APPENDIX A

A1. Cloning of PCR products using the pGEM®-T Easy Vector System (Promega Co., Madison, WI)

A1.1 Preparation of culture media and reagents used during the cloning procedures

A1.1.1 Luria-Bertani (LB) Medium (per Litre)

Bacto-tryptone (Difco, Detroit, MI)	10 g
Bacto-yeast extract (Difco)	5 g
NaCl (Merck, Darmstadt, Germany)	10 g
Deionised water	1 000 ml

Adjust the pH to 7.5 with NaOH (1 M) (Merck).

Sterilise by autoclaving for 20 min at 121°C.

A1.1.2 LB Solid Agar(per Litre)

Bacto-tryptone	10 g
Bacto-yeast extract	5 g
NaCl	10 g
Bacto agar (Difco)	15 g
Deionised water	1 000 ml

Adjust the pH to 7.5 with NaOH (1 M).

Sterilise by autoclaving for 20 min at 121°C.

A1.1.3 Preparation of LB/ampicillin/IPTG/X-Gal plates

Prepare LB solid agar as in A1.1.2. After the media has cooled to 50°C ampicillin (Sigma Chemical Co., St Louis, MO) was added to a final concentration of 100 µg/ml. The medium was supplemented with 0.5 mM isopropylthio-β-D-galactoside (IPTG) (Promega) and 80 µg/ml 5-bromo-4-chloro-3-indolyl- β-D-galactoside (X-Gal) (Promega). Thirty millilitres of LB/ampicillin/IPTG/X-Gal

medium was poured into 90 mm petri dishes (Concorde Plastics, Johannesburg, South Africa) and allowed to solidify. The plates were stored at 4°C.

A1.1.4 Mg^{2+} (2 M) stock

$MgCl_2.6H_2O$ (Sigma)	20.33 g
$MgSO_4.7H_2O$ (Merck)	24.65 g

Add deionised water to 100 ml and filter-sterilise.

A1.1.5 SOC Media (per 100 ml)

Bacto-tryptone	2 g
Bacto-yeast extract	0.5 g
NaCl (1 M)	1 ml
KCl (1 M) (Merck)	0.25 ml
Deionised water	97 ml

Sterilise by autoclaving for 20 min at 121°C and cool to room temperature (23°C). Add Mg^{2+} (2 M) filter-sterilised stock (1.1.4) and filter-sterilised glucose (2 M) (Sigma) solution, both to a final concentration of 20 mM. The final pH should be 7.0.

A1.2 Ligation using 2× Rapid Ligation Buffer

The pGEM®-T Easy Vector and control insert DNA were briefly centrifuged to collect the contents at the bottom of the tubes. The 2× Rapid Ligation Buffer was vortexed before each use and 0.5 ml microcentrifuge tubes (Eppendorf-Netheler-Hinz, Hamburg, Germany), known to have low DNA-binding capacity, were used for the ligation reactions. The preparation of the ligation reactions are summarised in Table A.1. The reactions were mixed by pipetting, followed by incubation for 1 h at room temperature (23°C). Alternatively, the reactions could be incubated overnight at 4°C to obtain the maximum number of transformants.

Table A.1 Preparation of ligation reactions

Reagents	Standard reaction	Positive Control	Negative Control
2× Rapid Ligation Buffer	5 µl	5 µl	5 µl
pGEM®-T Easy Vector (50 ng)	1 µl	1 µl	1 µl
PCR product	3 µl	-	-
Control insert DNA	-	2 µl	-
T4 DNA Ligase (3 Weiss units/µl)	1 µl	1 µl	1 µl
Deionised water to a final volume of	10 µl	10 µl	10 µl

A1.3 Protocol for transformations using the pGEM®-T Easy Vector ligation reactions

Eschirichia coli JM109 High Efficiency Competent Cells (Promega) were used for the transformations. The tubes containing the ligation reactions were briefly centrifuged to collect the contents at the bottom of the tube. Two microlitres of each ligation reaction was transferred to a sterile 1.5 ml microcentrifuge tube (Eppendorf) on ice. Another tube was set up on ice containing 0.1 ng of uncut plasmid for the determination of the transformation efficiency of the JM109 competent cells. The JM109 competent cells were thawed on ice (approximately 5 min) and gently mixed by flicking the tube. Fifty microlitres of JM109 cells were carefully transferred into each ligation reaction tube (100 µl of cells for the determination of transformation efficiency). The tubes were gently mixed and incubated on ice for 20 min. The cells were heat-shocked for 45-50 s in a heating block at exactly 42°C without shaking and returned to ice for 2 min. SOC medium (950 µl) (A1.1.5) was added to the tubes containing cells transformed with the ligation reactions, while 900 µl of SOC medium (1.1.5) was added to the tube containing cells transformed with uncut plasmid. The tubes were incubated for 1.5 h at 37°C in a shake incubator (150 rpm). Each transformation culture (100 µl) was plated onto duplicate LB/ampicillin/IPTG/X-Gal plates (A1.1.3). A 1:10 dilution was made with SOC medium of the tube containing the cells transformed with uncut plasmid DNA and 100 µl plated onto duplicate LB/ampicillin/IPTG/X-Gal plates (A1.1.3). The plates were incubated for 16-24 h at 37°C. Successful cloning of an insert in the pGEM®-T Easy

Vector interrupts the coding sequence of β-galactosidase, producing white colonies, which were used for further analysis.

A1.4 Calculation of transformation efficiency

The following formula was used to calculate the transformation efficiency of the JM109 cells:

$$\frac{\text{Average number of white colonies obtained}}{0.01 \text{ ng of plasmid DNA}}$$

(Note: If the transformation efficiency is lower than 1×10^8 cfu/µg DNA, fresh cells must be prepared.)

A2 Isolation of recombinant plasmid DNA

A2.1 Preparation of solutions used during the isolation of recombinant plasmid DNA

A2.1.1 7 M Guanidine-HCl Solution

Guanidine-HCl (Promega)	66.9 g
Nuclease-free water (Promega)	50 ml

Dissolve the Guanidine-HCl and adjust the solution to a final volume of 100 ml by adding nuclease-free water.

A2.1.2 4/40 Wash Solution

7 M Guanidine-HCl solution (A2.1.1)	30 ml
100% Isopropanol (Merck)	20 ml

Mix and store at 20-25°C.

A2.1.3 80% ethanol wash solution

100% Ethanol (Merck)	160 ml
Deionised water	40 ml

Store at 20-25°C.

A2.2 Purification of plasmid DNA using the Wizard® PureFection Plasmid DNA Purification System (Promega)

Each white colony obtained after cloning was incubated overnight at 37°C in 10 ml of LB medium (A1.1.1) containing ampicillin to a final concentration of 100 µg/ml. The bacterial cultures were centrifuged at 10 000 × *g* (Sorvall Super T21) for 5 min at room temperature (23°C). The supernatant was discarded and the pellets resuspended in 1.25 ml of Cell Resuspension Solution. The resuspended cells were transferred to sterile 15 ml centrifuge tubes (Corning Inc., Corning, NY). Cell Lysis Solution (1.25 ml) was added to the tubes and mixed by inverting the tubes 6-8 times to ensure cell lysis, followed by incubation for 5 min at room temperature (23°C). Neutralisation Solution (1.75 ml) was added and the tubes mixed immediately by inverting the tubes 6-8 times. The bacterial lysate was centrifuged at 10 000 × *g* (Sorvall Super T21) for 20 min at room temperature (23°C). This centrifugation step was repeated and the supernatant transferred to new 15 ml centrifuge tubes. Endotoxin Removal Resin (0.25 ml) was added to each tube and mixed vigorously. The tubes were incubated for 10 min at room temperature (23°C), shaking the tubes several times during the incubation period. The tubes were placed onto the MagneSil™ Magnetic Separation Unit (Promega) and the solution was allowed to clear for 30 s. The tubes were kept on the magnet while the supernatant was transferred to a new 15 ml centrifuge tube, after which the resin was discarded. Guanidine Thiocyanate (5 M) (1 ml) was added to the supernatant-containing tubes and mixed vigorously. The MagneSil™ Paramagnetic Particles (0.75 ml) were added to each tube and mixed vigorously. The tubes were incubated for 3 min at room temperature (23°C). The tubes were placed onto the magnetic unit and the solution allowed to clear for 30 s. The supernatant was discarded and the particles washed by adding 1 ml of the 4/40 wash solution (A2.1.2). The particles were resuspended by

vortexing for 10 s. The tubes were replaced onto the magnetic unit and the solution was allowed to clear for 30 s. The supernatant was discarded and the particles washed with 2.5 ml of the 80% ethanol wash solution (A2.1.3). The particles were resuspended by vortexing for 10 s. The tubes were replaced onto the magnetic unit and the solution was allowed to clear for 30 s. The supernatant was discarded. This washing step was repeated 3 times, and after the third wash, the tubes were left open. The particles were allowed to dry while the tubes remained on the magnetic unit. The tubes were removed from the magnetic unit and 1.2 ml of nuclease-free water was added to each tube. The particles were resuspended by vortexing for 10 s, followed by an incubation period of 1 min at room temperature (23°C). The tubes were replaced onto the magnetic unit and the solution allowed to clear for 3 minute to capture all particles. The supernatant was transferred to new 15 ml centrifuge tubes.

A2.3 Precipitation of the eluted DNA

The volume of the above-mentioned supernatant was determined and 0.5 volumes of ammonium acetate (7.5 M) added per volume of supernatant. Absolute ethanol (2.5 volumes) (Merck) was added to each tube and mixed well. The tubes were centrifuged (Sorvall Super T21) at 14 000 × *g* for 15 min at room temperature (23°C). The supernatant was aspirated using a 9-inch Pasteur pipette (Copan Innovation, Italy). The translucent DNA pellets were rinsed in 2 ml of 70% ethanol (Merck) and centrifuged at 14 000 × *g* (Sorvall Super T21) for 5 min. The ethanol was aspirated and the pellets allowed to air-dry for 3-5 min to allow the residual ethanol to evaporate. The DNA pellets were resuspended in 300 µl of nuclease-free water (Promega).

A2.4 Molecular detection of the HEV-like fragments in the purified plasmids

The HEV-like fragments were detected by polymerase chain reaction (PCR) amplification based on the procedure described by Erker *et al.*, 1999. Promega manufactured all the reagents used in the PCR, unless otherwise stated. The PCR was performed in a reaction volume of 49 µl containing 76.5 mM KCl, 15.3 mM Tris-HCl

(pH 9.0), 0.15% Triton X-100, 3.8 mM $MgCl_2$, 0.6 mM PCR nucleotide mix, 10 pmol of primer HEVORF2con-a1 (5'-CTTGTTCRTGYTGGTTRTCATAATC-3') (Sigma-Aldrich Co., NSW, Australia), 10 pmol of primer HEVORF2con-s1 (5'-GACAGAATTRATTTCGTCGGCTGG-3') (Sigma) and 2.5 units of Taq polymerase. After 35 cycles (20 s at 94°C, 30 s at 55°C and 30 s at 72°C) and an additional incubation for 10 min at 72°C (Px2 Thermal Cycler), 20 µl of the PCR mixture was analysed by agarose gel electrophoresis (2%) (Seakem LE agarose, Bioproducts, USA). The ethidium bromide-stained bands were visualised on an UV Transilluminator (UVP ImageStore 5000). The amplicons obtained were sequenced using a Spectrumedix SCE2410 genetic analysis system with the ABI BigDye Terminator cycle sequencing kit version 3.1 (Applied Biosystems) as specified by the manufacturer.

A2.5 References

Erker JC, Desai SM and Mushahwar IK (1999). Rapid detection of hepatitis E virus RNA by reverse transcription-polymerase chain reaction using universal oligonucleotide primers. *Journal of Virological Methods* **81**, 109-113.

APPENDIX B

The nucleotide sequences within the ORF2 region of the South African HEV strains detected during this study are shown below:

RSA - 1 (GenBank Accession No: AY621662)

```
1    gacagaattg atttcgtcgg ctggcgatca gctgttctac tctcgtcccg
51   tcgtctcagc caatggcgag ctgactgtta agctgtatac atctgtagag
101  aatgctcagc aggataaggg tatcgcaatc ccgcatgata ttgacctcgg
151  agagtctcgt gtggttattc aggattatga caaccaacac gaacaag
```

RSA - 2 (GenBank Accession No: AY621663)

```
1    gacagaaaag atttcgtcgg ctggtggcca gctgttctac tcccgtcccg
51   tcgtctcagc caatggcgag ccgacggtta agctgtatac atctgtagag
101  aatgctcagc aggataaggg tatcgcaatc ccgcatgata ttgacctcgg
151  agagtctcgt gtggttattc aggattatga caaccaacac gaacaag
```

swRSA - 1 (GenBank Accession No: AY621664)

```
1    gacagaattg atttcgtcgg ctgggggcca gttgttctac tcccgcccag
51   tagtctcagc caatggcgag ccgactgtca agttatatac atctgttgag
101  aatgcgcagc aggacaaggg gattgccatc ccacacgata tagatctggg
151  tgattcccgt gtggtcatcc aggattatga caaccagcac gaacaag
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swRSA - 2 (GenBank Accession No: AY621665)

```
1    gacagaattg atttcgtcgg ctggtggcca gctgttctac tcccgtcccg
51   tcgtctcagc caatggcgag ccgacagtga agcattatac atcagtcgag
101  aatgctcagc aggataaggg tatagctgtt tcacgcaata ccgaccttag
151  tgagtctcga gttgttatct tagattatga taaccagcat gagcagg
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