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Short communication

Rotavirus-associated diarrhoea in foals in Greece

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ABSTRACT

Severe outbreaks of diarrhoeic syndrome occurred in young foals at the same stud farm during two consecutive breeding periods namely spring 2006 and 2007. Rotavirus-like particles were detected by electron microscopy in the faeces of the affected foals and group A rotavirus infection was confirmed by Reverse-Transcription (RT)-PCR with selected sets of rotavirus-specific primers. Sequence analysis of the genes encoding the outer capsid rotavirus proteins VP7 and VP4 enabled classification of the viruses as G3AP[12] and revealed that the viruses were highly similar to recently reported equine rotavirus strains circulating in Europe. All Greek equine rotavirus isolates were genetically identical, suggesting persistence of the same viral strain in the stud farm, over the two consecutive foaling periods.

1. Introduction

Group A rotaviruses (GARVs) are the main cause of diarrhoea in foals up to 3 months of age and are also important enteric pathogens in children (Browning and Begg, 1996; Imagawa et al., 1991, 1998; Parashar et al., 2003). GARVs are non-enveloped icosahedral particles, composed of 11 segments of double-stranded (ds) RNA enclosed in a triple layered protein-capsid (Ciarlet and Estes, 2002). The two outer capsid proteins, VP7 and VP4, independently elicit neutralizing antibodies, induce protective immunity and are used to classify rotaviruses into G (for Glycoprotein) and P (for Protease-sensitive) types, respectively (Ciarlet and Estes, 2002). Thus far, 23 G types and 32 P types have been identified in humans and animals (Ursu et al., 2009; Collins et al., unpublished data).

GARV infections have severe economic impact on stud farming, due to morbidity and mortality of the affected foals. GARV-induced disease is characterised by watery diarrhoea, anorexia, abdominal pain, dehydration and depression (Imagawa et al., 1984).

Equine GARVs have been identified in the faeces of foals with diarrhoeic syndrome in Britain (Flewett et al., 1975), the United States of America (Conner and Darligton, 1980), Australia (Tzipori and Walker, 1978), New Zealand (Durham et al., 1979), Ireland (Collins et al., 2008) and Japan (Imagawa et al., 1981). Serological data have also been reported in several countries, indicating that equine GARVs are ubiquitous (Conner and Darligton, 1980; Goto et al., 1981; Eichorn and Huan, 1987).

Antigenic/genetic characterization of equine GARVs have revealed that the majority of strains are either G3P[12] or G14P[12] (Browning and Begg, 1996; Isa et al., 1996; Tsunemitsu et al., 2001; Elschner et al., 2005; Collins et al., 2008). Inactivated vaccines based on strains H2 and HO-5, G3P[12], have been developed for the prevention of rotavirus-associated diarrhoea in foals.

In this study we describe severe outbreaks of diarrheic syndrome in foals born in the same stud farm in Greece during two consecutive breeding periods, namely spring 2006 and 2007. The strains were analyzed by sequence

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analysis of the main antigenic determinants, VP7 and VP4, in order to acquire information on the antigenic/genetic diversity.

2. Materials and methods

2.1. Origin of the samples

Faecal samples were collected from 2 to 6 months old foals, suffering from severe diarrhoea, bred on a stud farm consisting of 50 mares and situated in the Attica region. Outbreaks of diarrhoea took place during the foaling period in two consecutive years (May–June 2006 and 2007). Similar outbreaks were reported simultaneously in surrounding stud farms in the same region. All the farm's foals were lactated naturally and were vaccinated against *C. tetani*. Farmed mares were subjected to regular vaccinations programs, excluding however, immunization against rotavirus. A total of fouteen faecal samples (six collected in 2006 and eight in 2007) were analyzed.

2.2. GARV detection using negative staining transmission electron microscopy (nsTEM) and ELISA

Faecal samples collected from foals were processed for nsTEM observation. Briefly, the faeces were diluted 1:10 in distilled water, vortexed and centrifuged for 20 min at $4000 \times g$ and again for 10 min at $9300 \times g$ for clarification. The supernatant was then ultracentrifuged for 15 min at $82{,}000 \times g$. After negative staining with 2% sodium phosphotungstate (pH 6.8), samples were examined using a TEM Philips CM10. All the samples were also screened using a sandwich ELISA assay, specific for GARV detection, as described previously (Lavazza, 1989).

2.3. RT-PCR amplification of the VP7 gene and of the VP8* portion of the VP4 gene

Total RNA was extracted from faecal samples using the QIAamp® viral RNA kit (Qiagen GmbH, Hilden, Germany) according to manufacturers' instructions. Extracted dsRNA was denatured in 50% DMSO at 97 °C for 5 min and was immediately cooled on ice. For VP7 gene amplification, Beg9/End9 primers set was used (Gouvea et al., 1990), in a one-step Reverse-Transcription (RT)-PCR protocol using Superscript III one-step RT-PCR kit (Invitrogen Ltd., Paisley, UK). For amplification of the VP8* subunit of the VP4 gene, Con 2 and Con 3 primers and the same commercial kit were used (Gentsch et al., 1992).

2.4. Sequence analysis of the VP7 and VP4 genes of equine GARV

Six GARV strains were selected for sequencing. PCR products with primers Beg9/End9 and Con 2/Con 3 were purified using a QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany) and were sequenced commercially at MWG-Biotech (MWG-biotech, Ebersberg, Germany). Sequences were assembled, edited and analyzed using the Bioedit software package version 2.1 (Hall, 1999). Preliminary analysis was performed by sequencing alignment by using web-based BLAST (http://www.ncbi.nlm.nih.gov/BLAST) and FASTA (http://www.ebi.ac.uk/fasta33) programs. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 2.1 (Arizona State University, USA) (Kumar et al., 2001). Phylogenetic trees, based on the VP7 and VP8 gene sequences, were elaborated with both parsimony and distance methods, supplying a statistical support with bootstrapping over 100 replicates.

2.5. GenBank accession numbers

The partial sequences of the VP8* subunit of the VP4 of strains 412/07-1/07/Gr, 412/07-2/07/Gr, 412/07-8/07/Gr, 412/07-9/06/Gr, 412/07-11/06/Gr and 412/07-13/06/Gr have been registered in GenBank under the accession numbers GQ266662, GQ266663, GQ266664, GQ266665, GQ266666 and GQ266667, respectively. The partial sequences of the VP7 of strains 412/07-1/07/Gr, 412/07-2/07/Gr, 412/07-8/07/Gr, 412/07-9/06/Gr, 412/07-11/06/Gr and 412/07-13/06/Gr have also been registered in GenBank under the accession numbers GQ266668, GQ266669, GQ266670, GQ266671, GQ266672 and GQ266673, respectively.

3. Results

3.1. nsTEM, ELISA and RT-PCR

By nsTEM, rotavirus-like particles were observed in the faeces of 4/10 animals. Ten out of fourteen samples were positive using ELISA. RNA was detected in ten out of fourteen samples using RT-PCR with primers sets specific for GARV.

3.2. Sequence analysis of the VP7 and VP4 genes

Sequence analysis of the VP7 gene revealed that the Greek equine GARVs displayed >99% nt identity to each other, suggesting a clonal origin. By BLAST and FASTA analysis, the highest genetic similarity (97.6 nt and 99.0% aa) was found with German and Irish equine strains (Elschner et al., 2005; Collins et al., 2008), G3 serotype and subtype A. Phylogenetic analysis (Fig. 1) showed that the G3 Greek strains were clustered with G3A equine rotaviruses (H2-like), along with recently reported European rotaviruses. An alignment of the VP7 antigenic regions A (aa 87–101), B (aa 141–152), C (aa 208–224) and F (aa 235–242) was made (Fig. 2) (Dyall-Smith et al., 1986; Nishikawa et al., 1989; Kobayashi et al., 1991; Ciarlet et al., 1997). The Greek strains were highly conserved with respect to the G3A German strain 4766G3, with a single amino acid substitution, 87-T → I, in region A. In contrast, they differed from the reference G3A strain H2, used in the majority of vaccines, in region C (213A → S and 217T → E) and region F (242T → A) and from the G3B strain HO-5 in 6 residues in region A, C and F (Fig. 2).

In the VP8* subunit of the VP4 gene, the Greek P[12] strains displayed high identity (>99% nt) to each other (Fig. 3). By FASTA and BLAST searches, the highest nucleotide (98.9%) and amino acid identity (98.8%) were