THE LANCET Infectious Diseases

Supplementary webappendix

This webappendix formed part of the original submission and has been peer reviewed. We post it as supplied by the authors.

Supplement to: Grigg MJ, William T, Menon J, et al. Artesunate–mefloquine versus chloroquine for treatment of uncomplicated *Plasmodium knowlesi* malaria in Malaysia (ACT KNOW): an open-label, randomised controlled trial. *Lancet Infect Dis* 2015; published online Nov 18. http://dx.doi.org/10.1016/S1473-3099(15)00415-6.

pkS25 RTPCR

Subject blood samples (1ml) were stored in RNAprotect (QIAGEN) (1:5). Samples were centrifuged at 15,000xg for 6 minutes to remove preservation buffer supernatant. RNA extraction was performed using PureLink Mini RNA kit (Invitrogen). Blood pellets (approximately 200ul) were re-suspended in 600ul of lysis buffer containing 1% 2-mercaptoethanol and vortexed. Each sample was then spiked with 1ul of extraction control (Bioline) before centrifugation at 20,000 g. Supernatant was transferred into a clean 2ml tube with the addition of 800ul of 70% ethanol and mixed. Samples were then applied to columns (PureLink Mini RNA kit; Invitrogen) and washed with wash buffer 1 (350ul). On-column DNase digestion was peformed as per the protocol for 15 minutes. Columns were then washed with wash buffer 1 (350ul), followed by 2 addition washes with buffer 2 (500ul) before elution. Each total RNA sample was eluted in two volumes of 20ul and immediately processed for cDNA synthesis. PrimeScript RT Reagent Kit with gDNA Eraser (Takara) was used to synthesize cDNA for each RNA sample. In each reaction, 7ul of total RNA was used and the manufacturer's protocol followed. An additional minus reverse transcriptase control as prepared for each sample to control for completion of removal of genomic DNA.

cDNA was run in duplex real time semi-qualitative PCR reactions for the detection of *pkS25* (FAM) and for the extraction control target (Cy5). Primers and probe sequences for *pkS25* are in the table below. The BioRad CFX-384 PCR machine was used for all PCR amplification of patient samples with cycling condition outlined in the table below. Each reaction contained 1.5ul of template in duplicate in a total volume of 15ul.

Forward Primer	5' CAGCACAGCAGAGTACATACA 3'
Reverse Primer	5' TTCACCAATACATTCACCACT 3'
Probe	56-FAM/AGGATGGAA/ZEN/CTTGTGTTGTTGATG/3IABkFQ

A standard curve was produced using an RNA run-off of pGEMT easy plasmid containing the PkS25 gene (Promega pGEM T easy kit, Bioline T7 RNA synthesis kit) following the manufacturer's protocols. RNA was then reverse transcribed into cDNA using PrimeScript RT Reagent Kit with gDNA Eraser (Takara). To produce the first standard, sythesised cDNA was diluted 1:1000 in RNase free water then a ten-fold dilution to complete the standard curve. Dilutions of each concentration were stored at -80^{0} until used.

PCR conditions-

	original conc.	f.c.	per rx	1 MM
QuantiNova	2		7.5	**7.5**
MgCl2	50	6mM	1.35	**1.35**
H2O			3.43	**3.425**
For (s25)	10	100.00	0.35	**0.35**
Rev (s25)	10	100.00	0.35	**0.35**
Probe (s25)	10	100.000	0.175	**0.175**
EC MIX	25	1	0.35	**0.35**
total			15	**13.5**
template	1.5		**check**	**13.5**

BioRad CFX 384

Step	**Time**	**Temp**	
Activation	15min	95°C	
Denaturation	15sec	95°C	Repeat for 40 cycles
Annealing	30sec	60°C	
Extension	20sec	72°C	