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I. Galindo-Cardiel, M. Fernández-Jiménez, L. Luján, J. Buesa, J. Espada, et al.. Novel group A rotavirus G8 P[1] as primary cause of an ovine diarrheic syndrome outbreak in weaned lambs. *Veterinary Microbiology*, 2011, 149 (3-4), pp.467. 10.1016/j.vetmic.2010.12.006 . hal-00683503

HAL Id: hal-00683503

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Submitted on 29 Mar 2012

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Accepted Manuscript

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PII: S0378-1135(10)00579-1
DOI: doi:10.1016/j.vetmic.2010.12.006
Reference: VETMIC 5118

To appear in: *VETMIC*

Received date: 8-7-2010
Revised date: 19-11-2010
Accepted date: 7-12-2010

Please cite this article as: Galindo-Cardiel, I., Fernández-Jiménez, M., Luján, L., Buesa, J., Espada, J., Fantova, E., Blanco, J., Segalés, J., Badiola, J.J., Novel group A rotavirus G8 P[1] as primary cause of an ovine diarrheic syndrome outbreak in weaned lambs, *Veterinary Microbiology* (2010), doi:10.1016/j.vetmic.2010.12.006

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1 **Novel group A rotavirus G8 P[1] as primary cause of an ovine diarrheic syndrome**
2 **outbreak in weaned lambs**

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20

21 **Abstract**

22 Rotavirus is a worldwide major cause of diarrhea outbreaks in neonatal ruminants. An
23 outbreak of ovine diarrheic syndrome (ODS) in 50-75 days-old lambs (weaned lambs)
24 is described. Fecal immunochromatography and intestinal immunohistochemistry for
25 rotavirus group A were performed. In addition, semi-nested multiplex RT-PCR for G

1 and P rotavirus genotyping in combination with sequencing were performed, to support
2 the diagnosis and identify the viral strain. A novel ovine rotavirus group A G8 P[1]
3 strain was determined as the main cause of the ODS observed, whereas other pathogens
4 were ruled out.

5

6 **Keywords:** Ovine diarrheic syndrome; rotavirus group A; weaned lambs

7

8 The economical management of ovine and other ruminant farms is seriously
9 compromised by postnatal (less than 10 days-old) mortality associated to several
10 infectious agents such as rotavirus (Holland, 1990, Muñoz *et al.*, 1995, Dahma *et al.*,
11 2009). The rotavirus genus is divided into seven antigenically distinct groups (A to G)
12 (Desselberger *et al.* 2009). They are classified by sequencing of the major structural
13 protein VP6, which have a role in viral transcription (Dhama *et al.*, 2009). The
14 protective immunity obtained by neutralizing antibodies is elicited by two outer capsid
15 proteins, VP4 and VP7, which act as major virulence factors. Both proteins are
16 associated with P and G serotype specificity, respectively (Desselberger *et al.* 2009,
17 Dhama *et al.*, 2009). VP4 helps the virus to attach to host cells and VP7 provides the
18 protective outer capsid layer (Desselberger *et al.* 2009). Sheep are one of the few
19 species in which group B rotaviruses appear to be more prevalent in neonatal lambs
20 than group A rotaviruses (Fitzgerald *et al.*, 1995).

21 Rotavirus infections have a very short incubation period and affected animals
22 can manifest profuse diarrhea and severe dehydration. Moreover, concurrent infection
23 with secondary pathogens may increase clinical severity (Dhama *et al.*, 2009).
24 Experimental infections with rotavirus in lambs have been performed (Van Pinxteren *et*
25 *al.*, 1998), but descriptions of natural rotavirus infections are scarce and results are not

1 always conclusive (Tzipori *et al*, 1983, Moon *et al*, 1997, Van Metre *et al*, 2000). In this
2 report, we describe a new ovine group A rotavirus G8 P[1] strain (OVgAR) associated
3 to an outbreak of ovine diarrheic syndrome (ODS) in 50 to 90 days old Rasa Aragonesa
4 lambs. Immunochromatography (IC), immunohistochemistry (IHC), and a multiplex
5 reverse transcriptase-polymerase chain reaction (RT-PCR) were performed to support
6 the diagnosis and to characterize the new viral strain.

7 An outbreak of acute ODS was reported in November 2008 in an high sanitary
8 status farm of Rasa Aragonesa sheep in Zaragoza (North-eastern Spain) under a semi-
9 intensive 3 lambing/2 years production system. All affected lambs belonged to the
10 September 2008 lambing period and they were housed in pens up to 1 square meter per
11 animal. No ODS outbreaks were noticed in the farm or in neighborhood flocks during
12 the 5 years previous to the present outbreak. No management or feeding practices
13 changes were recorded in the year previous to the disease outbreak.

14 A total of 192 out of 203, 50 to 60 day-old lambs (95%) were mild to severely
15 ODS-affected in an outbreak which lasted 10 days in total. Mortality associated with the
16 ODS was 17% (35 out of 203 lambs). A total of 25 ODS-affected and 5 apparently-
17 healthy lambs were studied as follows. Clinical severity was scored by using the
18 following parameters: (1) attitude/ lamb activity (0 = apparently healthy, 1 = slightly
19 decreased, 2 = moderately decreased, 3 = severely decreased), (2) rectal temperature (0
20 = below 40 C, 1 = above 40 C) and (3) diarrhea (0 = not present, 1 = present). These
21 partial scores were summed up, yielding a total cumulative clinical index that reflected
22 apparent health (score = 0), the presence of mild (score = 1-2), moderate (score = 3-4)
23 or severe (score = 5) clinical severity. Fecal swabs were cultured (aerobes, anaerobes
24 and fungi) and a zinc sulphate flotation test for larvae and oocysts were performed.
25 Seven protozoa-positive fecal samples were speciated (sporulation test) for *Eimeria spp.*

1 An immunocromatographic test for rotavirus group A (Spin-Rotavirus, SPINREACT
2 S.A.U, Girona, Spain) was performed in feces following manufacturer's instructions.
3 Clinical severity and pathogen detection results in these 30 lambs are shown in Table 1.

4 Five lambs severely affected by ODS were necropsied and samples of tissues
5 were formalin fixed and paraffin-embedded. Consecutive sections of each paraffin-
6 embedded block were processed for haematoxylin&eosin and for IHC. Main
7 pathological diagnosis consisted in segmental (proximal) moderate to severe catharral
8 enteritis (5/5 lambs) with diffuse severe epithelial stunting and blunting (5/5), diffuse
9 mild to moderate epithelial apical vacuolation (5/5), multifocal mild epithelial cryptal
10 hyperplasia (3/5) and diffuse mild lacteal dilatation (3/5). No enteric bacteria adhered to
11 microvilli were observed in any of these lambs. Moreover, diffuse severe mesenteric
12 lymphoid hypertrophy was observed (5/5). Other secondary lesions recorded were
13 multifocal moderate subacute necrotizing hepatitis (2/5), and multifocal mild subacute
14 catharral-purulent bronchopneumonia (2/5). Feces from the 5 necropsied lambs were
15 OVRGA-positive by IC. In addition, *Cryptosporidium parvum* was detected in two
16 lambs and no oocysts of *Coccidia spp.* were cultured.

17 The small intestines from all necropsied lambs were processed for VP6 rotaviral
18 antigen by IHC. Briefly, OVRGA antigen retrieval was performed using EDTA Buffer
19 (1mM EDTA, 0.05% Tween 20, pH 8.0). Polyclonal rabbit anti-rotavirus (Dako, ref.
20 A0082) served as the primary antibody (1:250), peroxidase-conjugated avidine complex
21 (ABC peroxidase staining kit, Thermo Scientific, ref 32020, 1:100) was utilized as the
22 visualization system and 3,3'-diaminobenzidine (DAB) was used as the chromogen.
23 Negative control procedures included omission of primary antibody. PCR- and IHC-
24 positive and negative intestinal tissues from lamb and pig were used as positive and
25 negative controls respectively. OVRGA-positive diagnosis was assessed based on the

1 amount and distribution of antigen in positive-stained apical enteric epithelium and
2 macrophages of the lamina propria. All samples were OVRGA-positive IHC. The number
3 of OVRGA-positive cells found by IHC was directly related with the severity of the
4 individual enteritis.

5 Isolated *Escherichia coli* strains from the 5 ODS-affected necropsied lambs were
6 serotyped and genotyped for enterotoxigenic (LT, STa, STb, K88, P987, K99 and F41),
7 verotoxigenic (VT1 and VT2), atypical enteropathogenic (*eae*) and adhesine F17
8 virulence factors (ETEC, VTEC, aEPEC and F17EC) as described elsewhere (Rey *et al.*,
9 2003). Serotyping and genotyping results of the detected *Escherichia coli* strains are
10 presented in Table 2.

11 Two frozen OVRGA-positive fecal samples (by IHC and IC) from necropsied
12 lambs were used for genotyping the rotavirus strain. RNA was extracted by using
13 RNAwiz™ reagent (Ambion), following the manufacturer's instructions. Reverse
14 transcription with random primers and PCR with rotavirus VP6 gene-specific primers
15 were performed (Gouvea *et al.*, 1990). Both fecal samples were VP6-positive PCR (type
16 I2). G- and P-genotyping was carried out by semi-nested multiplex PCR with G- and P-
17 types oligonucleotide primers previously described (Gouvea *et al.*, 1990, Gentsch *et al.*,
18 1992, Iturriza-Gómara *et al.*, 2000) and the OVRGA isolate was identified as a G8 P[1]
19 strain. One PCR amplicon per each isolate (3 in total) were purified with the QIAquick
20 Gel Extraction Kit (Qiagen) and sequencing was performed by the dideoxynucleotide
21 chain termination method with the ABI Prism BigDye terminator cycle sequencing
22 reaction kit (Applied Biosystems) on an ABI Prism 3100 automated sequencer (Applied
23 Biosystems). The sequencing was performed with the forward or reverse primers used
24 for the RT-PCR. BLAST analysis was carried out with the VP6 (GenBank Accession

1 No. FN675938), VP4 (Accession No. FN675939) and VP7 (Accession No. FN675937)
2 gene sequences obtained.

3 Sequence alignment was performed with Clustal X (version 2.0) and the
4 phylogenetic relationships of the strain described in the present study with other
5 reference rotavirus strains were assessed employing the software MEGA version 4.0
6 using the neighbor-joining method (Tamura et al., 2007). The robustness of the
7 taxonomic relationships was evaluated by bootstrap analysis (1,000 replications).
8 Reference strains for different VP7 G-, VP4 P- and VP6 I-genotypes were selected from
9 GenBank (Figure 1).

10 SAS 9.1.3 software was used (SAS Institute Inc., Cary, NC, USA) to perform
11 the ANOVA test. Clinical severity and pathogen detection were considered as variables.
12 Significant association between group A rotavirus-infection and presence of ODS was
13 the only association detected ($p = .026$, significant at $p < .05$; Table 1).

14 This ODS outbreak significantly correlates with the detection of the newly-
15 described rotavirus strain whereas other pathogens isolated did not. Based on this
16 correlation and the quick spread of the clinical process, high morbidity, limited
17 mortality, compatible pathology and pathogen detection (IC, IHC, RT-PCR), the
18 rotaviral etiology is strongly supported as the primary cause of this outbreak, following
19 accepted evidence-based veterinary medicine approach (Gay, 2006).

20 The phylogenetic analyses confirmed the VP6, VP4 and VP7 genotypes
21 identification previously obtained by RT-PCR, sequencing and BLAST analysis.
22 Interestingly, 96% identity was found between the OVRGA VP6 gene sequence, a
23 rotavirus group A sable antelope G6P[14] (Accession No. FJ495131.1) and a sheep
24 rotavirus G8P[14] strain previously isolated in Zaragoza (Spain) (Accession No.
25 EF554152.1) (Ciarlet *et al.*, 2008, Matthijnsens *et al.*, 2009). A 90% of identity

1 between OVRGA VP4 gene sequence obtained and the VP4 sequenced genes selected
2 for the analysis (Figure 1) was found. Moreover, OVRGA VP7 gene sequence obtained
3 and the sheep rotavirus G8P[14] showed the higher inter-sequences identity founded in
4 this study, a 98%. VP4 and VP7 are the main antigenic determinants for rotaviruses, but
5 only one of the OVRGA-positive isolate was positive for VP4 gene. The different
6 sensitivity of the primers used in the semi-nested multiplex PCR performed was
7 considered to be the cause of such findings (Gouvea *et al*, 1990, Gentsch *et al*, 1992).
8 However, genomic diversity within the VP4 gene of other rotavirus strains has been
9 related with failures of VP4 amplification using RT-PCR (Iturriza-Gómara *et al*, 2000).

10 The main difference was that these described strains were isolated from animals
11 which were into the described range of age for rotavirus infection (i.e., less than 30
12 days-old) (Holland, 1990, Dhama *et al.*, 2009). On the contrary, the strain described in
13 this paper mainly affects 50-75 days-old lambs (weaned lambs). However, few ovine
14 strains have been isolated and characterized to date (Ciarlet *et al*, 2008, Chen *et al*,
15 2009) possibly due to the lack of routine surveillance for rotaviral infections in lambs.
16 Further epidemiological studies should be carried out to clearly demonstrate the pattern
17 of variation between rotaviral strains.

18 *Escherichia coli* strains isolated in this study were not considered as the most
19 likely ODS-related primary etiology due to the lack of significant correlation with the
20 clinical process and to the high variability among the isolates. This phenomenon has
21 already been pointed out by others authors (Nagy and Fekete, 2005). However,
22 *Escherichia coli* strains forming attaching–effacing lesions (*eae*-positive) can cause
23 diarrhea due to their intimate association with intestinal epithelial cells (Moon, 1997)
24 but these lesions have not been observed in our work. However, its role as possible
25 complicating pathogen should be studied in further studies. Other pathogens detected in

1 this study (*Cryptosporidium parvum*, *Coccidia spp.*, *Campylobacter spp.*) did not
2 demonstrate to be the most likely ODS-outbreak related primary pathogen, although it is
3 assumed that they likely cause severe clinical complications (Holland, 1990, Van Metre
4 *et al*, 2000).

5 In conclusion, epidemiological, pathological and laboratory evidence supports
6 that a novel group A rotavirus G8 P[1] strain described in this paper was the main cause
7 of an ODS outbreak in 50-75 days-old lambs (weaned lambs). Further experimental
8 work using rotavirus and *E. coli* in lambs is being carried out by our group at the
9 moment.

10

11 **Acknowledgements**

12 The authors thank M. Pérez and E. Salazar for their support and observations in
13 the pathological analysis of the data.

14

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Table 1. Pathogen detection in 25 lambs affected by Ovine Diarrheic Syndrome and 5 apparently healthy lambs. Results are expressed as total number of animals.

Pathogen	Healthy (5)	Clinical severity			TOTAL (30)	SIG*
		Mild (10)	Moderate (4)	Severe (11)		
Rotavirus	0	2	1	11	14	p=.026
<i>Cryptosporidium spp.</i>	1	0	2	3	6	N. S. [§]
<i>Coccidium spp.</i>	0	0	2	2	4	N. S.
<i>Campylobacter spp.</i>	0	2	2	3	7	N. S.
<i>Proteus mirabilis</i>	0	1	1	2	4	N. S.
<i>Clostridium perfringens</i>	0	1	0	2	3	N. S.
Other bacteria	0	0	1	1	2	N. S.

* SIG: Statistical signification of pathogen by ANOVA

[§] N. S.: Not significant

Table 2. Serotyping and genotyping of *Escherichia coli* strains isolated in five severe ODS-affected necropsied lambs.

Lamb	Strain	Serotyping*	<i>eae</i> [#]	F17 [‡]	Reference number [§]
1	S1	ONT	+	-	eae-12073
2	S2	ONT	+	-	eae-12074
3	S3	-	-	+	F17-CNL
	S4	ONT	+	-	eae-12075
4	S5	O23	-	+	F17-12076
	S6	O4 (1/1280), O15 (1/640)	+	-	eae-12077
5	S7	-	-	+	F17-CNL
	S8	O4 (1/640), O15 (1/640)	+	-	eae-12078

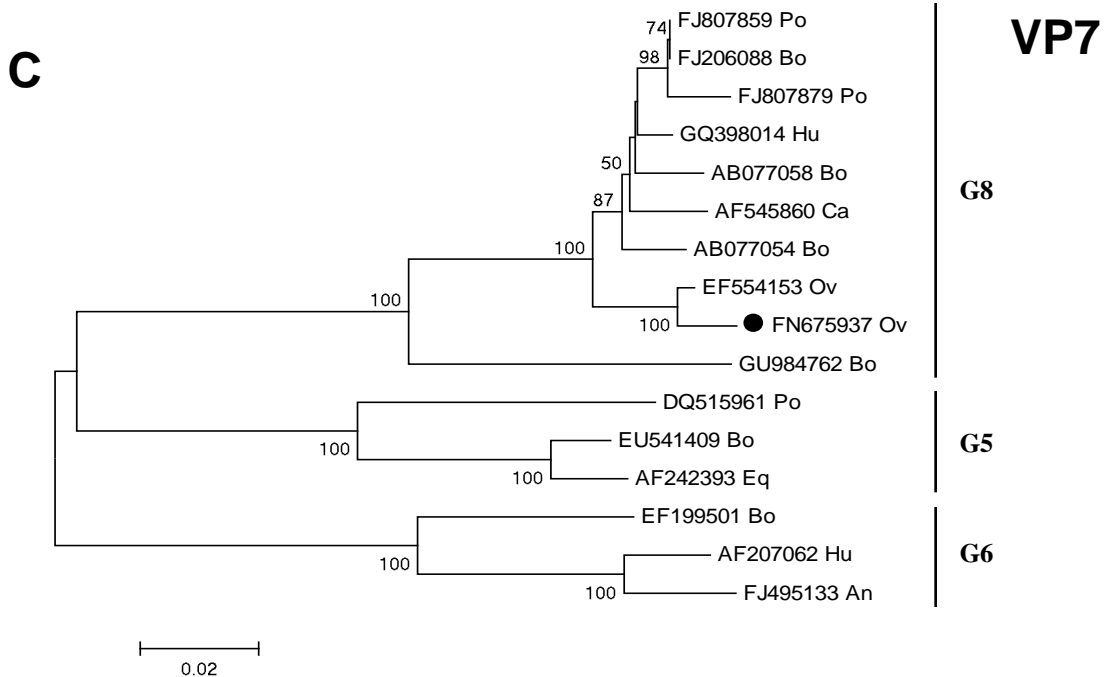
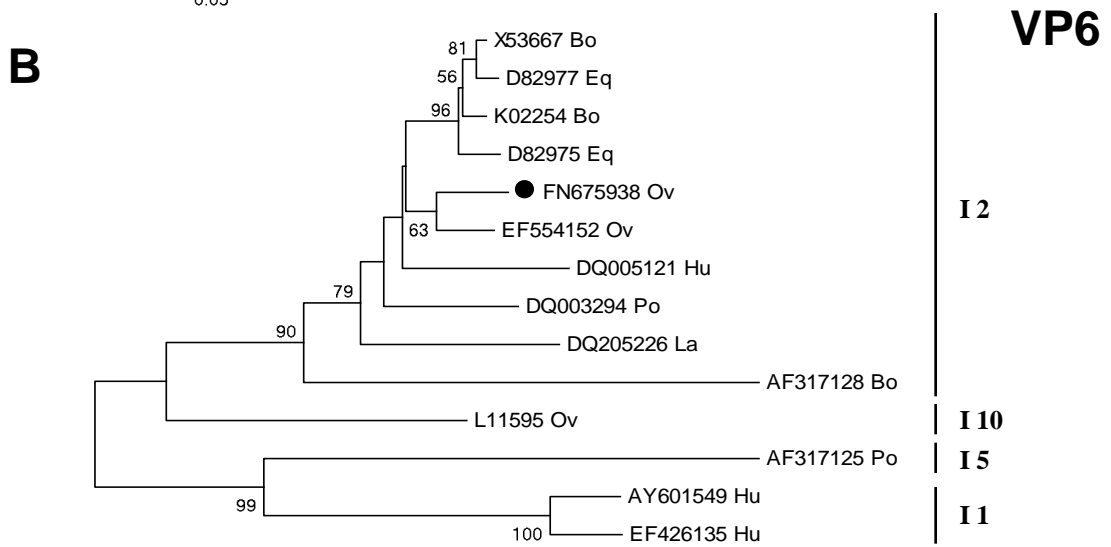
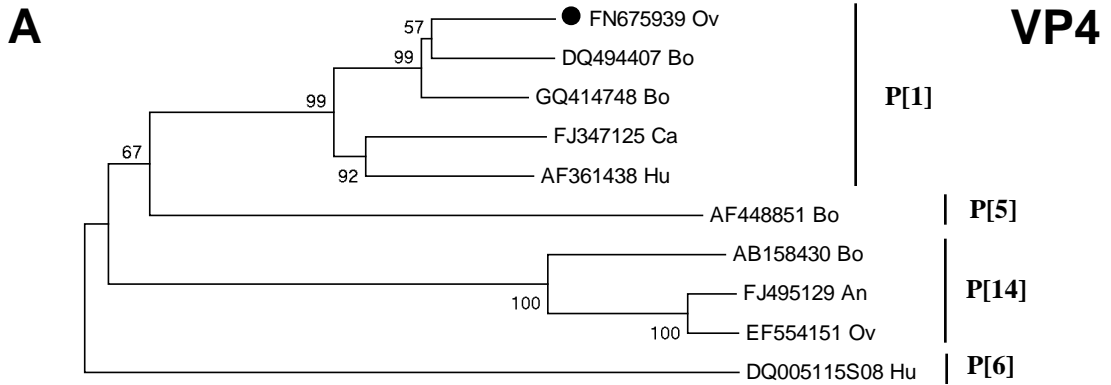
* Serotyping: Results given as serotype detected (ONT, O4, O15 and O23) with serotype dilution added in brackets when it is relevant.

[#] *eae*: intimin gene responsible for attaching effacing lesions

[‡] F17: adhesine fimbriae 17 gen

[§]Reference number given by the Reference Laboratory of *Escherichia coli* at the University of Santiago de Compostela in Lugo (Spain)

Figure



1 **Figure Legends**

2

3 **Figure 1.** Phylogenetic dendrograms showing the relationships between the VP4 (A),
4 VP6 (B) and VP7 (C) nucleotide sequences of the novel ovine rotavirus strain described
5 in this study (indicated in all phylogenetic trees with a filled circle) with other reference
6 rotavirus strains. The trees were generated according to the neighbor-joining method
7 based on partial sequences of the VP4, VP6 and VP7, respectively. Bootstrap analyses
8 were carried for 1000 replicates of the data set and the values above 50 are written as a
9 percentile beside the appropriate branch. The scale indicates the genetic distance
10 between the isolates calculated by the Jukes-Cantor method. Reference strains for the
11 different genotypes were selected from GenBank and their accession numbers are
12 shown in the figure. Abbreviations: An, antelope; Ca, camelid; Eq, equine; Hu, human;
13 La, lapine; Ov, ovine; Po, porcine.

14

15