



Rotavirus Detection and Isolation from Chickens With or Without Symptoms

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■ Keywords

Enteric viruses, rotavirus, isolation, MA104 cells, chicken.

■ Acknowledgments

The authors are grateful to FAPESP and PIBIC – CNPq for the grants given to Villarreal LYB, Uliana G, Valenzuela C and Chacón JL.

Arrived: January / 2006

Approved: September / 2006

ABSTRACT

Rotaviruses have been identified as one of the main etiological agents of diarrhea and enteritis in mammals, including humans, and in avian species. Few studies have been published about enteric viruses in Brazilian poultry, including those related to rotavirus infection. Such studies demonstrate significant occurrence and the importance of enteric viruses in poultry presenting intestinal problems. Enteric viruses are the primary cause of injuries to the gut, allowing other agents, especially bacteria, to attach, to penetrate, and to replicate in the enteric tissue, leading to further damage. The aim of the present study was to detect rotavirus in the intestinal contents of layers and broilers by polyacrylamide gel electrophoresis (PAGE) and virus isolation in MA-104 cell culture. A total of 45.3% of all samples were positive to rotavirus; rotavirus frequencies were 48.7% in samples from flocks with diarrhea, 46.4% in flocks with delayed growth, and 30% in asymptomatic flocks. It was possible to isolate rotavirus in MA-104 cells from the nine rotavirus-positive randomly chosen samples. These results indicate that rotavirus may have an important role in pathogenesis of enteric disease.

INTRODUCTION

Rotaviruses have been identified as one of the main etiological agents of diarrhea and enteritis in mammals, including humans (Tzipori, 1985), and in avian species (Bergeland *et al.*, 1977; Jones *et al.*, 1979). Rotaviruses are classified as a genus of the *Reoviridae* family, having a different morphology that easily allows distinguishing them from other enteric viruses and a characteristic 11-segmented RNA. This virus has been isolated from a wide variety of avian species, including turkeys, chickens, and pheasants (Gough *et al.*, 1985; Gough *et al.*, 1986; McNulty, 2003; Reynolds *et al.*, 1987a; Reynolds *et al.*, 1987b; Theil *et al.*, 1986). Rotaviruses that possess an antigenic A group are considered typical of mammals, while typical avian rotaviruses are those designated as groups D, F, and G (McNulty, 2003, McNulty *et al.*, 1981). However, avian rotaviruses from group A have already been isolated from the intestinal contents of chickens, turkeys, and other avian species (Sugiyama *et al.*, 2004; Brüßow *et al.*, 1992).

In field conditions, rotavirus infections in poultry may induce subclinical manifestations, or they may be associated with enteritis, dehydration, anorexia, low weight gain, and increased mortality (McNulty, 2003; Tamehiro *et al.*, 2003).

Symptoms of rotavirus infection may vary from a mild disease in young chickens to a more severe manifestation in 12 to 21-day-old chickens, characterized by unrest, litter ingestion, watery feces, wet litter, and severe diarrhea (Barnes, 1997).



Few studies have been published related to enteric viruses in Brazilian poultry, including those related to rotavirus infection. Such studies demonstrate significant occurrence and the importance of enteric viruses in poultry presenting enteric problems (Alfieri *et al.*, 1988; Alfieri *et al.*, 1989a; Alfieri *et al.*, 1989b).

This study aimed at isolating avian rotavirus field strains in MA-104 cells in order to determine its electrophoretic profile, and to discuss the importance of rotavirus in chickens with delayed growth and diarrhea.

MATERIALS AND METHODS

Sample collection

Between April 2004 and July 2005, 128 samples of intestinal contents were collected during necropsy of poultry (layers and broilers). Twenty eight of these samples were from chickens with delayed growth, 80 from birds presenting diarrhea and high feed conversion ratio, and 20 samples from asymptomatic flocks (without diarrhea and delayed growth). These samples came from different Brazilian states (CE, MG, PA, PR, RS, RJ, SP, and SC) with bird ages varying from 36 to 43 days. Each sample consisted of a pool of the intestinal contents of five birds, randomly chosen in each flock.

The samples were submitted to the Avian Pathology Laboratory (LABOR) FMVZ-USP under refrigeration. The enteric contents were collected, processed as 20% suspensions in PBS 0.01 M pH 7.2, and clarified at 12,000 x g/ 30 minutes at 4°C in order to obtain the supernatant, which was kept frozen at -80°C until the remaining processing was performed.

Polyacrylamide gel electrophoresis (PAGE)

RNA extraction from fecal suspensions was performed with phenol-chloroform, followed by discontinuous polyacrylamide gel electrophoresis at 3.5%/7.5% dyed with silver nitrate (Herring *et al.*, 1982). Samples classified as positive were those presenting RNA band migration patterns similar to those observed with the NCDV rotavirus strain (White *et al.*, 1970), included as a positive control.

Isolation in cell culture

Nine samples diagnosed as positive to rotavirus by PAGE were selected for cultivation in 48-hour-old confluent monolayer of MA-104 cell (Rhesus monkey kidney) in 25 cm² plastic flasks.

The supernatants of fecal suspensions were filtered

with 0.22 µm Millex-pore (Millipore™) filters and added up with virus activation solution (VAS), consisting on 5 mg/mL crystalline trypsin (Sigma™) in MEM – EAGLE medium (Cultilab™) in a 4:1 proportion in order to cleave VP4 to VP5 and VP8 (Arias *et al.*, 1996) and incubated at 37°C for 30 minutes.

Cell culture growth medium was discarded and monolayers were rinsed with sterile PBS 0.01 M pH 7.4. Next, 1 mL of the inoculum (sample treated with VAS) was incubated at 37°C for 60 minutes, and then the maintenance medium (MEM-EAGLE Cultilab™) with crystalline trypsin (Sigma™) at 5 µg/ml was added without discarding the inoculum, as described by Rodriguez *et al.* (2004).

The flasks were then incubated at 37°C, and the monolayers were observed until cytopathic effect appeared. Those monolayers presenting cytopathic effect after incubation for up to 96 hours were frozen at -80°C, and submitted to at least five serial passages.

All passages presenting cytopathic effect resembling rotavirus infection (cell rounding and cell elongation with gradual cell and monolayers destruction similar to a “string of pearls”) were monitored by PAGE in order to determine if the observed effect was actually caused by rotavirus and not by the trypsin.

Statistical analysis

PAGE results were categorized according to the disease status of the flocks, *i.e.*, flocks only with diarrhea, flocks only with delayed growth, flocks with diarrhea and delayed growth (diseased flocks), and asymptomatic flocks. All categories were compared with the Chi-square test using the Minitab® Release 14.1 software (© 1972 - 2003 Minitab Inc.), with one degree of freedom and α -level of 0.05.

RESULTS

Fifty-eight samples out of the 128 tested by PAGE (45.3%) were positive for rotavirus. Rotavirus frequencies were 48.7% among samples from flocks with diarrhea, 46.4% among flocks with delayed growth, and 30% among asymptomatic flocks (Table 1).

Statistical analysis for rotavirus detection revealed no significant differences among samples from flocks with diarrhea and asymptomatic flocks ($p=0.132$), flocks with delayed growth and asymptomatic flocks ($p=0.251$), flocks with diarrhea and flocks with delayed growth ($p=0.832$), and diseased and asymptomatic flocks ($p=0.134$).



Table 1 - Results of rotavirus detection by polyacrylamide gel electrophoresis (PAGE) in the intestinal contents of layers and broilers with diarrhea, delayed growth, or without symptoms (asymptomatic).

Symptoms	Presence of Rotavirus / Symptoms	Percentage
Diarrhea	39 / 80	48.8 %
Delayed growth	13 / 28	46.4%
Asymptomatic	6 / 20	30.0%
TOTAL	58/128	45.3%

The electropherotype obtained from the majority of the fecal samples did not show sufficient resolution to determine the rotavirus group.

It was possible to isolate rotavirus in MA-104 cells from the nine randomly chosen rotavirus-positive samples; these rotavirus strains were named AR-01 to AR-09.

In the first and second passages, all inoculated samples showed low intensity cytopathic effect, characteristic of rotavirus after 48 to 60 hours of inoculation, reaching the maximum effect 5 days post-inoculation.

By the fifth passage, an intense cytopathic effect was observed after 24 hours of inoculation, leading to the complete destruction of the monolayers two days after inoculation.

PAGE used for monitoring each passage was positive for all samples in all passages, presenting an electropherotype similar to group A rotaviruses, with the intensity of the bands increasing according to the successive passages. Figure 1 shows an example of PAGE results for strain AR-01 from the original intestinal content sample until the fifth passage in MA-104 cells.

DISCUSSION

Rotavirus with an electropherotype similar to that described for Group A rotaviruses was detected in layer hens and broilers with diarrhea and delayed growth, as well as in asymptomatic chickens, using polyacrylamide gel electrophoresis and cell culture isolation.

In poultry, both in layer hens and broilers, rotavirus has already been established as the etiological agent of enteritis, originated from viral replication in intestinal epithelium, resulting in diarrhea and nutrient malabsorption (Snodgrass *et al.*, 1986), which causes an increase in feed conversion ratio and large economic losses to poultry industry (Barnes, 1997).

The finding of rotavirus in healthy poultry can be explained most probably by the fact that these chickens were in the beginning of the infection period, already eliminating the virus in feces, but still in the incubation

period of two to five days. The hypothesis of the incubation period as an explanation for the presence of rotavirus in healthy poultry is supported by the absence of a significant difference between asymptomatic flocks and flocks with diarrhea ($p=0.132$), delayed growth ($p=0.251$), and the category "diseased flocks" ($p=0.134$), which included birds both with diarrhea and delayed growth. Another possibility is that the chickens had already gone through the clinical period of the disease, had recovered, but were still eliminating the virus (McNulty, 2003).

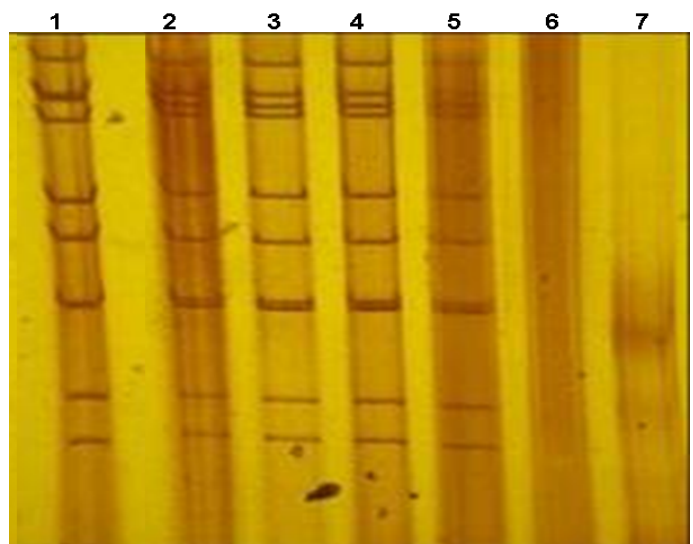


Figure 1 - Example of a polyacrylamide gel electrophoresis (PAGE) image showing the electropherotype of one of nine avian rotavirus strains similar to Group A rotaviruses isolated from the intestinal contents of chicken in MA-104 cell line. Lane # 1=NCDV rotavirus reference strain; Lanes #s 2 to 6: 5th, 4th, 3rd, 2nd and 1st passages of the avian rotavirus strain (strain AR-01); Lane 7: strain AR-01 as detected directly from the intestinal content sample, prior to the first passage in MA-104 cells.

It is still possible that virus-associated factors, such as virulence, as previously established in mammalian rotaviruses caused by genetic reassortment events (Estes, 1996), as well as host-associated factors, such as natural resistance to rotavirus due to different genetic poultry lines, may have accounted for the different patterns of infection observed herein.



In addition, predisposing factors, such as diseases attributed to other pathogens like coronaviruses, reoviruses, enteroviruses, and adenoviruses (Dea & Tijssen, 1988; Hayhow & Saif, 1993); bacteria such as *Escherichia coli* and *Salmonella* spp (Porter, 1998); physiological stress; and toxic and environmental factors, such as temperature, ventilation, and husbandry, may directly interfere in the resolution of the disease after rotavirus infection. However, the importance of these asymptomatic chickens is that they are constantly shedding the virus, without being clinically detected, and disseminate the virus to susceptible chickens.

Polyacrylamide gel electrophoresis to demonstrate the viral RNA segment is a sensitive, fast, and low-cost technique, which can be easily implemented in low-outfitted laboratories and, as shown in the present study, may be used as a screening procedure for rotavirus infection in poultry.

However, it is not always possible to obtain the visualization of the 11-viral segments for a definitive diagnosis. In the present study, the association of isolation in cell culture and PAGE technique was successful to enhance the visualization of rotavirus electropherotypes.

In the nine samples examined, from which rotavirus was isolated in cell culture, it was possible to improve the resolution of the electropherotype by increasing 11-RNA bands intensity as compared to those observed in the same fecal samples before the isolation.

Although cell culture isolation did not increase the number of positive samples in the present study, it can be used in association with PAGE in order to improve the resolution of this method in cases of poorly-defined electropherotypes.

While rotavirus isolation frequently requires from four to six passages before a positive sample can be detected (Kang *et al.*, 1986), in the cases presented here it was shown that two passages were sufficient to detect the virus. Moreover, it was already shown that MA-104 cell line is efficient for isolating avian rotavirus in field samples (Yason & Schat, 1986).

In this study a high frequency of rotavirus in Brazilian poultry was found using PAGE, indicating that this virus, although neglected as an important putative pathogen of poultry, may have a role in the pathogenesis of enteric disease of layers and broilers.

Further studies are needed in order to assess the real impact of rotavirus on Brazilian poultry, such as those focused on the genetic diversity of the detected strains and the effects after experimental inoculation of birds.

As a conclusion, it can be suggested that both diarrhea and the low performance presented by the birds studied here are due, at least in part, to the presence of rotavirus, which is largely disseminated in the Brazilian flocks, suggestion that rotavirus infection in broilers should be considered an important pathogen in a single manifestation or in association with another pathogen contributing to enteric problem onset.

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