulations of *E. coli* MG1655. A clear differentiation was observed between droplets with and without bacterial growth at 12, 18 and 24 hours. In this case, maximum swelling of droplets without bacterial growth was seen at 12, 18 and 24 hours as shown in Fig. 7c, which further separated droplets with and without bacterial growth at 24 hours. The results of this Example 2 clearly showed that the microfluidic device could be used to detect a subpopulation of growing bacteria.

As shown in Figs. 7a-7c, droplet shrinkage could be detected with all tested ratios of filled-to-empty droplets and the number of droplets displaying shrinkage compared well with the expected number of cell-containing droplets, as calculated at the encapsulation stage. Strikingly, even when only 1% of the droplets contained *E. coli* MG1655, this growth could be detected.

EXAMPLE 3

Example 3 was performed to ensure that the droplet-microfluidic device 10 did not report false negatives by performing control experiments using susceptible *E. coli* MG1655.

In this Example 3, the microfluidic device as disclosed in Example 1 was used to run experiments with susceptible *E. coli* MG1655 against two antibiotics cefotaxime (CTX) with a concentration of 2 mg/L, and gentamicin (GEN) with a concentration of 0.5 mg/L. In these experiments, *E. coli* MG1655 in MH broth media with antibiotic was used as the aqueous phase with 10^5 CFU/mL. In both these experiments, *E. coli* MG1655 did not grow in the presence of antibiotics (CTX and GEN). Figs. 8a and 8b illustrate the results following analysis of 2619 and 2628 droplets, respectively, for 24 hours. As is shown in Figs. 8a and 8b, there were 0 droplets with growth and shrinkage. These figures demonstrate that there was no growth and associated shrinkage in any of the droplets when susceptible bacteria were exposed to high antibiotic levels (above the minimum inhibitory concentration (MIC)). This Example 3 showed that susceptible bacteria in the presence of antibiotics did not grow in the droplets and there was no shrinkage of droplets when bacteria did not grow.

EXAMPLE 4

Example 4 was performed to confirm that a difference in droplet size could be observed between droplets containing growing bacteria and droplets containing non-growing bacteria. This was performed to explore if a resistant subpopulation could be detected when co-encapsulated with a population of susceptible bacterial cells.

In this Example 4, susceptible *E. coli* MG1655 were spiked with different frequencies (10^{-3}, 10^{-5}, and 10^{-6}) of resistant bacteria to detect the subpopulation of resistant bacteria. These spiked-up populations were tested in a microfluidic device according to Example 1 against three different kinds of antibiotics namely CTX, GEN, and tetracycline (TET).

First, *E. coli* MG1655 were spiked with CTX-resistant *E. coli* strain DA62594 of different frequencies (10^{-3}, 10^{-5}, and 10^{-6}) in presence of 2 mg/L CTX. In this experiment, *E. coli* MG1655+DA62594+2 mg/L CTX in MH broth was used as the aqueous phase to generate droplets in light mineral oil in the microfluidic device of Example 1. Based on the initial CFU used and droplet size, in this experiment, each droplet encapsulated around 1000 to 5000 bacteria. Figs. 9a-9c plot normalized droplet size against time to detect subpopulations with frequencies of 10^{-3}, 10^{-5} and 10^{-6}, respectively. To detect the subpopulation frequency of 10^{-3}, a CFU of 2.5×10^7/mL was used, we analyzed N_d = 1519 and found droplets with shrinkage and bacterial growth N_{GS} = 204. For detecting subpopulations with frequencies of 10^{-5} and 10^{-6}, a CFU of 5×10^8 /mL was used. For 10^{-5}, we analyzed N_d = 1649 and found droplets with shrinkage and bacterial growth N_{GS} = 60. For 10^{-6}, we analyzed N_d = 2611 and found droplets with shrinkage and bacterial growth N_{GS} = 37.

Secondly, *E. coli* MG1655 were spiked with GEN-resistant *E. coli* strain DA63654 of different frequencies (10^{-5}, and 10^{-6}) in the presence of 4 mg/L GEN. In this experiment, *E. coli* MG1655+DA63654+4 mg/L GEN in MH broth was used as the aqueous phase to generate droplets in light mineral oil in the microfluidic device of Example 1. Based on the initial CFU used and droplet size, in this experiment, each droplet encapsulated around 1000 to 5000 bacteria. Figs. 9d and 9e plot normalized droplet size against time to detect subpopulations with frequencies of 10^{-5} and 10^{-6}, respectively. For detecting subpopulations with frequencies of 10^{-5} and 10^{-6}, a CFU of 5×10^{8} /mL was used. For 10^{-5}, we analyzed N_d = 2534 and found droplets with shrinkage and bacterial growth N_{GS} = 123. For 10^{-6}, we analyzed N_d = 1757 and found droplets with shrinkage and bacterial growth N_{GS} = 3.

Thirdly, *E. coli* MG1655 were spiked with TET-resistant *E. coli* strain DA34827 of different frequencies (10^{-5}, and 10^{-6}) in the presence of 5 mg/L TET. In this experiment, *E. coli* MG1655+DA34827+5 mg/L TET in MH broth was used as the aqueous phase to generate droplets in light mineral oil in the microfluidic device of Example 1. Based on the initial CFU used and droplet size, in this experiment, each droplet encapsulated around 1000 to 5000 bacteria. Figs. 9f and 9g plot normalized droplet size against time to detect subpopulations with frequencies of 10^{-5} and 10^{-6}, respectively. For detecting subpopulations with frequencies of 10^{-5} and 10^{-6}, a CFU of 5×10^{8} /mL was used. For 10^{-5}, we analyzed N_d = 2077 and found droplets with shrinkage and bacterial growth N_{GS} =170. For 10^{-6}, we analyzed N_d = 2520 and found droplets with shrinkage and bacterial growth N_{GS} = 80.

Based on determinations of CFUs/mL for the susceptible and resistant populations before mixing, we knew the expected frequencies of resistant bacteria encapsulated. To calculate the frequency of resistant bacteria detected in the experiment we used the following formula:

number of droplets with shrinkage / (total number of droplets × average number of bacteria per droplet)

This allowed us to assess how well the experiments determined the frequency of the resistant subpopulation by comparing the spiked-up frequency to that experimentally observed. Table 1 summarizes these numbers, and as can be seen the frequencies are in good agreement with each other.

Table 1: Data showing the number of droplets analyzed (N_d), droplets with shrinkage (N_{GS}), average, and total volume of droplets analyzed in the case of susceptible MG1655 with different frequencies of resistant

E. coli subjected to CTX, GEN, and TET respectively. Spiked-up frequencies and experimentally calculated frequencies are compared.

Droplet content	N_d	N_{GS}	Bacteria encapsulated per droplet	Spiked frequency	Experimentally calculated frequency
MG1655+DA62594 2 mg/L CTX	1519	204	65	10^{-3}	2.0×10^{-3}
MG1655+DA62594 2 mg/L CTX	1649	60	1630	10^{-5}	2.2×10^{-5}
MG1655+DA62594 2 mg/L CTX	2611	37	2350	10^{-6}	6.0×10^{-6}
MG1655+DA3654 4 mg/L GEN	2534	123	2000	10^{-5}	2.4×10^{-5}
MG1655+DA3654 4 mg/L GEN	1757	3	2320	10^{-6}	7.4×10^{-7}
MG1655+DA34827 5 mg/L TET	2077	170	2290	10^{-5}	3.5×10^{-5}
MG1655+DA34827 5 mg/L TET	2520	80	2605	10^{-6}	1.5×10^{-5}

The experimentally calculated frequency slightly overestimated the spiked frequency, primarily due to one reason. Thus, despite statistically calculating the average number of bacteria per droplet, variation can arise due to bacterial sedimentation within the tubing connecting the syringe pump to the microfluidic chip. Taken together, these experiments show that we can correctly detect the resistant subpopulations even when their frequency is as low as 10^{-6}.

EXAMPLE 5

In this Example 5, clinically heteroresistant strains against two antibiotics CTX and GEN were used, wherein each strain was tested two times to ensure the reproducibility of the results.

First, *E. coli* DA63082 were tested against 2 mg/L CTX. In this experiment, *E. coli* DA63082+2 mg/L CTX in MH broth was used as the aqueous phase to generate droplets in light mineral oil in the microfluidic device of Example 1. To detect the resistant subpopulation, a CFU of 10^8 /mL was used. In n_1 N_d = 2614

were analyzed and droplets with shrinkage and bacterial growth were found N_{GS} = 38 and in n_2 N_d = 2982 were analyzed and droplets with shrinkage and bacterial growth were found N_{GS} = 67 as shown in Fig. 10a. The second clinically heteroresistant strain tested against CTX was *E. coli* DA63744. In this experiment, *E. coli* DA63744+2 mg/L CTX in MH broth was used as the aqueous phase to generate droplets in light mineral oil in the microfluidic device of Example 1. To detect the resistant subpopulation, a CFU of 10^8 /mL was used. In n_1 N_d = 3058 were analyzed and droplets with shrinkage and bacterial growth were found N_{GS} = 46 and in n_2 N_d = 3176 were analyzed and droplets with shrinkage and bacterial growth were found N_{GS} = 71 as shown in Fig. 10b.

Secondly, *E. coli* DA63082 were tested against 0.5 mg/L GEN. In this experiment, *E. coli* DA63082+0.5 mg/L GEN in MH broth was used as the aqueous phase to generate droplets in light mineral oil in the microfluidic device of Example 1. To detect the resistant subpopulation, a CFU of 5×10^8 /mL was used. In n_1 N_d = 2767 were analyzed and droplets with shrinkage and bacterial growth were found N_{GS} = 82 and in n_2 N_d = 2101 were analyzed and droplets with shrinkage and bacterial growth were found N_{GS} = 57 as shown in Fig. 10c. The second clinically heteroresistant strain tested against GEN was *E. coli* DA63340. In this experiment, *E. coli* DA63340+0.5 mg/L GEN in MH broth was used as the aqueous phase to generate droplets in light mineral oil in the microfluidic device of Example 1. To detect the resistant subpopulation, a CFU of 5×10^8 /mL was used. In n_1 N_d = 2377 were analyzed and droplets with shrinkage and bacterial growth were found N_{GS} = 123 and in n_2 N_d = 2172 were analyzed and droplets with shrinkage and bacterial growth were found N_{GS} = 140 as shown in Fig. 10d.

As summarized in Figs. 10a-10d and Table 2, the number of droplets imaged and analyzed (N_d) and the number of droplets with bacterial growth and associated shrinkage (N_{GS}) varied between 2101 to 3176 and 38 to 140, respectively. The frequencies of HR determined by PAP tests to the values from the experiments were compared and are presented in Table 2. As can be seen in Table 2 there was some minor variation, but overall, there was good agreement.

Table 2: Data showing the number of droplets analyzed (N_d), droplets with shrinkage (N_{GS}), and bacteria encapsulated per droplet in the case of clinical isolates of *E. coli* showing HR against CTX and GEN in

case of single and multiplex chip. PAP test frequencies are compared to the experimentally calculated values.

Droplet content		N_d	N_{GS}	Bacteria encapsulated per droplet	PAP test	Experimentally calculated frequency
Detecting resistant subpopulation HR clinical isolates in *E. coli* against single antibiotics						
DA63082 + 2mg/L CTX	N_1	2614	38	469	9.4×10^{-6}	3.1×10^{-5}
	N_2	2982	67	468		4.8×10^{-5}
DA63744 + 2mg/L CTX	N_1	3058	46	458	2.2×10^{-6}	3.3×10^{-5}
	N_2	3637	71	434		4.5×10^{-5}
DA63082 + 0.5 mg/L GEN	N_1	2767	82	2297	1.0×10^{-5}	1.3×10^{-5}
	N_2	2101	57	1051		2.6×10^{-5}
DA63340 + 0.5 mg/L GEN	N_1	2377	123	1622	2.0×10^{-5}	3.2×10^{-5}
	N_2	2172	140	1631		4.0×10^{-5}
Detecting resistant subpopulation HR clinical isolates in *E. coli* against multiple antibiotics						
Non-HR strain DA63333						
DA63333 (control)	N_1	523	43	0.37	N/A	N/A
	N_2	1121	53	0.32		N/A
DA63333 + 2mg/L CTX	N_1	607	0	182	$< 10^{-7}$	0
	N_2	1493	0	147		0
DA63333 + 0.5 mg/L GEN	N_1	515	0	985	$< 10^{-7}$	0
	N_2	1679	0	735		0
CTX HR strain DA63660						
DA63660 (Control)	N_1	2549	217	0.36	N/A	N/A
	N_2	9788	170	0.08		N/A
DA63660 + 2mg/L CTX	N_1	5260	59	197	2.6×10^{-5}	5.6×10^{-5}
	N_2	7705	53	121		5.7×10^{-5}
DA63660 + 0.5 mg/L GEN	N_1	573	0	2260	$< 10^{-7}$	0
	N_2	1076	0	2335		0
GEN HR strain DA62906						
DA62906 (Control)	N_1	1712	98	0.46	N/A	N/A
	N_2	1590	57	0.44		N/A

DA62906 + 2mg/L CTX	N_1	1797	0	483	$< 10^{-7}$	0
	N_2	2114	0	481		0
DA62906 + 0.5 mg/L GEN	N_1	2451	83	2080	2.0×10^{-5}	1.6×10^{-5}
	N_2	2788	58	2065		1.0×10^{-5}

EXAMPLE 6

To analyze the same clinical strains simultaneously against multiple antibiotics, we developed a multiplex chip comprised of a joint oil inlet and three independent aqueous phase inlets to generate and store three different kinds of droplets. For this set of experiments, we encapsulated (i) the clinical *E. coli* strain to be analyzed at 10^8 CFU/mL in 2 mg/L CTX, (ii) 5×10^8 CFU/mL of the clinical *E. coli* strain in 0.5 mg/L GEN and (iii) 10^5 CFU/mL in MH broth (control) to test if the strains were HR against CTX or GEN or non-HR as shown schematically in Fig. 14. All these experiments were performed two times (N_1 and N_2) and plotted side by side in Figs. 15a-15c. The experiments are described in more detail in the following subsections.

Detecting non-HR strain DA63333

In the first set of experiments, we used a clinically isolated non-HR strain DA63333 in the multiplex chip. The number of droplets in all the experiments varied from 523 to 1679 as shown in Fig. 15a and Table 2. Only the control experiments showed shrinkage of ~40-50 of the analyzed droplets. There was no growth and shrinkage observed in the case of CTX and GEN droplets, similarly to what was observed in the PAP test, demonstrating that the DA63333 strain does not display HR against CTX and GEN.

Detecting CTX HR strain DA63660

In the second set of experiments, we used the clinical CTX HR strain DA63660 in the multiplex chip following the same procedure. Here, the number of droplets in all the experiments varied from 573 to 9788 as shown in Table 2 and the control experiments showed shrinkage of 217 and 170 droplets respectively in N_1 and N_2. The two experiments involving DA63660 encapsulated with 2 mg/L CTX resulted in shrinkage of 59 and 53 droplets, as shown in Fig. 15b. Using the number of droplets showing shrinkage, we calculated the experimentally observed frequency of resistant bacteria and compared it with the PAP test results. Both assays were in good agreement in demonstrating CTX HR in this strain. There was no growth and shrinkage observed in the case of DA63660 encapsulated in 0.5 mg/L GEN as plotted in Fig. 15b, which is similar to the result from the PAP test.

Detecting GEN HR strain DA62906

In the third set of experiments, we used the clinical GEN HR strain DA62906 in the multiplex chip. The number of droplets in all the experiments varied from 1590 to 2788 as shown in Table 2. The control experiments showed shrinkage of 98 and 57 droplets respectively in N_1 and N_2. There was no growth and shrinkage observed in the case of DA62906 encapsulated in 2 mg/L CTX as plotted in Fig. 15c, which was similar to the PAP test. The two experiments involving DA62906 encapsulated in 0.5 mg/L GEN resulted in shrinkage of 83 and 58 droplets as plotted in Fig. 15c. From the number of droplets showing shrinkage, we calculated the experimentally observed frequency of resistant cells and compared it with PAP test results. Also in this case there was good agreement between the assays in demonstrating GEN HR in this *E. coli* strain.

Taken together, the multiplex chip could distinguish between non-HR, CTX HR, and GEN HR strains from a single experimental run.

EXAMPLE 7

To determine how *E. coli* grows in different sizes of droplets, we used the procedure as shown in Fig. 16. Droplets of different sizes (a total of 15 different sizes of droplets in the range of 20 µm to 200 µm in diameter) were generated using standard flow-focusing devices as shown in Fig. 16a. Light mineral oil with 3% PGPR as a surfactant was used as a continuous phase and MG1655 in MH broth media was used as a dispersed phase to generate droplets continuously. For droplet generation, the initial CFU per mL of MG1655 in MH broth was calculated for different sizes of the droplets generated to have a single/double encapsulation of MG1655 cells per droplet. These generated droplets were then transferred to a total of 6 different Eppendorf® tubes for different incubation times (0, 2, 3, 4, 8, and 24 hours) as shown in Fig. 16b. After the incubation time in each case, protocols as shown in Fig. 16c were performed. Finally, the resulting media was put on an agar plate for 24 hours at 37°C to determine the final CFU.

After 24 hours on agar plates, colonies of MG1655 were counted and plotted in Fig. 17a. To normalize the growth, we divided CFU at all the time points by CFU at 0 hours as shown in Fig. 17b. It shows that in droplet sizes below 130 µm, MG1655 growth became stagnant after 8 hours, while in the case of droplet sizes above 140 µm, MG1655 growth was still in the exponential phase. Taken together, we concluded that for having substantial growth of MG1655 over a longer period, it is preferred to use a droplet diameter over 140 µm, such as in the range of 180 to 220 µm.

EXAMPLE 8

We used a flow-focusing device to generate droplets of various sizes. The flow-focusing devices of various sizes were fabricated using standard soft lithography. Firstly, a Cr/glass mask was fabricated using a mask writer, and a total of 15 designs were printed on the mask. Following that, a Si wafer was cleaned using a plasma oven, and SU8 2050 was spin-coated to a thickness of 80 µm followed by standard pre-baking and post-baking protocols. The pattern was transferred to the SU8 with a mask aligner using UV lithography and the non-exposed SU8 was developed with a standard SU8 developer. The developed SU8 mold was then hard-baked at 120°C. The PDMS Sylgard 184 along with the curing agent in the ratio of 10:1 was molded on the fabricated SU8 master and cured in an oven at 65°C. Once cured, inlets and outlets were punched and the PDMS devices were bonded to a glass slide using a plasma oven.

EXAMPLE 9

Encapsulation of *E. coli* or any cells in droplets is governed by Poisson distribution and it depends on the initial concentration of cells (CFU/mL) and size of the droplet. We wanted to encapsulate 2000 or more CFU per droplet in the case of Gentamicin (GEN) and Tetracyclin (TET). We have therefore used 5×10^8 CFU/mL when detecting resistant frequencies of 10^{-5} and 10^{-6}. In the case of analyzing HR for Cefotaxime (CTX), we wanted to encapsulate 400 or more CFU per droplet and therefore used 10^8 CFU/mL for detecting resistant frequencies of 10^{-5} and 10^{-6}. When detecting resistant cells at 10^{-3} frequency we used only 2.5×10^7 CFU/mL at the encapsulation stage.

The detailed calculations are as follows: When a 5×10^8 CFU/mL of *E. coli* in MH broth is compartmentalized in a droplet of 4.7 nL size, the average number of CFU per droplet (λ) can be calculated as:

$$\lambda = \frac{5 \times 10^8\ CFU}{mL} \times \frac{4.7\ nL}{droplet} = 2350\ CFU/droplet$$

The same formula is used to calculate the average CFU per droplet in all cases. The λ obtained like this is used to calculate the experimentally observed HR frequency as follows:

$$Experimental\ HR\ frequency = \frac{N_{GS}}{N_d \times \lambda}$$

N_{GS} is the number of droplets showing bacterial growth and shrinkage, while N_d is the number of droplets analyzed.

EXAMPLE 10

This example investigated the usage of texture features in detecting heteroresistance in a bacterial population.

Microfluidic device design, fabrication, and setup

The microfluidic device consisted of two parts: the droplet generation part was a standard T-junction with an inlet of oil (HFE-7500 with 2% Fluosurf) as a surfactant having a channel width of 160 µm and an inlet of aqueous phase *(Acinetobacter baumannii* in Mueller-Hinton (MH) broth + antibiotics) with a 100 µm wide channel. The height of all channels was 160 µm. The droplet generation section generated droplets in the range of 180 to 220 µm in diameter. The droplet storage and incubation section could hold about 2000 to 3500 droplets at the present capacity of the microfluidic device. In the case of the multiplex chip, similar dimensions were used along with multiple droplet generators and incubation chambers. The microfluidic device mold was 3D printed using an ASIGA printer. The master mold was then used for PDMS casting with a 10:1 ratio of base to curing agent from Sylgard 184. Then the PDMS replica was punched at inlets and outlets with a 1 mm punch (Welltec) and bonded to a glass slide (Epredia) with a plasma oven (Diener Electronic, Germany). Syringe pumps (Cetoni, Nemesys pumps, Germany) were used to drive all fluids and the whole microfluidic setup was kept inside the stage-top incubator (OKOlab) maintained at 37°C and 80% humidity for the experiments.

Bacterial strains and culturing conditions

MH medium (Difco) was used for growth in broth and agar plates. Antibiotics (amikacin) were purchased from Sigma-Aldrich. For every experiment, bacteria frozen in 10% DMSO stocks were streaked on MH agar plates and incubated at 37°C overnight. Single colonies from the platers were inoculated in 1 mL MH broth, incubated at 37°C, 199 rpm agitation, overnight, and subsequently used for each experiment. Antibiotic stocks were prepared fresh from powder stock (Sigma Aldrich) in sterile PBS (Sigma-Aldrich) and used within 16 h of preparation.

Microscopy

We used an Olympus inverted microscope (IX73, Olympus) with a 10x objective, a motorized stage for x-y-z direction, and a camera (C11440, HAMAMTSU) for imaging of the droplets over 24 hours. The microfluidic device was placed in a stage-top incubator (OKOlab) with controlled temperature and humidity. Once droplets in the incubation chamber reached a stable state, each location was marked with a motorized stage in the x-y direction. Each location was then imaged every hour for the next 24 hours.

Image processing pipeline

The images that were obtained every hour were processed using a custom computational pipeline implemented in Python to determine the shrinkage in droplet diameter due to bacterial growth. Briefly, raw grayscale images were smoothened (Gaussian 31×31 kernel, standard deviation: 5), opened (21×21 kernel), min-max-normalized, and converted to unsigned 8-bit integer format. Droplet boundaries were identified by thresholding pre-processed images using Otsu's method (three classes: "droplet boundary", "other droplet content", "background"). The binary "droplet boundaries" masks were subjected to a circle Hough transform, droplet centroids were located by identifying peaks in accumulator-normalized Hough space, and maximum droplet radii were computed using a Euclidean distance transform of the "droplet boundaries" masks. Preliminary binary droplet masks were generated by labeling both "droplet boundaries" and "droplet disks" (given by droplet centroids and maximum radii) as regions corresponding to droplets. Droplets were then segmented based on a Euclidean distance transform of the preliminary droplet masks using a droplet centroid-seeded Watershed algorithm. Droplets touching the border of an image were excluded. Droplets were then quantified by computing mean intensities and gray-level co-occurrence matrix (GLCM, Haralick et al., Textural features for image classification, *IEEE Transactions on Systems, Man and Cybernetics* 3(6): 610-621, 1973) texture features namely contrast, dissimilarity (DIS), homogeneity (HOM), angular second moment (ASM), energy, and correlation. By correlating experimental images with the texture features we observed that homogeneity and correlation captured the bacterial growth accurately. Hence, we plotted these two parameters for the texture-based analysis. Droplet label masks were exported as TIFF files, and tabular droplet information (accumulator values, centroids, radii) was saved in CSV format. For downstream analysis, droplet sizes were normalized by the average droplet diameter at 0 hours and plotted against time every 6 hours for 24 to 30 hours.

The results are presented in Table 3 and in Fig. 18.

Table 3: Data showing the number of droplets analyzed (N_d), droplets with bacterial growth using homogeneity (N_{GH}), droplets with bacterial growth using correlation (N_{GC}) and bacteria encapsulated per droplet in the case of clinical isolates of *A. Baumannii* (DA33098) showing heteroresistance against AMK. PAP test frequencies were compared to the experimentally calculated values.

Droplet content		**N_d**	**N_{GH}**	**N_{GC}**	**Bacteria encapsulated per droplet**	**PAP test**	**Calculated frequency with homogeneity**	**Calculated frequency with correlation**

DA33098 + 8 mg/L AMK	N_1	2066	398	406	2457	10^{-3}	7.84×10^{-5}	8×10^{-5}
	N_2	667	194	214	2885		10^{-4}	1.11×10^{-4}

The embodiments described above are to be understood as a few illustrative examples of the present invention. It will be understood by those skilled in the art that various modifications, combinations and changes may be made to the embodiments without departing from the scope of the present invention. In particular, different part solutions in the different embodiments can be combined in other configurations, where technically possible.

CLAIMS

1. A method of detecting heteroresistance in a microbial population of microbes, the method comprises:

generating (S1) droplets comprising at least one microbe and an antimicrobial agent;

incubating (S2) the droplets in conditions supporting growth of the microbes in the droplets;

monitoring (S3) the size of the droplets during incubation; and

detecting (S4) heteroresistance to the antimicrobial agent in the microbial population based on detection of a shrinkage in size of at least one droplet comprising at least one microbe but no shrinkage in size of at least one other droplet comprising at least one microbe.

2. The method according to claim 1, wherein generating (S1) droplets comprises generating (S1) droplets comprising at least one microbe and the antimicrobial agent in a culture medium.

3. The method according to claim 2, wherein generating (S1) droplets comprises:

flowing (S10) a continuous phase comprising an oil in a microfluidic channel (14);

flowing (S11) a dispersed phase comprising the microbes and the antimicrobial agent in the culture medium in a perpendicular microfluidic channel (12); and

generating (S12) the droplets at a T-junction (15) between the microfluidic channel (14) and the perpendicular microfluidic channel (12).

4. The method according to any one of claims 1 to 3, wherein generating (S1) droplets comprises generating (S1) microbe-containing droplets comprising, on average, at least 10 microbes per microbe-containing droplet, preferably at least 25 microbes per microbe-containing droplet, more preferably at least 50 microbes per microbe-containing droplet, and most preferably from 100 up to 3000 microbes per microbe-containing droplet.

5. The method according to any one of claims 1 to 4, wherein

generating (S1) droplets comprises generating (S1) microbe-containing droplets comprising at least one microbe per microbe-containing droplet and empty droplets that do not contain any microbe; and

the microbe-containing droplets constitute less than 75 %, preferably less than 50 %, more preferably less than 25 %, and most preferably less than 10 %, of the generated droplets.

6. The method according to any one of claims 1 to 5, wherein

generating (S1) droplets comprises generating (S1) the droplets comprising at least one microbe and the antimicrobial agent at a droplet generator (19) of a microfluidic device (10);

incubating (S2) the droplets comprises incubating (S2) the droplets in conditions supporting growth of the microbes in the droplets in an incubation chamber (17) of the microfluidic device (10) in fluid connection with the droplet generator (19); and

monitoring (S3) the size comprises monitoring (S3) the size of the droplets during incubation in the incubation chamber (17).

7. The method according to any one of claims 1 to 6, wherein generating (S1) droplets comprises generating (S1) droplets comprising the at least one microbe and the antimicrobial agent and having an average diameter selected within an interval of from 125 up to 300 µm, preferably selected within an interval of from 105 up to 275 µm, and more preferably selected within an interval of from 175 up to 225 µm, such as selected within an interval of from 180 to 220 µm.

8. The method according to any one of claims 1 to 7, wherein generating (S1) droplets comprises generating (S1) droplets comprising the at least one microbe and the antimicrobial agent and having an average volume selected within an interval of from 1 up to 15 nL, preferably selected within an interval of from 2 up to 10 nL, and more preferably selected within an interval of from 4 up to 8.5 nL.

9. The method according to any one of claims 1 to 8, wherein incubating (S2) the droplets comprises incubating (S2) the droplets in conditions supporting growth of the microbes in the droplets while maintaining droplets in physical contact with neighboring droplets.

10. The method according to any one of claims 1 to 9, wherein monitoring (S3) the size comprises taking (S20) at least one image of the droplets during incubation.

11. The method according to any one of claims 1 to 10, wherein detecting (S4) heteroresistance comprises detecting (S4) heteroresistance to the antimicrobial agent in the microbial population based on detection of a reduction in diameter of at least one droplet comprising at least one microbe but no reduction in diameter of at least one other droplet comprising at least one microbe.

12. The method according to claim 11, wherein detecting (S4) heteroresistance comprises detecting (S4) heteroresistance to the antimicrobial agent in the microbial population based on detection of a

reduction in diameter of at least one droplet comprising at least one microbe but no change in diameter or an increase in diameter of at least one other droplet comprising at least one microbe.

13. The method according to any one of claims 1 to 12, further comprising:

determining (S30) an initial distribution of sizes of the droplets comprising at least one microbe prior to incubating the droplets or at a first time point during incubating the droplets; and

determining (S31) a subsequent distribution of sizes of the droplets comprising at least one microbe at a later time point during incubating the droplets, wherein

detecting (S4) heteroresistance comprises detecting (S4) heteroresistance to the antimicrobial agent in the microbial population based on a comparison of the subsequent distribution of sizes and the initial distribution of sizes.

14. The method according to any one of claims 1 to 12, wherein monitoring the size comprises:

identifying (S40) a plurality of droplets comprising at least one microbe and the antimicrobial agent;

for each identified droplet of the plurality of droplets:

determining (S41) an initial size of the identified droplet prior to incubating the droplets or at a first time point during incubating the droplets; and

determining (S42) a subsequent size of the identified droplet at a later time point during incubating the droplets, wherein

detecting (S4) heteroresistance comprises detecting (S4) heteroresistance to the antimicrobial agent in the microbial population based on pairwise comparisons of the subsequent sizes and the initial sizes for the identified droplets.

15. The method according to any one of claims 1 to 12, further comprising:

determining (S50) an average diameter of the droplets comprising at least one microbe prior to incubating the droplets or at a first time point during incubating the droplets; and

calculating (S51), for each droplet of a plurality of droplets comprising at least one microbe, a normalized droplet size representing a ratio between a diameter of the droplet and the average diameter, wherein

detecting (S4) heteroresistance comprises detecting (S4) heteroresistance to the antimicrobial agent in the microbial population based on a distribution of the normalized droplet sizes.

16. The method according to any one of claims 1 to 15, wherein the microbial population is a bacterial population of bacteria and the antimicrobial agent is an antibacterial agent.

17. The method according to any one of claims 1 to 16, further comprising determining, for at least a subset of the monitored droplets, at least one texture feature, preferably at least one gray level co-occurrence matrix (GLCM) texture feature, and more preferably GLCM correlation or homogeneity, wherein detecting (S4) heteroresistance comprises detecting (S4) heteroresistance to the antimicrobial agent in the microbial population based on detection of the shrinkage in size of at least one droplet comprising at least one microbe but no shrinkage in size of at least one other droplet comprising at least one microbe and based on the at least one texture feature.

18. A system (1) for detecting heteroresistance in a microbial population of microbes, the system (1) comprises:

a microfluidic device (10) comprising:

a sample inlet (11) configured to receive a sample comprising microbes and an antimicrobial agent in a culture medium;

an oil inlet (13) configured to receive an oil sample;

a droplet generator (19) in fluid connection with the sample inlet (11) and the oil inlet (13) and configured to generate droplets comprising at least one microbe and the antimicrobial agent in the culture medium; and

an incubation chamber (17) in fluid connection with the droplet generator (19) and configured to house a plurality of droplets in conditions supporting growth of the microbes in the droplets;

a camera (30) arranged to take images of the droplets in the incubation chamber (17);

a memory (22) configured to store program instructions; and

a processor (21) that, when executing the program instructions, is configured to:

process the at least one image to determine sizes of droplets in the incubation chamber (17); and

detect heteroresistance to the antimicrobial agent in the microbial population based on detection of a shrinkage in size of at least one droplet comprising at least one microbe but no shrinkage in size of at least one other droplet comprising at least one microbe.

19. The system according to claim 18, wherein the microfluidic device (10) comprises:

a microfluidic channel (14) in fluid connection with the oil inlet (13); and

a perpendicular microfluidic channel (12) in fluid connection with the sample inlet (11),

the droplet generator (19) comprises a T-junction (15) between the microfluidic channel (14) and the perpendicular microfluidic channel (12).

20. The system according to claim 18 or 19, wherein the microfluidic device (10) comprises a microfluidic meander channel (16) interconnecting the droplet generator (19) and the incubation chamber (17).

21. The system according to any one of claims 18 to 20, wherein the droplet generator (19) is configured to generate microbe-containing droplets comprising, on average, at least 10 microbes per microbe-containing droplet, preferably at least 25 microbes per microbe-containing droplet, more preferably at least 50 microbes per microbe-containing droplet, and most preferably from 100 up to 3000 microbes per microbe-containing droplet.

22. The system according to any one of claims 18 to 21, wherein

the droplet generator (19) is configured to generate microbe-containing droplets comprising at least one microbe per microbe-containing droplet and empty droplets that do not contain any microbe; and

the microbe-containing droplets constitute less than 75 %, preferably less than 50 %, more preferably less than 25 %, and most preferably less than 10 %, of the droplets.

23. The system according to any one of claims 18 to 22, wherein the droplet generator (19) is configured to generate droplets comprising the at least one microbe and the antimicrobial agent in the culture medium and having an average diameter selected within an interval of from 125 up to 300 µm, preferably selected within an interval of from 150 up to 275 µm, and more preferably selected within an interval of from 175 up to 225 µm, such as selected within an interval of from 180 up to 220 µm.

24. The system according to any one of claims 18 to 23, wherein the droplet generator (19) is configured to generate droplets comprising the at least one microbe and the antimicrobial agent in the culture medium and having an average volume selected within an interval of from 1 up to 15 nL, preferably selected within an interval of from 2 up to 10 nL, and more preferably selected within an interval of from 4 up to 8.5 nL.

25. The system according to any one of claims 18 to 24, wherein the incubation chamber (17) is configured to house the plurality of droplets in conditions supporting growth of the microbes in the droplets while maintaining droplets in physical contact with neighboring droplets.

26. The system according to any one of claims 18 to 25, wherein the processor (21) is, when executing the program instructions, configured to:

process the at least one image to determine diameters of droplets in the incubation chamber (17); and

detect heteroresistance to the antimicrobial agent in the microbial population based on detection of a reduction in diameter of at least one droplet comprising at least one microbe but no reduction in diameter of at least one other droplet comprising at least one microbe.

27. The system according to claim 26, wherein the processor (21) is, when executing the program instructions, configured to detect heteroresistance to the antimicrobial agent in the microbial population based on detection of a reduction in diameter of at least one droplet comprising at least one microbe but no change in diameter or an increase in diameter of at least one other droplet comprising at least one microbe.

28. The system according to any one of claims 18 to 27, wherein the processor (21) is, when executing the program instructions, configured to:

determine an initial distribution of sizes of the droplets comprising at least one microbe prior to incubating the droplets or at a first time point during incubating the droplets;

determine a subsequent distribution of sizes of the droplets comprising at least one microbe at a later time point during incubating the droplets; and

detect heteroresistance to the antimicrobial agent in the microbial population based on a comparison of the subsequent distribution of sizes and the initial distribution of sizes.

29. The system according to any one of claims 18 to 27, wherein the processor (21) is, when executing the program instructions, configured to:

identify a plurality of droplets comprising at least one microbe and an antimicrobial agent in the at least one image;

determine, for each identified droplet of the plurality of droplets, an initial size of the identified droplet prior to incubating the droplets or at a first time point during incubating the droplets;

determine, for each identified droplet of the plurality of droplets, a subsequent size of the identified droplet at a later time point during incubating the droplets; and

detect heteroresistance to the antimicrobial agent in the microbial population based on pairwise comparisons of the subsequent sizes and the initial sizes for identified droplets.

30. The system according to any one of claims 18 to 27, wherein the processor (21) is, when executing the program instructions, configured to:

determine, based on the at least one image, an average diameter of the droplets comprising at least one microbe prior to incubating the droplets or at a first time point during incubating the droplets;

calculate, for each droplet of a plurality of droplets comprising at least one microbe, a normalized droplet size representing a ratio between a diameter of the droplet and the average diameter; and

detect heteroresistance to the antimicrobial agent in the microbial population based on a distribution of the normalized droplet sizes.

31. The system according to any one of claims 18 to 30, wherein the microfluidic device (10) comprises:

multiple sample inlets (11A, 11B, 11C), wherein each sample inlet (11A, 11B, 11C) of the multiple sample inlets (11A, 11B, 11C) is configured to receive a respective sample comprising microbes and an antimicrobial agent in a culture medium;

multiple droplet generator (19A, 19B, 19C), wherein each droplet generator (19A, 19B, 19C) of the multiple droplet generators (19A, 19B, 19C) is in fluid connection with the oil inlet (13) and a respective sample inlet (11A, 11B, 11C) of the multiple sample inlets (11A, 11B, 11C) and configured to generate droplets comprising at least one microbe and the antimicrobial agent in the culture medium; and

multiple incubation chambers (17A, 17B, 17C), wherein each incubation chamber (17A, 17B, 17C) of the multiple incubation chambers (17A, 17B, 17C) is in fluid connection with a respective droplet generator (19A, 19B, 19C) of the multiple droplet generator (19A, 19B, 19C) and configured to house a plurality of droplets in conditions supporting growth of the microbes in the droplets.

32. The system according to any one of claims 18 to 31, wherein the processor (21) is, is, when executing the program instructions, configured to:

determine, for at least a subset of the monitored droplets, at least one texture feature, preferably at least one gray level co-occurrence matrix (GLCM) texture feature, and more preferably GLCM correlation or homogeneity; and

detect heteroresistance to the antimicrobial agent in the microbial population based on detection of the shrinkage in size of at least one droplet comprising at least one microbe but no shrinkage in size of at least one other droplet comprising at least one microbe and based on the at least one texture feature.