EXPLORING THE EPIDEMIOLOGY OF LAPINE ROTAVIRUSES: EVIDENCE FOR SPREADING OF ROTAVIRUSES DISPLAYING THE NEWLY-RECOGNISED P[22] VP4 GENE ALLELE IN ITALY

MARTELLA V.[1], LAVAZZA A.[2], TERIO V.[1], CAMARDA A.[1], CERIOLI M.[2], ELIA G.[1], PRATELLI A.[1], BUONAVOGLIA C[1].

[1] Dipartimento di Sanità e Benessere Animale. Università degli Studi di Bari. S.p. per Casamassima km 3 - 70010 Valenzano - Bari - Italia
[2] Instituto Zooprofilattico Sperimentale di Lombardia/Emilia Romagna, Via Bianchi 9, 25124 Brescia – Italia
v.martella@veterinaria.uniba.it

ABSTRACT

An epidemiological survey was carried out to investigate the distribution of the VP7 and VP4 antigenic specificities of lapine rotaviruses (LRV) in Italy. Rotaviruses were identified in rabbitries from different geographical regions of Northern and Southern Italy. The VP7 and VP4 specificities of the Italian LRV strains were determined by either PCR genotyping or sequence analysis. Almost all the strains (14 out of 16) were characterised as P[22],G3, confirming the presence of viruses resembling the newly-recognised P[22] LRVs in Italian rabbitries. The P[22] VP4s detected were about 87.3 to 91.5% identical at the amino acid level to the prototype P[22] LRV strains (160/01, 229/01 and 308/01), identified in 2001 in Italy. Only 1 strain was characterised as P[14],G3 and 1 sample was a mixed infection of P[14],G3 and P[22],G3 LRVs. These data are suggestive of an almost complete replacement of the P[14] allele by the P[22] in Italian rabbitries.

Key words: rotavirus, diarrhoea, rabbit, VP4, VP7.

INTRODUCTION

Group A rotaviruses, members of the *Reoviridae* family, are considered the main cause of acute viral gastroenteritis of humans and animals. Rotaviruses possess a genome consisting of 11 segments of double-stranded RNA (dsRNA) enclosed in a triple-layered capsid (ESTES, 2001). The two outer capsid proteins, VP4 and VP7, the main antigenic determinants, independently elicit neutralizing antibodies, and induce protective immunity (ESTES, 2001). Based on either antigenic or genetic characterization 15 VP7 gene alleles (each corresponding to a G serotype) and 23 VP4 gene alleles, P genotypes, have been recognised. Due to the lack of appropriate antibody reagents, out of the 23 P genotypes identified so far, only 14 P serotypes and 3 subtypes have been established (ESTES, 2001; HOSHINO *et al.,* 2002; MARTELLA *et al.,* 2003; LIPRANDI *et al.,*

2003) and a dual designation has been adopted for P serotypes (an open number) and P genotypes (a number in square brackets).

Analysis of the lapine rotavirus (LRV) strains isolated throughout the world has revealed a substantial antigenic/genetic homogeneity of LRVs. All the LRVs identified so far belong to the VP7 serotype G3 (CIARLET *et al.,* 1997a*;* CONNER *et al.,* 1988*;* PETRIC *et al.,* 1978*;* SATO *et al.,* 1982*;* THOULESS *et al.,* 1988) and to the VP4 serotype P11[14] (CIARLET *et al.,* 1997a; HOSHINO *et al.,* 2002). Recently, LRV strains (160/01, 229/01 and 308/01), belonging to a novel VP4 genotype, proposed as P[22], were detected in Italy (MARTELLA *et al.,* 2003). The detection of LRVs with a genetically different VP4 type from all the LRV strains identified previously rises the questions as to whether the distribution of the P[22] VP4 allele is geographically restricted to the areas where it was first identified or it is circulating in other regions. The aim of this study was to characterize molecularly the VP7 and VP4 genes of LRVs detected in diarrheic rabbits from different geographic regions of Italy to clarify the epidemiological role of the P[22] VP4 allele.

MATERIALS AND METHODS

Origin of samples and viruses.

Diarrheic faecal samples positive to rotavirus were collected from rabbits of 25 days - to 2-months of age. About 350 samples were screened for the presence of rotavirus infection during 1998-2003 and rotaviruses were detected in 62 samples (17.6%). Sixteen rotavirus-positive samples were selected randomly from 16 rabbitries, located in different geographical regions of Italy (Basilicata, Puglia, Lombardia, Veneto, Emilia Romagna). The presence of rotavirus was diagnosed by either electron microscopy or a commercial immuno-enzymatic assay specific for group A rotaviruses (*Rotascreen Dipstick, Microgen Bioproducts, Camberley, UK*).

RNA extraction, prediction of the VP7 and VP4 specificity by PCR genotyping and sequence analysis.

Viral dsRNA was extracted by adsorption on cellulose CF11 (WILDE *et al., 1990*) directly from the faecal samples. Determination of the VP7 specificities of all LRVs was achieved by PCR genotyping as previously described, with minor modifications (GOUVEA *et al.,* 1990; 1994a*;* DAS *et al.,* 1994; MARTELLA *et al.,* 2004). The full-length VP7 gene (1,062 bp) was reverse transcribed and amplified using the GeneAmp RNA PCR Core kit (*Applera Italia, Monza*). The cDNA synthesized was diluted 1:100 and used as template for a second PCR amplification, using AmpliTaq Gold® DNA polymerase (*Applera Italia, Monza*)**.** For the G-type characterization, different pools of G-type-specific primers (G1 to G6, G8 to G11) were used. Prediction of the VP4 specificity was achieved following the original typing strategy described by GENTSCH *at al*. (1992) and GOUVEA *et al*. (1994b). The VP8* sub-unit of the VP4 fragment was reverse transcribed and amplified using the primer pair *Con2-Con3*. The second PCR amplification was carried out with different pools of P-type-specific primers, including the most common human and animal P types (P[1], P[4], P[5], P[6], P[7], P[8], P[9], P[10], P[11]) and the lapine P types (P[14]

and P[22]). The sequence of the VP8* of 3 strains (1891/01, 1211/02 and 243/03-49) was determined. Sequence analysis was performed following purification of the PCR product on Ultrafree DA Columns (*Amicon Millipore, Bedford, USA*), using an ABI-PRISM 377 (*Applera Italia, Monza*).

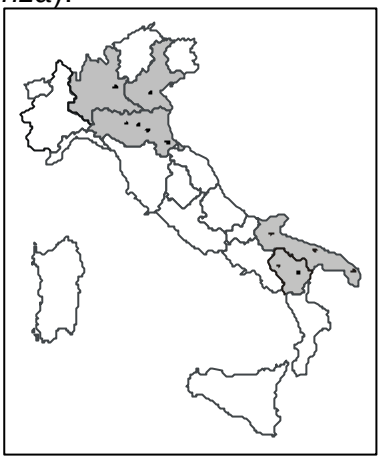

Figure 1. Origin of the viruses analysed in this study

RESULTS AND DISCUSSIONS

In this study, we characterized molecularly the VP7 and VP4 genes of LRVs identified in different rabbitries in Northern and Southern Italy between 1998 and 2003. Most of the Italian LRVs (14 out of 16) were classified as P[22],G3 (Table 1). Sequence analysis of the VP8* trypsin-cleavage fragment of the VP4 of the 1998-2003 LRV strains confirmed the specificity of the PCR genotyping assay. The highest degree of sequence identity, ranging from 87.3 to 91.5% (aa) and 87.6 to 89.6 (nt), was observed to the P[22] prototype strains 160/01, 229/01 and 308/01. Only 1 sample was found to contain a P[14],G3 strain, while another sample was found to contain a mixed population of viruses, P[14]+P[22],G3. All the Italian LRV strains displayed a VP7 of serotype G3 specificity. Such rotavirus G serotype has been described in a broad spectrum of animal species, including humans (NISHIKAWA *et al.,* 1989) but, so far, it is the only rotavirus VP7 specificity identified in rabbits (CIARLET *et al.,* 1997a*;* MARTELLA *et al.,* 2003). Thus our data confirms the highly conservation of the VP7 gene in lapine rotaviruses.

As regards the VP4, rotaviruses with a P[14] specificity were identified only in 2 rabbitries. So far, P[14] rotaviruses have been identified from rabbits in several countries (CIARLET *et al.,* 1997a*;* MARTELLA *et al.,* 2003). In humans, the P[14] specificity was first identified in Italy in the early 1990s in association with the unusual, bovine-like, G6 VP7 type (GERNA *et al.,* 1994) and, subsequently, it has been found with a sporadic pattern in G1, G6, G8 and G10 rotaviruses from Finland, Thailand, Australia, Egypt and Hungary (URASAWA *et al.,* 1993*;* PALOMBO *et al.* 1999*,* HOLMES *et al.,* 1999*;* MPHALELE *et al.,* 1999*;* BÁNYAI *et al.,* 2004). Interestingly, most of the LRVs analysed in the present study were characterised as P[22], i.e. the same VP4 specificity as the three prototype strains identified in Southern Italy in 2001 (MARTELLA *et al.,* 2003), suggesting that such VP4 genotype is widespread throughout the Italian territories, and it has been replacing,

during the last years, almost completely the P[14] allele in rabbits. The extent of sequence variation observed in the VP8* of the P[22] strains suggests repeated introduction of such viruses into Italian rabbit population and the existence of sub-genotypes. With a few exceptions, the finding that a novel rotavirus antigenic type is widespread in a population represents a unique epidemiological phenomenon, as, rotaviruses bearing novel/uncommon G and P types are usually identified with a sporadic pattern and only in restricted geographical/ecological settings.

Table 1: VP7 and VP4 specificities of the LRV strains analyzed in Italy. Designation of the VP4 is referred to the P genotype. The year and the place of identification are also reported.

Sample	G type	P type	Origin/Year	Reference
R-2	3	14	Japan, 1978	SATO *et al*., 1982
C-11	3	14	USA, 1983	THOULESS *et al*., 1986
ALA	3	14	USA, 1984	THOULESS *et al*., 1986
BAP-2	3	14	USA, 1988	CONNER *et al*., 1988
30/96	3	14	Bari, 1996	MARTELLA *et al.,* 2003
160/01	3	22	Matera, 2001	MARTELLA *et al.,* 2003
229/01	3	22	Foggia, 2001	MARTELLA *et al.,* 2003
308/01	3	22	Lecce, 2001	MARTELLA *et al*., 2003
1891/01	3	22	Padova, 2001	This study
170/03	3	22	Reggio Emilia, 2003	This study
753/98	3	22	Reggio Emilia, 1998	This study
1211/02	3	22	Modena, 2002	This study
1602/02	3	22	Brescia, 2002	This study
1601/02	3	22	Brescia, 2002	This study
1336/02	3	22	Reggio Emilia, 2002	This study
1070/02	3	22	Bologna, 2002	This study
370/01	3	22	Bologna, 2001	This study
1515/00	3	22	Forlì, 2001	This study
243/03-11	3	22	Potenza, 2001	This study
243/03-26	3	14	Foggia, 2002	This study
243/03-38	3	22	Lecce, 2002	This study
243/03-49	3	22	Matera, 2003	This study
293/03-4	3	22+14	Foggia, 2003	This study
1352120	3	22	Forlì, 2003	This study

CONCLUSIONS

Antibodies to rotavirus are detectable in most rabbits after 4 months of age, suggesting that rotavirus infection is endemic in commercial rabbitries. Passively-transferred antibodies protect young rabbits up to 2 months of age and rotavirus-associated disease is usually described after weaning, in 1 to 3 months old animals.

Rotavirus infection in rabbits has been largely investigated as the rabbit has been proposed as an animal model to assess active immunity and protection after rotavirus infection or vaccination (CIARLET *et al*., 1998b). Experimental infection has revealed that the disease, but not the infection, is age restricted (< 1 week) but that histopathological lesions may develop, in the absence of symptoms, even in adult rabbits (CIARLET *et al*., 1998b).

The finding that no specific pathogen may be constantly associated with rabbit enteropathies has led to the proposition that rabbit enteric diseases have a multi-factorial etiology, with synergic mechanisms that often enhance the pathogenicity of the various microorganism. From this perspective, it may be hypothesized that under field conditions rotaviruses seldom exert direct pathogenic activity, and, more frequently, they trigger the development of bacterial infections and/or other viral pathogens by inducing minimal alterations of the intestinal epithelium.

By analyzing several LRVs identified in different geographical areas of Italy, we observed the spread of viruses displaying the newly-recognised P[22] VP4 allele, suggesting that such P[22] type is of epidemiological relevance in Italy. Comprehension of antigenic/molecular diversity among domestic animal herd populations is of critical importance to comprehend the global ecology of rotaviruses and the mechanisms of rotavirus evolution. Furthermore, because of the serotype-specific nature of rotavirus-induced immunity, an exact comprehension of the G ad P types prevalence in the different animal species is essential to develop effective vaccines for the control of rotavirus-associated disease in humans and animals.

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