Laboratory protocols for the study “Whole genome analysis of selected human and animal rotaviruses identified in Uganda from 2012 to 2014 reveals complex genome re-assortment events between human, bovine, caprine and porcine strains”.

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Abstract

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Protocol

Preparation of feacal suspension

Step 1.

Human stool suspension(approximately 100 mg of stool) were suspended in 200 µl of Phosphate Buffered Saline Solution (PBS) or 200 µl of semi-formed stool was mixed with 150 µl of PBS.

Animal Feacal suspensions of 10-20% were prepared in PBS in 2 ml nunc tubes by adding 200ul of liquid sample or a bacteriological loop full (the size of a garden pea) from semi-solid samples to 2 ml of PBS. The 10% suspensions were stored at -70 °C.

RNA extraction using the guanidinium isothiocyanate

Step 2.

RNA was extracted using the guanidinium isothiocyanate (GTC)/ silica gel extraction of nucleic acid (The 'Boom' method) [1]. Two hundred (200) ul of faecal suspension prepared as above, was added to 1ml of lysis buffer-L6 buffer (annex) and 20ul of size fractionated silica (annex) in a 1.5ml screw-capped microcentrifuge tube. RNase-free distilled water was included in each run to act as a negative control. The mixture was vortexed for 10 seconds and incubated at room temperature for 15 minutes. Centrifugation for 15 seconds at 18000 g was then done. The supernatant was discarded as toxic waste and the pellet kept for the next step. The pellet was then washed with 1 ml of lysis buffer L2 (annex) by vortexing, then pelleted by centrifugation at 18000 g. The wash with L2 Buffer was

repeated once. The wash was then done twice with 1ml 70% ethanol and 1ml acetone once. After removal of the acetone (performed carefully to avoid pellet dislodging), centrifugation was done at 18000 g. The tubes were then placed with lid open at 56°C in a dry heating block for 5 minutes. Forty (40) ul of RNase free water were added to the pellet, vortexed and incubated at 56°C for 15 minutes to elute the nucleic acid from the silica. The pellet was centrifuged at 180000 g for 4 min and the supernatant was extracted (avoid disturbing the silica). This was then stored in a new microfuge tube at 4°C for 24h or at -70°C for longer.

RNA Extraction using TRIZOL LS Reagent (Invitrogen)

Step 3.

Human RV RNA was extracted using TRIZOL LS Reagent (Invitrogen). Approximately 100 mg of stool were suspended in 200ul of PBS or 200ul of semi-formed stool was mixed with 150ul of PBS. One ml of TRIZOL was added, vortex and let to stand for 5 minutes. Then 270ul of chloroform was added, the mixture was vortexed and let to stand for 3 minutes. The mixture was then centrifuged at 4°C for 20 minutes at 18000 g. One ml of ice-cold isopropanol was put in sterile tubes, in which the supernatant from the mixture was added, mixed by inversion and samples were let to stand for 5 minutes at room temperature. The RNA was precipitated by centrifugation of the mixture at room temperature for 30 minutes at 18000 g . The supernatant was discarded, and the pellet was dried by air for 10 minutes before suspending in 90ul of nuclease free water.

cDNA synthesis and PCR amplification of the entire rotavirus genome

Step 4.

cDNA synthesis, PCR amplification and purification of the entire rotavirus genome were carried out on the human samples that yielded more than 2 ng/µl dsRNA as described previously [2, 3]. This is detailed below:

RNA purification

30 ul of 8M Lithuim chloride was added to 90 ul of the double-stranded rotavirus RNA (dsRNA) extracted from each human and animal sample to remove single stranded RNAs. This was then incubated at 4°C overnight (16 hours). Samples were then spun for 30 minutes at 18000 g. The extracted dsRNA was then purified using a Min ELUTE gel extraction kit (Qiagen) following the manufacturer's instructions. The dsRNA was assessed using 1% Tris-borate Ethylenediaminetetracetic acid (TBE) agarose gel stained with ethidium bromide.

Oligoligation

The double stranded RNA was oligoligated using PC3-T7 (5'-p-GGATCCCGGGAATTCGGTAATACGACTCACTATATATTTTTATAGTGAGTCGTATTA-OH-3') loop primer and PC2 (5'-p-CCGAATTCCCGGGATCC-3') primer as previously described [2] . In detail: The PC3-T7 loop

(200ng) was ligated to dsRNA (0.4-200ng) in 50nM HEPES (NaoH, pH 8.0 (Sigma), 18nM $MgCl_2$ (Separations), 0.01% BSA (TaKaRa), 1mM ATP (Roche), 3 mM Dithiothrectal (DTT)(Roche), 10% DMSO (Sigma), 20% polyethyleneglycol $(PEG)_{6000}$ (BDH) and 30 U T4 RNA ligase (TaKaRa) in a final volume of 30ul. This was then incubated at 37°C for 16 hours for ligation to take place.

The ligated dsRNA was purified using the MinElute Gel extraction kit (Qiagen) following the manufacturer's instructions.

cDNA synthesis and PCR amplification of the rotavirus genome

The purified dsRNA (7ul) was denatured using 1ul of 300mM methylmercury hydroxide (Alfa Aesar). This was left to stand for 10-30 minutes at room temperature.

Reverse transcription of the dsRNA to synthesize cDNA: The reaction contained 50Mm Tris/HCl, pH 8.3 (Sigma), 10 mM $MgCl_2$ (Separations), 70Mm KCl(Sigma), 30 Mm β-mercaptolethanol(Sigma), 1 mMdNTPs(TaKaRa) and 15 U cloned AMV reverse transcriptase (Invitrogen). The reaction was incubated in a thermal cyckler at 42°C for 45 minutes followed by 55 °C for 15 minutes, and held at 10 °C.

The tubes were removed from the thermocycycler, vortexed and spun briefly. Then 3ul of Sodium hydroxide (Sigma) was added to end with 0.1M (aimed at removing the excess RNA. Then incubated at 65 °C for 20-30 minutes.

Six (6 ul) Tris/HCl 50nM, pH8.3 (Sigma) was then added to the mixture and incubated 65 °C for one hour (annealing of cDNA) .

cDNA was then amplified using primer PC2 under the following reaction conditions: one cycle at 72 °C for 2 minutes, followed by 25 cycles each of 94 °C for 2 minutes, 65 °C for 30 seconds and 72 °C for 4 minutes. This was followed by an extension step at 72 °C for 5 minutes. The PCR mixture contained 31ul of deionised water, 5ul of extracted Taq buffer, 4ul of dNTPs (10Mm/ul) 0.5ul primer (PC2), 4 ul $MgCl_2$ and 0.5 ul Taq enzyme (TaKaRa). Forty five (45)ul of PCR mixiture was added to 5 ul cDNA .

The amplified cDNA (2ul) was evaluated on a 1% TBE agarose gel stained with ethidium bromide.

Library preparation

Step 5.

The purified rotavirus cDNA PCR amplicons were subjected to standard bar-coding and library construction for Illumina sequencing using the Nextera XT DNA Library Preparation Kit by following the manufacturer's recommendations (Illumina Inc., CA, USA).

For all animal samples and human samples that yielded less than 2 ng/µl dsRNA, the ScriptSeq v2 RNA-Seq Library Preparation Kit (Epicentre, Chicago, IL, USA) was used, following the manufacture's instructions with the slight modification of an initial denaturation step (95 ºC for 5 min). When the concentration of RNA input was less than 50ng/µl, PCR cycles were increased up to 22 to obtain

sufficient product for sequencing. Each library was indexed with Illumina compatible barcodes to allow multiplexing.

ANNEX

Step 6.

1. NB. For this study we used commercial made L2 buffer, L6 buffer and size fractionated silica purchased from Severn Biotech Ltd, Unit 2 , Park lane, Kinderminiter, Worchestershire,UK)

However, L2 buffer, L6 buffer and size fractionated silica can also be reconstituted in the lab as below [4]:

L6 Buffer

Guanidium isothiocyanate (GTC) 60gm

0.1M Tris-HCL Ph 6.4 50ml

0.2M EDTA p H 8.0 11ml

Triton X-100 1.3gm

L2 Buffer

GTC 180gm

0.1M Tris-HCl pH 6.4 150ml

Preparation of size fractioned silica

Add Sixty (60) gm of silicon dioxide, SiO_2 (Sigma; S-5631) to demineralized water to a total volume of 500 ml in a measuring glass cylinder. Allow the silica to sediment under gravity for 24 hours at room temperature. Extract 430 ml of supernant and add demineralized water to 500 ml and shake

vigorously. Sedimentfor 24 hours at room temperature. Extract 440 ml of supernant and add 600ul HCl (32%, w/v) to adjust the silica suspension to pH 2.0.

References

Step 7.

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Step 8.