1 Supplemental Data

Figure S1. Antigen-specific IgG titers (in $\log_{10}$) from the serum samples of the mice (n = 8) intramuscularly immunized with PBS control (+), 10 µg cholera MEFA protein ($\triangledown$), or orogastrically immunized with Shanchol (100 µl, of the 1.5 ml adult human dose; ○), one primary and two boosters at a 3-wk interval, comparatively to the IgG titers from the mice intramuscularly immunized with 25 µg cholera MEFA protein at 2-wk interval (●). Mouse serum samples collected two weeks after the second booster were titrated for antigen-specific IgG responses in ELISAs, with two replicates. Mice IM immunized with 10 or 25 µg cholera MEFA protein developed the same levels of IgG response to each targeted antigen; these IgG titers were significantly greater than those from the mice orogastrically immunized with Shanchol except for TcpA (O1 El Tor and O139), based on Two-way ANOVA with the Bonferroni post hoc test. No anti-LPS response was detected. No antigen-specific IgG antibody responses were detected in the control mice.

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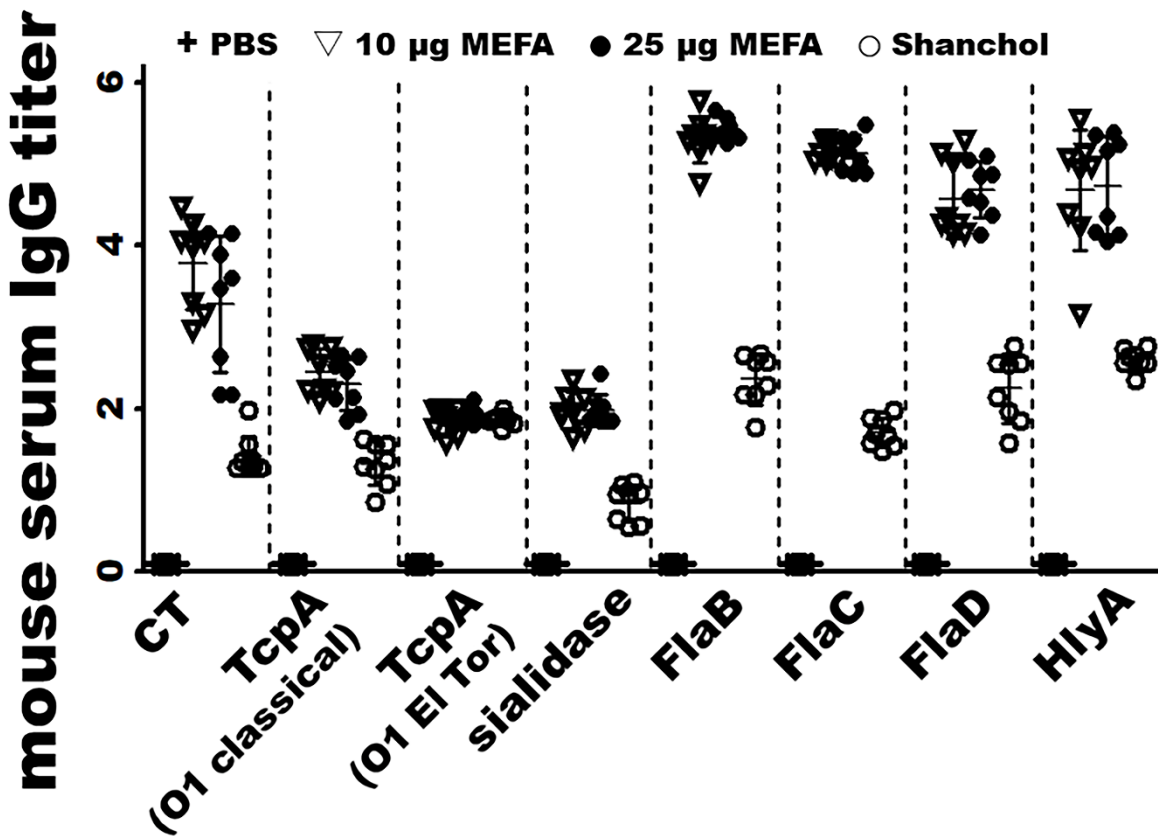
Figure S2. Microscopic images to show antibody inhibition activity against the motility of *Vibrio*

cholerae O1 El Tor N16961, O1 classical O395, O139 Bengal strain, and non-O1/non-O139 El

Tor 34-D 23 strain. Bacteria (10 µl, OD = 0.1), after incubation with PBS (no sera), the sera of the

mice IM immunized with PBS (control sera), or the sera of the mice IM immunized with cholera

MEFA protein and adjuvant dmLT (MEFA/dmLT sera), were observed under a Zeiss Axiovert

200M microscope with the Apotome Structured Illumination Optical Sectioning System. Images

were recorded using Axiocam 506 using a high-resolution black and white camera.

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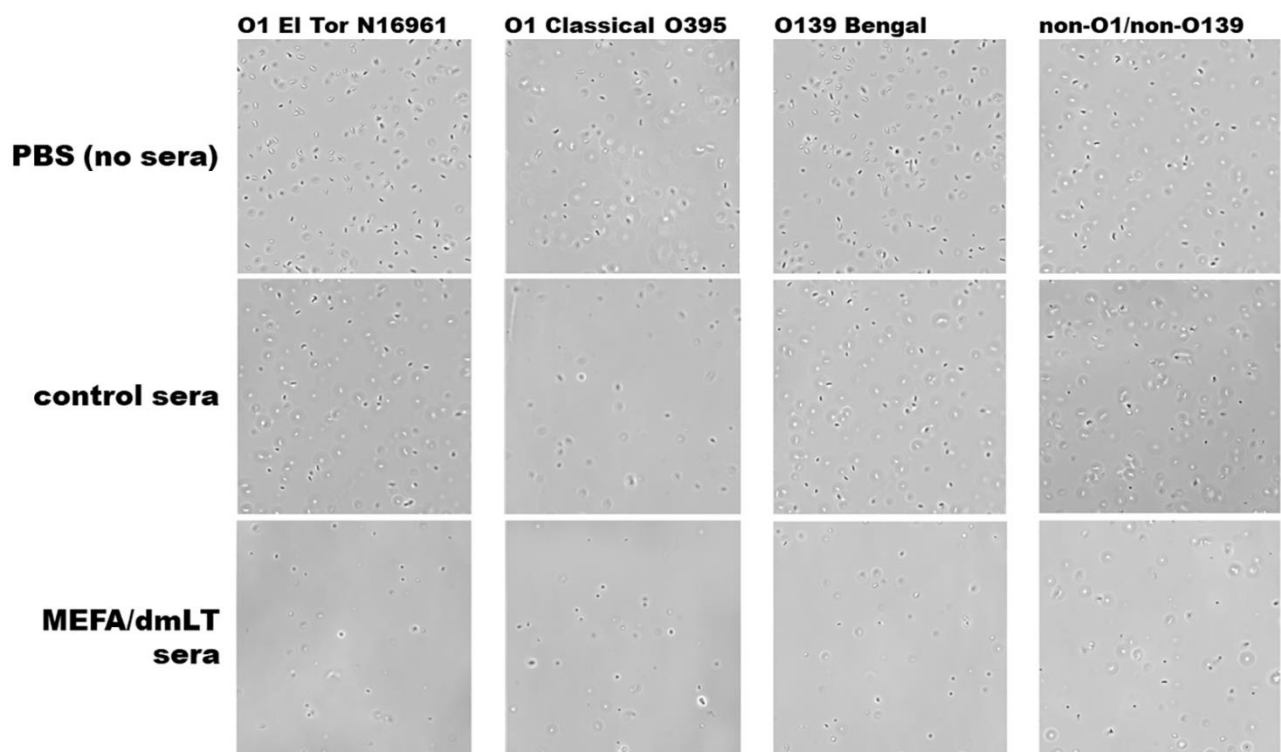
Figure S3. Rabbit IgA responses (OD_{650} readings in ELISA) from the cecum content supernatants of the adult rabbits (n=2, 5 replicates) IM immunized with 100 µg cholera MEFA protein (grey box), IM immunized with 100 µl PBS (white box), or orogastrically inoculated with Vaxchora (live attenuated *V. cholerae CVD 103-HgR*; $5x10^9$ CFUs in 1 ml PBS; black box); one primary and one booster at a two-week interval. Data indidated that IM immunization with cholera MEFA protein or orogastric inoculation of a live whole-cell cholera vaccine induced anigen-specific IgA responses in adult rabbits. ** and *** indicate a p-value of <0.01 and <0.001 respectively, based on Two-way ANOVA with the Bonferroni post hoc test.

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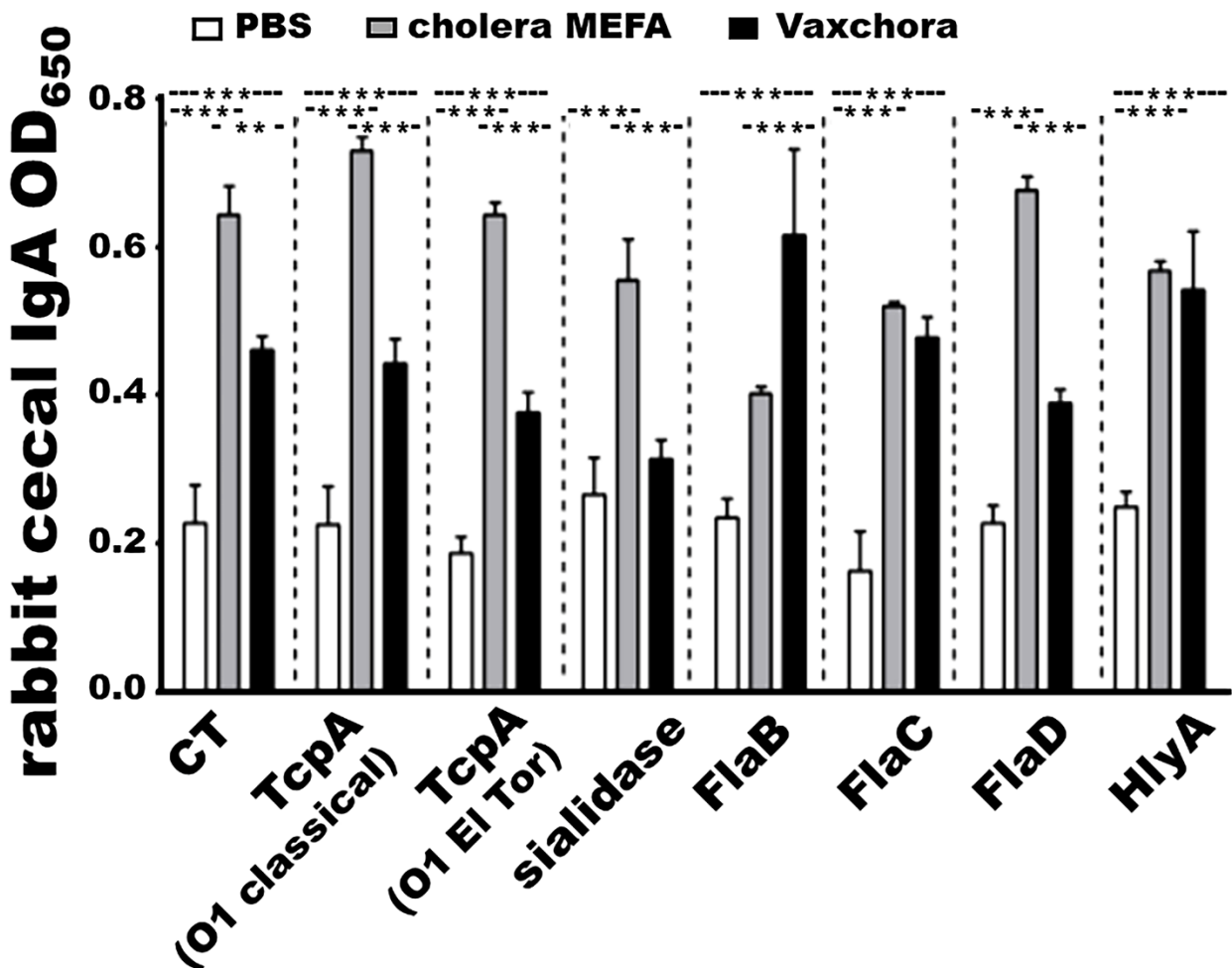
Figure S4. *Vibrio cholerae* O1 El Tor N16961 CFUs from the distal ilium segment of the adult rabbits (n = 2) after orogastric challenge with *V.cholerae* O1 El Tor N16961 (10^{10} CFUs, in 1ml PBS), with five replicates for each treatment. These rabbits were first IM immunized with 100 µg cholera MEFA protein (white box) or 100 µl PBS (black box), or orogastric immunization with Vaxchora (5×10^9 CFUs in 1 ml PBS; grey box), one primary and one booster, at the interval of two weeks. Data indicated that IM immunization of cholera MEFA protein or orogastic inoculation of a live cholera vaccine protected *Vibrio cholerae* colonization of small intestines in rabbits. ** indicates a p-value of <0.01, based on One-way ANOVA with Turkey's test.
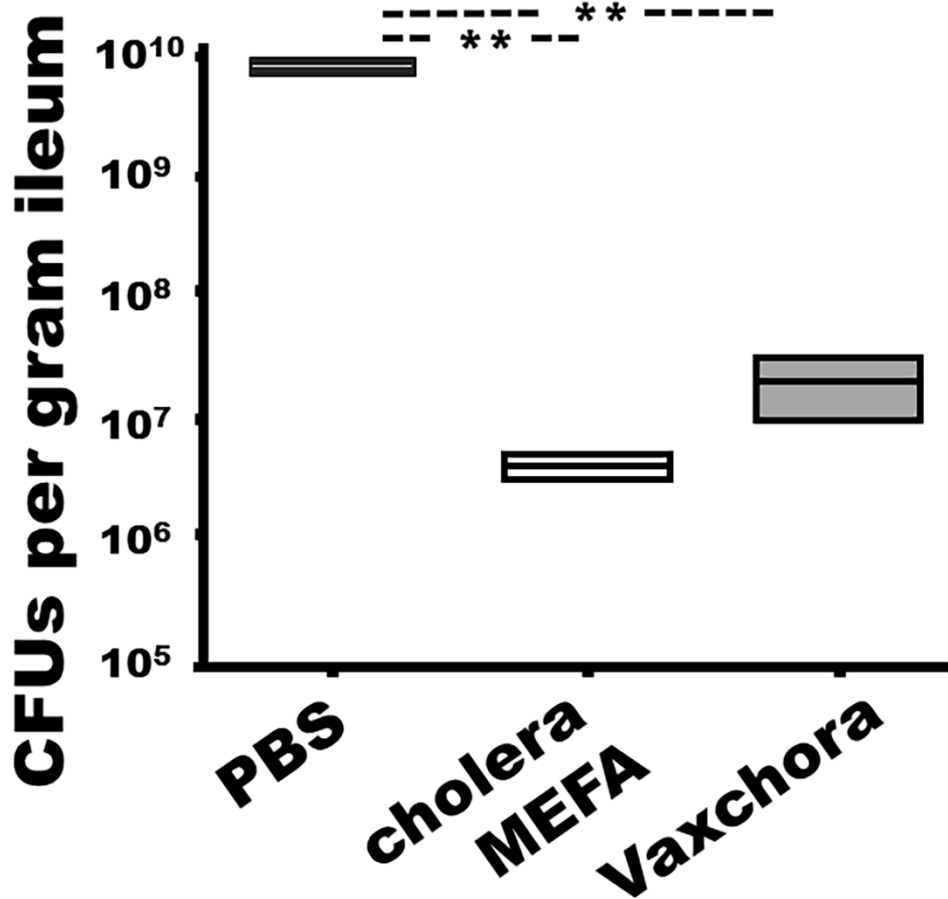
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71   **Movie S1.** Microscopic video clips to show antibody inhibition activity against motility of *Vibrio*

72 *cholerae* O1 El Tor N16961. Bacteria (10 µl, OD = 0.1), after incubation with PBS (no sera), or

73 the sera of the mice IM immunized with PBS (control sera) or the cholera MEFA protein and

74 adjuvant dmLT (MEFA/dmLT sera), were observed under a Zeiss Axiovert 200M microscope

75 with the Apotome Structured Illumination Optical Sectioning System. Video was recorded with

76 Axiocam 506 using a high-resolution black and white camera.

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78 **Table S1.** A list of *Vibrio cholerae* strains and recombinant *E. coli* strains used in this study. *V.*

79 *cholerae* serogroup strains acquired from BEI Resources (Biodefense and Emerging Infections

80 Research Resources Repository) were used as DNA templates for virulence gene cloning, *in vitro*

81 antibody function assays, and rabbit challenge studies. Recombinant virulence factor proteins

82 expressed by *E. coli* were used as ELISA coating antigens for antibody titration.

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Strains	Relevant properties	Sources
Vibrio cholerae N16961	O1, Inaba, El Tor	BEI NR-147 (ATCC 39315)
Vibrio cholerae O395	O1, Ogawa, Classical	BEI NR-9906 (ATCC 39541)
Vibrio cholerae MO45	O139 (Bengal)	BEI NR-144 (ATCC 51394)
Vibrio cholerae El Tor 34-D 23	O3 (non-O1/non-O139)	BEI NR-150 (ATCC 14731)
9746	'cholera MEFA' in pUC57, DH5α	this study
9747	'cholera MEFA' in pET28α, DH5α	this study
9748	'cholera MEFA' in pET28α, BL21(DE3)	this study
9764	'HlyA' in pET28α, BL21(DE3)	this study
9771	'TcpA (El Tor)' in pET28α, BL21(DE3)	this study
9779	'FlaC' in pET28α, BL21(DE3)	this study
9781	'FlaD' in pET28α, BL21(DE3)	this study
9808	'TcpA (Classical)' in pET28α, BL21(DE3)	this study

| 9863 | 'FlaB' in pET28α, BL21(DE3) | this study |
| 9866 | 'NanH (sialidase)' in pET28α, BL21(DE3) | this study |

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86   **Table S2**, PCR primers used in this study to amplify *V. cholerae* genes *tcpA* (O1 El Tor and

87   Classical), *flaB*, *flaC*, *flaD*, hemolysin A (*hlyA*), and sialidase (*nanH*).

primers	Nucleotide sequence (5' – 3')	PCR amplified region
TcpA-1	CCCATGGGCATGACATTACTCGAAGTAATCATTG	O1 El Tor and O139 *tcpA*
	TCACGGCCGTTAACTGTTACCAAAAGCTACTGTG	subunit gene
TcpA-2	CCGCCATGGATGCAATTATTAAAACAGC	O1 Classical *tcpA* subunit gene
	TCACGGCCGTTAACTGTTACCAAAAGCTACTG	
Fla B	CTAGCTAGCATGGCAATTAATGTAAACACG	flagellin B subunit gene
	TCACGGCCGTTATTATCCCAATAAGCTCAGAGCT	
Fla C	CTAGCTAGCATG GCGGTGAATGTAAACACC	flagellin C subunit gene
	TCACGGCCGCTAGCCTAGAAGAGCCAGTGC	
Fla D	CTAGCTAGCATGGCAGTGAATGTAAATAC	flagellin D subunit gene
	TCACGGCCGTTATTAACCCAACAGGCTGAGTG	
Nan H	CGGAATTCCGATGGCAACGGGTGACACTGAGTT	sialidase enzyme
	TCACGGCCGTTATTAGGTATCCCAAGTTATACC	
HlyA	CCGCCATGGATGCCAAAACTCAATCG	hemolysin gene
	TCACGGCCGGTTATCGACAGGAAAGGT	

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