SUPPLEMENTARY MATERIAL

Revisiting colistin susceptibility testing: Will adding calcium to Mueller-Hinton agar improve the detection of colistin resistance?

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Methods for Whole-genome sequencing

All *E. coli* and *K. pneumoniae* were investigated for colistin resistance mechanisms (*mcr* genes and point mutations in the genes related to the PmrA/PmrB system and the MgrB/PhoQ system) by WGS and detection of genetic variants by use of NCBI AMRFinderPlus. DNA was extracted using MagDEA Dx SV reagent and the magLEAD instrument (Precision System Science). Construction of size-selected 300 bp library was performed automatically by the AB Library builder system using 200 ng of input gDNA and the Ion Xpress™ Plus Library Kit for AB Library Builder™ System. Libraries were purified and quantified as previously described [1]. Libraries were normalised to a final concentration of 25 pM and prepared for sequencing with the Ion 54´30 Kit-Chef (Thermo Fisher Scientific, MA, USA) for 400 base-pair reads. Sequencing was performed on an Ion GeneStudio S5 Sequencing System (Thermo Fisher Scientific, MA, USA). Raw sequence reads were converted from uBAM to fastq using samtools (Version 1.10) [2]. Reads were filtered and trimmed using Fastp (Version 0.20.0) [3]. Following this, reads were assembled using CLC Assembly Cell (Version 5.2.0) according to the manufacturer's instructions. The assembled genomes were assigned a species using Mash (Version 2.2.2) and further processed using AMRFinderPlus (Version 3.8.4) [4-6]; this was performed with a database downloaded from NCBI, version 2020-11-09.1.

References

1. Beser J et al. Improving the genotyping resolution of Cryptosporidium hominis subtype IbA10G2 using one step PCR-based amplicon sequencing. Infect Genet Evol 2017;55:297-304.
2. Li H et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics 2009;25(16):2078-9.
3. Chen S, Zhou Y, Chen Y, Gu J. fastp: an ultra-fast all-in-one FASTQ preprocessor. Bioinformatics 2018;34(17):i884-i890.
4. Ondov BD et al. Mash Screen: high-throughput sequence containment estimation for genome discovery. Genome Biol 2019;20(1):232.
5. Ondov BD et al. Mash: fast genome and metagenome distance estimation using MinHash. Genome Biol 2016;17(1):132.
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Supplementary materials, Matuschek et al. Revisiting colistin susceptibility testing. Will adding calcium to Mueller-Hinton agar improve the detection of colistin resistance?

Table S1. Characteristics of the study isolates and information of in which phase of the study each isolate was included.

Code	Organism	Colistin MIC (mg/L)	Colistin resistance gene	Phase 1	Phase 2	Phase 3	Phase 4
COL 68	*Escherichia coli*	0.25	-			X	X
COL 01	*Escherichia coli*	0.5	-	X	X	X	X
COL 27	*Escherichia coli*	0.5	-	X	X	X	X
COL 69	*Escherichia coli*	0.5	-		X	X	X
COL 17	*Escherichia coli*	1	mcr-1	X	X	X	X
COL 77	*Escherichia coli*	1	-			X	X
COL 78	*Escherichia coli*	1	-			X	X
COL 76	*Escherichia coli*	2	mcr-1.1			X	X
COL 79	*Escherichia coli*	2	-			X	X
COL 04	*Escherichia coli*	4	mcr-1	X	X	X	X
COL 13	*Escherichia coli*	4	mcr-1	X	X	X	X
COL 21	*Escherichia coli*	4	mcr-1	X	X	X	X
COL 22	*Escherichia coli*	4	mcr-1	X	X	X	X
COL 23	*Escherichia coli*	4	mcr-1	X	X	X	X
COL 24	*Escherichia coli*	4	mcr-1	X		X	X
COL 25	*Escherichia coli*	4	mcr-1	X		X	X
COL 26	*Escherichia coli*	4	mcr-1	X		X	X
COL 18	*Escherichia coli*	8	mcr-1 + mcr-3	X	X	X	X
COL 81	*Escherichia coli*	8	-				X
COL 80	*Escherichia coli*	16	pmrB_L10R				X
COL 70	*Klebsiella pneumoniae*	0.5	-		X	X	X
COL 72	*Klebsiella pneumoniae*	0.5	pmrB_R256G			X	X
COL 73	*Klebsiella pneumoniae*	0.5	pmrB_R256G			X	X
COL 75	*Klebsiella pneumoniae*	0.5	-	X		X	X
COL 15	*Klebsiella pneumoniae*	1	pmrB_R256G	X	X	X	X
COL 74	*Klebsiella pneumoniae*	1	pmrB_R256G	X	X	X	X
COL 02	*Klebsiella pneumoniae*	2	pmrB_R256G	X	X	X	X
COL 36	*Klebsiella pneumoniae*	2	-	X	X	X	X
COL 05	*Klebsiella pneumoniae*	8	-	X	X	X	X
COL 19	*Klebsiella pneumoniae*	8	mcr-1	X	X	X	X
COL 33	*Klebsiella pneumoniae*	8	pmrB_R256G	X	X	X	X
COL 71	*Klebsiella pneumoniae*	8	pmrA_G53C	X	X	X	X
COL 03	*Klebsiella pneumoniae*	16	pmrB_R256G	X	X	X	X
COL 35	*Klebsiella pneumoniae*	16	mgrB_M27K	X		X	X
COL 37	*Klebsiella pneumoniae*	16	mgrB_M27K	X		X	X
COL 38	*Klebsiella pneumoniae*	16	-			X	X
COL 32	*Klebsiella pneumoniae*	32	-	X		X	X
COL 34	*Klebsiella pneumoniae*	32	pmrB_R256G	X		X	X
COL 29	*Pseudomonas aeruginosa*	0.25	NT	X			
COL 30	*Pseudomonas aeruginosa*	0.5	NT	X			
COL 31	*Pseudomonas aeruginosa*	1	NT	X			
COL 16	*Pseudomonas aeruginosa*	2	NT	X			
COL 64	*Pseudomonas aeruginosa*	2	NT	X			
COL 14	*Pseudomonas aeruginosa*	4	NT	X			
COL 58	*Pseudomonas aeruginosa*	4	NT	X			
COL 11	*Pseudomonas aeruginosa*	8	NT	X			
COL 49	*Pseudomonas aeruginosa*	8	NT	X			
COL 06	*Pseudomonas aeruginosa*	16	NT	X			
COL 08	*Pseudomonas aeruginosa*	32	NT	X			
COL 20	*Acinetobacter baumannii*	0.5	NT	X			
COL 10	*Acinetobacter baumannii*	1	NT	X			
COL 09	*Acinetobacter baumannii*	2	NT	X			
COL 12	*Acinetobacter baumannii*	16	NT	X			
COL 07	*Acinetobacter baumannii*	32	NT	X			
COL 52	*Acinetobacter berezinae*	2	NT	X			
COL 28	*Acinetobacter haemolyticus*	1	NT	X			
COL 53	*Acinetobacter lwoffii*	2	NT	X			

NT = Not Tested

Figure S1. Colistin Etest MICs on un-supplemented Mueller-Hinton (MH) agar (to the left) and MH agar supplemented with 5 mM Ca^{2+} (to the right) vs. reference broth microdilution.
EUCAST breakpoints are shown as lines for reference broth microdiltion and Etest on un-supplemented MH agar.

a) *Escerichia coli* (n=12)

Colistin Etest MIC (mg/L) Un-supplemented MH \ Colistin reference MIC (mg/L)	0.25	0.5	1	2	4	8	16	32
0.06		1						
0.125		1	1					
0.25								
0.5								
1								
2								
4				8		1		
8								
16								
32								
64								

Colistin Etest MICs (mg/L) MH + 5 mM Ca2+ \ Colistin reference MIC (mg/L)	0.25	0.5	1	2	4	8	16	32
0.06								
0.125			1					
0.25		2						
0.5								
1								
2								
4								
8								
16					7			
32					1	1		
64								

b) *Klebsiella pneumoniae* (n=14)

Colistin Etest MIC (mg/L) Un-supplemented MH \ Colistin reference MIC (mg/L)	0.25	0.5	1	2	4	8	16	32
0.06								
0.125		1						
0.25			1					
0.5			1					
1								
2				2				
4						4	3	
8								1
16								1
32								
64								

Colistin Etest MICs (mg/L) MH + 5 mM Ca2+ \ Colistin reference MIC (mg/L)	0.25	0.5	1	2	4	8	16	32
0.06								
0.125		1						
0.25							1	
0.5			2			1		
1								
2						1		1
4				1		1		
8				1			1	
16							1	1
32								
64						1		

c) *Pseudomonas aeruginosa* (n=11)

Colistin Etest MIC (mg/L) Un-supplemented MH \ Colistin reference MIC (mg/L)	0.25	0.5	1	2	4	8	16	32
0.06								
0.125	1							
0.25		1	1					
0.5				1	1	1		
1				1	1			
2								1
4						1	1	
8								
16								
32								
64								

Colistin Etest MICs (mg/L) MH + 5 mM Ca2+ \ Colistin reference MIC (mg/L)	0.25	0.5	1	2	4	8	16	32
0.06								
0.125	1							
0.25								
0.5								
1								
2								
4		1	1	1				
8				1	2	2	1	1
16								
32								
64								

d) Acinetobacter *spp.* (n=8)

Colistin Etest MIC (mg/L) Un-supplemented MH \ Colistin reference MIC (mg/L)	0.25	0.5	1	2	4	8	16	32
0.06								
0.125		1	1					
0.25			1	3			1	
0.5								
1								
2								
4								1
8								
16								
32								
64								

Colistin Etest MICs (mg/L) MH + 5 mM Ca2+ \ Colistin reference MIC (mg/L)	0.25	0.5	1	2	4	8	16	32
0.06								
0.125		1						
0.25								
0.5								
1			2	1				
2				2			1	
4								1
8								
16								
32								
64								

Figure S2. Inhibition zone diameter distributions for colistin 25 µg disks tested on un-supplemented Mueller-Hinton (MH) agar (to the left) and MH agar supplemented with 5 mM Ca^{2+} (to the right). Colistin reference MICs are shown as different colours of the bars. Green = susceptible. Orange and red = resistant.

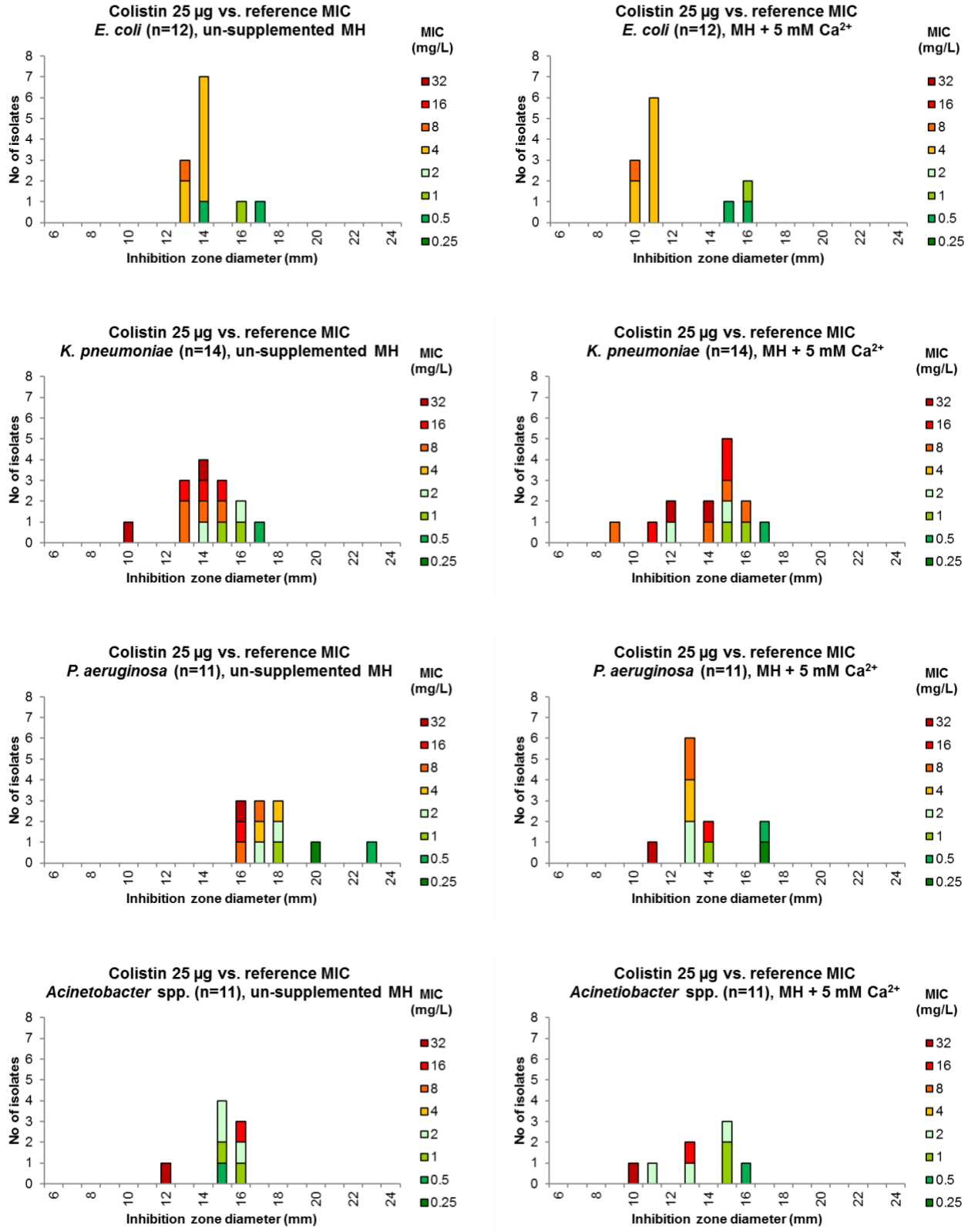

Figure S3. Inhibition zone diameter distributions for colistin 25 µg disks tested on un-supplemented Mueller-Hinton (MH) agar and MH agar supplemented with 5 mM Ca^{2+} for additional isolates of *E. coli* (to the left) and *K. pneumoniae* (to the right). Colistin reference MICs are shown as different colours of the bars. Green = susceptible. Orange and red = resistant.

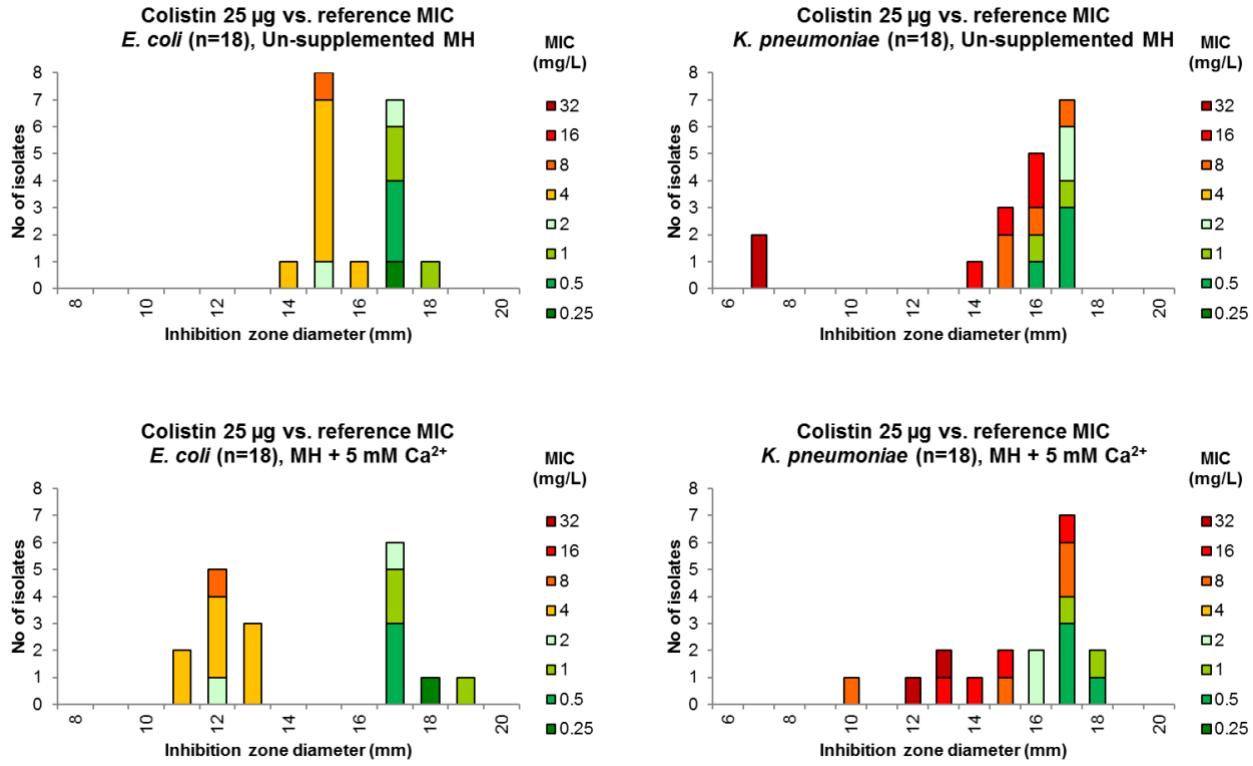

Figure S4. Inhibition zone diameter distributions for colistin 10 µg disks tested on un-supplemented Mueller-Hinton (MH) agar and MH agar supplemented with 5 mM Ca^{2+} for additional isolates of *E. coli* and *K. pneumoniae* on BBL (to the left) and Oxoid (to the right) Mueller-Hinton agar. Colistin reference MICs are shown as different colours of the bars. Green = susceptible. Orange and red = resistant.

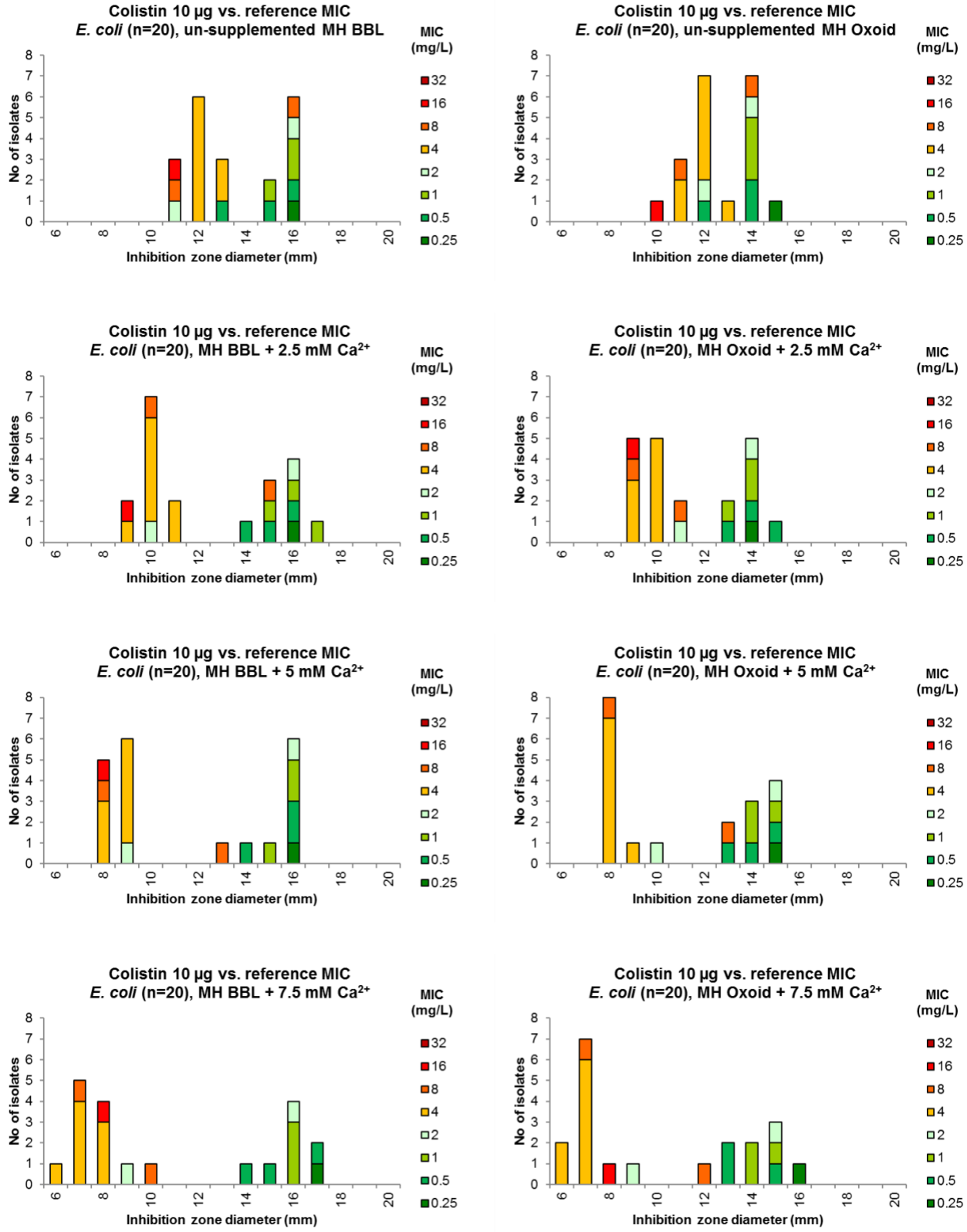

Figure S4 continued