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Cattle develop neutralizing antibodies to rotavirus serotypes which could not be isolated from faeces of symptomatic calves

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Neutralizing antibodies against 10 serotypes of rotavirus were measured in sera from different age groups of German cattle. Only five of 143 sera did not neutralize heterologous serotypes. Sera from 64 of 76 calves younger than 1 year neutralized bovine rotavirus NCDV (serotype 6). From these calves, sera 54, 26, 51, 24, 12, 10 and 37, in neutralized addition, the heterologous serotypes 1, 2, 3, 4, 5, 7 and 9, respectively. Thirty-eight of 46 rotavirus isolates from Bavarian calves with diarrhoea were serotyped by neutralization: 22, 2 and 14 isolates were typed as serotype 6, serotype 10 (B223) and a newly defined subtype of serotype 10 (V1005), respectively. All serotype 6 isolates and none of the serotype 10 or V1005-like viruses tested hybridized to a NCDV-specific cDNA probe. Eight isolates gave equivocal results by neutralization. We failed however to identify serotype 1, 2, 3, 4 or 8 bovine rotavirus isolates by neutralization with hyperimmune sera and dot blot hybridization with serotype-specific cDNA probes. Thus cross-reacting antibodies in cattle might not represent an anamnestic response, but the recognition of a cross-reacting neutralization epitope shared by many rotavirus serotypes.

Introduction

Rotaviruses are now recognized as the most important cause of severe viral gastroenteritis in humans (Kapikian & Chanock, 1990), and are also a major infectious cause of diarrhoea in calves (Acres *et al.*, 1977; de Leeuw *et al.*, 1980; McNulty, 1978; Woode & Bridger, 1975). Currently 11 serotypes of rotavirus have been defined (Estes & Cohen, 1989). Six serotypes (1, 2, 3, 4, 8 and 9) have been isolated from humans (Estes & Cohen, 1989). Most bovine rotaviruses (BRVs) that have been typed have belonged to serotype 6, but also serotype 10 and occasionally serotype 8 rotaviruses have been isolated from cattle (Bellinzoni *et al.*, 1989; Snodgrass *et al.*, 1984, 1990). In a survey of 85 British calf rotavirus isolates by cell culture neutralization, 91% belonged to serotype 6, 5% to serotype 10 and 1% to serotype 8 (Snodgrass *et al.*, 1984). Direct serotyping by ELISAs using monoclonal antibodies (MAbs) to VP7 of serotypes 6 and 10 identified 101 strains as serotype 6 and 11 strains as serotype 10 in 162 stool samples from British calves (Snodgrass *et al.*, 1990).

The serotypic diversity of the human rotaviruses (HRVs) has hampered the development of an efficient rotavirus vaccine for infants (Green *et al.*, 1990). Effective rotavirus vaccination has however been achieved in cattle by passive immunization through dam vaccination (Eichhorn *et al.*, 1982; McNulty & Logan, 1987; Snodgrass, 1986). A strong heterotypic immune response has been observed in cows (Brüssow *et al.*, 1988*a*; Snodgrass *et al.*, 1984) and might partly explain the efficiency of rotavirus vaccination in cattle. Single serotype vaccination of mature cows induced heterotypic milk and serum neutralizing antibodies against three BRV and four HRV serotypes (Brüssow *et al.*, 1988*a*; Snodgrass *et al.*, 1984). These puzzling results have been interpreted as an immune response to serotypes to which mature cows have been exposed in earlier life. We tested this hypothesis by investigating the age development of cross-neutralizing serum antibody in cattle and by screening BRV isolates for serological similarity with serotypes 1 to 4 rotaviruses.

Methods

Bovine serum samples. Rotavirus antibodies were quantitatively measured in a total of 143 bovine sera which were sent to the Institute of Medical Microbiology, Munich, Germany, in the years 1979 to 1983 [before the introduction of dam vaccination (Eichhorn *et al.*, 1982)] for diagnostic purposes unrelated to this study. Cases of calf diarrhoea

Table 1. *Prevalence of neutralizing antibodies to 10 rotavirus serotypes in different age groups of cattle*

		Percentage of sera neutralizing* the indicated rotavirus strain (serotype)										
		BRV					Heterologous serotypes					
Age group	*n*	NCDV† (6)	S-4‡	UK† (6)	678† (8)	B223 (10)	Wa (1)	DS-1 (2)	SA11 (3)	Hochi (4)	OSU (5)	Ty-3 (7)	WI61 (9)
0–1 m§	22	77	23	27	36	23	68 (95)	36 (50)	59 (100)	23 (45)	5 (14)	23 (32)	41 (95)
1–2 m	19	74	37	53	74	26	84 (100)	53 (74)	79 (95)	47 (74)	32 (37)	5 (21)	79 (100)
2–4 m	19	95	39	63	53	21	84 (100)	32 (47)	84 (89)	32 (42)	16 (32)	6 (17)	37 (100)
4–12 m	16	94	63	62	62	31	81 (88)	25 (50)	69 (88)	38 (56)	13 (31)	19 (19)	63 (100)
1–3 y‖	27	100	73	85	96	74	100 (100)	88 (100)	100 (100)	74 (93)	59 (89)	50 (65)	93 (100)
3–5 y	27	100	81	85	100	67	100 (100)	96 (100)	100 (100)	89 (96)	52 (70)	46 (85)	100 (100)
>5 y	13	100	85	100	100	84	100 (100)	100 (100)	100 (100)	100 (100)	73 (92)	62 (92)	100 (100)

* A serum was counted as neutralizing if a 1 : 90 or 1 : 30 (values in parenthesis) serum dilution neutralized 100 $TCID_{50}$ of the indicated rotavirus strain

† Quoted from Brüssow *et al.* (1990*a*).

‡ Reassortant rotavirus which derives 10 genes from NCDV and gene 4 from SA11 rotavirus (Offit *et al.*, 1986).

§ Month.

‖ Year.

were excluded from the study. Cattle, aged 4 days to 9 years, came from dairy farms situated within a 50 km radius of Munich. Animals more than 6 months old were almost exclusively females, whereas about half of the calves up to this age were males. The 143 sera were arranged into seven groups according to the age of the animals (see Table 1). Each serum sample represented a different animal. Ten additional foetal serum samples were collected from individual bovine foetuses at the abattoir in Munich; none showed a neutralizing activity at a 1 : 20 dilution.

Neutralizing test. Bovine sera were tested for neutralizing antibodies to the indicated rotavirus strain by the immunoperoxidase focus reduction test described by Gerna *et al.* (1984).

Briefly, MA-104 cells, which were grown in 96-well microtitre plates, were inoculated with 10^2 $TCID_{50}$ of the indicated rotavirus strain after the virus had been incubated for 1 h at 37 °C with 1 : 10, 1 : 30, 1 : 90, 1 : 270, 1 : 810, 1 : 2430 or 1 : 7290 serum dilution. Cells were incubated for 1 day, fixed with absolute ethanol and then reacted with a rabbit hyperimmune serum sample that was directed against SA11 single-shelled rotavirus particles. Finally a peroxidase-coupled goat antibody to rabbit IgG was added and intracellular viral antigen revealed by 3-amino-9-ethyl-carbazole.

Titres are expressed as the inverse of the maximum serum dilution that gave 50% or greater reduction in foci in the immunoperoxidase focus reduction test.

BRV isolation. Faecal samples were collected from 7- to 14-day-old calves with rotavirus diarrhoea from dairy farms around Munich during the years 1977 to 1983. They were adapted to cell culture as described previously (Bachmann & Hess, 1981). The bovine embryonic kidney cell line, Aubek, was grown in roller bottles or tubes, using 10% tryptose-phosphate broth and Earle's MEM supplemented with 5% foetal bovine serum (FBS). Cell cultures were infected 24 h after seeding. Inocula for cell cultures were prepared by diluting faecal samples 1 : 5 in 0·15 M-NaCl–0·05 M-phosphate buffer, containing 0·05% Tween-20. After homogenization by ultrasonic treatment (50 A for 60 s), the material was centrifuged at 2000 *g* for 30 min. The supernatant was treated with 200 μg/ml of streptomycin, 200 μg/ml of penicillin and 100 μg/ml of neomycin and was used as inoculum. The growth medium was decanted, and the cell cultures were washed once with PBS, pH 7·4. Inoculum (0·2 to 0·5 ml) was pipetted onto the cultures and was allowed to adsorb for 60 min at 37 °C. After washing with 2 ml of PBS, the viral medium was pipetted into the culture vessels containing Earle's MEM and 1% FBS to which 10% of a 0·0625% trypsin-0·025% EDTA in Ca^{2+}- and Mg^{2+}-free saline solution was added. Samples contaminated with bacteria after the isolation passage were treated with chloroform and then the isolation procedure was started again. Incubation was carried out in a roller apparatus for 3 to 7 days. Cultures were harvested by three cycles of freezing and thawing.

Serotyping of BRV isolates. Forty-six isolates grew sufficiently well on MA-104 cells to permit *in vitro* neutralization tests. As described previously (Brüssow *et al.*, 1987, 1990*b*) guinea-pig hyperimmune sera were raised against the different known BRV serotypes [NCDV, UK, 678, B223, V1005; neutralization titres (NT) ranged from 6400 to 12800] and against serotype 1 HRV Wa (NT 25600), serotype 2 HRV S-2 (NT 1600), serotype 3 rotavirus SA11 (NT 25600) and serotype 4 HRV Hochi (NT 6400). Preimmune titres of the guinea-pigs were < 50 against all rotaviruses.

Rabbit hyperimmune sera were raised against BRV UK, 678 and B223 as described previously (Brüssow *et al.*, 1990*b*); preimmune titres were < 10 against BRV UK. Two VP7-specific neutralizing MAbs, UK/7 and B223/3, were described previously (Snodgrass *et al.*, 1990). One-hundred $TCID_{50}$ of the BRV isolates was tested against this panel of hyperimmune sera and MAbs in the neutralization test of Gerna *et al.* (1984).

Reverse transcription (RT)/amplification and dot blot hybridization. Double-stranded RNA of CsCl-purified rotaviruses ranging from 500 ng to 1 μg was mixed with DMSO (final concentration 10%) and 1 μM of each primer in a total volume of 25 μl. Primer 1 corresponds to nucleotides 45 to 65 and primer 2 corresponds to nucleotides 1048 to 1062 of the conserved 5′ and 3′ ends of the negative and positive strands of segment 9, respectively.

This mixture was heated for 3 min at 95 °C and then cooled to 37 °C. One volume of 2 × RT/polymerase chain reaction (PCR) buffer and 3 μl enzyme mix were added to each tube. The reaction mixture was overlaid with 50 μl mineral oil. The 2 × RT/PCR buffer consisted of 60 mM-Tris–HCl pH 8·3, 110 mM-KCl, 3 mM-$MgCl_2$, 0·7 mM-DTT and 1 mM of each dNTP. Reverse transcriptase (Bioexcellence) and AmpliTaq polymerase (Perkin Elmer Cetus) were diluted in 1 × RT/PCR buffer so that 3 μl enzyme mix contained 1·2 units

AmpliTaq polymerase and 5 units reverse transcriptase. After incubation at 37 °C for 30 min the PCR was started immediately. The PCR programme consisted of 25 cycles of 93 °C (1 min), 55 °C (1 min) and 72 °C (2·5 min), and a final 10 min incubation at 72 °C.

The amplification product was electrophoresed on a 3% NuSieve GTG agarose gel. The cDNA was recovered by phenol extraction. The cDNA probe was labelled with [α-^{32}P]dATP using a commercial oligolabelling kit (Amersham).

Viral RNA was extracted from CsCl-purified BRV by phenol–chloroform and concentrated by ethanol precipitation. RNA ranging from 100 to 150 ng was applied to Hybond nylon membrane (Amersham) as described by Zheng *et al.* (1989). The nylon membrane was preincubated in 20 × SSC (1 × SSC is 0·15 M-NaCl plus 0·015 M-sodium citrate). Prehybridization, hybridization and washing of the nylon membrane were carried out as described by Zheng *et al.* (1989).

Results

Prevalence, titres and specificity of rotavirus neutralizing antibodies in bovine sera

The levels of neutralizing antibody to rotavirus serotypes 1 (Wa), 2 (DS-1), 3 (SA11), 4 (Hochi), 5 (OSU), 6 (NCDV), 7 (Ty-3), 8 (678), 9 (WI61) and 10 (B223) were measured in 143 sera from cattle belonging to different age groups. Prevalences are reported in Table 1 and geometric mean titres (GMTs) in Table 2. Sera from 64 of 76 calves (84%) younger than 1 year neutralized BRV NCDV. Interestingly, prevalences and GMTs of antibody to BRV UK are lower than those to BRV NCDV (Tables 1 and 2). This is also true for titres to the S-4 reassortant rotavirus (Tables 1 and 2) which contains 10 NCDV-derived genes, but an SA11-derived VP4 (Offit *et al.*, 1986). Of the 64 sera with neutralizing activity to BRV NCDV, 54 (84%), 26 (41%), 51 (80%), 24 (38%), 12 (19%), 10 (16%) and 37 (58%) sera neutralized in addition the heterologous serotypes 1, 2, 3, 4, 5, 7 and 9, respectively. There was a substantial prevalence increase when a serum was counted as neutralizing if a 1 : 30 but not 1 : 90 dilution neutralized the indicated rotavirus strain (Table 1). All 67 animals older than 1 year neutralized BRV NCDV; 67 (100%), 60 (90%), 67 (100%), 57 (85%), 39 (58%), 33 (49%) and 63 (94%) sera neutralized in addition the heterologous serotypes 1, 2, 3, 4, 5, 7 and 9, respectively. Overall we identified only five sera that did not cross-neutralize heterologous serotypes. In all age groups the majority of the neutralizing sera showed multiple reactivities with heterologous rotavirus serotypes (Table 3). In Fig. 1 antibody titres to BRV NCDV are plotted against titres to simian rotavirus SA11 for all individual bovine sera. Both in calves and in cattle older than 1 year, neutralizing antibody titres were nearly as high against the heterologous serotype 3 simian rotavirus SA11 as against the homologous serotype 6 BRV NCDV. We verified that the fourth gene of our SA11 strain did not comigrate with the fourth gene of NCDV rotavirus, but migrated as reported for the authentic SA11-SEM strain (Nishikawa *et al.*, 1988).

Serotype 6 BRV isolates

Twenty-two of 46 BRV isolates (48%) were neutralized with a high titre (NT >1000) by a serotype 6-specific rabbit hyperimmune serum (Table 4). All 22 isolates were also neutralized with a high titre by a guinea-pig hyperimmune serum to BRV UK (serotype 6, data not shown). In addition all but three (1050/78, 1150/78 and 274/83) of these 22 isolates were neutralized with high titres (NT $\geqslant 3200$) by a serotype 6-specific MAb (Table 4). Furthermore this MAb did not react with any isolate not neutralized by the serotype 6-specific hyperimmune serum. Only one (1150/78) of these 22 isolates was neutralized with a titre of $\geqslant 800$ by a serotype 10-specific rabbit hyperimmune serum and none was neutralized by a V1005-specific guinea-pig hyperimmune serum or a MAb to serotype 10. Six of the 22 strains were also neutralized with a titre of $\geqslant 800$ by a serotype 8-specific rabbit hyperimmune serum.

Table 2. *Geometric mean titre of neutralizing antibodies to 10 rotavirus serotypes in different age groups of cattle*

		GMT to the indicated rotavirus strain (serotype)											
		BRV					Heterologous serotypes						
Age group	*n*	NCDV† (6)	S-4*	UK† (6)	678† (8)	B223 (10)	Wa (1)	DS-1 (2)	SA11 (3)	Hochi (4)	OSU (5)	Ty-3 (7)	WI61 (9)
0–1 m	22	122	12	16	42	30	73	32	112	30	15	9	100
1–2 m	19	152	25	50	80	58	110	71	141	56	35	5	251
2–4 m	19	257	43	56	45	28	102	35	112	31	22	2	100
4–12 m	16	369	71	105	113	45	91	39	125	31	17	3	141
1–3 y	27	603	145	167	277	282	214	354	398	141	125	37	562
3–5 y	27	480	206	227	544	224	263	1000	501	177	100	47	794
>5 y	13	622	393	419	578	447	213	501	707	398	177	81	1000

* Reassortant rotavirus which derives 10 genes from NCDV and gene 4 from SA11 rotavirus (Offit *et al.*, 1986).

† Quoted from Brüssow *et al.* (1990*a*).

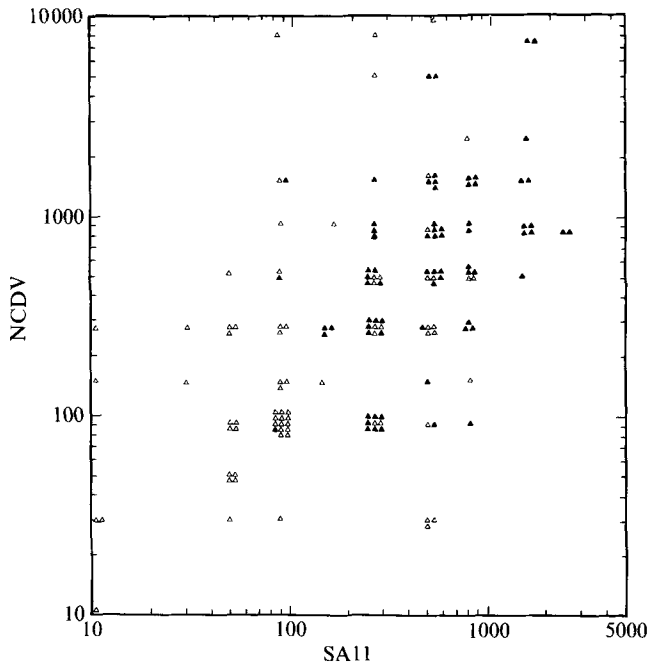

Fig. 1. For 76 calves younger than 1 year (△) and 67 animals older than 1 year (▲) the serum neutralizing antibody titre to BRV NCDV is plotted against the titre to SA11 rotavirus (serotype 3).

Table 3. *Specificity of neutralizing antibody in different age groups of cattle*

Age group	Number of sera neutralizing at least one BRV strain*	Number of sera also neutralizing the indicated number of heterologous rotavirus serotypes†			
		None	One	Two	Three or more
0–1 m	17	1 (0)	5 (0)	3 (0)	8 (22)
1–2 m	17	1 (0)	0 (0)	2 (0)	14 (19)
2–4 m	18	2 (0)	0 (0)	6 (2)	10 (17)
4–12 m	15	1 (0)	2 (1)	3 (0)	9 (15)
1–3 y	27	0	0	1 (0)	26 (27)
3–5 y	27	0	0	0	27 (27)
>5 y	13	0	0	0	13 (13)

* A serum was counted as positive if a 1:90 serum dilution neutralized at least one of the following four BRVs: NCDV, UK, 678, V1005.

† A serum was counted as positive if a 1:90 or 1:30 (values in parenthesis) serum dilution neutralized one of the following heterologous rotavirus serotypes: Wa, DS-1, SA11, Hochi, OSU, Ty-3, WI61.

Serotype 10 BRV isolates

Two isolates (216/78 and 103/79) were neutralized by a serotype 10-specific MAb; both isolates were also neutralized with a titre of 1600 and 400, respectively, by serotype 10-specific rabbit hyperimmune serum (Table 4). Overall only three isolates were neutralized by this serum with NTs >400.

V1005-like BRV isolates

Twenty-two of the 46 isolates (48%) could not be serotyped by MAbs and rabbit hyperimmune sera. Previously we have identified a BRV V1005, a new subtype of serotype 10 rotavirus (Brüssow *et al.*, 1990*b*). Interestingly 14 isolates (30%) that could not be typed and one of the two serotype 10 isolates were neutralized with high titres (NT >1000) by a V1005-specific guinea-pig hyperimmune serum (Table 4), whereas the 22 putative serotype 6 isolates were not neutralized at all (NT ≤100). Ten of the putative V1005-like isolates showed no significant neutralization by rabbit hyperimmune sera to serotypes 6 and 8. Notably only one of the 15 isolates neutralized by V1005-specific guinea-pig antiserum was neutralized by the MAb to serotype 10 (216/78) and only two (216/78 and 314/78) were neutralized with titres >400 by serotype 10-specific rabbit hyperimmune serum.

Untypable rotavirus isolates

Eight BRV isolates (17%) could not be serotyped by the antisera and MAbs described in Table 4. Therefore all isolates were retested against guinea-pig hyperimmune sera raised against BRV UK, 678, B223 and V1005 (Table 5). Six strains were significantly neutralized by a guinea-pig serum to BRV UK, two strains were significantly neutralized by a serum to BRV 678 and one strain was significantly neutralized by a serum to BRV B223. Notably none of the eight strains was significantly neutralized by guinea-pig sera to serotype 1 to 4 rotaviruses (Table 5). Neutralization was taken as being significant if less than a 20-fold titre difference was observed in comparison with the homologous titre. We raised a guinea-pig hyperimmune serum to isolate 28/78, one of the untypable rotavirus isolates. This serum neutralized BRV NCDV with a titre of 6400, but titres were <50 against serotype 1 to 4 rotaviruses. Four of the untypable isolates reacted under stringent hybridization conditions with a gene 9-specific DNA probe of BRV NCDV (Table 4 and below).

Screening for serotype 1 to 4 bovine isolates

None of the 38 typed and the eight untyped isolates was significantly neutralized by guinea-pig hyperimmune sera to serotype 1 and 2 rotaviruses (Table 5 and data not shown). Only one isolate (675/78) was significantly neutralized by a guinea-pig hyperimmune serum to serotype 3 (titre 800, homologous titre 12000), but isolate

Table 4. *Serological characterization of BRV isolates from Germany by using the neutralization test and dot blot hybridization*

Isolate Code/year	Titre* of hyperimmune antiserum† or MAbs‡ raised against the indicated rotavirus serotype						Serological diagnosis	Hybridization with NCDV- and B223-specific cDNA probe§		Hybridization diagnosis
	R/6	R/8	R/10	G/V1005	MAb/6	MAb/10		NCDV	B223	
627/77‖	600	300	<100	<100	<50	<50	?	++	(+)	6
666/77‖	200	100	100	<100	<100	<100	?	+	(+)	6?
808/77	600	300	400	100	<50	<50	?	NT	NT	NT
1429/77	>12000	400	100	<100	>6400	<50	ST 6	++	−	6
10/78	400	800	400	1600	<50	<50	V1005?	−	−	?
28/78	400	400	600	<100	<50	<50	?	−	−	?
124/78	6400	200	100	<100	>6400	<100	ST 6	++	−	6
216/78	100	200	1600	1600	<100	6400	ST10	−	++	10
303/78	>12000	1600	200	<100	>6400	50	ST 6	+	−	6
314/78	100	100	600	2400	<100	<100	V1005	−	(+)	10?
566/78	>12000	800	200	<100	>6400	<100	ST 6	NT	NT	NT
601/78‖	8000	300	<100	<100	>12000	<100	ST 6	++	−	6
623/78‖	6400	100	100	<100	>6400	<50	ST 6	++	−	6
675/78	>12000	300	<100	<100	>12000	<100	ST 6	++	−	6
915/78‖	200	400	<100	<100	<100	<100	?	+	(+)	6?
935/78	200	100	100	<100	<50	<50	?	NT	NT	NT
955/78	1600	<100	400	<100	3200	<50	ST 6	++	(+)	6
1050/78	6400	100	100	<100	<100	<100	ST 6	++	−	6
1150/78	>12000	800	800	<100	<50	<50	ST 6	+	−	6
1237/78	>12000	200	<100	<100	3200	<50	ST 6	++	(+)	6
1311/78	>12000	400	400	<100	>6400	<50	ST 6	NT	NT	NT
1334/78	800	800	100	3200	<50	<50	V1005	−	(+)	10?
1446/78	>12000	400	<100	<100	>12000	<100	ST 6	++	−	6
103/79	100	200	400	800	<50	1600	ST10	NT	NT	NT
415/79	>12000	400	100	<100	>6400	<50	ST 6	NT	NT	NT
470/79	100	200	200	3200	<50	<50	V1005	−	(+)	10?
522/79	12000	300	300	<100	>12000	<100	ST 6	++	−	6
616/79	200	200	300	3200	<50	<50	V1005	−	(+)	10?
638/79	400	400	400	6400	<100	<100	V1005	−	−	?
681/79	200	200	400	3200	<50	<50	V1005	NT	NT	NT
718/79‖	400	400	400	6400	<100	<100	V1005	−	(+)	10?
734/79‖	100	100	200	3200	<50	<50	V1005	−	−	?
770/79	>12000	800	300	<100	>6400	<100	ST 6	+	−	6
437/80‖	800	800	100	<100	<50	<50	?	+	(+)	6?
474/80‖	400	400	400	6400	<100	<100	V1005	−	−	?
479/80	200	400	400	3200	<50	<50	V1005	−	−	?
196/81	100	200	100	6400	<50	<50	V1005	NT	NT	NT
445/82	>12000	400	100	<100	>6400	<50	ST 6	NT	NT	NT
468/82	>12000	800	400	<100	>6400	<50	ST 6	++	−	6
951/82	400	400	200	1600	<50	100	V1005	NT	NT	NT
125/83	>12000	200	100	<100	>12000	<100	ST 6	+	−	6
274/83	6400	400	100	<100	<50	<50	ST 6	++	−	6
595/83‖	400	800	<100	<100	<50	<50	?	−	−	?
709/83‖	3200	800	−	<100	−	−	ST 6	+	(+)	6?
768/83	100	400	400	1600	<50	<50	V1005	NT	NT	NT
238/84	>12000	800	200	<100	>6400	100	ST 6	++	−	6
Control viruses										
UK	>12000	400	400	<100	>6400	<50	ST 6			
NCDV	>12000	400	400	<100	>6400	100	ST 6			
678	400	>12000	100	<100	<50	<50	ST 8			
B223	200	200	3200	800	<50	3200	ST 10			
V1005	200	400	200	6400	<50	50	V1005			
OSU (ST 5)	100	100	400	<100	<50	<50	Unrelated			

* Titres are expressed as the inverse of the maximum serum dilution that gave 50% or greater reduction in foci in the immunoperoxidase focus reduction test described by Gerna *et al.* (1984).

† R/6, R/8, R/10: rabbit hyperimmune sera to BRV UK (serotype 6), 678 (serotype 8) and B223 (serotype 10), respectively. G/V1005: guinea-pig hyperimmune serum to BRV V1005 (Brüssow *et al.*, 1987, 1990*b*)

‡ MAb/6 and MAb/10: murine MAbs to rotavirus UK and B223, respectively (Snodgrass *et al.*, 1990)

§ Rating of hybridization is: ++, strong; +, medium; (+), weak; −, negative; NT, Not tested.

‖ More than 11 RNA segments were revealed by silver staining of the polyacrylamide gel.

Table 5. *Serological analysis of eight untypable BRV isolates from Germany*

Isolate	Titre* of guinea-pig hyperimmune sera raised against the indicated rotavirus serotype (strain)							
Code/year	6 (UK)	8 (678)	10 (B223)	10 (V1005)	1 (Wa)	2 (S-2)	3 (SA11)	4 (Hochi)
627/77†	800	800	<100	<100	<100	<100	<100	<100
666/77†	300	200	<100	<100	<100	<100	<100	<100
808/77	3200	800	300	100	<100	100	<100	100
28/78	800	400	200	<100	<100	<100	<100	<100
915/78†	400	400	<100	<100	<100	<100	400	100
935/78	1200	300	<100	<100	<100	<100	<100	<100
437/80†	800	400	<100	<100	<100	<100	<100	<100
595/83†	800	400	<100	<100	<100	<100	<100	<100
Homologous virus	12800	12800	6400	6400	12800	1600	12800	12800

* See Table 5 for specifications.

† More than 11 RNA segments were revealed by PAGE.

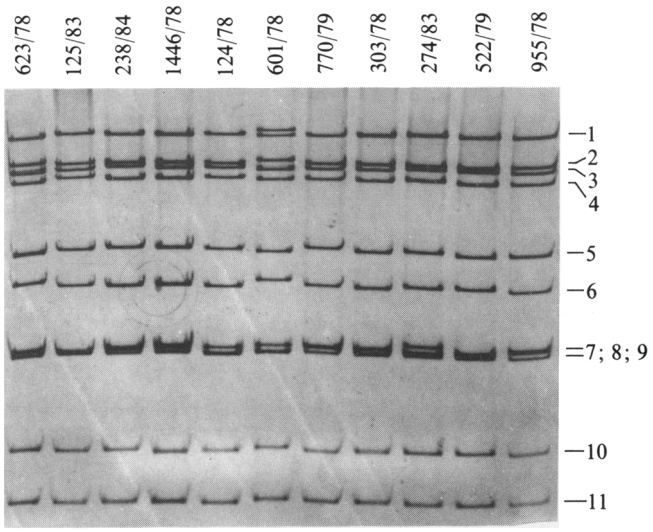

Fig. 2. Polyacrylamide gel showing a comparative genome analysis of 11 bovine isolates analysed in the study. The individual isolates are identified by their code number/year of isolation (see Table 4). The order of the genome segments is given on the right. Electrophoresis and silver staining were done according to Herring *et al.* (1982). All isolates were serotype 6 both by serology and hybridization.

675/78 is clearly a serotype 6 strain (Table 4). Only one isolate (1334/78) was neutralized by a guinea-pig serum to serotype 4 (titre 800, homologous titre 12000). Isolate 1334/78 was also neutralized with a high titre by guinea-pig serum to V1005 rotavirus.

Viral RNA

Single-shelled viral particles could be purified from 35 isolates for the preparation of genomic RNA. By PAGE the isolates showed similar, but in many cases not identical electropherotypes (Fig. 2). Eleven isolates showed more than 11 genome segments (Table 4). Viral genomic RNA was then used for dot blot hybridization with a DNA probe complementary to gene 9 of BRV NCDV. This cDNA probe hybridized to serotype 6 rotavirus (both NCDV and UK), but not to serotype 1 to 4 and 8 to 10 rotavirus (data not shown). Twenty-two (63%) of the 35 isolates analysed hybridized with this cDNA (Fig. 3). All isolates that were typed as serotype 6 by serology and four of the untypable isolates hybridized to this probe, whereas none of the isolates that were typed as serotype 10 or V1005-like by neutralization test gave a positive signal (Table 4).

A DNA probe complementary to gene segment 9 of BRV B223 hybridized strongly to isolate 216/78 confirming the serological identification as a B223-like serotype 10 (Fig. 3 and Table 4). It hybridized weakly to a further 12 isolates, of which four were serologically untypable, five were by serology V1005-like and three were serotype 6 by serology (Fig. 3 and Table 4). None of these 35 isolates hybridized above background with a specific DNA probe against serotype 1 (Wa), 2 (S-2), 3 (YO) and 8 (69M) rotaviruses (data not shown).

Discussion

In our survey of 46 German BRV isolates we identified 22 serotype 6, two serotype 10 and no serotype 8 BRV strains. Hybridization with an NCDV- and B223-specific cDNA probe confirmed the serological results. Twenty-two isolates did not fit into the current scheme of BRV serology. Recently we characterized a BRV, V1005, isolated from the study area (Bachmann & Hess, 1981) which showed a one-way cross-reaction with

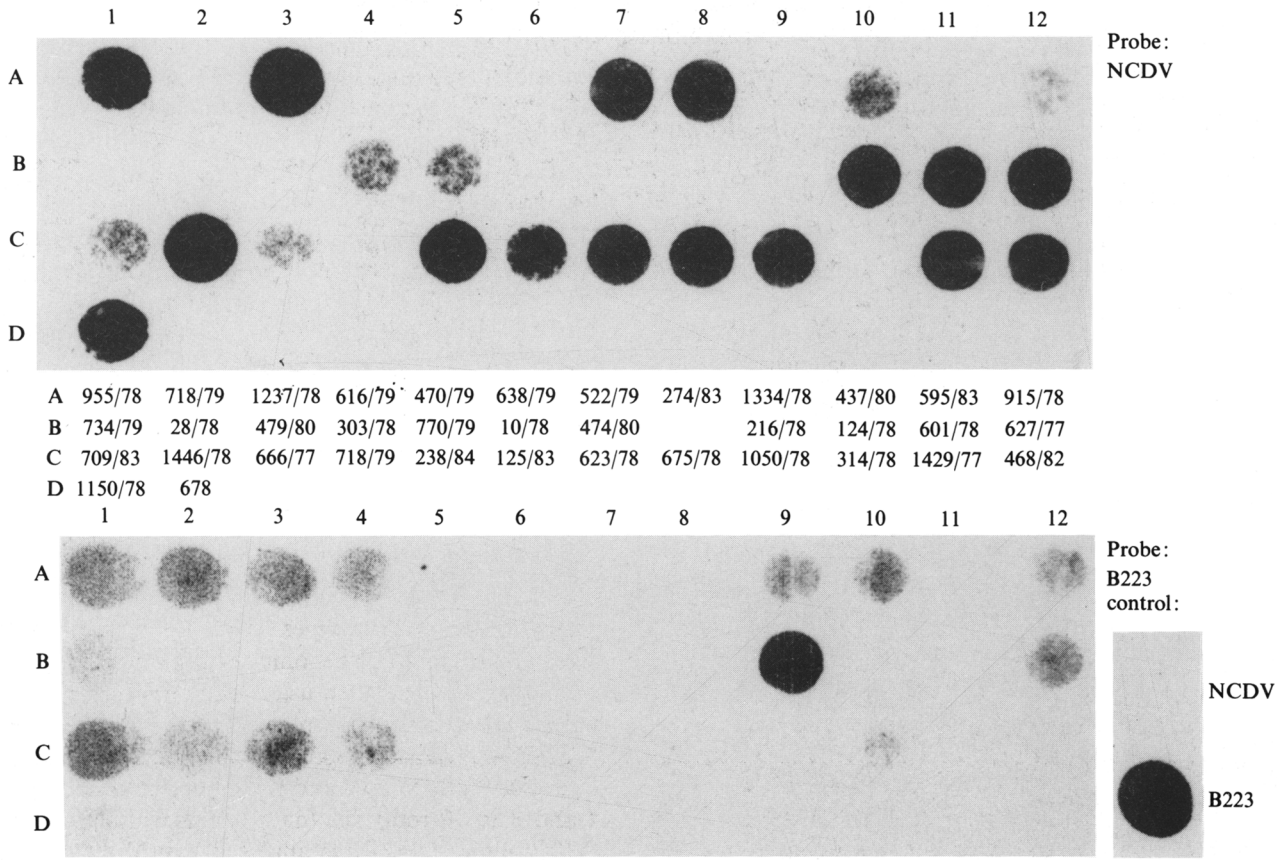

Fig. 3. Differentiation of BRV isolates by dot hybridization with cDNA probes of gene segment 9 of BRV NCDV and B223. The rotavirus isolates in rows A to D and columns 1 to 12 are identified by their code number. Dot D2 shows the serotype 8 BRV 678 reference strain (Snodgrass *et al.*, 1990). Control hybridization of the gene 9-specific B223 probe against B223 and NCDV RNA is shown at the lower right side.

serotype 10 BRV B223, but which was not neutralized by a set of MAbs to VP7 of B223 rotavirus (Brüssow *et al.*, 1990*b*). Fourteen of the untypable isolates were neutralized by a guinea-pig hyperimmune serum previously shown to be specific for BRV V1005 (Brüssow *et al.*, 1990*b*). None of these 14 isolates hybridized with the NCDV-specific cDNA probe and, interestingly, none showed a strong hybridization signal with the B223-specific cDNA probe. These data demonstrate that two subtypes of serotype 10 BRVs have to be distinguished. B223-like serotype 10 isolates represented only 4% of the German isolates and 5 to 10% of the British isolates (Snodgrass *et al.*, 1984, 1990). On the other hand, V1005-like serotype 10 isolates might account for up to 30% of the German isolates. None of these 35 isolates hybridized above background with a specific DNA probe against serotype 1 (Wa), 2 (S-2), 3 (YO) and 8 (69M) rotavirus. Biochemical evidence showed that BRV V1005 shares VP4 with BRV UK (Brüssow *et al.*, 1990*b*). This relatedness cannot, however, explain the high prevalence of V1005-like isolates, as we found that the German isolates were either UK-like or V1005-like, but never related to both. This high prevalence of non-serotype 6 rotaviruses in cattle is surprising in view of the high prevalence of serotype 6 rotaviruses in Britain (Snodgrass *et al.*, 1984, 1990) and the predominance of serotype 6-reactive sera in cattle from the study area (Brüssow *et al.*, 1990*a*). A relatively high level of non-serotype 6 rotaviruses (28%) was also detected in Argentinian dairy herds, but their serological identity was not defined (Bellinzoni *et al.*, 1989).

BRV isolates antigenically related to the major HRV serotypes have been reported in Argentinian herds (Blackhall *et al.*, 1990). These strains could explain the existence of heterotypic antibodies in cattle. However our data do not confirm their conclusions, as we could not identify serotype 1 to 4 rotaviruses in faeces of young calves with rotavirus diarrhoea. A negative result should,

however, be interpreted with caution. Serotyping of animal rotavirus strains is complicated by the fact that infection of animals with two rotavirus strains is not rare in the field. In fact PAGE of stool samples from calves of the Munich area revealed more than 11 RNA segments in a substantial number of cases (Krauss, 1985). Upon adaptation to cell culture growth, a decrease to 11 genome segments was observed, whereas no appearance of supernumerary genome segments after cell culture growth was observed (W. Eichhorn, unpublished results). Hypothetically, serotypes 1 to 4 of BRV could escape detection if they are less readily adapted to cell culture. However, direct serotyping of BRVs in 162 faecal samples of calves with diarrhoea failed to reveal serotypes 2, 3 and 5 in calves and serotype 1 reactivity was equivocal (Snodgrass *et al.*, 1990). Our data do not exclude the possibility that BRV serotypes 1 to 4 escaped detection because they were responsible for subclinical infections or infections in animals older than 2 weeks. There is however no published evidence supporting such assumptions, and good reason to suppose that cattle are not naturally infected by HRVs (Garbarg-Chenon *et al.*, 1986; Ryder *et al.*, 1986).

According to our results neutralizing antibody to heterologous rotavirus serotypes in bovine sera do not reflect a previous exposure of cattle to these serotypes. There is some precedence for these observations. Synthesis of cross-reacting antibody to heterologous rotavirus serotypes seems to be the rule rather than the exception after primary symptomatic rotavirus infection of infants (Brüssow *et al.*, 1988*b*, 1990*c*; Gerna *et al.*, 1990; Puerto *et al.*, 1987). This was however not observed after single serotype vaccination of young infants (Green *et al.*, 1990), whereas induction of cross-reactive antibodies was observed after vaccination both of human adults and mature cows (Brüssow *et al.*, 1988*a*; Green *et al.*, 1990; Snodgrass *et al.*, 1984). Interestingly, paired sera from German children hospitalized with symptomatic primary rotavirus gastroenteritis demonstrated a titre increase to VP4 protein more frequently than to VP7 protein (Brüssow *et al.*, 1990*c*). Furthermore, VP4-specific neutralization epitopes shared by several rotavirus serotypes have been identified by MAbs (Taniguchi *et al.*, 1987). Previously we observed that GMTs to BRV UK were substantially lower than those to BRV NCDV (Brüssow *et al.*, 1990*a*). Both strains share a nearly identical VP7 protein (Glass *et al.*, 1985) but have antigenically distinct VP4 proteins (Hoshino *et al.*, 1985; Kantharidis *et al.*, 1988; Nishikawa *et al.*, 1988), which could account for the observed titre difference. In addition, titres to reassortant rotavirus S-4 were also lower than titres to parental NCDV rotavirus. Reassortant S-4 contains 10 NCDV-derived genes, but its VP4 was replaced by a SA11-derived VP4 (Offit *et al.*, 1986). Although suggestive, these observations do not prove that a substantial part of the NCDV neutralizing antibody of bovine sera is directed against VP4 of the NCDV strain as background proteins can have a large effect on recognition of VP4 and VP7 proteins by antibody in reassortants (Chen *et al.*, 1989).

It seems unlikely that cross-reacting antibody is mainly directed against cross-reacting epitopes on VP4. Antibody levels against the S-4 reassortant containing SA11-specific VP4 are in young cattle lower than antibody levels against SA11 rotavirus. In addition Northern blot hybridization of ^{32}P-labelled RNA probes prepared from BRV strains NCDV and UK to denatured genomic RNAs of a panel of rotavirus strains did not reveal hybridization with the fourth gene segment of serotype 1, 4, 8 and 9 HRVs and simian rotavirus SA11 (G. Gerna *et al.*, unpublished results). This observation excludes the hypothesis that VP4 from serotype 6 BRV strains is closely related to VP4 of HRV or simian rotavirus strains. In addition no significant hybridization was observed between the ^{32}P-labelled RNA probe from both serotype 6 BRV strains and the gene 7–9 complex of the six HRV serotypes and simian rotavirus SA11 (G. Gerna *et al.*, unpublished results).

Previous work with escape variants (Knowlton & Ward, 1985) might provide an interpretation for our puzzling results. Exposure of serotype 3 rotavirus SA11 to polyclonal neutralizing antibody permitted selection of variants. Interestingly, five out of 12 antisera against the variants recognized BRV NCDV as the same serotype. The authors concluded that different serotypes of rotavirus share immunorecessive neutralizing epitopes. Recently, we observed that about 10% of children from a seroepidemiological survey showed serum antibody to heterologous animal rotavirus serotypes (Brüssow *et al.*, 1991). Their prevalence increased with the age of the children and the number of HRV serotypes neutralized, but it did not differ between urban and rural areas. It is thus conceivable that children and cattle recognize after repeated oral rotavirus exposure these hypothetical immunorecessive neutralization epitopes. Cattle differ from children by nearly all calves showing these cross-reacting antibodies. Note that calves are infected with rotaviruses at a younger age (Woode & Bridger, 1975) and that calves live in a more contaminated environment than children, favouring repeated rotavirus infections. Analysis of the bovine sera by an epitope-blocking immunoassay (Green *et al.*, 1990) will hopefully shed more light on the polypeptide and epitope specificity of these sera.

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