Occurrence and genetic diversity of rotavirus A in faeces of diarrheic calves submitted to a veterinary laboratory in Spain

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Abstract

A total of 237 faecal specimens from diarrheic calves younger than two months were collected and submitted for diagnosis of enteropathogens over a two-year period (2017-2018) to a veterinary laboratory. Samples originated from 193 dairy and beef farms in 29 provinces distributed throughout Spain, and were tested for the occurrence of three target enteric pathogens by reverse transcription real-time PCR (RT-qPCR): bovine rotavirus A (RVA), *Cryptosporidium parvum* and bovine coronavirus (BCoV). RT-PCR and nucleotide sequencing analysis were used to determine the G (VP7 gene) and P (VP4 gene) genotypes of 26 specimens positive for RVA. A total of 188 specimens (79.3%) were positive for at least one of the three target enteric pathogens, and 101 samples (42.6%) harbored mixed infections. The individual prevalence was 57.8%, 50.6% and 23.6% for *C. parvum*, RVA and BCoV, respectively. Molecular analysis of selected RVA strains revealed the presence of the G6, G10, G3, P[5] and P[11] genotypes, with the combinations G6P[5] and G6P[11] being the most prevalent. Alignments of nucleotide sequences of the VP7 and VP4 markers showed a high frequency of single nucleotide polymorphisms (SNPs), with up to 294 SNPs found in 869bp of sequence at the G6 genotype (0.338 SNPs/nt), which reveals the extensive genetic diversity of RVA strains. Phylogenetic analysis of the VP7 gene of the G6 strains revealed four distinct lineages, with most strains clustering in the G6-IV lineage. The discrepancies between the RVA genotypes circulating in the sampled cattle farms and the genotypes contained in commercial vaccines currently available in Spain are discussed. We believe that this is the first study on the molecular characterization of rotavirus infecting cattle in Spain.

Key words: Species A rotavirus; *Cryptosporidium parvum*; coronavirus; cattle; occurrence; genetic diversity; Spain

1. Introduction

Neonatal calf diarrhea is the most commonly reported disease in both dairy and beef herds (Meganck et al., 2015). In Europe, morbidity rates from 19.1% to 74.4% have been reported, and this disease is considered the main cause of death among un-weaned calves (Bartels et al., 2010; Żychlińska-Buczek et al., 2015; Johnson et al., 2017). Diarrhea in neonatal calves is a multifactorial disease influenced by infectious agents in combination with the environment and management practices. Multiple pathogens can contribute to this condition, including common (rotavirus, coronavirus, *Cryptosporidium parvum*, *Escherichia coli*, *Salmonella* spp., *Clostridium perfringens*) and emerging agents (enterovirus, torovirus, norovirus and nebovirus), although *C*. *parvum* and rotavirus are the dominant pathogens (Cho and Yoon, 2014). The simultaneous presence of more than one of these pathogens is frequent and may lead to complications and death (Chauhan et al., 2008).

Rotavirus is among the leading causes of gastroenteritis and diarrhea in humans and a broad range of animal hosts. A total of ten rotavirus species (A−J) have been established based on genetic and antigenic differences in the inner capsid VP6 protein (https://talk.ictvonline.org/taxonomy). Species A rotaviruses (RVA) are recognized as the most important members of the genus in both human and veterinary medicine, and a major cause of economic losses in cattle (Otto et al., 2015). Sequence-based genotyping targeting the genes encoding the VP7 and VP4 proteins have become the most common

current method for RVA genotyping. VP7 is a glycosylated protein and assigns the G type, whereas VP4 is a protease-sensitive polypeptide and assigns the P type (Midgley et al., 2012). To date, 36G and 51P genotypes are recognized by the Rotavirus Classification Working Group (RCWG, 2017). In cattle, at least 12 G types (G1–G3, G5, G6, G8, G10, G11, G15, G17, G21 and G24) and 11 P types (P[1], P[3], P[5−7], P[11], P[14], P[17], P[21], P[29] and P[33]) have been reported, but genotypes G6 (57%), G10 (21%), and G8 (3%) as well as P[5] (26%), P[11] (21%) and P[1] (2%) are the most prevalent in calves (Dóró et al., 2015). Phylogenetic analyses have classified the G6 genotype into five (I-V) distinct lineages. Briefly, lineages I and IV contain human and bovine rotavirus strains, respectively. Lineages II, III and V are composed by both human and animal strains (Badaracco et al., 2013).

Cattle production is of great economic significance in Spain, which has the fifth largest bovine population in the European Union after France, Germany, United Kingdom and Ireland (https://ec.europa.eu/eurostat). In 2018, a total of 6.5 million head of cattle were declared in Spain by 14,051 dairy farmers which provided more than 7 million tons of milk, and 20,357 beef cattle feedlots which provided just over a quarter (25.1%) of the veal meat in the European Union (https://www.mapa.gob.es/es). In spite of this, the impact of neonatal calf diarrhea in Spanish farms is not well documented and most studies on morbidity rates of infectious agents are limited to individual pathogens. *Cryptosporidium* has been reported to play a major role in the northern region of the country, where prevalence rates over 50% have been found in diarrheic calves (Castro-Hermida et al., 2002; Quílez et al., 2008). Some studies in north-western Spain have showed that Shiga toxin-producing *Escherichia coli* strains are also frequently isolated in calves with diarrhea (Blanco et al., 2004). However, the significance of rotavirus infection

has received limited attention and no data on its genetic diversity in cattle in Spain are available. A study in the central area of the country showed that rotaviruses were the second most prevalent enteropathogen among diarrheic calves (42.7%), and coinfection with *Cryptosporidium* was the most common combination (De la Fuente et al., 1998). In this study, faecal specimens submitted to a veterinary laboratory were used to determine the occurrence and genetic variability of RVA in diarrheic calves throughout Spain. Faecal samples were also analyzed for two other major enteropathogens included in the diagnostic panel for calf diarrhea: *C*. *parvum* and bovine coronavirus (BCoV).

2. Material and methods

2.1. Samples

A total of 237 faecal specimens from diarrheic calves younger than two months submitted for diagnosis of enteropathogens to a veterinary laboratory (Exopol S.L., Spain) were used. Specimens were received from January 2017 to December 2018 in the form of faeces from individual calves sampled directly from the rectum (n: 148) or faecal pools from several calves (n: 89). Samples originated from 193 farms in 29 provinces distributed throughout Spain (Figure 1). A total of 120 stool specimens originated from 101 beef herds (mean, 1.19 ± 0.72 specimens/farm) and 117 stool samples originated from 92 dairy herds (mean, 1.27 ± 0.66 specimens/farm). Most farms submitted a single (n: 163) or two (n: 24) specimens and the remaining six farms submitted three to five samples. On most of the farms submitting more than one specimen (26/30), repeated submissions were due to different diarrheic outbreaks, usually with at least one month apart.

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126 **2.2. Nucleic acid extraction and RT-qPCR**

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128 The commercial kit, MagMAX™ Pathogen *RNA*/DNA (Thermo Fisher Scientific) with
129 an automated magnetic particle processor (KingFisher Flex; Thermo Fisher Scientific)
130 was used for extraction of nucleic acids according to the manufacturer's instructions.
131 After extraction all specimens were tested by real-time PCR (RT-qPCR) using
132 commercial kits (EXOone qPCR kits, Exopol S.L.) for specific detection of bovine
133 species A rotavirus (RVA), bovine coronavirus and *C*. *parvum*. These assays target the
134 rotavirus non-structural protein NSP3, coronavirus nucleocapside protein and *C*. *parvum*
135 actin genes, respectively.

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137 **2.3. G and P genotyping of RVA**

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139 A subset of 26 rotavirus strains from dairy (n: 9) and beef (n: 17) calves from 26 farms in
140 12 provinces was characterized by nucleotide sequencing of VP7 and VP4 genes (Figure
141 1). These samples were selected for genotyping based on the cycle threshold (Cq<28) and
142 their geographic origin in order to analyze samples from different regions. Amplification
143 of both genes was performed using protocols described previously with the following
144 modifications. For G-typing, primers VP7F/VP7R (Fujii et al., 2012) or
145 Bov9Com5/Bov9Com3 (Isegawa et al., 1993) were used with annealing temperatures of
146 56°C and 52°C respectively. The expected amplicon size for the VP7F/VP7R and
147 Bov9Com5/Bov9Com3 set of primers, were 1,062 and 1,010 base pairs (bp), respectively.
148 For P-typing, primers Con2/Con3 (Gentsch et al., 1992) or Bov4com5/Bov4Com3
149 (Isegawa et al., 1993) were used with an annealing temperature of 50°C. The expected

amplicon size for these primer pairs was 876 bp and 860 bp, respectively. Each sample underwent amplification with the first of the above-mentioned primer pairs for VP7 and VP4 genes. The PCR products were analyzed on 1.5% agarose gels and visualized under UV light. If no amplicon was observed or if the amplicon was not of the expected length, the amplification was attempted with the alternative primer pairs. PCR products of positive samples were purified and sequenced at STABvida laboratories (Caparica, Portugal) by Sanger sequencing with the same primers used for amplification.

2.4. Sequence analysis

Nucleotide sequences were aligned against each other and with reference sequences retrieved from GenBank using Clustal WW and edited with BioEdit version 7.2.5 (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Sequences were analyzed for similarity using BLASTN and BLASTP searches at the NCBI databases (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic trees for VP7 and VP4 sequences were constructed by the Maximum Likelihood method of the Kimura two-parameter model in BioEdit version 7.2.5. The statistical reliability was checked using 1,000 bootstrap replicates.

2.5. Nucleotide sequence accession numbers

Representative sequences of both VP7 and VP4 markers from this study were deposited in the GenBank database under accession numbers MT02529 to MT025330.

2.6. Statistical analysis

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176 Chi-square tests were used to compare the occurrence of the three target enteropathogens
177 in faecal specimens. A $P<0.05$ value was required for significance.

178

179 **3. Results**

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181 **3.1. Occurrence of enteropathogens**

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183 The number of specimens and farms testing positive for any of the three enteropathogens
184 surveyed is shown in Table 1. A total of 120 stool samples (50.6%; 95%CI: 44.3–56.9%)
185 from 106 farms (54.9%; 95%CI: 47.8–61.7%) in 25 provinces were positive for species
186 A rotavirus. *C. parvum* infection was detected in 137 specimens (57.8%; 95%CI: 51.4–
187 63.9%) from 121 farms (62.7%; 95%CI: 55.6–69.2%) in 26 provinces. The presence of
188 rotavirus and/or *C. parvum* was identified in more than 76% of samples (n: 181) and both
189 pathogens were significantly more prevalent than coronavirus ($P<0.0001$), which was
190 found in 56 faecal specimens (23.6%; 95%CI: 18.6–29.4%) from 51 farms (26.4%;
191 95%CI: 20.7–33.1%) in 18 provinces. The frequency of positive samples was higher in
192 specimens from dairy calves than in samples from beef calves for *Cryptosporidium*
193 (61.5% *versus* 54.2%), rotavirus (56.4% *versus* 45%) and coronavirus (29.9% *versus*
194 17.5%), although differences were statistically significant only for coronavirus (P: 0.02).
195 Mixed infections by two or more of these pathogens was found in 101 samples (42.6%).
196 A total of 49 stool specimens (21.1%) tested negative for the enteropathogens analyzed
197 in this study (Table 1). The percentages hardly differed when only specimens from
198 individual calves (n: 148) were taken as the basis for occurrence analysis: a high detection
199 frequency was seen for *C. parvum* [84 calves (56.7%) from 75 farms (61%)] and rotavirus

200 [70 calves (47.3%) from 62 farms (50.4%)], and coronavirus infection was less common
201 [32 calves (21.6%) from 29 farms (23.6%)].
202
203 **3.2. Rotavirus genotypes**
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205 A total of 21 of the 26 specimens used for RVA genotyping were successfully
206 characterized at both the VP7 (G) and VP4 (P) genes, with three different G and two
207 different P genotypes being identified. Among the G genotypes, G6 was the most
208 common (n: 20), followed by G10 (n: 4) and G3 (n: 1). In the case of the P genotypes, 11
209 specimens were found to be P[5] and the remaining 11 were P[11]. Five specimens were
210 not successfully amplified at either the VP7 (n: 1) or the VP4 (n: 4) gene after repeated
211 attempts, and these specimens were considered untypeable. Five different combinations
212 of G and P genotypes were identified with G6P[5] and G6P[11] being the most common.
213 The combination G6P[5] predominated in beef calves (8/17 = 47%) while G6P[11] was
214 more common in dairy calves (5/9 = 55,5%). Nevertheless, this trend was not considered
215 conclusive due to the small number of specimens genotyped (Table 2).
216
217 Nucleotide sequence comparison of strains at the VP7 gene revealed a large diversity.
218 The highest number of single nucleotide polymorphisms (SNPs) was found at the G6
219 genotype of the VP7 marker, with alignments identifying up to 294 SNPs in 869 bp of
220 sequence (0.338 SNPs/nt). To prevent any bias related to the difference in the number of
221 samples, and index of 294/869/17 (SNPs/nt/sample) was calculated (0.0199). A similar
222 procedure provided an index of 0.030 SNPs/nt/sample (111/924/4) at the G10 genotype
223 of the VP7 marker. Comparison of nucleotide sequences of the VP4 gene showed a higher
224 variability at the P[5] genotype (125/767/8 = 0.0203 SNPs/nt/sample) as compared to the

P[11] genotype (80/764/8 = 0.0130 SNPs/nt/sample). The Maximum Likelihood analysis does not clearly cluster samples collected from nearby farms, indicating no strong phylogeographic structure. Moreover, the different VP7 and VP4 genotypes are not related to the latitude or the longitude of the collection points.

Phylogenetic analyses based on the VP7 gene of G6 rotaviruses identified four lineages. Most strains (14/18) clustered in the G6-IV lineage and demonstrated nucleotide identity higher than 92% to each other, and higher than 94% with strains contained in the vaccines available in Spain. The remaining four strains were allocated to lineages G6-II, G6-V (one strain each) and G6-III (two strains), and showed a nucleotide similarity lower than 86% with all the other and vaccine strains (Table 3, Figure 2, Supplementary Figures S1 and S2) (Jamnikar-Ciglenecki et al., 2016). In contrast, phylogenetic analysis based on the VP4 gene showed that RVA strains from this study were not clustered in lineages previously described into P[5] and P[11] genotypes for Argentinean rotavirus strains from cattle (Badaracco et al. (2013). Strains of the P[5] genotype showed nucleotide identity higher than 92% to each other, and higher than 90% with the vaccine strain available in Spain. RVA strains of the P[11] genotype demonstrated nucleotide similarity higher than 95% to each other (Tables 4 and 5, Figure 3, Supplementary Figure S3).

4. Discussion

Neonatal diarrhea is one of the major problems for cattle farms around the world, accounting for up to 50% of the mortality in pre-weaned dairy calves (Potter, 2011). There are no reported data on the negative impact of this condition in cattle herds in Spain, but the economic losses in other European countries such as Norway, which has a

250 significantly lower livestock production, were estimated to be US$ 10 million in 2006
251 (Østerås et al., 2007). In this study, we investigated the occurrence of three common
252 enteropathogens in specimens from diarrheic calves submitted by veterinarians to an
253 animal health laboratory. Commercial real-time RT-qPCR assays were used for a rapid
254 diagnosis of these agents. These molecular methods have been proven to be an excellent
255 tool for the detection of several enteric pathogens, and have gradually replaced other
256 diagnostic tests with the advantage of high sensitivity and specificity (Cho and Yoon,
257 2014). Many specimens were tested as faecal pools, which means that laboratory-
258 confirmed cases were considered to represent farm-level infection and not the individual
259 prevalence. Nevertheless, the percentages of positive specimens and farms hardly differed
260 when only specimens from individual calves were used for prevalence estimation.
261
262 Nearly 80% of specimens (188/237) were positive for at least one of the three target
263 enteric pathogens, indicating that they are usually involved in the etiology of infectious
264 calf diarrhea. The remaining diarrheic stool specimens (just over 20%) were negative for
265 the three target agents, although common bacterial (*E. coli* K99, *C. perfringens*), or other
266 viral pathogens (norovirus, torovirus, nebovirus) were not analyzed in this study.
267 Likewise, the role of non-infectious factors (inclement weather, poor sanitation, adequate
268 colostrum intake, etc) was not excluded. *C. parvum* and rotavirus were the most common
269 (57.8% and 50.6% of faecal specimens, respectively) and widespread pathogens (62.7%
270 and 54.9% of farms, respectively), with over 75% of calves being infected by one or both.
271 Previous studies in Europe have shown that *Cryptosporidium* and rotavirus were the two
272 most prevalent enteropathogens in diarrheic calves in Belgium (31% and 20%,
273 respectively), Sweden (11% and 24%), Switzerland (55% and 59%) and The Netherlands
274 (27.8% and 17.7) (de Graaf et al., 1999; Björkman et al., 2003; Lanz Uhde et al., 2008;

275 Bartels et al., 2010). Infections by bovine coronavirus were significantly less prevalent in
276 this study, although they were still found in approximately a quarter of faecal samples
277 and farms, which indicates that coronavirus also plays a significant role in calf diarrhea.
278 In fact, studies conducted in other European countries have reported substantially lower
279 detection rates for coronavirus in diarrheic suckling calves (3.4–8%) (de Graaf et al.,
280 1999; Björkman et al., 2003; Lanz Uhde et al., 2008; Bartels et al., 2010).
281
282 The three target enteric pathogens were most prevalent in specimens from dairy calves,
283 although differences with beef calves were statistically significant only for coronavirus.
284 Epidemiological studies in the Czech Republic have previously reported lower
285 *Cryptosporidium* infection prevalence in beef calves than in dairy calves, which was
286 linked to differences in breeding technology (Kvác et al., 2006). An overall low
287 *Cryptosporidium* prevalence was also seen in beef calves in Belgium, although
288 differences were attributed to the significantly higher age of the beef calves compared to
289 dairy calves sampled (Geurden et al. 2007). In contrast, the proportion of samples positive
290 to rotavirus in Brazilian farms was significantly higher in calves from beef herds, a
291 finding that the authors related to differences in management practices (Alfieri et al.
292 2006).
293
294 The occurrence of *Cryptosporidium* correlates well with other studies in diarrheic
295 neonatal calves from dairy farms in northern Spain, recording similar percentages of
296 infected calves (47.9–57.8%) using microscopy methods (Castro-Hermida et al., 2002;
297 Quílez et al., 2008). *Cryptosporidium* and rotavirus were also the most commonly
298 detected agents in a previous study in central Spain (52.3% and 42.7% positive samples
299 respectively), where mixed infections by both microorganisms were found in 21.6% of

300 the calves (De la Fuente et al., 1998). Studies in Europe and Australia have showed that
301 multiple pathogens are frequently reported in diarrheic calves (40–71% of samples), and
302 this has been linked with more severe clinical signs (Bazeley, 2003; Lanz Uhde et al.,
303 2008; Izzo et al., 2011). A recent study of calf diarrhea in India showed that the majority
304 of the samples (90%) showed mixed infections ranging from a combination of two to five
305 agents (Brar et al., 2017). In the current study, concurrent infections with two of the three
306 pathogens were more common than single infections (101 and 87 samples respectively)
307 and 10% of specimens contained all the three target pathogens. This finding indicates that
308 most calves were exposed to a diverse pathogenic load, which may result in a more severe
309 disease (Peek et al., 2018). The high frequency of co-infections also supports the
310 suggestion that control of calf diarrhea should be focused on hygienic measures and
311 improvement of the husbandry management system (Cho et al., 2013).
312
313 The present study also demonstrates a large genetic diversity of rotavirus circulating in
314 sampled cattle farms in Spain. A total of five combinations were identified in 21
315 specimens successfully typed, but a remarkable variability was seen among the strains
316 allocated to each particular genotype, especially for the VP7 marker. Genotype G6 was
317 by far the most prevalent and widely distributed G rotavirus type followed by G10.
318 Phylogenetic analysis of the VP7 gene allocated most strains to the G6-IV lineage, which
319 is the most usual lineage of bovine RVA according to the classification provided by
320 Jamnikar-Ciglenecki et al. (2016). The lineage includes the G6P[5] (strain RVA/Cow-
321 tc/GBR/UK/1973/G6P[5]) and G6P[1] (strains RVA/cow-tc/USA/NCDV/1967/G6P[1]
322 prototypes, which are present in commercial vaccines available in Spain. Nevertheless, a
323 significant ratio of strains (4/18) exhibited a much lower (< 86%) nucleotide identity with
324 the other strains and clustered into three other lineages (II, III, V), which contain both

human and bovine RV strains (Jamnikar-Ciglenecki et al., 2016). Analysis of the VP4 gene revealed that P types were more evenly distributed and two genotypes (P5 and P11) showed an identical occurrence rate. Lineages described by Badaracco et al. (2013) in RVA from Argentinian cattle were used for phylogenetic analysis of this marker, and no clear phylogenetic relationships with RVA strains from this study were found.

Recent reviews on rotavirus have revealed differences in the genotype distribution according to the host and some studies have reported fluctuations in the G and P type prevalence over time (Papp et al., 2013). The most frequent cattle genotypes belong to VP7 types G6 and G10, and VP4 types P5 and P11, with combinations G6P[5], G6P[11] and G10P[11] being predominant in many areas worldwide (Papp et al., 2013; Dóró et al., 2015) and this is in agreement with the results of the current study. The G-P combination G6P[5] was the most common rotavirus circulating in cattle in France (Kaplon et al., 2013), Ireland (Collins et al., 2014), Germany (Otto et al., 2015) and Iran (Pourasgari et al., 2016). In contrast, G6P[11] was the most common VP7/VP4 combination in calves in Italy (Monini et al., 2008), Turkey (Alkan et al., 2010) and Tunisia (Hassine-Zaafrane et al., 2014). Genotype combination G10P[11] predominated in diarrheic calves in other areas of Iran (Madadgar et al., 2015), India (Ahmed et al., 2017), Argentina (Badaracco et al., 2013) and Brazil (da Silva Medeiros et al., 2019).

It is worth mentioning that rotavirus G10P[11] is frequently associated with asymptomatic and symptomatic infection in Indian children and has been related to zoonotic transmission (Iturriza-Gomara et al., 2004; Ramani et al., 2009). This is also the genotype combination selected to be included in commercial vaccines in some countries (Rocha et al., 2017). The discrepancy between the RVA genotypes found in the

commercial vaccines and RVA strains circulating in cattle herds has been reported as a contributing factor to explain the lack of protection in some vaccinated herds, which suggests that only the combinations more common in the geographical area of interest should be present in the vaccines (Alkan et al., 2010; da Silva Medeiros et al., 2015; Rocha et al., 2017). The lack of information about rotavirus vaccination in the cattle farms sampled constitutes a limitation of this study. Nevertheless, it is significant to note that only one of the three commercial vaccines available in Spain contains the genotype RVA/Cow-tc/GBR/UK/1973/G6P[5], matching the most prevalent VP7/VP4 combination found in this study. The other commercial vaccines currently available in Spain contain the genotype RVA/Cow-tc/USA/NCDV/1967/G6P[1], which is a VP7/VP4 combination not detected in this study.

The current study highlights the role of rotavirus and *C. parvum* as major pathogens in the etiology of calf diarrhea in the Spanish farms sampled, with coronavirus playing a minor but not insignificant role. The molecular analysis revealed the genetic variability of rotavirus strains circulating in these cattle farms, with two predominant genotype combinations G6P[5] and G6P[11], but a high frequency of single nucleotide polymorphisms, especially at the VP7 marker. Further investigations with additional specimens are required to confirm these observations, with regard to genotype combinations to be incorporated into future vaccines. To the best of our knowledge, this is the first study on the molecular characterization of rotavirus from cattle farms in Spain.

Acknowledgments

We thank Dr. Demetris Savva (School of Biological Sciences, University of Reading, UK) for a thoughtful and careful review of the manuscript.

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Figure 1. Map of Spain showing the provinces (shaded) with cattle farms submitting samples for diagnosis. Some RVA strains from the twelve provinces marked with an asterisk were genotyped at the VP7 and VP4 genes.

Figure 2. Phylogenetic analysis of RVA strains of the G6 genotype examined in the current study (marked with an asterisk) to representative strains of different G6 lineages and strains RVA/cow-tc/USA/NCDV/1967/G6P[1] and RVA/Cow-tc/GBR/UK/1973/G6P[5] contained in the vaccines available in Spain. Lineages described by Jamnikar-Ciglenecki et al. (2016) are indicated. Maximum Likelihood analysis based on genetic distances calculated by the Kimura two-parameter model and a bootstrap value of 1,000.

Figure 3. Phylogenetic analysis of RVA strains of the P[5] and P[11] genotypes examined in the current study (marked with an asterisk) to representative strains of different P[5] and P[11] lineages. The strain RVA/Cow-tc/GBR/UK/1973/G6P[5] contained in the vaccines available in Spain is included. Lineages described by Badaracco et al. (2013) in RVA strains from Argentinian cattle are indicated. Maximum Likelihood analysis based on genetic distances calculated by the Kimura two-parameter model and a bootstrap value of 1,000.

Supplementary Figure S1. Alignment of predicted partial amino acid sequences (G6 genotype of the VP7 gene) of RVA strains found in the current and strain RVA/Cow-tc/GBR/UK/1973/G6P[5] contained in commercial vaccines available in Spain

Supplementary Figure S2. Alignment of predicted partial amino acid sequences (G6 genotype of the VP7 gene) of RVA strains found in the current and strain RVA/Cow-tc/USA/NCDV/1967/G6P[1] contained in commercial vaccines available in Spain

Supplementary Figure S3. Alignment of predicted partial amino acid sequences (P[5] genotype of the VP4 gene) of RVA strains found in the current and strain RVA/Cow-tc/GBR/UK/1973/G6P[5] contained in commercial vaccines available in Spain

Figure 1

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Figure 2

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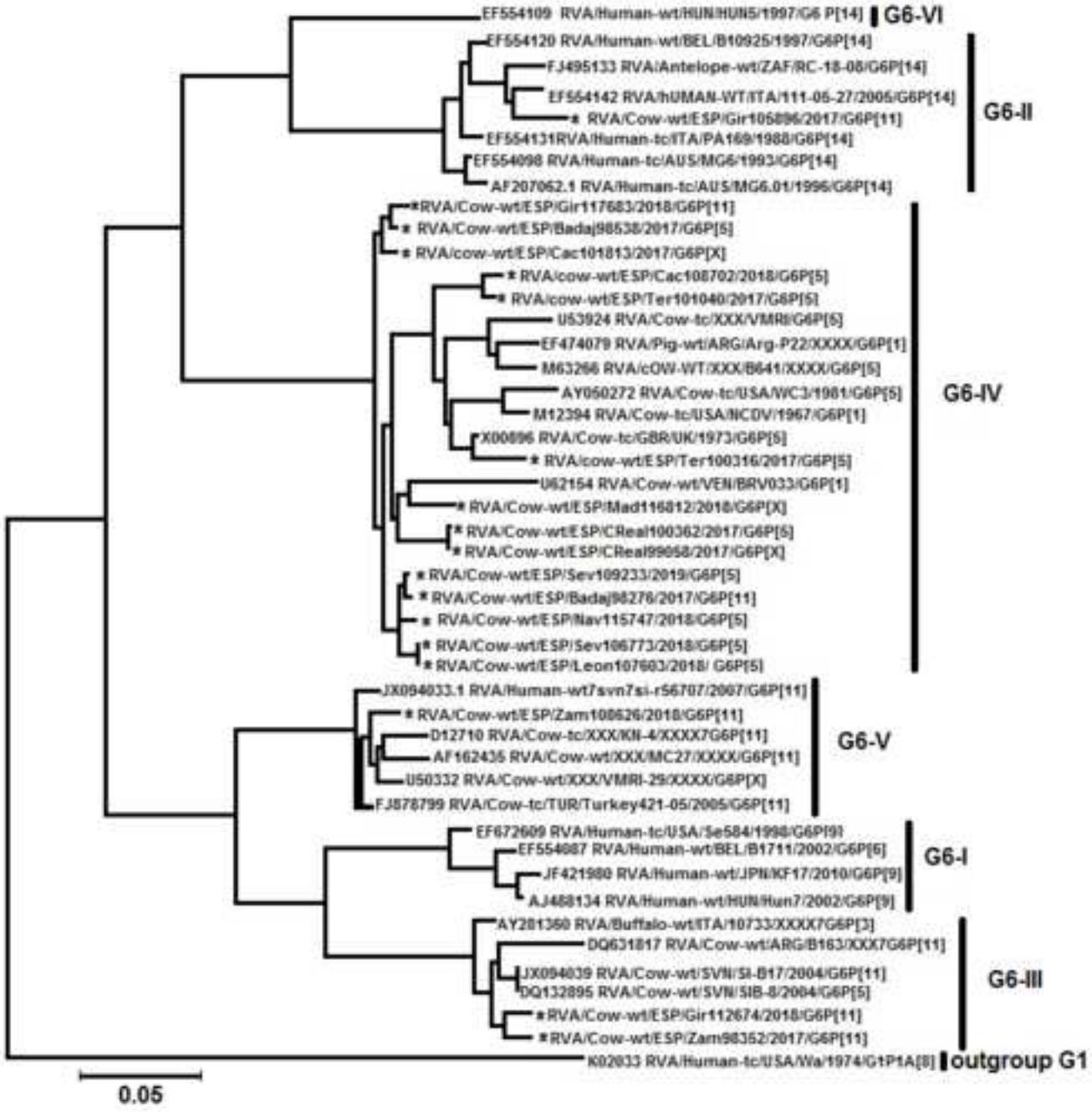

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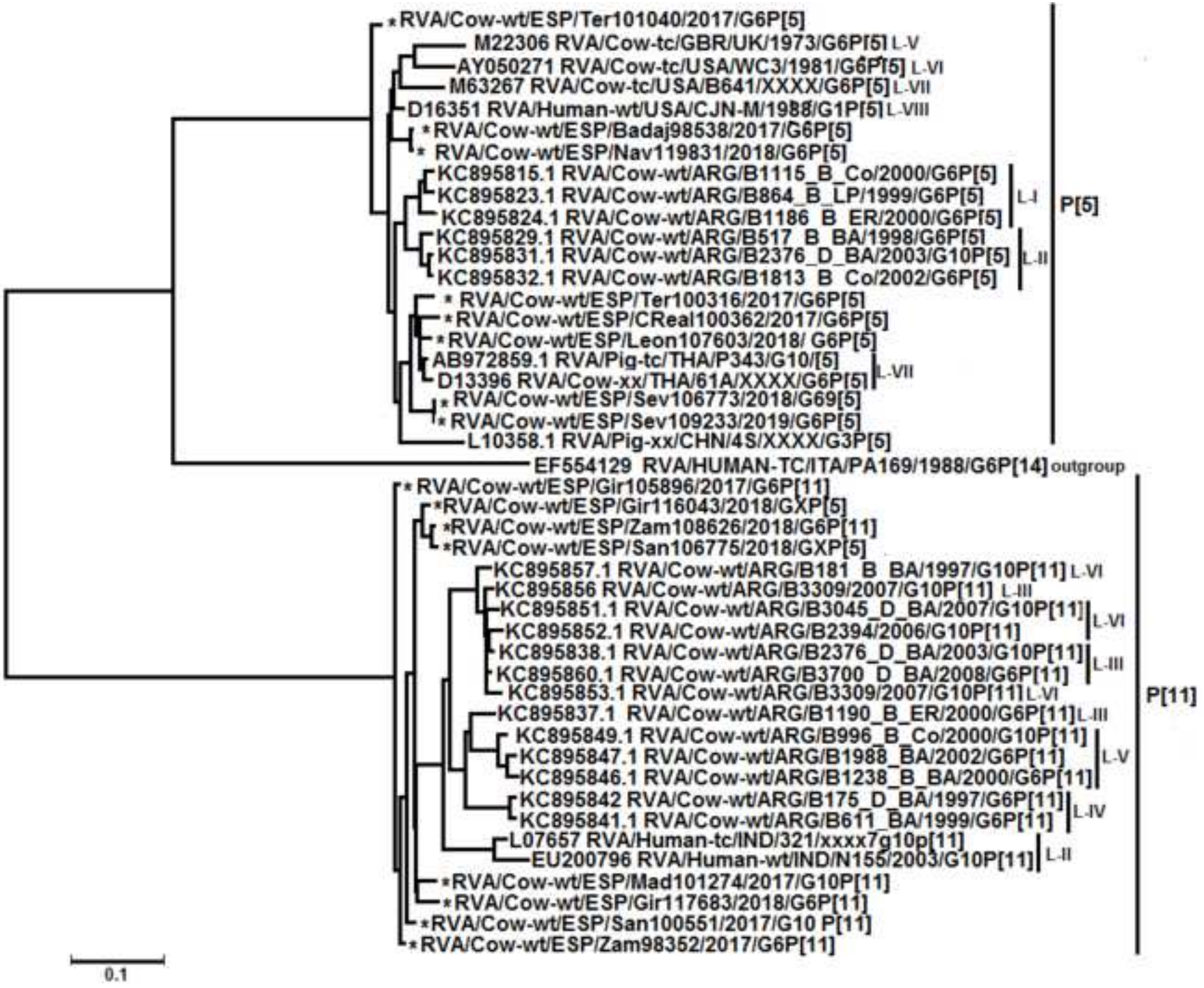

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Supplementary Figure S1

Supplementary Figure S2

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