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DETECTION OF RABBIT ROTAVIRUS BY POLYMERASE CHAIN REACTION IN FAECES AND COMPARISON OF GENE 9 SEQUENCE BETWEEN TWO ISOLATED STRAINS

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ABSTRACT

In described rotaviroses, clinical signs reveal soft diarrhoea, fluid and gas in the small intestine, caecum and colon. These observations mentioned by many authors appear close to the clinical signs described in epizootic rabbit enterocolitis (ERE). In the purpose of establishing a possible link between rotavirus pathogenicity and ERE we searched for the presence of rotaviruses in rabbits with ERE. Two techniques were used on faecal samples : enzyme immunoassay (EIA) and Reverse Transcriptase PCR. Rotaviruses were only detected in some stool specimens while all of the samples were able to reproduce ERE in specified pathogen free rabbits. Two strains, isolated before and during the epizooty of ERE, were compared by sequencing rotavirus Vp 7 gene. While the electrophoretypes of these two strains were different, a very moderate genetic variability was detected. but was not sufficient to differentiate the two strains. Our results indicate that the direct role of rotavirus in ERE is questionable.

INTRODUCTION

Rotaviruses are major etiological agents of acute viral gastro-enteritis in the young of a wide range of mammalian and avian species. Our investigation on the rabbit epizootic enterocolitis (ERE) which appeared in France at the beginning of 1997 (Licois *et al.* 1998, 1999) led us to consider the possible role of rotaviruses in the aetiology of the disease. Rotaviruses from rabbits have been described by many authors (Peeters *et al.* 1984, Rizzi *et al.* 1995, Thouless *et al.* 1996 and Ciarlet *et al.* 1997). In an epidemiological study, Peeters *et al.* in 1984 found a peak incidence of rotavirus from 36 to 42 days of age. Clinical signs and gross examination at necropsy revealed some similarities between rabbit rotavirus diseases and ERE. Indeed, the ERE is characterized by a high mortality rate (>30%) and a mild but highly liquid diarrhoea preceded by a reduction of food intake (Licois, 1998). Animals are bloated. Gross lesions are mainly a distension of the whole intestinal tract including the stomach which is filled with gas and fluid. The caecum is sometimes impacted and in some animals, clear, gelatinous mucus can be observed. No inflammation or congestion of the intestine are visible Which makes ERE strictly different from the other known intestinal diseases of the rabbit.

The aim of the study was first to detect rotaviruses from animals deriving from different rabbitries, using a reverse transcriptase-PCR assay (RT-PCR) and enzyme immunoassay (EIA). The genomes of two rabbits rotavirus strains isolated before and during the epizooty of ERE were compared by sequencing Vp7 gene and analysing RNA electrophoretypes.

MATERIALS AND METHODS

Faecal specimens. Faecal samples originating from 5 different areas of France (Côtes d'Armor, Charente-Maritime, Maine-et-Loire, Loire-Atlantique and Yvelines), were obtained

from rabbits with ERE in 1998 and 1999. All have been proved to reproduce ERE in specified pathogen free (SPF) rabbits. They were systematically tested by both EIA and RT-PCR to detect rotaviruses. Furthermore, two different specimens were used as negative controls in this experiment. The first one was obtained from New-Zealand White rabbits reared at INRA (Tours, France) under specific pathogen-free conditions. These animals were regularly checked for the absence of coccidia, helminth, *Encephalitozoon cuniculi*, *Pasteurella multocida*, and pathogenic strains of *Escherichia coli* (Coudert *et al*. 1988). The second control specimen derived from SPF rabbits purchased in a commercial supplier located in Ain (France).

Two samples originating from Rambouillet and Charente-Maritime have been purified after serial passages in rhesus monkey kidney cells (MA 104) using methods previously described (Estes *et al,.*1979). Rotavirus strain from Rambouillet (LA/RR510) was isolated from the intestinal content of a diarrhoeic rabbit which died in 1996 (before the epizooty of ERE). This strain was compared with the Charente Maritime one (Mg), isolated during the epizooty of ERE, on the basis of the sequence of their Vp7.

Extraction of RNA from faeces. For RT-PCR, approximately one gram faeces specimen (fresh or frozen) was added to 9 ml of sterile phosphate-buffer saline (PBS 0,05M, pH 7,4) and mixed by inverting and vortexing the tube for 5 to 10 min then centrifuged (10 000 X *g*) for 5 min to collect the upper phase. The samples were treated as described by the manufacturer, using the QIAamp DNA stool kit of Qiagen (Orsay, France).

Enzyme immunoassay (EIA). The test is routinely used to detect the major inner capsid protein (Vp6) present in group A rotaviruses, in human faecal samples. The samples were treated as described in the IDEIA^{TM} Rotavirus kit (J2L ELITECH, Labarthe Inard, France).

Primers. Oligonucleotides were selected from published sequences of Rotavirus gene 9 which encodes Vp7 (Gouvea *et al.* 1990). The primers Beg9 and End9 were chosen because they produce full-length copies of gene 9 from any group A Rotavirus strains. The nucleotides (nt.) positions and sequences of theses primers (5' to 3') were as follow : Beg9 (nt. 1 to 28), GGCTTTAAAAGAGAGAATTTCCGTCTGG ; End9 (nt. 1062 to 1036), GGTCACATCA-TACAATTCTAATCTAAG.

Reverse transcriptase and polymerase Chain Reaction amplification (RT-PCR). Ten µl of extract containing double-stranded RNA (dsRNA) were denatured by heating at 97°C for 10 min and quickly cooled on ice. A two-amplification procedure with the Promega kit (Charbonière, France), was used. Briefly, 2,5µl of dsRNA denatured was added to a reaction mix composed of 14µl of nuclease-free water, 5µl of AMV/T*fl* 5X reaction buffer, 0,5 of dNTP mix (10mM), 0,5µl of each primers (20µM), 1µl of $MgSO_4$ (25mM), 5U of Avian Myeloblastosis virus and 5U of thermostable T*fl* DNA polymerase from *Thermus flavus*. The samples were mixed and subjected to one cycle of reverse transcriptase (42°C, 45 min) and 35 cycles of PCR, in a thermal cycler (MWG-BIOTECH, France). Each PCR cycle was as follow: 30 sec at 94°C for the denaturing step, 1 min at 50°C for annealing and 2 min at 68°C for polymerization. A cooling cycle was used to bring the samples to 4°C. Twenty microliters of the amplified reaction was electrophoresed in a 1% agarose gel (Eurobio, Les Ulis, France) in TBE buffer (pH 8) for 2 hours at a constant voltage (100V). Products were stained with ethidium bromide. The amplified fragments were determined using the DNA molecular weight marker smart ladder (Eurogentec, Belgium). A positive identification is characterized by the presence of a specific 1026-bp band.

DNA Sequencing and sequence comparisons. The specific RT-PCR amplifications of Vp7 observed which the La/RR510an Mg strains were cloned into pGEM-T vector (Promega, France). Then, the gene encoding the capsid Vp 7 proteins was sequenced by using the

dideoxy chain termination technique (Sanger *et al*. 1980) and a fluorescent 373A automated DNA sequencer (Applied Biosystems). The predicted amino acid sequence was compared to known rotavirus strain described by Ciarlet *et al*. 1997 (Seq_rota). Sequencing data were analysed using the FASTA program of the Genetics Computer Groups (GCG) package (Pearson and Lipman, 1988).

Extraction and electrophoresis of RNA. Rotavirus double-stranded RNA of the La/RR510and Charente-Maritime (Mg) strains were extracted from cells culture as described in the first paragraph. For electrophoretyping of the rotavirus RNA, the buffer (1:3 v/v) used was composed of EDTA disodium salt dehydrate (5mM), Tris (20mM) and SDS (5mM). The samples were then centrifuged at 3000X*g* for 10 min. and the supernatant transferred and added to an equal volume of chloroform-phenol (2:1 v/v). The mix was vortexed for 30 sec. and centrifuged at 1200X*g* for 15 min. at 5°C. Concentration was achieved by ultrafiltering the sample solution through an anisotropic membrane by centrifugation 30 min. at 1000X*g* (Centricon YM-100, Millipore, France). A Laemmli discontinuous system was used (Laemmli *et al.*, 1970). A 10% polyacrylamide separating gel and 4% stacking gel were used. Forty µl of RNA extract mixed with 10µl of 80mM EDTA disodium salt, sucrose (75mM) and bromophenol blue (1mM) were loaded on gel and electrophoresed at 25mA/gel for 16 hours. The gel was silver-stained by using a Bio-Rad kit (Bio-Rad, Paris, France).

RESULTS AND DISCUSSION

Detection of rotaviruses from stool specimens.

EIA and RT-PCR gave equivalent results (Table 1). Rotaviruses were detected in the stool specimens from Côtes d'Armor and in the three samples from Charente Maritime but in only 2/5 of those from Yvelines. Moreover we were unable to detect rotaviruses from the samples of Maine-et-Loire and of Loire-Atlantique while they were associated with ERE and preliminary used to reproduce the disease.

Table 1. Detection of rotavirus by RT-PCR and enzyme immunoassay from different breeding stocks and in control animals. Number of positive sample / number of tested ones.

Sample	Origin	Number of samples	EIA*	RT-PCR**
Rabbits with ERE	Loire-Atlantique	6	0/6	0/6
	Yvelines	5	2/5	2/5
	Charente-Maritime	3	3/3	3/3
	Côtes d'Armor	1	1/1	1/1
	Maine-et-Loire	1	0/1	0/1
Negative feacal control	Tours	2	0/2	0/2
	Ain	3	0/3	0/3

* The samples were treated as described in the IDEIA™ Rotavirus kit (J2L ELITECH, Labarthe Inard, France).
** Oligonucleotides used for RT-PCR were selected of rotavirus gene 9 which encodes Vp7 (Gouvea *et al.* 1990).

Nevertheless, it must be kept in mind that a negative result does not exclude the possibility of rotavirus infection. We have established the threshold of both methods used. For EIA, it was 10^5 particles per gram of feces whereas RT-PCR limit of the detection was estimated at 10^4 particles per gram of fecal matter. For control subjects, stools were systematically tested by both EIA and reverse transcriptase (RT)-PCR. In this present study, RNA purifications were performed with stool nucleic acid extraction kit to obtain a better detection by RT-PCR. This amplification technique seems to be suitable to reveal rotavirus in specimens but

depends on procedure of extraction to eliminate inhibitors. Indeed, the amplifications might be hampered by an apparent inhibition derived from faecal extracts as the bilirubin and bile salts (Wilde *et al.* 1990).

Sequence comparisons (Figure 1).

The amplified products from rabbit-derived rotavirus strains (La/RR510and Mg) were sequenced and compared to the homologous sequences of rabbit-derived rotavirus described by Ciarlet *et al*. (1997) (Figure 1). A high degree of amino acid identity (98%) over 326-aa of the same protein was found between both La/RR510and Mg strains. The four minor differents in amino acid sequence (aa-66, 113, 123, 238) between these two strains showed that Vp7 was highly conserved in rabbit rotaviruses isolated before and during the epizooty of ERE.

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            1                                                    50
  Seq_rota. MYGIEYTTaL TFLISFILLN YILKSLTRMM DFVIYRFLFV IViLSPLLKA
Seq_protMg. MYGIEYTTVL TFLISFILLN YILKSLTRMM DFVIYRFLFV IVVLSPLLKA
Seq_protRY. MYGIEYTTVL TFLISFILLN YILKSLTRMM DFVIYRFLFV IVVLSPLLKA
  Consensus MYGIEYTTVL TFLISFILLN YILKSLTRMM DFVIYRFLFV IVVLSPLLKA

            51                                                  100
  Seq_rota. QNYGINLPIT GSMDsAYANS TQEETFLTST LCLYYPTEAA aEINDNSWKD
Seq_protMg. QNYGINLPIT GSMDTvYANS TQEETFLTST LCLYYPTEAA TEINDNSWKD
Seq_protRY. QNYGINLPIT GSMDTAYANS TQEETFLTST LCLYYPTEAA TEINDNSWKD
  Consensus QNYGINLPIT GSMDTAYANS TQEETFLTST LCLYYPTEAA TEINDNSWKD

            101                                                 150
  Seq_rota. TLSQLFLTKG WPTGSIYFRE YTDIVSFSVD PQLYCDYNVV LMKYDtTLQL
Seq_protMg. TLSQLFLTKG WPTGSIYFRE YTnIVSFSVD PQLYCDYNVV LMKYDATLQL
Seq_protRY. TLSQLFLTKG WPaGSIYFRE YTDIVSFSVD PQLYCDYNVV LMKYDAaLQL
  Consensus TLSQLFLTKG WPTGSIYFRE YTDIVSFSVD PQLYCDYNVV LMKYDATLQL

            151                                                 200
  Seq_rota. DMSELADLIL NEWLCNPMDI TLYYYQQTDE tNKWISMGSS CTIKVCPvNT
Seq_protMg. DMSELADLIL NEWLCNPMDI TLYYYQQTDE ANKWISMGSS CTIKVCPLNT
Seq_protRY. DMSELADLIL NEWLCNPMDI TLYYYQQTDE ANKWISMGSS CTIKVCPLNT
  Consensus DMSELADLIL NEWLCNPMDI TLYYYQQTDE ANKWISMGSS CTIKVCPLNT

            201                                                 250
  Seq_rota. QTLGIGCLTT nVaTFEEVAT AEKLVITDVV DGVNHKLDVT TATCTIRNCK
Seq_protMg. QTLGIGCLTT DVTTFEEVAT AEKLVITDVV DGVNHKLnVT TATCTIRNCK
Seq_protRY. QTLGIGCLTT DVTTFEEVAT AEKLVITDVV DGVNHKLDVT TATCTIRNCK
  Consensus QTLGIGCLTT DVTTFEEVAT AEKLVITDVV DGVNHKLDVT TATCTIRNCK

            251                                                 300
  Seq_rota. KiGPRENVAV IQVGGSDvLD ITADPTTAPQ TERMMRINWK KWWQVFYTVV
Seq_protMg. KLGPRENVAV IQVGGSDILD ITADPTTAPQ TERMMRINWK KWWQVFYTVV
Seq_protRY. KLGPRENVAV IQVGGSDILD ITADPTTAPQ TERMMRINWK KWWQVFYTVV
  Consensus KLGPRENVAV IQVGGSDILD ITADPTTAPQ TERMMRINWK KWWQVFYTVV

            301                    326
  Seq_rota. DYVNQIIQLM SKRSRSLNSA AFYYRV
Seq_protMg. DYVNQIIQLM SKRSRSLNSA AFYYRV
Seq_protRY. DYVNQIIQLM SKRSRSLNSA AFYYRV
  Consensus DYVNQIIQLM SKRSRSLNSA AFYYRV
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Figure 1. Amino acid sequences of 326-aa of the rotavirus encoding the Vp 7 protein from rabbit. The sequence Seq_ rota was described by Ciarlet *et al.* 1997 from rabbit rotavirus strains isolated in outbreak of diarrhoea in USA rabbit colony. The sequence Seq_protMg derived from rabbit during epizootic enterocolitis (ERE) isolated in Charente-Maritime (Seq_protMg) and the sequence Seq_protRY derived from rabbit before the apparition of ERE in France.

Rabbit rotavirus RNA electrophoretype.

Two distinct RNA patterns typically belonging to group A rotavirus with 4 segment groups (Lourenco *et al.*, 1981) were distinguished (Figure 2). Differences were identified in group III and Group IV. According to Gouvea *et al.* (1990), Vp7 is encoded by gene segment 9 or segment 8 (depending on the strain) which are precisely located in group III. Variations have ever been described by Estes *et al*. (1984) with rotaviruses from different humans. This analysis has proved to be a valuable means of distinguishing rotavirus strains.

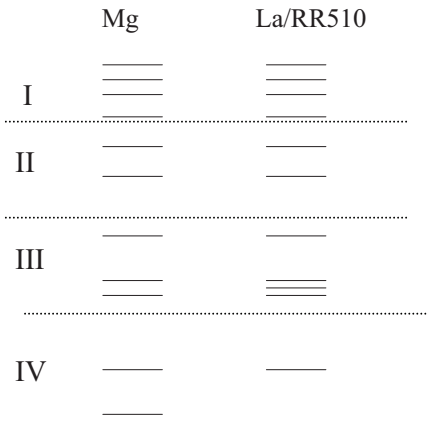

Figure 2. Schematic patterns of migration of rotavirus strains Mg and La/RR510dsRNA. All reported group I, group II, group III and group IV are shown as they are described in previous article of Lourenco *et al.* (1981).

In conclusion, ERE is at the present time the most important disease causing severe mortality in commercial rabbitries but the infectious agent is still unknown. Rotaviroses are common diarrhoeal diseases in breeding stocks. Thus we considered the possible role of rotavirus in the aetiology of the ERE. Among the samples associated with ERE, rotaviruses were not systematically found. In addition, no marked differences of the sequence of the Vp7 gene between La/RR510and Mg strains have been observed. The La/RR510strain has experimentally been inoculated to SPF rabbits without inducing ERE (unpublished data) and the Mg strain will be tested in a short time. For the moment, while electrophoretypes of these two strain are different, the results of our study indicates that the direct role of rotavirus in ERE is questionable. The research should be angled to other responsible agents of ERE in rabbits including bacteria and virus.

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