Attenuation and protection efficacy of ORF C gene-deleted recombinant of infectious laryngotracheitis virus

Maricarmen Garcia,1 S. J. Spatz,2 Y. Cheng,1,2 S. M. Riblet,1 J. D. Volkening3 and G. H. Schneiders1

1Poultry Diagnostic and Research Center, Department of Population Health, College of Veterinary Medicine, University of Georgia, 953 College Station Rd, Athens, GA 30602, USA
2US National Poultry Research Center, Agricultural Research Service, United States Department of Agriculture, 934 College Station Rd, Athens, GA 30602, USA
3BASE2BIO, 3461 Wayne Street, Madison, WI 53714, USA

Infectious laryngotracheitis (ILT) is a highly contagious respiratory disease of chickens caused by infectious laryngotracheitis virus (ILTV). The disease is controlled by the use of live-attenuated vaccines. Previously we reported the complete nucleotide sequence of the ILTV vaccine strain (TCO) and identified a nonsense mutation in the gene encoding the ORF C protein. This suggested that the ORF C protein might be associated with viral virulence. To investigate this, an ILTV recombinant with a deletion in the gene encoding ORF C was constructed using the genome of the virulent United States Department of Agriculture (USDA) challenge strain (USDAch). Compared to the parental virus, the ΔORF C recombinant replicated in chicken kidney (CK) cells with similar kinetics and generated similar titres. This demonstrated that the ORF C deletion had no deleterious effects on replication efficacy in vitro. In chickens, the recombinant induced only minor microscopic tracheal lesions when inoculated via the intratracheal/ocular route, while the parental strain induced moderate to severe microscopic tracheal lesions, even though virus load in the tracheas were comparable. Groups of chickens vaccinated via eye-drop with the ΔORFC-ILTV were protected to levels comparable to those elicited by TCO vaccination. To our knowledge, this is the first report that demonstrates the suitability of ΔORFC as a live-attenuated vaccine to prevent the losses caused by ILTV.

INTRODUCTION

Infectious laryngotracheitis (ILT) is a respiratory disease of chickens that results in severe production losses to the poultry industry (Bagust et al., 2000; Dufour-Zavala, 2008). The aetiological agent of the disease is Gallid herpesvirus 1 (GaHV-1) commonly referred to as infectious laryngotracheitis virus (ILTV). Together with the psittacid herpesvirus 1 (PsHV-1), ILTV belongs to the genus Iltovirus, subfamily Alphaherpesvirinae of the Herpesviridae family (Davison, 2010). Severe forms of the disease are characterized by: bird apathy, severe conjunctivitis, increased nasal discharge, respiratory rales, gasping, marked dyspnea and expectoration of bloody mucus. Severe epizootics are characterized by high morbidity and moderate mortality rates. The principal method to control the disease is through mass vaccination with live-attenuated vaccines or recombinant viral vectored vaccines. Live-attenuated vaccines originated from virulent field strain viruses that circulated during the late 50 s to early 60 s in the USA (Garcia et al., 2013a). These strains were attenuated by serial passages in either embryonated chicken eggs (chicken embryo origin, CEO) or in chicken tissue culture (tissue culture origin, TCO) (Gelenczei & Marty, 1965; Samberg & Aronovici, 1969). Viral vector vaccines expressing ILTV glycoproteins in turkey herpesvirus (HVT) and fowlpox virus (FPV) (Davison et al., 2006; Johnson et al., 2010; Tong et al., 2001; Vagnozzi et al., 2012a) are commercially available. The hallmark of these recombinant vaccines is their safety, as they do not transmit from vaccinated to non-vaccinated chickens (Armour & Garcia, 2014). Experimentally it has been
demonstrated that although recombinant viral vectored vaccines do reduce clinical signs of the disease, they fail to prevent viral shedding. On the other hand, live-attenuated vaccines are more efficacious in reducing clinical signs and viral shedding (Johnson, et al., 2010; Vagnozzi et al., 2012a) but can still transmit from vaccinated to non-vaccinated chickens (Rodriguez et al., 2007). It has been speculated that incomplete en mass vaccination, particularly with CEO vaccines, results in long-term persistence of selective vaccine subpopulations (García & Riblet, 2001; García et al., 2013b). Subsequently these strains regain virulence and are responsible for outbreaks of the disease worldwide (Menendez et al., 2014). In order to obtain more stable attenuated strains of ILTV, deletion of genes related to virulence has been extensively pursued (Fuchs et al., 2007). Twenty individual genes have been successfully deleted from the ILTV genome resulting in recombinants with a wide range of growth defects in vitro (Fuchs et al., 2007; Mundt et al., 2011; Pavlova et al., 2010, 2013). Among the 20 gene-deleted ILTV strains generated, only seven have been evaluated in vivo for attenuation and protection efficacy. Individually, these mutants contained deletions in the genes encoding a nuclear protein of unknown function (U1_0 gene), (Veits et al., 2003b), thymidine kinase (U1_23) (Han et al., 2002; Schnitzlein et al., 1995), dUTPase (U1_50) (Fuchs et al., 2000), glycoprotein G (U3_4) (Devlin et al., 2006), glycoprotein J (U5_5) (Fuchs et al., 2005; Mundt et al., 2011), glycoprotein C (U1_44) (Pavlova et al., 2010), and the tegument protein (U1_47) (Helferich et al., 2007). Growth kinetics analysis of these gene deletion recombinants indicated that they are non-essential for virus replication in cell culture (Fuchs et al., 2007). In chickens the ΔU1_50 (dUTPase) (Fuchs et al., 2000) and ΔU1_44 (gC) (Pavlova et al., 2010) gene-deleted recombinants still exhibited some degree of virulence, while the ΔU3_4 (gG) (Devlin et al., 2006), ΔU1_47 (tegument protein) (Helferich et al., 2007), ΔU1_23 (thymidine kinase) (Han et al., 2002), ΔU1_0 (nuclear protein) (Veits et al., 2003b), and ΔU1_5 (gL) (Fuchs et al., 2005) recombinants exhibited moderate to marked degrees of attenuation.

Deletion of individual genes within a cluster of five ORFs unique to iltoviruses (ORFs A through E) located at the 5’ end of the unique long (U1) region of the genome showed that deletion of individual ORFs (A, B, C, D and E) did not affect viral replication in cell culture (Veits et al., 2003a). However, recombinants with deletions in two sets of gene blocks (ORFs A, B and C or C, D and E) exhibited significant growth defects in vitro. Attempts to generate an ILTV recombinant lacking all five ORFs were not successful. Although the precise functions of the proteins encoded by these ORFs remain unknown, they might possess significant but redundant functions critical for virus replication (Veits et al., 2003a).

Among the five ORFs that were dispensable for virus replication in vitro, the gene encoding ORF C is of particular interest since complete genome sequence analysis of the TCO vaccine strain revealed that it contained a nonsense mutation near the 3’ end of ORF C. If translated, this gene would generate a truncated ORF C polypeptide of only 303 aa (García et al., 2013b). While the polypeptide encoded by ORF C from a wild-type strain is 335 aa long (Veits et al., 2003a). Rabbit monospecific polyclonal antiserum against bacterial fusion proteins identified the protein product of the ORF C gene as a 37.4 kDa protein. Although the ORF C gene was classified as a late expression gene, partial inhibition of ORF C transcription in the absence of protein synthesis suggests that its transcription maybe subjected to more complex yet unknown control (Mahmoudian et al., 2012; Veits et al., 2003a). Immunocytochemical analysis indicated that the ORF C protein mainly localizes within the cytoplasm, but a small fraction localizes to the nucleus of infected cells (Veits et al., 2003a). Although a recombinant with deletion of the ORF C gene has been previously described, the degree of attenuation of a ΔORF C recombinant derived from a well-characterized virulent ILTV strain has not been evaluated. The objective of this study was to generate an ORF C-deficient ILTV recombinant, using the virulent USDAch strain as its parent, and evaluate its attenuation and protection efficacy as a potential live-attenuated vaccine against ILT.

RESULTS

BΔORFC strain genotype and phenotype

Deletion of the ORF C gene from the USDAch strain genome was achieved through homologous recombination using classic marker-rescue technique. The ORF C gene was replaced by GFP expression cassette (Fig. 1a) and the ΔORFC virus was rescued and plaque purified (Fig. 1b). Subsequently, the GFP expression cassette was removed and the BΔORFC was rescued and plaque purified (Fig. 1c). The disruption of the ORF C locus in ΔORFC and BΔORFC viruses was initially confirmed by PCR analysis. The sizes of the PCR products for GAORFC, parental USDAch and BΔORFC templates were 4.7, 1.78 and 0.7 Kb, respectively, as expected. The PCR product size obtained for BΔORFC indicated the absence of 1.0 Kb, which corresponds to the length of the gene encoding ORF C (Fig. 2a). Double indirect immunofluorescence staining of infected cells was used to define the phenotype of the recombinant. The formation of multinucleated (blue) syncytial cells in chicken kidney (CK) cells infected with USDAch (Fig. 2b) and BΔORFC (Fig. 2c) in contrast to mock-inoculated CK cells (Fig. 2d) indicated that the ORF C protein was indeed dispensable for virus replication. The ORF C protein specific staining (green) could only be detected in the cytoplasm of cells infected with the USDAch parental strain (Fig. 2b) and not in the cytoplasm of multinucleated cells infected with BΔORFC (Fig. 2c), while the cytoplasm of cells infected with either USDAch (Fig. 2b) or BΔORFC (Fig. 2c) stained purple with a mAb specific for glycoprotein C. As expected, there was no staining for glycoprotein C or ORF C protein in mock-infected CK cells (Fig. 2d). Therefore,
end of the U₅2 polypeptide in BΔORFC is unlikely to contribute significantly to its attenuated phenotype.

An inconsequential synonymous mutation for glutamic acid was identified in the U₄1.45 polypeptide. Non-synonymous mutations that resulted in amino acids substitutions with similar physicochemical properties were identified in two polypeptides (U₄1.49 and U₄1.36). A non-synonymous mutation in the BΔORFC ICP4 gene resulted in the substitution of an amino acid with distinctly different physicochemical properties. However, this Pro¹⁹⁹ Ala substitution was found in other vaccine and virulent strains as well, suggesting that the rigidity of the ICP4 structure at this position is not important for its function and is not associated with strain attenuation. Four intragenic and 14 intergenic immunofluorescence staining of infected CK cells with ORF C monospecific polyclonal antisera confirmed the absence of ORF C expression in BΔORFC-infected cells.

BΔORFC and parental USDAch strain genome comparison

The full-length genome sequence of BΔORFC was determined in order to ascertain whether other secondary mutations, in addition to the deletion of the ORF C locus, were inadvertently introduced during the recombination process. Comparison of the BΔORFC and USDAch strain (GenBank accession no. JN542533) clearly delineated the boundaries of the ORF C deletion within the BΔORFC genome. However, other minor nucleotide insertion-deletions (INDELs) and single nucleotide polymorphisms (SNPs) were observed (Table S1, available in the online Supplementary Material). Briefly, the BΔORFC genome contained a frame-shift mutation, single nucleotide [A] deletion at position 638 in the U₅2 ORF, which resulted in an elongated polypeptide of 229 aa for BΔORFC, while the USDAch genome encoded a 217 aa polypeptide. The U₅2 genes have been shown to be non-essential for in vitro growth for several alphaherpesviruses and are often deleted during the generation of bacterial artificial chromosomes (BACs) containing the genomes of avian herpesviruses. However, restoration of the U₅2 gene has been reported to improve replication in vitro and in vivo for reconstituted viruses derived from the Marek’s disease BAC known as pRB-1B5 (Zhao et al., 2008). We speculate that the additional 12 aa at the very
INDELS were identified in the genome of BΔORF C (Table S1).

Recombinant BΔORF C and parental USDAch strain growth kinetics and plaque formation

Growth and plaque formation diameter of recombinant BΔORF C and the parental USDAch strain were measured in the leghorn male hepatoma (LMH) cell line (Fig. 3). At 24 h post-inoculation the extracellular virus titres of BΔORF C began to increase and attained a mean value of 0.82 (log_{10} TCID_{50}) while virus titre of USDAch strain remained undetectable. From 48 to 96 h post-inoculation, extracellular virus titres for BΔORF C and USDAch increased exponentially at a similar rate. Intracellular virus titres were first detected at 24 h post-inoculation and increased exponentially for both BΔORF C and USDAch from 48 to 96 h post-inoculation. Extracellular virus titres were consistently lower than intracellular virus titres for the two viruses (Fig. 3a). Overall, there were no statistical differences in growth kinetics between the BΔORF C recombinant and its parental USDAch strain, as the BΔORF C recombinant reached comparable extracellular and intracellular virus titres. The mean diameter of plaques formed in LMH cells by BΔORF C and the parental USDAch strain was determined 4 days post-inoculation. The diameter of 50 and 43 viral plaques was measured for recombinant BΔORF C and parental USDAch strain, respectively. The mean plaque diameter for BΔORF C was 1014 µm (+/- 134 µm). The mean plaque diameter for USDAch was 1834 µm (+/- 407 µm) (Fig. 3b). The BΔORF C recombinant virus produced a significantly smaller plaque (P<0.0001) as compared with the parental USDAch strain.

Fig. 3. Growth kinetics and plaque formation analysis. (a) LMH cells were inoculated with BΔORF C or USDAch strain. Viral progeny from supernatants represent cell-free (CF) virus and viral progeny from cells represents cell-associated (CA) virus. Viral titres in CK cells are expressed as the mean TCID_{50} ml^{-1} (log_{10}). Open circle represents mean viral titre of BΔORF C-F, filled triangle BΔORF C-CA, open square USDAch-CF, inverted filled triangle USDAch-CA, and bars represent standard deviation. Two-way ANOVA of CA and CF titres for each strain compared at 0, 24, 48, 72 and 96 h post-inoculation showed a significant increase in titres (P<0.0001), while CA and CF viral titres for parental USDAch and recombinant BΔORF C were similar (P=0.7067) when compared at the same time point. (b) Plaque diameter in LMH formed by BΔORF C and USDAch was measured using Image J software. The mean plaque diameter (µm) for BΔORF C and USDAch are presented in columns and bars represent the standard deviation. Paired t-test analysis indicated that the mean diameter of plaques formed by the recombinant BΔORF C were significantly smaller (P<0.0001) than plaques formed by the parental USDAch strain indicated by different superscript letters (a or b).

Attenuation and protection efficacy of BΔORF C strain after intra-tracheal/ocular administration

The objective of the first experiment was to determine the degree of attenuation of the BΔORF C recombinant as compared to the parental USDAch strain in specific pathogen-free (SPF) chickens inoculated via the intra-tracheal/ocular route. Four days post-inoculation, the group of chickens inoculated with the parental USDAch strain showed significantly higher clinical sign scores, including four mortalities, than those of the BΔORF C-inoculated (P<0.0001) or the mock-inoculated (P<0.0001) groups of chickens. On the other hand, no differences in clinical sign scores were observed between BΔORF C-inoculated and the mock-inoculated groups of chickens (P=0.35) (Fig. 4a). Tracheas from BΔORF C-inoculated chickens collected at 6 days post-inoculation showed minor microscopic tracheal lesions compared with moderate to severe microscopic tracheal lesions induced by USDAch inoculation (P<0.0001) as indicated by mean tracheal scores (Fig. 4b). Trachea virus load in chickens inoculated with the BΔORF C virus was not statistically different to virus load from that in chickens inoculated with the parental USDAch strain (P=0.39) (Fig. 4c).

The second experiment ascertained whether chickens inoculated via the intra-tracheal/ocular route with the BΔORF C recombinant were protected against challenge with strain 63140, previously shown to be more virulent than the USDAch strain (Vagnozzi et al., 2015). The group of chickens inoculated with the BΔORF C recombinant at 3 weeks of age and challenged at 5 weeks of age showed no mortalities and had significantly lower clinical sign scores that the non-vaccinated/challenged (NVx-Ch) group of chickens.
Fig. 4. Evaluation of BΔORFC virus attenuation as compared to its parental USDAch strain. SPF chickens were intra-tracheal-ocular inoculated with BΔORFC, parental USDAch strain, or cell culture media (mock) at 21 days-of-age. (a) Clinical signs score for individual chickens at 4 days post-inoculation per group are shown. Median clinical signs scores for Mock (0), BΔORFC (1) and USDAch (2) are depicted by horizontal lines and range by vertical lines. (b) Individual microscopic lesion score for tracheas collected at 6 days post-inoculation from Mock, BΔORFC and USDAch groups are shown. Median tracheal lesion score for Mock (0), BΔORFC (1) and USDAch (5) groups are depicted by horizontal lines and range by vertical lines. (c) Individual tracheal ORFC USDAch and Mock virus genome load (log_{10} 2^{-\Delta\Delta C_t}) at 6 days post-inoculation are shown. Mean tracheal genome load of Mock (0), BΔORFC (4.60) and USDAch (4.99) groups are depicted by horizontal lines and standard deviations by vertical lines. The one-way ANOVA Kruskal–Wallis test and the Dunn test for multiple comparisons were performed at the 5% level of significance (P<0.008). Differences in clinical sign scores, trachea lesion scores and trachea genome load are indicated by superscript letters (a, b or c).

(P<0.0001), that showed one mortality and increased clinical signs of the disease. On the other hand, clinical sign scores for the BΔORFC-Ch group of chickens were not different from those observed for the non-vaccinated/non-challenged (NVx-NCh) group of chickens (P=0.99) (Fig. 5a). The challenge virus load in tracheas and conjunctivae (Fig. 5b and c, respectively) of chickens inoculated with the BΔORFC recombinant were significantly lower than those of the NVx-Ch group of chickens (P<0.0001), indicating that inoculation with BΔORFC recombinant via the intra-tracheal-ocular route certainly influenced challenge virus shedding in trachea.

Replication and protection efficacy of BΔORFC strain after eye-drop administration

The replicative abilities of the BΔORFC recombinant relative to that of the commercial live-attenuated vaccines strains, CEO and TCO, were assessed at 5 days after eye-drop vaccination. Virus load of the BΔORFC recombinant in trachea when delivered at 2.7 (log_{10}) or 3.7 (log_{10}) doses was significantly lower than the CEO vaccine virus load (P<0.0001). However, trachea virus load for the BΔORFC recombinant was similar to that of the TCO-vaccinated groups of chickens (P=0.7767) (Fig. 6a).

The protection efficacy of BΔORFC administered at 2.7 and 3.7 (log_{10}) doses was compared to commercial ILTV live-attenuated vaccines, TCO and CEO, administered at the recommended full doses via eye-drop vaccination. At 3 weeks post-vaccination, vaccinated and non-vaccinated groups of chickens were challenged with virulent ILTV strain 63140. At 5 days post-challenge, at the peak of clinical signs (Fig. 6b), the CEO- and BΔORFC 3.7-vaccinated groups of chickens showed a significant decrease in clinical sign scores when compared with the TCO- and BΔORFC 2.7-vaccinated groups, and the non-vaccinated challenged (NVx-Ch) group of chickens (Fig. 6c). At the peak of clinical signs, four and two mortalities were recorded for the NVx-Ch and TCO-Ch groups of chickens, respectively. At 3 days post-challenge, the mean challenge virus load in tracheal swabs from vaccinees and controls was determined. The CEO-vaccinated group of chickens showed a significant (P=0.003) reduction in challenge virus load as compared with that of the non-vaccinated challenged (NVx-Ch) group of chickens (Fig. 6d). Challenge virus load detected for vaccinated groups, BΔORFC 2.7 (P=0.999), BΔORFC 3.7 (P=0.1496) and TCO (P=0.3930), were not significantly different from virus load of non-vaccinated challenged (NVx-Ch) chickens. Overall the challenge virus shedding in trachea indicates that both BΔORFC- and TCO-vaccinated groups reduce challenge virus load to similar levels, however this was not statistically different than that observed in non-vaccinated challenged chickens. Any reduction in individual virus load observed in BΔORFC or TCO vaccinees pales in comparison to the robust reduction observed within CEO-vaccinated chickens (Fig. 6d).

DISCUSSION

The present study provides in vitro and in vivo characterization of an ILTV recombinant (BΔORFC) containing a deletion in the ORF C gene, a conserved Iltovirus-specific gene (Veits et al., 2003a). Deletion of the ORF C gene from the genome of the United States Department of Agriculture (USDA) ILTV challenge strain (USDAch) was achieved through homologous recombination, and the absence of
ORF C protein expression was verified by indirect immunofluorescence. In agreement with Veits et al. (2003a) we confirmed that the ORF C gene is indeed dispensable for in vitro replication and provide evidence that the BΔORFC recombinant replicates as well as the parental USDAch strain. Also we confirmed that the absence of ORF C expression negatively influenced cell-to-cell spread in LMH cells as indicated by the smaller plaques formed by BΔORFC recombinant virus as compared to the parental USDAch strain.

Comparative genomics of BΔORFC and parental strain USDAch revealed that in addition to the ORF C gene deletion, several INDELs and SNPs were identified within the BΔORFC genome (see Table S1). These additional mutations of the BΔORFC genome are not found in any of the ILTV genomes that are currently published in the GenBank database. However, at present it is not clear whether these differences are due to unwanted second site mutations acquired during mutagenesis or to genome heterogeneity of the parental USDAch strain. Therefore at this point we cannot exclude the possibility that these secondary mutations influence the attenuation of the recombinant BΔORFC strain. Full genome sequencing of the USDAch parental strain utilized in this study is warranted.

The level of attenuation attained by BΔORFC was clearly evidenced when chickens inoculated with the ΔORF C recombinant via the intra-tracheal/ocular route failed to present clinical signs. Furthermore, microscopic examination of tracheas from infected chickens showed mild tracheal lesions as compared to lesions in the tracheas of USDAch-infected chickens. Similar to the BΔORFC strain, gene-deleted ILTV recombinants deficient in thymidine kinase (UL23) (Hans et al., 2002) and dUTPase (UL50) (Fuchs et al., 2000) also induced only minor microscopic tracheal lesions. Surprisingly, despite the lack of severe trachea lesions, the replicative capacity of the BΔORFC recombinant in trachea was comparable to that of the parental USDAch strain as indicated by similar viral genome load in trachea.

The protection efficacy of the ORF C gene deletion recombinant was evaluated using two compatible challenge models and different immunization routes with two scoring criteria: protection against clinical signs and reduction in challenge virus shedding. However, this method is not without a caveat. Granted that it is convenient in a clinical setting, but because of its invasive nature this vaccination route is not economically feasible for poultry. For ‘real world’ considerations, other administration methods (e.g.

![Fig. 5. Protection efficacy of BΔORFC when administered via intra-tracheal/ocular route. SPF chickens were intra-tracheal-ocular inoculated with BΔORFC at 3 weeks of age and challenged with virulent ILTV strain 63140 at 5 weeks of age. (a) Clinical signs score for individual chickens at 5 days post-challenge are presented. Median clinical signs scores for NVx-NCh (0), BΔORFC (0) and USDAch (3) are depicted by horizontal lines and range by vertical lines. (b) Individual trachea genome load at 5 days post-challenge are shown. Mean trachea genome load (log10 2^{-\Delta\Delta Ct}) of NVx-NCh (0.25), BΔORFC-Ch (0.41) and NVx-Ch (3.39) groups are depicted by horizontal lines and standard deviations by vertical lines. (c) Individual conjunctiva genome load (log10 2^{-\Delta\Delta Ct}) at 5 days post-challenge are shown. Mean conjunctiva genome load of NVx-NCh (0), BΔORFC-Ch (0.28) and NVx-Ch (4.32) groups are depicted by horizontal lines and standard deviations by vertical lines. The one-way ANOVA Kruskal–Wallis and the Dunn test for multiple comparisons were performed at the 5 % level of significance (P<0.005). Clinical sign scores and viral genome load changes are indicated with different superscript letters (a and b).](http://gv.microbiologyresearch.org)
spray, drinking water, eye drops, etc.) must be examined. Eye-drop vaccination, although labour intensive, is one such method for accurate mucosal delivery of live-attenuated vaccines to poultry (Marangon & Busani, 2006).

Protection induced by BΔORFC after eye-drop administration was evaluated based on the decrease of clinical signs and the decrease in virus load in the trachea after challenge with virulent strain. Previously it has been reported that these two parameters could differentiate the higher level of protection induced by the CEO vaccine compared to that of the TCO vaccine (Vagnozzi et al., 2012). Chickens immunized with the higher dose of BΔORFC [3.7 (log_{10}) TCID_{50}] showed a reduction in clinical signs comparable to that observed in the CEO-vaccinated chickens, however the reduction of challenge virus load in trachea was not as significant as that in the CEO vaccinees. On the other hand, reductions in challenge virus load in chickens immunized with either dose of BΔORFC [3.7 and 2.7 (log_{10}) TCID_{50}] were comparable to those induced by TCO vaccination. At the lower dosage, BΔORFC behaved similar to the TCO vaccines with respect to protection against clinical signs. This was not unexpected since TCO and the parental strain (USDAch), used to generate BΔORFC, are closely related phylogenetically with 99.91 % sequence identity and

![Fig. 6. Replication and protection efficacy of BΔORFC and live-attenuated CEO and TCO vaccines after eye-drop administration. (a) Individual trachea genome load (log_{10} 2^{-\Delta\Delta Ct}) 5 days after eye-drop vaccination with BΔORFC, CEO and TCO were determined. Mean virus genome load for vaccinated groups of chickens, BΔORFC 2.7 (0), BΔORFC 3.7 (0), CEO (3.80) and TCO (1.20), are depicted by horizontal lines and standard deviations by vertical lines. (b) Mean clinical signs scores to at days 3 to 6 post-challenge for vaccinated and non-vaccinated groups of chickens. (c) Clinical signs score for individual chickens at 5 days post-challenge are shown. Mean clinical signs scores for NVx-NCh (0.13), BΔORFC 2.7-Ch (3.25), BΔORFC 3.7-Ch (0.90), CEO-Ch (0.71), TCO-Ch (2.90) and NVx-Ch (4.30) groups of chickens are depicted by horizontal lines and range by vertical lines. (d) Individual trachea genome load (log_{10} 2^{-\Delta\Delta Ct}) at 3 days post-challenge is shown. Mean trachea viral genome load for NVx-NCh (0), BΔORFC 2.7-Ch (3.99), BΔORFC 3.7-Ch (3.17), CEO-Ch (1.35), TCO-Ch (2.97) and NVx-Ch (4.74) groups are depicted by horizontal lines and standard deviations by vertical lines. The one-way ANOVA Kruskal–Wallis test and the Dunn test for multiple comparisons were performed at the 5 % level of significance (P<0.005). Clinical sign scores and viral genome load changes among groups are highlighted with different single or combinations of superscript letters.

M. Garcia and others
97.53% sequence similarities (García et al., 2013). Therefore, the parallels in the level of attenuation for both viruses and the similar level of protection observed in BΔORFC vaccinees receiving the lower dose of vaccine are most likely associated to ORF C expression and their close genetic relatedness.

Interestingly, the BΔORFC virus load in tracheae after eye-drop vaccination was slightly lower than that in the tracheae of the TCO vaccinees but statistically lower than the CEO vaccine. In contrast to CEO and TCO, the low virus load of BΔORFC in tracheae after eye-drop vaccination further documents the attenuated phenotype of this strain.

Independent studies had shown that ILTV vaccine candidates, ΔTK (thymidine kinase) (Hans et al., 2002) and ΔgG (glycoprotein G) (Devlin et al., 2008) attained more robust protection after challenge when they were delivered via the intra-tracheal route than when delivered via eye-drop. Hans et al. (2002) showed that increasing the eye-drop dose of the ΔTK-deficient recombinant increased the protection efficacy to levels similar to those observed for chickens that received the ΔTK-deficient recombinant via the intra-tracheal route. Future studies will focus on determining the optimal dosage for eye-drop immunization with the BΔORFC recombinant in order to increase the protection efficacy of the recombinant virus while maintaining a balance between safety and protection. Additionally, the potential for en mass administration via water, spray, as well as in ovo vaccination, is currently being evaluated.

In summary, the ORF C locus was successfully deleted from the ILTV genome without negatively influencing viral replication in vitro. The lack of ORF C expression contributed to virus attenuation as indicated by the lack of tracheae pathology after intra-tracheal inoculation and low virus load in tracheae after eye-drop inoculation. The ORF C-deleted ILTV strain induced protection comparable to that elicited by TCO vaccination when applied via eye-drop. Although the function of this unique Iltovirus gene remains unknown, the findings from this study suggest a role of ORF C in ILTV virulence and demonstrate the suitability of BΔORFC as a live-attenuated vaccine against ILT.

**METHODS**

**Viruses and cells.** ΔORF C recombinant viruses were derived from the virulent USDA challenge strain (USDACH) and propagated in primary chicken kidney (CK) cells. The leghorn male hepatoma (LMH) cell line (Kawaguchi et al., 1987) was used for marker rescue of recombinants, replication kinetics and plaque assay analysis.

**Construction of recombinant viruses.** For construction of recombinant viruses, initially the ORF C was replaced by GFP expression cassette and the recombinant virus expressing GFP was rescued (ΔORFC) (Fig. 1a, b). The GΔORFC recombinant virus was propagated and through homologous recombination the GFP cassette was deleted from the GΔORFC genome and the BΔORFC virus was rescued (Fig. 1c). Schematics of recombinant plasmids that were utilized for the construction of the ORF C recombinant viruses are shown in Fig. S1. Initially the pBK-CMVgfpGFP (Strategene) was used as a template to PCR-amplify the GFP and kanamycin genes using primers containing restriction enzyme sites shown in Table S2. A 2.2 Kb PCR product containing the GFP gene and a 1.8 Kb PCR product containing the kanamycin gene were fused at the BstEII sites using fusion PCR. This resulted in the generation of a 4.0 Kb fusion PCR product flanked by an Ascl restriction site at its 5' end and a BsrGI site at its 3' end. The PCR product was digested with Ascl and BsrGI and cloned into a modified pIDT-SMART-Box2C (IDT) to create plasmid pIDT-SMARTBox2_gfpKan_C (Fig. S1a). The recombinants were selected on kanamycin (30 μg ml⁻¹) and ampicillin (100 μg ml⁻¹) plates and screened for GFP expression, since both eukaryotic and prokaryotic promoters control the expression of kanamycin expression. In order to delete the ORF C locus, sequences flanking the ORF C gene of the USDAch strain (GenBank accession no. JN542534) were synthesized and cloned. Briefly, a 135 bp (USDAch genome coordinates 23812: 23946) sequence located at the 3' end of the ORF B gene, that flanks the left arm of the ORF C gene, was synthesized and cloned into a modified pIDT-SMART-Box2C AMP vector containing two loxP sequences separated by BgII and BsrGI restriction enzyme sites, and upstream of BssWI and PacI sites, generating a plasmid named pSMART_ORFCTrunc. The GFP/Kan cassette from pIDT-SMARTBox2_gfpKan_C plasmid was excised using BgII and BsrGI and inserted into BgII/BsrGI-digested pSMART-ORFC-Trunc to generate a plasmid named pSMARTORFC-Trunc_gfp (Fig. S1b). A 541 (USDAch genome coordinates 24955: 25495) base-pair fragment located at the 5' end of ORF D gene, that flanks the right arm of ORF C gene, was amplified using primers ORF D fwpacI and ORF D rev BssWI (Table S2). This PCR product was digested with BssWI and PacI and cloned into the BssWI/PacI-digested pSMARTORFC-Trunc_gfp vector creating the donor plasmid named pdelORFC (Fig. S1c). The nucleotide sequence of pdelORFC was verified using Sanger dyeoxy sequencing with a battery of specific primers. Viral genomic DNA from USDAch strain propagated in CK cells was isolated as previously described (Volkening & Spatz, 2009). Swol-linearized donor plasmid pdelORFC and USDAch genomic DNA were transfected in LMH cells using the Mirus transfection reagents as previously described (Mundt et al., 2011), transfected cells were incubated at 39°C for 5 days. Recombinant viruses expressing GFP in LMH cells were isolated and plaque purified in CK cells. One candidate recombinant virus was named GΔORFC. Using fusion cloning, a donor plasmid with sequences homologous to the 3’ terminus of ORF B and 5’ terminus of the ORF D gene was constructed to eliminate the GFP cassette from the GΔORFC genome (Fig. S1d). Brieﬂy, a fragment located at the 3’ end of ORF B gene (USDAch genome coordinates 23668–23944) was amplified with primers ORF B fswXba I and ORF B rev Xmal (Table S2). The PCR product was digested with Xbal and Xmal. A fragment located at the 5’ end of ORF D (USDAch genome coordinates 24977–25553) was amplified with primers ORFD fsw Xmal and ORFD rev EcoRI (Table S2). The PCR product was digested with Xmal and EcoRI. The ORF B and ORF D amplicons were cloned into the Xbal–EcoRI-digested pUC19 plasmid using a two insert to one vector scheme with standard T4 DNA ligase. Escherichia coli DH5α cells were transformed using electroporation and recombinants plasmid named pUBD#12 was selected on plates with 50 μg ml⁻¹ ampicillin (Fig. S1e). The pUBD#12 plasmid was further characterized using Sanger sequencing. In order to
rescue a recombinant virus without the GFP cassette, the EcoRI-linearized pUBD412 plasmid and GAORFC virions DNA were transfected into LMH cells using the Mirus transfection reagents as previously described (Mundt et al., 2011). Fluorescence microscopy was used to identify viral plaques that did not emit fluorescence. After five rounds of plaque purification, non-fluorescent viruses (BΔORFC) were expanded in CK cells.

**Full genome analysis of recombinant virus BΔORFC.** The complete genome sequences of GAORFC and BΔORFC recombinants were determined using MiSeq next generation sequencing (Illumina) and compared to the genome of the previously published parent USDAch strain (GenBank accession no. JN542534). The GenBank accession numbers for the GAORFC and BΔORFC genomes are KX165321 and KX165320, respectively. To rapidly confirm the disruption in the ORF C locus, a PCR scheme was developed. The PCR assay utilized forward primer PCR fwd ORF B (USDAch reference strain coordinates 23869–23889) and reverse primer PCR rev ORF D (USDAch reference strain coordinates 25657–25656) whose sequences flanked the ORF C gene (Table S2). Viral genomic DNA was isolated from CK cells infected with GAORFC, BΔORFC and the parental USDAch strain as previously described (Volkengen & Spatz 2009).

**Indirect immunofluorescence.** The lack of ORF C expression was confirmed by indirect immunofluorescence. Briefly, eight-well chamber slides containing 4 × 10^4 cells per well were inoculated with USDAch strain and BΔORFC at an m.o.i. of 0.002 or were mock-inoculated with cell culture media. Two days post-inoculation, the cell supernatants were removed and the cells were washed with cold 1 × PBS, then fixed with 4% paraformaldehyde (300 µl per well) at room temperature. Cells were permeabilized with 0.2% Triton X for 5 min, rinsed with 1 × PBS. The cells were then blocked with 1% filtered goat serum. After blocking, cells were incubated for 1 h at room temperature with a combination of FITC anti-gC mAb (Mundt et al., 2011) and ORF-C rabbit polyclonal (kindly provided by Dr Walter Fuchs, Friedrich-Loeffler-Institute, Greifswald-Insel Riems, Germany) at 1:100 and 1:250 dilutions in PBS, respectively. After incubation slides were washed with 1 × PBS and incubated with secondary antibody anti-mouse Alexa Fluor 633 (α-mouse 633) and anti-rabbit Alexa Fluor 488 (α-rabbit 488), both at a dilution of 1:400, with DAPI at 1:1000 dilution. The slides were washed three times, rinsed in water, dried and mounted with Vectashield (Vector Laboratories). Slides were inspected by confocal LASER scan microscopy (CLSM) using the LSM 510 LASER confocal microscopy system (Zeiss) at the AHRC Cell Imaging Core Facility, University of Georgia.

**Growth kinetics and plaque formation.** Plaque size formation and replication kinetics of the BΔORFC strain and its parental USDAch strain were analysed in LMH cells. Briefly, LMH cells in suspension were seeded into a 0.5% gelatin-treated six-well-plate, 4 × 10^5 cells diluted in 2.5 ml per well, and incubated at 37°C for 24 h. The medium was removed from the cell layer and cells were inoculated at m.o.i. of 0.001 with BΔORFC or the parental USDAch strain. Virus was adsorbed for 60 min at 39°C. For plaque assay, the inoculum was removed after adsorption and cells were rinsed once with media and overlaid with semi-solid methacel primary culture media containing 2% calf serum and antibiotic-antimycotic. Cells were then incubated at 39°C for 4 days, after which the media was removed and the cells were fixed with 2% formaldehyde for 60 min at room temperature. The solution was discarded and the fixed cells were washed thoroughly under streaming water. Finally, the cells were stained with 1% crystal violet in 50% ethanol for 15 min, washed again and dried. Plaques were manually counted and plaque diameters were measured using the ImageJ software (Schneider et al., 2012). For growth kinetics, cells and supernatants from three independent wells were collected at 0, 24, 48, 72 and 96 h post-inoculation for each virus. Viral titres were determined in CK cells as previously described (Rodriguez et al., 2007). Viral titres for viruses within the cell culture supernatants (extracellular) and those associated with infected LMH cells (intracellular) were determined and expressed as the mean log_10 TCID_50 ml^-1. The BΔORFC recombinant virus and USDAch parental strain utilized in animal experiments were propagated in CK cells.

**Animal experiments.** Three chicken experiments were conducted. The objective of the first experiment was to determine the degree of attenuation of the BΔORFC virus in specific pathogen-free (SPF) chickens as compared to the parental USDAch strain. Briefly, a total of 215, 3-week-old, SPF chickens (Merial Select) were distributed into seven colony houses (Poultry Diagnostic Research Center, Athens, GA). At 3 weeks of age, 70 chickens were inoculated intratracheally (100 µl) and in the eye conjunctiva (50 µl each eye) with a dose of 3.16 (log_10 TCID_50 per chicken of the BΔORFC recombinant or the parental USDAch strain. The remaining 75 chickens were mock-inoculated with tissue culture media. Clinical signs were scored from days 4 to 7 post-inoculation as previously described by Vagnozzi et al. (2012a). At 6 days post-inoculation, tracheal swabs were collected from each group of chickens and analysed by real-time PCR to determine trachea virus load. At 6 days post-inoculation, 35 chickens from the BΔORFC- and USDAch-inoculated groups, and 20 chickens from the mock-inoculated group were euthanized. Tracheae were removed, formaldehyde (10%) fixed, paraffin embedded and sections were hematoxin & eosin stained. After microscopic examination, trachea lesion index scores were assigned to each trachea section as previously described (Guy et al., 1990).

The objective of the second experiment was to determine whether chickens inoculated with the BΔORFC strain via intra-trachea/ocular routes were protected against challenge with ILTV virulent strain 63140. Briefly, a total of 60 chickens, 35 of which were previously vaccinated at 3 weeks of age with BΔORFC virus, and 25 chickens sham-vaccinated as indicated above, were challenged (Ch) with the virulent ILTV strain 63140/C/08/BR at 5 weeks of age. The challenge virus was administered in a total volume of 200 µl. One hundred microlitres were applied intratracheally and 100 µl via eye drop (50 µl on each eye) at a dose of 3.5 (log_10) TCID_50. A third group of 25 chickens remained unchallenged (NCh). The three groups of chickens were identified as BΔORFC-Ch, non-vaccinated-challenge (NVx-Ch) and non-vaccinated-non-challenge (NVx-NCh). The level of protection induced by BΔORFC was assessed by evaluation of clinical signs and trachea and conjunctiva virus load 5 days after challenge in BΔORFC-vaccinated/challenge and non-vaccinated/challenge (BΔORFC-Ch v. NVx-Ch) groups of chickens as described below.

The objective of the third experiment was to evaluate the protection efficacy elicited by BΔORFC virus as compared to that afforded by chicken embryo origin (CEO) and tissue culture origin (TCO) live-attenuated ILTV vaccines when applied via eye drop. Sufficiently, four groups of 12 to 15 2-week-old SPF chickens were vaccinated with one dose of CEO vaccine [2.8 (log_10) TCID_50], one dose of TCO vaccine [3.5 (log_10) TCID_50], and BΔORFC at 2.7 or 3.7 (log_10) TCID_50. Two groups of 12 to 15 SPF chickens were sham-vaccinated via eye drop with cell culture media. At 5 days post-vaccination, tracheal swabs were collected from 10 to 15 chickens from each group. Tracheal swabs were analysed by real-time PCR to quantify vaccine virus load. Three weeks post-vaccination, CEO-, TCO-, BΔORFC 2.7-, BΔORFC 3.7-vaccinated and non-vaccinated groups of chickens were challenged as described above. The levels of protection induced by the experimental vaccine BΔORFC and commercial live-attenuated vaccines, TCO and CEO, were again assessed by comparing clinical signs and virus load between vaccinated and non-vaccinated groups of chickens after challenge. Clinical signs were scored from 3 to 6 days post-challenge, and challenge virus load were quantified by real-time PCR from tracheal swabs collected 3 days post-challenge. All animal experiments conducted in this study were performed under...
the Animal Use Proposal A2012 07-016-Y3-A3 approved by the Animal Care and Use Committee (IACUC) in accordance with regulations of the Office of the Vice President for Research at the University of Georgia.

**DNA extraction.** Tracheal swabs were placed in 2 ml tubes containing 1× PBS solution with 2% antibiotic-antimycotic (100×, Invitrogen) and 2% newborn calf serum. Swabs were stored at −80°C until processing. DNA was extracted using MegaZorb DNA mini-prep kit (Promega) following the manufacturer’s recommendations.

**Clinical sign scores.** Clinical signs of conjunctivitis, respiratory distress and lethargy were scored as previously described by Vagnozzi et al. (2012a). Clinical sign categories were evaluated in individual chickens and given a score. No clinical signs was given a score of 0; mild, a score of 1; moderate, a score of 2; and severe, a score of 3. A clinical sign score was calculated for each chicken and a mean clinical sign score was assigned for each group of chickens at each time point. At the time point where the peak of clinical sign was observed, median clinical sign score for each group was compared.

**Microscopic examination of tracheal lesions.** Tracheal microscopic lesions were scored from 0 to 5 as previously described (Guy et al., 1990). Briefly, normal epithelium was scored as 0; mild to moderate lymphocyte infiltration was scored as 1; mucosa thickened by marked cell infiltration and/or few foci of syncytia with intra-nuclear inclusion bodies was scored as 2; moderate to marked lymphocyte infiltration, together with numerous syncytia with intra-nuclear inclusion bodies was scored as 3; absence of normal epithelium and mucosal surface covered by a thin layer of basal cells, syncytia and intra-nuclear inclusion bodies occasionally present was scored as 4; no epithelium remaining with syncytia and intra-nuclear inclusion bodies rarely found was scored as 5. Median tracheal lesion scores for groups of chickens were calculated and compared.

**Quantitative real-time PCR for ILTV in tracheal swabs.** ILTV genomes were quantified by real-time PCR in a duplex assay as previously described (Vagnozzi et al., 2012b). The relative amount of viral DNA was expressed as log_{10} 2ΔΔCt (Livak & Schmittgen, 2001), where ΔΔCt is the amount of the ILTV target gene normalized against the housekeeping gene – chicken collagen.

**Statistical analysis.** The two-way ANOVA was utilized to highlight differences in mean viral titres of BΔORFC and its parental USDAch strain when grown in LMH cells. The t-test was utilized to compare viral plaque diameter formed by BΔORFC and the parental USDAch strain. The one-way ANOVA Kruskal–Wallis test (P<0.05) was used to highlight differences in: (1) median clinical signs scores among groups of chickens inoculated via the intra-tracheal route with BΔORFC and the parental USDAch strain; (2) median clinical signs scores among BΔORFC-, CE0- or TCO-vaccinated groups of chickens post-challenge; (3) median trachea lesion scores among groups of chickens inoculated intra-tracheally with BΔORFC and the parental USDAch strain; (4) mean viral genome load in trachea from chickens that received the BΔORFC and parental USDAch strain via the intra-tracheal route, viral genome load in trachea from chickens that received BΔORFC, CEO or TCO vaccines via eye drop, and viral genome load in trachea and conjunctiva from vaccinated and non-vaccinated groups of chickens after challenge with virulent ILTV strain. Statistical analysis was performed with the GraphPad PRISM 6.0 software (GraphPad Software).

**ACKNOWLEDGEMENTS**

This project was funded by the US Poultry Harold E. Ford Foundation (project #683) and by the USDA–Agricultural Research Service (CRIS project #6040-32000-065-00D). The authors would like to thank Dr Laszlo Zsak (US National Poultry Research Center, USDA-ARS) for helpful suggestions during the course of this project.

**REFERENCES**


strains chicken embryo origin (CEO) and tissue culture origin (TCO). Virology 440, 64–74.


