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A two-hour procedure for determining the susceptibility of enterococci and staphylococci to antibiotics by a colourimetric method

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ABSTRACT

Introduction. Rapid determination of the antibiotic susceptibility test in bacteria remains a challenge for Clinical Microbiology laboratories.

Methods. An improvement in the colorimetric antimicrobial susceptibility testing performed with resazurin in enterococci and staphylococci has been carried out. The design of method was performed using two collection strains, which have a known susceptibility. This procedure was then validated against standard commercial methods on 15 strains of staphylococci and 15 strains of enterococci from patients.

Results. The essential agreement between the colorimetric method and commercial methods (E-test, MicroScan and VITEK2) was 100%.

Conclusion. Resazurin allows us to obtain a reliable antibiotic susceptibility test in staphylococci and enterococci in less than two hours.

Keywords: resazurin; rapid antibiotic susceptibility test; bacteriology

Un procedimiento de dos horas para determinar la sensibilidad de estafilococos y enterococos a los antibióticos en dos horas mediante un método colorimétrico

RESUMEN

Introducción. La realización de un antibiograma rápido sigue siendo un reto para los laboratorios de Microbiología Clínica.

Métodos. Se ha realizado una mejora en el antibiograma colorimétrico realizado mediante resazurina en estafilococos y enterococos. El diseño del método se realizó mediante el uso de dos cepas de colección que presentan una sensibilidad conocida. Este procedimiento se validó posteriormente frente a los métodos comerciales mediante el procesamiento de 15 cepas de estafilococos y 15 de enterococos aisladas de pacientes.

Resultados. Se ha obtenido un 100% de concordancia entre la sensibilidad obtenida mediante resazurina y la obtenida mediante los métodos comerciales (E-test, MicroScan and VITEK2).

Conclusión. Mediante el uso de resazurina es posible obtener un antibiograma en estafilococos y enterococos en menos de dos horas de forma fiable.

Palabras clave: resazurina; antibiograma rápido; bacteriología

INTRODUCTION

In clinical microbiology laboratories, the most widely used methods to obtain antibiotic susceptibility tests (AST) are based on the observation of bacterial growth when the bacteria, previously isolated in culture plates, are incubated in the presence of the antibiotic being tested. Within these methods, disc diffusion and antimicrobial gradient methods (e.g. E-test strips) require a time of 17-24 hours to yield a result[1]. Also, broth microdilution has been used in various automated equipment; among which there is the VITEK2® (biomérieux, Marcy l'Etoile, France) and the Phoenix® (BD Biosciences, Franklin Lakes, NJ, USA) systems that provide the fastest results with an average time of nine hours[2].

All the aforementioned methodologies have two drawbacks. Ideally, results should be obtained faster, since early administration of an appropriate antibiotic for the treatment of bacterial infections improves patients' outcome, lowering hospitalization costs[3]. Besides, the values of minimum inhibitory concentration (MIC) or the inhibition zone diameter provided are not reproducible[4,5]. In order to overcome these difficulties, various methodologies, such as flow cytometry, molecular detection techniques, bioluminescence, chemiluminescence, nephelometry, microarrays or colourimetric methods have been introduced in clinical microbiology laboratories. Within colourimetric methods, resazurin is a well-known and easy-

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to handle probe for determining cell viability[6]. Resazurin is an oxidised form of a redox indicator that is blue in colour and non-fluorescent. When incubated with non-viable cells, it is not reduced and maintains its blue colour. On the other hand, when incubated with viable cells, which contain reductants substances, such as NADPH or NADH, resazurin is reduced to resorufin that is red and fluorescent. This colour change can be detected using both fluorescence and optical density measuring instruments, or with the naked eye[7]. When this feature of resazurin was applied to AST, the time required to achieve results ranged from four to 24 hours[7-10]. In order to optimize this procedure, the aim of the present work is to perform a faster AST from gram-positive bacteria obtained in culture plates using resazurin.

MATERIAL AND METHODS

Experimental design of the antimicrobial susceptibility test. Standard antimicrobial powder of vancomycin, oxacillin, amikacin, ciprofloxacin and ampicillin were obtained from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions were prepared according to the protocol proposed by the EUCAST[11], sterilized by filtration using a MILLEX GS 0.22 µm membrane filter (EMD® Millipore Corporation, Billerica, MA, USA) and stored at -80ºC. Further dilutions of antibiotic were carried out before use.

Resazurin powder (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in distilled water at a concentration of 0.1% and sterilised by filtration using a MILLEX GS 0.22 µm membrane filter (EMD® Millipore Corporation, Billerica, MA, USA). Finally, the dissolution was stored in darkness at +4ºC until use.

The influence of both bacterial inoculum and resazurin concentration on the time required to obtain red colour was studied using the strains *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 29213. From colonies of these strains, a 3-McFarland-standard bacterial suspension was prepared. Adequate aliquots of these suspensions were added into tubes containing 3 ml of cation-adjusted Mueller-Hinton broth (CAMHB) (Difco, Sparks, MD, USA) with 0.0002% or 0.0003% resazurin in order to achieve bacterial concentrations of 10^4, 10^5, 10^6, 10^7 and 10^8 CFU/ml. In addition, a bacteria-free tube was used as a negative control tube. Tubes were incubated in a water bath at 35ºC for three hours. During this time, two methods of lecture were applied after every fifteen minutes of incubation. In the first one, the colour of the medium was read visually; growth in each tube was indicated by a colour change from blue to red. In the second one, measurements of optical density at both 570 and 600 nm were performed (Smart Spec™ Plus, Bio-Rad, Hercules, CA, USA) and the percentage of the reduced form of resazurin was calculated in accordance with the manufacturer's technical insert[12] by applying the next equation:

$$\text{Percent reduced form of resazurin} = \frac{(\varepsilon_{ox})_{\lambda_2} \cdot A_{\blacklozenge_1} t - (\varepsilon_{ox})_{\lambda_1} \cdot A_{\lambda_2} t}{(\varepsilon_{red})_{\lambda_1} \cdot A_{\lambda_2} c - (\varepsilon_{red})_{\lambda_2} \cdot A_{\lambda_1} c} \cdot 100$$

where

λ_1:570 nm, λ_2:600 nm

$(\varepsilon_{ox})\lambda_2$: 117216

$(\varepsilon_{ox})\lambda_1$: 80586

$(\varepsilon_{red})\lambda_1$:155677

$(\varepsilon_{red})\lambda_2$:14652

$A\lambda_1 t$: observed optical density reading for test tube.

$A\lambda_2 t$: observed optical density reading for test tube.

$A\lambda_2 c$: observed optical density for negative control tube.

$A\lambda_1 c$: observed optical density for negative control tube.

In order to set up the experimental conditions of the AST, the strain *E. faecalis* ATCC 29212 was tested against ampicillin, vancomycin and ciprofloxacin, and *S. aureus* ATCC 29213 was tested against amikacin, vancomycin, oxacillin, and ciprofloxacin. All antibiotics were studied at EUCAST breakpoints concentrations of sensitivity and resistance[13]. Moreover, each strain was evaluated at three bacterial concentrations: 10^6, 10^7 and 10^8 CFU/ml. These bacterial concentrations were achieved in the same way as described previously. The medium used to perform the AST was 3 ml of CAMHB with 0.0003% resazurin and antibiotic. For oxacillin, the medium was supplemented with 2% NaCl. In addition, control tubes without antibiotics were included in all experiments and bacteria-free tube was used as negative control tube. Tubes were incubated in a water bath at 35ºC for three hours. Finally, tubes were measured in the same way as described previously and a new procedure for determining of the susceptibility of staphylococci and enterococci to antibiotics was proposed.

Validation procedure. The procedure was validated with 30 clinical strains isolated at University Clinic Hospital of Valladolid (Spain), which were identified from colonies obtained in culture plates using MALDI-TOF (Bruker Daltonik GmbH, Bremen, Germany). In brief, 15 enterococci (10 *Enterococcus faecalis* and 5 *Enterococcus faecium*) were tested against vancomycin, ampicillin and ciprofloxacin, and 15 staphylococci (5 *Staphylococcus aureus*, 5 *Staphylococcus epidermidis*, 3 *Staphylococcus hominis*, 1 *Staphylococcus lugdunensis* and 1 *Staphylococcus haemolyticus*) were tested against amikacin, vancomycin, oxacillin, and ciprofloxacin. All antibiotics were studied at EUCAST breakpoints concentrations of susceptibility.

The results of susceptibility obtained from colonies grown in culture plates by means of the commercial methods VITEK2 (bioMérieux, Marcy l'Etoile, France), MicroScan (Siemens, Tarrytown, NY, USA) and E-test (bioMérieux, Marcy l'Etoile, France), applying the criteria published by the EUCAST, were considered as the gold standard. Finally, results obtained by resazurin were compared to the gold standard according to FDA criteria[14]; agreements and disagreements among the susceptibility values obtained were classified as agreements, very major errors (false susceptibility), major errors (false resistance), or minor errors (susceptible/resistant versus intermediate susceptibility).

RESULTS

Results obtained from collection strains. When the strains *E. faecalis* ATCC 29212 and *S. aureus* ATCC 29213 were incubated without antibiotic, inoculums of 10^4 and 10^5 CFU/ml were not able to produce the colour change from blue to red after three hours of incubation. On the other hand, inoculums of 10^6, 10^7 and 10^8 CFU/ml of these strains required a determined period of time to produce the colour red. Specifically, bacterial concentrations of 10^6 CFU/ml produced the colour change during the third hour of incubation, bacterial concentrations of 10^7 CFU/ml produced it during the second hour of incubation, and bacterial concentrations of 10^8 CFU/ml produced it during the first hour of incubation. Moreover, these times were the same with both resazurin concentrations tested, but the intensity of red colour obtained with 0.0003% resazurin was visually more intense than the obtained with 0.0002% resazurin. When the colour red was appreciated visually, the percentage of the reduced form of resazurin was higher than 17%, irrespective of the resazurin concentration tested.

When we performed the AST using the collection strains, inoculums of 10^6 CFU/ml provided the colour change during the third hour of incubation and the results obtained during this period of time are shown in table 1. With these conditions, all AST were correctly obtained because the strains *E. faecalis* ATCC 29212, *S. aureus* ATCC 29213, which are susceptible to the antibiotic being incubated, were not able to produce the colour change. Inoculums of 10^7 CFU/ml of the collection strains provided the colour change from blue to red during the second hour of incubation and the results obtained during this period of time are shown in table 2. Also, with these conditions, all AST were correctly obtained for the same reason aforementioned. Inoculums of 10^8 CFU/ml of the collection strains provided the colour change of the medium during the first hour of incubation and the results obtained during this

Table 1 Percentage of the reduced form of resazurin obtained from collection strains with an inoculum of 10^6 CFU/ml

Strain tested	Antibiotic tested	Incubation time (minutes)												Susceptibility by resazurin
		135			150			165			180			
		WA	S	R	WA	S	R	WA	S	R	WA	S	R	
E. faecalis 29212	Ampicillin	9.61	3.71	2.88	13.44	6.38	5.89	20.25	7.21	7.86	34.01	10.33	9.95	Susceptible
	Vancomycin	10.64	2.73	1.77	15.15	5.87	4.68	24.47	8.26	9.52	39.20	11.89	11.37	Susceptible
	Ciprofloxacin	7.40	3.64	3.11	12.91	5.82	6.47	21.23	8.76	8.34	36.87	10.19	10.45	Susceptible
S. aureus 29213	Amikacin	7.42	5.51	4.44	9.48	6.20	6.31	15.92	7.69	9.25	24.22	9.63	9.14	Susceptible
	Vancomycin	6.92	4.32	4.71	8.10	5.82	6.15	14.72	8.76	7.30	23.98	10.73	10.90	Susceptible
	Oxacillin	6.28	4.72	3.53	10.47	6.53	5.29	14.67	8.35	9.95	23.06	10.12	9.62	Susceptible
	Ciprofloxacin	8.89	4.06	4.17	9.23	5.85	6.58	13.30	7.28	7.12	25.71	12.76	12.19	Susceptible

WA: tube without antibiotic; S: tube with antibiotic at breakpoint concentration of susceptibility; R: tube with antibiotic at breakpoint concentration of resistance

Table 2 Percentage of the reduced form of resazurin obtained from collection strains with an inoculum of 10^7 CFU/ml

Strain tested	Antibiotic tested	Incubation time (minutes)												Susceptibility by resazurin
		75			90			105			120			
		WA	S	R	WA	S	R	WA	S	R	WA	S	R	
E. faecalis 29212	Ampicillin	19.73	5.12	5.92	35.04	8.93	9.85	45.52	11.13	11.33	55.30	14.51	14.37	Susceptible
	Vancomycin	20.68	4.34	4.60	38.77	9.58	9.95	47.49	9.05	9.71	58.01	15.25	15.19	Susceptible
	Ciprofloxacin	20.85	5.70	5.48	36.38	8.61	8.78	43.19	10.11	9.25	57.40	13.73	12.82	Susceptible
S. aureus 29213	Amikacin	7.34	6.54	7.63	10.22	8.65	8.76	22.81	12.15	12.84	37.97	12.13	11.54	Susceptible
	Vancomycin	6.25	6.01	6.23	12.73	10.92	10.86	24.90	11.08	12.95	37.72	13.72	12.38	Susceptible
	Oxacillin	7.46	6.27	6.32	13.66	9.68	11.53	23.33	10.54	9.03	35.28	10.04	9.99	Susceptible
	Ciprofloxacin	7.32	7.21	6.12	11.53	9.30	9.99	24.37	12.57	12.88	40.93	13.31	13.13	Susceptible

WA: tube without antibiotic; S: tube with antibiotic at breakpoint concentration of susceptibility; R: tube with antibiotic at breakpoint concentration of resistance

Table 3 **Percentage of the reduced form of resazurin obtained from collection strains with an inoculum of 10^8 CFU/ml**

Strain tested	Antibiotic tested	Incubation time (minutes)												Susceptibility by resazurin
		15			30			45			60			
		WA	S	R	WA	S	R	WA	S	R	WA	S	R	
E. faecalis 29212	Ampicillin	7.99	8.36	7.42	14.25	14.13	15.39	28.41	26.55	23.81	41.03	30.61	33.59	Resistant
	Vancomycin	6.23	9.27	9.88	13.75	14.37	13.21	30.55	25.34	27.12	42. 07	33.38	38.11	Resistant
	Ciprofloxacin	7.68	7.85	7.54	12.19	14.20	14.97	33.29	34.37	30.51	47.43	45.09	44.65	Resistant
S. aureus 29213	Amikacin	5.44	6.52	6.21	9.07	12.87	11.96	14.30	13.55	14.71	26.86	29.17	32.82	Resistant
	Vancomycin	5.32	5.18	7.26	9.94	9.80	10.36	15.15	12.13	15.07	27.92	27.16	31.79	Resistant
	Oxacillin	7.47	6.82	5.54	10.03	10.59	12.28	14.62	14.77	11.83	29.12	24.52	27.57	Resistant
	Ciprofloxacin	6.75	7.66	7.25	9.34	10.18	11.95	15.46	13.27	15.52	30.02	32.94	33.76	Resistant

WA: tube without antibiotic; S: tube with antibiotic at breakpoint concentration of susceptibility; R: tube with antibiotic at breakpoint concentration of resistance

period of time are shown in table 3. With these conditions, all collection strains tested provided an incorrect AST because all susceptible strains to the antibiotics being incubated were able to produce colour change. In all these cases, when the colour red was appreciated visually, the percentage of the reduced form of resazurin was also higher than 17%.

On the basis of the results obtained from collection strains, the variables of the AST proposed were defined as follows: inoculum of 10^7 CFU/ml of staphylococci and enterococci, two hours of incubation and 0.0003% resazurin.

Results obtained from clinical strains. For validation of the proposed AST against commercial methods, 15 clinical strains of staphylococci and 15 of enterococci were processed. The results of the AST performed by commercial methods (VITEK2, MicroScan and E-test) from colonies obtained in culture plates showed 100% concordance among them. Results obtained from clinical strains of staphylococci and enterococci are shown in tables 4 and 5 respectively. Applying the proposed method, all AST were correctly obtained. As in all other such previous cases, when the colour red was appreciated visually, the percentage of the reduced form of resazurin was also higher than 17%.

DISCUSSION

Recently, there has been an increased effort to develop rapid and low-cost methods for AST. One method that meets is resazurin. In addition, solutions of resazurin can be easily prepared and, according manufacturer's manual[12], can be stored at room temperature in the darkness for a year without losing optical properties, thus requiring only a small amount of storage space. Moreover, the results obtained with resazurin can be interpreted easily, by naked eye or using a fluorometer or spectrophotometer. For these reasons, resazurin could be extensively used. On the other hand, molecular methods are expensive and require technical equipment, and so they can be used only in developed centres[9].

Resazurin concentrations of 0.0002% and 0.0003% were selected because absorbance values obtained during the experiments were within the linearity range of the spectrophotometer. For the same bacterial inoculum incubated without antibiotic, resazurin concentration values did not alter the time needed for the bacteria to produce the colour change, which means that the value of resazurin concentration did not produce differences in the time required to produce the colour change. However, we decided to use resazurin at 0.0003% because the intensity of the colour red produced was more intense than that produced with 0.0002%.

E. faecalis ATCC 29212 and *S. aureus* ATCC 29213 strains were used in the experimental design of the AST because they have a known antibiotic susceptibility and are commonly used in quality control of susceptibility testing. These strains, which are susceptible to the antibiotics tested, produced a colour change from blue to red when it were incubated with antibiotic at inoculums higher than 10^7 CFU/ml. Therefore, and an incorrect AST was obtained since growth in each tube was indicated by a colour change from blue to red (table 3). Thus, the maximum inoculum of these strains that provided a reliable AST was 10^7 CFU/ml and, as consequence, results could be obtained in less than hours. Concentrations lower than 10^7 CFU/ml of these strains also provided a reliable AST, but the time needed to obtain results was more than two hours (table 1).

The proposed AST was validated with 30 clinical strains of staphylococci and enterococci using inoculums of 10^7 CFU/ml. Applying our method, a 100% concordance between the results of susceptibility obtained by resazurin and by the commercial methods (VITEK2, MicroScan and E-test) was obtained in less than two hours (tables 4 and 5). Our results were the same to that previously reported in enterococci tested against vancomycin and in *S. aureus* tested against vancomycin, oxacillin and cefoxitin[8-10]. However, in these cases, the time required to obtain results ranged from four to six hours. This was because bacteria were incubated with antibiotic for several hours, later resazurin was added, and one hour or two hours

Table 4 | **Percentage of the reduced form of resazurin obtained from 15 clinical strains of staphylococci**

Bacteria (number of strain)	Antibiotic tested	Susceptibility by commercial methods (E-test MIC: mg/L)	Incubation time (minutes)												Susceptibility by resazurin
			75			90			105			120			
			WA	S	R	WA	S	R	WA	S	R	WA	S	R	
S. aureus (1)	Amikacin	Susceptible (0.06)	10.59	5.12	6.49	19.19	9.54	9.36	31.38	12.17	10.26	39.33	13.71	12.80	Susceptible
	Vancomycin	Susceptible (1)	15.62	7.34	6.85	24.11	10.49	10.21	39.90	13.53	13.71	50.77	16.23	15.18	Susceptible
	Oxacillin	Susceptible (0.5)	13.13	8.40	7.35	21.85	10.18	11.55	33.93	12.44	11.83	41.18	15.36	16.37	Susceptible
	Ciprofloxacin	Susceptible(0.008)	10.84	6.83	7.21	19.94	9.33	10.18	28.46	11.93	10.80	38.46	12.87	13.49	Susceptible
S. aureus (2)	Amikacin	Susceptible (1)	8.61	6.83	5.71	14.63	11.16	10.70	21.69	13.60	12.35	33.05	15.95	14.22	Susceptible
	Vancomycin	Susceptible(2)	7.17	7.26	6.78	15.47	10.81	11.63	24.11	13.72	12.18	35.81	14.22	15.95	Susceptible
	Oxacillin	Resistant (>256)	9.45	5.78	6.27	11.20	12.27	11.29	19.47	20.88	22.37	31.54	29.08	33.21	Resistant
	Ciprofloxacin	Resistant (4)	8.56	7.55	7.99	13.58	13.92	14.37	18.75	19.46	20.29	27.74	25.16	26.78	Resistant
S. aureus (3)	Amikacin	Susceptible (1)	3.19	3.13	4.63	9.24	10.81	9.95	15.52	12.39	12.54	23.10	13.55	14.03	Susceptible
	Vancomycin	Susceptible(1)	4.69	4.74	5.23	8.17	8.34	8.50	12.77	9.57	9.65	20.79	13.89	11.94	Susceptible
	Oxacillin	Resistant (8)	5.37	5.15	5.19	8.31	10.60	9.38	12.13	11.21	12.81	22.07	19.46	19.59	Resistant
	Ciprofloxacin	Susceptible (0.015)	6.03	4.27	6.30	10.56	7.88	8.91	13.73	12.97	11.28	21.11	17.57	16.14	Susceptible
S. aureus (4)	Amikacin	Susceptible (2)	4.77	4.95	4.61	8.80	7.66	6.16	15.81	10.33	11.74	24.49	10.93	12.26	Susceptible
	Vancomycin	Susceptible (1)	5.52	4.21	6.34	9.42	7.77	7.43	13.29	10.85	9.99	20.02	12.36	12.75	Susceptible
	Oxacillin	Susceptible (0.32)	6.25	7.94	5.56	10.76	9.45	9.32	16.11	12.91	11.83	24.42	14.51	15.33	Susceptible
	Ciprofloxacin	Susceptible(0.06)	4.89	5.10	5.24	9.13	5.53	4.02	12.64	7.28	6.55	19.23	9.36	10.68	Susceptible
S. aureus (5)	Amikacin	Susceptible (0.015)	6.19	6.34	6.97	10.25	8.15	7.89	15.47	12.77	11.79	21.22	14.44	14.12	Susceptible
	Vancomycin	Susceptible (0.5)	7.64	6.19	5.37	11.86	7.22	7.71	16.48	14.09	13.28	24.66	15.89	13.41	Susceptible
	Oxacillin	Susceptible(0.32)	8.19	7.83	7.21	12.68	10.99	9.95	15.26	12.43	12.25	22.34	13.29	14.31	Susceptible
	Ciprofloxacin	Susceptible (1)	6.49	3.71	3.44	9.37	4.53	5.12	13.74	9.26	9.92	19.27	13.11	12.85	Susceptible
S. epidermidis (6)	Amikacin	Susceptible(1)	7.40	5.27	7.31	12.59	10.11	10.25	16.17	12.78	14.36	21.81	14.93	15.54	Susceptible
	Vancomycin	Susceptible (1)	7.16	6.59	6.93	11.01	11.48	8.39	15.95	8.10	10.91	20.28	11.33	12.79	Susceptible
	Oxacillin	Resistant (8)	6.99	5.38	4.13	9.54	7.77	7.68	12.42	10.39	9.55	19.23	12.85	12.41	Resistant
	Ciprofloxacin	Susceptible(0.015)	5.11	4.69	3.48	8.29	5.25	5.88	11.93	6.42	7.18	18.91	9.79	10.83	Susceptible
S. epidermidis (7)	Amikacin	Susceptible (0.06)	9.74	7.12	7.25	13.61	9.99	10.03	19.32	10.89	10.15	22.76	13.24	14.24	Susceptible
	Vancomycin	Susceptible (0.5)	8.67	6.14	7.78	12.34	8.56	8.93	20.80	12.41	11.71	28.35	15.66	14.16	Susceptible
	Oxacillin	Susceptible(0.25)	8.22	5.87	6.22	13.79	7.33	6.78	19.15	9.57	9.23	25.84	12.33	13.58	Susceptible
	Ciprofloxacin	Susceptible (0.06)	9.63	4.76	5.83	13.37	6.93	7.19	19.10	12.32	11.81	20.39	13.12	13.79	Susceptible
S. epidermidis (8)	Amikacin	Susceptible(1)	5.70	4.43	4.39	10.24	6.54	7.32	14.66	9.57	9.33	20.24	12.19	12.02	Susceptible
	Vancomycin	Susceptible (2)	6.31	6.91	4.52	9.77	8.27	8.16	15.72	10.80	11.16	22.01	14.90	15.11	Susceptible
	Oxacillin	Resistant (>256)	6.65	7.18	5.77	11.54	10.62	10.77	14.59	15.68	14.32	21.35	22.66	20.59	Resistant
	Ciprofloxacin	Susceptible(1)	5.83	3.46	3.94	10.72	7.33	7.42	16.11	8.72	9.98	25.68	12.13	14.41	Susceptible
S. epidermidis (9)	Amikacin	Susceptible (0.12)	4.76	3.11	4.59	9.32	6.81	5.53	13.82	10.55	9.27	20.76	11.29	13.38	Susceptible
	Vancomycin	Susceptible(1)	5.18	5.52	5.32	10.55	8.19	9.15	15.65	12.21	11.76	21.64	14.88	15.47	Susceptible
	Oxacillin	Resistant (16)	3.21	4.63	6.25	9.78	9.53	9.48	14.81	13.37	14.51	20.17	20.64	20.25	Resistant
	Ciprofloxacin	Susceptible (0.03)	6.55	5.34	5.87	9.02	8.76	9.86	13.35	10.61	10.65	20.15	13.33	14.66	Susceptible

Table 4 **Percentage of the reduced form of resazurin obtained from 15 clinical strains of staphylococci (cont.)**

Bacteria (number of strain)	Antibiotic tested	Susceptibility by commercial methods (E-test MIC: mg/L)	Incubation time (minutes)												Susceptibility by resazurin
			75			90			105			120			
			WA	S	R	WA	S	R	WA	S	R	WA	S	R	
S. epidermidis (10)	Amikacin	Susceptible (1)	5.34	3.29	3.94	9.43	5.34	5.27	14.49	8.45	8.13	22.59	14.61	14.17	Susceptible
	Vancomycin	Susceptible (2)	6.72	5.39	6.41	10.29	7.23	8.40	15.44	10.27	9.57	20.82	12.17	13.28	Susceptible
	Oxacillin	Resistant (>256)	5.18	5.42	5.63	10.89	10.71	11.38	14.13	13.34	13.80	23.73	21.42	20.33	Resistant
	Ciprofloxacin	Susceptible (0.24)	7.94	6.74	5.91	12.32	8.36	8.67	16.28	12.92	12.41	25.30	15.93	14.19	Susceptible
S. hominis (11)	Amikacin	Susceptible (0.5)	4.39	5.58	5.21	10.47	9.82	8.38	16.12	12.47	13.11	25.17	14.88	15.84	Susceptible
	Vancomycin	Susceptible (1)	5.74	6.23	3.86	9.36	10.47	9.04	15.83	13.35	13.39	24.84	15.72	15.73	Susceptible
	Oxacillin	Resistant (32)	5.16	7.82	7.31	10.50	11.29	11.85	15.54	12.64	12.88	26.05	25.95	26.46	Resistant
	Ciprofloxacin	Susceptible (1)	6.22	4.19	3.99	9.28	10.51	10.60	16.91	15.39	15.23	23.18	22.50	21.24	Susceptible
S. hominis (12)	Amikacin	Susceptible (1)	9.31	7.52	6.27	15.91	9.23	9.49	24.77	11.32	11.82	30.22	14.96	15.17	Susceptible
	Vancomycin	Susceptible (2)	11.04	7.19	6.62	14.76	10.19	9.57	22.32	12.49	12.42	31.56	16.13	16.24	Susceptible
	Oxacillin	Susceptible(0.125)	10.73	6.43	7.84	14.12	9.90	8.01	23.66	14.72	13.15	32.78	15.27	14.52	Susceptible
	Ciprofloxacin	Susceptible (0.008)	10.68	8.55	8.51	15.47	10.38	10.25	22.72	12.21	12.94	31.63	15.36	15.66	Susceptible
S. hominis (13)	Amikacin	Susceptible (0.5)	4.14	4.28	5.10	8.85	6.13	6.34	12.53	9.27	10.19	20.45	13.54	13.31	Susceptible
	Vancomycin	Susceptible (1)	3.36	3.69	4.72	9.26	7.26	8.93	13.15	10.94	9.77	19.19	14.21	13.15	Susceptible
	Oxacillin	Resistant (>256)	4.95	5.35	5.44	8.68	8.42	8.63	12.68	12.26	13.81	21.64	22.78	20.63	Resistant
	Ciprofloxacin	Resistant (>32)	4.73	6.12	4.98	9.75	8.33	9.75	15.18	14.79	13.63	20.95	21.39	20.42	Resistant
S. lugdunensis (14)	Amikacin	Susceptible (0.06)	11.71	8.94	7.20	19.41	10.09	10.46	29.66	12.94	11.29	38.28	15.63	15.64	Susceptible
	Vancomycin	Susceptible (0.5)	14.16	7.11	7.13	22.55	9.75	10.70	30.31	13.11	13.87	39.37	16.14	15.37	Susceptible
	Oxacillin	Susceptible (0.32)	15.50	8.04	9.24	20.27	11.81	11.32	33.27	13.23	12.24	41.67	14.31	14.51	Susceptible
	Ciprofloxacin	Susceptible(0.06)	13.34	6.64	6.83	21.71	9.53	9.24	32.78	11.44	13.11	40.07	14.62	15.74	Susceptible
S. haemolyticus (15)	Amikacin	Susceptible (1)	5.49	4.53	4.26	11.83	5.16	8.95	16.45	8.97	11.81	22.13	14.57	14.63	Susceptible
	Vancomycin	Susceptible (0.5)	6.62	4.16	5.86	9.94	7.62	7.87	15.28	10.84	12.38	24.86	15.39	16.48	Susceptible
	Oxacillin	Susceptible (0.125)	5.10	5.86	6.38	10.11	8.31	8.39	16.36	13.77	12.76	24.42	16.21	15.13	Susceptible
	Ciprofloxacin	Susceptible (0.24)	6.75	5.48	5.63	10.25	8.95	9.28	16.76	13.14	13.94	25.74	15.70	15.89	Susceptible

WA: tube without antibiotic; S: tube with antibiotic at breakpoint concentration of susceptibility; R: tube with antibiotic at breakpoint concentration of resistance

later after adding resazurin, results could be interpreted. In this study, in order to reduce the time required to perform the AST, bacteria, resazurin and antibiotic were incubated together. This procedure allowed us to obtain reliable results in less than two hours in staphylococci and enterococci.

In both AST performed using collection and clinical strains, when a colour change from blue to red was observe the percentage of the reduced form of resazurin was, in all cases, higher than 17% (tables 1-5). By testing more strains, we think that it could be possible to define a criterion of susceptibility using the optical density readings. This fact might facilitate the automation of the assay for routine use in clinical microbiology laboratories because the AST could be interpreted by a spectrophotometer.

In addition, resazurin is a potential alternative method for performing a fast and reliable AST for other microorganisms. In the case of *Mycobacterium tuberculosis*, it is possible to obtain results with an incubation of, at least, eight days[15-18]. In yeasts, results can be achieved with an incubation up to 48 hours[19-22]. Finally, resazurin, in a period of time ranged from 24 to 53 hours, allows determining the cytotoxicity of different drugs against parasites, such as *Leishmania* spp. and *Trypanosoma cruzi* [23-25].

Since MALDI-TOF has revolutionized clinical microbiology laboratories due to the fact that bacterial identification can be obtained in some minutes[26], resazurin could be used in commercial panels with a large inoculum of staphylococci, enterococci to provide a faster AST without the need to introduce another methodology in the laboratory. Moreover, given that the change from blue (no growth) to red (growth) is ob-

Table 5 **Percentage of the reduced form of resazurin obtained from 15 clinical strains of enterococci**

Bacteria (number of strain)	Antibiotic tested	Susceptibility by commercial methods (E-test MIC: mg/L)	Incubation time (minutes)												Susceptibility by resazurin
			75			90			105			120			
			WA	S	R	WA	S	R	WA	S	R	WA	S	R	
E. faecalis (1)	Ampicillin	Susceptible (0.5)	15.12	6.17	7.38	21.87	10.55	11.38	34.79	13.15	12.71	45.41	15.28	15.77	Susceptible
	Vancomycin	Susceptible (1)	15.31	7.80	7.13	22.73	10.91	9.26	31.20	12.06	12.42	40.98	16.93	15.47	Susceptible
	Ciprofloxacin	Susceptible (2)	15.15	5.27	6.42	22.24	9.13	9.82	30.42	13.87	13.29	42.35	14.34	15.22	Susceptible
E. faecalis (2)	Ampicillin	Susceptible (2)	13.21	7.43	6.19	19.19	11.84	10.49	29.11	13.18	12.68	37.79	13.66	13.28	Susceptible
	Vancomycin	Susceptible (2)	12.10	6.46	6.62	18.88	9.77	9.70	27.87	12.61	10.62	35.58	15.91	14.12	Susceptible
	Ciprofloxacin	Resistant (>32)	14.95	13.24	14.72	20.17	21.93	20.64	28.33	28.32	28.59	37.96	39.19	34.27	Resistant
E. faecalis (3)	Ampicillin	Susceptible (1)	19.48	6.44	6.81	29.63	10.23	9.79	38.39	11.21	11.51	43.19	15.20	14.51	Susceptible
	Vancomycin	Susceptible (0.5)	18.71	6.26	5.68	29.44	8.97	9.58	41.19	13.72	13.86	50.28	16.89	14.13	Susceptible
	Ciprofloxacin	Susceptible (1)	21.90	5.70	7.93	28.14	9.80	9.39	40.62	12.82	13.43	52.14	15.93	15.66	Susceptible
E. faecalis (4)	Ampicillin	Resistant (>256)	12.43	8.63	8.37	22.69	21.54	20.96	31.48	30.74	32.11	43.33	40.27	39.26	Resistant
	Vancomycin	Susceptible (2)	11.54	7.59	6.31	20.06	10.68	10.45	29.56	12.83	14.27	37.69	15.64	15.78	Susceptible
	Ciprofloxacin	Resistant (>32)	11.75	10.70	10.47	21.91	22.11	22.93	30.67	29.39	26.84	38.81	37.31	38.31	Resistant
E. faecalis (5)	Ampicillin	Susceptible (2)	18.95	5.34	5.10	24.28	7.25	7.02	35.18	12.58	11.18	47.26	15.73	14.71	Susceptible
	Vancomycin	Susceptible (2)	19.27	3.29	4.29	25.11	5.14	5.76	33.83	12.44	9.94	43.41	14.84	13.05	Susceptible
	Ciprofloxacin	Susceptible (0.06)	18.96	4.12	3.12	27.74	7.85	6.88	34.11	10.82	10.73	45.49	13.62	13.48	Susceptible
E. faecalis (6)	Ampicillin	Susceptible (1)	5.04	3.41	3.28	12.51	6.55	7.75	19.93	10.19	10.97	33.84	15.29	15.63	Susceptible
	Vancomycin	Susceptible (2)	6.11	4.36	4.71	11.19	6.68	8.22	20.84	11.13	12.73	34.32	15.68	14.16	Susceptible
	Ciprofloxacin	Susceptible (2)	5.87	5.45	3.83	13.65	8.44	7.81	21.30	9.28	9.22	34.53	15.42	14.31	Susceptible
E. faecalis (7)	Ampicillin	Susceptible (1)	12.59	4.48	5.62	20.16	7.94	6.34	31.65	12.72	11.39	40.80	15.59	14.40	Susceptible
	Vancomycin	Susceptible (0.5)	12.56	3.75	3.97	20.37	6.39	6.12	32.26	12.54	12.18	45.74	14.38	15.98	Susceptible
	Ciprofloxacin	Susceptible (1)	12.88	5.11	4.64	19.84	7.47	7.23	30.32	11.17	10.59	39.36	14.89	13.17	Susceptible
E. faecalis (8)	Ampicillin	Resistant (16)	19.16	20.51	19.94	29.27	31.26	30.87	40.26	41.12	41.62	53.88	50.47	52.12	Resistant
	Vancomycin	Susceptible (2)	20.48	4.42	4.37	28.12	7.31	8.55	43.57	11.48	10.79	56.55	14.78	15.29	Susceptible
	Ciprofloxacin	Resistant (>32)	18.84	19.18	19.28	31.55	28.12	28.26	44.40	40.23	39.82	51.47	49.51	51.66	Resistant
E. faecalis (9)	Ampicillin	Susceptible(1)	5.34	5.73	5.85	11.76	8.42	8.40	19.78	10.92	11.58	32.75	15.92	14.53	Susceptible
	Vancomycin	Susceptible (1)	4.79	4.50	3.13	12.33	8.67	7.48	20.94	12.11	12.93	29.28	14.49	15.46	Susceptible
	Ciprofloxacin	Susceptible (1)	6.32	5.13	4.80	10.69	9.29	6.57	18.12	11.27	9.14	30.32	16.77	14.23	Susceptible
E. faecalis (10)	Ampicillin	Susceptible (2)	12.97	8.71	5.17	20.28	10.97	9.24	31.38	12.16	11.98	42.68	16.12	14.79	Susceptible
	Vancomycin	Susceptible(2)	13.73	6.93	7.85	20.62	11.34	9.05	30.72	12.48	12.21	45.43	15.28	15.36	Susceptible
	Ciprofloxacin	Susceptible(0.5)	13.22	8.39	8.92	21.59	10.74	11.84	36.47	11.53	12.55	49.29	13.86	13.27	Susceptible
E. faecium (11)	Ampicillin	Resistant (>256)	20.82	20.68	21.17	32.27	33.26	33.31	44.29	40.94	40.89	52.13	49.77	47.15	Resistant
	Vancomycin	Susceptible(2)	22.45	5.27	5.29	32.58	8.75	7.19	45.64	9.11	11.29	57.41	13.63	13.71	Susceptible
	Ciprofloxacin	Susceptible (2)	20.26	5.59	4.42	33.74	7.99	7.52	40.57	12.84	10.74	50.18	15.95	12.23	Susceptible

Table 5 **Percentage of the reduced form of resazurin obtained from 15 clinical strains of enterococci (cont.)**

Bacteria (number of strain)	Antibiotic tested	Susceptibility by commercial methods (E-test MIC: mg/L)	Incubation time (minutes)												Susceptibility by resazurin
			75			90			105			120			
			WA	S	R	WA	S	R	WA	S	R	WA	S	R	
E. faecium (12)	Ampicillin	Resistant (>256)	11.88	10.36	12.16	20.98	19.83	19.83	32.88	30.43	29.28	44.16	39.33	41.34	Resistant
	Vancomycin	Susceptible (2)	12.78	5.33	7.10	19.26	8.19	8.46	31.27	11.81	12.10	40.84	14.28	15.11	Susceptible
	Ciprofloxacin	Susceptible (1)	10.27	6.41	4.35	22.03	8.64	9.28	30.14	12.59	13.64	43.58	15.13	15.67	Susceptible
E. faecium (13)	Ampicillin	Resistant (>256)	19.19	18.26	18.93	29.34	28.67	27.15	38.12	35.15	36.49	47.76	48.34	48.24	Resistant
	Vancomycin	Susceptible (1)	20.32	6.47	5.54	32.26	9.86	8.72	36.88	12.41	12.26	46.95	14.72	16.48	Susceptible
	Ciprofloxacin	Susceptible (1)	22.97	4.35	4.33	33.57	7.29	10.16	40.14	11.67	11.75	50.28	15.76	15.71	Susceptible
E. faecium (14)	Ampicillin	Susceptible (0.5)	11.43	8.83	7.87	19.26	10.45	10.64	28.81	12.72	13.19	35.81	15.84	16.65	Susceptible
	Vancomycin	Susceptible (2)	12.99	10.27	11.22	18.83	12.17	12.58	29.42	13.13	14.64	38.43	16.21	15.18	Susceptible
	Ciprofloxacin	Susceptible (1)	10.55	9.34	9.23	22.52	11.59	12.44	32.28	13.46	13.82	40.11	16.44	16.38	Susceptible
E. faecium (15)	Ampicillin	Susceptible (1)	11.63	6.28	7.22	22.58	10.30	9.71	33.27	13.12	11.63	40.35	14.18	15.63	Susceptible
	Vancomycin	Susceptible (1)	11.77	5.63	7.48	20.03	8.29	8.62	34.84	12.46	12.82	44.71	15.23	13.52	Susceptible
	Ciprofloxacin	Susceptible (0.06)	12.31	7.47	7.59	25.72	10.88	9.16	33.93	11.39	12.25	42.11	13.42	15.38	Susceptible

WA: tube without antibiotic; S: tube with antibiotic at breakpoint concentration of susceptibility; R: tube with antibiotic at breakpoint concentration of resistance

vious, even for wells with reduced or smalls rates of growth, resazurin could overcome problems frequently encountered in conventional broth microdilution tests, such as inoculums sedimentation or very scant or transparent growth, which occur with some species of bacteria[7].

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