Papers and Articles

Cell culture-grown putative bovine respiratory torovirus identified as a coronavirus

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A putative bovine respiratory torovirus (BRTV) was propagated in bovine fetal diploid lung and human colonic tumour cells, and fringed pleomorphic particles were detected in the culture supernatants by electron microscopy. Antisera directed against a bovine (Breda strain) and equine (Berne strain) torovirus failed to react with BRTV-infected cells in immunofluorescence assays and did not neutralise BRTV. No toroviral RNA was found in the supernatants of infected cells by means of a reverse transcriptase-polymerase chain reaction with torovirus-specific primers. On the other hand, bovine coronavirus-specific antisera and monoclonal antibodies did neutralise the cytopathic effects, and coronaviral antigen was detected in the cultures by immunofluorescence. Furthermore, bovine coronavirus RNA was detected in the supernatants of BRTV-infected cells after nucleic acid amplification. It is concluded that the cytopathic BRTV isolate is a coronavirus.

ATTEMPTS to propagate toroviruses in cell culture have been unsuccessful, with the notable exception of a single isolation from the horse (Weiss and others 1983). When Vanopdenbosch and others (1991, 1992a) reported the in vitro adaptation of a torovirus from the respiratory tract of a calf with pneumonia, it was greeted as an important finding: both the isolation of a bovine torovirus and its inferred pathogenic properties are of veterinary relevance.

Toroviruses form a genus in the family Coronaviridae (Pringle 1992) of the recently established order Nidovirales (de Vries and others 1997). Virions appear as pleomorphic, enveloped particles with a single or double (Cornelissen and others 1997) fringe of spikes (peplomers) at their surface and a characteristic tubular nucleocapsid of helicoidal symmetry that can confer a doughnut-shape to the virion (Weiss and others 1983). Because of its growth in cell culture, the Berne strain of the equine torovirus has been studied extensively (Snijder and Horzinek 1993, de Vries and others 1997).

Toroviruses infect horses, cattle, pigs and possibly human beings and cats (Woode and others 1982, Beards and others 1984, Weiss and others 1984, Scott and others 1987, Muir and others 1990, Koopmans and Horzinek 1994, Kroneman and others 1998). The high prevalence of seropositive animals indicates that the infection is widespread in horse and cattle populations (Weiss and others 1984, Brown and others 1987, 1988, Woode 1987, Koopmans and others 1989). No clinical disease has been associated with torovirus infection in horses, but the bovine torovirus is an enteric pathogen causing mild to profuse diarrhoea in experimentally or naturally infected young calves (Woode and others 1982, Koopmans and others 1990) and possibly in adult cattle (Koopmans and others 1991b, Scott and others 1996). Some reports have suggested that torovirus infections are also responsible for upper respiratory tract illness in calves (Woode and others 1982, 1985, Vanopdenbosch and others 1992a, b, Koopmans and Horzinek 1994).

Vanopdenbosch and others (1992a) recovered a putative isolate of bovine respiratory torovirus (BRTV) from a calf that had died from neonatal pneumonia (Vanopdenbosch and others 1991). By using torovirus antisera in an immunofluorescence assay the authors detected stained cells in tissue sections of the lung and trachea. Inoculation of a lung homogenate into cultures of different cell types was followed by a cytopathic effect (cpe), and 'torovirus-like' particles were observed by electron microscopy.

The in vitro cultivation of a bovine torovirus would not only allow the detailed molecular characterisation of a second torovirus, but would also facilitate the production of antigen for diagnostic assays. Moreover, if the virus were found to be pathogenic, it would justify the development of a vaccine. This paper describes attempts to confirm the published data and to characterise the cytopathogenic agent present in the BRTV isolate. The studies were conducted independently in two laboratories, and indicate that the cytopathogenic virus replicating in cell culture is a coronavirus.

Materials and methods

Viruses, cells and antisera

The BRTV isolate provided by Vanopdenbosch had been passaged six or eight times in Madin-Darby Bovine Kidney (MDBK) cells (BRTV P6 and P8, respectively). The Mebus strain of the bovine coronavirus was used throughout the study. Bovine fetal diploid lung (BFDL) cells were obtained from the ID-DLO, Lelystad, and all other cell lines were purchased from the America Type Culture Collection. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco Laboratories) or Glasgow's MEM supplemented with 10 per cent fetal calf serum (FCS) and antibiotics. A field serum sampled from a cow served as the BRTV reference serum (BαBRTV, Vanopdenbosch and others 1992a); sera from gnotobiotic calves infected experimentally with bovine coronavirus (GC78) or bovine torovirus strain Breda 2 (GC76) were provided by G. Woode (Woode and others 1982). The rabbit immune serum against bovine coronavirus was prepared by D. Brian; all

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TABLE 1: Summary of the growth of the putative bovine respiratory torovirus on different cell lines determined by microscopical examination of the development of a cytopathic effect (cpe) and serotyping immunofluorescence

Cell line†	cpe*	Serum‡	Immunofluorescence BFDL	HCT-8	MDBK§
MDBK	-	RαBCV	+	ND	ND
PD5	-	BαBRTV	±	+	±
CRFK	-	GC76	-	ND	-
BEL	-	RαMHV	+	ND	ND
BHK	-	GC78	+	ND	+
FEF	-	RαBEV	-	-	-
JCK	-	RαBRV	-	-	ND
RK13	-	mAb αBEV S	-	ND	-
Caco-2	-	ChαBCV	ND	+	ND
VERO	-	mAb αBCV S	ND	+	ND
HCT-8	+	BαRota	ND	-	ND
BFDL	+	PαBVD	-	ND	ND
RV	-	BαBVD	ND	-	ND
SK	-				

*cpe, starting two to four days after infection, characterised by syncytium formation, cytoplasmic vacuolisation, and rounding and detachment of the cells
†Abbreviations used for the cell lines: MDBK, Madin Darby bovine kidney cells; Caco-2, A human colon carcinoma cell line; VERO, Monkey kidney cells; BEL, Bovine epithelial lung cells; HCT-8, A human colonic tumour cell line identical to the human rectal tumour cell line HRT18; RV, Bovine kidney cells; BHK, Hamster kidney cells; SK, Swine kidney cells; BFDL, Bovine fetal diploid lung cells; RK13, Rabbit kidney cells; CRFK, Crandell feline kidney cells; PD5, Porcine kidney cells, FEF, Feline embryonic fibroblasts; JCK, A calf kidney cell line
‡Abbreviations used for the sera: RαBCV, Rabbit anti BCV serum; BαBRTV, Bovine field serum containing antibodies against BRTV reference serum; GC76, Serum of a gnotobiotic calf immunised with sucrose-gradient purified bovine torovirus strain Breda; RαMHV, Rabbit serum against mouse hepatitis virus-A59; GC78, Hyperimmune serum of a gnotobiotic calf raised against purified bovine coronavirus; RαBEV, Rabbit serum against purified ETV strain Berne; RαBRV, Rabbit serum against purified bovine torovirus strain Breda; mAb αBEV S, Monoclonal antibody against the S protein of BEV; ChαBCV, Chicken serum against bovine coronavirus; mAb αBCV S, Monoclonal antibody against the S protein of BVC; BαRota, Bovine serum anti-rotavirus; PαBVD, BαBVD, respectively porcine and bovine serum anti-BVD
§BRTV-infected cells, trypsinised and spotted on 10-well glass slides
ND Not determined

the other sera listed in Table 1 were produced in one of the laboratories involved in this study (Rottier and others 1981, Koopmans and others 1986, 1990, Kaeffer and others 1989).

Propagation of the BRTV isolate

Supernatants of BRTV P6 and P8 were used as the inoculum of various cell lines. Confluent monolayers of cells were rinsed with PBS-DEAE (50 mg/litre) and either infected with 0·5 ml of virus or mock-infected with DMEM. At one hour after infection, 3·5 ml of DMEM with or without FCS was added and incubation was continued at 37°C. In an attempt to enhance the viral infectivity, the medium was supplemented with different concentrations of trypsin, neuraminidase or pancreatin (Storz and others 1981, Weiss and Horzinek 1986, St Cyr-Coats and others 1988). The monolayers were monitored daily for cpe and tested for the presence of virus by indirect immunofluorescence.

Serology

For the indirect immunofluorescence assay, cell monolayers grown on 12 mm coverslips were either inoculated with BRTV culture supernatant or mock-inoculated. One to four days after infection, the cells were fixed and examined for evidence of infection as described by Wellemans and others (1979). Glass microscope slides carrying BRTV-infected MDBK cells (Vanopdenbosch and others 1992a) were tested in the same manner. The cells were incubated for one hour with a range of antisera, washed and stained for one hour with the appropriate antispecies fluorescein isothiocyanate conjugate. After three final washes of five minutes each, the cells were mounted in Fluorsave (Calbiochem) and examined with an Olympus BH2-RFCA microscope.

TABLE 2: Oligonucleotide primers used for the reverse transcription-polymerase chain reaction

Primer	Nucleotide sequence (5′ to 3′)	Orientation	Position*
172	TCTGGGGTAGGCCTAAT	Antisense	654 - 661
173	AATGGCACTGAAGACTCTAAT	Sense	421 - 441
174	CATAACATCTTACATGGAGAC	Antisense	448 - 471
292	GTCGCTACCTTTACGATTAAGGT	Sense	618 - 639
293	AGCAACCTTGTGGTTGGTCTGT	Sense	494 - 515
294	CTTACATGGAGACACTCAACCA	Antisense	609 - 630
295	CTACTTCTGGGGTAGGCCTAAT	Antisense	645 - 666
S1s†	TGCTATACCGAATGGTAGG	Sense	1185 - 1203
S1as†	GTAAACCGATAACCAGTGG	Antisense	1203 - 2333

*Position counted from the start codon of the N gene of equine torovirus strain Berne (Snijder and others 1989)
†Designed on the basis of sequences from the S gene of bovine coronavirus (Rekik and Dea 1994)

For the virus neutralisation assay cells were grown to sub-confluent monolayers in 96-well microtitre plates. Serial two-fold dilutions of the serum were pre-incubated with approximately 100 TCID50 of virus for one hour at 37°C before adding the mixture to the cells. The plates were incubated at 37°C in an atmosphere of 5 per cent carbon dioxide for four days and monitored for cpe. Neutralisation end points were taken as the reciprocal of the highest serum dilution at which complete neutralisation was observed.

Electron microscopy

BFDL cells were infected with BRTV P8 as described above. Twelve hours after the development of an incipient cpe the culture supernatant was harvested, centrifuged for 10 minutes at 1000 *g*, and the virus was sedimented at 200,000 *g* for two hours. The pellet was taken up in a small volume of twice-distilled water, and 5 µl of the suspension was absorbed on to a formvar/carbon-coated copper grid. The supernatant of equine torovirus-infected equine dermis cells was treated in parallel. After staining for 30 seconds in 2 per cent potassium phosphotungstate the grids were examined in a Philips CM10 electron microscope.

Reverse transcriptase-polymerase chain reaction (RT-PCR) and sequence analysis

A RT-PCR was used to detect torovirus RNA in the tissue culture supernatant of BRTV-infected cells, as described by Koopmans and others (1991a), using oligonucleotide primers designed after the 3′ untranslated regions of the genomes of the Breda strain of bovine torovirus and the Berne strain of equine torovirus (primers 172 and 173 or 292 and 295, Table 2). (Koopmans and others 1991a, L. A. H. M. Cornelissen and R. J. de Groot, unpublished observations). A second set of oligonucleotides (primers 173 and 174 or 293 and 294) corresponding to internal sequences of the predicted toroviral RT-PCR product was used to amplify torovirus sequences in a nested PCR. Culture supernatant of mock-infected and equine torovirus-infected equine dermis cells served as negative and positive controls, respectively. Bovine coronavirus sequences, corresponding to nucleotides 1185 to 2333 of the S gene, were amplified in a RT-PCR assay as described by Rekik and Dea (1994) by using oligonucleotide primers S1s and S1as (Table 2). The PCR product was cloned into the PC GENE vector and the sequence was analysed using the T7 DNA polymerase sequencing kit according to the instructions of the manufacturer (Pharmacia Biochemicals).

Results

Cell culture propagation of BRTV

Established cell lines of different species were examined for their ability to support the growth of BRTV (Table 1). In most cell

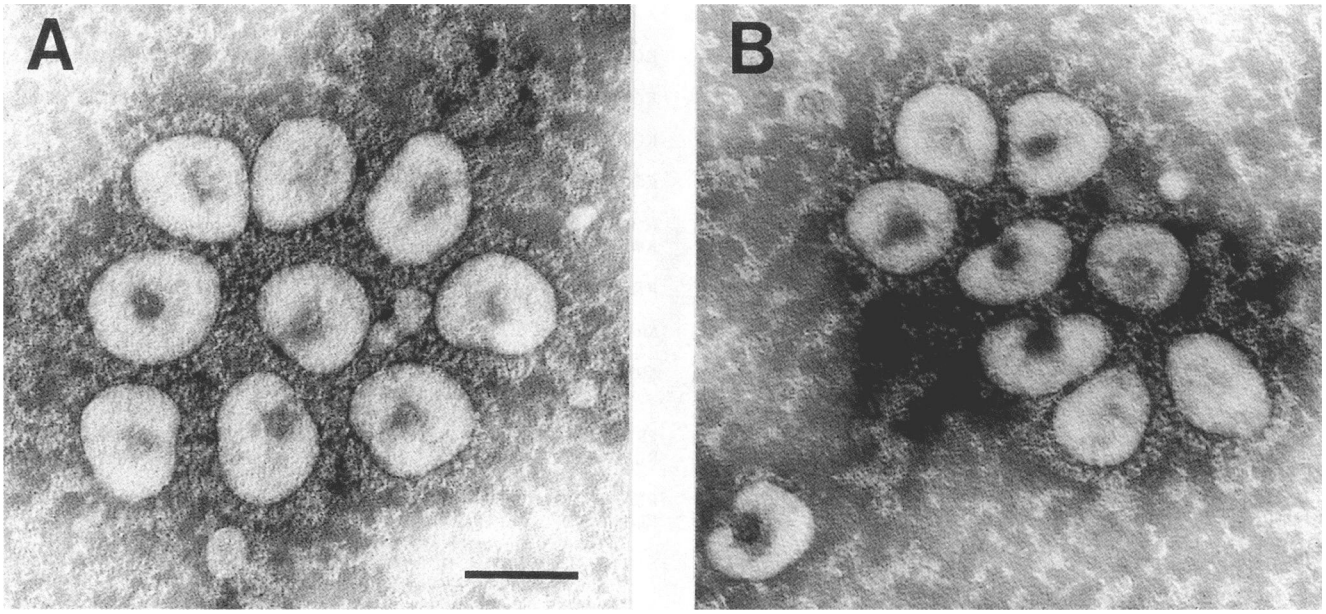

FIG 1: Electron microscopy of virus particles negatively stained with phosphotungstic acid. A) Pleomorphic particles are present in the supernatant of BRTV-infected BFDL cells. B) For comparison, negatively-stained equine torovirus particles are shown. Bar = 100 nm

types, including those that had been found susceptible by Vanopdenbosch and others (1992a), no cpe was observed. In monolayers of both HCT-8 and BFDL cells a cpe occurred two to four days after infection which was characterised by syncytium formation, cytoplasmic vacuolisation, and rounding and detachment of the cells.

In the supernatant of BRTV-infected BFDL cells pleomorphic virions approximately 120 nm in diameter were detected in electron microscope preparations; they were indistinguishable from equine torovirus particles analysed in parallel (Fig 1). Torovirus-like, kidney-shaped structures were also occasionally seen.

Serology

Because a diagnosis of torovirus cannot be based on the morphology of the virions alone, an attempt was made to obtain serological evidence that the cpe was due to a torovirus infection. Immune sera, including those originally used to demonstrate the presence of torovirus in the respiratory tract of affected calves, were used in immunofluorescence assays on BFDL and HCT-8 monolayers. No positive cells were detected, and the presence of torovirus antigens could not be demonstrated with the available sera.

However, rabbit sera directed against mouse hepatitis virus (RαMHV), and bovine coronavirus (RαBCV), a chicken serum against bovine coronavirus (ChαBCV), the serum of a bovine coronavirus-inoculated gnotobiotic calf (GC78) and a monoclonal antibody recognising the bovine coronavirus S protein (mAb αBCV-S) all produced a diffuse cytoplasmic staining (Table 1). MDBK cells infected with BRTV showed distinct fluorescence only when incubated with the bovine coronavirus-specific serum GC78.

The BRTV reference serum (BαBRTV) gave a weak fluorescence in BRTV-infected BFDL and HCT-8 cells as well as in the infected MDBK cells (Table 1). This serum did not react with equine torovirus but did result in a positive immunofluorescence when applied to bovine coronavirus-infected monolayers (not shown in the table).

To further characterise the BRTV isolate, neutralisation assays were used. An equine torovirus-neutralising rabbit serum did not significantly inhibit the cpe in BRTV-infected cultures. In contrast, virus infectivity was neutralised by ChαBCV and mAb αBCV-S. Furthermore, the reference serum BαBRTV showed neutralising activity against bovine coronavirus, which confirmed the presence of coronaviral antibodies in this serum.

Nucleic acid amplification and sequence analysis

Because no torovirus antigens could be detected, attempts were made to detect toroviral RNA. For this purpose a RT-PCR was applied, using oligonucleotide primers derived from the highly conserved 3´-terminal region of the genome.

No torovirus-specific sequences were amplified; however, a 1146 bp product was obtained when bovine coronavirus-specific oligonucleotides were used. Sequence analysis confirmed that it was part of the spike gene of bovine coronavirus. A comparison between the nucleotide sequence of BRTV and nucleotides 1187 to 1653 of the S gene of the Mebus strain of bovine coronavirus revealed 99 per cent identity.

Discussion

Vanopdenbosch and others (1991, 1992a) reported the isolation of a BRTV that would grow in cells from a wide range of host species. In the present study only the BFDL and HCT-8 cell lines displayed a cpe after infection with BRTV. Under the electron microscope, pleomorphic particles with conspicuous surface projections were observed in the supernatants of infected cultures. Toroviruses closely resemble coronaviruses in negatively stained preparations (Woode and others 1982, Weiss and others 1983, Beards and others 1986), and other assays are required to distinguish between these viruses.

Torovirus antigens were not detected by immunofluorescence and nor was the BRTV-induced cpe inhibited by equine torovirus-neutralising antibodies. There is serological cross-reactivity, at the level of cross-neutralisation, between the equine, bovine and porcine toroviruses (Woode and others 1982, Weiss and others 1983, Koopmans and others 1986, Kroneman and others 1998), but the existence of antigenically unrelated toroviruses cannot be ruled out. The culture supernatants were therefore also examined for the presence of toroviral RNA, using an RT-PCR with primers targeted at the highly conserved 3´-terminal genomic region of the known toroviruses. These primers had been used for the amplification of RNA from equine and bovine toroviruses, and from toroviruses recently discovered in pigs (Kroneman and others 1998). Again, no amplification products were detected, and the presence of a torovirus in the BRTV material could not be confirmed.

However, BRTV-infected cells appeared to react with antisera directed against bovine coronavirus and the mouse hepatitis coro-

navirus. Although of common evolutionary descent, coronaviruses and toroviruses are antigenically unrelated; furthermore, only minor antigenic variations have been found between different strains of bovine coronavirus (Woode and others 1982, Weiss and others 1983, Rekik and Dea 1994, Tsunemitsu and Saif 1995). Since the cytopathic properties of BRTV were neutralised by sera containing antibody against bovine coronavirus, the cpe must be attributed to the replication of a coronavirus and not to that of a torovirus. This assumption was confirmed by the amplification of spike gene sequences of bovine coronavirus.

The source of the bovine coronavirus in the BRTV isolate is unclear. It is assumed that it had already been present in the early passages, since both the laboratories involved in this study worked independently and obtained the same results. Most probably the respiratory tract of the calf from which the sample was taken had been infected with bovine coronavirus. Although this coronavirus is most commonly associated with acute diarrhoea in neonatal calves, it also has a tropism for the respiratory tract (McNulty and others 1984, Reynolds and others 1985, Saif and others 1986). However, Vanopdenbosch and others (1992a) reported a scattered cytoplasmic fluorescence in early passages of BRTV-infected cells when using the torovirus-specific GC76 antiserum; later, a different staining pattern was obtained when using a guinea pig anti-BRTV serum or bovine sera from naturally infected cattle (BαBRTV). With these sera five times more cells were positive, and they showed a more diffuse fluorescence. It is concluded that this staining was most probably due to the replication of bovine coronavirus in the cultures. The cytopathogenic virus was clearly identified as a coronavirus and no evidence of a torovirus was found in cultures of passage 6 and later.

These data do not exclude the possibility that a torovirus had infected the respiratory tract of the calf from which the BRTV isolate was made. In fact, this is not improbable. When using bovine torovirus-specific immune serum GC76 (Woode and others 1982) and mAbs directed against the S protein of equine torovirus (Kaeffer and others 1989), Vanopdenbosch and others (1991) had reported fluorescent cells in respiratory tissue. These sera were devoid of coronaviral antibody. Furthermore, torovirus-infected calves may develop respiratory signs (Woode and others 1985, Koopmans and others 1989), and toroviruses may therefore have a tropism for respiratory tissue.

In view of the chance element involved in virus isolation the authors consider that endless attempts to culture toroviruses in vitro are not justified; a single cytopathogenic equine isolate, the Berne strain, has been obtained in what must have been thousands of isolation attempts. Molecular techniques need to be marshalled for the study of toroviruses and their role in bovine respiratory disease.

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Abstract

Treatment of canine nasal aspergillosis with enilconazole

THE antimycotic agent enilconazole, as a 10 per cent suspension, was used to treat four dogs with nasal aspergillosis by a new non-invasive technique. The dogs were anaesthetised and intubated and the suspension was left in the nostrils for one hour. None of the dogs responded to a single treatment. One dog died from an acute septic response to pyelonephritis and bacterial endocarditis eight days after a second treatment. One dog responded completely to a second treatment but in the other two dogs an extensive and profuse fungal growth developed. This method of applying enilconazole appears to have poor therapeutic efficacy and exacerbated the fungal growth in two of the dogs.

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Notes