Discrimination between transmissible gastroenteritis virus isolates

Brief Report

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Summary. Twenty TGEV isolates were compared by sequencing a 393–414 nucleotide stretch near the 5' end of the S gene, after amplification by RT-PCR. This part of the S gene is known to show considerable variation between porcine, canine and feline coronaviruses and is completely deleted from porcine respiratory coronaviruses. The discrimination achieved by nucleotide sequence analysis was compared with that obtained by monoclonal antibody typing. The viruses could be split into several clusters, and recent isolates of TGEV from England, The Netherlands and Belgium showed the greatest differences compared to earlier reference types. However, not all viruses with unique isolation histories were distinct, suggesting either genetic stability over many years, laboratory cross-contaminations or repeated introductions of similar viruses into the field. Firm conclusions on evolutionary trends cannot be drawn without obtaining a larger number of isolates, preferably from outbreaks with known epidemiological links. The sequences of some field isolates from the 1980s contained both nucleotide deletions and insertions. The latter included a short sequence of fourteen nucleotides with identity to a region of the TGEV polymerase gene.

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Transmissible gastroenteritis (TGE) is a highly contagious pig disease that may cause serious losses, especially in unweaned piglets [17]. The causative virus, TGEV, is a member of the *Coronaviridae*, and has a large, single-stranded, positive sense RNA genome. The virion is enveloped, and covered in projections caused by a glycosylated spike protein (S), of approximately 200 kDa. The S protein is a major target for antibodies, and monoclonal antibodies (mAbs) have helped to define four principal antigenic sites, one of which

(site A) is involved in antibody-mediated virus neutralisation [3]. Porcine respiratory coronavirus (PRCV) is a respiratory variant of TGEV, that has a deletion of over 670 nucleotides near the 5' end of the S gene. This virus has become widespread in recent years, and its emergence has been associated with a reduction in the incidence and severity of cases of TGEV [14].

Whilst numerous studies have examined antigenic differences between TGE viruses, only a small number of TGEV isolates have been compared genetically [16]. Here, we report nucleotide sequence comparisons of amplified products from 20 TGEV isolates obtained from eight countries between 1946 and 1996 (Table 1).

Viral RNA for RT-PCR was extracted from cell lysates by the acid-phenol method [18], or from culture supernatants using QIAamp HCV kits (Qiagen). Uninfected cell and water controls were processed concomitantly to check for unexpected amplifications. Establishment of the RT-PCR method has been described elsewhere (Paton et al., in press). The target region of 886

Table 1. Viruses sequenced in this study

TGE virus	Country of isolation	Date isolation	Passage level	Cell types grown in ^a
64-216	England	1964	+2 ^c	PPK
70-772	England	1970	>10	PPK, PK15
83-3 289	England	1983	14	PPK
84-3 658	England	1984	14	PPK
85-45 210	England	1985	4	PPK
96-1 933	England	1996	4	LLCPKI
V344 ^b (98-1-Pm)	Bulgaria	nk	24	PPK
V345 ^b (98-3-BA)	Bulgaria	nk	5	PPK, SPEV
V346 ^b (98-4-TI)	Bulgaria	nk	4	PPK, SPEV
V347 ^b (98-5-IG)	Bulgaria	nk	22	PPK, SPEV
V355 ^b (LKN)	Russia	nk	+2	PPK
V91 ^b (Purdue)	USA	1946	42	PPK
IA-136	USA	1990s	+2	ST
IA-139	USA	1990s	+2	ST
KS-204	USA	1990s	+2	ST
Slagharen	Netherlands	1986	+3	nk
Erica	Netherlands	1986	+3	nk
V63	Belgium	1988	6	ST
V66 ^b (SH)	Japan	1962	56	PPK
V126 ^b (M42)	Czech	1968	64	PPK

^aCell types grown in other than LLC PK1 (where known)

^bAccession numbers at Brno Collection of Animal Pathogenic Microorganisms (original strain designation in parenthesis)

^c+2 indicates passaged twice at authors' laboratory, but previous passage level unknown

PPK Primary pig kidney cells, SPEV pig kidney cell line from Moscow, ST swine testis, nk not known

nucleotides straddles a large deletion (672–681 nucleotides) found exclusively in the S gene of isolates of PRCV, but not TGEV. Two mAb binding sites (B and C) have been mapped to this deleted region of the S protein [9]. The forward primer (F1121, 5'-TATTTGTGGTYTTGGTYGTAATGC) is equivalent to nucleotide 11–344 of the S protein gene of TGEV, and the reverse primer (R1122, 5'-GGCTGTTTGGTAACTAATTTRCCA) is complementary to nucleotides 896–873. Reverse transcription used random hexamers and M-MLV reverse transcriptase. The PCR used Taq polymerase and a temperature profile with 32 cycles (45 s at 95 °C, 1 min at 50 °C and 2 min at 72 °C) followed by a final extension time of 5 min at 72 °C.

For each of 20 TGEV isolates, RT-PCR was performed in triplicate, and amplified products were pooled and cleaned using Wizard PCR prep columns (Promega). The PCR templates were sequenced using a FS dye primer kit (Perkin Elmer) and internal primers F1174 (5'-CGCAATAATAGTAATGACCTTTAT) and R950 (5'-TCAAACCAACCAAGGTCTACAA), identical or complementary to S protein gene nucleotides 208–231 and 684–665. In instances when the sequence data was ambiguous the external primer, R1122 was also used. Sequencing reactions were visualised by the use of a commercial service (University of Durham, UK) which employed an ABI 373 automated sequencer (Perkin Elmer/ABI). Raw sequencing data was edited and aligned using the DNASTAR (DNASTAR Inc.) and GCG [4] computer packages. All sequences used for comparative purposes were confirmed by sequencing in both directions. Aligned sequence data was compared with previously published data and phylogenetic trees were constructed by maximum likelihood, using the DNAML program from the PHYLIP package [6].

For mAb typing, TGEV infected LLCPK1 cultures in 96 well microtitre plates were fixed at 12–72 h post infection, before cytopathic effect became extensive. The fixative, 20% acetone in PBS, was applied for 10 min and then plates were dried under a bench lamp at 37 °C for 3–5 h. Immunostaining was carried out either immediately or after thawing plates stored at –20 °C in sealed plastic bags. Optimised dilutions of antisera or mAbs were applied and these were detected by an appropriate anti-species peroxidase conjugate (Dako) and the substrate 3-amino 9-ethyl carbazole [10]. The mAbs were courtesy of D. Garwes (produced using TGEV 70–772; DA3, 4B1, 3C1, 7A6, 7C6, 9D2 [7, 8]), L. Enjuanes (produced using TGEV Purdue; 1D.B12 [11]) and A. van Nieuwstadt (produced using TGEV Purdue; 57.22, 57.110 [19]). All isolates were recognised by mAbs DA3 (anti-nucleocapsid), 3C1, 7C6 and 9D2 (anti-S). The pattern of reactivity with mAbs able to discriminate between the isolates (all anti-S) is shown in Table 2. Reliable typing results could not be obtained for the 96–1993 isolate due to its poor growth in cell culture.

The length of sequence obtained for each TGEV isolate ranged from 393 to 414 nucleotides, depending on the presence of insertions and or deletions. The new data were aligned with the published sequences of the prototype Purdue isolate (nucleotides 250–648) and that of TGEVs 70–772, Toy56, Neb72, Miller [16] and TF1 [2], as shown in Fig. 1. Our own sequence of Purdue, from

Table 2. Reactivity of discriminatory mAbs

	Monoclonal antibodies				
	4B1	57.22	57.110	1D.B12	7A6
TGEVs					
64–216	+	++	+/-	-	+
70–772	++	++	++	++	+
83–3 289	++	++	-	-	-
84–3 658	++	-	-	-	+
85–45 210 ^a	+	NT	+	+	-
V345 (98-3-BA)	+	++	++	-	-
V355 (LUK)	+	++	++	-	-
Slagharen	-	++	++	++	-
Erica	-	++	++	-	-
V63	-	++	++	++	-

^aTested by IFA rather than IPX

NT Not tested; - no staining; +/- weak staining; + positive staining; ++ strong staining

The following were recognised by all the above mAbs except 7A6: V344, V346, V347, V355, V91, IA-136, KS-204, V66, V126

a virus obtained via The Czech Republic (V91) differed at one nucleotide from the published sequence. Similarly, a previous sequence of 70–772 [1] differed at a single nucleotide compared to our own data. Four isolates had either additional or deleted nucleotides, none of which altered the reading frame of the S gene. The English isolate 83–3289 had a deletion of six nucleotides between position 286 and 291, overlapping antigenic site B [9]. The two Dutch isolates Erica and Slagharen, shared the same insertion of 15 nucleotides between positions 496 and 497. The two nucleotides preceding the insertion and the first twelve nucleotides of the insertion itself, are identical in sequence to a region within the published polymerase Ia gene of the Purdue isolate of TGEV (nucleotides 1 054–1 065 [5]). The last three nucleotides of the insert are the same as those at positions 494–496 which immediately precede the insertion. The Belgian isolate V63 had the most complex rearrangement, in which five nucleotides were inserted, also after position 496, followed by a deletion of eleven nucleotides at position 497–507. The five nucleotides inserted into V63 were the same as the first five nucleotides inserted into the Dutch isolates Slagharen and Erica. The 5' end of the S protein of TGEV is that which differs greatest from canine and feline coronaviruses which otherwise have sequences very similar to those of TGEV. PRCV and other coronaviruses have been found to contain large deletions in this part of the S gene [14, 15], whilst it has been reported that the S protein of the Purdue and Nebraska 72 isolates from America were two amino acids shorter than in other TGE viruses [16]. Our new data provide further evidence for the plasticity of this region of the S gene. The three isolates, Erica, Slagharen and V63 all share similar

rearrangements to one another and are geographically and temporally linked, suggesting that the changes arose *in vivo* in a common precursor. In the case of the two isolates, Erica and Slagharen, template switching between the polymerase and S gene of the TGEV genome could account for the insertion, since there is a region of secondary structure predicted in front of the insertion site, and the same three nucleotides are present before the insertion and at its end.

The greatest sequence dissimilarity was between isolates 96-1933 and Slagharen with 35 nucleotide sequence differences (91% identity), discounting the 15 nucleotide insertion in Slagharen. All of the isolates were more closely related to one another than to the related coronaviruses from dogs and cats. If insertions and deletions are excluded, approximately 60% of nucleotide positions where sequence differences are observed give rise to amino acid changes. Alignment of the deduced amino acid sequences showed greatest variability around amino acid positions 97 and 144, previously shown to be part of mAb binding site B [9]. Interestingly, mAb 1D.B12, previously shown to bind to site B, showed a different reactivity with two isolates (64-216 and 84-3 658) that had no sequence differences, suggesting that some other region may influence this antigenic site.

Figure 2 shows the similarity of the isolates to one another deduced by comparison of the nucleotide sequences. A number of clusters of similar

Fig. 2. Maximum likelihood phylogenetic tree of the viruses used in this study and some published sequences. Viruses with identical sequences have been omitted (see legend to Fig. 1). The country of origin and year of isolation (where known) is given in parenthesis. *BE* Belgium, *BU* Bulgaria; *JP* Japan; *NL* Netherlands; *TA* Taiwan; *UK* United Kingdom; *US* United States

isolates are evident. The largest cluster includes isolates similar or identical to the prototype Purdue virus. These viruses were isolated in Japan in 1962 (V66), in Czechoslovakia in 1968 (V126), in Bulgaria (V347), and in USA in the 1990's (IA-136 and KS-204). The next closest group, included two viruses with a distinctive respiratory tropism, one isolated in Japan in 1956 (Toy56), the other isolated in Russia in the 1980's (V355). Three English viruses (64-216, 70-772, 84-3 658) isolated between 1964 and 1984 had identical sequences and were similar (3 nucleotide differences) to the published sequence of the 1965 Miller isolate from the USA. Another US isolate from the 1990s (IA-139) was identical to Miller. The three isolates from The Netherlands and Belgium were a clearly distinct lineage. The remaining three viruses from England were isolated between 1983 and 1996 and show an increasing divergence from the other TGEVs over time. The sequence obtained for the 96-1 933 virus was identical when obtained from amplified cell culture material (passage 4) or directly from faeces.

The phylogenetic tree shown in Fig. 2 reveals a number of clusters of similar viruses, but in several cases there is no obvious correlation between clustering and the date or place of isolation. Some viruses are identical or nearly identical over the region examined despite being apparently isolated many years apart, and even from different continents. For instance the large group of viruses closely related to the prototype Purdue isolate. Other examples were IA-139 and Miller viruses and the 64-216, 70-772 and 84-3 658 viruses isolated in US and England respectively, in each case over a time period of approximately 20 years. To be certain that these identities did not arise from cross-contamination during RT-PCR procedures, we repeated the sequencing on all viruses appearing genetically identical, using freshly obtained amplicons derived from an earlier passage of the virus. Where possible, sequencing was repeated after direct amplification from virus stocks as received from abroad. Further support that the similarities identified were genuine, is provided by the mAb typing, which generally suggested the same virus groupings, and which was performed on the same virus stocks used for the RT-PCR. All of the above-mentioned Purdue-like group of viruses reacted identically with the mAbs, whilst the similarity of the three English viruses, 64-261, 70-772 and 84-3 658 was demonstrated by their unique reactivity with the mAb 7A6.

Other than extreme evolutionary stability, the reintroduction of laboratory isolates into the field or the contamination of laboratory isolation cultures are possible explanations for the observed sequence identity of some isolates. In the case of the Purdue-like group of viruses, use in live vaccines could have caused reintroduction to the field, whilst in vitro contamination could have been associated with use as laboratory reference strains, for instance in virus-serum neutralisation tests.

In contrast to these examples of apparent evolutionary stasis, the European viruses isolated in the last ten years are much more distinct, implying either a different origin or else a more rapid accumulation of mutations. It is tempting to

speculate that a more rapid rate of evolution could somehow be caused by the spread of PRCV. However, since the sequenced region is deleted from PRCV, immunological cross-reactivity between PRCV and TGEV seems an unlikely cause. Since few outbreaks of TGE have been confirmed in Britain in recent years, it was uncertain where the 96–1933 isolate responsible for a recent episode of TGE [12] had come from. Interestingly, there is a unique nucleotide at position 426, present in all of the English isolates as well as in the sequence of TF1 from Taiwan, an isolate that appears phylogenetically closest to English isolate 83–3289. This also suggests a common origin, but in the absence of a representative set of sequence data from viruses circulating elsewhere outside Britain, no firm conclusions on the immediate origin of any of these viruses is possible. As well as obtaining S gene sequence data from more viruses, there is a need to compare other parts of the TGEV genome using phylogenetic analysis, in order to check for evidence of recombination between strains, a known feature of coronaviruses [13].

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