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Replication-independent reduction in the number and diversity of recombinant progeny viruses in chickens vaccinated with an attenuated infectious laryngotracheitis vaccine

Carlos A. Loncoman *, Carol A. Hartley, Mauricio J.C. Coppo, Glenn F. Browning, José A. Quinteros, Andrés Diaz-Méndez, Dulari Thilakarathne, Omid Fakhri, Paola K. Vaz, Joanne M. Devlin

Asia-Pacific Centre for Animal Health, Department of Veterinary Biosciences, Melbourne Veterinary School, Faculty of Veterinary and Agricultural Sciences, University of Melbourne, Parkville, Victoria 3010, Australia

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ABSTRACT

Recombination is closely linked with virus replication and is an important mechanism that contributes to genome diversification and evolution in alphaherpesviruses. Infectious laryngotracheitis (ILTV; *Gallid alphaherpesvirus 1*) is an alphaherpesvirus that causes respiratory disease in poultry. In the past, natural (field) recombination events between different strains of ILTV generated virulent recombinant viruses that have caused severe disease and economic loss in poultry industries. In this study, chickens were vaccinated with attenuated ILTV vaccines to examine the effect of vaccination on viral recombination and diversity following subsequent co-inoculation with two field strains of ILTV. Two of the vaccines (SA2 and A20) prevented ILTV replication in the trachea after challenge, but the level of viral replication after co-infection in birds that received the Serva ILTV vaccine strain did not differ from that of the mock-vaccinated (control) birds. Even though the levels of viral replication were similar in the two groups, the number of recombinant progeny viruses and the level of viral diversity were significantly lower in the Serva-vaccinated birds than in mock-vaccinated birds. In both the mock-vaccinated and Serva-vaccinated groups, a high proportion of recombinant viruses were detected in naïve in-contact chickens that were housed with the co-inoculated birds. Our results indicate that vaccination can limit the number and diversity of recombinant progeny viruses in a manner that is independent of the level of virus replication. It is possible that immune responses induced by vaccination can select for virus genotypes that replicate well under the pressure of the host immune response.

1. Introduction

Infectious laryngotracheitis virus (ILTV – *Gallid alphaherpesvirus 1*), is an alphaherpesvirus that causes respiratory disease in poultry worldwide. It causes major economic losses as a result of mortality and decreases in weight gain and egg production [1]. Attenuated vaccines are widely used and are generally effective at controlling clinical disease, but many do not entirely prevent ILTV replication, as measured by viral titres after challenge [2]. Attenuated vaccines also have other limitations, including the capacity to undergo recombination to generate new virus strains [3]. This was first observed in Australian poultry when natural recombination between two attenuated ILTV vaccine strains generated virulent, recombinant field viruses that spread to cause outbreaks of severe disease in commercial flocks [4]. Extensive ILTV recombination has subsequently been demonstrated *in vivo* under field [4–6] and laboratory conditions [7,8]. Both vaccine strains and field strains of ILTV have been involved in recombination events [4–6,8].

Recombination is an important mechanism contributing to genome diversification and evolution in ILTV, and other alphaherpesviruses [9]. Herpesviruses have double stranded linear DNA genomes and have complex viral DNA replication machinery, including a DNA polymerase with a highly efficient proofreading capacity, resulting in low spontaneous mutation rates [10–12]. The slow rate of accumulation of point mutations accentuates the role of recombination as a major evolutionary force facilitating persistence of alphaherpesviruses and even driving increases in virulence over time [4,13,14]. Herpesvirus recombination is closely linked with replication and can only occur when a host cell is infected with two viruses at the same time (co-infection). The molecular mechanisms involved in alphaherpesvirus recombination are not well understood, but in *Human alphaherpesvirus 1* (HSV-1) ICP8 (a single strand annealing protein) and the gene pro-

* Corresponding author.
E-mail address: cloncoman@student.unimelb.edu.au (C.A. Loncoman).

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duct of UL12 (an exonuclease) are hypothesised to function together to catalyze recombination, in a process analogous to that described for lambda bacteriophages [15–19].

Experimental co-infection of specific pathogen free (SPF) chickens with ILTV is a convenient model for studying alphaherpesvirus recombination in a natural host. It also has direct relevance for poultry industries, in which ILTV recombination threatens animal health. Any measure that might reduce the rate of recombination in the field could have considerable impact on the evolution of more virulent strains of ILTV. Therefore, this study sought to examine the effects of vaccination on ILTV recombination and viral diversity over time to better understand alphaherpesvirus recombination and to determine if vaccination could be used to limit viral recombination.

2. Material and methods

2.1. Viruses and cell culture

The V1-99 and CSW-1 strains of ILTV, previously described [20], were used as the parental viruses for *in vivo* co-inoculation. Three commercial live-attenuated ILTV vaccines were used. The SA2 vaccine (Zoetis, Australia) was derived from an Australian field strain isolated in 1950 [21,22]. The A20 vaccine (Zoetis, Australia) was produced by serial passage of the SA2 ILTV strain in primary chick embryo cell cultures [23]. The Serva vaccine strain (MSD Animal Health) is an European origin strain that was first registered in Australia in 2006 [24]. Viruses were propagated and titrated in chicken hepatocellular carcinoma (LMH) cells [25]. Titration was performed using a plaque assay, as described previously [26]. Virus isolation and purification from clinical material collected during the *in vivo* experiment were performed in LMH cell monolayers cultured in growth medium (GM) consisting of Dulbecco's Minimal Essential Medium (DMEM) supplemented with amphotericin B (0.005 mg/ml), gentamicin (0.05 mg/ml), and co-trimoxazole (0.01 mg sulfamethoxazole/ml and 0.002 mg trimethoprim/ml), 10% v/v fetal bovine serum (FBS) and 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.7).

2.2. In vivo co-inoculation experiment

This experiment was undertaken with approval from the Animal Ethics Committee of the Faculty of Veterinary and Agricultural Sciences, The University of Melbourne (approval number 1413401.1). This committee follows the Australian Code for the Care and Use of Animals for Scientific Purposes [27]. Sixty birds, at two-week of age, were obtained from Australian SPF Services Pty Ltd. Four groups of ten chickens were directly vaccinated (or mock-vaccinated) and were then directly co-inoculated. Twenty birds were used as in-contact chickens for each vaccinated group (five birds per group). Groups were housed in separate isolator units and were provided with water and food *ad libitum*. Birds were vaccinated at two-week of age with Serva, SA2 or A20 vaccines, or were mock-vaccinated with sterile vaccine diluent (mock-vaccinated group). Each vaccine was prepared according to the manufacturer's instructions. All chickens were vaccinated (or mock-vaccinated) via eye-drop using 30 μL of vaccine preparation or sterile media. At five weeks of age, all vaccinated or mock-vaccinated chickens were co-inoculated by intra-tracheal inoculation with 300 μL of a mixture of the CSW-1 and V1-99 strains of ILTV containing $10^{3.0}$ plaque forming units (PFU) of each strain [8]. Immediately after co-inoculation, five naïve (unvaccinated, un-inoculated) chickens of the same age (five weeks old) were introduced into each isolator as in-contact animals. All birds were monitored for eight days. At 2, 4, 6 and 8 days after co-inoculation, tracheal swabs were collected and placed into 1 mL of viral transport medium (DMEM, 3% v/v FBS and 0.1 μg/mL of ampicillin) and processed for virus isolation and purification. Prior to storage at −80 °C, 200 μL aliquots of the tracheal swab suspension were collected and stored separately at −20 °C for DNA extraction using a Corbett X-tractor robot and VX DNA extraction kit (QIAxtractor-Qiagen) following the manufacturer's recommendations. ILTV genome copy numbers were quantified using a qPCR as described previously [28].

2.3. Virus isolation and purification

Progeny viruses were isolated and purified as described previously [7]. Serial 10-fold dilutions from tracheal swabs were used to inoculate LMH cell monolayers in 6-well plates. After 1 h of incubation at 37 °C, the cell monolayer was covered with semi-solid methyl-cellulose overlay medium (1% w/v methylcellulose in DMEM containing 1% v/v FBS, 10 mM HEPES 7.7, 50 μg/mL gentamicin, 1x Cotrimoxazole and 5 μg/mL amphotericin B) and incubated at 37 °C in a humidified atmosphere of 5% v/v CO_2. After incubation for 24–48 h, up to 20 plaques were picked from each sample from each chicken. Three rounds of plaque purification were performed, with one freeze/thaw cycle between each round.

2.4. SNP genotyping assay and examination of viral diversity

To characterise the viral progeny and identify recombinants, DNA from each plaque-purified virus was extracted and used as template in a TaqMan SNP genotyping assay that targeted six unique SNPs distributed along the ILTV genomes. The SNP were separated by a maximum of 30 kilobase pairs (kbp), and a minimum of 2 kbp and were selected following alignment of whole genome sequences of CSW-1 and V1-99 ILTV (Genbank accession JX646899 and JX646898, respectively) as described previously [7]. Any samples that contained a mixed population of viruses (i.e. both SNPs present at any of the six locations) were not included in further analyses. To measure diversity, we first identified each recombinant using a unique genotype pattern code as described previously [8]. These genotype pattern codes were then analysed in RStudio 0.99.902 using the VeganR [29] and BiodiversityR [30] packages. VeganR calculates diversity indices, which are used to perform ecological diversity measurements in communities [31]. BiodiversityR was used to generate Renyi profiles [30].

3. Results

3.1. Bird survival, virus genome quantification and virus isolation

The survival rate eight days after co-inoculation was 100% in directly inoculated birds (10/10) and in-contact birds (5/5) in the A20- and SA2-vaccinated groups (Supplementary Fig. 1). In the Serva-vaccinated group the survival rate was 60% (6/10) in the directly inoculated birds and 100% (5/5) in the in-contact birds. In the mock-vaccinated group the directly inoculated birds had a survival rate of 30% (3/10), while the survival rate in the in-contact birds was 100% (5/5) (Supplementary Fig. 1).

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vaccine.2018.08.012.

Following co-inoculation, high concentrations of virus were detected by qPCR in the tracheal swabs collected from birds in the mock-vaccinated and Serva-vaccinated groups. In these groups, peak concentrations were seen earlier in the directly inoculated birds (day 4) than in the in-contact birds (day 6) (Fig. 1). No significant differences in virus concentration were found between the