Journal of General Virology (2016), **97**, 1829–1840 DOI 10.1099/jgv.0.000478

Hepatitis E virus (HEV)-1 harbouring HEV-4 non-structural protein (ORF1) replicates in transfected porcine kidney cells

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Hepatitis E virus (HEV) is a causative agent of acute hepatitis and a major public health problem in India. There are four mammalian HEV genotypes worldwide. In India, genotype 1 (HEV-1) is restricted to humans whereas genotype 4 (HEV-4) circulates in pigs. Studies from our laboratory have shown that HEV-4 (swine) virus can establish experimental infection in rhesus monkeys; however, HEV-1 (human) virus cannot infect pigs. Viral and/or cellular factors responsible for this host specificity are not yet known. We developed 12 different genotype 1–4 chimeric full genome clones with pSK-HEV2 as the backbone and by replacing structural (ORF2 and ORF3), non-structural (ORF1) and non-coding regions (NCR) with corresponding segments from the HEV-4 clone. S10-3 (human hepatoma) and PK-15 (pig kidney) cells were transfected with transcripts generated from the above clones to test their replication competence. Transfected cells were monitored for successful virus replication by detecting replicative intermediate RNA and capsid protein (immunofluorescence assay). All the chimeric constructs were able to replicate in S10-3 cells. However, only two chimeric clones, HEV-1 (HEV-4 5′NCR-ORF1) and HEV-1 (HEV-4 ORF1), containing 5′NCR-ORF1 and ORF1 regions from the HEV-4 clone, respectively, were able to replicate in PK-15 cells. We demonstrate for the first time the crucial role of ORF1 polyprotein in crossing the species barrier at the cellular level. These results indicate the importance of interactions between ORF1 protein domains and host cell specific factors during HEV replication and the critical role of cellular factors as post-entry barrier/s in virus establishment.

Received 7 December 2015
Accepted 8 April 2016

INTRODUCTION

Hepatitis E virus (HEV) belongs to the genus *Orthohepevirus* in the family *Hepeviridae*. The species *Orthohepevirus A* includes all viral variants known to infect humans (presently known as genotypes 1–4) (Smith *et al.*, 2014). The HEV genome is a single stranded positive sense polyadenylated RNA. Except avian HEV (6.8 kb), the average genome size is ~7.2 kb. The genome is capped at its 5′ end and contains three overlapping ORF – namely ORF1, ORF2 and ORF3 (Tam *et al.*, 1991). The viral genome is flanked by short 5′ and 3′ UTRs and a conserved 58 nt region within ORF1; these elements are likely to fold into conserved stem–loop and hairpin structures. Genotypes 1 and 2 are restricted to humans, while genotypes 3 and 4 are zoonotic (Pavio *et al.*, 2010; Meng, 2011).

The genotype 3 strain of human HEV can infect pigs and the genotype 3 swine HEV strain is able to establish successful infection in non-human primates. However, genotype 1 or 2 human HEV strains are not able to infect pigs under experimental conditions (Meng *et al.*, 1998a). Successful experimental infections of non-human primates with genotype 3 and 4 swine HEV further prove the ability of these viruses to infect humans (Meng *et al.*, 1998b; Arankalle *et al.*, 2006). Vice versa, pigs can be experimentally infected with genotype 3 (Halbur *et al.*, 2001) and genotype 4 (Feagins *et al.*, 2008) human HEV strains. These studies show that pigs are refractory to human genotype 1 and 2 HEV.

Incorporation of 3′ non-coding region (NCR) sequences of the swine strain of HEV (genotype 3) into the genotype 1 human strain does not enable the chimeric virus to infect

Four supplementry tables are available with the online Supplementary Material.

swine (Emerson *et al.*, 2001). Feagins *et al.* (2011) developed chimeric virus constructs by swapping genomic regions of genotype 1, 3 and 4 viruses and demonstrated that chimeric viruses containing the ORF2 gene, either alone or in combination with its adjacent 5′ junction region (JR) and 3′ NCR from genotype 4 human HEV, in the backbone of genotype 3 swine HEV are replication-competent in Huh7 cells and infectious in HepG2/C3A cells and in pigs. However, chimeric viruses containing the JR+ORF2 +3′NCR of either genotype 3 or 4 HEV in the backbone of genotype 1 human HEV failed to infect pigs, suggesting the possible role of other genomic regions such as 5′ NCR and ORF1 in HEV cross-species infection. This study was further extended by Córdoba *et al.* (2012). They constructed two chimeric viruses: (a) JR+ORF2+3′NCR of genotype 1 human HEV in the genotype 4 human HEV infectious clone backbone, and (b) JR+ORF2+3′NCR of genotype 1 human HEV in the genotype 3 swine HEV infectious clone backbone. They also tested infectivity of chimeras (c) JR+ORF2+3′NCR of genotype 4 human HEV in the genotype 1 human HEV infectious clone backbone, and (d) JR+ORF2+3′NCR of genotype 3 swine HEV in genotype 1 human HEV infectious clone backbone, developed earlier (Feagins *et al.*, 2011), by direct intra-hepatic inoculation of the transcripts in pigs. All four constructs were replication-competent in Huh7 cells. However, the RNA transcripts from chimeras made with genotype 1 virus backbone, failed to infect pigs. Moreover, the two chimeras developed with genotype 3 and 4 virus backbones also failed to replicate in pigs, except for one pig. The species specificity of HEV with respect to viral genomic regions still remains elusive.

In this study, we constructed an infectious cDNA clone from a genotype 4 (swine) virus which is currently circulating in India. Further, we constructed chimeric clones by replacing different genomic regions of the parental genotype 1 construct with that of the genotype 4 virus and checked replication competence of the clones in human and swine cells. We conclude that the non-structural (ORF1) region may play an important role in deciding species specificity.

RESULTS

Construction of genotype 4 full genome clone and its replication competence *in vitro*

The full genome clone HEV-4FG had T7 promoter at the 5′ end and Bam HI site (for linearization immediately downstream to viral poly A sequence) at the 3′ end. Since the cDNA clone would be used as a donor for genotype 4 genes for construction of chimeras it was essential to test the replication competence and infectious particle generation of the clone. For that, S10-3 cells were used as they are known to support replication of both genotype 1 and genotype 4 HEV. RNA generated from pSK-HEV2 was used as a positive control during these experiments. Cells were transfected with *in vitro* transcribed capped RNA and processed for the detection of capsid protein by immunofluorescence assay (IFA) on the 12th day post-transfection. Positive fluorescence, though at a lower level (about 0.5–1 % positive cells) as compared to pSK-HEV2 (about 3–4 % positive cells) (data not shown) could be detected, indicating replication competence of the HEV-4FG. Further, to check for the infectivity of generated HEV particles, HepG2/C3A cells were infected with the transfected S10-3 cell lysates (12 days post-transfection). Cells were processed both for negative sense RNA (nsRNA) assay on the 6th day (post-infection) and for IFA, on the 9th day. Infected HepG2/C3A cells were positive in both IFA and nsRNA assay indicating successful generation of infectious virus particles (results not shown). However, virus was not released into the supernatant and remained cell associated which was also seen for the pSK-HEV2 generated virus. This confirmed successful generation of an infectious cDNA clone of genotype 4 HEV. With these results it was decided that this clone would be used as a parent genotype 4 clone for developing genotype 1/4 chimeric constructs.

Screening of cell lines by transfecting them with HEV genotype 1 and 4 *in vitro* transcripts

We were in search of a cell line that would support replication of HEV-4 and at the same time would not be permissive for HEV-1. We reasoned that the chimera that would replicate in this cell line would indicate the genomic region responsible for crossing the species barrier. We used detection of nsRNA, replicative intermediates to screen the cell lines. The major reasons were:

1. In our hands we found it to be more sensitive compared with IFA.
2. Cells negative for replicative intermediates would be negative for IFA, so could be excluded from the experimental set up.

Human cell lines already demonstrated to support HEV replication were used for this experiment (human liver origin: HepG2, PLC/PRF/5, Huh7, S10-3 and HepG2/C3A; human lung origin: A549; human colon: Caco2). In the absence of commercial cell lines of swine liver origin, PK-15 (kidney), ESK-4 (embryo) and ST (testes) were screened. PK-15 cells have already been demonstrated to be non-permissive to HEV-1 replication. We thought it would be interesting to check their susceptibility to HEV-4. Susceptibility to HEV-4 and not to HEV-1 would indicate an ideal host for testing chimeras. ESK-4 and ST cells are susceptible to bovine viral diarrhea virus 1 and swine enteroviruses, respectively (Technical sheet, ATCC). We reasoned that since HEV is also an enteric virus, the said cell lines could be susceptible to HEV. Cells were transfected with capped transcripts obtained from both pSK-HEV2 and HEV-4FG clones. Detection of nsRNA intermediates was performed 6, 9 and 12 days post-transfection. All the human cells were positive on the 6th day for pSK-HEV2; positivity could be seen until the 12th day, when the experiment was terminated. All three porcine cell lines transfected with genotype 1 transcripts remained negative in the

Table 1. nsRNA positivity in mammalian cells on the 9th day post-transfection

Cell lines	HEV1FG or pSK-HEV2	HEV-4FG
HepG2	+	–
PLC/PRF/5	+	+
Huh7	+	+
S10-3	+	+
Caco2	+	–
A549	+	+
HepG2/C3A	+	+
PK-15	–	+
ESK-4	–	+
ST	–	–

nsRNA detection assay until 12th day post-transfection. HEV-4 showed positive signals in PLC/PRF/5, Huh7, S10-3, HepG2/C3A, A549, PK-15 and ESK-4 cells on day 9 and remained positive on day 12 (Table 1). HEV-4 could not establish successful replication in Caco2, HepG2 as these cells remained negative until 12 days post-transfection. This entire experiment was carried out in three sets. However, these two cell lines remained consistently negative. ST cells were extremely sensitive for transfection with RNA, showed considerable cell death and were excluded from further experiments. It has been previously shown that S10-3 cells support efficient replication upon transfection with HEV recombinant genomes [produce infectious virus particles and nearly mimic natural replication cycle of the virus (Graff *et al.*, 2006)]. Therefore, we decided to use these human cells further to check the replication competence of chimeric HEV clones. Replicative intermediates could be detected much more consistently in PK-15 cells compared with ESK-4. So, PK-15 was used as model porcine cell system.

Evaluation of replication potential of intergenotypic chimeras *in vitro*

It is known that genotype 4 HEV has the ability to cross species barriers (pig to human or vice versa), whereas genotype 1 HEV is restricted to humans. We felt that development of a chimeric virus with the backbone of the genotype 1 genome would provide insight into the genomic region/s of genotype 4 virus which help in crossing the species barrier. Twelve intergenotypic chimeric constructs were made by replacing: structural region (ORF2 and ORF3); non-structural region (ORF1); and regulatory regions 5′ non-coding region (5′NCR), 3′ non-coding region (3′NCR),

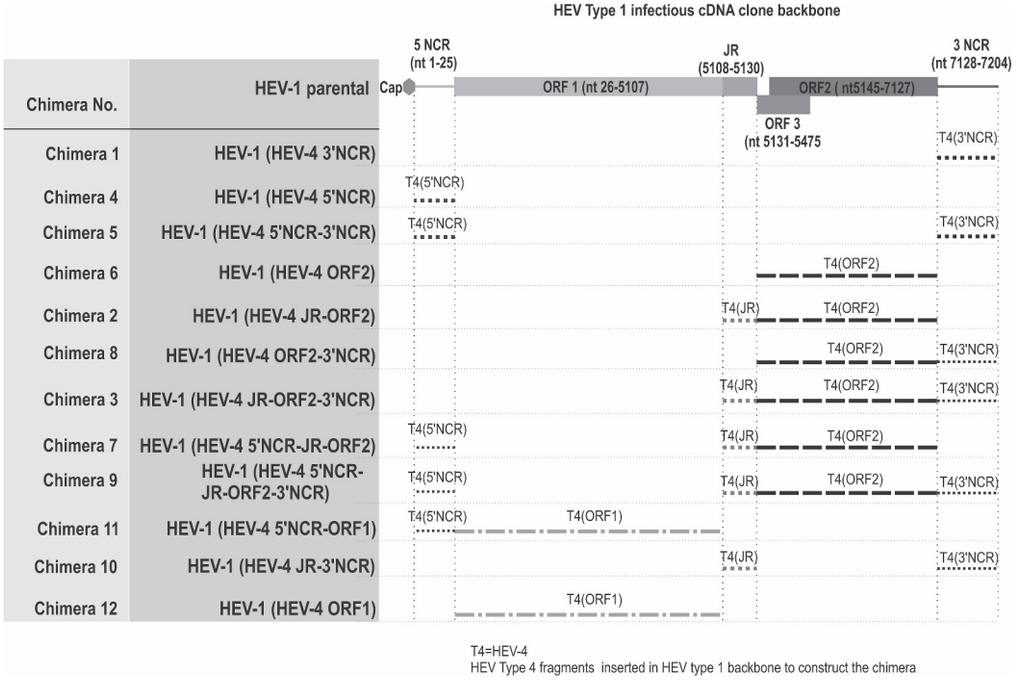

Fig. 1. Schematic diagram showing chimeric constructs and respective genomic region replacements: HEV genotype 1 infectious cDNA clone (pSK-HEV2) was used as the backbone for construction of chimeras. Genomic regions of genotype 1 were replaced with respective regions of genotype 4 to construct 12 chimeras. Regions of HEV genotype 4 in different chimeras are: Chimera 1: HEV-1 (HEV-4 3′NCR), Chimera 2: HEV-1 (HEV-4 JR-ORF2), Chimera 3: HEV-1 (HEV-4 JR-ORF2-3′NCR), Chimera 4: HEV-1 (HEV-4 5′NCR), Chimera 5: HEV-1 (HEV-4 5′NCR-3′NCR), Chimera 6: HEV-1 (HEV-4 ORF2), Chimera 7: HEV-1 (HEV-4 5′NCR-JR-ORF2), Chimera 8: HEV-1 (HEV-4 ORF2-3′NCR), Chimera 9: HEV-1 (HEV-4 5′NCR-JR-ORF2-3′NCR), Chimera 10: HEV1 (HEV-4 JR-3'NCR), Chimera 11: HEV-1 (HEV-4 5'NCR-ORF1), Chimera 12: HEV-1 (HEV-4 ORF1).

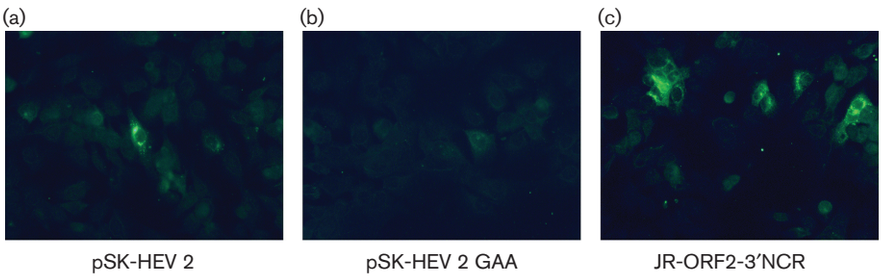

Fig. 2. Representative picture of immunofluorescence staining (IFA): IFA for detection of HEV ORF2 protein was performed on S10-3 cells transfected with capped RNA transcripts from parental and chimeric clones. The representative picture demonstrates IFA positivity for pSK-HEV2 (a) and chimera: HEV-1 (HEV-4 JR-ORF2-3′NCR) (c), pSK-HEV2 GAA (b) served as the negative control.

junction region (JR) of the HEV genotype 1 virus genome with corresponding regions from the genotype 4 virus (Fig. 1). PK-15 and S10-3 cells were transfected with capped transcripts obtained from the constructs and processed for IFA, 12 days post-transfection. HEV-4FG and pSK-HEV2, parent constructs were used in all experiments as controls and respective RNA dependent RNA polymerase (RdRp) GAA mutants were used as negative controls for the experiments.

We observed IFA positivity in S10-3 cells with all the chimeras, though the percentage IFA positivity differed significantly. These observations suggest that genomic regions of genotype 1 HEV can be replaced with that of genotype 4 without hampering the *in vitro* replicative potential. RdRp GAA mutants of both genotype 1 and 4 parental virus constructs remained negative until 12 days in both the cells. All the constructs, except HEV-4FG, HEV-1 (HEV-4 5′NCR-ORF1) and HEV-1 (HEV-4 ORF1) remained negative in IFA until 12 days post-transfection in PK-15 cells (Fig. 2, Table 2).

HEV-4 non-structural proteins encoded by ORF1 are important for replication in porcine cells

The observation of differential replication competence of different constructs in S10-3 and PK-15 cells was thought to be due to two reasons:

1. Capsid protein synthesis is at a low level.
2. Kidney cells (not being the primary site of infection) are not conducive to a full replication cycle.

Table 2. Detection of HEV by IFA in mammalian cells on 12th day post-transfection

Chimera	Genotype 4 region	S10-3 cells	PK-15 cells
pSK-HEV2	HEV-1 parental	+	–
HEV-4FG	HEV-4 parental	+	+
pSK-HEV 2 GAA	HEV-1 RdRp GAA mutant	–	–
HEV-4FG GAA	HEV-4 RdRp GAA mutant	–	–
Chimera 1	HEV-1 (HEV-4 3′NCR)	+	–
Chimera 2	HEV-1 (HEV-4 JR-ORF2)	+	–
Chimera 3	HEV-1 (HEV-4 JR-ORF2-3′NCR)	+	–
Chimera 4	HEV-1 (HEV-4 5′NCR)	+	–
Chimera 5	HEV-1 (HEV-4 5′NCR-3′NCR)	+	–
Chimera 6	HEV-1 (HEV-4 ORF2)	+	–
Chimera 7	HEV-1 (HEV-4 5′NCR-JR-ORF2)	+	–
Chimera 8	HEV-1 (HEV-4 ORF2-3′NCR)	+	–
Chimera 9	HEV-1 (HEV-4 5′NCR-JR-ORF2-3′NCR)	+	–
Chimera 10	HEV-1 (HEV-4 JR-3′NCR)	+	–
Chimera 11	HEV-1 (HEV-4 5′NCR-ORF1)	+	+
Chimera 12	HEV-1 (HEV-4 ORF1)	+	+

HEV-1 denotes the backbone of genotype 1 HEV and bracket denotes the genotype 4 fragments inserted into the parental backbone.

Table 3. Detection of HEV by nsRNA in mammalian cells on the 9th day post-transfection

Chimera	Genotype 4 region	S103 cells	PK-15 cells
pSK-HEV2	Genotype 1 parental	+	–
HEV1FG	Genotype 1 parental	+	–
HEV-4FG	Genotype 4 parental	+	+
pSK-HEV 2 GAA	Genotype 1 RdRp GAA mutant	–	–
HEV-4FG GAA	Genotype 4 RdRp GAA mutant	–	–
Chimera 1	HEV-1 (HEV-4 3′NCR)	+	–
Chimera 2	HEV-1 (HEV-4 JR-ORF2)	+	–
Chimera 3	HEV-1 (HEV-4 JR-ORF2-3′NCR)	+	–
Chimera 4	HEV-1 (HEV-4 5′NCR)	+	–
Chimera 5	HEV-1 (HEV-4 5′NCR-3′NCR)	+	–
Chimera 6	HEV-1 (HEV-4 ORF2)	+	–
Chimera 7	HEV-1 (HEV-4 5′NCR-JR-ORF2)	+	–
Chimera 8	HEV-1 (HEV-4 ORF2-3′NCR)	+	–
Chimera 9	HEV-1 (HEV-4 5′NCR-JR-ORF2-3′NCR)	+	–
Chimera 10	HEV-1 (HEV-4 JR-3′NCR)	+	–
Chimera 11	HEV-1 (HEV-4 5′NCR-ORF1)	+	+
Chimera 12	HEV-1 (HEV-4 ORF1)	+	+

To confirm the latter possibility, detection of replicative intermediates was tried out. S10-3 and PK-15 cells were transfected in parallel with 2 μg of RNA of all the constructs. Total RNA was isolated from the transfected cells on the 9th day post-transfection and processed for nsRNA detection. For that, RNA was first processed for real time PCR assay to quantify positive sense RNA in the sample. The sample was diluted to reduce positive sense RNA copy number to 10^5 copies per reaction. In accordance to IFA results, except for the GAA RdRp mutants (of pSK-HEV2 and HEV-4FG), all parental and chimeric constructs showed positivity in S10-3 cells, again indicating that they could establish successful replication. For PK-15 cells, again HEV-4FG, and the chimeras HEV-1 (HEV-4 5′NCR-ORF1) and HEV-1 (HEV-4 ORF1) gave positive results (Fig. 3, Table 3).

Fig. 3. nsRNA assay of constructs transfected in PK-15 cells. Constructs were transfected in PK-15 cells and assayed for presence of replicative intermediates on the 9th day post-transfection. Lane 1: 100 bp ladder, Lane 2: HEV-4FG, Lane 3: HEV-4FG GAA, Lane 4: Blank, Lane 5: Chimera 10: HEV-1 (HEV-4 5′NCR-ORF1), Lane 6: Blank, Lane 7: pSK-HEV2.

The chimera with 5′NCR of the genotype 4 genome, either alone or in combination with other genotype 4 regions, did not yield positive results in PK-15 cells, indicating the importance of ORF1 in crossing the species barrier.

5′ and 3′ NCRs dictate replication efficiency

The chimeras developed by replacing ORF1, ORF2/3 encoding regions and the *cis*-regulatory genomic elements, 5′NCR, 3′NCR and putative sub-genomic promoter (JR) (currently called the junction region rather than the sub-genomic promoter since it is not functionally confirmed) with HEV-4 sequences showed different replication efficiencies when analysed using IFA. To have a more unbiased quantitative measurement of the replicative competence of chimeras developed in this study, we carried out ANOVA analysis using IFA. The null hypothesis that the data was normally distributed was rejected after data analysis (JarqueBera = 241.739; $P<0.0001$). Leven's test of homogeneity of variance suggested that the variances were equal across the groups ($P=1.77\times10^{-11}$). Since variance tests were found to be significant, we used a non-parametric Kruskal–Wallis test with sequential Bonferroni correction for the comparison of the percentage of HEV-positive cells for different constructs. The

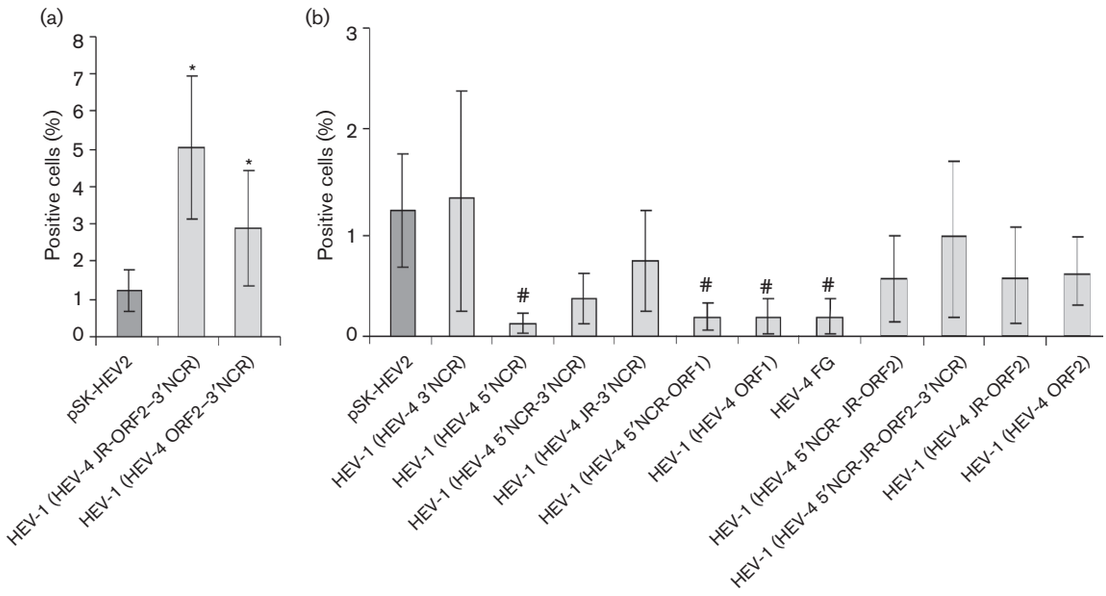

Fig. 4. Mean percentage of IFA positive cells (S10-3) on the 12th day post-transfection. S10-3 cells were transfected with capped RNA transcripts of parental and chimeric constructs and HEV ORF2 was detected using IFA staining. Cells positive for ORF2 were counted for each construct, and replication efficiency was compared with pSK-HEV-2 construct. NCRs (both 5′ and 3′) dictate the replication efficiencies of the chimeric constructs. (a) Chimeras having higher replication potential than parental construct. (b) Chimeras having lower replication potential than parental construct. *, Significantly higher replication efficiency than pSK-HEV2; Mann–Whitney U test, Bonferroni correction $P<0.0001$; #, significantly lower replication efficiency than pSK-HEV2; Mann–Whitney U test, Bonferroni correction $P<0.0001$.

Kruskal–Wallis test showed significant differences in the percentage of HEV-positive S10-3 cells [H (χ^2) = 112.5; Hc (tie corrected) = 115.9; $P = 1.28\times10^{-18}$].

Cells transfected with the constructs HEV-1 (HEV-4 JR-ORF2-3′NCR) and HEV-1 (HEV-4 ORF2-3′NCR) showed significantly higher HEV-positive cells (Mann–Whitney U test; Bonferroni correction $P< 0.0001$ each) as compared with other constructs used in this study (Fig. 4). HEV-1 (HEV-4 5′NCR), HEV-1 (HEV-4 5′NCR-ORF1), HEV-1 (HEV-4 ORF1) and HEV-4FG showed significantly lower replication efficiencies as compared with pSK-HEV2 (Mann–Whitney U test, Bonferroni correction $P<0.0001$ each). Swapping of ORF2, JR-ORF2, JR-3′NCR or 3′NCR [chimeras HEV-1 (HEV-4 ORF2), HEV-1 (HEV-4 JR-ORF2), HEV-1 (HEV-4 JR-3′NCR) and HEV-1 (HEV-4 3′NCR)] did not change the replication efficiencies of the clones significantly. There was a significant reduction in the replication efficiency of the HEV-1 (HEV-4 5′NCR) clone compared to the HEV-1 (HEV-4 3′NCR) clone, however inclusion of both 5′ and 3′NCR in the chimera HEV-1 (HEV-4 5′-3′NCR) improved replication. Comparable replication efficiencies of the chimeras HEV-1 (HEV-4 5′NCR-JR-ORF2-3′NCR), HEV-1 (HEV-4 JR-3′NCR) and pSK-HEV2, but lower level efficiencies of the chimeras HEV-1 (HEV-4 5′NCR-JR-ORF2) and HEV-1 (HEV-4 JR-ORF2) suggest a minor role of the junction region in deciding the host-specific replication efficiency. Significantly higher replication efficiencies of the chimeras HEV-1 (HEV-4 JR-ORF2-3′NCR) and HEV-1 (HEV-4 ORF2-3′NCR), indicated a critical role of 3′NCR in regulating host-specific replication. However, replication of the chimera containing 3′NCR alone, HEV-1 (HEV-4 3′NCR), was comparable to pSK-HEV2, and significantly less than the HEV-1 (HEV-4 JR-ORF2-3′NCR) and HEV-1 (HEV-4 ORF2-3′NCR) clones.

Infectivity of virus particles generated by transfecting S10-3 cells

Though S10-3 cells exhibited good transfection efficiency it was not possible to use these cells for evaluating infectivity of virus that was generated after transfection. We have previously infected several cell lines of human origin such as HepG2, PLC/PRF/5, Huh7, S10-3, Caco2 and A549 with genotype 1 virus purified from human stool samples to see their permissiveness (Devhare *et al.*, 2013) and noticed that HepG2/C3A cells become positive for negative strand 8 h post-infection and IFA positive on the 6th day. HepG2/C3A cells have been successfully used by other researchers for HEV infectivity studies (Emerson *et al.*, 2010; Feagins *et al.*, 2011). Virus particles generated from the chimera HEV-1 (HEV-4 ORF1) would have capsid protein of genotype 1. We hypothesized that infection of PK-15 cells with this virus would help us determine whether the host restriction of

Table 4. Detection of HEV using IFA and nsRNA in infected mammalian cells

Construct	Detection of HEV by IFA		Detection of HEV by nsRNA	
	HepG2/C3A	PK-15	HepG2/C3A	PK-15
pSK-HEV2	+	–	+	–
HEV-1 (HEV-4 ORF2-3′NCR)	–	–	+	–
HEV-4FG	–	–	+	+
HEV-1 (HEV-4 JR-ORF2-3′NCR)	+	–	+	–
HEV-1 (HEV-4 ORF1)	–	–	–	–

genotype 1 virus is at the receptor level or whether it is dependent upon the host cell environment (host cell proteins). It was also essential to check whether PK-15 cells were capable of generating infectious viral particles. To analyse this, we decided to transfect PK-15 and S10-3 cells and evaluate the infectivity of the generated virus particles in PK-15 and HepG2/C3A cells. For that, S10-3 and PK-15 cells were transfected (T25 cm^2 flask for each construct) with transcripts generated from the parental genotype 1 and 4 clones and three chimeras, two replicating with significantly higher efficiency compared with pSK-HEV2: HEV-1 (HEV-4 JR-ORF2-3′NCR) and HEV-1 (HEV-4 ORF2-3′NCR) and the third chimera, HEV-1 (HEV-4 ORF1), which was successfully replicating in PK-15 cells. Cells were harvested on the 12th day post-transfection, and the cleared lysates were used for infecting both PK-15 and HepG2/C3A cells. Infected cells were monitored by both nsRNA assay as well as IFA for the virus replication.

HepG2/C3A cells infected with the lysates of S10-3 cells transfected with the parent construct pSK-HEV2 and the chimeric construct HEV-1 (HEV-4 JR-ORF2-3′NCR) were positive for both nsRNA and IFA. HEV-4FG and HEV-1 (HEV-4 ORF2-3′NCR) were positive only for nsRNA. Chimera HEV-1 (HEV-4 ORF1) was negative for both (Table 4).

None of the constructs showed IFA positivity in PK-15 cells. However, PK-15 cells infected with HEV-4FG were nsRNA positive indicating successful virus entry. However, PK-15 cells infected with virus particles generated with the chimera HEV-1 (HEV-4 ORF1) were negative in both IFA and nsRNA. HepG2/C3A and PK-15 cells infected with lysates prepared from transfected PK-15 cells also did not show any positivity for nsRNA and IFA.

With these results it was not possible to conclude whether particles generated from HEV-1 (HEV-4 ORF1) chimera were unable to enter into the PK-15 cells or whether very few virus particles were generated in S10-3/PK-15 cells and they were unable to establish successful infection in PK-15 cells.

DISCUSSION

Successful replication of a virus in its host is a complex process which involves multilevel interactions. Viruses and hosts evolve together and this co-evolution often leads to species specificity. The presence of specific attachment proteins/receptors and availability of a complex pool of cellular factors required for viral replication decide the permissiveness of a virus host. Though the structural proteins are involved in receptor binding (capsid/envelope) and are important in entering host cells, non-structural proteins involved in replication can also contribute significantly to deciding host/tissue specificity. Lack of an appropriate cell culture system or small animal model has been the major hurdle to studying the molecular mechanisms of HEV replication, tissue/species specificity, cell surface receptors and immune-pathogenesis of HEV. Genotype 3 and 4 HEV strains were shown to grow efficiently in PLC/PRF5 cells (Tanaka *et al.*, 2007, 2009); however, genotype 1 virus still lacks an efficient cell culture system. Infectious cDNA clones have been developed successfully for the genotypes 1, 3 and 4 of the mammalian strains of HEV (Panda *et al.*, 2000; Emerson *et al.*, 2001; Yamada *et al.*, 2009; Córdoba *et al.*, 2012) and used to shed some light on HEV replication. There are a number of studies that have addressed the issue of viral determinants and host specificity in HEV (Córdoba *et al.*, 2012; Feagins *et al.*, 2011; Graff *et al.*, 2005), but the question is still open. A recent report by Nguyen *et al.* (2014) suggested that the host restriction for the genotype 1 virus could be due to restricted entry into the host cells and efficiency of the virus to generate ORF2 protein in the given host. Lack of compatibility between cell surface receptors and the receptor binding region (rcp) (456–605 aa) in the capsid protein of the virus was suggested to be the deciding factor for successful infection. Nguyen *et al.* (2014) used an efficiently replicating genotype 3 virus replicon (P6) developed from the Kernow C-1 virus (Shukla *et al.*, 2012) for developing chimeric genotype 1/3 viral genomes. P6 has a 171 nt insertion (from human S17 ribosomal protein encoding gene) in the Hyper Variable Region (HVR) region. It was previously reported by this group that P6 virus isolated from a chronic HEV patient can cross the species barrier (Shukla *et al.*, 2011). Further, it was shown that the SAR55/S17 chimera can replicate in LLC-PK (pig) cells. Use of a P6 virus clone to develop the SAR55/P6 (genotype 1/3) chimeras in the first place was not a very suitable model to study species specificity since the virus had the ability to infect cells from a broad spectrum of

species, ranging from rodent to primate. Further, selective growth of the SAR55/P6-rcp chimera in HepG2/C3A cells but not in LLC-PK cells suggested a possible role for additional factor/s other than just the receptor binding region in the capsid protein in deciding host specificity.

We attempted to answer this question by constructing genotype 1/4 chimeras. In the first phase, our aim was to construct a replication competent cDNA clone of HEV genotype 4 and then construct chimeras by replacing parts of genotype 1 with corresponding parts of genotype 4 and check their species specificity using human and swine cell lines.

Construction of full genome infectious clones had many difficulties, the major one being single nucleotide mutations introduced during conventional cloning, which in turn led to reduced/total loss of replication efficiency. In addition to mutations in the ORF1 region, we found an additional eight mutations in the ORF2 encoding region of HEV-4 FG (synonymous: T5775C, T7152 and non-synonymous: A5267G, A5801G, T6355A, C6719T, T6899C, T6929C). We considered that as ORF1 is the non-structural region it would contribute more to replication competence compared with ORF2. So we repaired the mutations in ORF1 to its prototype. However, Emerson *et al.* (2013) have recently reported the presence of two highly conserved stem–loop structures (SL), ISL1 and ISL2, in the centre of the ORF2 encoding region that are essential for capsid protein synthesis. Silent mutations in this region were shown to have a negative effect on capsid protein synthesis. Though none of the ORF2 mutations observed by us coincide with the SL structures, we still believe that removal of mutations in the ORF2 encoding region would increase replication efficiency of the HEV-4 FG replicon construct.

HEV is a positive sense RNA virus and replication proceeds through a negative strand replicative intermediate. Positive strand sub-genomic RNA generated from these replicative intermediates are translated to generate viral capsid protein. Hence, detection of viral capsid protein and/or replicative intermediates would indicate successful replication. For the detection of replicative intermediate RNA, we employed a newly developed assay using tagged primer based PCR (Chatterjee *et al.*, 2012). Use of tagged primers prevented self-priming during cDNA synthesis. RNA virus genomes often contain functionally active RNA structures that are critical during various stages of viral replication. Secondary structures in such RNA elements play essential regulatory roles during translation, RNA replication and assembly of virions. These viral regulatory elements are also responsible for interacting with various cellular proteins at different stages of replication. *Cis*-acting 5′ and 3′ non-coding genomic regions are important signatures of RNA viral genomes which help viral RdRp to discriminate between cellular and viral mRNAs. RNA secondary structures are sensitive to point mutations which may cause large changes in the secondary structures. Graff *et al.* (2005) have shown that there is a significant decrease in HEV replication with a single nucleotide change at 7106 nt in the 3′NCR of pSK-HEV2, proving that sequences or structures in the 3′ terminal region are critical for HEV replication. Similarly, with specific binding of the ORF2 protein to the 5′end of the HEV genome, the importance of 5′NCR in encapsidation has also been demonstrated (Surjit *et al.*, 2004).

Considering these facts, we developed HEV-1/HEV-4 chimeras by replacing ORF1, ORF2/3 encoding regions and the *cis*-regulatory genomic elements, 5′NCR, 3′NCR and JR with HEV-4 sequences. Replication efficiencies of these chimeras in S10-3 cells, as seen with the IFA, were not similar. Replacement of ORF2, JR-ORF2, JR-3′NCR, 3′NCR [chimeras HEV-1 (HEV-4 ORF2), HEV-1 (HEV-4 JR-ORF2), HEV-1 (HEV-4 JR-3′NCR), HEV-1 (HEV-4 3′NCR)] did not change replication efficiencies of the clones. However, chimeras HEV-1 (HEV-4 JR-ORF2-3′NCR) and HEV-1 (HEV-4 ORF2-3′NCR) were replicating with higher efficiency than the parental clone pSK-HEV2. When compared with the HEV-1 (HEV-4 3′NCR) clone, there was a significant reduction in replication efficiency of the HEV-1 (HEV-4 5′NCR) clone, indicating a major role for 5′NCR during virus replication. It is likely that specific host proteins interact with the 3′end of the negative sense antigenome to help initiate synthesis of genomic positive sense molecules.

Improvement in the replication efficiency after inclusion of 3′NCR in the chimera HEV-1 (HEV-4 5′-3′ NCR) further complicated the analysis. We speculate that the HEV 5′- and 3′NCRs may either directly interact with each other or via viral/host-cell proteins during replication. Significantly higher replication efficiencies of the chimeras HEV-1 (HEV-4 JR-ORF2-3′NCR) and HEV-1 (HEV-4 ORF2-3′NCR) indicated a critical role for 3′NCR in regulating host-specific replication. However, since replication of the chimera HEV-1 (HEV-4 3′NCR) was significantly lower than the HEV-1 (HEV-4 JR-ORF2-3′NCR) and HEV-1 (HEV-4 ORF2-3′NCR) clones, we speculate that binding of the host proteins was probably more effective in the presence of respective ORF2 sequences from the same genotype (probably via RNA elements such as SL1 and SL2).

The role of the junction region cannot be completely neglected since chimera HEV-1 (HEV-4 JR-ORF2-3′NCR) replicated more efficiently than the HEV-1 (HEV-4 ORF2-3′NCR). The junction region has been shown to influence HEV replication (Cao *et al.*, 2010), but one more chimera HEV-1 (HEV-4 JR) would perhaps shed more light on this matter.

These results indicate that host proteins can bind to 3′NCR alone and/or require compatible 5′- and 3′NCR. Our results suggest compatibility of 5′NCR of the genotype 1 and 3′NCR of the genotype 4 viral genomes. Reverse interaction is probably not very successful, as the chimera HEV-1 (HEV-4 5′NCR) replicated with very low efficiency. Other chimeras, such as the HEV-1 (HEV-4 3′NCR-5′NCR) and HEV-1 (HEV-4 5′NCR-JR-ORF2-3′NCR), harbouring

5′NCR could replicate with better efficiency, perhaps due to the presence of compatible genotype 4 NC regions. The clone HEV-1 (HEV-4 5′NCR-ORF1) probably replicated with very low efficiency due to the presence of non-compatible 5′NCR.

HEV-4FG was positive for both nsRNA and IFA in transfected PK-15 cells. Similarly, HEV-1 (HEV-4 5′NCR-ORF1) and HEV-1 (HEV-4 ORF1) were also positive for both IFA and nsRNA in PK-15 cells upon transfection. These results compel us to conclude that ORF1 may be the region responsible for deciding the species barrier. ORF1 encodes non-structural proteins of the virus and viral non-structural proteins are known to interact with host cell proteins and modulate host cell environment (Ayllon *et al.*, 2015; Marascio *et al.*, 2014). When PK-15 and HepG2/C3A cells were infected with virions generated from the above three constructs, only the HEV-4FG construct showed positive results (only nsRNA positive), while other constructs remained completely negative. These results indicate that HEV-1 (HEV-4 5′NCR-ORF1) and HEV-1 (HEV-4 ORF1) constructs probably generated a very small number of virions which were not able to establish successful infection in new cells. We still believe that using a porcine liver cell line would shed better light.

Even though *in vitro* transcribed RNA was rendered negative for template DNA, quantitative PCR was not helpful. Present cell culture systems for genotype 1 HEV are not robust and the presence of a large amount of input RNA in cell transfections leads to high background of this RNA and its degradation products. Any marginal change due to newly synthesized RNA is not significant and hence we did not use real-time PCR to evaluate replication efficiencies of the replicons. Further, virus particles of genotype 1 HEV generated after RNA transfections remain cell-associated (Emerson *et al.*, 2006). So we resorted to IFA and statistics as our tool to compare replication efficiencies of the constructs. Focus forming assay (variant of IFA) has been successfully used for comparing the infectivity titres of hepatitis E virions recently (Nguyen *et al.*, 2014).

pSK-HEV2 is known not to infect swine cells (Nguyen *et al.*, 2014). Our observation was similar. It was surprising that HEV-1 (HEV-4 JR-ORF2-3′NCR) virus behaved similarly, as it had the capsid region of genotype 4 and was expected to infect PK-15 cells, if the restriction was at the capsid protein level (receptor binding). This chimeric virus also failed to establish replication when we directly transfected PK-15 cells with the capped genomes. However, at this juncture we cannot comment on the receptor-based barrier as none of our chimeras were able to infect PK-15 cells. We speculate that the barrier could be both at the receptor level and non-conducive cell milieu. It would be interesting to see how the HEV-4 FG and the chimeras HEV-1 (HEV-4 5′NCR-ORF1), HEV-1 (HEV-4 ORF1) behave in an efficient pig cell culture system. It would be worthwhile to see infectivity of these clones in pigs by direct intra-hepatic inoculations.

METHODS

Ethics statement. All experimental protocols were reviewed and approved by the Institutional Biosafety Committee (IBSC) of the National Institute of Virology (NIV), Pune, India. All experiments were carried out in strict accordance with good laboratory practices as defined by the IBSC in BSL2 laboratory.

Viral strains and cell lines. The following cell lines were purchased from American Type Culture Collection (Manassas, VA): HepG2, PLC/PRF/5 (Catalogue no. CRL-8024), HepG2/C3A (Catalogue no. CRL-10741), A549 (Catalogue no. CCL-185), Caco2 (Catalogue no. HTB-37), PK-15 (Catalogue no. CCL-33), ESK-4 (Catalogue no. CL-184) and ST cells (Catalogue no. CRL-1746). Huh7 cells were purchased from the Japanese Collection of Research Bioresources Cell Bank (Japan). S10-3 cells (clone of Huh7 cells) and full genome infectious cDNA clone pSK-HEV2 were from NIH (a gift from Suzanne U. Emerson, NIAID, NIH, Bethesda, MD, USA). The majority of the cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) containing 10 % FBS (Invitrogen), 100 U ml^{-1} penicillin and 100 µg ml^{-1} streptomycin. Cells were grown at 37 °C in the presence of 5 % CO_2. Transfected/infected cells were incubated at 34.5 °C. Stool suspension containing Indian swine HEV (GenBank accession no. AY723745) was used as the source of genotype 4 virus.

Construction of HEV genotype 4 full genome clone. Total RNA was isolated from 10 % stool suspension of a pig stool sample containing genotype 4 virus. Five overlapping fragments encompassing the entire viral genome were amplified by PCR using five different sets of PCR primers (Table S1, available in the online Supplementary Materials) and TA-cloned into pGEMT-EASY vector (Promega, USA). Clone numbers 1, 2, 3, 4 and 5 contained fragments representing 1–387, 102–2177, 1848–4694, 3924–6317 and 5728–7214 nt in the genotype 4 genome, respectively. Fragment 1 (1–387) was initially amplified from source using 5′RACE (Ambion, USA). It was later re-amplified with forward primer T7F containing a T7 promoter sequence at the extreme 5′ end of the viral genome. For the assembly of the full genome clone, TA clones of the individual fragments were assembled stepwise using unique restriction sites in the genome. Briefly, clone 1 and 2 were assembled using PstI and SmaI sites to generate clone 6. Clone 3 and clone 6 were assembled using KpnI and NotI sites to generate clone 7. Clone 4 and clone 5 were processed with KpnI and PstI and ligated to generate clone 8. Clone 7 and 8 were linearized with PstI, treated with shrimp alkaline phosphatase and ligated to generate the full genome clone, HEV-4FG. The clone was confirmed by sequencing. The sequence was compared with the original sequence which was directly generated from PCR amplicons and mutations were corrected by carrying out multiple, stepwise site-directed mutagenesis reactions.

Construction of replication-deficient full genome clones. RNA-dependent RNA polymerase (RdRp) mutants (GDD to GAA) were constructed for the parental full genome constructs pSK-HEV2 and HEV-4FG. Mutations in this conserved motif have been shown to abolish HEV replication (Emerson *et al.*, 2004) and thus were used as negative controls.

Construction of chimeras. For the development of genotype 1/4 chimeric constructs, genotype 1 complete cDNA clone pSK-HEV2 was used as the backbone. Genotype 4 HEV full-genome clone was used as the template for amplifying different genomic regions which were replaced from the pSK-HEV2 (Fig. 1). Multiple strategies such as site-directed mutagenesis, fusion PCR and unique restriction sites were used to facilitate the replacement of designated genes of genotype 1 (HEV-1) virus with corresponding genes from the genotype 4 virus (HEV-4). To construct Chimera 1: HEV-1 (HEV-4 3′NCR) 3′NCR of genotype 1 genome was replaced with the corresponding region of HEV-4. Chimera 4: HEV-1 (HEV-4 5′NCR) was constructed by site-directed mutagenesis

of the original pSK-HEV2 (HEV-1) clone. Chimera 5: HEV-1 (HEV-4 5′NCR-3′NCR) was developed by site-directed mutagenesis of Chimera 1. Junction region (JR), ORF3 and ORF2 with or without 3′NCR were replaced in the place of corresponding regions of HEV-1 clone to produce Chimera 2: HEV-1 (HEV-4 JR-ORF2) and Chimera 3: HEV-1 (HEV-4 JR-ORF2-3′NCR). Site-directed mutagenesis was performed on the Chimeras 2 and 3 to generate Chimera 7: HEV-1 (HEV-4 5′NCR-JR-ORF2) and Chimera 9: HEV-1 (HEV-4 5′NCR-JR-ORF2-3′NCR), respectively. To generate Chimera 6: HEV-1 (HEV-4 ORF2) and Chimera 8: HEV-1 (HEV-4 ORF2-3′NCR), the junction region of chimera 2 and 3 constructs was changed back to HEV-1. ORF1 along with adjacent 5′NCR was replaced with the corresponding region of HEV-4 to construct Chimera 11: HEV-1 (HEV-4 5′NCR-ORF1). Chimera 10: HEV-1 (HEV-4 JR-3′NCR) and Chimera 12: HEV-1 (HEV-4 ORF1) were constructed by carrying out site-directed mutagenesis of the Chimera 1 and Chimera 11 constructs, respectively. All generated constructs were confirmed by sequencing before proceeding with the further experiments.

Generation of full length RNA. The full genome chimeric virus encoding plasmids were linearized utilizing restriction enzyme sites *Bgl*II (pSK-HEV2 and Chimeras) or *Bam*HI (HEV-4FG), present at the 3′ end of the respective full genome clones. The linearized DNA was used as the template to generate full genome long transcripts using T7 promoter incorporated at the 5′ end of the HEV genome with *in vitro* transcription kit (mMESSAGE m Machine T7 ultra kit, Ambion, USA). Briefly, 11 µl 2× NTP/ARCA mix, 2.0 µl 10× buffer, 2.0 µl enzyme mix were mixed with 5.0 µl template DNA (2.0 µg) and incubated at 37 °C for 2 h. Plasmid DNA was removed by adding 5 µl (1 unit/µl) of RQ1 RNase-free DNase I (Promega, USA) and incubated at 37 °C for 30 min. RNA was precipitated using 7.5 M LiCl and the pellet was washed with 70 % ethanol, air dried and dissolved. A second round of DNase treatment was given with Turbo DNase (2 units/µl) (Ambion, USA) at 37 °C for 1 h for complete removal of the DNA template wherever required. RNA was purified by phenol chloroform extraction and dissolved in RNase-free water. The concentration of RNA was determined spectrophotometrically using Nanodrop (ND-1000) at 260 nm; RNA integrity was checked by denaturing agarose gel electrophoresis. RNA was aliquoted and stored at −80 °C until required.

Degradation of template DNA. Reports suggest incomplete degradation of template DNA in the presence of excess of product RNA in transcription mixtures. To confirm this possibility, equal amounts of RNA (2 µg) used for transfection was processed for nested PCR (using primers for negative strand detection) without using reverse transcriptase. Suitable positive controls were also processed simultaneously. Only after confirming complete removal of template DNA (negative for PCR), was RNA used for cell transfection.

Transfection of cell lines with *in vitro* transcribed RNA. Cells (0.75×10^5/well) were plated in a 24-well plate (Corning, USA) 24 h prior to transfection and incubated at 37 °C. Cells were washed three times with Opti-MEM (Invitrogen, Life Technologies, USA), 2 µg of *in vitro* transcribed RNA was mixed with 2 µl of 1,2-dimyristyl Rosenthal inhibitor ether (DMRIE-C) transfection reagent (Invitrogen, USA) in 200 µl of Opti-MEM and layered on cells. Cells were incubated at 34.5 °C for 5 h. Transfection mixture was replaced with complete medium without antibiotics and further incubation was carried out at 34.5 °C. All transfections were carried out in triplicate.

Immunofluorescence assay (IFA). To check replication competence of constructs, transfected cells were checked for the presence of capsid protein by IFA. Assays were performed 12 days post-transfection. For that, transfected cells were trypsinized on day 11 and plated in each well of an eight-well chambered glass slide (Nunc, Germany). After incubation for 24 h, medium was removed, the cell layer was washed once in 1× PBS and fixed in acetone at room temperature for 2 min. Cells were hydrated in 1× PBS followed by layering with 200 µl of the blocking buffer (Super block) (Pierce, USA) and incubation at room temperature for 30 min in a humid chamber. Polyclonal primary antibody (anti-HEV IgG antibody positive: human serum) was diluted (1:100) in blocking buffer (0.5 % BSA, 0.5 % milk powder and 0.1 % Triton X-100 in PBS), added and incubation was carried out at room temperature for 30 min. The human serum (primary antibody) was selected after screening in ELISAs which used recombinant ORF2 and ORF3 proteins, respectively, as coating antigens. The serum was positive only for anti-ORF2 and negative for anti-ORF3 antibodies. After incubation with the primary antibody, cells were washed and the secondary antibody, anti-human IgG tagged with Alexa Fluor 488 in blocking buffer [1 : 2000 dilution (stock: 2 mg ml^{-1})], was overlaid on the cells. After incubating at room temperature for 30 min, and after washing cells, a coverslip was laid with 10 µl of mounting fluid (Chemicon) and analysed under inverted fluorescence microscope (Axioscope, Carl Zeiss, Imager A.2, Germany) at 200× magnification. Parameters were set to differentiate between positive and negative IFA signals. For each slide 15–20 fields were randomly selected and images were taken. Image acquisition was carried out using 'ProgRes capture Pro 2.8'.

Quantitative PCR. Total cellular RNA was extracted from transfected and infected cells with Ribopure RNA extraction kit (Ambion, Life technologies, USA) as per the manufacturer's instructions. RNA was eluted in nuclease-free water. Quantitation of RNA was done using Nanodrop at 260 nm. RNA was aliquoted and stored at −80 °C until required. HEV RNA copies in the samples were determined by Taqman Real-time PCR assay using 7300 Real Time PCR system (Applied Biosystems, CA, USA) as described previously (Arankalle *et al.*, 2009). Separate sets of primers, probes specific for pSK-HEV2 and HEV-4, were used for different constructs as required (Table S2 and Table S3).

Detection of replicative intermediates (negative sense RNA) in the transfected/infected cells. Total cellular RNA was extracted from cells using Ribopure RNA extraction kit (Ambion) and detection of negative sense RNA (nsRNA) (replicative intermediate) was done as described previously (Chatterjee *et al.*, 2012) using tagged primer-based reverse-transcription PCR. A separate set of primers, specific for pSK-HEV2 and HEV-4, were used (Table S4). Before proceeding with the nsRNA detection, all RNA samples were processed for positive sense HEV RNA quantitation. Samples were diluted to reduce input positive sense RNA copies to $\leq10^5$ copies/reaction to avoid false positivity.

Preparation of cell lysates and *in vitro* infectivity assays. Confluent monolayers of cells transfected with RNA (25 cm^2 flask) were harvested 12 days post-transfection by trypsinization, centrifuged for 2 min at 2000 rpm. Supernatant was aspirated, and the cell pellet was stored at −80 °C. For preparing cell lysate, frozen cell pellets were extracted at room temperature by adding 0.45 ml of water and vortexing vigorously until the pellet dispersed and the solution became cloudy. The sample was vortexed once or twice more in the next 10 min, 0.05 ml of 10× PBS was added, and debris was removed by centrifugation at 16 000 ***g*** for 2 min. HepG2/C3A and PK-15 cells were plated (0.75×10^6 per well) on an eight-well glass chamber slide (Nunc, Denmark) and 24-well plate, 24 h prior to infection. Next day, cells were washed three times with incomplete DMEM and then incubated with 100 µl of cell lysate at 34.5 °C in a 5 % CO_2 atmosphere for 5 h. Liquid was aspirated and replaced with 0.4 ml of growth medium containing antibiotics. Cells from 24-well plate were harvested on the 6th day for RNA negative strand detection. Chamber slides were processed for IFA on the 7th day. Cells were infected keeping the cell-to-cell ratio the same (cell lysate generated from a well of a 24-well plate was used for the infection of cells seeded in a single well of a 24-well plate).

Statistical analysis. The Jarque-Bera test was used to check normality of data (number of HEV-positive cells per 100 screened cells). The homogeneity of comparison groups was tested using Leven's test of homogeneity of variance. Comparison of 'percent HEV positivity' of 11 constructs in cells was carried out using the Kruskal–Wallis test. Pairwise comparison of percentage HEV-positive cells was carried out using the Mann–Whitney U test, with the Bonferroni adjustment to the probabilities (as 55 comparisons were made, we used 0.05/55 = 0.00054 as our cut-off value).

REFERENCES

Arankalle, V. A., Chobe, L. P. & Chadha, M. S. (2006). Type-IV Indian swine HEV infects rhesus monkeys. *J Viral Hepat* **13**, 742–745.

Arankalle, V. A., Lole, K. S., Deshmukh, T. M., Chobe, L. P. & Gandhe, S. S. (2007). Evaluation of human (genotype 1) and swine (genotype 4)-ORF2-based ELISAs for anti-HEV IgM and IgG detection in an endemic country and search for type 4 human HEV infections. *J Viral Hepat* **14**, 435–445.

Arankalle, V. A., Lole, K. S., Deshmukh, T. M., Srivastava, S. & Shaligram, U. S. (2009). Challenge studies in Rhesus monkeys immunized with candidate hepatitis E vaccines: DNA, DNA-prime-protein-boost and DNA-protein encapsulated in liposomes. *Vaccine* **27**, 1032–1039.

Ayllon, J. & García-Sastre, A. (2015). The NS1 protein: a multitasking virulence factor. *Curr Top Microbiol Immunol* **386**, 73–107.

Cao, D., Huang, Y. W. & Meng, X. J. (2010). The nucleotides on the stem–loop RNA structure in the junction region of the hepatitis E virus genome are critical for virus replication. *J Virol* **84**, 13040–13044.

Chatterjee, S. N., Devhare, P. B. & Lole, K. S. (2012). Detection of negative-sense RNA in packaged hepatitis E virions by use of an improved strand-specific reverse transcription-PCR method. *J Clin Microbiol* **50**, 1467–1470.

Córdoba, L., Feagins, A. R., Opriessnig, T., Cossaboom, C. M., Dryman, B. A., Huang, Y. W. & Meng, X. J. (2012). Rescue of a genotype 4 human hepatitis E virus from cloned cDNA and characterization of intergenotypic chimeric viruses in cultured human liver cells and in pigs. *J Gen Virol* **93**, 2183–2194.

Devhare, P. B., Chatterjee, S. N., Arankalle, V. A. & Lole, K. S. (2013). Analysis of antiviral response in human epithelial cells infected with hepatitis E virus. *PLoS One* **8**, e63793.

Emerson, S. U., Zhang, M., Meng, X.-J., Nguyen, H., St. Claire, M., Govindarajan, S., Huang, Y. K. & Purcell, R. H, Meng, X. J. (2001). Recombinant hepatitis E virus genomes infectious for primates: importance of capping and discovery of a *cis*-reactive element. *Proc Natl Acad Sci U S A* **98**, 15270–15275.

Emerson, S. U., Nguyen, H., Graff, J., Stephany, D. A., Brockington, A. & Purcell, R. H. (2004). In vitro replication of hepatitis E virus (HEV) genomes and of an HEV replicon expressing green fluorescent protein. *J Virol* **78**, 4838–4846.

Emerson, S. U., Nguyen, H., Torian, U. & Purcell, R. H. (2006). ORF3 protein of hepatitis E virus is not required for replication, virion assembly, or infection of hepatoma cells in vitro. *J Virol* **80**, 10457–10464.

Emerson, S. U., Nguyen, H. T., Torian, U., Burke, D., Engle, R. & Purcell, R. H. (2010). Release of genotype 1 hepatitis E virus from cultured hepatoma and polarized intestinal cells depends on open reading frame 3 protein and requires an intact PXXP motif. *J Virol* **84**, 9059–9069.

Emerson, S. U., Nguyen, H. T., Torian, U., Mather, K. & Firth, A. E. (2013). An essential RNA element resides in a central region of hepatitis E virus ORF2. *J Gen Virol* **94**, 1468–1476.

Feagins, A. R., Opriessnig, T., Huang, Y. W., Halbur, P. G. & Meng, X. J. (2008). Cross-species infection of specific-pathogen-free pigs by a genotype 4 strain of human hepatitis E virus. *J Med Virol* **80**, 1379–1380.

Feagins, A. R., Córdoba, L., Sanford, B. J., Dryman, B. A., Huang, Y. W., LeRoith, T., Emerson, S. U. & Meng, X. J. (2011). Intergenotypic chimeric hepatitis E viruses (HEVs) with the genotype 4 human HEV capsid gene in the backbone of genotype 3 swine HEV are infectious in pigs. *Virus Res* **156**, 141–146.

Graff, J., Nguyen, H., Kasorndorkbua, C., Halbur, P. G., St Claire, M., Purcell, R. H. & Emerson, S. U. (2005). In vitro and in vivo mutational analysis of the 3′-terminal regions of hepatitis E virus genomes and replicons. *J Virol* **79**, 1017–1026.

Graff, J., Torian, U., Nguyen, H. & Emerson, S. U. (2006). A bicistronic subgenomic mRNA encodes both the ORF2 and ORF3 proteins of hepatitis E virus. *J Virol* **80**, 5919–5926.

Halbur, P. G., Kasorndorkbua, C., Gilbert, C., Guenette, D., Potters, M. B., Purcell, R. H., Emerson, S. U., Toth, T. E. & Meng, X. J. (2001). Comparative pathogenesis of infection of pigs with hepatitis E viruses recovered from a pig and a human. *J Clin Microbiol* **39**, 918–923.

Marascio, N., Torti, C., Liberto, M. & Focà, A. (2014). Update on different aspects of HCV variability: focus on NS5B polymerase. *BMC Infect Dis* **14**, S1.

Meng, X.-J. (2011). From barnyard to food table: the omnipresence of hepatitis E virus and risk for zoonotic infection and food safety. *Virus Res* **161**, 23–30.

Meng, X.-J., Halbur, P. G., Haynes, J. S., Tsareva, T. S., Bruna, J. D., Royer, R. L., Purcell, R. H. & Emerson, S. U. (1998a). Experimental infection of pigs with the newly identified swine hepatitis E virus (swine HEV), but not with human strains of HEV. *Arch Virol* **143**, 1405–1415.

Meng, X. J., Halbur, P. G., Shapiro, M. S., Govindarajan, S., Bruna, J. D., Mushahwar, I. K., Purcell, R. H. & Emerson, S. U. (1998b). Genetic and experimental evidence for cross species infection by swine hepatitis E virus. *J Virol* **72**, 9714–9721.

Nguyen, H. T., Shukla, P., Torian, U., Faulk, K. & Emerson, S. U. (2014). Hepatitis E virus genotype 1 infection of swine kidney cells in vitro is inhibited at multiple levels. *J Virol* **88**, 868–877.

Panda, S. K., Ansari, I. H., Durgapal, H., Agrawal, S. & Jameel, S. (2000). The in vitro-synthesized RNA from a cDNA clone of hepatitis E virus is infectious. *J Virol* **74**, 2430–2437.

Pavio, N., Meng, X. J. & Renou, C. (2010). Zoonotic hepatitis E: animal reservoirs and emerging risks. *Vet Res* **41**, 46.

Shukla, P., Nguyen, H. T., Faulk, K., Mather, K., Torian, U., Engle, R. E. & Emerson, S. U. (2012). Adaptation of a genotype 3 hepatitis E virus to efficient growth in cell culture depends on an inserted human gene segment acquired by recombination. *J Virol* **86**, 5697–5707.

Shukla, P., Nguyen, H. T., Torian, U., Engle, R. E., Faulk, K., Dalton, H. R., Bendall, R. P., Keane, F. E., Purcell, R. H. & other authors (2011). Cross-species infections of cultured cells by hepatitis E virus and discovery of an infectious virus-host recombinant. *Proc Natl Acad Sci U S A* **108**, 2438–2443.

Smith, D. B., Simmonds, P., International Committee on Taxonomy of Viruses Hepeviridae Study Group., Jameel, S., Emerson, S. U., Harrison, T. J., Meng, X. J., Okamoto, H., Van der Poel, W. H. & Purdy, M. A (2014). Consensus proposals for classification of the family Hepeviridae. *J Gen Virol* **95**, 2223–2232.

Surjit, M., Jameel, S. & Lal, S. K. (2004). The ORF2 protein of hepatitis E virus binds the 5′ region of viral RNA. *J Virol* **78**, 320–328.

Tam, A. W., Smith, M. M., Guerra, M. E., Huang, C. C., Bradley, D. W., Fry, K. E. & Reyes, G. R. (1991). Hepatitis E virus (HEV): molecular cloning and sequencing of the full-length viral genome. *Virology* **185**, 120–131.

Tanaka, T., Takahashi, M., Kusano, E. & Okamoto, H. (2007). Development and evaluation of an efficient cell-culture system for hepatitis E virus. *J Gen Virol* **88**, 903–911.

Tanaka, T., Takahashi, M., Takahashi, H., Ichiyama, K., Hoshino, Y., Nagashima, S., Mizuo, H. & Okamoto, H. (2009). Development and characterization of a genotype 4 hepatitis E virus cell culture system using a HE-JF5/15F strain recovered from a fulminant hepatitis patient. *J Clin Microbiol* **47**, 1906–1910.

Yamada, K., Takahashi, M., Hoshino, Y., Takahashi, H., Ichiyama, K., Tanaka, T. & Okamoto, H. (2009). Construction of an infectious cDNA clone of hepatitis E virus strain JE03-1760F that can propagate efficiently in cultured cells. *J Gen Virol* **90**, 457–462.