DOI: 10.1590/1808-1657v73p1012006

SCIENTIFIC COMMUNICATION

AN ENTERIC CORONAVIRUS IN A 3-DAY-OLD DIARRHEIC FOAL

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ABSTRACT

Coronaviruses are of special interest in diarrhea of horses, once they cause disease in foals and in the adult. This study aimed to evaluate the existence of coronavirus, rotavirus, protozoa and bacteria in stool of a 3-day old foal suffering from acute diarrhea. A nested PCR assay for the RNA-dependent RNA-polymerase gene was applied to coronavirus detection and PAGE, sucrose flotation test and classical bacteriology for rotavirus, protozoa and bacteria detection, respectively. An enteric group II coronavirus was found with no concurrent infections. The role of coronavirus in this clinical case is discussed, as well as possible transmission routes.

KEY WORDS: Coronavirus, newborn, diarrhea, foal.

RESUMO

UM CORONAVÍRUS ENTÉRICO EM UM POTRO DE 3 DIAS DE IDADE COM DIARRÉIA. Coronavírus têm um interesse especial em cavalos, uma vez que causam doenças em potros e adultos. Este estudo objetivou avaliar a existência de coronavírus, rotavírus, protozoários e bactérias em fezes de um potro com 3 dias de idade sofrendo de diarréia aguda. Uma reação de nested-RT-PCR para o gene da RNA-polimerase RNA-dependente foi aplicada para a detecção de coronavírus e as provas de PAGE, teste de flutuação em sacarose e bacteriologia clássica foram aplicadas para a pesquisa de rotavírus, protozoários e bactérias, respectivamente. Um coronavírus entérico do grupo II foi encontrado, sem outras infecções ou infestações simultâneas. O papel dos coronavírus neste caso clínico é discutido no presente artigo, bem como as possíveis vias de transmissão.

PALAVRAS-CHAVE: Coronavírus, neonatos, diarréia, potro.

Diarrhea in young horses has already been associated with *Cryptosporidium* sp., *Escherichia coli*, *Proteus* sp., rotavirus and coronavirus (Durhan et al., 1979; Mair et al., 1990), but the latter is of special interest, since it may also cause an acute enteric syndrome in equine, first named Potomac fever (Huang et al., 1983).

Coronaviruses are enveloped round-shaped viruses, about 220 nm in diameter, with six or five structural proteins (N, M, sM, HE, S and I) and a positive-sense single-stranded RNA making up the helical nucleocapsid in association with protein N, classified in the order *Nidovirales*, family *Coronaviridae*, which comprises the genera *Coronavirus* and *Torovirus*. Coronaviruses are sub-grouped in three antigenic and genetic groups, but no equine coronavirus has yet been demarcated in this genus (Lai & Cavanagh, 1997; van Regenmortel et al., 2000). Although viruses in the same group share antigenic properties, high species specificity is a striking feature (Kuo, 2000).

This study aimed to evaluate the role of coronavirus, rotavirus, bacteria and protozoa in stool of a diarrheic foal suffering from acute diarrhea.

Sample

A stool sample was collected from a 3-day-old female foal suffering of an acute and severe diarrhea

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in São Paulo State, Southern Brazil, in October 2002. The sample was collected from the rectum and stored at -80º C.

Coronavirus detection

The sample was prepared as a suspension in phosphate buffer saline solution 0.01M/BSA 0.1% pH 7.2 (PBS) and clarified by centrifugation (12.000xg / 30' at 4º C). An RT-PCR assay targeted to amplify a 136-bp fragment of group II coronaviruses RNA-dependent RNA-polymerase gene (*RdRp*) was applied as described by Brandão et al. (2005). Bovine coronavirus Kakegawa strain (Akashi et al., 1980) was used as positive and PBS as negative control.

c-DNA synthesis - Total RNA from the stool suspension was extracted with TRIzol reagent and 7 µL of each extracted RNA re-suspended in DEPC-treated water was denatured at 95ºC for 5 minutes and added to c-DNA mix containing 1 x First Strand Buffer, 1 mM of each dNTP, 10 mM DTT, 1 pmol/µL of each primer (4Bm and 2Bp as described by Stephensen et al. (1999) and 200U M-MLV Reverse Transcriptase for a 20 µL final reaction. Reverse transcription was carried out at 42 ºC / 60´.

First-round amplification and nested PCR - Next, 5 µL of c-DNA were added to the PCR mix (1 x PCR Buffer, 0.2 mM of each dNTP, 0.5 pmol/µL of each primer [4Bm and 2Bp], 1.5 mM $MgCl_2$, 25.25 µL ultra-pure water and 1.25U Taq DNA polymerase for a 50 µL final reaction) and submitted to 6 cycles of 94º C / 1', 40º C / 2' and 72º C / 1', 36 cycles of 94º C / 1', 50º C / 1.5' and 72º C / 1', followed by 72º C / 10' for final extension.

Second-round amplification was carried out with 5 µL of first PCR product added to the PCR mix (1 x PCR Buffer, 0.2mM of each dNTP, 0.5 pmol/µL of each CV2L and CV2U primers, 1.5 mM $MgCl_2$, 25.25 µL ultra-pure water and 1.25U Taq DNA polymerase) and submitted to 26 cycles of 94º C / 1', 54.8º C / 1.5' and 72º C / 1' followed by 72º C / 10' for final extension.

An ultra-pure water-containing tube was used as a nested negative control, mix was also added and it was submitted to thermocycler to monitor amplicon contamination. Furthermore, in order to avoid any laboratory contamination, each step (RNA extraction, reverse transcription and PCR, nested and electrophoresis) was carried out in separate rooms with separate materials.

Ten microliters of the nested product were analyzed in 1.5% agarose gel electrophoresis stained with 0.5 µg/mL ethidium bromide.

Rotavirus detection

The stool sample was searched for rotavirus 11-segmented RNA in PAGE (polyacrylamide gel electrophoresis) according to Herring et al. (1982). Total RNA was extracted with phenol/clorophormium from 20% fecal suspensions in PBS, precipitated with ethanol and resolved in 3.5% / 7.5% discontinuous polyacrylamide gel under 20 mA for 2 hours and stained with silver. The NCDV rotavirus strain (White et al., 1970) was included as positive and PBS as negative control.

Protozoa detection

Oocysts of *Crypstosporidium* sp. and *Eimeria* sp. and cysts of *Giardia* sp. where searched with the sucrose flotation test (specific gravity 1.205) (Ogassawara & Benassi, 1980).

Bacteriological analysis

Bacteria that might have had a role in the onset of the diarrhea observed in this foal, e. g., *Salmonella*, *E. coli*, *Proteus* etc where searched by classical bacteriological methods according to the Ministry of Agriculture, Fisheries and Food (1984). Briefly, the stool sample was seeded in MacConkey and blood agar in Petri dishes with a slope and incubated at 37º C, the growth of colonies screened up to 24 hours.

The stool sample from the diarrheic foal was found positive to group II coronavirus in the PCR targeting the *RdRp* gene, evidenced by the appearance of the predicted 136-bp fragment in 1.5% agarose gel electrophoresis, as seen to the Kakegawa positive control. Neither the nested negative control nor the PBS included as negative control showed spurious bands or contamination.

The sample was negative for rotavirus in PAGE, since rotavirus RNA electropherotype, as found for the NCDV positive control, was not seen. No bacterial colonies grew either on MacConkey or in blood agars after 24 of incubation. Furthermore, the sucrose flotation test evidenced no protozoa in this stool sample.

An enteric group II coronavirus was found in a stool sample of a newborn foal suffering of an acute and severe diarrhea. Regarding the finding that no concurrent infections by rotavirus, protozoa or bacteria were found in the sample, coronavirus might be implicated in the primary etiology of the pathologic processes that lead to the development of clinical symptoms.

Newborn mammals, unless fed colostrum in the first few hours after birth, are prone to suffer from a large variety of acute diseases, especially those affecting the enteric and respiratory tracts, since passively transferred local immunity is a remarkable feature to avoid adherence, adsorption and infection in these sites by agents such as coronavirus.

Thus, if the foal here reported was not able to receive enough amounts of protective colostrum,

coronavirus would have had free access to the intestinal *villi* of the small intestine, its preferential site of infection (Brandão et al., 2001), replicated in the enterocytes and caused desquamation of the mucosa, leading to malabsortive diarrhea.

Feeding habits of newborn foals are restricted to suckling milk and they do not graze. In calves, coronavirus may infect the intestinal tract both by the nasal and oral routes (Heckert et al., 1991). Thus, once the foal probably did not ingest plants contaminated with coronavirus by grazing, the nasal route is a possible way infection happened. Stool ingestion, be it by coprophagia or incidentally, also needs to be considered.

Sources of infection such as other diseased foals or even healthy adults eliminating the virus may pose a risk to immunologically naive animals. Furthermore, despite the species-specificity of Coronaviruses (Kuo, 2000) an inter-species transmission of coronavirus might have occurred and the foal may have acquired a coronavirus from calves, pigs or other animals.

The finding of a group II coronavirus in equines is in agreement with the report by Guy et al. (2000), who classified a coronavirus isolated from a diarrheic foal as a group II coronavirus based on IFAT, serum-virus neutralization assay and N gene sequencing.

The genealogic relationship among the virus here reported and other coronaviruses already found in horses remains to be answered based, for instance, on sequencing of the *RdRp* and also of envelope proteins genes. This might help understanding virus diversity and depict the route of transmission.

Regarding the results reported here, coronavirus, already assigned a role in adult and young horses must from now on be thought as also involved in cases of diarrhea in newborn foals.

ACKNOWLEDGMENTS

This research was supported by FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo). The authors are grateful to Mr. Alexandre Abelardo Sanches for his technical support.

References

Akashi, H.; Inaba, Y.; Miura, Y.; Tokuhisha, S.; Sato, K.; Satoda, K. Properties of a coronavirus isolated from a cow with epizootic diarrhea. *Veterinary Microbiology*, v.5, p.265-276, 1980.

Brandão, P.E.; Gregori, F.; Heinemann, M.B.; Lima, C.H.A.; Rodriguez, C.A.R.; Ruiz, V.L.A.; Jerez, J. A. Animal coronaviruses. *Virus Reviews & Research*, v.6, p.7-13, 2001.

Brandão, P.E.; Gregori, F.; Villarreal, L.Y.B.; Rodriguez, C.A.R; Soares, RM; Jerez, J.A. A nested polymerase chain reaction to bovine coronavirus diagnosis. *Virus Reviews & Research*, v.10, n.1, p.45-49, 2005.

Durham, P.J.K.; Stevenson, B.J.; Farquharson, B.C. Rotavirus and coronavirus associated diarrhoea in domestic animals. *New Zealand Veterinary Journal*, v.27, p.30-32, 1979.

Guy, J.S.; Breslin, J.J.; Breuhaus, B.; Vivrette, S.; Smith L.G. Characterization of a coronavirus isolated from a diarrheic foal. *Journal of Clinical Microbiology*, v.38, p.4523-4526, 2000.

Heckert, R.A.; Saif, L.J.; Myers, G.W.; Agnes, A.G. Epidemiologic factors and isotype-specific antibody responses in serum and mucosal secretions of dairy calves with bovine coronavirus respiratory tract and enteric tract infections. *American Journal of Veterinary Research*, v.52, p.845-851, 1991.

Herring, A.J.; Inglis, N.F.; Oeh, C.K.; Snodgrass, D.R.; Menzies, J.D. Rapid diagnosis of rotavirus infection by direct detection of viral nucleic acid in silver-stained polyacrylamide gels. *Journal of Clinical Microbiology*, v.16, p.473-477, 1982.

Huang, J.C.M.; Wright, S.L.; Shipley, W.D. Isolation of coronavirus-like agent from horses suffering from acute equine diarrhoea syndrome. *Veterinary Record*, v.17, p.262-263, 1983.

Kuo, L.; Godeke, G.; Raamsman, M.J.B.; Masters, P.S.; Rottier, P.J.M. Retargeting of coronavirus by substitution of the spike glycoprotein ectodomain: crossing the host cell species barrier. *Journal of Virology*, v.74, p.1393-1406, 2000.

Lai, M.C.M. & Cavanagh, D. The molecular biology of coronaviruses. *Advances in Virus Research*, v.48, p.1-100, 1997.

Mair, T.S.; Taylor, F.G.R.; Harbour, D.A.; Pearson, G.R. Concurrent cryptosporidium and coronavirus infections in an Arabian foal with combined immunodeficiency syndrome. *Veterinary Record*, v.10, p.127-130, 1990.

Ministry of Agriculture, Fisheries and Food. *Manual of veterinary investigation laboratory techiniques*. London: Her Majesty's Stationery Office, 1984. v.1.

Ogassawara, S. & Benassi, S. Infecção experimental de gatos com coração de bovino parasitado por *Sarcocystis* sp. *Arquivos do Instituto Biológico*, São Paulo, v.47, p.27-32, 1980.

Stephensen, C.B.; Casebolt, D.B.; Gangopadhyay, N.N. Phylogenetic analysis of a highly conserved region of the polymerase gene from 11 coronaviruses and development of a consensus polymerase chain reaction assay. *Virus Research*, v.60, p.181-189, 1999.

van Regenmortel, M.H.V.; Fauquet, C.M.; Bishop, D.H.L.; Carstens, E.B.; Estes, M.K.; Lemon, S.M.; Maniloff, J.; Mayo, M.A.; McGeoch, D.J.; Pringle, C.R.; Wickner, R.B. Virus Taxonomy: The classification and nomenclature of viruses. *The Seventh Report of the International Committee on Taxonomy of Viruses. San Diego*: Academic Press, 2000.

White, R.G.; Mebus, C.A.; Twiehaus, M.J. Incidence of herds infected with a neonatal Calf Diarrhea Virus (NCDV). *Veterinary Medicine, Small Animal Clinician*, v.65, p.487-490, 1970.

Received on 31/12/05
Accepted on 27/3/06