Supplementary Materials

Dynamics of HEV Antibodies and Development of a Multi-factorial Model to Improve the Diagnosis of Current HEV Infection in Resource-limited Settings

Validation of the laboratory-developed test (LDT) of HEV RNA detection

The in-house developed HEV RNA RT-PCR assay was validated against the commercial Promotor® HEV RNA detection kit (ACON, Hangzhou, China), which was approved by the National Medical Products Administration (NMPA) of China to provide a qualitative dichotomous positive/negative diagnostic result. For each method, the reproducibility was assessed by calculating the coefficient of variation (CV) of the threshold cycle (C*t*) obtained for each standard dilution tested in 5 replicates. The CV was found to be inferior to 2% (Table S1). The LDT yielded good dilution linearity at HEV RNA levels within 3.73–7.73 $\log_{10}$copies/mL. The standard curve of the LDT gave a slope of -3.2534, with Y-intercept of 48.083 and a R^2 value of 0.9979.

The limit of detection (LOD) was determined using serial 3-fold dilutions of HEV RNA standard in nuclease-free water to give 4.73, 4.25, 3.77, 3.30, 2.82 and 2.34 $\log_{10}$copies/mL (5 replicates tested by the LDT and 3 replicates tested by the Promoter® assay of each concentration). Probit analysis predicted the 95% LOD of 1849 (95% CI: 258-13234) copies/mL for the Promoter® assay and 2295 (95% CI: 1.6-3265472) copies/mL for the LDT (Table S2).

HEV RNA presence was tested in 28 clinical samples in parallel by each assay and 100% qualitative agreement (negative or positive) was reached across specimens. The viral loads in 22 HEV RNA-positive samples measured by each assay are shown in Fig. S1. The results by the Promoter® assay and the LDT were linearly associated

and correlated (R^2=0.8611, $p < 0.0001$) (Fig. S1A). Bland-Altman analysis indicated that the LDT gave a slightly higher viral load than did the Promoter® assay. The mean [Promoter®-LDT] difference was – 0.76 log_{10}copies/mL (Fig. S1B). Notably, the other 6 samples with positive anti-HEV IgM but negative HEV RNA determined by the LDT were confirmed negative by the Promoter® assay.

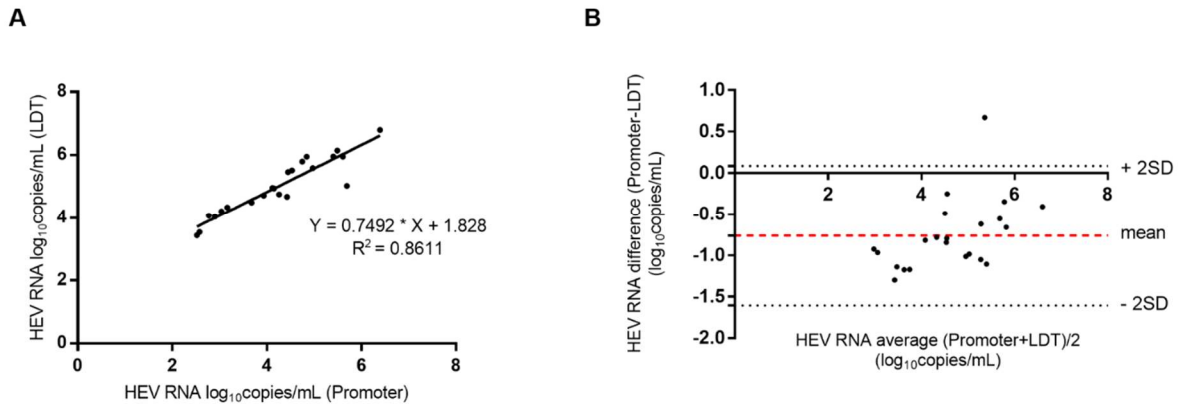

Fig S1 Agreement between HEV RNA concentrations measured by the LDT and the Promoter® assay. (A) Linear regression analysis of HEV RNA concentrations determined by the LDT and the Promoter® assay. (B) Bland-Altman plot for bias analysis between the LDT and the Promoter® assay. The red dashed line represents the mean of the difference (n = – 0.76 log_{10}copies/mL) and black dotted lines show the mean ± 2 standard deviation.

Table S1 Detection results of 10-fold dilutions of HEV RNA standard

HEV RNA concentration		Promoter®				LDT			
copies/mL	log_{10}(copies/mL)	Detected/Tested	Mean C*t*	SD	CV%	Detected/Tested	Mean Ct	SD	CV%
5.35E+07	7.73	5/5	18.95	0.24	1.27	5/5	22.80	0.13	0.57
5.35E+06	6.73	5/5	22.40	0.19	0.85	5/5	26.20	0.22	0.84
5.35E+05	5.73	5/5	25.75	0.14	0.54	5/5	29.55	0.25	0.85
5.35E+04	4.73	5/5	29.02	0.39	1.34	5/5	33.02	0.20	0.60
5.35E+03	3.73	5/5	32.51	0.50	1.54	5/5	35.66	0.54	1.51
5.35E+02	2.73	3/5	35.49	0.71	2.00	0/5	/	/	/

LDT: laboratory-developed test; C*t*: cycle threshold; SD: standard deviation; CV: coefficient of variation

Table S2 Detection limit of the RT-PCR assays

HEV RNA concentration		Promoter®		LDT	
copies/mL	log_{10}copies/mL	Detected/Tested	% Detected	Detected/Tested	% Detected
5.35E+04	4.73	3/3	100.0	5/5	100.0
1.78E+04	4.25	3/3	100.0	5/5	100.0
5.94E+03	3.77	3/3	100.0	5/5	100.0
1.98E+03	3.30	3/3	100.0	4/5	80.0
6.60E+02	2.82	2/3	67.7	0/5	0.0
2.20E+02	2.34	1/3	33.3	0/5	0.0
7.34E+01	1.87	0/3	0.0	0/5	0.0

Probit value (95% detection rate) for the Promoter® assay = 1849 copies/mL (95% CI: 258.4-13234).

Probit value (95% detection rate) for the LDT assay = 2295 copies/mL (95% CI: 1.612-3265472).

Table S3 HEV Ag and HEV RNA levels in sample A and sample B diluted with either positive or negative anti-HEV IgG serum

	IgG (COI)	IgM (COI)	HEV Ag (S/CO)	HEV RNA (log_{10}copies/mL)
IgG positive pooled sera	16.7	0.32	0.08	(-)
IgG negative pooled sera	0.02	0.05	0.08	(-)
Sample #A	7.81	42.29	37.11	7.44
#A_1/10 diluted by IgG_positive sera	/	/	0.07	6.20
#A_1/100 diluted by IgG_positive sera	/	/	0.09	5.23
#A_1/1000 diluted by IgG_positive sera	/	/	0.06	4.29
#A_1/10 diluted by IgG_negative sera	/	/	23.79	6.35
#A_1/100 diluted by IgG_negative sera	/	/	4.21	5.43
#A_1/1000 diluted by IgG_negative sera	/	/	0.41	3.96
Sample #B	4.47	12.06	34.81	7.91
#B_1/10 diluted by IgG_positive sera	/	/	0.06	7.32
#B_1/100 diluted by IgG_positive sera	/	/	0.08	6.50
#B_1/1000 diluted by IgG_positive sera	/	/	0.08	5.42
#B_1/10 diluted by IgG_negative sera	/	/	16	7.39
#B_1/100 diluted by IgG_negative sera	/	/	1.81	6.36
#B_1/1000 diluted by IgG_negative sera	/	/	0.15	5.28

Table S4 Multivariate stepwise logistic regression analysis of factors associated with HEV RNA presence

Variables	Current HEV infection					
	univariate analysis			multivariate analysis		
	β coefficient	OR (95% CI)	*p* value	β coefficient	OR (95% CI)	*p* value
Age	0.017	1.02 (1.00–1.04)	0.098	–	–	–
Gender	0.276	1.32 (0.76–2.29)	0.328	–	–	–
Log_{10}(Ag)	3.04	20.88 (8.62-50.57)	< 0.001	3.08	25.52 (4.75-136.97)	< 0.001
Log_{10}(IgG)	-0.97	0.38 (0.11-1.34)	0.132	–	–	–
Log_{10}(IgM)	2.94	18.86 (8.47-41.96)	< 0.001	5.14	175.57 (19.78- > 999.99)	< 0.001
$\sqrt{ALT/ULN}$	1.61	5.00 (2.98-8.40)	< 0.001	2.64	12.28(2.09-72.01)	< 0.001

OR: odds ratio; CI: confidence interval; Ag: antigen; ALT: alanine aminotransferase; ULN: upper limit of normal