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## Molecular characterization of bovine rotavirus strains circulating in Northern Italy, 2003–2005

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1 **Molecular characterization of bovine rotavirus strains circulating in Northern Italy, 2003-**  
2 **2005.**

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1 **Abstract**

2 A total of 232 stools collected from calves with rotavirus infection in herds located in Northern Italy  
3 from 2003 to 2005 was investigated. Determination of the rotavirus G- and P-types was carried out  
4 using nested RT-PCR. G6 was the most prevalent genotype, accounting for 78.5% of samples, G10  
5 accounted for 9.9% of samples and viruses of G8 type were found in 4.7% of samples. In 3% of  
6 samples, viruses were not classified due to concomitant infection with more G type strains, whereas  
7 viruses in 3.9% of samples could not be characterized with any of the G-specific primers used in  
8 this study. Most common P-types were P[11] and P[5], accounting for 65.1% and 25%,  
9 respectively. In 2.6% of cases, samples reacted with multiple P-specific primers; no P[1] serotype  
10 was identified. The G6P[11] combination was predominant throughout the study period, i.e. 52.5%  
11 in 2003, 50% in 2004 and 40% in 2005. The incidence of G6P[5] increased from 13.1% in 2003 to  
12 27% in 2004 and 25.5% in 2005. The G10P[11] combination decreased markedly from 18% in  
13 2003 to 2.6% in 2004, rising again to 7.3% in 2005. G8P[11] viruses were similarly present in 2003  
14 (5%) and 2004 (4.3%), declining slightly in 2005 (1.8%).

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16 Keywords: Rotavirus; Genotyping; Bovine; Italy

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## 1 **Introduction**

2 Group A rotaviruses are a major cause of acute gastroenteritis in the young of humans and many  
3 animal species (Glass et al., 1997; Estes et al., 2007). Rotaviruses possess a genome of 11 segments  
4 of double-stranded RNA inside a triple-layer protein capsid (Estes et al., 2007). The middle layer  
5 protein, VP6, defines the serogroup (A-G), while the outer capsid proteins, VP4 and VP7, define  
6 the G (VP7) and P (VP4) serotypes, elicit serotype specific neutralizing antibodies and are involved  
7 in immunity (Estes et al., 2007). Currently, rotavirus genotyping by amplification of VP7 (Gouvea  
8 et al., 1990, 1994b) and VP4 (Gouvea et al., 1994a; Gentsch et al., 1992) gene fragments using  
9 serotype-specific primers is largely used in epidemiological studies (Estes et al., 2007). Based on  
10 genetic characterization and phylogenetic analyses, at least 15 VP7 and 27 VP4 genotypes are  
11 recognized (Martella et al., 2005, 2007), several of which infect both humans and animals (Estes et  
12 al., 2007). Rotavirus strains with serotypes G6, G8, and G10, in association with VP4 types P6[1],  
13 P7[5], and P8[11] are common in cattle, although G1, G3, and G11 strains are detected  
14 sporadically. Unusual strains with G5, G7 and P[14], P[17] and P[21] genotypes have also been  
15 described (El-Attar et al., 2002; Rao et al., 2000; Fukai et al., 2002; Estes et al., 2007).

16 Typical rotavirus types in humans are G1-4, and G9, combined to P[8] or P[4]. Strains with  
17 common bovine G types, such as G8 and G10, have been identified in children with diarrhoea,  
18 suggesting a zoonotic transmission (Iturriza-Gomara et al., 2004; Cook et al., 2004). Therefore,  
19 surveillance of rotavirus G and P types is important to understand rotavirus ecology, interspecies  
20 transmission and adaptation to new hosts. Furthermore, the recent introduction of two live human  
21 vaccines in many countries, including Europe and North America, might exert a selective pressure  
22 against circulating rotaviruses particularly those sharing the same G1-4 and P[8] specificities as the  
23 vaccine strains (Desselberger et al., 2006).

24 We previously reported the genetic characterization of bovine rotaviruses collected in 1994-1998 in  
25 Northern Italy (Falcone et al., 1999), showing that G6P[5] and G10P[11] accounted for most  
26 strains. In the present study, we investigated bovine rotavirus isolated in 2003-2005 in the same  
27 area to verify possible changes on the circulation of G and P genotypes.

28

## 29 **Materials and methods**

### 30 **Samples**

31 A total of 232 stools from calves with diarrhoea collected in herds in Northern Italy were studied.  
32 Rotavirus diagnosis was performed by ELISA (Cordioli and Lavazza 1992), in some samples was  
33 also confirmed by direct electron microscopy (EM).

### 34 **RNA extraction**

1 Samples were suspended in ddH<sub>2</sub>O (10% vol/vol), and clarified by centrifugation at 6000 x g for 5  
2 min. Rotavirus RNA was purified from 100 µl of suspension with the QIAamp RNA Mini kit  
3 (Qiagen), and eluted in RNase-free water containing 200U/ml of RNase inhibitor. As control for  
4 genotyping a set of well-characterized cell culture-adapted RV strains were used: Wa (G1P[8]),  
5 NCDV(G6P[1]), UK (G6P[5]), B223 (G10P[11]), and 678 (G8P[5]), OSU (G5P[7]).

#### 6 **RT-PCR of VP7 and VP4 genes**

7 For G- and P-typing, the protocols described by Gouvea et al. (1990; 1994a,b) and Gentsch et al.,  
8 (1992) were used with some modifications. Reverse transcription (RT) and first PCR amplification  
9 were performed in a one-tube format with the Access RT-PCR System (Promega). For  
10 amplification of full-length VP7 (1,062 bp) gene (Gouvea et al., 1990), 5 µl viral dsRNA were  
11 denatured (97°C, 5 min) with 0.5µM of Beg9-End9 primers; primers Con2-Con3 were used for  
12 amplification of a 876 bp VP4-specific region (Gentsch et al., 1992). The denatured RNA was  
13 added to a RT-PCR mixture containing the AMV/Tfl PCR buffer 5x, 1mM MgSO<sub>4</sub>, 0.2mM dNTPs,  
14 and 0.1 U/µl each of AMV Reverse Transcriptase and Tfl DNA Polymerase. Synthesis of cDNA  
15 was achieved at 45°C for 45 min, 94°C for 2 min. The product was then subjected to 30 cycles at  
16 94°C for 30 sec, 51°C for 1 min, and 68°C for 1 min 15 sec.

17 For G-typing, 2 µl of RT-PCR product, diluted 1:100 in H<sub>2</sub>O, were used as the template for the  
18 second PCR amplification in 10x PCR buffer II (KCl, 50mM; Tris-HCl, 10mM [pH 8.3]),  
19 containing MgCl<sub>2</sub> (2mM), dNTPs (200µM), 0.02 U/µl of AmpliTaq Gold DNA polymerase (Perkin-  
20 Elmer), and primers specific for bovine rotavirus (50nM each). These included primers for G5, G6,  
21 G8, G10, and G11 types and sBeg9 (Gouvea et al 1994b; Martella, personal communication).  
22 Samples resulting negative were further analyzed with primers specific for human genotypes G1,  
23 G2, G12 and End9 (Gouvea et al., 1990, Gray, personal communication). The mixtures were  
24 subjected to 30 cycles at 94°C for 1 min, 45°C for 2 min, and 72°C for 1 min. PCR products were  
25 analyzed on a 1.5% agarose gel in TAE (40mM Tris-acetate, 1mM EDTA [pH 8]), stained with  
26 ethidium bromide.

27 For P-typing, the second PCR (94°C, 1 min; 55°C, 2 min; 72°C, 1 min; 30 cycles) was performed  
28 with primer Con2 or Con3 and primers specific for genotypes P[6], P[7], P[1], P[5], and P[11]  
29 (Gentsch et al., 1992; Gouvea et al., 1994a, Martella et al. 2006).

#### 30 **DNA sequencing**

31 A subset (37) of rotavirus strains were characterized by nucleotide sequencing of PCR products.  
32 Amplified DNA was purified by using the Wizard SV Gel and PCR Clean-Up System (Promega),  
33 and sequenced using an ABI sequencer apparatus (model ABI3730XL; Applied Biosystems).

1 Multiple alignments were performed using DNASIS MAX v.2. Sequences were compared against  
2 the GenBank database using the BLAST program.

### 4 **Results**

5 Samples were collected from cattle farms located in Northern Italy regions Lombardia and Emilia-  
6 Romagna. Farms included both dairy and beef herds, and were sampled during 2003-2005. A total  
7 of 232 field samples from cases of neonatal calf diarrhea, positive for rotavirus by group A ELISA  
8 and/or EM, were also analyzed by VP4 and VP7 RT/PCR. Second round nested-PCR was  
9 performed to characterize rotavirus G and P types of -positive samples. The results of typing are  
10 shown in Table 1. The G type was determined in 223 (96.1%) samples. The incidence of G types  
11 was 78.5% for G6, 4.7% for G8, 9.9% for G10; mixed types were found in 3% of cases. Nine  
12 specimens (3.9%) remained untypable. In 2004, G10 was replaced as the second most common type  
13 by other genotypes, decreasing from 21.3 % in 2003 to 3.4% in 2004, increasing again to 11% in  
14 2005. Instead, G8 viruses remained constant throughout the study (3.6-5.2%). In 2004, the number  
15 of mixed types increased to 4.3% and was 3.6% in 2005. The untyped specimens were 6.5% in  
16 2003 and 9.1% in 2005, whereas all rotavirus strains in 2004 were G-genotyped.

17 The P genotype was determined in 215 (92.7%) cases. The incidence of P genotypes was 65.1% for  
18 P[11], 25% for P[5], 2.6% mixed, and 7.3% untyped. The incidence of each P type varied less from  
19 year to year than the incidences of G types. Overall, P[11] was the most frequent type throughout  
20 the study, varying from 80.3% of strains in 2003 to 60.3% in 2004 and 58.2% in 2005. The decrease  
21 of P[11] type was balanced by the increase of either P[5] viruses or viruses with a mixed genotype.  
22 P[5] was 16.4% in 2003, 28.5% in 2004 and 27.3% in 2005, and mixed types occurred in 1.6% of  
23 cases in 2003, 1.7% in 2004 and 5.4% in 2005. Untyped samples were 1.6% in 2003, 9.5% in 2004  
24 and 9.1% in 2005.

25 The combinations of G and P types are reported in Table 1. G6P[11] combination remained the  
26 predominant one although it decreased along years. Conversely, the incidence of G6P[5] increased  
27 from 13.1% of 2003 to 27% in 2004 and 25.5% in 2005. G10P[11] strains sharply decreased from  
28 18% in 2003 to 2.6% in 2004, being 7.3% in 2005. G8P[11] viruses were constant in 2003 (5%) and  
29 2004 (4.3%) declining in 2005 (1.8%). As previously shown by others (Falcone et al., 1999, Fukai  
30 et al., 2002), rotaviruses with a P[5] type exhibited most frequently a G6 type, whereas  
31 combinations of P[5] with either G10 or G8 occurred less frequently. To confirm results of  
32 genotyping RT-PCR, 37 samples from all G- (20 G6, 11 G8 and 6 G10) and P- (10 P[11] and 10  
33 P[5]) types identified were further analyzed by nucleotide sequencing of the DNA obtained from G-  
34 and/or P-typing PCR. Sequence comparisons revealed a close relationship between the VP7 and

1 VP4 genes of strains found in our study and bovine strains reported in GenBank (95% to 99%  
2 sequence similarity, data not shown).

3 The samples which resulted negatives at both G and P genotyping (0.8%) did not yield any visible  
4 band after first round amplification, and could not be examined further. In 20 cases where only the  
5 G or P type was determined by PCR, the amount of DNA after the first amplification was not  
6 sufficient to allow sequencing, and the complete genotype could not be determined. These DNAs  
7 were then subjected to a second round of genotyping PCR with primers for the most prevalent  
8 human rotavirus types, but none yielded a positive result.

9

## 10 **Discussion**

11 In this study we determined the P and G types of rotaviruses circulating in bovine farms in the  
12 North of Italy, an area encompassing 30% of the whole cattle population of the country. The RT-  
13 PCR based genotyping method used was further confirmed to be a useful epidemiological tool for  
14 examining strain diversity (Desselberger, et al., 2001), although it has gaps in encompassing novel  
15 or uncommon rotavirus genotypes and mixed infections. Previous reports (Fukai et al., 2002; Rao et  
16 al., 2000) have shown that most bovine rotavirus strains belong to G6, G10, [P5], and [P11]  
17 genotypes. Also, a study on bovine strains conducted in Italy in the late 90s revealed that G6P[5]  
18 was very common (38.3%), followed by G10P[11] (31.5%) (Falcone et al., 1999). The results of the  
19 present study confirm that bovine rotavirus diversity in Northern Italy is limited to a narrow range  
20 of bovine-specific genotypes. Compared to human rotavirus studies indicating a great variation of  
21 predominant strains between years in a same area (Desselberger et al., 2006), the molecular  
22 epidemiology of bovine rotavirus in Italy seems not to change remarkably with time. In fact, our  
23 data altogether confirm that most bovine strains in each of the three years investigated presented a  
24 G6 VP7 in combination with either a P[11] or P[5] VP4. A G8 VP7 in association with P[5] or  
25 P[11] seems to be rare in bovines as recently reported in Southern Italy (Pisanelli et al., 2005).  
26 Compared to our previous findings in the 1990's, the relative frequencies of different VP4 and VP7  
27 types appear to have changed remarkably. In fact, in 1994-8 the predominant combination was  
28 G6P[5] (Falcone et al., 1999) while in the present study G6P[11] became predominant. Another  
29 major finding is the decrease of the G10P[11] combination from 2003 to 2004. This type was  
30 frequent in 1994-8 representing approximately 30% of viruses (Falcone et al., 1999) whilst it  
31 accounted for less than 3% of strains in 2004. These observations confirm previous reports (Fukai et  
32 al., 2002; Pisanelli et al., 2005) that the incidence of G and P types may change significantly with  
33 time. Whether this reflects changes in serotype-specific herd immunity is unclear. In fact, it has  
34 been proposed that despite differences in serotypes, other cross-reactive antigens or antigenic sites



1 might ensure broad spectrum immunity in animals infected with a single rotavirus type (Blutt et al.,  
2 2006).

3 In the present study, a limited number of strains could not be G-typed. Three of them might be  
4 ELISA false-positive since they were also P-untypable, and no DNA was visible after the first  
5 round RT-PCR. Concerning P-typing, a higher number (17) of samples remained untyped; this  
6 finding may either reflect sequence variation within gene 4 (Martella et al., 2006) or suggest the  
7 emergence of novel rotavirus genotypes (Fukai et al., 2007).

8 Sequential replacement of rotavirus antigenic types has been described during a long-term survey in  
9 a bovine herd in Italy (Martella et al., 2003), and a periodic shift on the VP4 and VP7 specificities  
10 has been observed for bovine rotavirus in Japan (Fukai et al., 2002) and human rotaviruses in  
11 Southern America (Timenetsky Mdo et al., 1994), possibly related to mechanisms of antigenic  
12 escape.

13 Presently, three killed or live modified vaccines are used in Italy to prevent neonatal rotavirus  
14 diarrhoea of ruminants, belonging to G5P[7], G6P[1] or G6P[5], respectively (Pisanelli et al.,  
15 2005). Rotavirus genotype surveillance in cattle to detect emerging strains is important to monitor  
16 circulating strains in the farming population and optimize current animal vaccines. In addition, data  
17 might help identify animal rotavirus strains with a possible zoonotic potential (Cook et al., 2004),  
18 although such an event has not been reported in Italy. Most molecular epidemiology studies on  
19 human rotavirus in Italy have shown conventional human G and P types, similarly to the rest of  
20 Europe (Van Damme et al., 2007). However, atypical genotypes such as G8, G10 and G12 have  
21 been recently reported in children with diarrhoea in Central Italy (Ansaldi et al., 2007) even if their  
22 possible animal origin was not addressed. Differently than in the Netherlands (van der Heide,  
23 2005), the occurrence of interspecies transmission of rotaviruses between cattle and humans has  
24 been reported in Slovenia (Steyer et al., 2007), confirming earlier reports of zoonotic bovine  
25 rotaviruses from India (Kang et al., 2005).

26

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31 rotavirus strains of human and animal origin”), and from the European Commission, DG Research  
32 Quality of Life Program 6th Framework (EVENT, SP22-CT-2004-502571).

33

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Table 1. Relative frequencies of various combinations of G and P genotypes of group A rotavirus detected in bovines, Italy 2003-2005

Year	No. of strains of genotype (%)										Total
	G6[P11]	G6[P5]	G10[P11]	G8[P11]	G10[P5]	G8[P5]	Mixed	G-untyped	P-untyped	G/P untyped	
1994-8*	23 (15.4)	57 (38.3)	47 (31.5)	5 (3.4)	4 (2.7)	2 (1.3)	5 (3.4)	6 (4%)	0	0	149
2003	32 (52.5)	8 (13.1)	11 (18)	3 (5)	2 (3.3)	0	1 (1.6)	3 (4.9)	0	1 (1.6)	61
2004	58 (50)	31 (26.7)	3 (2.6)	5 (4.3)	1 (0.9)	1 (0.9)	6 (5.1)	0	11 (9.5)	0	116
2005	22 (40)	14 (25.5)	4 (7.3)	1 (1.8)	1 (1.8)	0	5 (9)	3 (5.5)	3 (5.5)	2 (3.6)	55
Total (2003-5)	112 (48.3)	53 (22.8)	18 (7.8)	9 (3.9)	4 (1.7)	1 (0.4)	12 (5.2)	6 (2.6)	14 (6)	3 (1.3)	232

\*Data for 1994-8 are "rearranged" from Falcone et al., 1999 and reported for comparison.