HAMLET, a protein complex from human milk kills and enhances the activity of antibiotics against pathogenic *Streptococci*

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Supplemental Material

Table S1. Pneumococcal strains used in the study

Strain	Serotype	Antibiotic Resistance (*gene*)*	Source**
S. pneumoniae			
D39	2	None	(1)
D39 Δ*dldh* (C0832)	2	Erythromycin (*ermB*)	(2)
D39 Δ*stkP*	2	Kanamycin (*aphA3*)	(3)
D39 Δ*pspC*	2	Tetracycline (*tetM*)	(4)
D39 Δ*pitB*	2	Chloramphenicol (*cat*)	(5)
D39-Sm	2	Streptomycin (*rpsL*)	This study
SP670	6A	Penicillin G	(6)
ATCC 49619	19F	None	(7) EUCAST
3974	14	Erythromycin (*ermB*)	EUCAST
7545	14	Erythromycin (*mef* + *msrD*)	EUCAST
12627	6B		EUCAST
13331	14		EUCAST
17476	23A		EUCAST
1947	19F	Trimethoprim-Sulfoxide (R)	EUCAST
16467	6A		EUCAST
17446	16A/F	Trimethoprim-Sulfoxide (I)	EUCAST
18091	23F		EUCAST
4269	3	Fluoroquinolone	EUCAST
13985	19F	Tetracycline	EUCAST
17144	19F		EUCAST
6701	4	Penicillin (I)	EUCAST
16998	6B		EUCAST
12116	35B	Penicillin (I), Cephalosporine / Ampicillin (R)	EUCAST
16031	35A	Penicillin, Cephalosporine	EUCAST
19000	35B		EUCAST
18120	6A		EUCAST
4393	23F		EUCAST

* I = Intermediate resistance; R = Resistant

** EUCAST; The European Committee on Antimicrobial Susceptibility Testing

Table S2. Primers used for Erm-resistance determination.

Gene name	Primer name	Primer sequence (5' to 3')	Reference
ermB	ErmB-F	GGTACCATGAACAAAAATATAAAATATTCTC	This study
	ErmB-R	GGATCCTTATTTCCTCCCGTTAAATAATAG	This study
	ErmB-F2	GAAAAGGTACTCAACCAAATA	(8)
	ErmB-R2	AGTAACGGTACTTAAATTGTTTAC	(8)
ermTR	ErmTR-F	GGGTCAGGAAAAGGACATT	(9)
	ErmTR-R	CATTCGCATGCTTCAGC	This study
ermT	ErmT-F	CCGCCATTGAAATAGATCCT	(10)
	ErmT-R	GCTTGATAAAATTGGTTTTTGGA	(10)
mefA/mefE	MefAE-F	AGGGCAAGCAGTATCATTAATCA	(10)
	MefAE-R	CTGCAAAGACTGACTATAGCCT	(10)
mefA (GBS)	MefA-F	AGTATCATTAATCACTAGTGC	(8)
	MefA-R	TTCTTCTGGTACTAAAAGTGG	(8)
linB	LinB-F	CCTACCTATTGTTTGTGGAA	(11)
	LinB-R	ATAACGTTACTCTCCTATTC	(11)
msrD	MsrD-F	TTGGACGAAGTAACTCTG	(12)
	MsrD-R (GAS)	GCTTGTCTCTTACGTTC	This study
	MsrD-R (SPN)	GCTTGGCTCTTACGTTC	(12)

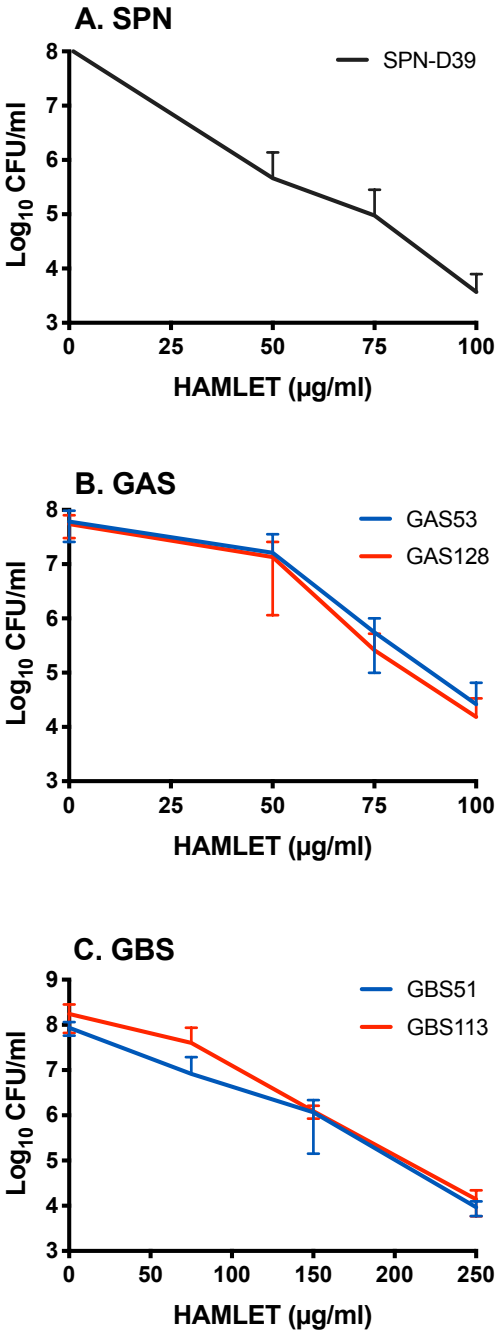

Figure S1. Bactericidal activity of HAMLET against *S. pneumoniae*, GAS and GBS. Bacteria were grown in THY, washed and resuspended in PBS with 25 mM glucose to keep the bacteria energized. The bacterial suspensions were prepared to obtain a starting concentration of approximately 1 x 10^8 CFU/ml. Then, increasing concentrations of HAMLET were added to wells of each bacterial strain and the bacteria were allowed to incubate for 1 hour at 37°C. Bacteria were then serially diluted and dilutions were plated onto agar that were allowed to grow for 24-48 hours to detect colonies. The viable CFU counts were counted and the concentration as CFU/ml was calculated and depicted in the graphs. The results represent the mean data from at least 3 separate experiments with standard deviations.

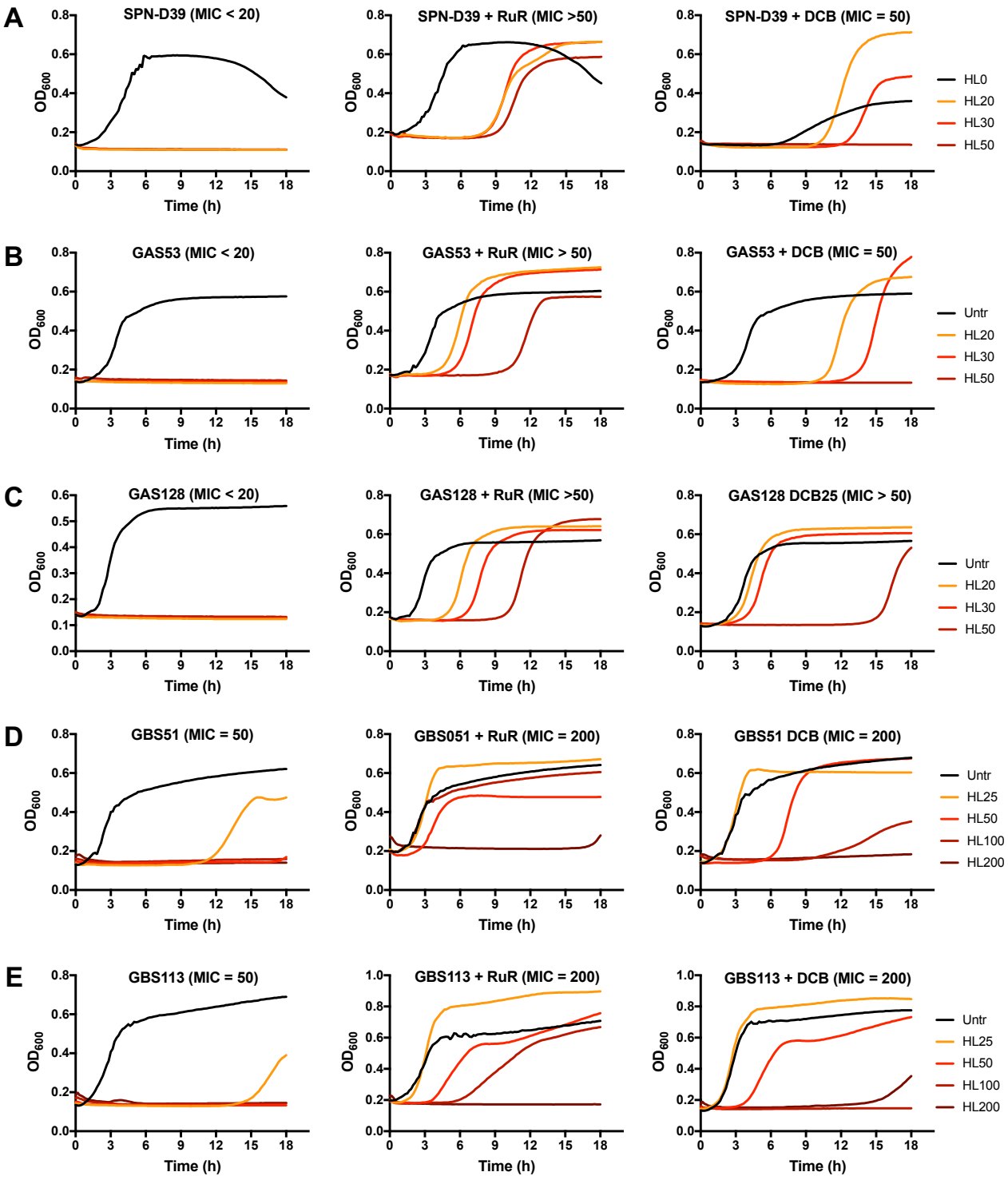

Figure S2. Inhibition of MIC growth curves of pathogenic *Streptococci* with ion transport inhibitors. MIC growth curves are shown for (A) D39, (B and C) GAS clinical isolates 53 and 128, and (D and E) GBS clinical isolates 51 and 113 in the presence of increasing concentrations of HAMLET

(left) and Ruthenium red (30 μM; middle) or Dichlorobenzamil (25 μM; right). Bacteria were grown in the presence of HAMLET alone or in the presence of inhibitors for 18 h at 37°C with the absorbance at 600 nm (OD_{600}) recorded every 10 min. The lowest concentration of HAMLET where no growth was detected over 18 h was considered the MIC value. The figure shows a representative growth curve for each strain. However, the MIC values are based on three separate experiments with samples run in duplicate wells.

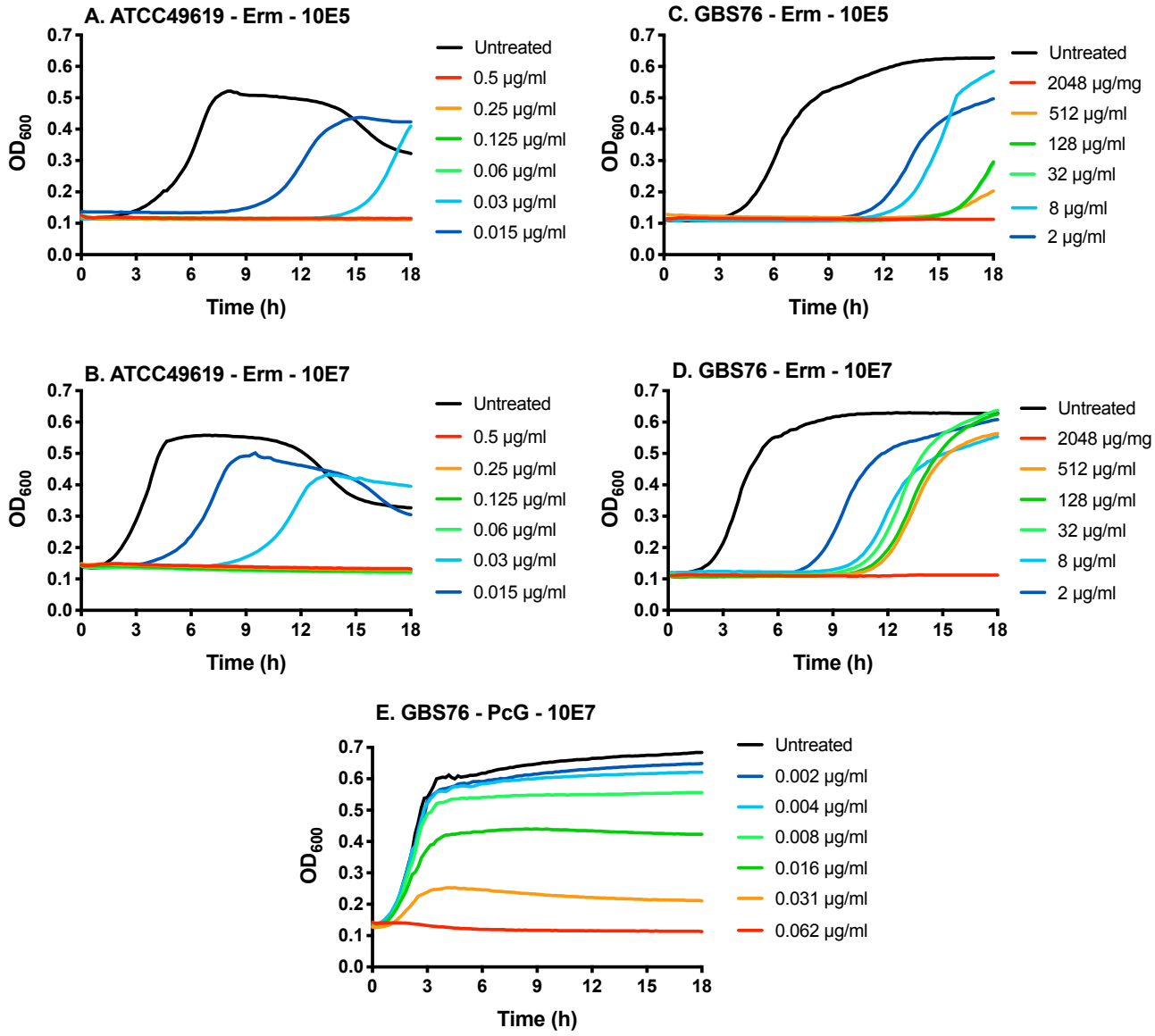

Figure S3. Validation of MIC assays using different inoculum concentrations. MIC growth curves are shown for *S. pneumoniae* ATCC49619 or GBS strain 76 exposed to various erythromycin concentrations with the bacterial inoculum being 10^5 CFU/ml (A and C, respectively) or 10^7 CFU/ml (B and D, respectively). The growth curves show a right shift in the presence of sub-MIC concentration of erythromycin that is less substantial at the higher inoculum, without the MIC for erythromycin being affected. Exposure of GAS 76 to penicillin G (E) resulted in a concentration-dependent decrease in maximal optical density, without a right-shift of the growth curve.

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