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RESEARCH ARTICLE

Sequence and phylogenetic analysis of chicken reoviruses in China

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Abstract

Avian reovirus (ARV) has been responsible for many cases of chicken tenosynovitis in China in recent years, causing high morbidity among layer and broiler chickens. To study the degree of genetic divergence and evolution among ARVs, the full-length nucleotide sequences of the σC-encoding gene of eight ARV field isolates and the entire coding-region sequences of four isolates were determined and analyzed. The sequence analysis revealed that the eight σC-encoding genes shared 99.0–99.9% nucleotide sequence identity with each other and over 99% with the chicken reovirus reference strain S1133. However, the nucleotide sequences of the eight σC-encoding genes varied extensively from that of isolate AVS-B (GenBank accession no. FR694197), with only 55.5% identity. A sequence analysis of the whole ARV-coding region showed some nucleotide substitutions in the open reading frames encoding λA, λB, λC, μA, μB, μNS, σC, σA, σB, and σNS in the field strains. A phylogenetic analysis showed that all eight isolates clustered in group I with S1133, but that four field isolates shared less homology with strain S1133 than the others, indicating that they had been evolved in the field. We also studied the pathogenicity of two strains. No characteristic lesions were observed in vaccinated chickens, and no virus was detected in sampled tissues. However, an enzyme-linked immunosorbent assay revealed significant differences between the antibody responses of the inoculated groups and the negative controls. These results revealed that Chinese isolates shared the highest sequence homologies with S1133, grouped together in one cluster. Although the vaccination against ARV is used in farms, the pathogens still persist in Chinese poultry flocks.

Keywords: avian reovirus, chicken, genome, S1, homology, phylogenetic analysis, pathogenicity

1. Introduction

Avian reovirus (ARV) is a member of the genus *Orthoreovirus* in the family *Reoviridae*. Its genome of 10 RNA segments is divided into three size classes, designated large (L1, L2, L3), medium (M1, M2, M3), and small (S1, S2, S3, S4) (Spandidos and Graham 1976; Schnitzer 1985). At least 10 structural proteins and five nonstructural proteins encoded by ARV have been described (Benavente and

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Martinez-Costas 2007; Banyai *et al.* 2011). The L, M, and S genome segments express three λ (λA, λB, λC), three μ (μA, μB, μNS), and four σ (σC, σA, σB, σNS) primary translation products, respectively. Additional proteins, p10 and p17, are encoded by the first two cistrons of the ARV tricistronic S1 gene. The σC protein encoded by the S1 gene is a minor component of the outer capsid of the virion, is the cell attachment protein of ARV, and can cause cellular apoptosis *in vitro*. The σC protein is also the protein that predominantly induced the production of neutralizing antibodies against ARV. Because of the powerful immunogenicity of σC, the S1 gene has become the basis of ARV genotyping.

ARV infections are responsible for significant economic losses in the poultry industry worldwide (Xie *et al.* 1997). In chickens, ARV is associated with tenosynovitis, runting-stunting syndrome, myocarditis, hepatitis, respiratory diseases, and even central nervous system infections (Liu *et al.* 2003; Van de Zande and Kuhn 2007; Banyai *et al.* 2011). Live attenuated and inactivated vaccines are readily available to prevent ARV infection (Schnitzer 1985; Endo-Munoz 1990; Mallo *et al.* 1991). They are considered the safest available vaccines and are used widely (Van der Heide *et al.* 1983). Viral tenosynovitis is often controlled in broilers by the antibodies transferred to the progeny after the vaccination of the maternal fowl (Troxler *et al.* 2013), although the full effect is only achieved when the progeny are challenged with a homologous serotype (Rau *et al.* 1980; Wood *et al.* 1986).

However, recent studies based on the phylogenetic analysis of the immunogenic σC protein have indicated the existence of many genotypic variants, which have been classified into a distinct cluster within the ARV (Liu *et al.* 2003). These new ARV isolates seem to be distinct from the strains used in vaccines (Van Loon *et al.* 2001; Teng *et al.* 2013).

In China, several field isolates of ARV have been isolated from different provinces and the number of cases of ARV infection diagnosed by the Diagnostic Center of Livestock and Poultry Epidemic Disease (DCLPED) at China Agricultural University has increased since 2008. In this study, we examined the evolutionary relatedness of different ARV genes to understand whether diverse ARV genomes are present in China. If so, this might explain the outbreaks of ARV in free-range and standard broilers. We analyzed the nucleotide sequences of the S1 genomic segment from eight ARV field strains provided by the DCLPED, which were isolated at different times and geographic locations. We then selected four of these isolates and determined the complete nucleotide sequences of the viral genomes, and investigated their genomic organization and their relationships with other ARVs. Two isolates (SD09-1 and LN09-1) from different regions were selected to investigate their pathogenicity in specific-pathogen-free (SPF) chickens.

2. Materials and methods

2.1. Viral strains

Eight ARV strains (BJ10-1, HB09-1, HLJ09-1, LN10-1, HB10-1, LN09-1, SD09-1, and SD10-1) isolated from the tendons of diseased chickens with tenosynovitis/arthritis in different provinces of China in 2008–2010 were examined in this study (Table 1). These isolates were passaged three times by inoculating the embryo yolk sacs of 6-day-old SPF embryonated eggs with the viruses. After the embryos had died, usually at 3–5 days postinfection (dpi), the chorioallantoic membrane (CAM) was collected and homogenized in phosphate-buffered saline (PBS; 20%, w/v) containing antibiotic (1 000 U mL^{-1} gentamicin), and then split into 1.5-mL aliquots and stored at −80°C. Briefly, each virus was titrated in embryonated eggs using serial dilutions from 10^{-1} to 10^{-7}, with five eggs per dilution. All eggs were illuminated daily in a dark room to determine embryo viability. The $10^{5.0}$ median embryo infective doses (EID_{50}) of each ARV isolate was analyzed using the formula of Reed and Muench (1938). Infected CAM homogenates with titers of $>10^{5.0}$ EID_{50}/0.2 mL were used as the sources of virus for infection.

2.2. Primer design and RNA preparation

Based on the nucleotide sequence of ARV isolate S1113 (GenBank accession no. L39002), 21 pairs of specific primers (Table 2) were designed to amplify the σC-encoding gene and the complete genomes of the four ARV isolates HB10-1, LN09-1, SD09-1, and SD10-1. Total RNA was extracted from 250 μL of clarified embryo homogenate using the TranZol RNA Extraction Kit (TransGen, Beijing, China), according to the manufacturer's instructions.

2.3. RT-PCR

To determine the nucleotide sequences of the genomic segments of the ARV isolates, the purified genomic double-stranded RNA from these isolates was used to generate cDNA clones with RT-PCR. The primers used to amplify the S-class, M-class, and L-class genes were located at the 5′ and 3′ noncoding regions, and encompassed the λa, λB, λC, μA, μB, μNS, σC, σA, σB, and σNS ORFs. The sequences and locations of the primers are shown in Table 2. In the RT reaction, 5 μL of extracted RNA, 2 μL of dNTP mix (10 mol L^{-1} each; Vigorous, Beijing, China), 5 μL of RNase-free water, and 20 pmol of specific primer were heated at 97°C for 5 min, and then placed in an ice bath for 5 min. To this reaction mixture were added 4 μL of 5′ first-strand buffer, 1 μL of dithiothreitol (100 mol L^{-1}), 1 μL of 200 U mL^{-1} Super-