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Short communication

Detection of transmissible gastroenteritis virus by RT-PCR and differentiation from porcine respiratory coronavirus

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Abstract

An RT-PCR method was developed that amplified genetic material from the 5′ end of the S protein gene of both transmissible gastroenteritis virus (TGEV) and porcine respiratory coronavirus (PRCV), but discriminated between the two by the size of the product generated. A number of restriction endonuclease enzymes were assessed for recognition of the amplicons so produced. The assay was shown to detect viral RNA from all of the 26 different TGEV and PRCV isolates examined, covering a period from 1946 to 1996. Detection of TGEV in clinical specimens was possible using a spin column method to extract RNA and sensitivity was compared to virus isolation and antigen detection ELISA. The method could provide a means of confirming positive results from immunological screening tests such as FAT and ELISA, reducing the need for virus isolation and convalescent serology. © 1997 Elsevier Science B.V.

Keywords: Gastroenteritis virus; Respiratory coronavirus; Transmissible gastroenteritis

Transmissible gastroenteritis (TGE) is a highly contagious pig disease that has been reported from many parts of the world including America, Europe and Asia. The causative virus, TGEV, is a member of the coronaviridae, and has a large, single-strand, positive sense RNA genome. Since the mid 1980s, a variant respiratory form of the TGE virus known as porcine respiratory coronavirus (PRCV), has become common in pigs in Europe and has more recently been reported from North America and Asia (Pensaert et al., 1986; Wesley et al., 1990). It generally causes mild disease under experimental conditions. In Europe, the emergence of PRCV has been associated with a reduction in the incidence and severity of cases

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of TGE. Compared to TGEV, PRCV has a deletion of between 672 and 681 nucleotides near the 5′ end of the S gene, resulting in the loss of some antigenic sites on the S protein (Laude et al., 1993), and of sialic acid binding activity (Schultze et al., 1996).

Rapid methods for the detection of TGEV are important because of the highly contagious nature of the disease. Since the virus is often difficult to adapt to growth in cell cultures, the most widely used techniques are immunological, principally a fluorescent antibody test (FAT) for cryostat sections of intestine and an antigen ELISA for detection of virus in faeces (Paton, 1992). Differentiation of TGEV from the closely related PRCV is possible using anti-S protein monoclonal antibodies (mAbs) directed against non-neutralising antigenic sites that are lacking in PRCV (Garwes et al., 1988; Callebaut et al., 1988). There are also reports of the use of DNA probes and of RT-PCR as detectors of TGEV RNA (Bae et al., 1991; Vaughn et al., 1996; Wesley et al., 1991; Jackwood et al., 1995), but the methods were not shown to be suitable for the direct detection of virus in clinical samples. An in situ hybridization method for detection of TGEV and PRCV has been reported, but requires tissues rather than faeces (Sirinarumitr et al., 1996). Here we report the development of an RT-PCR method for the detection and differentiation of TGEV/PRCV.

A total of 25 archived viruses were examined in this study, mainly European, but also including isolates from North America and Japan (Table 1). The viruses had been passaged to varying extents (> 60 in some cases) in a variety of cell cultures. To prepare stocks for this study, viruses were passaged in a pig kidney cell line (LLCPK1, Flow), maintained in modified Eagle's medium with 10% foetal calf serum.

Samples used for RT-PCR were extracted from either cell lysates or cell culture supernatants. Uninfected cell controls were always processed concomitantly. In the case of cell lysates, cultures were processed at 7–36 h post infection or mock-infection, depending on the extent of virus growth, and total RNA was extracted by the acid-phenol method (Stallcup and Washington, 1983). For cell culture supernatants, RNA was recovered using commercially available spin columns (QIAamp HCV kits, Qiagen).

A pair of oligonucleotide primers was designed to amplify the 5′ end of the gene encoding the S protein of TGEV/PRCV. The target region straddles a large deletion (672–681 nucleotides) found exclusively in isolates of PRCV, but not TGEV. The forward primer (F1121, 5′-TATTTGTGGTYTTGGTYGTAATGC) is equivalent to nucleotide 11–34 of the S protein gene of TGEV, and the reverse primer (R1122, 5′-GGCTGTTTGGTAACTAATTTRCCA) is complementary to nucleotides 896–873. The primers were chosen by analysis of an alignment of TGEV/PRCV sequences available in GenBank. The predicted size of the amplified product is 886 bp for TGEV and 205–214 bp for PRCV.

Reverse transcription was in 20 μl volumes, using 2 μl of sample, 0.5 μl of random hexamers (Pharmacia, 50 pmol), 1 μl of dNTPs (40 mM), 4 μl of 5X RT buffer (Promega), 0.5 μl of RNAsin (20 U, Promega) and 0.5 μl of M-MLV reverse transcriptase (100 U, Promega). Samples were incubated at 37°C for 30 min and then at 95°C for 5 min, before cooling to 4°C. PCR was in 100μl volumes, using 10 μl of 10 × buffer (500 mM KCl, 100 mM Tris–Cl pH 8.3, 15 mM $MgCl_2$, 0.1% gelatin), 1 μl dNTPs (40 mM), 1 μl Triton X100 (10%), 2 μl cDNA, 0.5 μl of each primer (50 μM) and 0.5 μl of TAq Polymerase (2.5 U, Promega). The temperature profile was 32 cycles of 45 s at 95°C, 1 min at 50°C and 2 min at 72°C. There was then a final extension time of 5 min at 72°C.

A 5 μl aliquot of each PCR product was visualised by agarose gel electrophoresis (1% agarose, 100 volts for 30 min, 0.8 μg/ml ethidium bromide included in gel) and subsequent U.V. transillumination. An RT-PCR product of the size expected for TGEV or PRCV was amplified from total RNA extracted from cells infected with each of the viruses examined in this way (Fig. 1). The large difference in the size of amplicon for TGEV and PRCV is obvious. The slightly larger size of amplicon obtained with American PRCV isolates, compared to European ones was also evident if the amplicons were run on a polyacrylamide gel (data not shown).

Table 1
Viruses used in this study

Virus	Country of isolation	Date isolated	Source (and reference)
TGEVs			
64–216	England	1964	CVL†, (Cartwright et al., 1965)
70–772	England	1970	CVL, (Garwes et al., 1987)
83–3289	England	1983	CVL
84–3658	England	1984	CVL
85–45210	England	1985	CVL
96–1933	England	1996	CVL, (Jones and Paton, 1996)
V344* (98-1-Pm)	Bulgaria	nk§	Valicek
V345* (98-3-BA)	Bulgaria	nk	Valicek
V346* (98-4-TI)	Bulgaria	nk	Valicek
V347* (98-5-IG)	Bulgaria	nk	Valicek
V348* (98-6-Mr)	Bulgaria	nk	Valicek
V349* (98-7-Tr)	Bulgaria	nk	Valicek
V355* (LNK)	Russia	nk	Valicek
V91* (Purdue)	USA	1946	Valicek
IA–136	USA	1990s	Frey, (Vaughn and Paul, 1993)
IA–139	USA	1990s	Frey, (Vaughn and Paul, 1993)
KS–204	USA	1990s	Frey, (Vaughn and Paul, 1993)
Slagharen	Netherlands	1986	van Nieuwstadt, (Van Nieuwstadt and Boonstra, 1992)
Erica	Netherlands	1986	van Nieuwstadt, (Van Nieuwstadt and Boonstra, 1992)
V63	Belgium	1988	van Reeth
V66* (SH)	Japan	1962	Valicek, (Harada et al., 1963)
V126* (M42)	Czech	1968	Valicek, (Stepanek et al., 1969)
PRCVs			
86–135308	England	1986	CVL, (O'Toole et al., 1989)
V70	Belgium	1992	van Reeth
IA–1894	USA	1992	Frey, (Vaughn et al., 1994)
Indiana 89	USA	1989	Frey, (Wesley et al., 1990)

*Accession numbers at Brno Collection of Animal Pathogenic Microorganisms (original strain designation in parenthesis).
†CVL, Central Veterinary Laboratory (the authors' laboratory). §not known.

The sensitivity of the RT-PCR for virus detection in culture fluids was compared with cell culture virus isolation. Virus titres were calculated as 50% tissue culture infectious doses ($TCID_{50}$) by titrating virus stocks in tenfold steps, and assaying for cell culture infectivity by viral cytopathic effect (cpe) and immunostaining of cultures fixed in 20% acteone (Holm Jensen, 1981). Immunostaining used a polyclonal antiserum against TGEV 64–216, a rabbit anti-porcine peroxidase conjugate (Dako) and the substrate 3-amino 9-ethyl carbazole. The supernatant fluids from cell cultures infected with TGEV strains 70–772 and Slagharen had cell culture infectivity titres of $10^{4.75}$ and $10^{2.25}$ per 50 μl, whilst with RT-PCR a visible band was detectable from samples diluted to 10^{-4} and 10^{-2} respectively.

The technique's application to clinical samples was evaluated on specimens obtained following investigation of a pig herd that showed typical signs of epizootic TGE (Jones and Paton, 1996). The TGE virus isolated from the case was designated 96–1933. Two faecal samples and three samples of small intestine were obtained from three-week-old pigs with diarrhoea. A sample of intestine was homogenised in PBS and fed to four five-day-old piglets held in isolation and fed on milk replacer. Profuse diarrhoea began within 18 h in all pigs and 8 faeces samples were collected over the next 6 h, after which the pigs were euthanased. Faeces samples from the field case and the experimentally infected pigs were all TGEV positive in a routinely used ELISA (Paton, 1992). The dilution at which TGEV could be

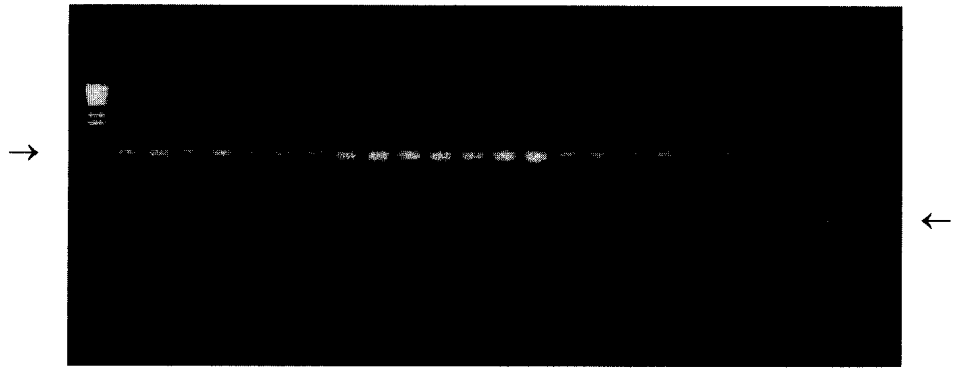

Fig. 1. Specificity of RT-PCR for different TGEV and PRCV isolates. Ethidium stained agarose gel showing RT-PCR amplicons from 24 isolates. Lane A, molecular weight marker; Lanes B-U: TGEV isolates IA–139, V345, Slagharen, Erica, V63, 84–3658, 64–216, 83–3289, V344, V346, V347, V348, V349, V355, V66, V126, V91, IA–136, KS–204, 70–772; lanes V–Y: PRCV isolates Indiana 89, IA–1894, V70, 135308; lane Z: extraction from uninfected cell control.

detected in ELISA was between 10^{-2} and 10^{-4}. FAT on cryostat sections of intestine, using a commercial source of TGEV antiserum (VMRD, Inc.), confirmed TGEV antigen in two out of three of the field samples and in all four pigs inoculated experimentally. Clarified, 10% homogenates of intestine and faeces from both the field outbreak and the subsequent experimentally-induced cases were used to inoculate cell cultures for attempted virus isolation as previously described (Paton, 1992). Cell cultures used included secondary pig kidney, LLCPK1, secondary canine kidney and a canine rectal tumour cell line (A72). In each case, except for the secondary pig kidney cultures, virus isolation was attempted with and without added trypsin (10 μg/ml, with daily medium renewal). The 96–1933 isolate grew very poorly in the cell types assessed. No cpe was evident in any culture after four blind passages, with or without added trypsin, but immunostaining revealed a small number of infected plaques in cells within the A72 and LLCPK1 cultures. Tenfold dilution of the inoculum from the third cell culture pass lead to complete loss of detectable infectivity at the fourth passage.

For RT-PCR, samples of faeces were diluted 1 in 10 in a disruption buffer containing 500 mM Tris–Cl, pH 8.3, with 2% PVP-40, 1% PEG 6000, 140 mM NaCl, and 0.05% Tween 20 (Rowhani et al., 1995), vortexed and left to stand at room temperature for 10 min. Earlier experiments had shown an increase in sensitivity of up to ten-fold, when disruption buffer was used instead of PBS (data not shown). The suspensions were clarified by centrifugation at 2000 g for 2 min and 140 μl of supernatant was used in QIAamp spin-column kits, according to the manufacturer's recommendation. Ten per cent intestinal homogenates in PBS plus antibiotics were clarified by centrifugation at 3000 g for 10 min. Thereafter, 140 μl aliquots were processed in QIAamp kits as above. All of the faeces samples were positive by RT-PCR, and the same two out of three pigs from the source outbreak for 96–1933 were positive for TGEV using both FAT on cryostat sections of intestine and RT-PCR on gut homogenates. To assess the sensitivity of the RT-PCR, three positive faeces samples were diluted in ten fold steps in disruption buffer, prior to extraction of RNA. The dilutions at which TGEV could be detected in the three samples were 10^{-5}, 10^{-5}, and 10^{-6} for RT-PCR, compared to 10^{-2}, 10^{-4}, and 10^{-2} respectively for ELISA.

To confirm the authenticity of the amplified products restriction endonuclease (RE) analysis was assessed using each of three enzymes (*Hae*III, *Hinf*I and *Sau*3AI) for TGEV and the enzyme Sau3AI for PRCV. These enzymes were predicted to cut the amplified region of the Purdue strain of TGEV. The DNA was extracted from a 50-μl aliquot of each PCR reaction using Wizard prep columns (Promega) and a one tenth volume of this was digested for 1 h in a 20-μl volume using the manufacturer's recommended digestion buffer (Promega). Half of the digest was then examined by agarose electrophoresis, as above, except using 2% high resolution agarose (MetaPhor, Flowgen) and electrophoresis at 80 volts for 45 min. The restriction enzyme *Hae*III produced the predicted cut into two fragments of approximately 520 and 380 bp for all TGEV isolates (Fig. 2). The other enzymes did not give a consistent pattern (data not shown). *Hinf*I gave extra bands with two isolates (83–3289 and 96–1933), whilst *Sau*3AI gave extra bands with 96–1933 and did not cut 84–3658, nor the PRCV isolate Indiana 89.

Isolation of TGEV in cell cultures is a slow and unreliable diagnostic method. The process may require multiple blind passages to be made before characteristic cpe is evident, although this period may be somewhat shortened by immunostaining of the cell monolayer with TGEV-specific reagents. It has been observed that some isolates are more readily adapted to cell culture than others and in a large proportion of cases, success is very elusive (Vaughn and Paul, 1993). In the current study, clinical samples from pigs infected with the 96–1933 isolate did not grow well in a variety of cell cultures, despite evidence from other assays, including pig inoculation, that infectious virus was present. Attempts to improve yield by trypsin treatment, using a method modified from that of Honda et al. (1990) had no beneficial effect.

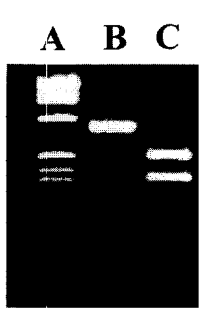

Fig. 2. Restriction digests of RT-PCR products. Ethidium stained agarose gel showing undigested and digested amplicons of a representative TGEV isolate. Lane A,: molecular weight markers; lanes B, C: amplified DNA from TGEV 83–3289. Lane B: no restriction enzyme; lane C: *Hae*III digest.

To avoid the difficulties of virus isolation in routine diagnosis, immunoassays such as FAT and ELISA have been developed for the detection of TGEV antigens in gut sections or faeces (Pensaert et al., 1968; Bernard et al., 1986; Van Nieuwstadt et al., 1988). However, in many European countries, TGEV has been either absent or present at very low levels in the last ten years. In such circumstances, a positive diagnosis of TGEV becomes more significant, and it may be necessary to confirm diagnoses made by antigen detection tests using an independent assay procedure. Serology is an option, but suffers from two disadvantages. Firstly, rapid diagnosis may not be possible, due to the need to allow time for convalescence and antibody development. Secondly, it usually requires a repeat visit to the farm to be made, for the appropriate samples to be collected. There are reports of the development of nucleic acid probes for detection of TGEV RNA (Bae et al., 1991; Vaughn et al., 1996; Wesley et al., 1991), but the methods did not describe direct use on clinical samples and may lack sensitivity for such work.

It would be desirable to assess the RT-PCR method on a larger number of clinical samples, including ones from older pigs. From a small number of titrations, the RT-PCR method appeared more sensitive than ELISA, but slightly less sensitive than virus isolations. The sensitivity of the RT-PCR may have been reduced by using only two of 50 μl of extracted RNA and again only two of 20 μl of the RT reaction mix. Nevertheless, a recent field isolate (96–1933) that was not readily grown in cell culture was detected with a strong RT-PCR signal, using faeces or intestine submitted from the field case. Another TGEV isolate (85–45210) could not be recovered in cell culture from an archived frozen stock of liquid culture supernatant, but an RT-PCR product was amplified. Furthermore, sequencing revealed that the amplicons obtained from each of these two viruses were unique (data not shown).

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