

## Molecular and Serological Analyses of Two Bovine Rotaviruses (B-11 and B-60) Causing Calf Scours in Australia

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Received 6 May 1991/Accepted 27 September 1991

Fecal specimens from 78 calves involved in outbreaks of calf diarrhea which occurred in three farms in Victoria, Australia, in 1988 were analyzed for rotaviruses. Thirty-eight samples were positive for group A virus antigen by enzyme-linked immunosorbent assay, and 20 of these contained viral double-stranded RNAs that could be detected by polyacrylamide gel electrophoresis. Two major electropherotypes could be observed, and a representative isolate of each electropherotype (isolates B-11 and B-60) was successfully adapted to grow in MA104 cells. Sequencing of the VP7 genes directly from RNA transcripts of fecal and cell culture-adapted viruses demonstrated that no base changes occurred in this gene upon adaptation to growth in MA104 cells. Sequencing also revealed that the VP7 protein of B-60 was closely related to G serotype 6 (G6) strains, whereas the B-11 sequence was significantly different from all previously published sequences except the recently reported VP7 sequences of bovine isolates 61A and B223, particularly across the antigenic regions A, B, and C. The other strains most closely related to B-11 by VP7 amino acid sequence analysis were G4 porcine strains BMI-1 and BEN-144 and G8 human strain 69M. Serotyping of B-11 and B-60 gave results that were in good agreement with the sequencing data. Hyperimmune typing sera clearly identified B-60 as a member of G6, whereas the B-11 strain reacted to moderate titers only with antisera to some G10 strains. Antiserum raised against B-11 neutralized some strains of G10 and cross-reacted with porcine G4 type isolates BMI-1 and BEN-144 but not with other G4 strains or with rotaviruses of other mammalian G serotypes. Northern blot hybridization showed that B-11 was closely related to the recently reported bovine G10 strain B223, and they both possessed a similar segment 4 that was different from that of either UK bovine or NCDV rotavirus.

Rotaviruses are one of the most important infectious agents known to cause gastroenteritis in young animals and children (15, 30, 36, 39, 55, 60). Heavy losses as a result of rotavirus infections in animal husbandry industries are well documented (15, 47, 60).

The intact rotavirus particle consists of 11 double-stranded RNA (dsRNA) segments enclosed in a double-shelled capsid (30, 36). The outer capsid is composed of a major glycoprotein (VP7) and a minor protein (VP4), and both proteins are involved in antibody-mediated virus neutralization (31). VP4, which is encoded by genome segment 4, determines P serotype specificity, whereas VP7, the product of segment 7, 8, or 9, depending on the virus, defines the G serotype specificity (19). Comparative studies on the amino acid sequences of VP7 proteins of various G serotypes have revealed six discrete regions which vary significantly between G serotypes but which are highly conserved within each serotype (21, 26). Studies with monoclonal antibodies have positively identified the neutralization epitopes in three of these regions, and they were designated antigenic regions A, B, and C (17, 53). The inner capsid contains the rotavirus group antigens VP6 and VP2 and is also associated with viral RNA polymerase function (19, 28, 59).

Traditional methods of virus serotyping, such as neutralization and enzyme-linked immunosorbent assays (ELISA), remain important, although new techniques with great potential, like nucleic acid hybridization, RNA sequencing, and the polymerase chain reaction, are being developed (24, 27, 52, 58, 64). Currently, 11 G serotypes based on VP7

glycoproteins have been classified; and potential candidates, G serotype 12 (G12), G13, and G14, have been reported previously (8, 9, 19, 50, 57, 62). G1, G2, and G9 have been found only in humans; G3, G4, and G8 have been found in both humans and animals, while G5, G6, G7, G10, and G11 are exclusively found in animals. Bovine rotaviruses generally belong to G6 or G10 and have been isolated from calves in most parts of the world (6, 40, 54, 61). A few isolates of bovine rotaviruses that belong to G8 were also reported recently (46). The rotavirus P serotypes determined by VP4 have not been well characterized. So far, three P types have been identified among bovine rotaviruses, with those of NCDV, UK bovine, and B223 representing each of them (38). In Australia, the widespread occurrence of bovine rotaviruses was discovered by gel electrophoresis of dsRNAs in 1978, but further studies have been limited (2, 49). Recent outbreaks of calf diarrhea have inflicted heavy losses in dairy and beef calves and prompted us to investigate the etiologic agents. In 1988, a total of 78 samples were collected during outbreaks of diarrhea in three farms in Victoria, Australia, and they were subjected to molecular and serological analyses.

In this study, we report the isolation and characterization of the predominant G serotypes of bovine rotaviruses from these outbreaks. Two isolates (B-11 and B-60) were adapted to growth in cell culture, their G serotypes were assessed, the sequences of their VP7 genes were determined, and their segments 4 were compared. While the B-60 strain was clearly identified as G6, similar to UK bovine rotavirus, strain B-11 was similar to the recently described bovine strains 61A and B223 in its VP7, but it differed from UK bovine or NCDV rotavirus in its VP4 (35, 44, 56, 63). In

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addition, B-11 was found to be serologically related to porcine G4 strains by one-way cross-neutralization.

## MATERIALS AND METHODS

**Collection of specimens and detection of rotaviruses by ELISA.** Seventy-eight fecal samples were individually collected during outbreaks of calf diarrhea in three separate farms in Victoria, Australia, in 1988. One fecal and two MA104 cell-adapted rotavirus samples were also kindly provided by the Veterinary Research Institute in Adelaide, Australia, about 800 km away from the farms from which our other samples were collected. Clarified suspensions (10%) in Eagle's minimum media supplemented with 2 µg of gentamicin per ml and amphotericin B were used for the detection of rotavirus group A antigen by ELISA with hyperimmune Northern Ireland calf rotavirus antisera by the methods of Beards et al. (5). Strong positive samples, which gave optical density values of  $\geq 2$ , were selected for virus isolation. In addition, all samples were screened by polyacrylamide gel electrophoresis (PAGE) as described previously (29) to identify the migration patterns of dsRNAs and to identify possible non-group A bovine rotaviruses.

**Adaptation of rotaviruses to growth in MA104 cells.** Two strains, B-11 and B-60, from fecal samples positive for rotavirus RNA by ELISA and PAGE were successfully adapted to MA104 cells. The cells were grown in Eagle's minimal essential medium with 0.02 N *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 2 µg of gentamicin per ml, amphotericin B, and 10% fetal calf serum. Before inoculation, the cells were washed with maintenance medium to remove the fetal calf serum. Virus inocula were pretreated with porcine trypsin (10 µg/ml) for 0.5 h at 37°C. After 1 h of adsorption at 37°C, the inocula were replaced with serum-free maintenance medium supplemented with HEPES, antibiotics, and 0.5 µg of porcine trypsin per ml. The cell-adapted strains were subjected to plaque purification three times before further amplification. Attempts to grow the other sample strains in cell culture were not successful.

**Antisera, monoclonal antibodies, and reference viruses.** Antiserum to B-11 was made in a rabbit as described previously (4). The additional reference hyperimmune sera used were against RV-5, SA-11, and UK bovine (I. Lazdins, Department of Microbiology, University of Melbourne, Australia); BEN-144 and TFR-41 (H. S. Nagesha); B37 and F45 (M. J. Albert, Royal Children's Hospital, Victoria, Australia); and B223, E4046, V1005, and K923 (D. R. Snodgrass).

VP7-specific neutralizing monoclonal antibodies against G1 human strain RV-4 (RV-4:1 and RV-4:2) (14), SA11 (A10, A11, and B8) (17), CRW-8 (C3/1) (41), BEN-144 (B2/4) (41), UK (H7) (55a), and rhesus rotavirus (57-8) (37) were also tested.

The reference rotavirus strains used were human Wa (G1); human RV-5 (G2); simian SA-11 (G3); porcine BMI-1 (G4); reassortant BUK (BEN-144 × UK bovine, containing BEN-144 VP7; G4 porcine); porcine TFR-41 (G5); UK bovine (G6); bovine NCDV (G6); human B37 (G8); human F45 (G9); and bovine B223, E4046, K923, and V1005 (G10).

**Immunofluorescence and neutralization tests.** Immunofluorescence and neutralization tests were performed in microtiter plates as described previously (42). Immunofluorescence tests were used to monitor the virus titers at each passage, and neutralization tests were used to serotype the virus isolates. Neutralization titers were expressed as reciprocals of the highest antibody dilution which reduced by 50%

the number of the immunofluorescent foci (5, 13, 42). Two viruses were considered serotypically different if the ratio between the homologous and the heterologous neutralizing titers was greater than or equal to 20, whereas if the ratio was less than 20, they were regarded to be antigenically related and were assigned to the same serotype (32).

**Virus purification and transcription.** Viruses were purified from fecal samples and from infected cells by previously described methods (35). In cases of small amounts of samples, like the ones from Adelaide, viruses were directly pelleted for transcription without going through a glycerol gradient. Transcription was carried out as described by Cohen et al. (12), except that sodium acetate was not added to the reaction.

**Reverse transcriptase sequencing.** The dideoxy chain termination sequencing method adapted for reverse transcriptase was used to sequence viral transcripts (17, 33, 51). Synthesis and preparation of sequencing oligonucleotides were done as described elsewhere (17).

**Northern blot hybridization.** Northern blot hybridization was performed as described by Nakagomi et al. (43). Briefly, genomic dsRNAs were fractionated in a 10% discontinuous polyacrylamide gel and were alkaline blotted onto a Zeta Probe membrane (Bio-Rad). Single-stranded RNA probes (mRNA) were prepared by *in vitro* transcription of single-shelled rotavirus particles (12). Prehybridizations and actual hybridizations were carried out in 30 ml of buffer containing 2.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 50% deionized formamide, 1% sodium dodecyl sulfate (SDS), 5× Denhardt solution, and 1 mg of sonicated salmon sperm DNA at 55°C for 3 and 20 h, respectively. After hybridization, the membranes were washed four times in 1× SSC-0.1% SDS at room temperature, twice in 2× SSC-0.1% SDS at 55°C, and twice in 1× SSC at 55°C. The membranes were then blot dried and exposed to X-ray film at -60°C.

**Nucleotide sequence accession numbers.** The nucleotide sequence accession numbers for B-11 and B-60 VP7 sequences in GenBank are M64679 and M64680, respectively.

## RESULTS

**ELISA and PAGE of fecal specimens.** Of 78 fecal samples collected in Victoria, 38 were positive for rotavirus group

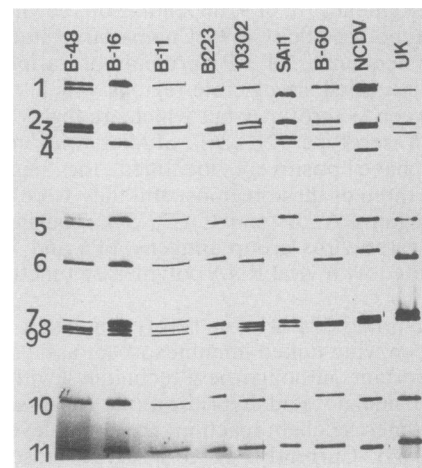


FIG. 1. Polyacrylamide gel analysis of rotavirus dsRNAs extracted from fecal samples and infected cells (as described in the text).

TABLE 1. Fluorescent focus neutralization test

Rotavirus strain	G sero-type	Titer with antiserum against <sup>a</sup> :								
		Wa	RV-5	SA11	BEN-144	TFR-41	UK	B37	F45	B-11
Wa	1	<b>32,000</b>	NT	NT	NT	NT	NT	NT	NT	≤200
RV-5	2	NT	<b>204,800</b>	NT	NT	NT	<200	NT	NT	≤200
SA11	3	NT	NT	<b>800,00</b>	150	≤200	200	NT	NT	≤200
BUK	4	NT	NT	NT	<b>200,000</b>	200	200	NT	NT	25,600
BMI-1	4	NT	NT	NT	<b>200,000</b>	200	200	NT	NT	25,600
TFR-41	5	NT	NT	NT	NT	<b>200,000</b>	<200	NT	NT	≤200
UK	6	≤200	≤200	≤800	≤800	<200	<b>740,000</b>	≤800	200	<200
B-60	6	<200	<200	<800	<200	200	<b>400,000</b>	<200	<200	≤200
B37	8	NT	NT	NT	NT	NT	NT	<b>94,000</b>	NT	<200
F45	9	NT	NT	NT	NT	NT	NT	NT	<b>25,600</b>	≤200
B-11	10	≤200	≤200	≤200	≤200	≤200	≤200	≤200	<200	<b>51,200</b>

<sup>a</sup> The numbers are the reciprocals of antibody dilutions that gave a 50% reduction in the numbers of fluorescent foci. Two neutralizing monoclonal antibodies made against RV-4, RV-4:1 and RV-4:2, were used (14). Homologous titer are given in boldface type. The underlined data were extracted from previously published reports (1, 14, 42). NT, not tested.

antigen after screening by ELISA, and 20 of these displayed dsRNA patterns typical of group A rotaviruses by PAGE. The rest of the ELISA-positive and -negative fecal samples did not exhibit any sign of dsRNA bands even after they were concentrated. The discrepancies between ELISA and PAGE may mean that too few virus particles were present in the sample or that viruses had broken down. No non-group A bovine rotavirus RNA migration patterns were found. Although samples containing more than one virus electropherotype were common, the isolates could be roughly grouped into two electropherotypes, with B-11 and B-60 serving as representative electropherotypes (Fig. 1). B-11 genomic dsRNAs migrated slightly differently from those of B223 and differently from those of B-60 in segments 1, 2, 3, 4, 7, 8, and 9 (Fig. 1).

**Adaptation to cell culture and serological analysis.** Strains B-11 and B-60 were adapted to MA104 cells, and both could easily be grown to high titers ( $10^7$  and  $10^6$  focus-forming units per ml, respectively). Attempts to cultivate the other isolates in MA104 cells were not successful, even though viruses directly purified from the fecal samples gave good yields of transcripts (data not shown). Neutralization tests showed that B-60 reacted with UK bovine rotavirus antiserum to a high titer (Table 1), and thus, it was considered to be a member of the same G serotype, i.e., G6. The other virus, B-11, was neutralized by antisera to some G10 rotavirus strains (Table 2) but not by antisera to rotaviruses of serotype G1 to G9 of animal and human origin (Table 1). B-11 antiserum raised in a rabbit reacted with G10 strains and cross-reacted with G4 porcine isolates BMI-1 and BUK to high titers (Table 1). The three samples from Adelaide were also serotyped; two of them were like B-11, and the

other one was like B-60 (data not shown). VP7-specific neutralizing monoclonal antibodies against RV-4 (RV-4:1 and RV-4:2), SA11 (A10, A11, and B8), CRW8 (C3/1), BEN-144 (B2/4), UK (H7), and rhesus rotavirus (57-8) were also tested, but none of them showed significant titers against B-11 (Table 2).

**VP7 sequences.** Sequencing was performed to confirm the serological relationships of B-11 and B-60 determined by neutralization tests. The VP7 genes of B-11 and B-60 were completely sequenced and found to share the common features of the previously published VP7 genes (19). They were both 1,062 nucleotides long with two in-phase potential initiation codons and a common termination codon (Fig. 2). Nucleotide sequence homologies were about 80% between the B-11 VP7 gene and those of the reported rotaviruses except G10 bovine isolates 61A and B223, which shared over 90% similarities with B-11 in their VP7 genes. Particularly high homologies (about 95%) were found between B-60 and G6 strains UK and NCDV (18, 21).

The deduced amino acid sequence of B-11 VP7 protein indicated that it possessed two potential glycosylation sites at amino acid positions 69 and 238. The potential carbohydrate attachment sites at residue 145 in G10 strains, at residue 146 in G2 strains, and at residue 318 in G6 UK bovine isolate were not found in B-11. The B-11 VP7 amino acid homology with that of G10 strain B223 was markedly high (97.8%); however, five of seven amino acid changes were found in the antigenic B and C regions (Fig. 3). One of the two remaining amino acid variations was found at residue position 165, at which a generally conserved cysteine was replaced by a serine in B223, and the other was at amino acid residue 238, which resulted in B-11 possessing a poten-

TABLE 2. Antigenic characterization of G10 strains with G10 rotavirus antisera and monoclonal antibodies<sup>a</sup>

Virus	Titer with antisera to:					Titer with monoclonal antibody:						
	B-11	B223	E4046	V1005	K923	57/8	C3/1	B2/4	A10	A11	B8	H7
B-11	<u>1,280</u>	160	320	<10	1,280	<10	<100	<100	<100	<100	<100	<100
B223	80	<u>2,560</u>	10,240	<10	10,240	81,920	NT	NT	NT	NT	NT	NT
E4046	20	<u>5,120</u>	10,240	40	10,240	5,120	NT	NT	NT	NT	NT	NT
V1005	<10	640	10,240	<u>640</u>	10,240	<10	NT	NT	NT	NT	NT	NT
K923	<10	640	2,560	40	<u>5,120</u>	20	NT	NT	NT	NT	NT	NT

<sup>a</sup> The tests with antisera to B-11, B223, E4046, V1005, and K923 and monoclonal antibody 57/8 were performed in Britain. B-11 antiserum was freeze-dried and sent from Australia. The homologous titers are underlined. NT, not tested.

	10	20	30	40	50	60	70	80	90	100	
B-11	GGCTTTAAAA	GCGAGAATTT	CCGTTTGGCT	AGCGGTTAGC	<u>TCCTTTTAAAT</u>	<u>GTATGGTATT</u>	GAATATACCA	CATTCTAAT	CTATTTGATA	TCAATTATAT	
	*****	*****	*****	*****	*****	*****	*****	*** **	** **	* **	
B-60	GGCTTTAAAA	GCGAGAATTT	CCGTTTGGCT	AGCGGTTAGC	<u>TCCTTTTAAAT</u>	<u>GTATGGTATT</u>	GAATATACCA	CAATTCTAAT	CTTCTTGACA	TCGATTACAT	
	110	120	130	140	150	160	170	180	190	200	
B-11	TACTTAATTA	CATATTAAG	AGTATAACTA	<u>GAATGATGGA</u>	CTACATAATT	TACAAATTTT	TGCTTATAGT	CACAATTAAT	TCAATTGTTG	TTAATGCACA	
	* * * * *	* * * * *	*****	*****	*****	*****	*****	** *	* * *	* * * * *	
B-60	TGTTGAACTA	TATCTTGAAA	TCAATAACGA	<u>GAATAATGGA</u>	CTATATAAAT	TACAGGTTTC	TGCTTATAGT	GACTGTCTTG	GCCACCATGA	TAAATGCACA	
	210	220	230	240	250	260	270	280	290	300	
B-11	AAATTACGGT	ATCAATTTAC	CAATAACCGG	ATCAATGGAT	ACGTCATATG	TGAATGCTAC	TAAAGATGAG	CCATTTCTAA	CATCAACATT	ATGCTATATC	
	*****	*****	*****	*****	*****	** **	** **	*****	*****	*****	
B-60	AAACTATGGA	GTGAATCTGC	CAATTACAGG	TTCGATGGAT	ACTGTATATG	CAAACCTCTAC	GCAAAGTGAG	CCATTTTTTAA	CATCAACTCT	TTGTTTGTAT	
	310	320	330	340	350	360	370	380	390	400	
B-11	TATCCAACAG	AAGCTAGGAC	AGAAATAAAC	GATAATGAAT	GGACAAGTAC	GTTGTGCGAG	CTGTTCTTAA	CAAAGGGATG	GCCGACTGGA	TCCGTATATT	
	*****	** *	*****	*****	*****	*****	*****	*****	*****	*****	
B-60	TATCCTGTTG	AGGCATCAAA	CGAATAGCT	GATACCGAAT	GGAAGATAC	CTTATCACAA	CTGTTTTTAA	CGAAGGGATG	GCCAACTGGA	TCGTTATATC	
	410	420	430	440	450	460	470	480	490	500	
B-11	TTAAAGAATA	TGATGATATA	GCCACTTTTT	CAGTGGATCC	ACAATTGTAT	TGTGACTATA	ACATAGTTTT	AATGAGATAC	AATTCGAACA	TAGAAGTGA	
	*****	** **	** * *	* * * * *	* * * * *	* * * * *	* * * * *	*****	*****	*****	
B-60	TTAAAGAATA	TGCTGATATA	GCGGCCTTTT	CTGTGGAGCC	ACAGTTATAC	TGTGATTATA	ATTTAGTTTT	AATGAAATAT	GATTCTACAC	AGGAACTAGA	
	510	520	530	540	550	560	570	580	590	600	
B-11	TATGTCGGAA	TTGGCAAATC	TAATATTGAA	TGAATGGCTA	TGCAATCCAA	TGGACATTAC	ATTGTACTAT	TACCAACAGA	CGGATGAGCC	AAACAATGG	
	*****	*****	*****	*****	*****	*****	*****	*****	*****	*****	
B-60	TATGTCGAA	CTGGCCGATC	TTATACTAAA	CGAATGGCTG	TGCAATCCAA	TGGGAATAAC	GCTGTATTAT	TATCAACAGA	CAGATGAGGC	AAATAAATGG	
	610	620	630	640	650	660	670	680	690	700	
B-11	ATAGCAATGG	GACAATCATG	TACAATAAAA	GTGTGTCCAT	TGAATACCCA	AACGCTAGGA	ATAGGATGTC	AGACAACAGA	TACTGAAACC	TTTGAAGAG	
	*****	* * * * *	* * * * *	* * * * *	*****	*****	*****	*****	* * * * *	*****	
B-60	ATATCAATGG	GTTCTTCTTG	CACAGTCAAA	GTATGTCCGT	TGAATACGCA	AACACTTGGT	ATTGGATGTC	TAATAACTAA	TCCAGACACA	TTTGAACC	
	710	720	730	740	750	760	770	780	790	800	
B-11	TCGCAACGGC	TGAAAAATTG	GTAATTACTG	ATGTGGTTGA	CGGCGTAAAT	CACAAACTAA	ATGTTACTAC	TGCGACCTGT	ACTATCAGAA	ATTGTAAGAA	
	* * * * *	* * * * *	* * * * *	* * * * *	* * * * *	* * * * *	* * * * *	* * * * *	*****	* * * * *	
B-60	TTGCGACAAC	GGAGAAGTTA	GTGATTACGG	ATGTTGTAGA	TGGTGTCAAC	CATAAATTAA	ACGTCACAAC	AACAACATGC	ACTATACCGA	ACTGTAAGAA	
	810	820	830	840	850	860	870	880	890	900	
B-11	ATTGGGCCCA	AGAGAAAATG	TAGCAGTGAT	ACAAGTGGGT	GGTGCTGATA	TCCTTGATAT	AACATCTGAT	COGACGACCA	ATCCACAAAC	TGAACGGATG	
	*** **	*****	*****	*****	* * * * *	* * * * *	*****	*****	*****	** * **	
B-60	ATTAGGACCA	AGAGAGAACG	TAGCAGTCAT	ACAGGTAGGC	GCGCGAACA	TTTTAGACAT	CACAGCTGAC	CCAACGACTA	CGCCACAGAT	AGAGAGAATG	
	910	920	930	940	950	960	970	980	990	1000	
B-11	ATGCGGATAA	ATTGGAAGAA	ATGGTGCAA	GTGTTCTATA	CTATAGTTGA	TTACGTC AAC	CAAATGTGTC	AAGCAATGTC	TAAGAGGTCC	AGATCACTGA	
	*****	*****	*****	*****	* * * * *	*****	*****	*****	*****	*****	
B-60	ATGCGAATAA	ATTGGAAGAA	ATGGTGCAA	GTATTTTATA	CAGTAGTGA	TTACGTC AAT	CAGATAATTC	AAAAGATGTC	CAAAGATCT	AGATCACTTA	
	1010	1020	1030	1040	1050	1060					
B-11	ATTGAGCAGC	GTTTTATTAT	AGAGTGTAGA	TATTCTGTAG	ATTAGAGTTG	TATGATGTGA	CC				
	*** **	*****	*****	* * * * *	*****	*****	*****				
B-60	ATTGAGCAGC	GTTTTATTAT	AGAGTGTAGG	TGCATGTAG	ATTAGAGTTG	TATGATGTGA	CC				

FIG. 2. Comparison of the complete nucleotide sequences of B-11 (G10) and B-60 (G6). The initiation and termination codons are underlined. Asterisks indicate homologous bases.

tial glycosylation site at amino acid position 238 instead of at position 145 as in B223. The VP7 amino acid homologies between B-11 and the strains of the other known G serotypes fluctuated around 80% and was particularly low within the major antigenic regions (Fig. 3). However, close analysis of the A, B, and C antigenic regions revealed that the A region of B-11 VP7 showed some similarity to the corresponding region of the G4 porcine rotaviruses BEN-144 and BMI-1 (Fig. 4) (33), while the C region was most similar to that of the G8 strain 69M (Fig. 3). In the case of B-60 VP7, as indicated by the high nucleotide homologies, they were only 11 amino acid differences when it was compared with UK bovine rotavirus (G6), and none of these changes occurred in the well-studied antigenic regions (17) or at the potential glycosylation sites (Fig. 3). The VP7 sequence of B-60 agreed well with the serological data that B-60 was a G6 isolate.

The VP7 gene sequences of viral mRNAs from viruses

purified directly from fecal samples and from viruses that underwent eight passages in cell culture did not exhibit any nucleotide changes. A partial sequence spanning the antigenic A region and a sequence of the 3' termini of VP7 genes were also obtained from two isolates (10302 and 1698) sent from Adelaide (data not shown). The sequences were identical to those of B-11 VP7 (10302) or B-60 VP7 (1698) across the regions analyzed and indicated that similar strains are widespread in Australia.

**VP4 sequence analysis.** The VP4 genes of bovine rotavirus isolates B-60 and B-11 were subjected to nucleotide sequence analysis in order to determine their VP4 identities. The sequence of B-11 segment 4 has almost been completed, and about 350 bases of B-60 were sequenced around nucleotide positions 670 to 1020 (amino acid residues 220 to 395), which span the trypsin cleavage site in the VP4 gene (data not shown). Comparison of this portion of the B-60 sequence with the corresponding region of the UK bovine VP4 gene

	10	20	30	40	50	60	70	80								
B-11(10)	MYGIEYTTFL	IYLISIILLN	YILKSIIRMM	DYIIYKFLLI	VTITSIVVNA	QNYGINLPIT	GSMDTSYVNA	TKDEPFLTST								
WA (1)	I	F	V	I	R	L	AV	<u>T</u> <u>S</u> <u>OE</u> V								
RV-5 (2)	I	TI	T	NT	FR	L	IALI	PF RT MY L AV <u>T</u> <u>S</u> <u>SG</u>								
SA-11(3)	V	TF	T	L	I	F	R	F IV L PFLR A <u>A</u> <u>S</u> <u>OE</u>								
ST-3 (4)	V	F	FV	VS	T	IKI	RIAFV	IVVL VLS A <u>A</u> <u>S</u> <u>Q</u> NN V								
OSU (5)	V	TF	L	V	F	R	V	IVVLAPLIK P <u>M</u> <u>S</u> <u>TS</u> T								
B-60 (6)	I	F	T	T	I	R	VLATMI	V V <u>A</u> <u>S</u> <u>QS</u>								
UK (6)	I	F	T	T	I	R	V	VLATMI V <u>A</u> <u>S</u> <u>QS</u>								
69M (8)	T	TF	LLV	I	L	H	F	IV VTPF S N <u>Q</u> <u>V</u> <u>SNP</u>								
F45 (9)	V	TF	T	L	SA	F	RL	L IV V PF KT A <u>A</u> <u>L</u> <u>SOQ</u> T								
B223(10)																
YM (11)	I	T-	L	FIT	T	F	R	FV IVLAPF KT P <u>M</u> <u>S</u> <u>MS</u> T								
L26 (12)	I	TI	V	S	F	R	V	FV VLPFIK A <u>S</u> <u>QQ</u> S M								
L338(13)	I	TF	F	I	D	TI	TL	A	F	R	V	V	L	PLLT	ATA	<u>S</u> <u>OE</u> N V
F123(14)	C	I	TF	L	N	QLL	I	F	R	F	IVFL	PFLK	V	A	<u>S</u> <u>QENI</u>	

	90	A	100	110	120	130	140	B	150	160						
B-11(10)	LCLYYPTPEAR	TEINDNEWT	SLSQFLTKG	WPTGVS	YVYFKE	YDDIATFS	VD POLYCDYNIV	LMRYNSNIEL	DMSELANLIL							
WA (1)	S	Q	GD	KD	S	M	SN	VD	L	K	DQSL	D				
RV-5 (2)	A	KN	S	D	EN	I	D	NN	V	DNIS	A	D				
SA-11(3)	A		S	KD	E	TN	S	V	K	DATLQ	D	D				
ST-3 (4)	S	P	Q	S	T	KD	N	SNVLE	I	K	V	I	FV	GE	I	D
OSU (5)	N	A	A	TK	E	G	A	S	E	K	DG	LQ	G			
B-60 (6)	V	S	N	A	T	KD	L	A	A	E	L	K	D	TQ	D	
UK (6)	V	S	N	A	T	KD		T	A	E	L	K	D	TQ	D	
69M (8)	V	E	A	SS	KD	S	T	IN	K	A	S	D				
F45 (9)	A	S	Q	G	T	KD	T	S	I	V	K	D	TL	D		
B223(10)																
YM (11)	H	A	Q	A	DK	KD	S	T	S	K	DG	SQ	D			
L26 (12)	NSVT	T	PD	H	N	S	A	S	N	VQ	QNSLA	V	D			
L338(13)	N	VV	S	L	DS	KN	N	S	S	I	VVK	STELQ	I			
F123(14)	A	Q	D	SS	KD	I	A	L	T	TS	I	V	K	DEALQ	D	

	170	180	190	200	210	C	220	230	240									
B-11(10)	NEWLCNPM	DI TLYYYQQTDE	ANKWLIAMGQS	CTIKVCLPNT	QTLGIGCOTT	DTETFE	E	VAT	A	E	K	L	V	I	T	D	V	DGVNHLNVT
WA (1)	V	SG	S	S	S	V	NVDS	MI	EN	AV	I	I	L					
RV-5 (2)	S	SS	S	S	TD	V	K	VN	I	S	S	N	NI	IS				
SA-11(3)			S	S			L	AT	P			D						
ST-3 (4)			S	S	V		N	A	T	D	S	A	I	YI	I			
OSU (5)	M		S	T			S	INS	T	N	A	D						
B-60 (6)	G		S	S	V		LI	NPD	T	T								
UK (6)			S	S	V		LI	NPD	T	T								
69M (8)	A		S	D			L	T				Y	I					
F45 (9)							T	N	A	A		D						
B223(10)	S						N	G				D						
YM (11)			S	N			L	PT	S			D						
L26 (12)	V		S	D	V	M	T	VA	N			I						
L338(13)			S	T	V		L	D				I						
F123(14)			S	S			L	NVA	S	K	D	VCET						

	250	260	270	280	290	300	310	320	326							
B-11(10)	TATCTIRNCK	KLGP	RENVAV	IQVGGADILD	ITSDPTINPQ	TERMMRINWK	KWQV	FY	TIV	DYVNQIVQAM	SKRSRSLNSA	AFYYRV				
WA (1)	T			SNV	A	V				I	V					
RV-5 (2)	IN	N	I	PNA	A	V	VQ	I		V	I	VI	V	DA	I	
SA-11(3)				S	A	A				V	D	I	V			
ST-3 (4)	ST		I	SN	A	S		V		V	I	V		D	S	
OSU (5)	S			PN	A	A		R				V		D		
B-60 (6)	T			N	A	T	I			V		I	K	<u>S</u>		
UK (6)			I	NV	A	A				V		I	T	<u>S</u>		
69M (8)	T			SN	A	A				V		I	T	D	S	I
F45 (9)	T	R	I	SEV	A	A				V	I	V				
B223(10)																
YM (11)				SN	A	A						V			I	
L26 (12)	LN	Q		S	I	A	I		I	V	I	V			I	
L338(13)				R	STI	A	M			V	I	V		DAA	E	
F123(14)	T		I	S	A	A	IA			V	I	V		D	I	

FIG. 3. Deduced VP7 amino acid comparison of B-11 and B-60 with strains of G1 to G14 (G7 was unavailable). The VP7 sequences of Wa, RV-5, SA-11, OSU, UK, 69M, F45, 61A, B223, YM, L26, L338, and F123 are from previously published reports (7, 8, 9, 16, 18, 22, 25, 48, 50, 57, 63), and ST-3 VP7 was cloned and sequenced by C. P. Hum (33a). The antigenic sites are overlined and indicated with letters A, B, and C, while the potential glycosylation sites are underlined. The numbers in parentheses indicate the G serotypes.

	87	101
B-11	TEARTEINDNEWST	
BEN-144	Q	KD
BMI-1	Q	KD
ST-3	S P Q S T	KD
Gottfried	S P Q	KD

FIG. 4. Comparison of B-11 VP7 antigenic region A with those of G4 strains. Homologous amino acids were deleted to highlight the differences. BEN-144, BMI-1, and Gottfried sequences were from previously published reports (23, 33), while ST-3 VP7 was cloned and sequenced by C. P. Hum (33a).

showed only 87% nucleotide homology, but their amino acid homologies were about 96%. The sequence of the B-11 VP4 gene compared with the corresponding regions of the UK, NCDV, and C486 genes was remarkably different and further demonstrated that B-11 possesses a new bovine P type, as suggested by Matsuda et al. (38).

**Hybridization results.** Hybridization results confirmed that except for segment 3, B-60 was closely related to G6 UK bovine rotavirus (Fig. 5). The hybridization also revealed that B-11 and B223 were closely related in all the segments (Fig. 5). As suggested by the VP4 sequence comparison, Fig. 5 showed that B-11 and B223 had similar VP4 sequences that were different from those of UK bovine, B-60, NCDV, BMI-1, and SA11. Although we anticipated finding NCDV

VP4-related bovine rotaviruses in Australia, so far we have not come across one. In addition, Northern blot hybridization revealed that B-11 VP7 is encoded by gene segment 9 (Fig. 5). The identity of an additional band (band a) between segments 4 and 5 observed in the lane containing the UK bovine strain in the Northern hybridization (Fig. 5) was unclear.

## DISCUSSION

Bovine rotaviruses have been adapted to cell culture (3) and were previously shown to be mainly confined to G6 and G10, with NCDV and B223, respectively, serving as prototypes (40, 54, 61). Both G types of bovine rotaviruses are believed to be widespread (6, 40, 54). A few bovine isolates related to G8 were also reported recently (46, 54).

In our studies, we found that rotaviruses of two G serotypes predominated in Australian calves from which individual samples were obtained, with B-11 and B-60 representing each of them. Fecal samples manifesting more than one dsRNA electropherotype suggested that some calves may suffer multiple or mixed infections with group A rotaviruses.

The failure to adapt the other isolates to growth in MA104 cells was probably due to inhibitory agents in the fecal samples and/or damage to the viral outer capsid, which has important functions, such as affecting cell tropism, cell receptors, and penetration into the cell during virus infection (19, 20).

The major serotype-specific antigenic regions of VP7 would be expected to remain conserved between rotaviruses of the same G serotype. Green et al. (27) previously suggested a method based on the amino acid homologies of combined antigenic A and C regions to predict the G serotypes of rotaviruses; homology of greater than 85% was taken to indicate the same G serotype. In our studies, the amino acid homologies of combined A and C antigenic regions between B-11 and any of the reported mammalian rotaviruses of G1 to G9 and G11 to G14 were far below 85%, but over 90% homology was observed between B-11 and B223. In this respect, B-11 and B223 appeared to be well distinguished from other G serotypes and should be considered to have the same G serotype. By the same criteria, B-60 clearly fell into G6.

The neutralization and hybridization results further confirmed that B-60 is a member of G6 and demonstrated that UK bovine-like rotaviruses are widespread in the world (54). Northern blot hybridization revealed that all segments of B-11 and B223 are related, and the VP7 sequences confirmed their close relationship. The comparatively low cross-neutralization between B-11 and B223 is likely to be the result of amino acid differences in the B and C antigenic regions and of potential glycosylation location differences, such as those shown in SA11 mutants selected with VP7-specific neutralizing monoclonal antibodies (11). The unexpected one-way cross-reactivity observed between bovine B-11 and porcine BMI-1 and reassortant BUK could similarly be explained by their overall amino acid compositions, their similar A regions, and the different numbers of potential glycosylation sites. It seems that the antigenic region A may play a major part in VP7-mediated one-way cross-reactivity, because a virus like B-37 did not exhibit cross-reactivities with viruses that had the same antigenic C region (34).

Although the VP7 sequences of B-11 and B223 were very similar and their A regions were the same, the A region-directed broad-spectrum neutralizing monoclonal antibody 57-8 (37) reacted differently (Table 2). While it could neu-

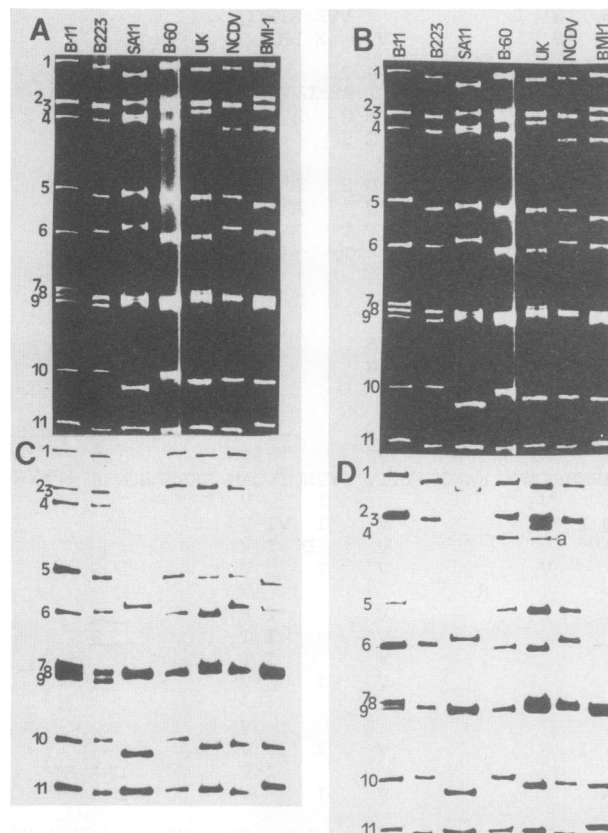


FIG. 5. Hybridization patterns of genomic dsRNAs of B-11, B223, SA-11, B-60, UK bovine, NCDV, and BMI-1 with B-11 (A and C) or UK bovine (B and D) single-stranded RNA probes. (A and B) Photographs of the gels after ethidium bromide staining and UV illumination; (C and D) the corresponding autoradiograms.

tralize B223 to a high titer (10), it did not react with B-11. The difference was possibly due to the shielding of epitopes in the A region by a carbohydrate attached to amino acid 238 in B-11 or to the two amino acid changes in the B-11 C region which could alter the tertiary structure of the epitopes, since the A and C regions are reportedly closely apposed on the native molecule (17).

It is noteworthy that the VP7 sequences of viral mRNAs from viruses purified directly from fecal samples and from viruses that underwent eight passages in cell culture did not exhibit any nucleotide changes within the VP7 gene. This may suggest that genetic drift may be a process that involves lengthy periods of time or that adaptation to cell culture selected mutations in other genes of the virus. In the case of the B-11 virus, it was probably brought into Australia in cattle from other continents by early European settlers. Because of strict quarantine procedures and the geographical isolation of the continent, B-11 may have altered its major antigenicity by genetic drifts over a long period of time. The relatively high VP4 nucleotide discrepancies in the partial sequences of B-60 and UK may also indicate that they were separated for a long time, while some conserved amino acid sequences could mean that these groups of amino acids were functionally essential.

Although the VP4 genes of B-11 and B223 seemed to be closely related, while they were different from the corresponding genes of either UK or NCDV by hybridization and sequence analyses, they behaved differently in relation to their growth characteristics in MA104 cells. B223 and related strains are reportedly very difficult to grow in MA104 cells (54), whereas B-11 could easily be grown to a high titer ( $10^7$  focus-forming units per ml). Since VP4 has been documented to be associated with viral growth (45), it will be interesting to compare the sequences of both VP4 genes to locate the region(s) that may affect cell tropism.

#### ACKNOWLEDGMENTS

We thank T. Fahey and I. Cunningham, Regional Veterinary Laboratory, Bendigo, Victoria, Australia, and the Adelaide Veterinary Institute for providing samples. We thank I. Lazdins and M. L. Dyall-Smith for helpful discussions. We also appreciate the help from J. Currie in making hyperimmune sera. J. Huang was supported by a Melbourne University scholarship.

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