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Characterisation of a recent virulent transmissible gastroenteritis virus from Britain with a deleted ORF 3a

Brief Report

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Summary. Analyses of transmissible gastroenteritis virus (TGEV) and porcine respiratory coronavirus (PRCV) isolates have suggested that tropism and pathogenicity are influenced by the spike protein and ORF 3. In general, enteric viruses (TGEV) have been shown to contain intact spike and ORF 3 genes, whilst respiratory isolates (PRCV) have major deletions within both regions. Virulence has been correlated to a functional ORF 3. Here, sequence analysis of a recent isolate of virulent TGEV, revealed a variant with an intact spike gene, but a large deletion in ORF 3a. This suggests that ORF 3a is not essential for enteric virulence.

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Transmissible gastroenteritis (TGE) has been reported in many parts of the world, including America, Asia and Europe. The disease is spread by the faeco-oral route and is characterised by vomiting, diarrhoea and high mortality in piglets. The causative agent is a coronavirus (TGEV), which is a member of the *Coronaviridae* family and belongs to the order Nidovirales. The virion is enveloped, encapsidating a relatively large single stranded RNA genome of positive polarity. During infection a number of subgenomic mRNAs are generated that encode for both the structural and non-structural polypeptides. The 3 major structural polypeptides are: a 200 kDa glycosylated surface spike protein (S); a 30 kDa glycosylated integral membrane protein (M); and a phosphorylated nucleoprotein (N) of approximately 45 kDa. These are encoded by ORFs 2, 5 and 6 respectively. In addition, an envelope protein (E), previously designated as the small membrane

*The nucleotide sequence of TGEV 96-1933 has been submitted to Genbank under accession number AF104420

(sM) protein, is encoded by ORF 4. The function of the ORF 3 products (3a and 3b) is not known, although a correlation between the presence of deletions and viral pathogenicity has been noted [6, 21].

Since the mid 1980's, an aerogenically spread, respiratory form of TGEV, known as porcine respiratory coronavirus (PRCV), has become very common in Europe and more recently in America and Asia [11, 22, 29, 27]. Under experimental conditions it causes sub-clinical to mild disease but unlike TGEV that is enteric, PRCV appears to replicate predominantly in the cells of the respiratory tract. At the nucleotide level the two viruses are very similar and consequently PRCV has been considered to be a variant of TGEV. There are 2 main differences between PRCV and TGEV genotypes. Firstly, PRCVs have a deletion within the amino-terminal region of the spike protein. Secondly, most PRCVs have deletions of varying sizes, within ORF 3a and/or 3b which render these mRNAs either undetectable or truncated. Comparisons between TGEV and PRCV isolates have thus concluded that both tropism and virulence could be attributed to deletions within the spike and ORF 3 genes [5, 17, 21, 28, 30, 31]. However, recent studies have also shown that the enteric tropism of TGEV can be lost without changes downstream of the spike gene [1].

In this study we report the presence of a novel TGEV variant in Britain and show for the first time a virulent TGEV lacking an intact 3a gene.

In 1996, a severe outbreak of TGE was confirmed in England [14]. Losses on the affected farm included 750 piglets and 5 sows. The virus (isolate no. 96–1933) grew very poorly in cell cultures [20]. Faecal and intestinal material from the affected herd was fed to four neonatal piglets resulting in severe TGE progressing to prostration within 24 h, and a high level of virus excretion [20]. Preliminary characterization based on sequencing 399 nucleotides of the 5′ end of the spike, revealed no nucleotide deletions, but that the virus was phylogenetically distinct from other TGEV isolates [19].

The 3′ third of the virus genome (approximately 8000 nucleotides) was amplified by RT-PCR in five segments, each of 1 628 – 1 936 nucleotides. First strand cDNA synthesis and PCR was carried out as described [20], using RNA extracted from the faeces of one of the above four experimentally infected piglets, and new PCR primers. The PCR primers are shown in Table 1a, and were designed from an alignment of Genbank sequences of TGEV (strains FS772/70, Purdue, and TF1) and canine coronavirus (strain INSAVC-1). Amplified fragments were separated in 1% agarose gels containing 0.2 μg/ml ethidium bromide prior to visualisation by UV transillumination. Each specific PCR was performed in triplicate, and the amplicons were pooled and then cleaned using Wizard PCR prep columns (Promega). PCR primers and additional internal primers (Table 1b) were used to sequence the appropriate amplicons in both directions using a FS dye primer kit (Perkin-Elmer). Sequencing reactions were visualised on an ABI 373 automated sequencer (Perkin-Elmer/ABI) through the use of a commercial service (University of Durham, UK). Each fragment was sequenced in its entirety in both directions. The raw data obtained was subsequently edited and aligned to published sequences using GCG and DNAStar (DNAStar Inc.) computer packages.

Table 1a. Oligonucleotides used for the PCR amplification of TGE strain 96-1033

Primer	Sequence	Position[a]	Product size
F153	GAA ATG GTC ATT GGA TTA CTA ARG A	153–177	1935 bp
R2088	CAA ATG AGA AAG GAC AAG TAC CAG	2087–2064	
F1959	ACC AAT TTT CAG TTT ATG TTC ATT C	1959–1983	1705 bp
R3663	TCA AGC CTA TTR TAA ATG TCA CTA A	3663–3639	
F3527	ACT GTT GCT AAA GCA TTG GYA AA	3527–3549	1845 bp
R5371	GGT GCA GCT CTG CCA TGT A	5370–5352	
F5143	TAC AAA CTT TAA GAC GTG TGT CGG	5143–5166	1686 bp
R6828	GGG GTT GAA GAA TGA AAG AGG TAT	6828–6805	
F6694	ATG GTA TAA CTA AAC TTC TAA ATG GCC	6694–6720	1628 bp
R8321	CCA GAC GTT AGC TCT TCC ATT G	8321–8300	

Table 1b. Sequence of oligonucleotide primers used for sequencing

Primer	Sequence	Position[a]
FS1174	CGC AAT AAT AGT AAT GAC CTT TAT	451–475
FS582	CTC CAT CAC AGT TAC AAC AA	580–599
FS988	GTT TCC AAT TGC ACT GAT CA	986–1005
FS1741	AAT GCT CTC AAC TTA CTG CTA	1719–1739
FS2611	AAA TGA TAT GAC TCG TGG CA	2590–2609
FS3185	TTA GGT ACA GTT GAC GAA GA	3164–3183
FS4075	TAT GTA TCA GCC TAG AGT GG	4054–4073
F5000	TGA TTG GTG GAC TTT TTC	4938–4955
F5570	CAG TTG AAC TTC TCA ATG GTG	5526–5546
FS5684	TTC CTA GGG CAT TGA CTG TC	5665–5684
F6200	TTC AGC TGG TTC GTG TAT GGC	6139–6159
FS6266	GTC CAG GTA TGT AAT GTT CG	6240–6259
FS7274	ACA GTC CAA TAA CAA GAA GG	7254–7273
F7750	GAA CAA TTC CTT CAG CAG ATT	7572–7592
FS7848	TGA TGA CAC ACA GGT TGA GA	7821–7840
RS512	TCC CAA TAC AAT GCT TTA AG	510–491
R950	TCA AAC CAC CAA AGG TCT ACA A	930–909
RS1444	AGT ACC GTG GTC CAT CAG TT	1442–1423
RS2449	TGC GCT TAC ATC ACA TGG AG	2467–2448
R2530	TTT AGA CCT AAC AAT TCA CTG	2538–2518
RS2950	CAG AAA CAA ATA ACA TGG AAT C	2969–2948
R3080	AAG TAT GTA TTT TAG ACC TTC	3088–3068
RS4110	CAT TAA CAA ACA GCA CAT CG	4127–4108
R4250	ATA GGT TGC ATT AAA AAT GTC	4261–4241
RS5083	TGC TGA ACT CTG GGT AAT AG	5102–5083
RS5714	TCA ACA GGA ACC AGA AAA TG	5733–5719
R5900	ATT CTT ATA GGC ATC GTA AGC	5855–5835
RS7071	GTT GGT TTG TTC ATG GCA C	7088–7070
R7350	AGC TTG CTC CAC ACT GTC ATC	7293–7273
RS7625	TTT GTG TGT GAA CGT GAC TT	7641–7622

[a] All of the above positions relate to the published TF1 sequence [6]

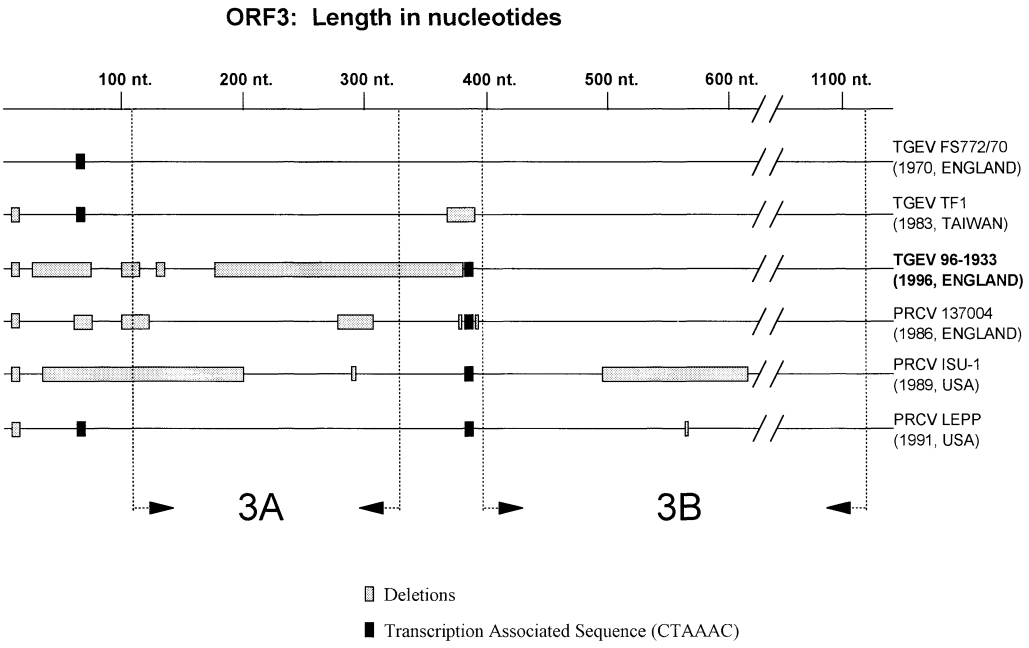

Fig. 1. Deletions observed in ORF 3 of 96-1933 compared to other TGEV/PRCV

The maximum likelihood method was used to prepare dendrograms of phylogenetic relatedness for each of the different genes that had been sequenced, using the DNAML program from the PHYLIP package [12].

Sequence comparisons indicated that all of the structural genes are TGEV-like. The spike gene (ORF 2, 4350 nucleotides) contains no insertions or deletions compared to that of the 1970 British TGEV isolate FS772/70 [4]. The spike genes of the two viruses show a nucleotide identity of 95.7% corresponding to 185 nt changes and an amino acid identity of 94.5% corresponding to 80 amino acid changes. Just downstream of the spike gene is a series of large deletions that obliterate the transcription signal and start codon of ORF 3a as well as 75% of ORF 3a itself (Fig. 1). The remaining ORFs from 3b onwards appear to be unaffected by deletions or insertions, other than a 3 nucleotide insertion in the E gene (ORF 4).

The appearance of the respiratory variant (PRCV) from 1984 onwards, coincided with the virtual disappearance of TGE in Europe. It has been suggested that the widespread occurrence of PRCV provides a measure of cross-protection against TGE. Sequence analysis of TGEVs and PRCVs have shown that the two variants are highly homologous except for two relatively small deleted regions within the PRCV genome. The first involves a 621–681 nt deletion within the amino-terminal spike gene. The spike gene has been shown to be the receptor binding component of the viral envelope [9, 13] binding to the aminopeptidase N (APN) which is a membrane bound metalloprotease [2, 10]. In addition, the spike gene also has sialic acid acid binding (HA) activity [16, 18] that is lacking

in PRCV [25]. This deleted region encompasses amino acids that encode for 2 of the 4 antigenic sites [8] and amino acids that are involved with HA activity [16] and enteropathogenicity [1, 3, 16]. The second difference between TGEVs and PRCVs, involves a number of deletions within ORF 3. In the virulent PRCV strains AR310 and Lepp, ORF 3 is intact except for one nucleotide deletion within 3b, whereas low virulent PRCVs have significantly altered ORF 3s [21]. Similarly for TGEV, a small plaque variant and two cell culture adapted strains that are non-pathogenic, have deletions or insertions that alter ORF 3. Consequently ORF 3 has been postulated as an important determinant for virulence [5–7, 17, 21, 28, 31].

The virulence of TGEV 96-1933 was demonstrated by the severity of the outbreak from which it originated and the experimentally induced disease in neonatal pigs. Genetic characterization confirmed, as with other TGEVs, that there was no deletion within the spike protein, consistent with the serological findings from the farm. However, a somewhat surprising result was the degree of variation at both the nucleotide and amino acid levels between the spike gene of 96-1933 and other TGEVs and PRCVs. Nearly half of the nucleotide changes resulted in amino acid substitutions compared to the earlier UK isolate FS772/70. Recombination is a major driving force in coronavirus evolution so we compared the 3′ 3 579 nucleotides of the spike protein from isolate 96-1933 to equivalent sequence from TGEVs, PRCVs and other coronaviruses (canine, feline, and human). Phylogenetic comparison using the 3′ 3 579 nucleotides of the spike gene, immediately downstream of the deleted region found in PRCVs demonstrated that the closest variant to 96-1933 is the virulent TGEV TF1 (Fig. 2), although it is still evolutionarily distinct. From Fig. 2 it can be seen that recombination has not occurred between this part of 96-1933 and other related coronaviruses from dogs, cats and humans. Although, sequence data on other structural genes is not available for so many viruses, additional phylogenetic comparisons using ORFs 3b, 4, 5 and 6 were also consistent with this finding (data not shown). Consequently, the origin of the virus is uncertain and requires further investigation.

The size of the deletions affecting ORF 3a is greater than has been reported in other TGEVs or even PRCVs (Fig. 1). Although deletions in this gene have been suspected to play an important role in viral attenuation [6, 21], this virus is fully virulent. Therefore, it appears that this ORF is not essential for enteric virulence and that virulence may be determined by more subtle genetic variations. This has recently been shown in part by Ballesteros et al. [1], where 2 amino acids were shown to be important in the loss of enteric tropism without changes downstream of the spike gene. A possibility remains that the virulence of the 1996 outbreak was due to a standard TGEV that co-circulated with the 96-1933 strain lacking ORF3a. This could not be formally excluded by analysis of plaque purified virus, due to the difficulties in growing the virus in vitro. However, only a single PCR band was observed for the amplicon encompassing ORF3 (data not shown). It has been shown that TGEV is difficult to grow in cell culture [26] especially with strains lacking ORF 3a [21, 30]. This may account for the difficulty of successfully adapting the 96-1933 isolate to growth in cell culture [20].

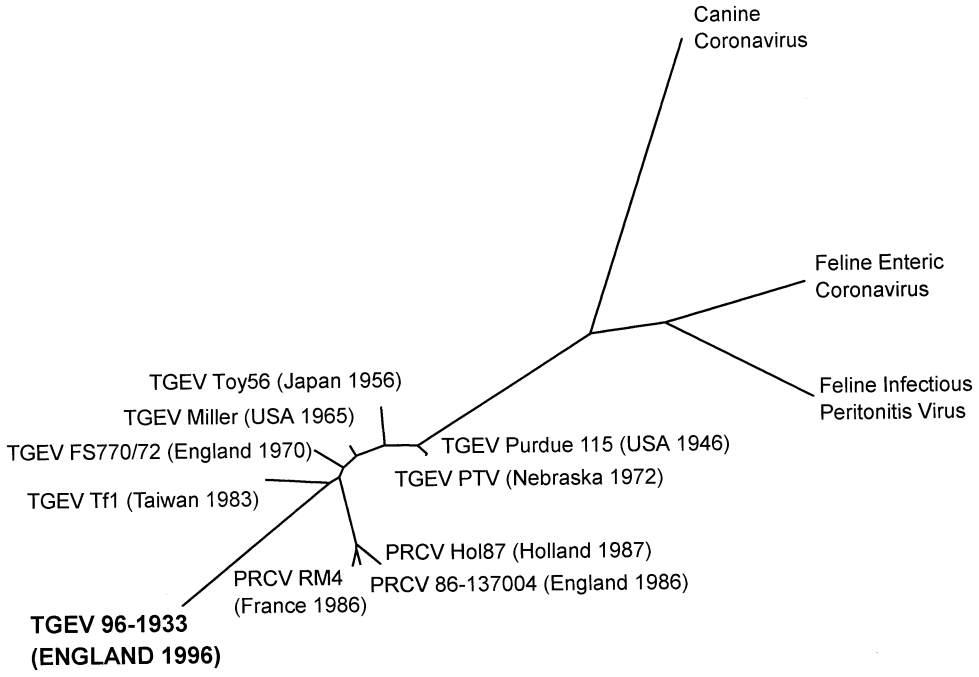

Fig. 2. Dendogram to show similarity of the 3′ end of the spike gene (3579 nt) for 96-1933 and other coronaviruses. Where applicable the country of origin and the year of isolation are given in parenthesis. GenBank Accession numbers: TGEV 96-1933 (AF104420), TGEV Toy56 (M94103), TGEV Miller (S51223), TGEV FS770/72 (X53128), TGEV Tf1 (Z35758), TGEV PTV (M94099), TGEV Purdue (D00118), PRCV RM4 (Z24675), PRCV 86-137004 (X60089), PRCV Hol87 (Z24675), Canine Coronavirus (X77047), Feline Enteric Coronavirus (X80799), and Feline Infectious Peritonitis Virus (X06170)

The remaining ORFs from 3b onwards appear to be unaffected by deletions or insertions, other than a 3 nucleotide insertion in the E gene (ORF 4). This insertion is also found in PRCV isolates RM4 and 86-137004, in the non-pathogenic Purdue strain of TGEV, but not in the virulent TGEV isolates TF1 and FS772/70. The possible significance of this could be addressed by examining this region in other field isolates.

PRCV has predominated in England since the late 1980's and is believed to have provided cross-protection to TGE due to the genetic similarities between the two viruses. Approximately 1 year prior to the TGE outbreak in Lincolnshire, serological screening had revealed antibodies to PRCV but not TGEV, suggesting that prior exposure of the herd to PRCV had not proved protective [15]. Subsequent TGE surveillance in England was increased, but further severe outbreaks of disease were not encountered. However, evidence of infection with TGEV was identified in several herds in which there was only mild enteric disease [15, 23]. It may be that the genetic differences in these TGEVs reduce the protection afforded by prior infection with PRCV. Characterisation of these viruses is awaited.

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