



- (51) **International Patent Classification:**
C12Q 1/18 (2006.01) *G01N 33/94* (2006.01)
G01N 33/04 (2006.01)
- (21) **International Application Number:**
PCT/EP2012/059330
- (22) **International Filing Date:**
21 May 2012 (21.05.2012)
- (25) **Filing Language:** English
- (26) **Publication Language:** English
- (30) **Priority Data:**
11166682.2 19 May 2011 (19.05.2011) EP
- (71) **Applicant (for all designated States except US):** **DSM IP Assets B.V.** [NL/NL]; Het Overloon 1, NL-6411 TE Heerlen (NL).
- (72) **Inventors; and**
- (75) **Inventors/Applicants (for US only):** **GRAAF, DE, Tim** [NL/NL]; P.O. Box 130, NL-6100 AC Echt (NL). **SANTEN, VAN, Laurentius Cornelis Adrianus** [NL/NL]; P.O. Box 130, NL-6100 AC Echt (NL). **LANGEVELD, Pieter Cornelis** [NL/NL]; P.O. Box 130, NL-6100 AC Echt (NL).
- (74) **Agents:** **BIERMANN, Jan** et al.; DSM Intellectual Property, P.O. Box 130, NL-6100 AC Echt (NL).

- (81) **Designated States** (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.
- (84) **Designated States** (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— with international search report (Art. 21(3))

(54) **Title:** METHOD FOR THE DETERMINATION OF THE PRESENCE OF AN ANTIBIOTIC IN A FLUID

(57) **Abstract:** The present invention provides a method and test for the determination of the presence or absence of an antibiotic in a sample such as milk.



WO 2012/156528 A1

METHOD FOR THE DETERMINATION OF THE PRESENCE OF AN ANTIBIOTIC IN A FLUID

Field of the invention

The present invention relates to a new and improved microbial growth inhibition test for the determination of the presence or absence of an antibiotic in a sample such as milk.

Background of the invention

Nowadays, antibiotics are frequently used in veterinary practice not only for treatment of bacterial infections, but also for prophylactic purposes to improve the productivity of foodstuffs. In recent years, this irresponsible misuse of antibiotics as a preventive measure has been a decisive factor favoring the growth of bacterial resistance.

Antibiotic residues are known to be among the most frequently detected contaminants in milk and dairy products and cause important problems in this industrial sector at economical level.

To prevent the negative impact of antibiotic residues on human health and on the entire ecosystem, microbial growth inhibition tests for the determination of the presence or absence of antibiotic residues in a sample have been developed. Examples of such tests have been described in EP 0 005 891A and EP 0 285 792A. The tests described therein are ready-to-use tests that make use of a test organism and an indicator molecule, for instance a pH- and/or redox-indicator. The general principle of the test is that, when an antibiotic is present in a sample in a concentration sufficient to inhibit growth of the test organism, the color of the indicator will stay the same, while, when no inhibition occurs, growth of the test organism is accompanied by the formation of acid or reduced metabolites or other phenomena that will induce an indicator signal.

The tests have been designed to respond to antibiotic residues at or above maximum residue limits (MRL). This gives a high degree of certainty that violative samples will be detected before entering the food chain. However, the problem with the tests currently distributed on the market is that they give positive results below violative

levels, leading to so-called false positives. Consequently, when using the currently marketed tests, there is a severe risk of rejecting milk that is still within legislative limits. The practical consequences thereof would be the unnecessary and unjustifiable disposal of milk, which is unacceptable to producers.

5 In WO 2005/118838 and WO 2005/049809 it has been described that a protein capable of binding a beta-lactam antibiotic, such as for example an antibody or a penicillin-binding protein, can be added to the sample or the test to reduce the sensitivity of the test towards beta-lactam antibiotics. There are however several disadvantages associated with the use of these proteins. First of all, the proteins need to be produced
10 and purified before use which is time consuming and expensive and therefore economical unattractive. Secondly, the proteins described in WO 2005/118838 and WO 2005/049809 are beta-lactam specific, making them unsuitable for reducing the sensitivity of the microbial growth inhibition tests to other families of antibiotics. Thirdly, the proteins bind to intact beta-lactam antibiotics. Therefore, the risk of false positive
15 resulting from non-intact beta-lactam antibiotics is still present. Fourthly, the proteins described in WO 2005/118838 and WO 2005/049809 cannot be used under all conditions. For instance, certain conditions might impede binding of the proteins to the beta-lactam antibiotics or might lead to degradation of the proteins. Consequently, the proteins cannot be used under each and every condition.

20 In view of the above, there is thus a need for an improved microbial growth inhibition test le that does not have the above-identified disadvantages.

Summary of the invention

25 It is an object of the present invention to provide a simple, inexpensive and easy-to-use, broadly applicable microbial growth inhibition test with a low amount of false positives for the determination of the presence or absence of an antibiotic in a sample such as milk. Surprisingly, it has been found that such a test can be provided when the microbial culture used in the test is prepared in the presence of an antibiotic.

Detailed description of the invention

30 In a first aspect the present invention is directed to a method for reducing the sensitivity of a microbial growth inhibition test for the determination of the presence or absence of an antibiotic in a sample to at least one family of antibiotics, the method comprising the steps of (a) preparing a test organism, (b) contacting a fluid sample with the

test organism, (c) growing the test organism, and (d) detecting the amount of growth of the test organism, wherein lack of growth reflects the presence of an antibiotic in the sample, characterized in that an antibiotic is present in step a and the sensitivity of the test to at least one family of antibiotics is reduced by the presence of the antibiotic in step a.

5 In step a of the above method the test organism or spores thereof are produced by growing the test organism or spores thereof in the presence of a growth medium comprising an antibiotic. Preferably, the medium is liquid. After growth of the test organism or spores thereof to sufficient amounts, the growth medium comprising the antibiotic is removed from the test organism or spores thereof. The obtained test organism or spores
10 thereof are then added to the test medium to prepare the microbial growth inhibition test. Suitable components that can be present in the test medium are described below. After preparation of the microbial growth inhibition test, a fluid sample is added to the test and the test can be performed according to conditions as set forth below.

In a further aspect the invention relates to a microbial growth inhibition test wherein
15 the test organism used in the test has been prepared in the presence of an antibiotic. In an embodiment the microbial growth inhibition test according to the invention is used for the determination of the presence or absence of an antibiotic and comprises a test organism, an indicator and a gelling agent, characterized in that the test organism has been prepared in the presence of an antibiotic and the sensitivity of the test to at least one family of antibiotics
20 is reduced compared to a test wherein the test organism has been prepared in the absence of an antibiotic. The below embodiments are applicable to all aspects of the invention.

In an embodiment of the invention the microbial growth inhibition test comprises an indicator. The term "indicator" refers to a substance used to measure (for example by change of colour or fluorescence) the condition of a test medium with respect to the
25 presence of a particular component (for example an acid, a base, oxidizing or reducing agents). Particularly useful are indicators that, upon changing from one state to the other, provide a visually detectable signal such as a change in color or fluorescence. The amount of indicator in the test medium is generally between 0.01 and 50 g/l test medium, preferably between 0.1 and 10 g/l, more preferably between 0.5 and 5 g/l, most
30 preferably between 1 and 3 g/l. The indicator may be a pH-indicator, a redox-indicator or a combination thereof. The term also may refer to two or more indicators. The skilled artisan will appreciate that many indicators are suitable for the purpose of the present invention. Examples of suitable indicators can be found in handbook H.J. Conn's Biological Stains, R.D. Lillie ed., Baltimore, 1969.

The test may have the form of a liquid, a solid or a gel-like matrix. In an embodiment of the invention the microbial growth inhibition test further comprises a gelling agent. The term "gelling agent" as used herein refers to a compound that assists in changing a mixture into a gel or taking on the form of a gel. Examples of suitable gelling agents in the various aspects of the present invention include, but are not limited to, agar, gelatin, alginic acid and salts thereof, carrageenan, locust bean gum (Carob gum), hydroxypropyl guar and derivatives thereof, processed eucheuma seaweed and the like. Agar is the preferred gelling agent. In an embodiment a test organism and an indicator and optionally other additional ingredients are introduced into an agar solution. The agar solution is allowed to solidify to form the test medium such that the test - organism stays alive, but cannot multiply because of e.g. low temperature. The amount of gelling agent in the test is generally between 1 and 200 g/l test medium, preferably between 2 and 50 g/l, more preferably between 5 and 20 g/l, most preferably between 7 and 15 g/l.

When the test medium has the form of a solid matrix, it may comprise a carrier material such as a ceramic, cotton, glass, a metal particle, a polymer in any shape or form, a silicate, a sponge, wool and the like. Alternatively, the test may have the form of a tablet, disc or paper filter comprising the test organism, indicator and optionally nutrient. The three constituents may be present in a single tablet, but also in two or more tablets. Of course, test systems combining test media in solid, liquid and/or gel-like form may be used.

Optionally, the microbial growth inhibition test may also contain nutrients, stabilizers, salts, buffers and/or viscosity-increasing agents. The term "nutrient" as used herein refers to a nutritive substance or ingredient that promotes and/or is required for the growth of the test organism. Suitable nutrients depend from the microorganism used in the test system. The test medium may comprise two or more different nutrients. They include, but are not limited to, assimilable carbon sources such as carbohydrates such as e.g. glucose, fructose, sucrose, lactose and dextrose; assimilable nitrogen sources such as amino acids such as e.g. peptone or tryptone; sources of vitamins and growth factors such as beef or yeast extract; and sources of minerals such as earth alkaline metal salts such as salts of e.g. barium or calcium. Suitable additional ingredients that may be present in the test according to the present invention are known to the person skilled in the art.

In an embodiment of the invention the test organism is selected from the group consisting of a *Bacillus* species, an *Escherichia* species and a *Streptococcus* species. In

a preferred embodiment of the invention the test organism is thermophilic. Examples are *Bacillus stearothermophilus* or *Streptococcus thermophilus*, with *Bacillus stearothermophilus* being preferred. These species may be introduced in the test as units capable of producing colonies, or Colony Forming Units (CFUs). The term "CFU" as used herein refers to the number of test organisms, spores of test organisms, partially germinated spores of test organisms, vegetative cells or any mixture thereof capable of producing colonies of organisms. The concentration of said CFUs is expressed as Colony Forming Units per ml of test medium (CFU/ml) and is usually in the range of 1×10^5 to 1×10^{12} CFU/ml, preferably 1×10^6 to 1×10^{10} CFU/ml, more preferably 2×10^6 to 1×10^9 CFU/ml, most preferably 5×10^6 to 1×10^8 CFU/ml, or still more preferably 5×10^6 to 2×10^7 CFU/ml.

The test organism is prepared before being added to the test medium. This is done by culturing the test organism in suitable conditions before the addition to the test medium. When, for example, a thermophilic test organism is prepared, said temperature is preferably between 40 and 70°C, more preferably between 50 and 65°C. Culturing of a test organism such as for instance *Bacillus stearothermophilus* may comprise different phases including germination, growth, sporulation and maturation. In an embodiment different conditions are applied in the different phases. Suitable conditions are known to the person skilled in the art. The medium used in the different phases may contain nutrients, stabilizers, salts, buffers and/or viscosity-increasing agents, to name just a few. Suitable nutrients include, but are not limited to, assimilable carbon sources such as carbohydrates such as e.g. glucose, fructose, sucrose, lactose and dextrose; assimilable nitrogen sources such as amino acids such as e.g. peptone or tryptone; sources of vitamins and growth factors such as beef or yeast extract; and sources of trace elements such as manganese, copper or zinc ;sources of earth alkaline metal salts such as salts of e.g. barium or calcium. Suitable additional ingredients that may be present in the test according to the present invention are known to the person skilled in the art. In an embodiment, germination, growth and/or sporulation take place in sub-merged cultures. Alternatively, germination, growth and/or sporulation take place in plates containing a gelling agent such as for instance agar.

In an embodiment of the invention the sample may be derived from a body liquid, an organ, meat or eggs. Antibiotics might also be present in food products in which these animal products are added as an ingredient. Examples of food products are milk; meat of cow, pig, poultry and fish; sea food such as shrimps; liver; processed meat products such as

sausages; ready-to-eat meals and baby food. Antibiotics might also be present in body liquids or animal tissues, which are suitable for examination by for example food inspection authorities. Examples are blood, kidney tissue or pre-urine obtained from the kidney and urine. Urine and blood are suitable for examination prior to slaughtering of the animal.

5 Antibiotics may also be present in waste water, water from any type industry, etc. In a preferred embodiment the sample is urine, blood, egg, honey, kidney, meat, liver, fish, shrimp, feed and/or milk, with milk being most preferred. In an embodiment the sample might not be fluid and fluid comprising the antibiotic(s) needs to be extracted from the sample.

10 In an embodiment of the invention the antibiotic present in the preparation of the test organism is a beta-lactam antibiotic. In a preferred embodiment of the invention the beta-lactam antibiotic is a cephalosporin or penicillin derivative. Examples of such derivatives are amoxicillin, ampicillin, cefadroxil, cefradine, ceftiofur, cephalixin, penicillin G, penicillin V and ticarcillin, to name just a few. In a preferred embodiment the beta-lactam antibiotic is a

15 penicillin derivative. In an embodiment of the invention the microbial culture is prepared in the presence of 10 ppb or less of the antibiotic, preferably 5 ppb or less, more preferably 4 ppb or less, even more preferably 3 ppb or less, and most preferably 2.5 ppb or less. In particular, the microbial culture is prepared in the presence of 0.1 to 10 ppb of the antibiotic, preferably 0.2 to 5 ppb of the antibiotic, more preferably 0.3 to 4 ppb of the antibiotic, even

20 more preferably 0.4 to 3 ppb of the antibiotic and most preferably 0.5 to 2.0 ppb of the antibiotic.

In an embodiment of the invention the at least one family of antibiotics is selected from the group consisting of the family of beta-lactam antibiotics, the family of tetracycline antibiotics, the family of sulfonamide antibiotics, the family of aminoglycoside antibiotics, and

25 the family of quinolone antibiotics. In a preferred embodiment of the invention the at least one family of antibiotics is selected from the group consisting of the family of beta-lactam antibiotics, the family of tetracycline antibiotics and the family of sulfonamide antibiotics. In an embodiment of the invention the presence of the antibiotic in the preparation of the test organism reduces the sensitivity of the test to at least two families of antibiotics. In another

30 embodiment of the invention the presence of the antibiotic in the preparation of the test organism reduces the sensitivity of the test to at least three families of antibiotics. It was established that addition of an antibiotic such as a beta-lactam antibiotic, e.g. penicillin, results in reduction of sensitivity of the microbial growth inhibition test for the family of beta-lactam antibiotics. Surprisingly, not only the sensitivity for these compounds could be

adjusted, simultaneously the sensitivity for other antibiotic families such as the family of tetracycline antibiotics and the family of sulfonamide antibiotics was also reduced.

Preferably, there is minimal or no germination and outgrowth of the test organism prior to the addition of fluid sample. This is achieved by storing and keeping the test under
5 conditions comprising an unfavorable temperature and/or an unfavorable pH-value and/or the absence of nutrients essential for germination and outgrowth of the test organism. Of course, the conditions should not cause irreversible damage to all CFUs present in the microbial growth inhibition test.

After contacting the fluid sample with the test organism, growth of the test organism
10 is allowed to take place during a period sufficiently long for the test organisms to grow in case no antibiotic is present. Growth is encouraged by adding nutrients, optionally before the contacting of said fluid sample, and/or raising the temperature, and/or providing for a pH-value at which the test organism is able to grow. Alternatively, these conditions may be established prior to contact of the fluid sample with the test organism.

The amount of growth of the test organism is detected by observing the presence
15 or absence of a change of the indicator. Lack of growth reflects the presence of an antibiotic in the sample. As indicated above, the presence or absence of an antibiotic is determined by the presence or absence of a change of the indicator(s) used. When, for example such a change is a color change, said color change may be observed visually.
20 However, said color change may also be determined using an arrangement that generates digital image data or an arrangement that generates analog image data and converts said analog image data into digital image data followed by interpretation of said digital image data by a computer processor. An example of such an arrangement, *i.e.* a sample-reading device such as a scanner coupled to a personal computer, is described
25 in WO 03/033728. Another example of such an arrangement, *i.e.* a combined sample incubating and sample-reading device (such as a scanner combined with an incubator) coupled to a personal computer is described in WO 2007/090683. Both documents are herewith incorporated by reference.

Optionally, certain test ingredients are sterilized and usually the pH of the test is
30 adjusted to the required value. Optionally, samples may be mixed (*e.g.* with other samples, but also with salts, buffering compounds, nutrients, stabilizers, enzymes, and the like), concentrated and/or diluted (*e.g.* with diluting liquids such as water, solvents, and the like) prior to addition to the test organism.

In an embodiment of the invention, the test organism is grown by incubating it for a predetermined period, preferably within a time span of 0.5 to 6 hours, more preferably between 0.75 to 5 hours, most preferably between 1.0 to 4 hours. Preferably the test organism is incubated at a predetermined temperature, preferably the optimal growth temperature of the test organism. When, for example, thermophilic test organisms are used, said temperature is preferably between 40 and 70°C, more preferably between 50 and 65°C, most preferably between 60 and 64°C. Optionally said reaction can be carried out with the aid of a thermostatic device. Alternatively, the time required for growth of the test organism is equal to the time that is required for a calibration sample with a known amount of antibiotic to induce a change in the indicator.

In an embodiment of the invention the test organism is present in one or more containers. Optionally, the containers are part of a kit which further comprises a sampling device. The containers may be test tubes of any shape and size and from any material available, provided that observation of indicator changes is possible. Also, the containers may be wells such as those incorporated in microtiter plates. A sampling device is a device with the aid of which fluid can be added to the microbial growth inhibition test. Examples include, but are not limited to, a container (optionally with volume markings) a syringe, a pipette or an automated pipetting system. Such a syringe or pipette may be designed in such a fashion that with only one mode of operation a predetermined volume can be withdrawn from the fluid sample to be analyzed. Optionally, systems known in the art with which more than one syringe or pipette can be operated with one single handling may be applied. Optionally, the kit further comprises means for sealing of said containers filled with test organism during incubation and/or an insert with instructions for use and/or a means for setting the time needed for incubation.

Optionally, the ratio of the sample to test medium exceeds 2:3 (0.68:1) (v/v). Preferably, said ratio is at least 20:27 (0.74:1) (v/v), more preferably said ratio is at least 25:27 (0.93:1) (v/v); most preferably said ratio is at least 2:1 (v/v). It has been found that there is no technical reason for an upper limit to the amount of sample. In practice, this volume should not exceed the maximum content of the container that holds the test medium. For example, in a 2 ml container having 0.2 ml test medium, no more than 1.8 ml of fluid sample should be added. In practice, containers for performing the method of the present invention have a volume that rarely exceeds 50 ml and hence the amount of fluid sample to be added shall not exceed 50 ml, preferably 10 ml, more preferably 5 ml, still more preferably 2 ml, most preferably 1 ml. Thus, in general, the upper limit of the ratio of

the volume of fluid sample to the volume of test medium is 250:1 (v/v), preferably 50:1 (v/v), more preferably 25:1 (v/v), still more preferably 10:1 (v/v), most preferably 5:1 (v/v). In a preferred embodiment the volume of the dilution/sample is greater than the volume of test medium.

5 Optionally, the kit further comprises a thermostatic device, with the aid of which samples can be kept at a pre-set temperature, such as the temperature at which the test organism shows sufficient growth. Preferably, said thermostatic device is designed in such a fashion that it can hold said containers filled with the test organism. Optionally, the thermostatic device is coupled to a means for setting the time needed for incubation
10 such that heating and/or cooling is stopped after lapse of a pre-set period.

 Optionally, the kit further comprises a data carrier loaded with a computer program suitable for instructing a computer to analyze digital data obtained from a sample-reading device. Said data carrier may be any carrier suitable for storing digital information such as a CD-ROM, a diskette, a DVD, a memory stick, a magnetic tape or
15 the like. Advantageously, said data carrier loaded with a computer program provides for easy access to the latest available computer programs suitable for use in the method of the present invention.

EXAMPLES

20

Example 1

Sensitivity of a microbial growth inhibition test for different antibiotics as a function of the amount of antibiotic that was present in the preparation of the test organism

 A strain of *Bacillus stearothermophilus* var. *calidolactis* was plated on plate count
25 agar, pH 8.0. The plate count agar was prepared according to manufacturers instructions. Next, 0, 1.0, 2.0 or 3.0 µg penicillin G per litre plate count agar was added to the plates. Plates were incubated at 52°C for 1-3 days. After incubation, a single colony was picked per plate and cultured on a new plate comprising plate count agar with penicillin G at the same concentration as the concentration that was used during the
30 first incubation. After incubation at 52°C for 1-3 days, the colonies were harvested per plate and re-suspended in 5 ml normal saline.

 The obtained spore solution was inoculated in a shake flask containing 500 ml sporulation medium. The spores were incubated at 55°C, 200 RPM for 3 days. After 3 days, the spores were stored at 2-7°C for 7 days. The spores were centrifuged at

3600xg. The obtained pellet was re-suspended in 10 ml sporulation medium by shaking thoroughly. The obtained spore suspension was pasteurized for 10 minutes at 80°C $\pm$ 0.5°C. Thereafter, microbial growth inhibition test were prepared essentially as described in EP 0 005 891A by using the spores prepared as described above.

5 The sensitivity of the test for various families of antibiotics was determined by analyzing raw milk samples spiked with different concentrations of various antibiotics. The tests were read at the moment at which an antibiotic-free sample changed color from purple to yellow. The results for the different antibiotics that were tested are shown in Table 1.

10

Table 1: Effect of different penicillin concentrations on the sensitivity of microbial growth inhibition tests towards different families of antibiotics.

Sensitivity of test (ppb)	Penicillin present during preparation of test organisms (ppb)		
	0	1	2
<u>Beta-lactams:</u>			
Amoxicillin	1.1	3.1	>4
Ampicillin	0.9	2.6	>4
Penicillin G	0.6	2.0	>4
Cloxacillin	5.9	29	>80
<u>Non-beta-lactams:</u>			
Sulfadiazine (Sulfonamide)	20	58	96
Oxytetracycline (Tetracycline)	128	212	203

15

The concentration of antibiotic at which there no longer occurred a change of color was determined as threshold value. The results clearly show that sensitivity towards at least three families of antibiotics (beta-lactam antibiotics, tetracycline antibiotics and sulfonamide antibiotics) can be reduced, when penicillin is present during the preparation of the test organisms.

CLAIMS

1. A method for reducing the sensitivity of a microbial growth inhibition test for the determination of the presence or absence of an antibiotic in a sample to at least one
5 family of antibiotics, the method comprising the steps of:
 - (a) preparing a test organism,
 - (b) contacting a fluid sample with the test organism,
 - (c) growing the test organism,
 - (d) detecting the amount of growth of the test organism, wherein lack of growth
10 reflects the presence of an antibiotic in the sample,characterized in that an antibiotic is present in step a and the sensitivity of the test to at least one family of antibiotics is reduced by the presence of the antibiotic in step a.
2. A method according to claim 1, characterized in that the microbial growth inhibition
15 test comprises an indicator.
3. A method according to claim 1 or 2, characterized in that the test organism is selected from the group consisting of a *Bacillus* species, an *Escherichia* species and a *Streptococcus* species.
20
4. A method according to any one of the claims 1-3, characterized in that the sample is milk.
5. A method according to any one of the claims 1-4, characterized in that the test
25 organism is thermophilic.
6. A method according to any one of the claims 1-5, characterized in that the antibiotic present in step a is a beta-lactam antibiotic.
- 30 7. A method according to claim 6, characterized in that the beta-lactam antibiotic is a penicillin derivative.
8. A method according to any one of the claims 1-7, characterized in that the at least one family of antibiotics is selected from the group consisting of the family of beta-lactam

antibiotics, the family of tetracycline antibiotics and the family of sulfonamide antibiotics.

- 5 9. A method according to any one of the claims 1-8, characterized in that the sensitivity of the test to at least two families of antibiotics is reduced by the presence of the antibiotic in step a.
- 10 10. A method according to any one of the claims 1-9, characterized in that the test organism is prepared in the presence of 10 ppb or less of the antibiotic.
11. A method according to claim 10, characterized in that the test organism is prepared in the presence of 0.5 to 2.0 ppb of the antibiotic.
- 15 12. A method according to any one of the claims 1-11, characterized in that the microbial growth inhibition test further comprises a gelling agent.
13. A method according to any one of the claims 2-12, characterized in that the indicator is a pH indicator and/or a redox indicator.
- 20 14. A microbial growth inhibition test for the determination of the presence or absence of an antibiotic comprising a test organism, an indicator and a gelling agent, characterized in that the test organism has been prepared in the presence of an antibiotic and the sensitivity of the test to at least one family of antibiotics is reduced compared to a test wherein the test organism has been prepared in the absence of an
- 25 antibiotic.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/059330

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/18 G01N33/04 G01N33/94
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q G01N

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2005/049809 A1 (CHARM SCIENCES INC [US]; SALTER ROBERT S [US]; SAUL STEVEN J [US]; ROS) 2 June 2005 (2005-06-02) cited in the application	1-8, 10-14
A	the whole document abstract page 2, paragraph 7 page 3, paragraph 10 - paragraph 11 page 6, paragraph 28 - paragraph 29 page 7, paragraph 31 - paragraph 32 page 12, paragraph 53 - page 14, paragraph 58; example 5; tables 1-3 claims 1,10 claims 13-22 claim 29 claim 51 claims 59,62-64 ----- -/-	9



Further documents are listed in the continuation of Box C.



See patent family annex.

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Date of the actual completion of the international search

8 June 2012

Date of mailing of the international search report

20/06/2012

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

Gall, Anne-Laure

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2012/059330

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DE 36 13 794 A1 (MUELLER FRANK JOACHIM) 29 October 1987 (1987-10-29) the whole document abstract column 1, line 42 - line 46 column 1, line 47 - line 54 column 1, line 54 - line 66 column 1, line 67 - column 2, line 12 column 2, line 31 - line 35 claim 1	1,2,4, 12,14
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