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Short communication

Molecular characterization of rotavirus isolated from alpaca (*Vicugna pacos*) crias with diarrhea in the Andean Region of Cusco, Peru

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ABSTRACT

Alpacas (*Vicugna pacos*), a species of South American camelids (SAC), suffer high morbidity and mortality from infectious diseases. Diarrhea is one of the leading causes of alpaca cria mortality in Peru and elsewhere. In order to develop appropriate control and/or treatment, it is necessary to identify infectious pathogens that cause diarrhea in crias. Rotavirus was isolated in cell culture from feces collected from crias with acute diarrhea that tested positive to rotaviral antigen by rapid immunochromatographic methods in an earlier study. The isolates were identified as rotaviruses by RT-PCR run with specific primers for human rotavirus VP7 coding sequences using total RNA extracted from cells displaying cytopathic effects as template. These alpaca isolates were further identified as group A rotaviruses by means of a VP6-specific PCR and were designated as ALRVA-K'ayra/Perú/3368-10 and ALRVA-K'ayra/Perú/3386-10. Molecular G and P typing, placed the former as G3/P11 and the latter as G3/P?. Sequence analysis of two genome segments (coding for VP4 and VP7) from the alpaca isolates revealed partial homologies to swine and human rotaviruses, respectively. These results demonstrate that rotaviruses are associated with a proportion of cases of diarrhea in crias, although prevalence and impact remain to be determined. The isolation of rotaviruses from alpaca crias with diarrhea will contribute positively to further understand the pathogen and its role in the diarrhea complex.

1. Introduction

Alpacas (*Vicugna pacos*) are economically important South American camelid (SACs) species raised for fiber and food by a significant proportion of dwellers of the Peruvian Andes. Numerous diseases have been recognized in these species and a variety of factors are involved in disease causation, including nutrition, genetics, and climate. Infectious pathogens are a major cause of morbidity, reproductive failure and mortality in SACs leading to production losses (Moro, 1971; Fernandez Baca, 1975; Ramirez et al., 1980; Kapil et al., 2009; Sharpe et al., 2009). Among infectious agents, viruses that are prevalent in other domestic species are transmitted to llamas and alpacas (Thedford and Johnson, 1989; Mattson 1994; Whitehead and Bedenice, 2009), however, their impact in morbidity and mortality remain unclear. Therefore, establishing causal relationships and measuring impact, are of utmost importance to implement appropriate and effective control measures.

Evidence of exposure and morbidity caused by pathogens originated in other domestic species has been documented in alpacas and llamas. Thus, there is serologic and immunochemical evidence of exposure to bovine respiratory, enteric and systemic viruses (Rivera et al., 1987; Rosadio et al., 1993; Puntel, 1997; Puntel et al., 1999; Leoni et al., 2001; Victorio et al., 2004; Cabello et al., 2006; Marcoppido et al., 2010; Rosadio et al., 2011). Enteric disease, caused by protozoa, enteric bacteria and enteric viruses, remains a leading cause of morbidity and mortality of SAC crias (Parreño et al., 2004; Constantini et al., 2001; Cebra et al., 2003; Palacios et al., 2005; Whitehead and Anderson 2006; Morales et al., 2007; Starkey et al., 2007; Twomey et al., 2012; Lopez et al., 2011; Marcoppido et al., 2011; Badaracco et al., 2013). The negative effects of enteric disease in alpaca production are significant and indicate a need for further investigation to establish more precisely roles and impact of enteric pathogens in morbidity and mortality associated with diarrhea.

While there is evidence of specific antibodies to rotavirus (Chang-Say et al., 1985; Rivera et al., 1987), and rotaviral antigens in alpaca crias with diarrhea (Morales et al., 2007, Lopez et al., 2011). there has not been reports of actual rotavirus isolation from the affected animals. The isolation and molecular characterization

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reported here further confirm an association of rotavirus with acute diarrhea in alpaca crias.

2. Materials and methods

2.1. Animals and Virus isolates

The alpaca herd of La Raya Research Center, Universidad Nacional de San Antonio Abad del Cusco (USAAC) in the province of Canchis, Department of Cusco was used for investigation in this study. La Raya is located in an area of intensive alpaca production. Fecal samples collected from crias with acute diarrhea in the herd screened in an earlier study (Lopez et al., 2011) for rotaviral antigen using an immunochromatrographic (ICG) kit kindly provided by the manufacturers (VetAll Laboratories Koyang-Ku, Gyeonggi-Do, South Korea) and stored at −80 °C were used. Two fecal samples positive for rotaviral antigen by ICG, were further processed for virus isolation. The isolates were identified by RT-PCR.

2.2. RT-PCR

RNA was extracted with Trizol Reagent from cell cultures showing CPE and from non-inoculated cell controls, as recommended by the manufacturer (InVitrogen, Carlsbad, CA). The RNA mixed with equal volumes of DMSO, was heated at 97 °C for 5 min, cooled down to 4 °C and then kept in ice until the reverse transcription (RT) reaction was conducted. The RT reaction was carried out using an iScript cDNA kit, as instructed (BioRad, Hercules, CA). Briefly, reaction mixtures contained 5× reaction buffer, reverse transcriptase, random hexamers, 50–100 ng of RNA template, and nuclease-free water in a 20 μl/reaction. The RT reaction was run at 25 °C for 5 min, followed by 42 °C for 45 min and the cDNA held at 4 °C. The cDNA was used as the template to amplify VP7 or VP6 coding genes using the Green master mix kit (Promega Madison, WI). Briefly, reactions contained Green master mix, cDNA template, and Beg 9 and End 9 (Table 1) as forward and reverse primer sets for VP7 coding sequence (Gouvea et al., 1990), and nuclease-free water in a 25 μl/reaction. The PCR reaction was run at 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min. To amplify the VP6 coding sequence, specific primers rot3 and rot5 (Table 1) (Elschner et al., 2002) were used. The amplicons separated by electrophoresis in 1% agarose gels were visualized by ethidium bromide intercalation. Bovine rotavirus (BRV) strain 130 BDV acquired from NVSL (Ames, Iowa) was utilized as reference positive control in RT-PCR.

2.3. Molecular and P typing

cDNAs obtained during RT reactions were amplified by PCR using primer cocktails containing Beg9, G6, G8, G10 G11 (cocktail 1), End9, G3, G4 (cocktail 2) for G typing and Con2 (Gentsch et al., 1992), P1, P5, P11 (cocktail 3) and Con3 (Gentsch et al., 1992), P10 (cocktail 4) for P typing. The G3 primer used was 5′CGTTTGAAGAAGTTGCAACAG 3′ (Gouvea et al., 1990) and the P11 primer used was 5′ GGAACGTATTCTAATCCGGTG 3′ (Gouvea et al., 1994). The PCR reaction and electrophoretic separation of amplicons were conducted essentially as described (Gouvea et al., 1990, 1994). Then based on band patterns subsequent PCRs were conducted with single G or P primers in combination with END9 or Beg9 or Con2 for G and P typing respectively. Primer sequences are listed in Table 1.

2.4. Sequencing

PCR products were purified using the AxyPrep™ PCR Cleanup Kit (Axygen Biosciences, Union City, CA) and sequenced at the University of Connecticut Biotechnology Center by the didedoxy-nucleotide chain termination method using an ABI-3500 Genetic Analyzer (Applied Biosystems). The sequences were analyzed using the Sequencher 5.0 software (Gene Codes Corporation Ann Arbor, MI) and compared against sequences in the GenBank database using the BLAST alignment tool.

3. Results and discussion

3.1. Alpaca cria isolates identified as rotavirus A by RT-PCR

The isolation of rotavirus confirms earlier serologic and antigen evidence of rotavirus infection reported in alpaca crias in Peru (Rivera et al., 1987, Morales et al., 2007, Lopez et al., 2011). Rotavirus RNA was identified by a VP7 specific RT-PCR in total RNA extracted from the two cell cultures showing CPE and these two virus isolates were designated thereafter ALRVA/K'ayra/Perú/3368-10 and ALRVA/K'ayra/Perú/3386-10 respectively. The reaction products for VP7 coding sequence from the two isolates, had similar migration in agarose gel electrophoresis than that of the NVSL BRV used as positive control (Fig. 1A). Control samples extracted and amplified under identical methods were negative. Furthermore, the two alpaca isolates also tested positive in VP6-specific RT PCRs, yielding amplicons of similar electrophoretic migration in agarose as those of the NVSL BRV positive control (Fig. 1B). These molecular data identified the two virus isolates as group A rotaviruses.

3.2. ALRVA/K'ayra/Perú/3368-10 is G3/P11 and ALRVA/K'ayra/Perú/3386-10 G3/P?

Molecular G typing yielded positive reactions and identified both isolates as type G3, and P typing identified ALRVA/K'ayra/Perú/3368-10 as P11 while for yet unknown reasons, P typing of ALRVA/K'ayra/Perú/3386-10 was inconclusive despite of several attempts (Fig. 1C). Molecular G and P typing of the isolates identified ALRVA/K'ayra/Perú/3368-10 as G3/P11 and isolate ALRVA/K'ayra/Perú/3386-10 while was also G3 positive, P typing remained elusive. The reasons of the inconclusive P typing for the latter are not understood at this juncture as the tests were repeated several times with different samples with similar results. Nevertheless, the isolation, identification and partial molecular characterization of rotavirus in fecal samples of alpaca crias with diarrhea will be instrumental for understanding the epidemiology,

Table 1
Primer sets utilized to identify and characterize Alpaca rotavirus isolates

Primer sets	Sequence	Target	Product bp	Ref.
Con3	5′TGGCTTCGCCATTTTTATAGACA3′	VP4	876	Gentsch et al., 1992
Con2	5′ATTTCGGACCATTTATAACC3′			
Beg 9	5′GGCTTTAAAAGAGAGAATTTCCGTCTGG3′	VP7	1062	Gouvea et al., 1990
End 9	5′GGTCACATCATACAATTCTAATCTAAG			
rot3	5′AAAGATGCTAGGGACAAAATTG3′	VP6	308	Elschner et al., 2002
rot5	5′TTCAGATTGTGGAGCTATTCCA3′			

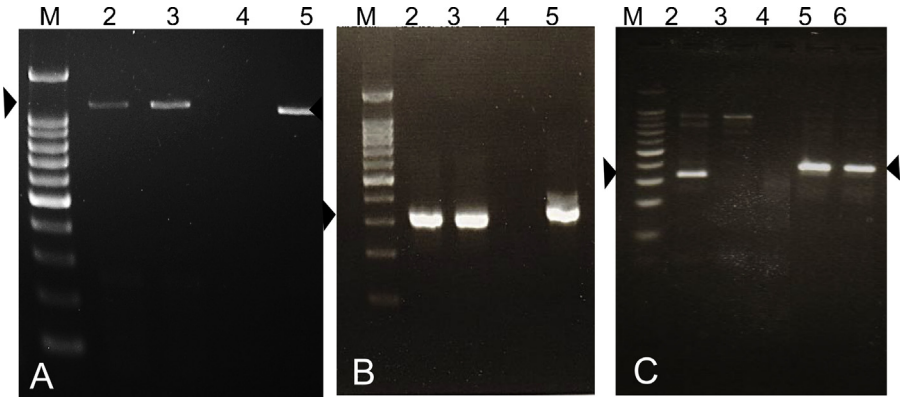

Fig. 1. Molecular characterization of alpaca rotavirus isolates. RNA extracted from cell culture isolates was amplified by RT-PCR using either VP7 sequence-specific primers (1A) or VP6 sequence specific primers (1B). (A and B) DNA ladder (M); ALRVA/K'ayra/Perú/3368-10, ALRVA/K'ayra/Perú/3386-10 (2, 3); negative control (4); NVSL bovine rotavirus (BRV) as positive control (5). (C) molecular G and P typing. ALRVA/K'ayra/ Perú/3368-10 and ALRVA/K'ayra/ Perú /3386-10 cDNAs amplified by PCR using P11/Con2 primer combination (2 and 3); negative control (4) or G3/END9 primer combination (5 and 6).

pathogenesis, and will be of significant value to establish the role of rotavirus relative to other pathogens in the diarrhea complex in alpaca crias more precisely. The finding that the rotavirus isolates appear to be type G3 is intriguing as this is a common type in humans and sequencing of the corresponding gene revealed a relatively high nucleotide homology (96%) with human rotavirus in this segment.

3.3. The alpaca rotavirus isolates had partial homologies to swine and human rotavirus

Sequencing of PCR-amplified fragments coding for VP4 and VP7 of the two alpaca isolates revealed homologies with swine and human rotaviruses. Interestingly, the alpaca rotavirus isolates showed 75% homology with porcine rotavirus VP4 coding sequence and up to 96 % homology with human rotavirus VP7 coding sequence (GeneBank accession numbers KT250941 and KT250942). The sequence relatedness of the two alpaca isolates with swine and human rotaviruses suggest that these may represent novel viruses resulting from interspecies transmission that may also involve zoonotic transmission (Matthijnssens et al., 2006; Parra et al., 2008; Martella et al., 2010; Parreño et al., 2004; O'shea et al., 2014) and swine may be an important reservoir in which new virus reassortants may originate (Parra et al., 2008). However, in order to draw phylogenetic or epidemiologic conclusions will require more extensive sequencing. Thus, additional comparative genetic analysis of all segments would be indicated to establish linkages and evolutionary relationships with rotaviruses from other species dwelling in the same ecologic niche including humans as recently reported with group A giraffe and vicugna rotaviruses (O'shea et al., 2014; Badaracco et al., 2013). The full or partial genome sequence analysis reported in rotaviruses recovered from these species, suggests that interspecies circulation of viruses within a particular genotype constellation is a likely transmission event (O'shea et al., 2014; Badaracco et al., 2013). Further extensive sequencing and phylogenetic analysis would be required to verify whether rotaviruses recovered from other SACs or any other artiodactyls or humans within the same area of our study share such genotype constellations with the alpaca rotavirus isolates.

The relative importance of several pathogens in diarrhea of crias has been documented in North America (Cebra et al., 2003; reviewed by Whitehead and Anderson, 2006). It appears, that coronavirus is one of the leading causes of diarrhea in crias followed by protozoal pathogens with a relatively lower incidence of rotavirus associated diarrhea (Cebra et al., 2003). Coronavirus-induced diarrhea has been identified in crias of llamas and alpacas and in adult animals in North America (Cebra et al., 2003; Genova et al., 2008). The relatedness of the genome sequence to bovine coronavirus suggests that the virus in alpacas is from bovine origin (Jin et al., 2007).

In contrast, an outbreak of acute diarrhea in guanacos (*Lama guanicoe*) in Patagonia (Argentina) with high morbidity and mortality was shown to be associated virologically and serologically with rotavirus while coronavirus was not a significant factor (Parreño et al., 2004). Previous serologic surveys in Argentinian llama farms found a 88% prevalence of antibody to rotavirus (Puntel et al., 1999). There is also evidence of antibodies to rotavirus (Rivera et al., 1987), or rotaviral antigen in crias with diarrhea in Peru (Morales et al., 2007, Lopez et al., 2011) in the Andes of Peru. Recent studies confirm rotaviral infection in guanaco crias by seroconversión and excretion of virus in feces (Marcoppido et al., 2011), and in vicuña by ELISA and molecular methods (Badaracco et al., 2013). These data indicate marked differences in frequency and distribution of enteric viruses in SACs in different geographical locations.

The present study reports the molecular characterization of rotaviruses isolated from feces of crias that had acute diarrhea and this strongly suggests a causal relationship. However, the prevalence and relative importance of rotavirus in the diarrhea complex in alpacas remains undetermined. Thus, further investigation is required to establish the actual impact of rotaviruses in the overall morbidity and mortality of alpaca crias.

4. Conclusions

Diarrhea is one of the leading causes of cria mortality. The isolation and molecular characterization of rotavirus from alpaca crias with acute diarrhea enabled us to further understand this important pathogen which potentially will contribute to develop appropriate control and/or treatment. Two rotavirus isolates were obtained from fecal samples by inoculation into cell monolayers. The isolates were identified as rotavirus by means of a RT-PCR with specific primers for human rotavirus. During identification, bovine rotavirus A was used as a reference. The alpaca isolates were identified as group A rotaviruses by VP6-specific PCR and were designated ALRVA/K'ayra/Perú/3368-10 and ALRVA/K'ayra/Perú/3386-10. Molecular G and P typing, placed the former as G3/P11 and the latter as G3/P?. Sequencing of the isolates segments coding for VP4 and VP7a revealed partial homologies to swine and human rotaviruses, respectively. These findings are of significant interest and call for further investigation to establish epidemiologic and

phylogenetic relationships with rotaviruses from other species dwelling in the same area including humans.

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