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ARTICLE

Standard Nutrient Agar 1 as a substitute for blood-supplemented Müller–Hinton agar for antibiograms in developing countries

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Abstract In the industrial world, the agar diffusion test is a standard procedure for the susceptibility testing of bacteria isolates. Beta-hemolytic Streptococcus spp. are tested with Müller–Hinton agar supplemented with 5 % blood, a socalled blood agar. The results are interpreted using standardized tables, which only exist for this type of nutrient matrix. Because of a number difficulties, both with respect to technicalissues and to manual skills, blood agar is not a feasible option in many developing countries. Beta-hemolytic Streptococcus spp. also grow on Standard Nutrient Agar 1 (StNA1). This suggests using that type of nutrient medium for running agar diffusion tests. However, there are no standardized tables that can be used for interpreting the diameters of the zones of inhibition on StNA1 1. Using the existing standardized tables for blood agar to interpret cultures on StNA1 1 would be of great benefit under such circumstances where blood agar is not available.With this in mind, we conducted comparative tests to evaluate the growth characteristics of beta-hemolytic Streptococcus spp. on StNA1 1 compared to Müller–Hinton agar supplemented with 5%sheep blood. In this study, we were able to show that betahemolytic Streptococcus spp. develop similar zones of inhibition on blood agar and on StNA1 1. Therefore, it is suggested that, for the interpretation of antibiograms of betahemolytic Streptococcus spp. performed on StNA1 1, the standard tables for blood agar can be used.

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Introduction

In the industrial world, the agar diffusion test or the Kirby–Bauer disk diffusion method is a standard procedure for the susceptibility testing of bacteria isolates. Bacteria to be tested are streaked uniformly across a culture plate. Paper disks impregnated with different antibiotics to be tested are placed on the surface of the gelatinous agar. During incubation of the agar plate, the antibiotics diffuse from the disks into the surrounding agar. An effective agent will inhibit bacterial growth, and the size of such a zone of inhibition (ZoI) around the disk is measured. The relative effectiveness of a compound is determined by comparing the diameter of the ZoI with values in standard tables.

However, the use of standard tables requires the culture plates to be filled correctly with standardized nutrition media. For example, Enterobacteriaceae, Pseudomonas spp., Staphylococcus spp., and Enterococcus spp. are tested on agar plates containing Müller–Hinton agar, which should have a level depth of 4 mm±0.5 mm [4]. Beta-hemolytic Streptococcus spp., however, are tested with Müller–Hinton agar supplemented with 5 % defibrinated blood [4], a so-called blood agar. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommends defibrinated horse blood for the susceptibility testing of beta-hemolytic Streptococcus spp., but defibrinated or citrated sheep blood can also be used [5, 8, 9]. Thus, antibiograms of beta-hemolytic Streptococcus spp. using agar diffusion tests require blood agar. Because of a number difficulties, both with respect to technical issues and to manual skills, blood agar is not a

feasible option in many developing countries. The first challenge is to collect and defibrinate animal blood aseptically. Another problem is the monitoring of animal health, which is highly important but difficult to achieve because of a lack of qualified and experienced people. Furthermore, without a blood bank, the storing of blood is practically impossible. The main problem, however, is the aseptic preparation the blood agar. The blood has to be blended with the agar matrix at temperatures below 40 °C, as blood will denaturate at higher temperatures. In industrial countries, there are laminar flow cabinets available for this step, but most laboratories in developing countries cannot afford these culture hoods. Sterilization of the filled plates is not an option, because low-temperature sterilizers, e.g., plasma sterilizers, gas sterilizers, or x-ray sterilizers, are also too expensive, too intricate, or too dangerous for use by lab technicians with insufficient training. In other words, agar diffusion tests cannot be conducted correctly in many developing countries.

For some time, a culture medium has been commercially available which acts as a universal nutrient matrix for many bacterial families. Standard Nutrient Agar 1 (StNA1), marketed by Carl Roth GmbH & Co. KG, Germany, can be used for culturing Enterobacteriaceae, Pseudomonas spp., Staphylococcus spp., and Streptococcus spp. [3]. Betahemolytic Streptococcus spp. grow on StNA1, which suggests conducting agar diffusion tests on StNA1. StNA1 is not temperature-sensitive and can be prepared and autoclaved like Müller–Hinton agar. Unfortunately, there are no standard tables available that can be used to interpret the diameters of the zones of inhibition on StNA1. The use of existing tables for interpreting the diameters on blood agar would require that beta-hemolytic Streptococcus spp. have the same, or at least very similar, growth characteristics on both of these nutrient media. If nearly equal growth characteristics of beta-hemolytic Streptococcus spp. on blood agar and on StNA1 can be demonstrated, already existing tables for interpreting the diameters on blood agar can be used to interpret the diameters of the zones of inhibition on StNA1.

With this in mind, we conducted 103 comparative tests to evaluate the growth characteristics of beta-hemolytic Streptococcus spp. on StNA1 compared to Müller–Hinton agar supplemented with 5 % sheep blood (B-MH). Our intention was to ascertain whether pathogenic betahemolytic streptococci show similar growth on StNA1 and on B-MH. One possible way to demonstrate this is to compare the diameters of the zones of inhibition on the two different agars. If there are no significant differences and no spikes, laboratories in developing countries can use easily preparable StNA1 and readily available standard tables dedicated to test beta-hemolytic Streptococcus spp. on B-MH for antibiograms.

Materials and methods

The study was conducted at the laboratory of the nongovernmental organization Globolab e.V., in Assling, Germany. The specimens were obtained from patients admitted to a cooperating hospital in Germany. Streaking and susceptibility testing was done at the Globolab e.V. laboratory. Sixteen beta-hemolytic streptococci strains were obtained. Strain numbers, species, and the sources of the specimens are shown in Table 1. Species classifying was performed with the API identification system Rapid ID 32 from bioMérieux.

Susceptibility tests

The streaking of specimens (three-sector streaking) and isolation of single colonies were performed on ColumbiaAgar+5% wether blood obtained from bioMérieux. This agar contains casein peptone and meat peptone (bovine and pig; 10.0 g/l), protein hydrolysate (bovine or pig; 10.0 g/l), heart peptone (bovine or pig; 3.0 g/l), maize starch (1.0 g/l), sodium chloride (5.0 g/l), blood (wether; 50 ml/l), and agar (13.5 g/l) [1]. After the isolation of single beta-hemolytic streptococci colonies, saline was inoculated with the colonies to make a suspension equal to a McFarland 0.5 turbidity standard. Each suspension was plated on B-MH as well as on StNA1. B-MH was obtained from bioMérieux. The ready-to-use plates contain casein peptone (bovine; 17.5 g/l), meat extract (bovine or pig; 2.0 g/l), potato starch (1.5 g/l), blood (sheep; 50 ml/l), and agar (17.0 g/l) [2].

Table 1 Tested Streptococcus spp.

Strain no.	No. of species	Source of specimen
01	Streptococcus agalactiae	Infected wound
02	Streptococcus dysgalactiae ssp. equisimilis	Blood culture
03	Streptococcus dysgalactiae ssp. equisimilis	Infected wound
04	Streptococcus dysgalactiae	Blood culture
05	Streptococcus dysgalactiae ssp. dysgalactiae	Infected wound
06	Streptococcus agalactiae	Urine
07	Streptococcus agalactiae	Urine
08	Streptococcus anginosus	Swab, soft tissue surgery
09	Streptococcus dysgalactiae ssp. equisimilis	Blood culture
10	Streptococcus dysgalactiae ssp. equisimilis	Infected wound
11	Streptococcus dysgalactiae	Glenohumeral joint
12	Streptococcus agalactiae	Cervix uteri
13	Streptococcus pyogenes	Blood culture
14	Streptococcus agalactiae	Infected wound
15	Streptococcus agalactiae	Urine
16	Streptococcus agalactiae	Urine

StNA1 was obtained from Carl Roth GmbH + Co. KG in the form of a granular base. Three different lots were obtained and the nutrient matrix was prepared at the Globolab e.V. laboratory, according the manufacturer's instructions. After cooking the agar, glass petri dishes were filled to a level depth of 4 mm±0.5 mm according to the guidelines of the EUCAST. The StNA1 matrix contains peptone (15.0 g/l), yeast extract (3.0 g/l), sodium chloride (6.0 g/l), dextrose (1.0 g/l), and agar (12.0 g/l) [3]. The StNA1 plates were autoclaved at 124 °C for 15 min. After sterilization, the nutrient medium was allowed to solidify and was subsequently dried in a Memmert UE 200 incubator.

The inoculated plates, B-MH as well as StNA1, were incubated in ambient air at 35 °C for 19 h in a Memmert UE 200 incubator. Antibiotic susceptibility testing was performed according to the EUCAST methods [4]. Antimicrobial susceptibility testing disks were obtained from Oxoid Deutschland GmbH. Zones of inhibition were measured from above the agar surface with a back light as well as with a dark background.

The following antibiotics were tested, each on B-MH and on StNA1:

Amoxicillin (AMX) on strains: 01, 03, 04, 05, 07, 08, 09, 10, 11, 12, 13, 14, 15, and 16
Ampicillin (AMP) on strains: 01, 02, 03, 04, 05, 06, 07, 08, 09, 10, 11, 12, 13, 14, 15, and 16
Ceftriaxone (CRO) on strains: 01, 02, 03, 04, 05, 06, 07, 08, 09, 10, 11, 12, 13, 14, 15, and 16
Ciprofloxacin (CIP)[1] on strains: 02, 06, 07, 11, 12, 13, 14, 15, and 16
Erythromycin (ERY) on strains: 01, 02, 03, 04, 05, 06, 07, 08, 09, 10, 11, 12, 13, 14, 15, and 16
Penicillin (PEN) on strains: 01, 02, 03, 04, 05, 06, 07, 08, 09, 10, 11, 12, 13, 14, 15, and 16
Vancomycin (VAN) on strains: 01, 03, 04, 05, 07, 08, 09, 10, 11, 12, 13, 14, 15, and 16
Amoxicillin–clavulanic acid (AMC) on strains: 07 and 11

Results

Statistical summary

As shown in Table 2, we found in 96.12 % (99 out of 103) of all cases that the absolute values of ZoI differences were

[1] CIP is not recommended for treating beta-hemolytic Streptococcus spp., but in this study, we analyzed and compared the growth behavior of these bacteria, not their resistances. For this reason, we decided to check also an antibiotic not common for treating beta-hemolytic Streptococcus spp.

Table 2 Comparison of antibiograms on Standard Nutrient Agar 1 (StNA1) and Müller–Hinton agar supplemented with 5 % sheep blood (B-MH) with various strains and antibiotic agents

Test no.	Strain no.	Antibiotic	ZoI (StNA1)	ZoI (B-MH)	ΔZoI
1	1	PEN	20	30	+10
2	1	AMP	30	30	0
3	1	AMX	32	32	0
4	1	CRO	32	32	0
5	1	VAN	18	18	0
6	1	ERY	26	26	0
7	2	PEN	32	30	−2
8	2	AMP	31	32	+1
9	2	CRO	27	30	+3
10	2	CIP	22	20	−2
11	2	ERY	21	22	+1
12	3	PEN	30	30	0
13	3	AMP	31	30	−1
14	3	AMX	31	30	−1
15	3	CRO	30	30	0
16	3	VAN	19	20	+1
17	3	ERY	18	20	+2
18	4	PEN	34	33	−1
19	4	AMP	32	30	−2
20	4	AMX	33	32	−1
21	4	CRO	32	30	−2
22	4	VAN	22	20	−2
23	4	ERY	32	29	−3
24	5	PEN	33	37	+4
25	5	AMP	31	32	+1
26	5	AMX	32	34	+2
27	5	CRO	32	35	+3
28	5	VAN	18	18	0
29	5	ERY	28	28	0
30	6	PEN	33	34	+1
31	6	AMP	35	35	0
32	6	CRO	35	35	0
33	6	CIP	23	24	+1
34	6	ERY	29	29	0
35	7	PEN	37	38	+1
36	7	AMP	38	37	−1
37	7	AMX	40	39	−1
38	7	CRO	38	38	0
39	7	CIP	29	28	−1
40	7	VAN	24	22	−2
41	7	ERY	28	30	+2
42	7	AMC	40	38	−2
43	8	PEN	23	22	−1
44	8	AMP	22	22	0
45	8	AMX	19	20	+1
46	8	CRO	19	20	+1
47	8	VAN	17	16	−1

Table 2 (continued)

Test no.	Strain no.	Antibiotic	ZoI (StNA1)	ZoI (B-MH)	ΔZoI
48	8	ERY	19	20	+1
49	9	PEN	30	30	0
50	9	AMP	32	33	+1
51	9	AMX	32	33	+1
52	9	CRO	30	28	−2
53	9	VAN	18	19	+1
54	9	ERY	28	30	+2
55	10	PEN	26	22	−4
56	10	AMP	28	24	−4
57	10	AMX	28	25	−3
58	10	CRO	28	25	−3
59	10	VAN	19	18	−1
60	10	ERY	26	25	−1
61	11	PEN	38	40	+2
62	11	AMP	40	40	0
63	11	AMX	45	46	+1
64	11	CRO	36	38	+2
65	11	CIP	24	26	+2
66	11	VAN	21	22	+1
67	11	ERY	29	30	+1
68	11	AMC	38	40	+2
69	12	PEN	43	45	+2
70	12	AMP	42	44	+2
71	12	AMX	43	45	+2
72	12	CRO	38	40	+2
73	12	CIP	28	29	+1
74	12	VAN	22	22	0
75	12	ERY	27	30	+3
76	13	PEN	41	40	−1
77	13	AMP	40	40	0
78	13	AMX	41	40	−1
79	13	CRO	39	40	+1
80	13	CIP	28	27	−1
81	13	VAN	25	24	−1
82	13	ERY	30	30	0
83	14	PEN	40	41	+1
84	14	AMP	40	40	0
85	14	AMX	40	41	+1
86	14	CRO	36	38	+2
87	14	CIP	25	27	+2
88	14	VAN	21	21	0
89	14	ERY	31	30	−1
90	15	PEN	44	43	−1
91	15	AMP	41	43	+2
92	15	AMX	44	46	+2
93	15	CRO	40	39	−1
94	15	CIP	28	31	+3
95	15	VAN	24	25	+1
96	15	ERY	5	5	0

Table 2 (continued)

Test no.	Strain no.	Antibiotic	ZoI (StNA1)	ZoI (B-MH)	ΔZoI
97	16	PEN	36	38	+2
98	16	AMP	35	37	+2
99	16	AMX	36	37	+1
100	16	CRO	36	37	+1
101	16	CIP	24	24	0
102	16	VAN	21	20	−1
103	16	ERY	32	32	0
∑ +33 mm					

smaller than or equal to 3 mm, and 89.32 % (92 out of 103) were smaller than or equal to 2 mm.

Even more significant than the above-mentioned absolute values of ZoI differences are the signed values of the ZoI differences, because, as is readily understood, a ZoI difference between two different culture media is an indication of either the inhibition (negative difference value) or increase (positive difference value) of growth. For this reason, the algebraic signs of the ZoI differences cannot be disregarded.

The sum of all differences between B-MH and StNA1 is +33 mm. Thus, the average of the ZoI differences between the two media is 0.32 mm, calculated on the basis of signed values (ΣZoI differences/no. of tests).

As also shown in Table 2, we found ZoI diameters between 5 and 46 mm on the B-MH plates. The average ZoI diameter on the B-MH plates (Σall ZoI on blood agar/ no. of all ZoI on blood agar) is 30.60 mm. Therefore, an average ZoI difference of 0.32 mm is 1.05 % of the average ZoI diameter on B-MH.

Finally, it has to be noted that there is no significant spike for any antibiotic agent. This means that there is no antibiotic agent causing an agent-specific idiosyncrasy on only one of the two reviewed nutrient media, B-MH and StNA1.

Interpretation of the statistical summary

Firstly, distinctions in biological systems are to be expected. In the present case, these distinctions should become manifest in different inhibition zone diameters. Secondly, the EUCAST recommend rulers, callipers, or automatic zone readers for measuring the ZoI diameters [4]. However, with the use of semi-opaque agar plates and frequently appearing blurred edges between bacterial growth and inhibition zones, measuring errors of a few millimeters are common [7]. Furthermore, with automatic zone readers, errors of up to 3 mm are accepted [6].

In summary, differences in the ZoI diameter of up to 2 or 3 mm are acceptable. In other words, significant differences in the growth behavior of beta-hemolytic Streptococcus spp. on B-MH compared to StNA1 must cause ZoI diameters

differences of more than 3 mm. The average ZoI difference of 0.32 mm between B-MH and StNA1 suggests that betahemolytic Streptococcus spp. show very similar growth behavior on B-MH and on StNA1.

Conclusion

In this study, we show that, in over 96 % of all cases, betahemolytic Streptococcus spp. develop a similar ZoI on BMH and on StNA1. The statistical average of ZoI differences between these two nutrient media is 0.32 mm, a value so small that it cannot be detected with a ruler, let alone the naked eye. Therefore, it is suggested that, for the interpretation of antibiograms performed on StNA1, the tables dedicated to test beta-hemolytic Streptococcus spp. on B-MH can be consulted.

Discussion

It goes without saying that our intention is not to replace blood agar with StNA1. Blood agar is always the better choice, because:

- Fastidious bacteria grow better on blood agar than on StNA1
- Different bacteria species can be distinguished easier on blood agar than on StNA1 due to their different appearance on this nutrient medium
- Hemolysis can only be observed on blood agar

On the other hand, it has to be noted that the reading of the ZoI is often easier on StNA1 than on B-MH, because StNA1 is more transparent and, therefore, the edges between bacterial growth and the inhibition zone can be discerned more easily. Moreover, as pointed out in the introduction, using blood agar is not always feasible.

In such cases where no blood agar is available for conducting antibiograms, StNA1 is an appropriate substitute for blood-supplemented Müller–Hinton agar.

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