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Differentiating infected from vaccinated animals, and among virulent prototypes of reovirus

Dana Goldenberg[a,b], Avishai Lublin[c], Ezra Rosenbluth[c], E. Dan Heller[b], Jacob Pitcovski[a,d,*]

[a] Migal – Galilee Technology Center, Kiryat Shmona, Israel
[b] Department of Animal Sciences, The Robert H. Smith Faculty of Agriculture, Food and Environment, The Hebrew University of Jerusalem, Rehovot, Israel
[c] Division of Avian and Fish Diseases, Kimron Veterinary Institute, Bet Dagan, Israel
[d] Department of Biotechnology, Tel-Hai Academic College, Kiryat Shmonah, Israel

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Birds are most susceptible to infection by avian reovirus, genus *Orthoreovirus* family *Reoviridae*, at a young age. Although chicks are protected by antibodies transferred from vaccinated maternal flocks, due to the many variants in the field, the efficiency of the vaccines is limited. The level of antibodies against viruses is generally determined by enzyme-linked immunosorbent assay (ELISA), using the whole virus as the antigen. This has some disadvantages: first, the test measures antibodies against all capsid proteins, most of which are irrelevant for neutralizing the virus, and as such does not reflect the real protection status; second, it is impossible to distinguish between vaccine- and infection-derived antibodies. In the case of a virus that changes frequently, a third disadvantage is the inability to distinguish among serotypes. The aim of this study was to develop a test that would address these concerns. Four prototypes of the avian reovirus protein sigma C were used as antigens on the ELISA plate. Sigma C is the main protein inducing neutralizing antibodies and the most variable among strains and isolates, and it is used for reovirus classification. This differentiating ELISA enabled distinguishing between vaccine and field strains of the virus, identifying the infection source, and in the case of vaccination, exclusively determining the level of protective antibodies. Whereas the whole virus detected antibodies against all strains, differentiating ELISA enabled differentiating between infected and vaccinated animals (DIVA) and in most cases, identifying the sigma C genotype. In a field study, a correlation was found between disease symptoms and antibodies identified against virulent strains in the flock. Thus virulent strains can be identified in the field, enabling adjustment of the relevant vaccines.

1. Introduction

Avian reovirus belongs to the genus *Orthoreovirus* in the family *Reoviridae*. Its genome consists of 10 double-stranded RNA segments. The RNA is packaged into a non-enveloped double concentric icosahedral capsid of 70–80 nm in diameter (Schnitzer et al., 1982; Spandidos and Graham, 1976). Avian reovirus in poultry induces arthritis, chronic respiratory diseases and malabsorption syndrome (Fahey and Crawley, 1954). Birds are most susceptible at a young age (Rosenberger et al., 1989). The RNA sequence of some avian reovirus strains has been determined, and the encoded proteins have been identified (Cashdollar et al., 1984; Liu et al., 1997; McCrae and Joklik, 1978; Schnitzer, 1985; Wickramasinghe et al., 1993). The sigma C protein is encoded in the third open reading frame of the S1 fragment (Schnitzer, 1985; Wickramasinghe et al., 1993) and functions in the virus's identification and binding to the target cell (Martinez-Costas et al., 1997). Sigma C is a relatively small protein of 326 aa (Shapouri et al., 1995), whose importance manifests itself at two levels. First, it induces the production of neutralizing antibodies that protect against challenge (Wu et al., 2005) and second, it is the most variable of the reovirus proteins (Liu and Giambrone, 1997). Thus, sigma C has been found suitable for comparisons among strains. Variants with broad antigenic diversity have been isolated all over the world. In a previous study based on sigma C sequences, all reported reovirus isolates were divided into four main groups with over 75% identity within a group (Goldenberg et al., 2010). Sequence analysis of three of the most common vaccine strains in the world (1133, 1733, and 2408) (Liu et al., 1997) and one Israeli vaccine (641) showed that they are all closely related and all belong to group I.

Chicks are protected by antibodies transferred from vaccinated maternal flocks. The most common vaccines are the attenuated strain or inactivated strains injected as a monovalent, bivalent or trivalent mixture (Rekik and Silim, 1992; Shapouri et al., 1995; van der Heide, 2000; Varela and Benavente, 1994). To detect the

* Corresponding author at: Migal – Galilee Technology Center, Kiryat Shmona, Israel. Tel.: +972 4 6953509; fax: +972 4 6944980.
E-mail address: jp@migal.org.il (J. Pitcovski).

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reovirus, several diagnostic assays based on the molecular amplification of different genomic regions of avian reovirus RNA have been developed (Lee et al., 1998; Liu and Giambrone, 1997; Shapouri et al., 1995; Xie et al., 1997; Yin et al., 1997). ELISA tests using the whole virus (Slaght et al., 1978), the recombinant avian reovirus protein sigma B or the non-structural protein sigma NS have been described (Chen et al., 2004; Shien et al., 2000; Xie et al., 2010). The strategy of using non-structural proteins in ELISA to differentiate infected from vaccinated animals (DIVA) has been used for foot-and-mouth disease (Fu et al., 2011), hepatitis C (Inoue et al., 1992), influenza (Ozaki et al., 2001) and avian reovirus (Xie et al., 2010). The limitation of using non-structural proteins is that it does not enable the exclusive detection of protective antibodies, or distinguishing among genotypes.

The present study had two aims: first, to identify infected birds as soon as possible, differentiating them from vaccinated birds; second, to establish a diagnostic test that would relate antibodies to a particular genotype, thereby enabling vaccination with the relevant strain.

2. Materials and methods

2.1. Virus source and growth

The viruses in this study, designated vaccine strains ISR5215, ISR5223, ISR528 and s1133, are representatives of four genotypes characterized in a previous study (Goldenberg et al., 2010). ISR5215, ISR5223, ISR528 were isolated from the tendon lesions of infected birds, and propagated as described previously (Goldenberg et al., 2010). Briefly, the tendon fluid from infected birds was injected into the yolk sac of 6–8-day-old specific-pathogen-free (SPF) (SPAFAS, Norwich, CT) embryos and the propagated virus was isolated from the allantoic fluid after 3–5 days. For ELISA, strains ISR5215 and s1133 were propagated in monkey kidney Vero cells. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 20 mM Hepes (pH 7.2). Cells were cultured in a 37 °C incubator supplied with 5% CO_2. Viruses were added at a cell confluence of 70–80%. When the cytopathic effect covered about 80% of the infected cells, the cells were frozen and thawed twice, and centrifuged at 3000 × *g* for 10 min to remove cellular debris. The supernatant was stored at −70 °C.

2.2. Bioinformatics analysis

Assembly and sequence manipulations were performed using Lasergene 99 v. 6.1 Dnastar. Multiple sequence alignment was carried out by MUSCLE (Edgar, 2004) with a Java viewer (http://www.ebi.ac.uk/Tools/muscle/index.html).

2.3. Sigma C production

Sigma C DNA of the four representatives was synthesized by GenScript (Piscataway, NJ, USA) and supplied in PUC 57 plasmid. The plasmid was transformed into *Escherichia coli* strain JM109 and propagated. The DNA was digested out of the plasmid by restriction enzymes BamHI and KpnI and ligated into pAL 781 plasmid (Invitrogen, San Diego, CA, USA), which was digested with the same enzymes. The recombinant plasmid was transformed into *E. coli* GI698 (Invitrogen). Bacterial colonies carrying the sigma C gene were detected by PCR with the primers pAL forward: 5′ TGTAAAACGACGGCCAGTGC 3′ and Trx reverse: 5′ TGTAAAACGACGGCCAGTGC 3′, isolated from an agarose gel, purified, and sequenced. Sequences were determined by Hy-Labs (Rehovot, Israel). Transformed *E. coli* GI698 were cultured in RM medium (per liter: 20 g of casamino acids, 3 g of monopotassium phosphate, 0.5 g of sodium chloride, 6 g of disodium phosphate, 1 g of ammonium chloride and 0.095 g magnesium chloride) at 30 °C. When the culture reached an OD600 of 0.6, gene expression was induced with 100 μg L-tryptophan/mL at 25 °C. The cells were harvested after 3 h induction by centrifugation at 12,000 × *g* for 30 min at 4 °C, washed, and suspended in lysis buffer (2.5 mM EDTA, 20 mM Tris–HCl, pH 8). The cells were disrupted by sonicating three times, 10 min each (Sonics, Taunton, MA, USA) and centrifuged (10,000 × *g* for 15 min at 4 °C). The supernatant and the pellet were collected separately. The expressed sigma C protein was detected in the pellet by 12% SDS-polyacrylamide gel electrophoresis (PAGE). The amount of sigma C was estimated by comparison with a standard curve of known amounts of bovine serum albumin run on the same gel.

2.4. Vaccination with whole viruses

2.4.1. Vaccine preparation

Virus was thawed and partially purified from embryo debris (4500 × *g*, 10 min, 4 °C). It was inactivated by adding formalin to a final concentration of 0.5% (v/v). Full inactivation was verified by injecting the treated virus into embryonated eggs and monitoring embryo viability. The virus solution was mixed 2:1 (v/v) with mineral oil as adjuvant. A mixture of the four virus representatives was composed of equal amounts of each virus. Therefore, the titer of the mixture was equal to the average titer of each virus, 10^6 to $10^{7.5}$ embryo lethal dose (ELD) 50/mL inactivated virus.

2.4.2. Vaccination procedure

Each bird was vaccinated intramuscularly at two sites in the pectoral muscles and subcutaneously under the leg skin near the distal tarsometatarsal bone with 1 mL of the above ELD 50/mL inactivated virus ($10^{6.6}$ ELD 50/mL on average). Anti-avian reovirus antibodies and disease signs typical of reovirus (arthritis, tenosynovitis or tendon cross syndrome towards the end of growth, i.e. 4 weeks of age until marketing/slaughter) were followed up in six commercial flocks. Representative results of three of those flocks are shown. Blood was taken at 21, 35 and 42 days of age. At 42 days of age, birds were tested for tendon cross syndrome by professional kashruth supervisors. The main signs of tendon cross syndrome are swelling and yellow or green coloration of the leg above the hock joint, sometimes with stiffness. In dissected legs, one or a few of the tendons are loose or torn following pulling, with bloody content or fibrinous exudates in some cases.

Anti-sigma C antibodies were determined by ELISA (as described in Section 2.5) using sera pooled from 6 to 10 birds from each flock at a final dilution of 1:4000.

2.5. Enzyme-linked immunosorbent assay (ELISA)

To test for antibodies to whole virus, avian reovirus strains ISR5215 and s1133 were used as antigen. Following propagation in Vero cells, the virus was purified on a sucrose gradient by ultracentrifugation. The working stock concentration of the virus in the ELISA was determined in a preliminary test, following 10-fold dilutions of the virus and detection by positive and negative control sera. Differentiation ELISA consisted of using sigma C protein as the antigen. In both cases, antigen was diluted in coating buffer (0.397 g Na_2CO_3, 0.732 g $NaHCO_3$, 250 mL DDW pH 9.6) and incubated on an ELISA plate (Nunc, Rochester, NY, USA) for 24 h at 4 °C. Each subsequent step was followed by three washes with 0.05% (w/v) Tween-20 in PBS and drying on a paper towel. Serum from birds vaccinated with strain ISR5215, ISR528, ISR5223 or s1133 was diluted in blocking buffer (5%, w/v skim milk, 0.05% Tween-20 in PBS) and incubated for 1 h. To test antibodies to whole virus,