EUCAST EUROPEAN COMMITTEE ON ANTIMICROBIAL SUSCEPTIBILITY TESTING
European Society of Clinical Microbiology and Infectious Diseases

Mueller-Hinton agar with 5% mechanically defibrinated horse blood and 20 mg/L β-NAD (MH-F) continues to be the EUCAST recommended medium for AST of fastidious organisms. However, plates where horse blood is exchanged for sheep blood (MH-S) can with one exception be used instead – the exception is *Haemophilus* spp.

EUCAST recommends MH-F agar with 5% mechanically defibrinated horse blood and 20 mg/L β-NAD for disk diffusion antimicrobial susceptibility testing of fastidious organisms. The range of organisms where MH-F is recommended is evident from breakpoint tables and method documents on the EUCAST website (https://www.eucast.org/). It includes streptococci, *Haemophilus influenzae, Campylobacter, Kingella* and many others but, because of inadequate growth, not *Neisseria* spp. or anaerobic bacteria.

In parts of the world colleagues report difficulties accessing the MH-F medium. This is either because of a lack of tradition in the use of horse blood, or because manufacturers of pre-poured plates are not offering the product locally. EUCAST has been asked to investigate alternative media for disk diffusion of fastidious organisms without recalibrating the entire EUCAST system. After having tested a variety of alternative media, we decided to look further into using Mueller-Hinton agar with 5% defibrinated sheep blood (MH-S) instead of MH-F. This medium is recommended by CLSI for some, but not all, fastidious organisms.

A shared project between the EUCAST Development Laboratory (EDL, Växjö, Sweden) and Statens Serum Institut (SSI, Copenhagen, Denmark) was set up to further investigate MH-S as a substitute for MH-F.

All species for which disk diffusion on MH-F is recommended (except *Corynebacterium diphtheriae* and *C. ulcerans,* which could not be tested due to limiting safety regulations) were tested against all antimicrobial agents with zone diameter breakpoints in the EUCAST Breakpoint Tables v. 15.0.

Haemophilus influenzae does not grow on MH-S and was excluded from further testing and analysis.

In phase 1, disk diffusion testing was performed on MH-F and MH-S in parallel. Inhibition zone diameters on both media were read by the same technician and growth on both media were assessed visually. Categorical agreement (comparing susceptibility categories) for MH-S was calculated using MH-F as reference (EUCAST Breakpoint Tables v. 15.0). Also, the correlation and bias between zone diameters on the two agars were calculated.

In phase 2, wild-type isolates for each species were tested with disk diffusion on MH-S using plates prepared from Mueller-Hinton agar from three manufacturers to produce wild-type zone diameter distributions.

Relevant Quality Control (QC) strains (*Streptococcus pneumoniae* ATCC 49619 were used for all species, except for *Campylobacter* spp. for which *Campylobacter* ATCC 33560 was used) were tested in parallel throughout the project.

Results and discussion
For ***H. influenzae***, MH-S does not support growth. It was known from previous experiments that exchanging the horse blood with sheep blood would not be enough to support growth, even when adding β-NAD at various concentrations. EUCAST will continue to insist on the use of MH-F for disk diffusion of *H. influenzae*.

For **all other species** where MH-F is currently recommended, MH-S supported equally good growth as MH-F.

Correlation between MH-F and MH-S. 45-degree analyses comparing inhibition zone diameters on MH-F and MH-S for each species/organism group tested are shown in **Figure 1**. In each graph, the percentage of zone diameters on MH-S being within ± 1 mm with the corresponding zone diameter on MH-F is shown, as well as the percentage of zone diameters being larger ("above") and smaller ("below") on MH-S. Bias is calculated as the difference in percent of zones being larger and smaller (negative bias = smaller zones on MH-S, positive bias = larger zones on MH-S).

Random categorical errors occurred for 8 of the 34 tested antimicrobial agents, and represented different antimicrobial classes and different species (**Table 1**). In general, the errors were related to individual isolates with zone diameters close to the breakpoints, and with few exceptions could not be attributed to either of the two, alternative media.

Comparison of MH agar base manufacturers. Inhibition zone diameters on MH-S prepared from MH agar from three manufacturers showed overall equal results. Wild-type zone diameter distributions on MH-S are available in a separate document and can be used by laboratories wanting to implement MH-S for disk diffusion of fastidious organisms.

Quality control strains. Mean inhibition zone diameters for *S. pneumoniae* ATCC 49619 tested on MH-F and MH-S tested in parallel are shown in **Table 2a**. Mean values were close to the target values for both media and no systematic difference was observed. The same was true when comparing results from testing on MH-S prepared from MH agar from three manufacturers (**Table 2b**).

Reading of zone diameters. Reading of zone diameters were performed according to EUCAST standard reading instructions for fastidious organisms, with one important exception. When reading zone diameters for Streptococcus groups A, B, C and G and trimethoprim-sulfamethoxazole on MH-S, growth within the inhibition zone had to be ignored and the outer zone diameter read to get the correct result.

Conclusions. Based on all results, differences between the media were small and EUCAST therefore suggest the following **conclusions and recommendations:**

- Since MH-F supports growth of more species than MH-S, EUCAST will continue to consider **MH-F the standard EUCAST medium for disk diffusion of fastidious organisms** unless otherwise specifically stated. This will be evident from the methods section on the website and at the top of species tabs in breakpoint tables. For preparation of MH-F agar plates, see "Preparation of agar plates and broth for EUCAST AST" at https://www.eucast.org/ast_of_bacteria/media_preparation.
- **For almost all species and agents (the exception being *Haemophilus* spp), MH-S can be used instead of MH-F**. The MH-S agar plates must contain 5% mechanically defibrinated sheep blood and have the same agar depth as MH-F (4.0 mm with a random

variation of ± 0.5 mm). However, EUCAST will not validate MH-S to the same extent as MH-F, and when using MH-S instead of MH-F, it is the responsibility of the user to validate the quality and usability of the medium, for all disks and species included in disk diffusion testing at the laboratory. This is primarily done by use of QC strains but also by comparing local distributions with those available from EUCAST.

- When using MH-S instead of MH-F, all other aspects of the EUCAST disk diffusion method (e.g. inoculum preparation, inoculation of plates, application of disks, disk potencies, incubation and reading of zone diameters) should be adhered to.
- Reading of zone diameters on MH-S should be performed according to EUCAST standard reading instructions for fastidious organisms, except for **Streptococcus groups A, B, C and G and trimethoprim-sulfamethoxazole**, for which growth within the zone should be ignored and the outer zone edge read.
- EUCAST encourages **the involvement of NACs** in investigations into the national availability and comparative costs of MH-F and MH-S. NACs can aid by coordinating testing and trouble shooting. NACs may decide to recommend the use of MH-F or MH-S on a national basis. We encourage users to perform further comparisons and validation of MH-S, using the MH agar and disks available locally and to report problems to EUCAST. The EDL can assist in trouble shooting.
- **EUCAST will, for each new organism or antimicrobial agent developed for testing on MH-F, perform a parallel, less extensive validation of MH-S** to disclose any major discrepancies between the two media.
- Problems with using MH-S, when noted and confirmed by EUCAST, will be listed as a **warning on the EUCAST** website (https://www.eucast.org/ast-of-bacteria/warnings).

Table 1. Categorical errors for disk diffusion on MH-S using disk diffusion on MH-F as reference. Errors were calculated based on EUCAST Breakpoint Tables v 15.0.

Species/organism group	No of isolates tested	PCG	AMP	MOX	NOR	MIN	TET	TED	TSU
Streptococcus groups A, B, C and G	60					1	3	1	5[3]
Streptococcus pneumoniae	20					1			1
Viridans group streptococci	50	2	1						
Moraxella catarrhalis	20								
Listeria monocytogenes	20	1							
Pasteurella multocida	20								2
Campylobacter jejuni and *C. coli*	40								
Corynebacterium spp.[1]	20	1		1					
Aerococcus sanguinicola and *A. urinae*	20				2				
Kingella kingae	20	1							
Categorical errors (%)[2]		**2.4**	**0.8**	**0.6**	**2.0**	**2.0**	**1.5**	**0.9**	**5.0**
Total number of isolates tested		**210**	**130**	**170**	**100**	**100**	**200**	**110**	**160**

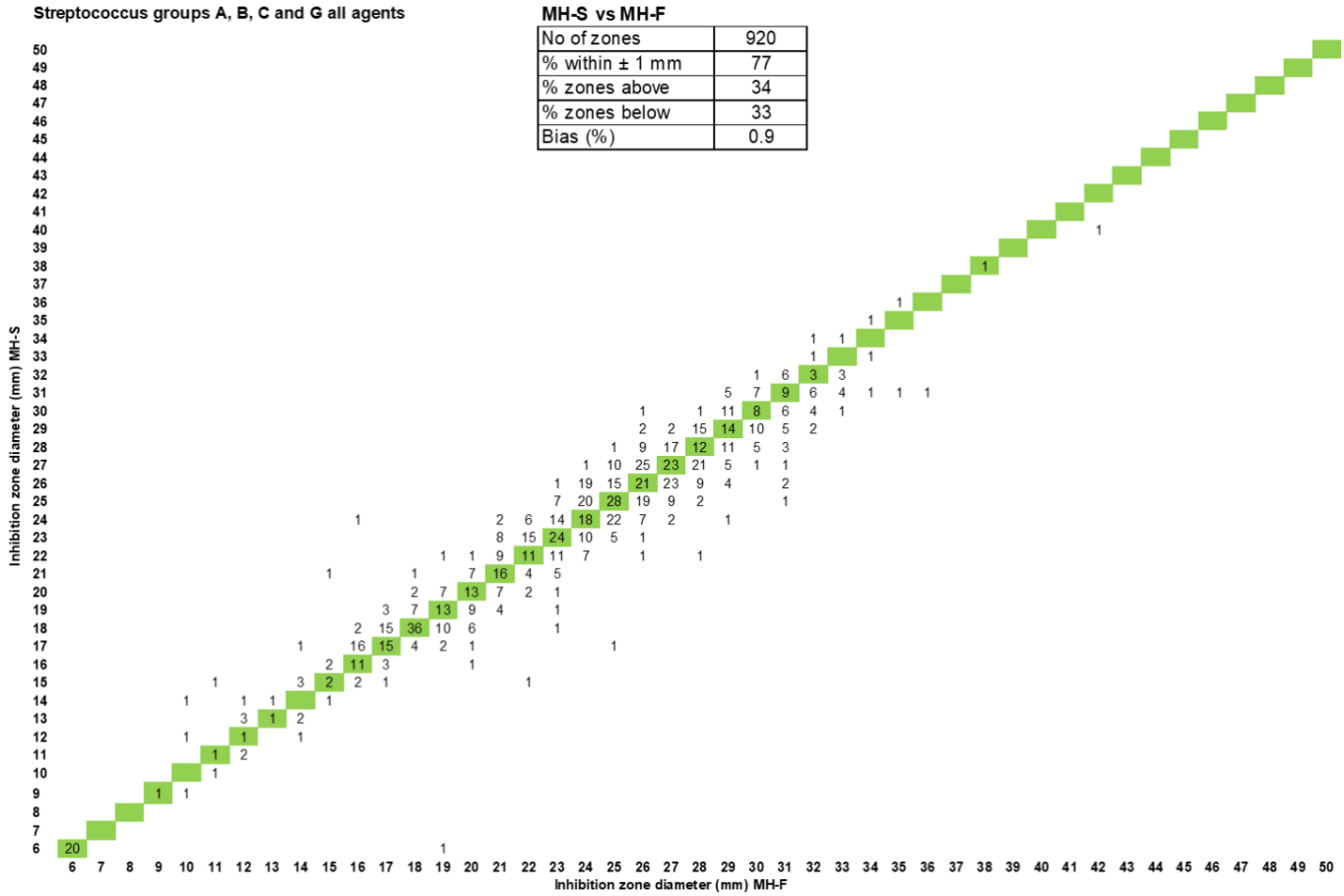
[1] Species other than *C. diphtheriae* and *C. ulcerans*.

[2] Based on the total number of isolates tested for each antimicrobial agent.

[3] When growth within the zone was igenored and the outer zone read.

PCG = Benzylpenicillin, AMP = Ampicillin, MOX = Moxifloxacin, NOR = Norfloxacin, MIN = Minocycline, TET = Tetracycline, TED = Tedizolid, TSU = Trimethoprim-sulfamethoxazole

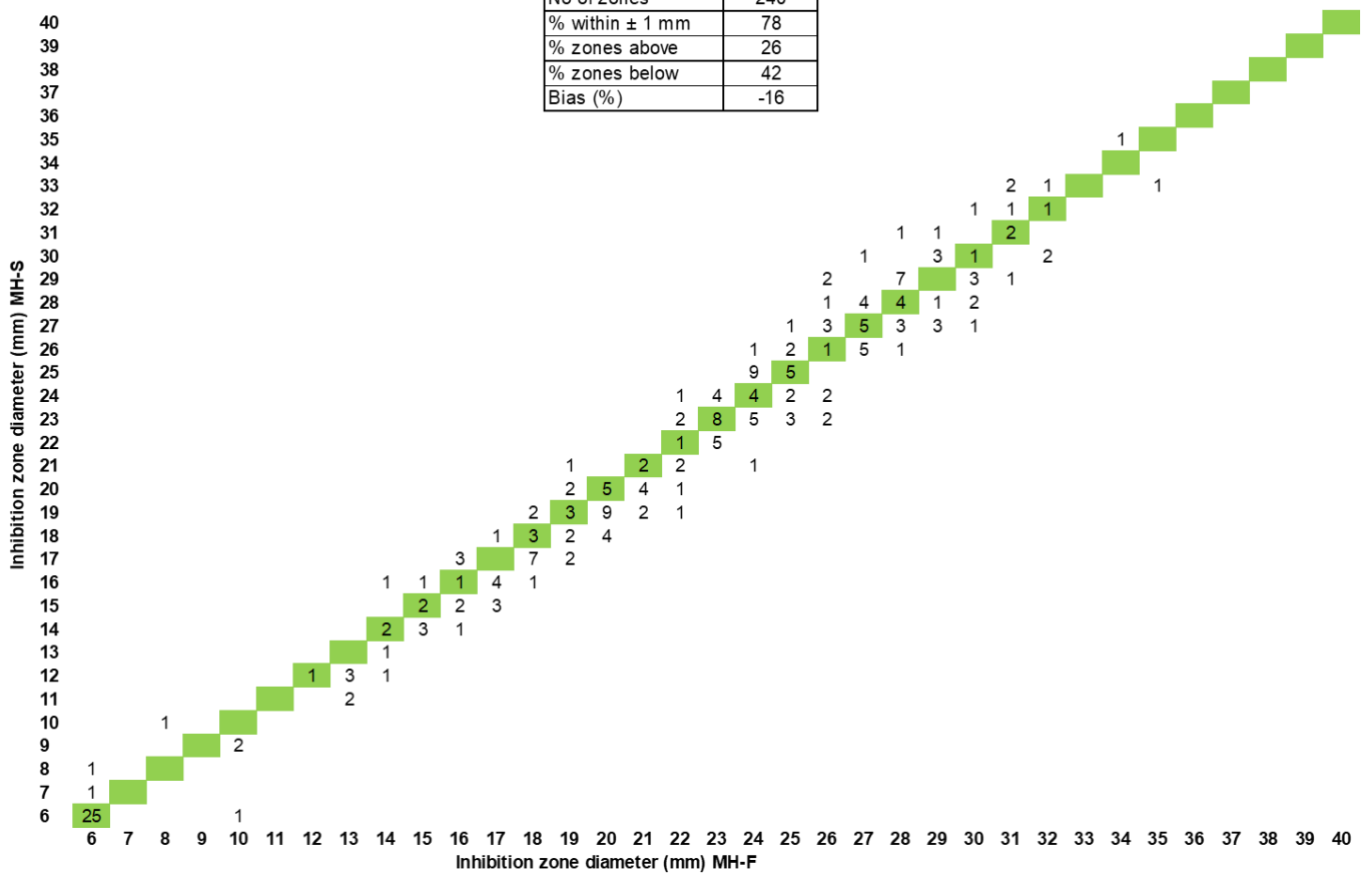
Figure 1. 45-degree tables comparing inhibition zone diameters on MH-S and MH-F for each species/organism group tested.



***Streptococcus pneumoniae* all agents**

MH-S vs MH-F

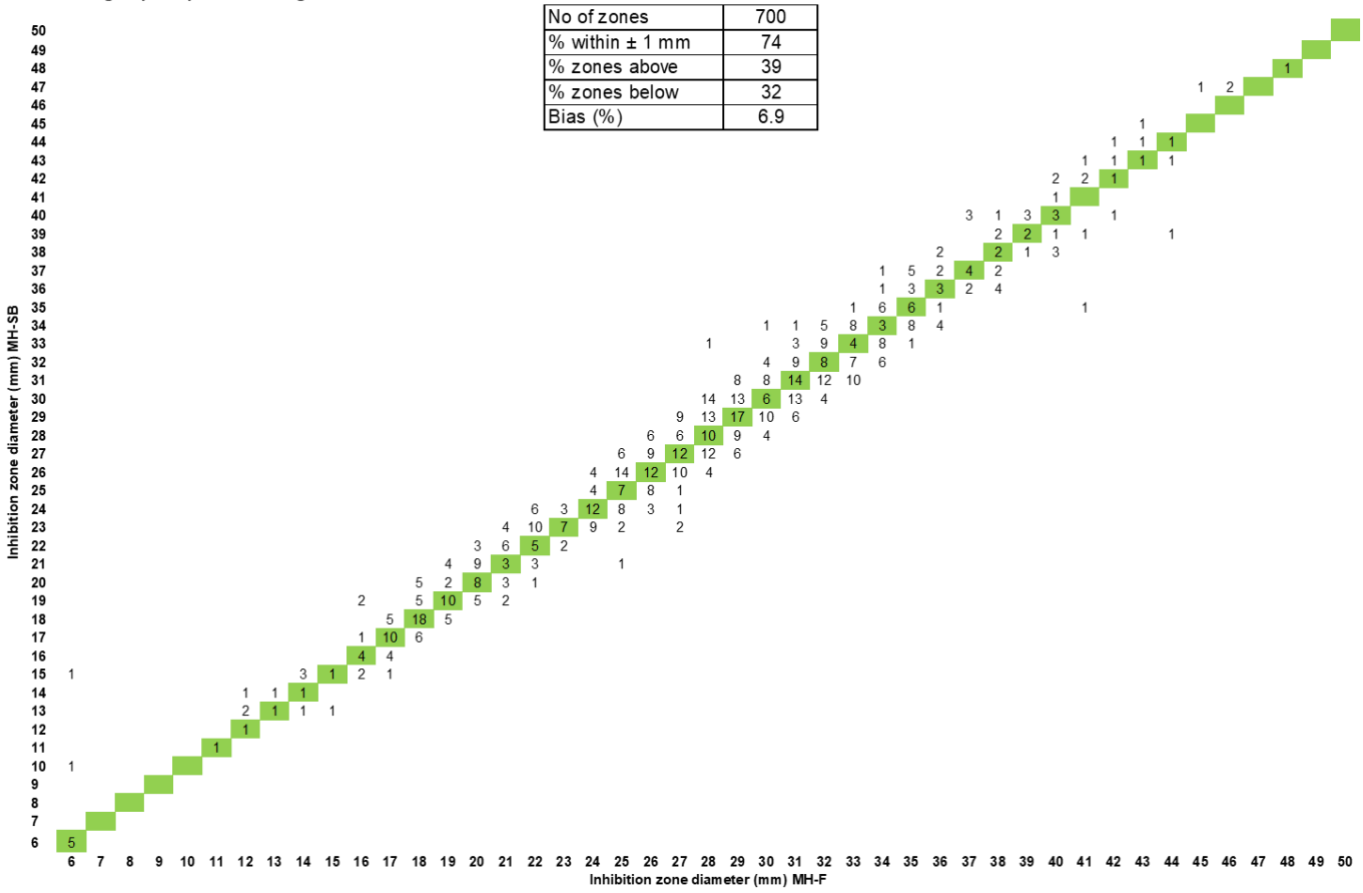
No of zones	240
% within ± 1 mm	78
% zones above	26
% zones below	42
Bias (%)	-16



Viridans group streptococci all agents

MH-S vs MH-F

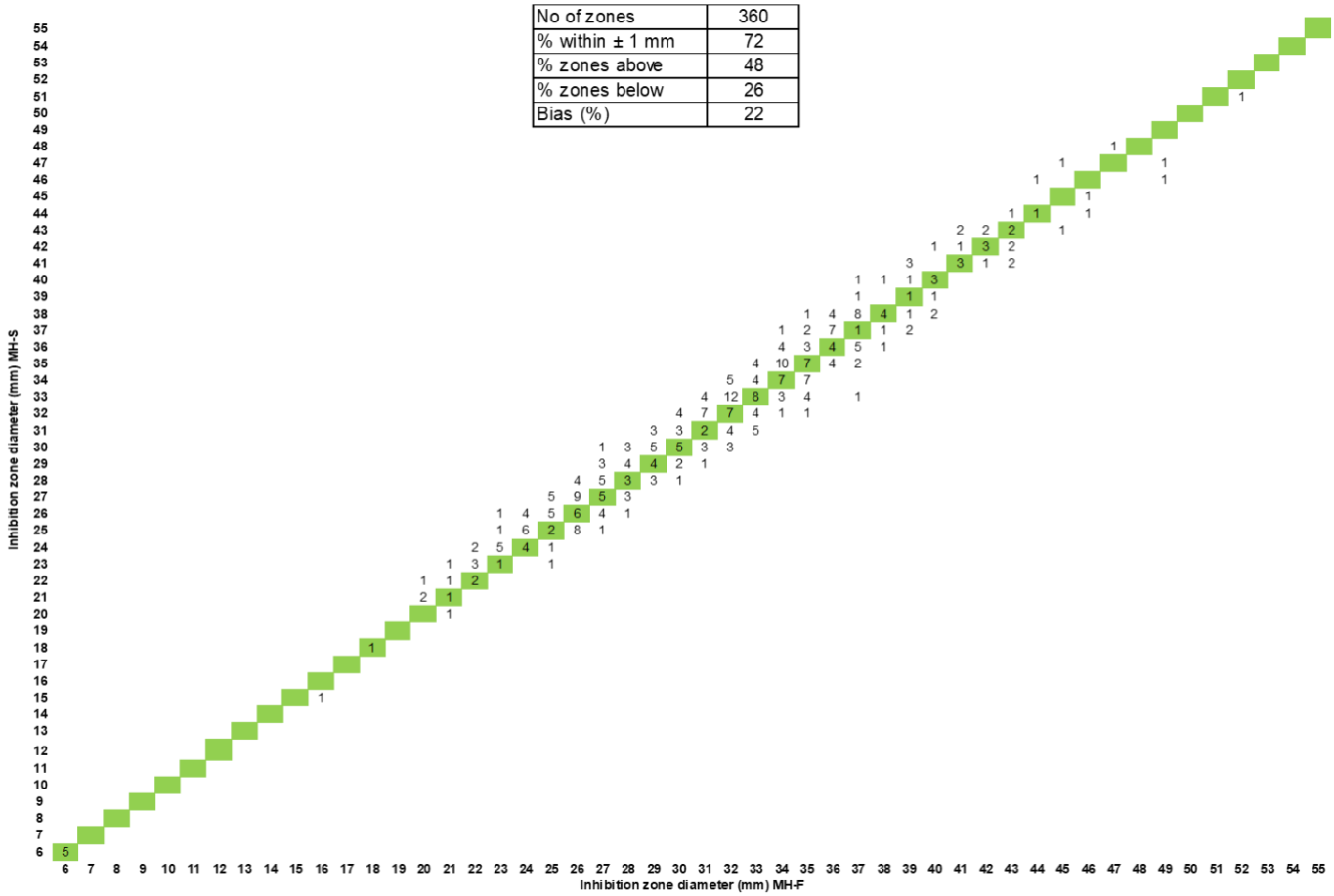
No of zones	700
% within ± 1 mm	74
% zones above	39
% zones below	32
Bias (%)	6.9



Moraxella catarrhalis **all agents**

MH-S vs MH-F

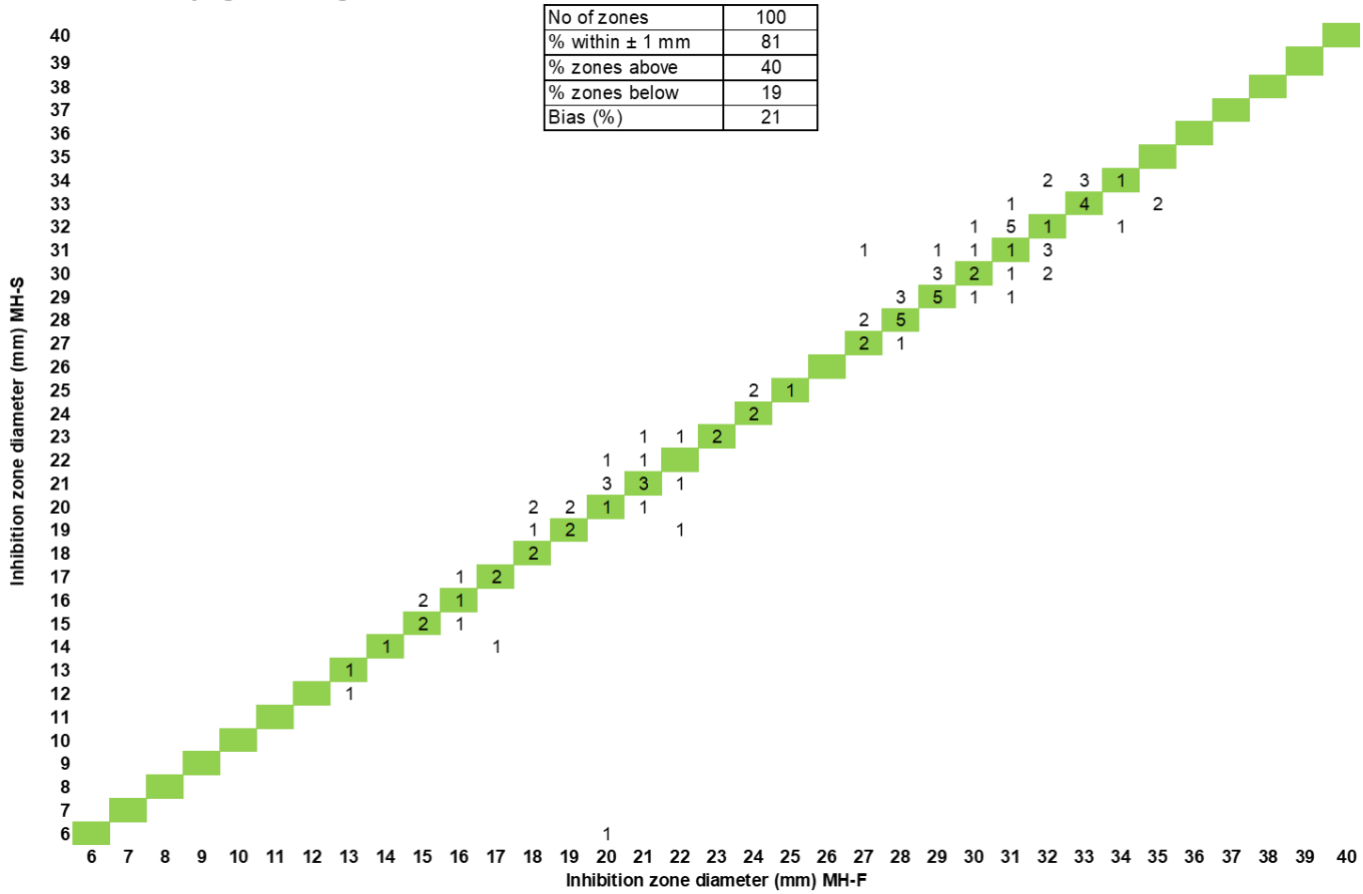
No of zones	360
% within ± 1 mm	72
% zones above	48
% zones below	26
Bias (%)	22



***Listeria monocytogenes* all agents**

MH-S vs MH-F

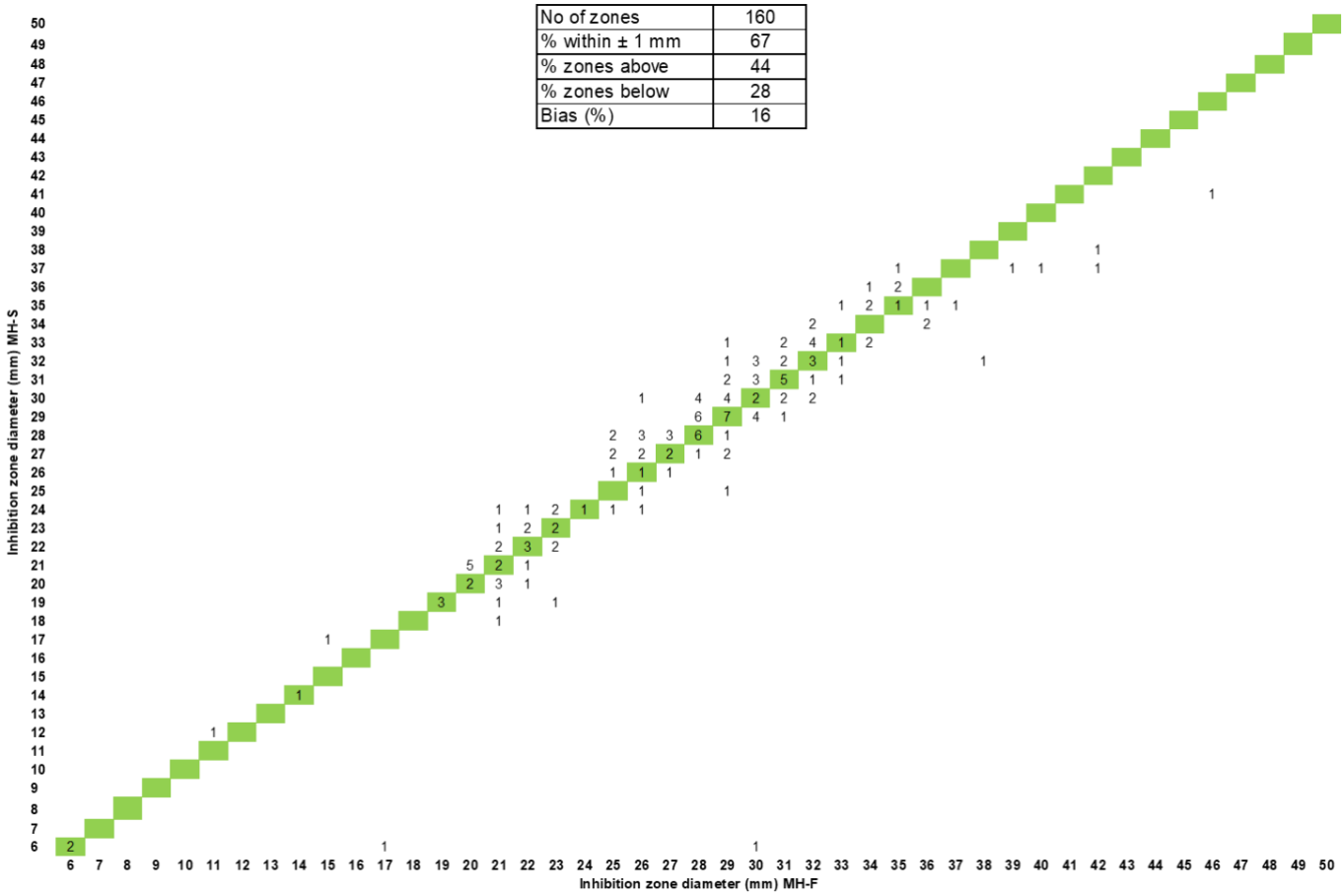
No of zones	100
% within ± 1 mm	81
% zones above	40
% zones below	19
Bias (%)	21



***Pasteurella multocida* all agents**

MH-S vs MH-F

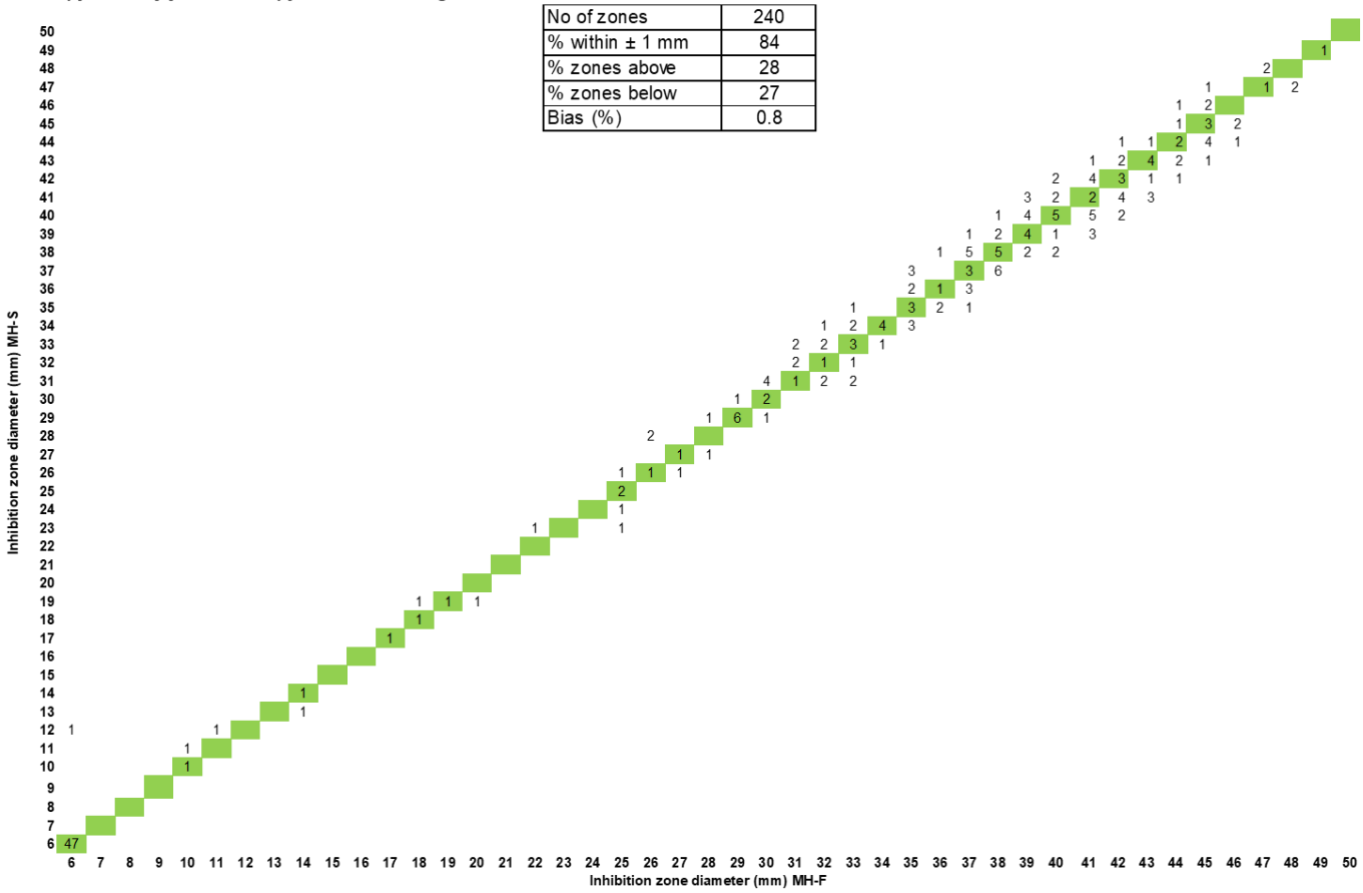
No of zones	160
% within ± 1 mm	67
% zones above	44
% zones below	28
Bias (%)	16



***Campylobacter jejuni and Campylobacter coli* all agents**

MH-S vs MH-F

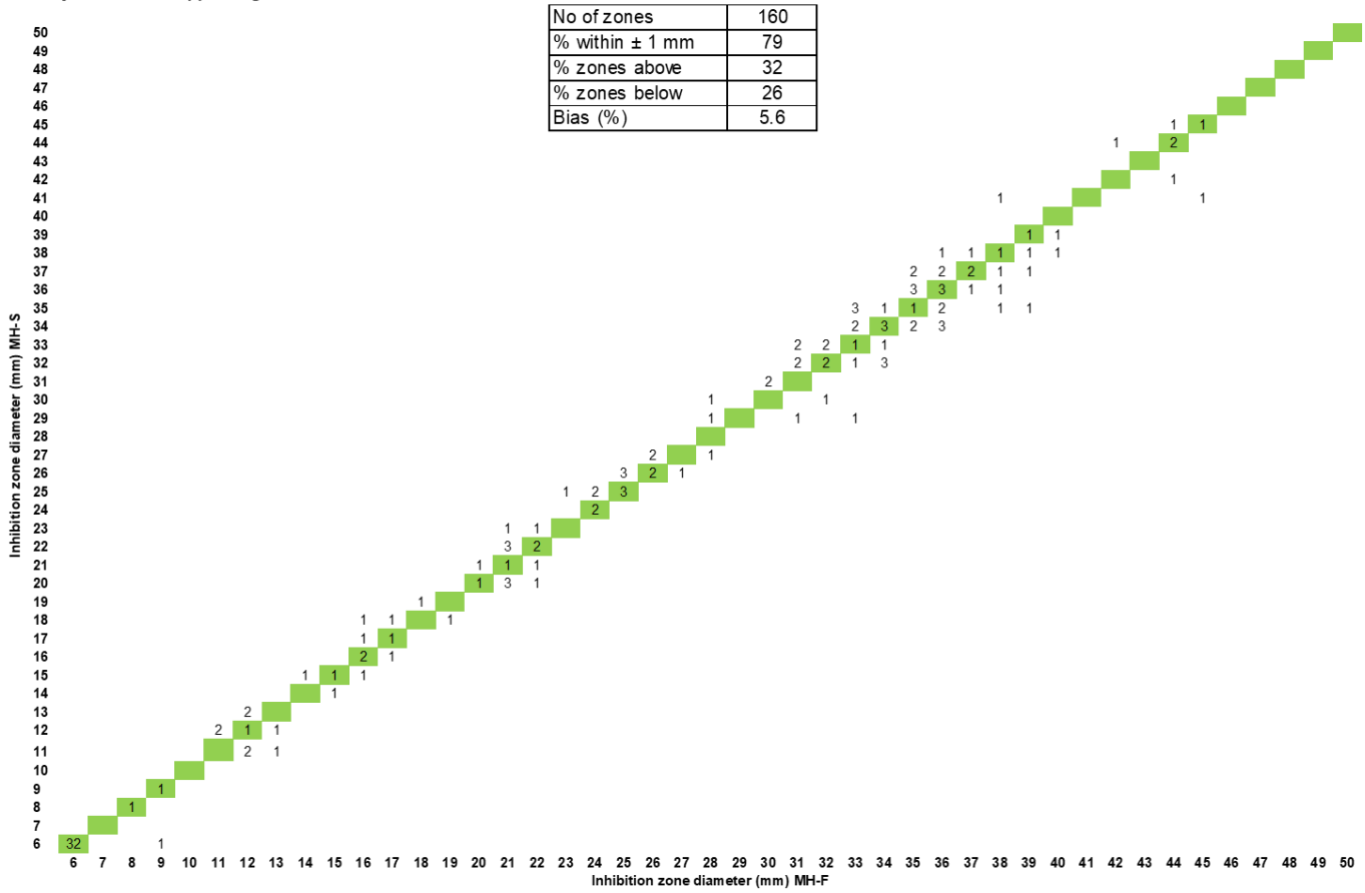
No of zones	240
% within ± 1 mm	84
% zones above	28
% zones below	27
Bias (%)	0.8



Corynebacterium* spp. *all agents

MH-S vs MH-F

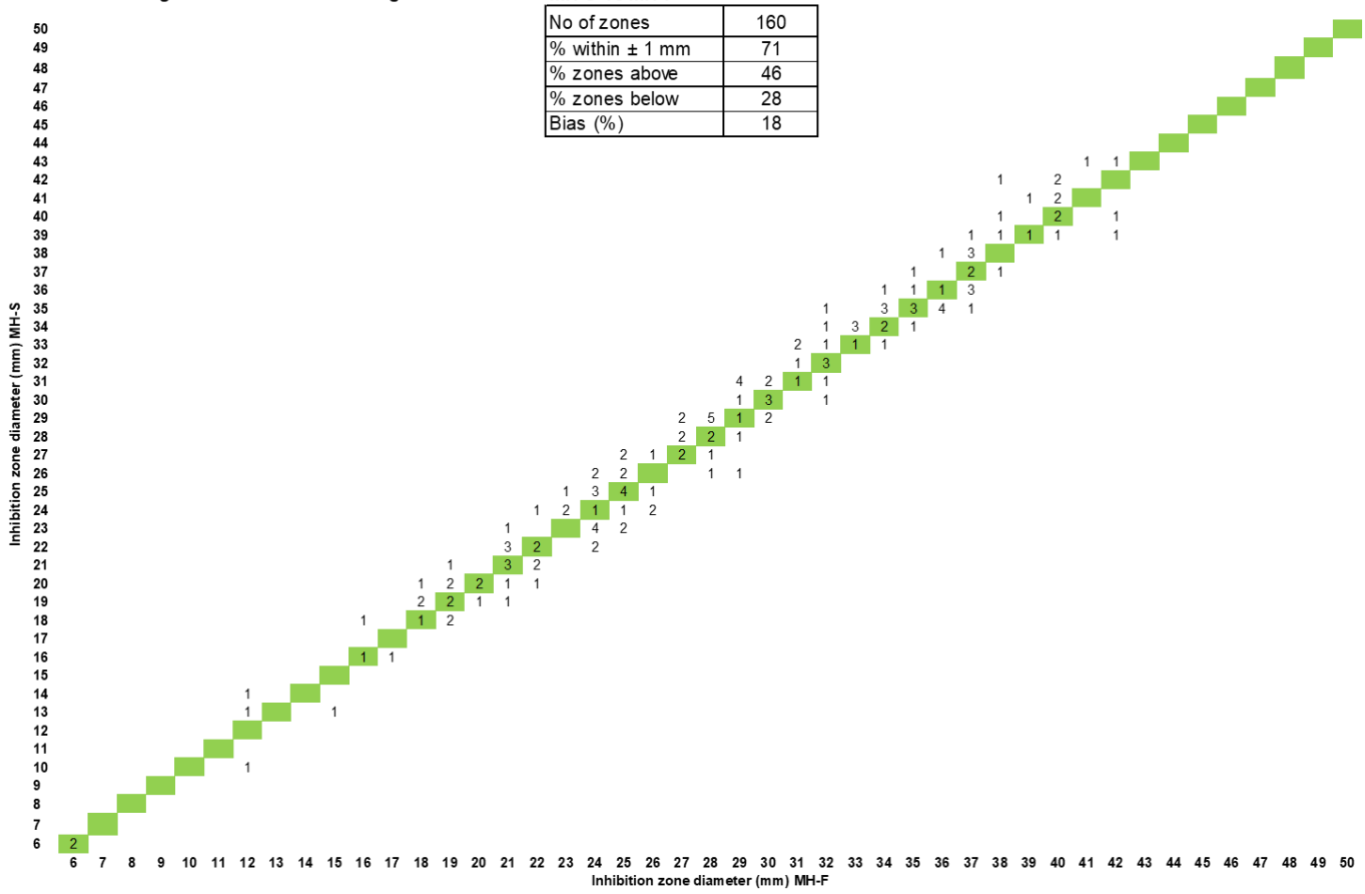
No of zones	160
% within ± 1 mm	79
% zones above	32
% zones below	26
Bias (%)	5.6



***Aerococcus sanguinicola* and *A. urinae* all agents**

MH-S vs MH-F

No of zones	160
% within ± 1 mm	71
% zones above	46
% zones below	28
Bias (%)	18



***Kingella kingae* all agents**

MH-S vs MH-F

No of zones	240
% within ± 1 mm	61
% zones above	41
% zones below	39
Bias (%)	2.5

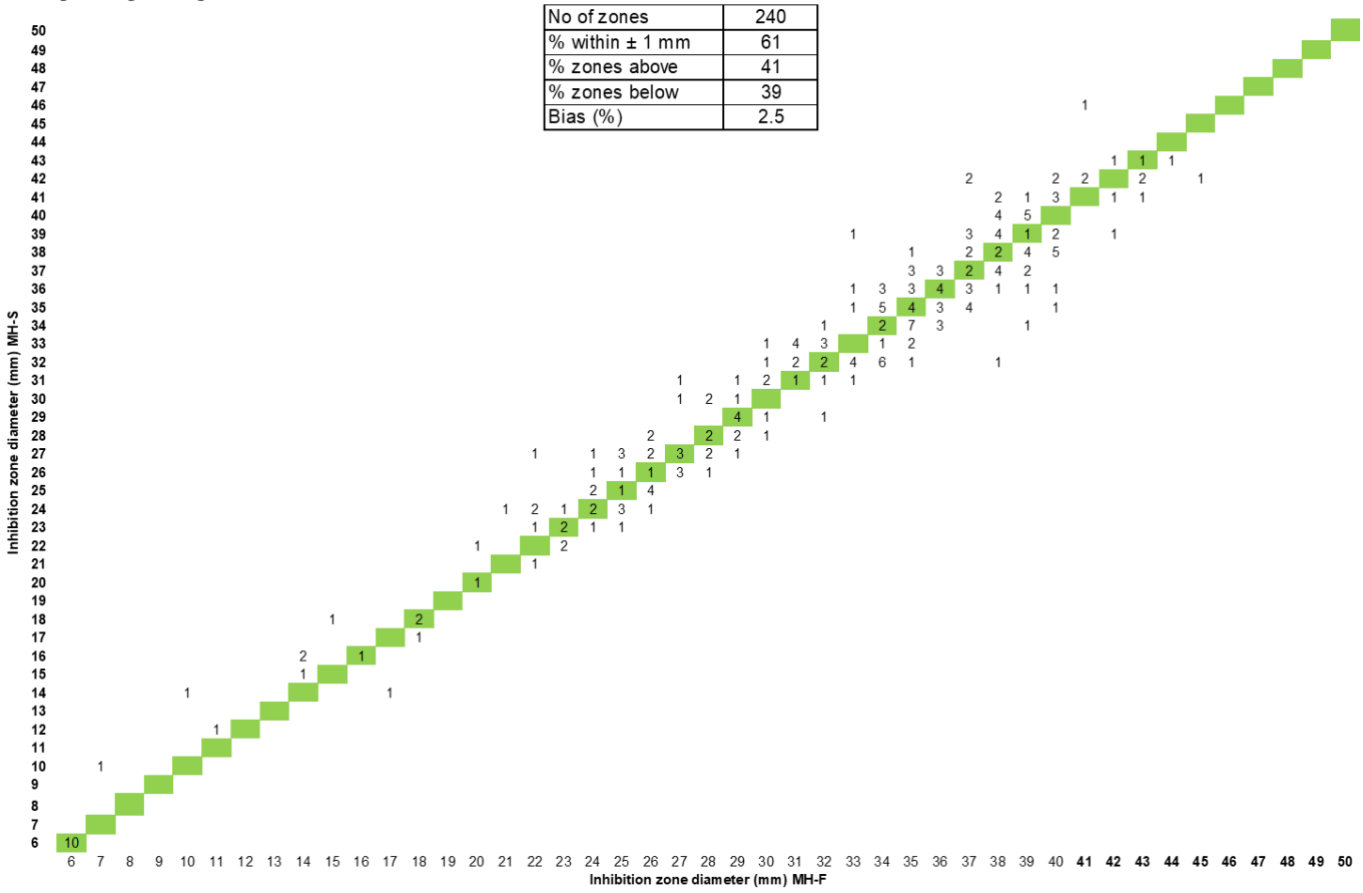


Table 2a. Mean inhibition zone diameters for *Streptococcus pneumoniae* ATCC 49619 from parallel tests on MH-S and MH-F.

Target and ranges according to EUCAST QC Tables v. 15.0, 2025, are listed for comparison.

Antimicrobial agent	No of tests per medium	Mean value (mm) per medium		Target	Range
		MH-S	MH-F		
Amoxicillin-clavulanic acid 2-1 µg	12	29	30	-	-
Ampicillin 2 µg	18	29	29	28	25-31
Benzylpenicillin 1 unit	36	17	18	19	16-22
Cefepime 30 µg	10	34	34	34	31-37
Cefixime 5 µg	12	20	22	-	-
Cefotaxime 5 µg	18	30	30	31	28-34
Ceftriaxone 30 µg	16	34	34	35	32-38
Cefuroxime 30 µg	16	32	32	31	28-34
Ciprofloxacin 5 µg	16	23	24	25	22-28
Clindamycin 2 µg	32	25	24	25	22-28
Eravacycline 20 µg	14	28	29	27	24-30
Ertapenem 10 µg	12	32	32	31	28-34
Erythromycin 15 µg	42	29	29	29	26-32
Imipenem 10 µg	12	38	38	38	34-42
Lefamulin 5 µg	8	20	19	18	15-21
Levofloxacin 5 µg	36	23	24	24	21-27
Linezolid 10 µg	26	26	25	26	23-29
Meropenem 10 µg	14	34	34	34	30-38
Mincycline 30 µg	28	27	28	28	25-31
Moxifloxacin 5 µg	36	27	27	27	24-30
Nalidixic acid 30 µg	6	6	6	-	-
Nitrofurantoin 100 µg	10	26	26	28	25-31
Norfloxacin 10 µg	26	20	21	21	18-24
Ofloxacin 5 µg	10	20	21	21	18-24
Oxacillin 1 µg	10	9	10	11	8-14
Rifampicin 5 µg	40	28	28	29	26-32
Tedizolid 2 µg	22	21	21	22	19-25
Teicoplanin 30 µg	30	20	21	21	18-24
Tetracycline 30 µg	38	30	30	31	28-34
Tigecycline 15 µg	16	26	27	27	24-30
Trimethoprim-sulfamethoxazole 1.25-23.75 µg	38	23	22	22	18-26
Vancomycin 5 µg	34	20	21	20	17-23

Mean value on target ± 1 mm
Mean value on target ± 2 mm

Table 2b. Mean inhibition zone diameters for *Streptococcus pneumoniae* ATCC 49619 from parallel tests on MH-S produced using MH agar from 3 manufacturers.

Targets and ranges according to EUCAST QC Tables v. 15.0, 2025, are listed for comparison.

Antimicrobial agent	No of tests per agar	Mean value (mm) per MH manufacturer			Target	Range
		BBL	Bio-Rad	Oxoid		
Amoxicillin-clavulanic acid 2-1 µg	13	29	29	29	-	-
Ampicillin 2 µg	13	28	27	28	28	25-31
Benzylpenicillin 1 unit	20	17	17	18	19	16-22
Cefepime 30 µg	13	34	33	34	34	31-37
Cefixime 5 µg	9	19	20	20	-	-
Cefotaxime 5 µg	11	30	29	29	31	28-34
Ceftriaxone 30 µg	7	34	33	34	35	32-38
Cefuroxime 30 µg	7	31	31	32	31	28-34
Ciprofloxacin 5 µg	13	25	25	24	25	22-28
Clindamycin 2 µg	13	25	24	24	25	22-28
Eravacycline 20 µg	10	28	28	28	27	24-30
Ertapenem 10 µg	3	32	32	32	31	28-34
Erythromycin 15 µg	16	29	29	29	29	26-32
Imipenem 10 µg	3	38	38	38	38	34-42
Lefamulin 5 µg	9	19	21	20	18	15-21
Levofloxacin 5 µg	10	25	24	24	24	21-27
Linezolid 10 µg	9	26	26	26	26	23-29
Meropenem 10 µg	9	33	33	34	34	30-38
Mincycline 30 µg	9	27	28	27	28	25-31
Moxifloxacin 5 µg	16	28	27	27	27	24-30
Nalidixic acid 30 µg	7	6	6	6	-	-
Nitrofurantoin 100 µg	12	28	26	27	28	25-31
Norfloxacin 10 µg	9	20	20	21	21	18-24
Ofloxacin 5 µg	3	21	21	21	21	18-24
Oxacillin 1 µg	9	11	11	12	11	8-14
Rifampicin 5 µg	16	28	28	28	29	26-32
Tedizolid 2 µg	13	23	23	22	22	19-25
Teicoplanin 30 µg	10	19	20	19	21	18-24
Tetracycline 30 µg	16	31	31	31	31	28-34
Tigecycline 15 µg	3	26	26	25	27	24-30
Trimethoprim-sulfamethoxazole 1.25-23.75 µg	16	23	22	23	22	18-26
Vancomycin 5 µg	16	20	20	19	20	17-23

Mean value on target ± 1 mm
Mean value on target ± 2 mm
Mean value on target ± 3 mm