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(71) Applicant: BAXTER DIAGNOSTICS INC. [US/US]; One Baxter Parkway, Deerfield, IL 60015 (US).

(72) Inventors: BADAL, Robert ; 7711 River Landing Drive, Sacramento, CA 95831 (US). KELLEY, Roger ; 11F Concord Road, West Milford, NJ 07480 (US). SAND, Theodore, T. ; 12745 Corte Rayito, Poway, CA 92064 (US). BASCOMB, Shoshana ; 4321 El Macero Drive, Davis, CA 95618 (US).

(74) Agents: BARTA, Kent, S. et al.; One Baxter Parkway, Deerfield, IL 60015 (US).

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(54) Title: METHOD AND COMPOSITION FOR DETERMINING ANTIMICROBIAL SUSCEPTIBILITY OF THE MAJORITY OF CLINICALLY SIGNIFICANT GRAM POSITIVE ORGANISMS

(57) Abstract

This invention relates to a method to determine susceptibility to antimicrobial agents of a majority of clinically significant Gram positive organisms. This invention also relates to a mixture of fluorogenic substrates used to detect the growth of Gram positive Bacteria.

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METHOD AND COMPOSITION FOR DETERMINING ANTIMICROBIAL SUSCEPTIBILITY OF THE MAJORITY OF CLINICALLY SIGNIFICANT GRAM POSITIVE ORGANISMS

Field of the Invention

This invention relates to a method to determine susceptibility to antimicrobial agents of a wide variety of clinically significant Gram positive organisms. This invention also relates to a mixture of fluorogenic substrates used to detect the growth of Gram positive bacteria.

Background of the Invention

Microorganisms isolated from patients and other sources are routinely tested for their susceptibility to antimicrobial agents and for their metabolic growth requirements. In particular, the minimum inhibitory concentrations (MIC's) of antimicrobial agents or the categorical interpretations (susceptible, moderately susceptible, or resistant) of microorganisms isolated from clinical sources are routinely determined.

Numerous methods and apparatus have been developed to conduct susceptibility tests. In particular, multi-compartmentalized devices, such as microdilution panels, with each compartment containing a specific quantity of an antimicrobial agent or a growth promoting material, such as a vitamin, are often used to determine growth and susceptibilities. These compartments generally contain, in additional to the agent investigated, growth supporting medium. These devices may be freshly prepared, frozen or dried for convenient storage.

To conduct susceptibility tests the above described devices are inoculated with a standardized microbiological inoculum and incubated until visible growth appears. This typically takes 15-24 hours. Generally, microbial growth in either a liquid medium or on the surface of a solid support medium is determined by direct visual recognition or by turbidimetric measurements. The endpoint of susceptibility tests is defined as the lowest concentration of an antimicrobial agent in which growth, when compared to that of the growth compartment, appears to be inhibited.

Commonly used susceptibility tests require 15-24 hours of incubation prior to the availability of results. Earlier receipt by the physician of accurate antimicrobial agent susceptibility information would result in better patient treatment. A number of methods have been published to obtain earlier susceptibility determinations. They may be divided into measurement of bacterial mass described in Patent Nos. 4,236,211 (Pfizer), 3,832,532 (Pfizer), G.B. Patent No. 1,554,134 (Pfizer), and indirect estimation of bacterial mass by measurement of enzymatic activity described in Patent No. 4,242,447 (Bio Research), fluorogenic measurement of phosphate, Patent No. 3,509,026 (Litton Systems Inc.), fluorogenic measurement of phosphotase, FR 2,504,679, use of methylumbelliferyl derivatives, EP 0,091,837B and in articlesdescribing the use of amido-coumarin derivatives for antibiotic susceptibility testing by M.R. Mateo, et al., Abstract Annual Meeting, American Society for Microbiology, 1980, C201, p308 and the measurement of metabolic activity by reduction of tetrazolium in microdilution panels by T. Urban and C. Jarstrand, J. Antimicro. Chemo (1981) 8, 363-369. All the following are about the use of a mixture of fluorogenic substrates: Nolte et al., J. Clin. Microbio. (1988) 26, 1079-1084, Staneck et al., J. Clin. Microbiol. (1985), 187-191, Doern et al., J. Clin. Micro. (1987), 1481-1485, and Staneck et al., J. Clin. Micro. (1988) v.26 1-7.

One particular method is the Sensititre™ system which has an instrument capable of automatically reading antimicrobial susceptibility microdilution trays. In this procedure, microbial growth is determined by the measurement of fluorescence produced by bacterial enzymatic action on fluorogenic substrates. The fluorescence signals are interpreted by the instrument and converted to MIC's. Staneck, (1985) Supra at 187. The Sensititre™ method involves the use of a fluorogenic substrate cocktail to detect the minimal inhibitory concentration for Gram positive and Gram negative bacteria based on a single measurement within five hours of the addition of the hydrolysable

fluorogenic substrates to the inoculum. It is disclosed that the fluorogenic substrates for this group of bacteria are selected from 7-(N)-(aminoacyl)-7-amido-4-methylcoumarin, 4 methyl-umbelliferyl nonanoate, 4-methylumbelliferyl phosphate. EP #0,091,837B.

Single measurement methods to predict or determine minimal inhibitory concentration using fluorogenic substrates, however, have been found to be unreliable. In order to determine accurate minimal inhibitory concentrations sufficient growth and utilization of the substrate must occur. A single measurement precludes the accurate determination of a minimal inhibitory concentration under optimum conditions because different bacterial species may obtain sufficient growth to determine minimal inhibitory concentrations at different times. A single early measurement may result in an inaccurate prediction of a minimal inhibitory concentration because of insufficient expression of resistance. A single late measurement may result in an inaccurate estimation of enzymatic activity in the antimicrobial agent containing compartment in relation to that of the growth compartment. After a certain fluorescence level is reached, the photometric detection system is unable to accurately determine fluorescence.Consequently, a need exists to develop a system to accurately predict minimal inhibitory concentrations for a wide range of Gram positive organisms using fluorogenic substrates in one standardized test system.

Summary of the Invention

The present invention involves a method for determining antimicrobial susceptibility of a wide variety of Gram positive organisms comprising: a) suspending and homogeneously mixing a sufficient number of morphologically similar colonies of the Gram positive organism in an aqueous suspending medium to prepare an inoculum having a turbidity equivalent to at least 0.5 McFarland Barium Sulfate, turbidity standard; b) diluting said inoculum in a sufficient amount of a growth supporting medium to achieve a nominal concentration of about 2×10^6 colony forming units/ml:

c) combining a portion of said inoculum with a sufficient amount of fluorogenic substrates consisting of: leucine-7-amido-4-methylcoumarin, phenylalanine-7-amido-4-methylcoumarin and 4-methylumbelliferyl phosphate and a predetermined amount of an antimicrobial agent to form a mixture; d) repeatedly monitoring fluorescence intensity of said mixture between about 3 1/2 to 15 hours after step 1(c) to detect a given increase in fluorescence intensity and e) comparing said detected fluorescence intensity with changes in fluorescence intensity of a control or controls to determine susceptibility to antimicrobial agents of said Gram positive organisms. A kit to conduct the above described method comprised of control, growth control, and antimicrobial agents in individualized compartments: said control compartments containing buffer, said growth control compartments containing buffer and fluorogenic concentrate, and said antimicrobial agent compartment containing buffer, antimicrobial agent and fluorogenic concentrate. All kit biochemical components are in a stable format, such as in the dehydrated state.

The invention differs from the procedure previously described for Gram positive bacteria used by Sensititre™ EP #0,091837 in three important aspects. Firstly, the mixture of fluorogenic enzyme substrates; secondly, in the scope of organisms covered; and thirdly, in the repeated measurement of fluorescence.

In the Sensititre™ procedure the substrates mixture consists of alanyl-7-amido-4-methylcoumarin, 4-methylumeblliferyl phosphate and 4-methylumbelliferyl nonanoate. The last compound is difficult to redissolve evenly after dehydration. Moreover, a special procedure of drying the substrate mixture on a solid carrier in combination with an emulsifying agent was the preferred method in the EP #0,091837 description. Removal of this compound from the mixture allows easy preparation and aliquoting of the fluorogenic substrate mixture. In the current invention phenylalanine-7-amido-4-methylcoumarin and leucine-7-amido-4-methylcoumarin provide a superior mixture to that described before because it removes the need for an emulsifying agent during drying

and the need of drying the fluorogenic enzyme substrates on a solid carrier. The current fluorogenic substrate mixture also allows for testing of a larger range of Gram positive species than previously described. Additionally, the use of the autoSCAN®-W/A allows monitoring of growth for between 3 1/2 and 15 hours, determination of minimum inhibitory concentration occurring only when sufficient growth has been achieved. This ensures that the minimum inhibitory concentration determinations of each isolate is determined at the optimal time.

Detailed Description

The invention combines three fluorogenic compounds to facilitate the detection of growth of the majority of clinically significant Gram positive organisms, staphylococci, streptococci, enterococci, and listeriae from between about 3 1/2-15 hours. The combination of the three compounds are added to the growth control and to every concentration of the antimicrobial agents compartments. Growth of the organism is detected by an increase in fluorescence units.

As the organism grows, enzymes are produced which hydrolyze the fluorogenic compound(s), thus releasing the fluorophore, which, when excited by light in the 340-370nm range, fluoresces in the 440-470nm range. If the organism does not grow, the fluorophore part is not released from the compound, and there is no increase in fluorescence at the selected wavelength. After the organism has grown for between 3 1/2-15 hour, the amount of fluorescence in the individual concentrations of the antimicrobial agents can be compared to the amount of fluorescence in the growth control, and the minimal inhibitory concentrations of each antimicrobial agent can be determined.

The advantage of utilizing a combination of fluorogenic compounds to detect growth, is the rapidity with which growth and consequently minimal inhibitory concentrations can be determined. Conventional minimal inhibitory concentration determinations, which rely on turbidity, require 15-24 hours of incubation prior to reading. Using a combination of certain compounds allows one

to determine minimum inhibitory concentrations for the majority of Gram positive organisms, however, within 3 1/2-15 hours.

Several combinations of fluorogenic compounds were tested and this combination detected growth of most species of Gram positive organisms. The compounds are leucine-7-amido-4-methylcoumarin, phenylalanine-7-amido-4-methylcoumarin, and 4-methylumbelliferyl phosphate. This combination detected the majority of clinically relevant species of staphylococci, streptococci, enterococci, and listeriae. See TABLE 1.

TABLE 1

Staphylococcus aureus	S.cohnii	S.epidermidis
S.haemolyticus	S.hominis	S.hyicus hyicus
S.intermedius	S.lentus	S.saprophyticus
S.sciuri	S.similans	S.xylosus
S.kloosii	S.caseolyticus	S.chromogenes
S.carnosus	S.caprae	S.gallinarum
Streptococcus pyogenes (Group A)		
St.agalactiae (Group B)	St.equi/equisimilis	St.zooepidemicus
St.bovis I	St.bovis II	St.equinus
St.mutans	St.sanguis I	St.sanguis II
St.anginosus/milleri	St.constellatus/milleri	
St.intermedius/milleri	St.mitis	St.morbillorum
St.salivarius	St.pneumoniae	Enterococcus faecalis
Ec.faecium	Ec.durans	Ec.avium
Listeria monocytogenes.		

In particular, in this assay as the organism grows it metabolizes one, two, or all three components of the fluorogenic concentrate. Metabolic activity results in the release of fluorophores, amino-4-methylcoumarin and/or 4-methylumbelliferone which, when excited by light in the 340-370 nm range, emit light (fluoresce) in the 440-470 nm range. As the organism multiplies, the amount of fluorescence increases. The amount of fluorescence in each concentration of the antimicrobial agents is compared to the amount of fluorescence in the growth control. Using a mathematical model based on discriminate functional analysis, the minimum inhibitory concentration of each antimicrobial agent, for the organism tested, can be determined. Utilization of a

discriminant function analysis model in microbiological pattern recognition is described in S. Bascomb, Computers in Taxonomy and Systematics, p.65-102 In T.N. Bryant and J.W.T. Wimpenny (Ed.), Computers in Microbiology, a Practical Approach (IRL Press, Oxford). In an alternative embodiment the minimal inhibitory concentration for each antimicrobial agent for the organism tested can be determined by a break point or threshold method or by linear regression analysis. J. McKie, et al., Antimicrobial Agents and Chemotherapy (1980) v.17, 813-823.

Generally, the invention involves making a stock solution containing the three fluorogenic compounds: leucine-7-amido-4-methylcoumarin (0.1M), phenylalanine-7-amido-4-methylcoumarin (0.05M) and 4-methylumbelliferyl phosphate (0.25M); dissolving the compounds in dimethylformamide; b) making the diluent which is HEPES buffer, 0.01M, pH 7.0 or a similar buffer. The antimicrobial agents are diluted in 0.01M buffer; and the fluorogenic concentrate (2ml/L) is added to the growth control, which contains only buffer, and to each concentration of the antimicrobial agents. Additionally, a control which contains only buffer is used in this assay. The final concentration of the fluorogenic components per compartment are 0.2mM leucine-7-amido-4-methylcoumarin, 0.1mM phenylalanine-7-amido-4-methylcoumarin, and 0.5mM methylumbelliferyl phosphate. These concentrations were selected to assure optimum enzyme activity throughout the growth period. One hundred fifteen microliters of each of said solutions are dispensed into individual compartments of a microdilution panel. The panels are dehydrated and packaged in foil wrapping with a packet of desiccant. This package is stored at 2-8°C.

The antimicrobial agents include: Amoxicillin/K, Clavulanate (Aug), Ampicillin (am), Ampicillin/Sulbactam (A/S), Cefamandole (Cfm), Cefazolin (Cfz), Cefotaxime (Cft), Ceftriaxone (Cax), Cefuroxime (Crm), Cephalothin (Cf), Chloramphenicol (C), Ciprofloxacin (Cp), Clindamycin (Cd), Erythromycin (E), Gentamicin (Gm), Gentamicin Synergy Screen (GmS) Imipenem (Imp),

Nitrofurantoin (Fd), Norfloxacin (Nx), Oxacillin (Ox), Penicillin (P), Rifampin (Rif), Streptomycin Synergy Screen (Sts) (high concentration streptomycin in well. The screen determines that a combination of streptomycin and another antibiotic will be affective.), Tetracycline (Te), Trimethoprim (T), Trimethoprim Sulfamethoxazole (T/S) and Vancomycin (Va).

Generally, the test procedure involves making a suspension of a Gram positive organism, which is equivalent to a 0.5 McFarland barium sulfate turbidity standard in a saline pluronic broth. Three hundred microliters of the suspension is diluted into 25 milliliters of the inoculum broth. The suspension is mixed. The dried panels are rehydrated with 115 microliters of the inoculum broth per suspension. Rapid Pos® Inoculum Broth (Baxter MicroScan). The panels are incubated at 35° ± 1°C in a non-CO_2 incubator. The panels are read in a fluorometer at designated times. MicroScan has an autoScan® W/A (Baxter MicroScan) instrument which incubates and reads the panels automatically at designated times.

The following table shows the distribution of reagents and inoculum in the growth control and antimicrobial agent wells.

TABLE 2

	Growth Well	Control Well	Antimicrobial Agent Well
Inoculum	+	+	+
Growth Medium	+	+	+
Fluorogenic Substrates	+	-	+
Antimicrobial Agent	-	-	+
Growth	+	+	-/+
Increase in Fluorescence	+	-	-/+

Using the above described assay, the majority of clinically significant Gram positive organisms, See TABLE 3, were tested to determine the efficacy of this test to determine the minimal inhibitory concentration of certain antimicrobial agents.

TABLE 3

ORGANISMS TESTED FOR EFFICACY

Organisms:	Number Tested
Staphylococcus aureus	187
Methicillin - Susceptible	(92)
Methicillin - Resistant	(95)
Coagulase - negative Staphylococci	144
Staphylococcus epidermidis	(49)
Staphylococcus saprophyticus	(15)
Other coagulase - negative Staphylococci	(80)
Group D Streptococci (Enterococci)	110
Beta Hemolytic Streptococci (St.pyogenes and St.agalactiae	77
Viridans Streptococci	29
Streptococcus pneumoniae	32
Listeria monocytogenes	16

For the organisms shown in Table 3, the following efficacy of this test was reported for the antimicrobial agents listed in Table 4. A 95.9% agreement with a reference minimal inhibitory concentration determination was obtained.

TABLE 4

EFFICACY SUMMARY REPORT

ANTIMICROBIC	TOTAL	+/-1 DILUTION	* VMJ	** MAJ	*** MIN	AGREEMENT
Ampicillin	592	571	6	4	4	96%
Ampicillin/Sulbactam	593	577	0	3	3	97%
Amoxicillin/ K. Clavulanate	591	572	11	0	0	97%
Clindamycin	593	540	19	14	20	91%
Gentamicin	345	327	7	6	4	95%
Imipenem	593	558	9	3	6	94%
Norfloxacin	593	585	3	3	0	99%
Oxacillin	319	289	5	15	0	91%
Penicillin	593	553	19	6	5	93%
Trimethoprim	440	430	7	3	0	98%
Trimethoprim/ Sulfamethoxazole	440	428	1	9	0	97%
Vancomycin	593	592	0	0	0	100%
Cefazolin	593	547	10	16	6	92%
Ceftriaxone	594	555	5	7	26	93%
Cefuroxime	595	552	10	10	10	93%
Cefotaxime	595	543	11	20	16	91%
Chloramphenicol	595	568	3	1	0	95%
Cefamandole	595	568	3	3	4	95%
Cephalothin	595	589	4	2	0	99%
Ciprofloxacin	595	588	0	1	6	99%
Erythromycin	595	566	11	4	13	95%
Nitrofurantoin	595	584	0	11	0	98%
Rifampin	595	586	5	4	0	98%
Tetracycline	595	566	10	4	5	95%
Streptomycin Synergy Screen	594	582	4	8	0	98%
Gentamicin Synergy Screen	593	587	2	4	0	99%
TOTAL	14,609	14,003	165	161	128	
PERCENT		95.9%	1.1%	1.1%	0.9%	95.9%

AGREEMENT (±1 dil) = 95.9%

*VMJ - Very Major Error - A very major error occurred when the isolate was categorized as susceptible by the test method and as resistant by the reference method.

**MAJ - Major Error - A major error occurred when the isolate was categorized as resistant by the test method and as susceptible by the reference method.

***MIN - Minor Error - A minor error occurred when the isolate was categorized as moderately susceptible by one method and as susceptible or resistant by the other method.

Examples of the invention are given below:

Example 1

A primary inoculum suspension of a bacterial isolate in saline-pluronic, equivalent to a 0.5 McParland turbidity standard is prepared. The final inoculum suspension is prepared by diluting a 0.3ml aliquot of the primary suspension in 25ml of Rapid Pos Inoculum Broth (B1015-14, Baxter Diagnostics Inc., MicroScan Division, West Sacramento, CA). A 115µl of the final inoculum is added to the growth compartment which contains dried material of fluorogenic enzyme substrates equivalent to 0.023mM leucine-7-amido-4-methylcoumarin, 0.012mM phenylalanine-7-amido-4-methylcoumarin and 0.058mM methylumbelliferyl phosphate and 1.15mM HEPES buffer at pH 7.0. The final inoculum is also added to a number of compartments containing dried material identical to that in the growth well and different concentrations of an antimicrobial agent. An aliquot of the final inoculum is also added to a compartment, containing only dried HEPES buffer, acting as a control. The inoculated multicompartment device (panel) is inserted in the autoSCAN®-W/A. The fluorescence of each compartment is measured at predetermined times and a Growth Index is calculated by dividing the fluorescence of the growth compartment by that of the negative control compartment. See Table 4.

TABLE 4

$$\text{Growth Factor} \qquad \frac{{}^{FL}\text{growth well}}{{}^{FL}\text{control well}}$$

$$\%\,{}^{FL}_{drug} = \frac{{}^{FL}\text{drug} - {}^{FL}\text{control}}{{}^{FL}\text{growth} - {}^{FL}\text{control}}$$

If the value of the Growth Index is equal to or exceeds a specified value, the Minimum Inhibitory Concentration for every antimicrobial agent present in the multicompartment device is calculated. Otherwise the device is returned to its position and measured again at the next read time. The read times in this

example are 3.5, 4.5, 5.5, 7.0, 8.0, 11.0, and 15.0 hours. For calculation of minimum inhibitory concentration, the percent fluorescence of compartments containing the different concentrations of the antimicrobial agent is calculated by subtracting from each compartment the fluorescence of the control well and dividing it by the delta fluorescence of the growth compartment, obtained by subtracting the fluorescence of the negative control compartment from the fluorescence of the growth compartment. The minimum inhibitory concentration is calculated using a mathematical model specific for each concentration range of the antimicrobial agent. Using a mathematical model based on discriminate functional analysis, the minimum inhibitory concentration of each antimicrobial agent, for the organism tested, can be determined. Utilization of a discriminate function analysis model in microbiological pattern recognition is described in S. Bascomb, Computers in Taxonomy and Systematics, p.65-102 In T.N. Bryant and J.W.T. Wimpenny (Ed.), Computers in Microbiology, a Practical Approach (IRL Press, Oxford). In an alternative embodiment the minimum inhibitory concentration for each antimicrobial agent for the organism tested can be determined by a breakpoint or threshold method or linear regression analysis. J. McKie, et al., Antimicrobial Agents and Chemotherapy (1980) v.17, 813-823.

In Example 1, the growth of Enterococcus faecalis in wells containing specified amounts of antimicrobial agnets is estimated by measurement of fluorescence. In Table 7, the percent fluorescence at different concentrations of antimicrobial agents is reported. For example, .12, .25, .5, 1, 2, 4 and 8 µg/mL of ampicillin (Am) were present in the microdilution wells.

The numbers 105, 105, 67, 7, 6, 5, 6 are percent fluorescence values as calculated in Table 4. These figures are used to determine susceptibility according to the methods discussed on pages 12 and 13.

TABLE 7

Minimum Inhibitory Concentration Determination

5	95	105	105	67	7
StS	GmS	.12Am	.25Am	.5Am	1Am
6	5	6	99	104	105
2Am	4Am	8Am	.03P	.06P	.12P
105	105	93	12	7	7
25P	.5P	1P	2P	4P	8P
104	105	3928	103	98	100
8Cfm	16Cfm	C	.250x	.50x	10x
99	95	90	91	85	79
20x	40x	80x	.5Te	1Te	2Te
71	62	37	105	102	104
4Te	8Te	128Te	2Cfz	4Cfz	8Cfz
100	103	7	61369	47805	85
16Cfz	8Cf	16Cf	G	OxG	2Crm
39	15	10	10	8	8
4Crm	8Crm	16Crm	4Cft	8Cft	16Cft
7	15	9	8	7	65
32Cft	4Cax	8Cax	16Cax	32Cax	1Imp
5	5	5	105	99	95
2Imp	4Imp	8Imp	1Gm	2Gm	4Gm
93	93	59	9	5	5
6Gm	8Gm	.25E	.5E	1E	2E
4	58	21	5	5	5
4E	1Rif	2Rif	2Va	4Va	8Va
2	88	83	75	71	5
16Va	.25Cd	5Cd	1Cd	2Cd	8A/S
5	84	94	6	6	6
16A/S	2T/S	8T/S	1Cp	2Cp	4Cp
6	6	6	5	86	21
4Nxn	8Nxn	2Aug	4Aug	32Fd	46Fd
9	6	4			
4C	8C	16C			

G = Growth Compartment
C = Control Compartment

TABLE 8

PL	PS	Ox	Cfz
N/R	N/R	N/R	>16
Cft	Gm	Va	Cp
>4	N/R	<2	<1
NxN	AmO	AmL	AmS
<4	0.5	N/R	N/R
Cf	Cd	E	Aug
16	>2	4	<2
Te	Fd	Crm	Imp
128	<32	>16	4
A/S	Cfm	C	Rif
<8	>16	<4	2
T/X	Sts		
>8	5		

In Table 8, the minimum inhibitory concentration for Example 1 are reported. For the antimicrobial agent, cephalothin, the organism was found to be susceptible at a concentration at 16 µg/mL. For ampicillin/sulbactans susceptibility was observed at less than 8 µg/mL while the organism was resistant to trimethoprim/sulfamethoxazole at greater than 8 µg/mL. The organism was susceptible to norfloxacin, cefotaxime and chloramphenic at less than 4 µg/mL, while susceptiblity at 4 µg/mL was observed of Imipenem and erythromycin. Additionally, the organism was found to be susceptible at less than 2 µg/mL of vancomycin and amoxicillin/K.Clavalanate and at 2 µg/mL of rifampin. The organism was resistant at greater than 2 µg/mL of clindamycin. The organism was found to be susceptible at less than 1 µg/mL of ciprofloxacin. The organism was found to be susceptible to 0.5 µg/mL of ampicillin. The organism was found to be susceptible at 128 µg/mL to tetracycline. The organism was found to be susceptible to less than 32 µg/mL of nitrofurantoine.

The organism was found to be resistant at greater than 16 µg/mL for cefazolin, cefuroxime, and cefamandole. Additionally, it was determined that this organism was susceptible to a Streptomycin Synergy Screen.

Variants or equivalents: one alternative would be to use other combinations of fluorogenic compounds. The combination described above worked the best and detected the greatest number of Gram positive organisms. Other compounds tested were alanine-7-amido-4-methylcoumarin and methionine-7-amido-4 methylcoumarin. The individual concentrations of the compounds or the ratio of compounds in the mixture could be varied.

WE CLAIM:

1. A method for determining susceptibility to antimicrobial agents of the majority of clinically significant gram positive organisms comprising:

   a. suspending and homogenously mixing a sufficient number of morphologically similar colonies of Gram positive organisms in an aqueous suspending medium to prepare an inoculum having a turbidity equivalent to at least 0.5 McFarland barium sulfate, turbidity standard;

   b. diluting said inoculum in a sufficient amount of growth supporting medium to achieve a nominal concentration of about 2 x 10^6 colony forming units/ml;

   c. combining a portion of said inoculum with a sufficient amount of fluorogenic substrates consisting of: leucine-7-amido-4-methylcoumarin, phenylalanine-7-amido-4-methyl coumarin and 4-methylumbelliferyl phosphate and predetermined amount of an antimicrobial agent to form a mixture;

   d. monitoring fluorescence intensity of said mixture between about 3 1/2 to 15 hours after step 1(c) to detect a given increase in fluorescence intensity; and

   e. comparing said detected fluorescence intensity with changes in fluorescence intensity of a control to determine susceptibility to antimicrobial agents of said Gram positive organisms.

2. The method of Claim 1 where said control consist of a growth control an a buffer control.

3. The method of Claim 1 wherein said Gram positive organisms are selected from the class consisting of: Staphylococcus aureus, S.cohnii, S.epidermidis, S.haemolyticus, S.hominis, S.hyicus-hyicus, S.intermedius, S.lentus, S.saprophyticus, S.sciuri, S.similans, S.xylosus, S.kloosii, S.caseolyticus, S.chromogenes, S.carnosus, S.caprae, and S.gallinarum, Streptococcus pyogenes (Group A), St.agalactiae (Group B), St.equi/equismilis, St.zooepidemicus, St.bovis I, St.bovisII, St.equinus, St.mutans, St.sanguis I, St.sanguis II, St.anginosus/milleri, St.constellatus/milleri, St.intermedius/milleri, St.mitis, St.morbillorum, St.salivarius, St.pneumoniae, Enterococcus faecalis, Ec.faecium, Ec.durans, Ec.avium, and Listeriae monocytogenes.

4. The method of Claim 1 wherein said antimicrobial agent is selected from the class consisting: Amoxicillin/K Clavulanate, Ampicillin, Ampicillin/Sulbactam, Cefamandole, Cefazolin, Cefotaxime, Ceftriaxone, Cefuroxime, Cephalothin, Chloramphenicol, Ciprofloxacin, Clindamycin, Erythromycin, Gentamicin, Gentamicin Synergy Screen, Imipenem, Nitrofurantoin, Norfloxacin, Oxacillin, Penicillin, Rifampin, Streptomycin Synergy Screen, Tetracycline, Trimethoprim, Trimethoprim/Sulfamethoxazole and Vancomycin.

5. Method for determining susceptibility to antimicrobial agents of the majority of clinically significant Gram positive organisms comprising:

a. suspending and homogenously mixing a sufficient number of morphologically similar colonies of Gram positive organism in an aqueous suspending medium to prepare an inoculum having a turbidity equivalent to at least 0.5 McFarland barium sulfate turbidity standard;

b. diluting said inoculum in a sufficient amount of growth supporting medium to achieve a nominal concentration of about 2 x 10^6 colony forming units/mL;

c. combining a portion of said inoculum with a sufficient amount of fluorogenic substrates consisting of: leucine-7-amido-4-methylcoumarin, phenylalanine-7-amido-4-methylcoumarin and 4-methylumbelliferyl phosphate, a predetermined amount of an antimicrobial agent, and sufficient amounts of buffer to stabilize said fluorogenic substrate in individual microwell dilution compartments to form antimicrobial agent wells;

d. combining a portion of said inoculum with a sufficient amount of fluorogenic substrates consisting of: leucine-7, amido-4-methylcoumarin, phenylalanine-7-amido-4-methylcoumarin and 4-methylumbelliferyl phosphate and a sufficient amount of buffer to stabilize said fluorogenic substrate in other individual microwell dilution compartments to form growth wells;

e. combining a portion of said inoculum with a sufficient amount of buffer to stabilize said fluorogenic substrate in individual microwell dilution compartments to form control wells.

f. repeatedly monitoring fluorescence intensity of said wells between about 3 1/2 to 15 hours after steps (c), (d) and (e); and

g. comparing said detected fluorescence intensities of said antimicrobial agent wells with said growth wells and control well to determine susceptibility to antimicrobial agent of said Gram positive organisms.

6. The method of Claim 5 wherein said Gram positive organisms are selected from the class consisting of: Staphylococcus aureus, S.cohnii, S.epidermidis, S.haemolyticus, S.hominis, S.hyicus-hyicus, S.intermedius, S.lentus, S.saprophyticus, S.sciuri, S.similans, S.xylosus, S.kloosii, S.caseolyticus, S.chromogenes, S.carnosus, S.caprae, and S.gallinarum, Streptococcus pyogenes (Group A), St.agalactiae (Group B), St.equi/equismilis, St.zooepidemicus, St.bovis I, St.bovisII, St.equinus, St.mutans, St.sanguis I, St.sanguis II, St.anginosus/milleri, St.constellatus/milleri, St.intermedius/milleri, St.mitis, St.morbillorum, St.salivarius, St.pneumoniae, Enterococcus faecalis, Ec.faecium, Ec.durans, Ec.avium, and Listeriae monocytogenes.

7. The method of Claim 5 wherein said antimicrobial agent is selected from the class consisting: Amoxicillin/K Clavulanate, Ampicillin, Ampicillin/Sulbactam, Cefamandole, Cefazolin, Cefotaxime, Ceftriaxone, Cefuroxime, Cephalothin, Chloramphenicol, Ciprofloxacin, Clindamycin, Erythromycin, Gentamicin, Gentamicin Synergy Screen, Imipenem, Nitrofurantoin, Norfloxacin, Oxacillin, Penicillin, Rifampin, Streptomycin Synergy Screen, Tetracycline, Trimethoprim, Trimethoprim/Sulfamethoxazole and Vancomycin.

8. A kit to conduct the method of Claim 1 comprising control, growth control, and antimicrobial agents in individualized compartments, said control compartments containing buffer, said growth control compartments containing buffer and fluorogenic concentrate and said antimicrobial agent compartment containing buffer, antimicrobial agent and fluorogenic concentrate.

9. The kit of Claim 8 wherein said fluorogenic substrates are concentrated.

10. The kit of Claim 8 where kit reagents are dehydrated.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/09720

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): C12Q 1/02
US CL : 435/32

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	435/29, 32, 34; 436/800

Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched[5]

APS; "Fluorescenc?," "Antimicrob"

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
A	US, A 4,812,409 (BABB et al) 14 March 1989, see entire document.	1-10
A	US, A 4,025,393 (HIRSCHFELD) 24 May 1977, see entire document.	1-10
A	US, A 4,812,393 (GOSWAMI et al.) 14 March 1989, see entire document.	1-10
A	US, A 3,957,583 (GIBSON et al) 18 May 1976, see entire document.	1-10
A	US, A 4,129,483 (BACHNER) 12 December 1978, see entire document.	1-10
A	US, A 4,916,060 (WEAVER) 10 April 1990, see entire document.	1-10
A	US, A 4,026,767 (SHIH et al.) 31 May 1977, see entire document.	1-10
A	US, A 4,126,516 (MESSING et al.) 21 November 1978, see entire document.	1-10
A	US, A 4,568,637 (KLEIN) 04 February 1986, see entire document.	1-10

* Special categories of cited documents:[15]

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search[2]	Date of Mailing of this International Search Report[2]
02 FEBRUARY 1992	08APR 1992
International Searching Authority[1]	Signature of Authorized Officer[20]
ISA/US	Jane Williams

Form PCT/ISA/210 (second sheet)(May 1986) ß

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	Journal of Clinical Microbology, Volume 26, No. 6, issued June 1988, Nolte et al., "Rapid and Overnight Miccrodilution Antibiotic Susceptibility Testing with the Sensitive Breakpoint Autoreader System", pages 1079-1084, see entire document.	1-10
A	Journal of Clinical Microbiology, Volume 22, No. 2, issued August 1985, Staneck et al., "Automated Reading of MIC Micrqdilution Trays Containing Fluorogenic Enzyme Substrates with the Sensitive Autoreader" pages 187-191, see entire document.	1-10

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE[1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _, because they relate to subject matter (1) not required to be searched by this Authority, namely:

2. ☐ Claim numbers _, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out (1), specifically:

3. ☐ Claim numbers _, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING[2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Search Authority did not invite payment of any additional fee.

Remark on protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
A	Journal of Clinical Microbiology, Voilume 26 No. 1, issued January 1988, Staneck et al, "Rapid MIC Testing with the Sensititre Autoreader" pages 1-7, see entire document.	1-10