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Biological and molecular characterization of ArkGA: A novel Arkansas serotype vaccine that is highly attenuated, efficacious, and protective against homologous challenge

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ABSTRACT

Almost all commercial poultry are vaccinated against avian coronavirus infectious bronchitis virus (IBV) using live attenuated vaccines mass administered by spray at day of hatch. Although many different types of IBV vaccines are used successfully, the ArkDPI serotype vaccine, when applied by spray, does not infect and replicate sufficiently to provide protection against homologous challenge. In this study, we examined a different Ark vaccine strain (Ark99), which is no longer used commercially due to its reactivity in one day old chicks, to determine if it could be further attenuated by passage in embryonated eggs but still provide adequate protection. Further attenuation of the Ark99 vaccine was achieved by passage in embryonated eggs but ArkGA P1, P20, and P40 (designated ArkGA after P1) were still too reactive to be suitable vaccine candidates. However, ArkGA P60 when given by spray had little or no vaccine reaction in one day old broiler chicks, and it induced protection from clinical signs and ciliostasis following homologous challenge. In addition, vaccinated and challenged birds had significantly less challenge virus, an important measure of protection, compared to non-vaccinated and challenged controls. The full-length genomes of viruses from egg passages 1, 20, 40, and 60 were sequenced using the Illumina platform and the data showed single nucleotide polymorphisms (SNPs) had accumulated in regions of the genome associated with viral replication, pathogenicity, and cell tropism. ArkGA P60 accumulated the most SNPs in key genes associated with pathogenicity (polyprotein gene 1ab) and cell tropism (spike gene), compared to previous passages, which likely resulted in its more attenuated phenotype. These results indicate that the ArkGA P60 vaccine is safe for spray vaccination of broiler chicks and induces suitable protection against challenge with pathogenic Ark-type virus.

1. Introduction

Avian infectious bronchitis virus (IBV) is a gammacoronavirus that causes an economically significant upper respiratory tract disease in chickens [1]. Because of its prevalence and infectivity, nearly all commercial poultry in the U.S. are vaccinated for IBV in a serotype-specific manner [2,3]. IBV vaccines are developed by passaging a pathogenic field virus in embryonated eggs until the virus has lost its pathogenicity in chickens. During these repeated rounds of embryo passage, the pathogenic field virus will accumulate mutations that result in an adaptation for replication in embryos. Conversely, the outcome of this adaptation is a decreased affinity for chicken tissues and therefore a reduced virulence in

Abbreviations: Ark99, Arkansas 99; ArkDPI, Arkansas Delmarva Poultry Industry; ArkGA, Arkansas Georgia; CAS, chorioallantoic sac; C_T, cycle threshold; EID_{50}, 50% embryo infective dose; IBV, infectious bronchitis virus; MHV, murine hepatitis virus; nsp2, nonstructural protein 2; nsp3, nonstructural protein 3; P, passage; PBS, phosphate-buffered saline; qRT-PCR, quantitative real-time reverse-transcriptase polymerase chain reaction; RT-PCR, reverse-transcriptase polymerase chain reaction; SARS-CoV, severe acute respiratory syndrome coronavirus; SD, standard deviation; SEM, standard error of mean; SNP, single nucleotide polymorphism; SPF, specific-pathogen free; US, United States; USDA, United States Department of Agriculture.

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chickens [4–6]. Live attenuated vaccines stimulate both humoral and cellular immunity, resulting in high levels of protection, and can be mass applied by spray [3,7]. Immunity resulting from vaccination with live attenuated IBV vaccines prevents replication of homologous virulent challenge virus within only a short time following vaccination [7].

Of the vaccines used in the U.S., the Arkansas Delmarva Poultry Industry (ArkDPI) serotype vaccine has been shown to be highly variable in its protective ability and is frequently isolated from vaccinated chicks [8–10]. Ideally, when mass applying an IBV vaccine, a high percentage of chicks should be infected with actively replicating virus (vaccine coverage) by days 7–10 post-vaccination, followed by a gradual decline in viral load. However, it has been shown that the ArkDPI vaccine has an atypical vaccine coverage and replication pattern when mass applied by spray, and previous data from our laboratory suggests that the percentage of chicks infected with vaccine virus by 10 days post-vaccination only reaches 15–25% [11,12]. Multiple replication cycles also occur in the bird (indicated by viral load and clinical signs in chicks), resulting in "rolling" reactions at different time points post-vaccination [13]. Our previous research has shown that to achieve an adequate proportion of infected chicks with ArkDPI vaccine and eliminate rolling replication cycles, a 100x dose is required [12].

The atypical vaccine coverage and cycling observed following ArkDPI vaccination is a product of the multiple minor genetic subpopulations in the vaccine bottle [14]. It has been previously shown that several serotypes of IBV vaccines contain genetic subpopulations and the subpopulations are often recovered in chickens following vaccination, even though these vaccines show a typical infection and replication cycle and protect from challenge [15]. With ArkDPI, the major population in the vaccine contains multiple, distinct amino acid changes in the spike protein that increase binding affinity in the embryonated egg but decrease binding affinity to mature chicken cells [9,16]. Conversely, the minor populations, which have the opposite spike protein binding profile, are more suited to infect and replicate in chickens [16,17]. However, these minor subpopulations are only a fraction of the total genetic population contained in the vaccine bottle. Thus, the proportion of infected chicks is very low and the time to reach peak infection and replication is delayed [12]. For these reasons, chickens do not develop adequate immunity following ArkDPI vaccination. Although using one of the viral subpopulations with binding affinity for chicken cells directly as a vaccine will induce a protective immune response, these subpopulations cannot be maintained through multiple passages in embryonated chicken eggs, which is required to propagate IBV vaccine. Research has been performed to homogenize the ArkDPI viral population by adapting it for growth in chicken embryo kidney cells, however this vaccine model has not yet been shown to be commercially feasible [18].

While ArkDPI is the only commercially available Ark-type IBV vaccine today, it is not the only Ark-type IBV vaccine ever produced. The Arkansas 99 (Ark99) strain was the first Ark-type virus to be attenuated for use as a vaccine. When originally mass applied in the field, it caused a severe vaccine reaction in young broilers, and was therefore discontinued when ArkDPI was developed [19,20]. The purpose of this study was to reevaluate the original and additionally attenuated Ark99 vaccine by multiple serial passaged in embryonated eggs as a potential Ark-type vaccine candidate. In addition, we investigated the mechanisms of attenuation of this vaccine by sequencing the genome and performing SNP analysis during the subsequent embryo passages. This study led to development of a new, more attenuated yet still efficacious vaccine strain designated Arkansas Georgia (ArkGA).

2. Materials and methods

2.1. Vaccine and challenge viruses

Ark99 vaccine is no longer produced nor is USDA license maintained by any vaccine manufacturer. An archived reference sample of live Ark99 vaccine was obtained from a commercial source and passaged once in 9-to-11 days of incubation specific-pathogen free (SPF) chicken embryos as described below. The University of Georgia egg-passaged virus, now designated ArkGA, was then used for further experimentation. Different egg passages, beginning at egg passage 1 (P1) and going to P60, were used in this study for consecutive experiments. A pathogenic Arkansas serotype challenge virus from our laboratory was also used in this study.

2.2. Embryonated chicken eggs and chickens

SPF embryonated chicken eggs were purchased from Charles River Laboratories (North Franklin, CT) and incubated to 9-to-11 days of development for virus passage, titration, and isolation experiments. Commercial non-vaccinated broiler chickens were used in the vaccination experiments as described below.

2.3. Virus attenuation

ArkGA was serially passaged 60 times by inoculating 9-to-11-day-old SPF embryonated chicken eggs in a 0.1 ml volume via the chorioallantoic sac (CAS) route [21]. Inoculated eggs were incubated at 37 °C for 48 h, at which point the embryos were humanely euthanized, and chorioallantoic fluid was collected for subsequent passage into additional 9-to-11-day-old embryos. Embryos were candled daily and mortality determined to be from non-viral origin was discarded.

2.4. Virus titration

Viruses were titrated at different egg passage levels using the following protocol: 10-fold serial dilutions of the virus were made in sterile deionized water and each dilution was inoculated into five 10-day-old embryonated SPF chicken eggs (0.1 ml/egg). Inoculated eggs were incubated at 37 °C for 7-days and embryos were examined for IBV-specific lesions. Embryo mortality within 24-h post-inoculation was considered nonspecific and not included in virus titer calculations. Virus titers were calculated by the method of Reed and Muench [22] and expressed as the 50% embryo infectious dose (EID_{50}). In addition to titration of embryo passages, vaccine and challenge viruses for the ArkGA P1 and P60 experiments were also titrated following dilution for inoculation into chickens to confirm the inoculation dose.

2.5. Experiment 1. Evaluation of infection and replication of ArkGA P1 and protection from challenge

One hundred one-day-old broiler chicks were vaccinated with the ArkGA P1 vaccine candidate in a 7 ml spray volume using a commercial vaccine spray cabinet and placed in an isolation house on fresh litter. Ten additional non-vaccinated chicks were placed in Horsfal-Bauer isolation units as controls. At 7, 10, 14, 17, 21, 24, and 28 days post-vaccination, all vaccinated chicks were swabbed in the choanal cleft for qRT-PCR analysis of viral load. Clinical signs corresponding to IBV vaccine reactions were also recorded on those days [3]. On day 30 post-vaccination, 20 vaccinated and 5 non-vaccinated chickens were challenged with pathogenic Arkansas serotype virus in a 0.1 ml eyedrop application, while an

additional 5 vaccinated and 5 non-vaccinated chickens were held as non-challenged controls. Five days post-challenge, clinical signs were scored and all chickens were swabbed and euthanized for necropsy. Tracheas were collected at necropsy for ciliostasis scoring.

2.6. Experiment 2. Evaluation of infection and replication of ArkGA P20, P40, and P60 vaccine candidates and protection from challenge

2.6.1. Trial 1. ArkGA P20

The ArkGA P1 vaccine candidate was further attenuated by 19 additional embryonated egg passages, yielding the ArkGA P20 vaccine candidate. One hundred one-day-old broiler chicks were vaccinated using a spray cabinet with the ArkGA P20 vaccine candidate in an 18 ml spray volume and placed in an isolation house on fresh litter. Ten additional non-vaccinated chicks were placed in isolators as controls. Swabs were taken at 3, 5, 7, 10, and 14 days post-vaccination to assess viral load and vaccine coverage in chicks, and clinical signs were recorded.

2.6.2. Trial 2. ArkGA P40

The ArkGA P20 vaccine candidate was passaged an additional 20 times in embryonated eggs to produce the ArkGA P40 vaccine candidate and another vaccination trial was conducted as described in Trial 1.

2.6.3. Trial 3. ArkGA P60

The ArkGA P40 vaccine candidate was passaged an additional 20 times in embryonated eggs to further attenuate the virus, producing ArkGA P60. One hundred one-day-old broiler chicks were spray vaccinated with the ArkGA P60 vaccine candidate in an 18 ml spray volume and placed in an isolation house on fresh litter. Ten additional non-vaccinated chicks were placed in isolation units as controls. At 3, 5, 7, 10, 14, 17, 21, 24, and 28 days post-vaccination, all vaccinated chicks were swabbed in the choanal cleft for qRT-PCR analysis of viral load as previously described. Clinical signs corresponding to vaccine reactions were also recorded on those days. On day 30 post-vaccination, 20 vaccinated and 5 non-vaccinated chickens were challenged with pathogenic Ark-type IBV in a 0.1 ml eyedrop application, while an additional 5 vaccinated and 5 non-vaccinated chickens were held non-challenged as controls. Five days post-challenge, clinical signs were recorded, and all chickens were swabbed in the choanal cleft palate and euthanized for necropsy. Tracheas were collected at necropsy for ciliostasis scoring.

2.7. Virus detection using quantitative real-time RT-PCR

Viral RNA was extracted from 50 μl of choanal swab fluid using the MagMAX-96 RNA Isolation Kit (Ambion Inc., Austin TX) on a KingFisher Flex magnetic particle processor (Thermo Scientific, Waltham, MA) per the manufacturer's protocol. Quantitative real-time RT-PCR (qRT-PCR) was conducted using an Applied Biosystems 7500 Fast Real-Time PCR System (Life Technologies, Carlsbad, CA) and the AgPath-ID™ One-Step RT-PCR kit (Ambion Inc.) per the manufacturer's recommendations. Primers and probe for the qRT-PCR were previously published [23] and consist of a forward primer IBV5′GU391 (5′-GCT TTT GAG CCT AGC GTT-3′), a reverse primer IBV5′GL533 (5′-GCC ATG TTG TCA CTG TCT ATT G-3′) and a Taqman® dual-labeled probe IBV5′G probe (5′ –FAM-CAC CAC CAG AAC CTG TCA CCT C-BHQ1-3′). Cycle-threshold (C_T) values above the limit of detection for each run (determined by a standard curve) were considered negative [24]. All positive samples were used to determine the total percent positive for each group. Post-challenge viral load data was presented as relative viral load values made between the groups within an experiment and were not absolute virus genome copy numbers.

2.8. Clinical sign scoring

Clinical signs were scored based on a method described by Jackwood et al. [25]. Scoring was conducted on a scale from 0 to 3, where 0 = negative, 1 = mild signs, 2 = watery eyes and some mucus in the nares, and 3 = watery eyes, mucus in the nares, and tracheal rales.

2.9. Ciliostasis scoring

Ciliostasis scoring was conducted by examining five rings approximately 1 mm thick cut from each chicken trachea representing the proximal, middle and distal portion. Cilia activity was observed with an inverted microscope (Olympus, Center Valley, PA). Scoring was conducted based on the method by Cook et al. wherein: 0, all cilia beating; 1, 75% of cilia beating; 2, 50% of cilia beating; 3, 25% of cilia beating; 4, no cilia beating. Each ring was scored by 3 individuals independently. Mean scores per group were calculated and relationships between groups were analyzed statistically [26].

2.10. Challenge virus detection in embryonated eggs

Routine virus isolation techniques were used for detection of IBV challenge virus in 9-to-11 days of incubation embryonated SPF chicken eggs. Briefly, 2 ml of ice-cold PBS were added to the choanal swab fluid to match the stipulations of the U.S. Code of Federal Regulations, title IX (9-CFR) [27]. PBS from the swabs was filter sterilized and 0.2 ml of each sample were inoculated into the CAS of 6 embryonated chicken eggs. Eggs were candled daily (24–72 h deaths were discarded) for 7 days and the number of deaths and embryo lesions consistent with IBV infection was recorded.

2.11. Genome sequencing

Complete genome sequencing was performed on ArkGA P1, P20, P40, and P60 to detect changes occurring within the viral genome during attenuation. Virus stock was filtered with a 0.2 μm syringe filter. Viral RNA was extracted from samples using the Direct-Zol RNA MiniPrep Kit (Zymo Research) and treated with DNase I (New England Biolabs). The SISPA method was used for random amplification of RNA as previously described [28]. Complementary DNA (cDNA) was synthesized using SuperScript IV (Invitrogen/Thermo Scientific). Double stranded cDNA (dsDNA) was generated from cDNA templates using Second Strand cDNA Synthesis Kit (Applied Biological Materials Inc.). Complete genome sequencing at a 50× depth of coverage was conducted using the Nextera XT DNA Sample Preparation Kit (Illumina) and MiSeq sequencer (Illumina) according to manufacturer's instructions. De Novo and directed assembly of genome sequences was carried out using the MIRA3 sequence assembler and Geneious r8 program (www.geneious.com). Non-synonymous substitutions in the assembled sequence reads were compared to consensus sequence at 5% of minimum variant frequency using Geneious r8 program. Whole genome consensus sequences were entered into the GenBank database, with accession numbers as follows: Ark99 pathogenic field virus MH779860, ArkGA P1 MH779856, ArkGA P20 MH779857, ArkGA P40 MH779858, ArkGA P60 MH779859.

2.12. Sequence analysis of the S1 gene of viral RNA isolated from vaccinated chickens

For ArkGA P1, P20, P40, and P60 vaccination trials, viral RNA from 5 choanal cleft palate swabs each from days 7, 10, and 14 post-vaccination was purified and amplified for sequencing of the S1 region of the genome. Briefly, viral RNA was purified using the Zymo Direct-zol RNA miniprep kit (Zymo Research, Irvine CA). S1 gene sequences were amplified by RT-PCR using the Titan One-Step RT-PCR system (Roche Diagnostics, Indianapolis, IN) and previously published primers: NEWS1OLIGO5′ [29] and Degenerate3′ [30]. RT-PCR reactions were analyzed on a 1% w/v agarose gel and bands of the correct size were excised and DNA was purified from the gel fragment using the GeneJET Gel Extraction Kit (Thermo Scientific, Waltham, MA). Sanger sequencing was performed by the Georgia Genomics Facility, University of Georgia, Athens, GA. The S1 sequences were assembled and compared using the DNAStar suite of programs (DNAStar, Madison WI). The S1 amino acid sequences of viral RNA isolated from vaccinated chickens was compared to the consensus S1 sequences of the vaccine virus for P1, P20, P40, and P60 obtained from whole genome sequencing to detect changes in the viral population after replication in chicken tissues.

2.13. Statistical analysis

Statistical analysis was performed using Prism v.6.0. (GraphPad Software, Inc., La Jolla, CA). For experiments 1 and 2, post-vaccination viral load determined by qRT-PCR was compared between all vaccinated chickens within each collection time point via analysis of the mean and standard error of the mean (SEM). Post-challenge clinical signs, ciliostasis, and viral load between challenge groups were analyzed using an alpha of 0.05 with ordinary one-way ANOVA and Tukey's multiple comparisons test.

3. Results

3.1. ArkGA P1

3.1.1. ArkGA P1 whole genome sequence analysis and comparison with Ark99 pathogenic field virus

The reference sequence used for comparison of the ArkGA passages was the full genome of the Ark99 pathogenic field virus (unpublished sequence). In the ArkGA P1 vaccine consensus sequence, mutations occurred in polyprotein 1a, the spike gene, and in a non-coding region (Fig. 1). Only the mutation occurring in the spike gene resulted in an amino acid change (Table 1).

3.1.2. ArkGA P1 vaccination study

ArkGA P1 was titrated in embryos prior to vaccination and was determined to have an EID_{50} of $1x10^6$/ml. Appropriate dilutions were performed to achieve the desired vaccination dose of $1x10^{3.5}$ EID_{50} per bird, and this was confirmed by back-titration in embryonating eggs. Fig. 2 shows viral load and vaccine infection rate (coverage) post-vaccination with ArkGA P1. Viral load in chickens was high by day 7 post-vaccination, and remained constant until 14-days post-vaccination, when it began to decrease (Fig. 2A). ArkGA P1 vaccine candidate coverage was 100% by day 7 post-vaccination and remained constant throughout the course of the experiment (Fig. 2B). Clinical signs were also recorded at these time points and tracheal rales were observed in 60% of the chicks vaccinated with ArkGA P1 vaccine candidate at 10 days post-vaccination, which is consistent with previous reports for Ark99.

Titration of pathogenic Ark type challenge virus showed an EID_{50} of $1x10^7$/ml. Challenge virus was diluted to a dose of $1x10^{3.5}$ per bird prior to inoculation and confirmed by back-titration in embryos. Data collected at five days post-challenge is shown in Fig. 3. All groups showed significantly less clinical signs than the non-vaccinated and challenged group (Fig. 3A), and all groups had significantly reduced ciliostasis scores compared to the non-vaccinated and challenged group (Fig. 3B), as expected. Relative viral load was also significantly reduced in all groups when compared to the non-vaccinated and challenged group (Fig. 3C). Plotting the Ct values from individual samples taken from each group shows that 4/20 chickens in the ArkGA P1 vaccinated and challenged group were positive by qRT-PCR (Fig. 3D). Virus isolation was not performed for this trial.

3.1.3. Analysis of the ArkGA P1 S1 sequence isolated from vaccinated birds

Table 2 shows important amino acid positions that were noted to change in the S1 gene region during the ArkGA passages. In the ArkGA P1 S1 sequence, there was no difference in viral sequence between the vaccine and the virus isolated from choanal cleft palate swabs of vaccinated chickens.

3.2. ArkGA P20

3.2.1. ArkGA P20 whole genome sequence analysis and comparison with previous ArkGA passages

Between P1 and P20, all 3 mutations that occurred between the Ark99 pathogenic field virus and ArkGA P1 were lost, and a new mutation was gained in a non-coding region of the P20 consensus sequence (Table 1, Fig. 1).

3.2.2. ArkGA P20 vaccination study

ArkGA P20 was titrated and shown to have an EID_{50} of $1 \times 10^{6.5}$/ml. As seen in ArkGA P1, for chicks vaccinated with ArkGA P20, viral load and vaccine coverage were typically high early post-vaccination (Fig. 4). However, thirty percent of chickens vaccinated with ArkGA P20 showed severe clinical signs (rales) on day 10 post-vaccination, which is reduced from the previous trial but still much higher than what would be accepted by the commercial poultry industry. Because of the excessive clinical signs seen post-vaccination, the ArkGA P20 vaccination trial was ended prior to challenge, and back-titration of the diluted vaccine was not performed.

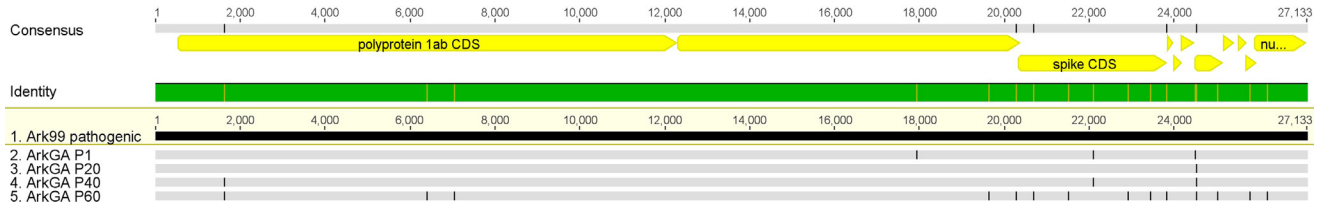

Fig. 1. Consensus sequences of the ArkGA IBV vaccine candidates in comparison to Ark99 pathogenic field reference strain.

Table 1
Consensus sequence SNPs compared to pathogenic Ark99 occuring with passage of ArkGA in embryonated eggs.

Passage	Nucleotide Position	Gene	Change	Codon Change	Protein Effect	Amino Acid Change
P1	17,939	Polyprotein 1ab	C → T	TTC → TTT	None	
	22,094	Spike	A → G	GAA → GGA	Substitution	E → G
	24,493	Non-coding	G → A		None	
P20	24,512	Non-coding	C → T		None	
P40	1,619	Polyprotein 1ab	A → C	GAG → GCG	Substitution	E → A
	22,081	Spike	C → T	CTT → TTT	Substitution	L → F
	24,512	Non-coding	C → T		None	
	27,567	Non-coding	C → T		None	
P60	1,619	Polyprotein 1ab	A → C	GAG → GCG	Substitution	E → A
	6,402	Polyprotein 1ab	C → T		None	
	7,040	Polyprotein 1ab	A → C	GAT → GCT	Substitution	D → A
	19,626	Polyprotein 1ab	C → T	CTT → TTT	Substitution	L → F
	20,269	Polyprotein 1ab	A → C	AAA → ACA	Substitution	K → T
	20,681	Spike	G → A	AGC → AAC	Substitution	S → N
	21,485	Spike	G → A	CGT → CAT	Substitution	R → H
	22,894	Spike	G → A	GTG → ATG	Substitution	V → M
	23,443	Spike	G → A	GAG → AAG	Substitution	E → K
	23,809	Spike	G → T	GAA → TAA	Truncation	E → Stop codon
	24,512	Non-coding	C → T		None	
	25,025	Membrane	G → A	CGT → CAT	Substitution	R → H
	25,766	5b	A → G	ATT → GTT	Substitution	I → V
	26,191	Nucleocapsid	G → C	GGA → CGA	Substitution	G → R
	27,567	Non-coding	C → T		None	

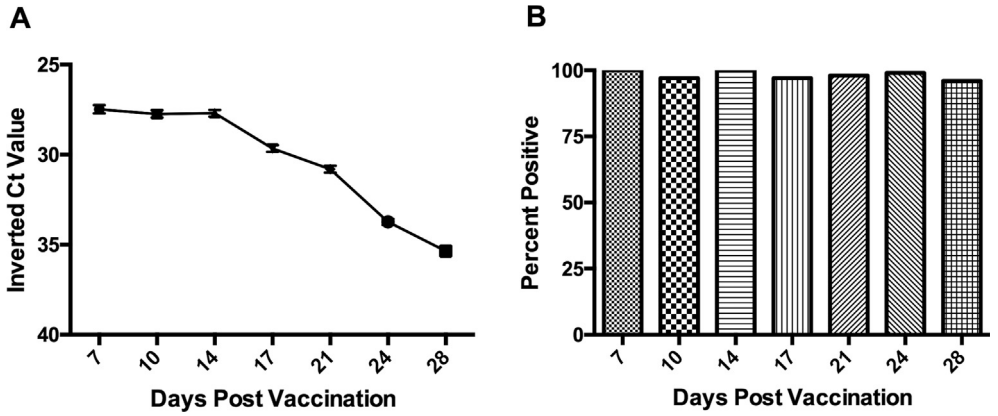

Fig. 2. Experiment 1. Viral load in chickens (A) and vaccine coverage (B) after spray vaccination with ArkGA P1 vaccine candidate. Ct = cycle threshold. Mean and SEM were determined for the Ct values within each time point post-vaccination. At each time point, 97 chickens were swabbed to obtain viral load (3 chickens died prior to day 7 due to natural causes).

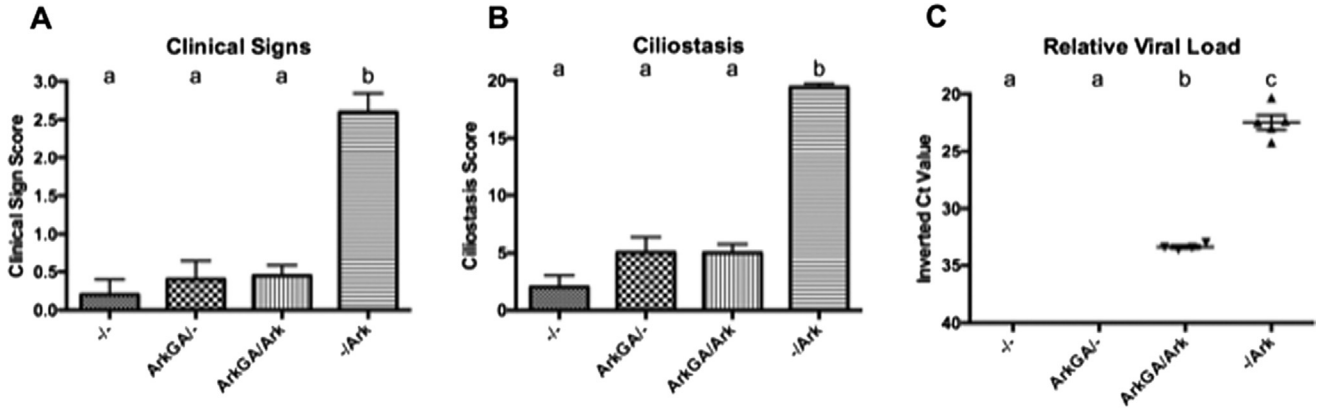

Fig. 3. Experiment 1. Clinical signs, ciliostasis scores, and viral loads in chickens post-challenge. Clinical sign scores were calculated based on severity where 0 = negative, 1 = mild signs, 2 = watery eyes and some mucus in the nares, and 3 = watery eyes, mucus in the nares and trachea (tracheal rales). Ct = cycle threshold. Clinical sign scores, ciliostasis scores, and relative viral load were compared between challenge groups using ordinary one-way ANOVA ($\alpha = 0.05$) with Tukey's multiple comparisons test. The -/-, ArkGA/-, and -/Ark groups each contained 5 chickens. The ArkGA/Ark group contained 20 chickens.

Table 2
S1 amino acid sequence comparison of ArkGA vaccine virus and viral RNA isolated from 5 choanal cleft palate swabs on days 7, 10, and 14 post-vaccination. Included are mutations recorded for amino acid positions in the S1 sequence that were maintained to ArkGA P60.

ArkGA Passage	S1 Amino Acid Position	Vaccine Amino Acid	Reisolated Vaccine Amino Acid and Frequency of Change	
P1	117	S	S	–
	198	K	K	–
	200	G	G	–
	385	R	R	–
P20	117	S	S	–
	198	K	T	20%
	200	G	D	7%
	385	R	R	–
P40	117	S	S	–
	198	K	T	67%
	200	G	D	60%
	385	R	R	–
P60	117	N	S	100%
	198	K	T	100%
	200	G	D	100%
	385	H	R	100%

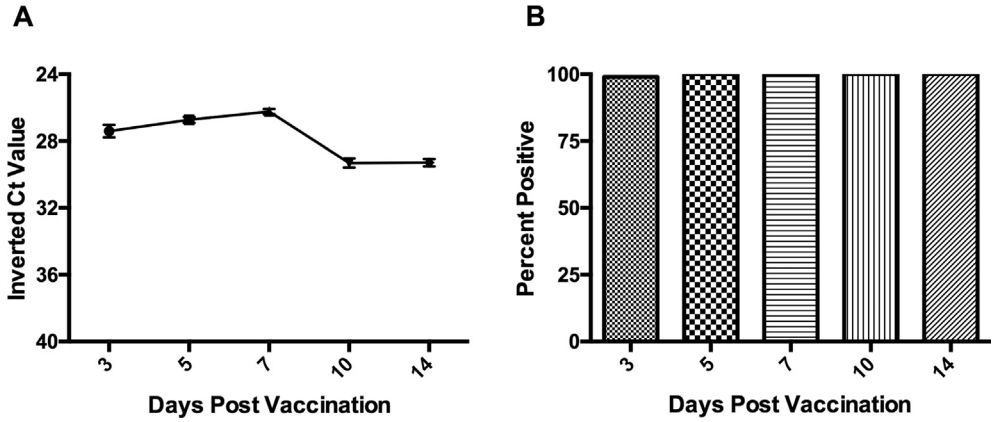

Fig. 4. Experiment 2. Trial 1. Viral loads in chickens (A) and vaccine coverage (B) post-vaccination with ArkGA P20. Ct = cycle threshold. Mean and SEM were determined for the Ct values within each time point post-vaccination. At each time point all 100 vaccinated chickens were swabbed.

3.2.3. Analysis of the ArkGA P20 S1 sequence isolated from vaccinated birds

Sanger sequencing of the S1 subunit of the spike gene in post-vaccination swabs showed two amino acid positions that changed between the vaccine and the swab viral material (Table 2). At amino acid position 198 where there was a lysine present in the vaccine, 20% of the swabs showed a threonine. At position 200, the vaccine had a glycine, but 7% of the swabs sequenced showed a change to aspartic acid.

3.3. ArkGA P40

3.3.1. ArkGA P40 whole genome sequence analysis and comparison with previous ArkGA passages

In ArkGA P40, the consensus whole genome sequence showed 4 mutations compared to the reference Ark99 field virus sequence. A change in the nonstructural protein 2 (nsp2) region of polyprotein 1a resulted in an amino acid substitution. A substitution also occurred in the S2 region of the spike gene. Two other SNPs were noted in non-coding regions; one of which was at nucleotide position 24,512 that was maintained from P20 (Table 1, Fig. 1).

3.3.2. ArkGA P40 vaccination study

Prior to vaccination, ArkGA P40 was titrated and determined to have an EID_{50} of 1×10^8/ml. In chicks vaccinated with ArkGA P40, viral load and vaccine coverage were lower at days 3 and 5 post-vaccination than in the P20 trial but peaked by day 7 post-vaccination (Fig. 5). Clinical signs were reduced to 10% of chicks with tracheal rales at 10 days post-vaccination, which was less than with P20, but was still considered too pathogenic for a commercial poultry vaccine. For this reason, the ArkGA P40 vaccination trial was terminated at this point, and back-titration of the diluted vaccine was not performed.

3.3.3. Analysis of the ArkGA P40 S1 sequence isolated from vaccinated birds

In ArkGA P40, the S1 sequence comparison between the vaccine virus and viral RNA from swabs of vaccinated birds showed changes in the same two amino acid positions, 198 and 200, that were seen in P20. In P40 the frequency of change was increased to 67% in position 198 and 60% in position 200 (Table 2).

3.4. ArkGA P60

3.4.1. ArkGA P60 whole genome sequence analysis and comparison with previous ArkGA passages

Numerous SNPs were seen in the ArkGA P60 consensus sequence compared to the Ark99 pathogenic field virus. Five SNPs occurred in polyprotein 1ab, 4 of which resulted in amino acid substitutions. The nsp2 SNP from P40 was maintained, and two polyprotein 1ab SNPs occurred in the nonstructural protein 3 (nsp3) region. In P60, SNPs were seen in S1 as well as S2 of the

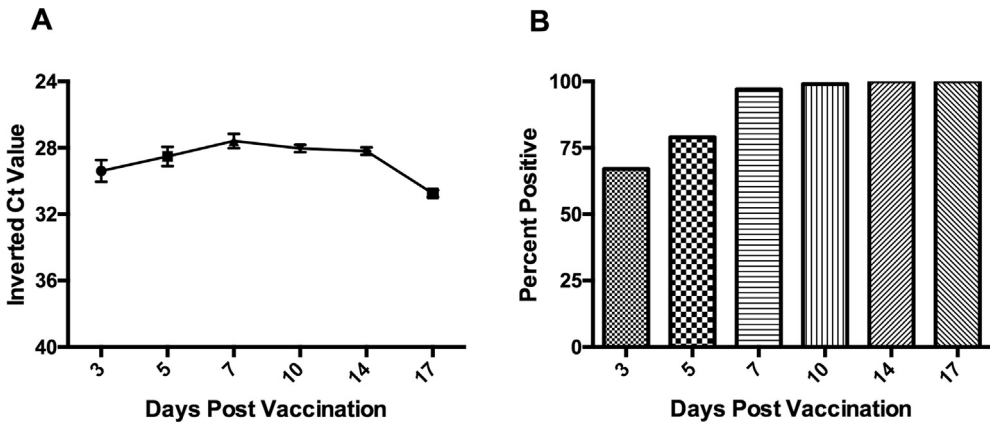

Fig. 5. Experiment 2. Trial 2. Viral loads in chickens (A) and vaccine coverage (B) post-vaccination with ArkGA P40. Ct = cycle threshold. Mean and SEM were determined for the Ct values within each time point post-vaccination. At each time point, 99 chickens were swabbed to obtain viral load (1 chicken died prior to day 3 due to natural causes).

spike gene, although the S2 SNP seen in P40 was not detected. Of note, a SNP was detected at nucleotide position 23,809 in S2 that generated a stop codon, resulting in truncation of S2 by 8 amino acids. Additional SNPs were detected towards the end of the genome, including in the membrane, 5b, and nucleocapsid proteins (Table 1, Fig. 1).

3.4.2. ArkGA P60 vaccination study

ArkGA P60 was diluted from a determined EID_{50} of 1×10^7/ml to a vaccination dose of $1 \times 10^{3.1}$ EID_{50} per bird, as shown by back-titration in embryos. ArkGA P60 viral load and vaccine coverage are shown in Fig. 6. Viral load in chicks was high soon after vaccination, though coverage was lower than expected on days 3 and 5 post-vaccination. By 7 days post-vaccination, coverage had reach 93% and peaked at 100% on day 14 post-vaccination. By 21 days post-vaccination, chickens began to clear the vaccine virus, indicated by reduced viral load and coverage (Fig. 6). Only 3% of chicks vaccinated with ArkGA P60 showed clinical signs (snicks), which was deemed acceptable for an IBV vaccine.

Back-titration of diluted Ark type challenge virus indicated a dose of $1 \times 10^{3.4}$ EID_{50} per bird. Data from five-days post-challenge with pathogenic Ark virus are presented in Fig. 7. All groups showed significantly reduced clinical signs (Fig. 7A), ciliostasis scores (Fig. 7B) and viral loads (Fig. 7C) compared to the non-vaccinated/challenged group.

When analyzing the individual viral load values, 5/20 of the vaccinated and challenged birds were positive by qRT-PCR. It should also be noted that the ArkGA P60 vaccinated/non-challenged group had 2/5 chickens positive for virus (Fig. 7D).

Virus isolation post challenge was consistent with the results found by qRT-PCR (Table 3). All of the non-vaccinated and non-challenged group swabs were negative for virus isolation. In the vaccinated/non-challenged group, one of the swabs was found to be positive with an embryo death at 120-h post-inoculation. All 5 of the other embryos in this set died by 72-h post-inoculation however, indicating a possible bacterial contamination in that sample. In the vaccinated/challenged group, 3/19 of the swabs were found to be positive for Ark-type challenge virus. All embryos in the 20th swab sample died at 48 h post-inoculation, so that sample could not be analyzed. All 5 of the non-vaccinated/challenged bird swabs were positive for IBV.

To ensure that virus isolation positives in challenged groups were indeed challenge virus and not residual vaccine, the spike gene of samples from both challenged groups was sequenced. In all instances, sequence matched the Arkansas challenge virus, indicating it was not residual vaccine. In the vaccinated/non-challenged group, no sequence could be obtained from qRT-PCR positive samples.

3.4.3. Analysis of the ArkGA P60 S1 sequence isolated from vaccinated birds

When comparing the S1 amino acid sequence between the ArkGA P60 vaccine and the virus re-isolated from vaccinated birds, multiple changes were seen. The two amino acid changes at positions 198 and 200 seen in P20 and P40 were 100% predominant in

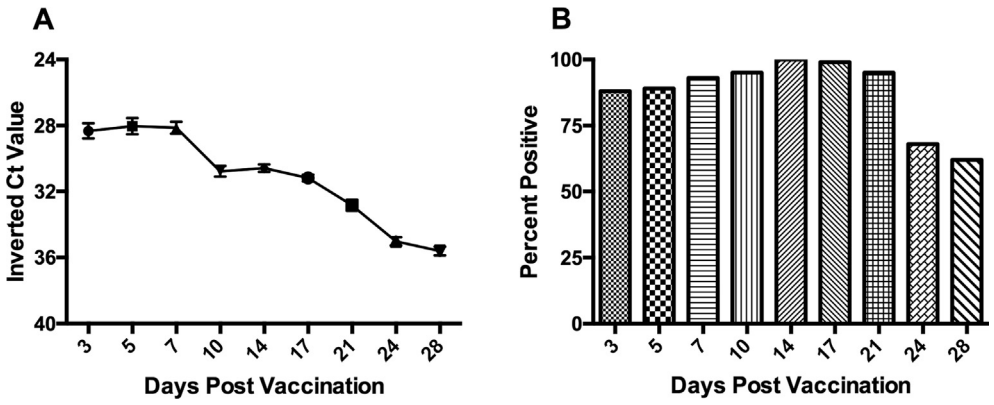

Fig. 6. Experiment 2. Trial 3. Viral loads in chickens and vaccine coverage post-vaccination with ArkGA P60. Ct = cycle threshold. Mean and SEM were determined for the Ct values within each time point post-vaccination. At each time point all 100 vaccinated chickens were swabbed.

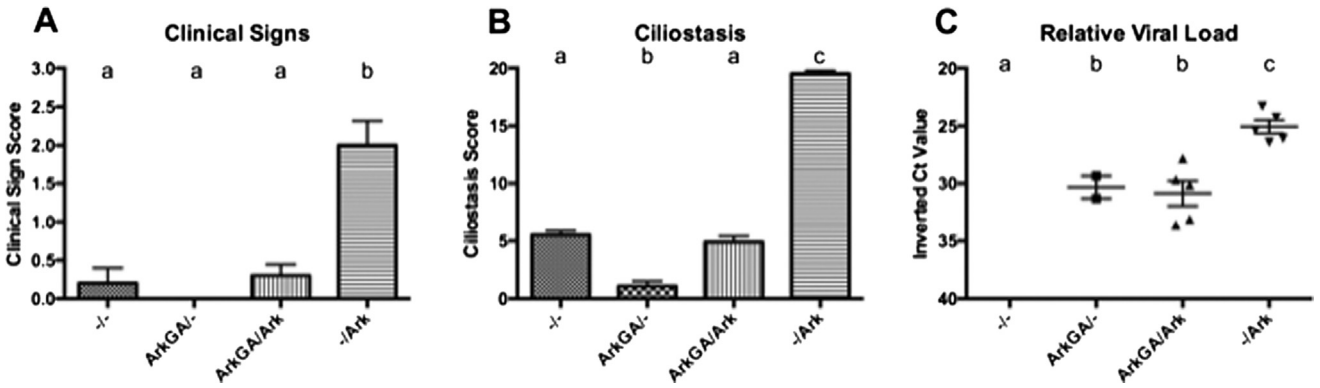

Fig. 7. Experiment 2. Trial 3. ArkGA P60 vaccinated and non-vaccinated clinical signs, ciliostasis scores, and viral loads in chickens post-challenge. Clinical sign scores were calculated based on severity where 0 = negative, 1 = mild signs, 2 = watery eyes and some mucus in the nares, and 3 = watery eyes, mucus in the nares and trachea (tracheal rales). Ct = cycle threshold. Clinical sign scores, ciliostasis scores, and relative viral load were compared between challenge groups using ordinary one-way ANOVA ($\alpha = 0.05$) with Tukey's multiple comparisons test. The -/-, ArkGA/-, and -/Ark groups each contained 5 chickens. The ArkGA/Ark group contained 20 chickens.

Table 3
Experiment 2. Trial 3. Pathogenic Ark-type challenge virus detection in embryonated eggs. Data are represented as the number embryos positive per total for classic IBV signs 7 days post inoculation.

Group	Chicken	ArkDPI
Non-Vaccinated/Non-Challenged	1	0/6[a]
	2	0/6
	3	0/6
	4	0/6
	5	0/6
Vaccinated/Non-Challenged	6	0/5
	7	0/6
	8	0/6
	9	0/6
	10	1/1
Vaccinated/Challenged	11	0/6
	12	0/4
	13	0/5
	14	0/6
	15	0/6
	16	0/6
	17	0/4
	18	0/6
	19	0/4
	20	0/5
	21	0/4
	22	1/6
	23	0/6
	24	0/3
	25	3/6
	26	0/5
	27	1/6
	28	0/3
	29	0/6
	30	0/6*
Non-Vaccinated/Challenged	31	3/6
	32	1/5
	33	1/6
	34	3/6
	35	5/5

[a] Number of embryos positive per total for classic IBV signs 7 days after inoculation.
* All 6 embryos died 48 h post-inoculation, presumably from bacterial contamination. Embryos did not show lesions of IBV when examined post-death.

re-isolated virus in P60. In addition, at amino acid positions 117 and 385, the P60 vaccine virus population had two substitutions occur compared to prior vaccine passages. The amino acids in virus isolated from swab material showed a reversion to previous vaccine sequences at these positions (Table 2).

4. Discussion

In this study, serial passage of the ArkGA vaccine virus in embryonated eggs resulted in numerous changes to the viral genome. Over 60 passages, the ArkGA virus accumulated 15 SNPs, 10 of which were located in the replicase and spike genes, which have been attributed to IBV attenuation [31]. Most notably, ArkGA P60 had SNPs in the nsp2, nsp3, and both S1 and S2 gene regions. Changes in nsp2 and nsp3, which are part of the viral replicase complex, have been shown to impact viral replication and pathogenesis [32–38]. In the spike gene, which is the major determinant of cell tropism and plays a role in viral attenuation [39,40], 5 amino acid changes occurred in the S1 and S2 subunits. One mutation in S2 resulted in truncation of spike by 8 amino acids, likely shortening the cytoplasmic domain. Changes in the cytoplasmic tail of IBV and murine hepatitis virus (MHV) have been shown to affect endocytosis signaling and regulation of the levels of spike at the surface of infected cells and may reduce infectivity [41,42]. In severe acute respiratory syndrome coronavirus (SARS-CoV), a truncation of 8 amino acids from the carboxyl terminus of S resulted in reductions in cell fusion and cell surface expression and faster endocytosis compared to wild type [43]. As this truncation was only seen in ArkGA P60 and not in previous passages, further investigation is needed to fully understand the ramifications of this truncation. Additionally, a mutation was detected in the nucleocapsid gene, which may be related to reduced replication efficiency [36].

The genomic changes seen in ArkGA over serial embryo passage can be correlated to the changes in performance dynamics seen in vaccinated chickens. During passage of ArkGA, the virus became more attenuated, showing reduced reactivity in vaccinated broiler chickens. Different passages of the ArkGA vaccine were evaluated for infection, replication, vaccine reaction, and efficacy in broiler chicks. Experimental vaccine and challenge trials showed that the ArkGA P1 vaccine had suitable infection and replication, and induced adequate protection from challenge, but was too pathogenic, causing a severe vaccine reaction in the majority of chicks. Further passages in embryonated eggs reduced the severity of the vaccine reaction to 30% for P20, 10% for P40, and 3% for P60. This further attenuation did not adversely affect infection or replication characteristics of the vaccine, as the relative viral load in chicks did not change throughout the trials.

ArkGA P60 vaccine coverage was slightly less than expected shortly after vaccination but reached 100% by day 14. This may be attributed to the S1 amino acid changes in position 117 and 385 seen between the vaccine and swab sequences in P60, as the S1 sequences re-isolated from chickens had reverted to the more

pathogenic P1 sequence. In the ArkGA P1 vaccine trial a higher vaccine coverage was seen, indicating that these amino acid positions may have an impact on rate of infection. It is also possible that the changes in the replicase gene and the S2 mutations impacted the performance of ArkGA P60.

Infection and replication patterns in all trials were predictable and "typical" of what would be expected of an IBV vaccine. This stands in contrast to the infection and replication cycles of the ArkDPI vaccine, which shows very low vaccine coverage and multiple replication cycles during the life of the bird following spray vaccination [13]. One explanation for this is in the S1 portion of the spike gene. The ArkGA P1 spike sequence contains a histidine at position 43, which has been previously shown to significantly increase spike protein binding to chicken tracheal tissues, and an asparagine deletion at position 344, which has been shown to influence the ability of antibodies to recognize the protein. Conversely, the ArkDPI S1 contains a tyrosine at position 43 and an asparagine at position 344, which may be attributed to its reduced ability to bind and replicate in chicken tissues [16]. The presence of a minor virus subpopulation capable of inducing a protective immune response in ArkDPI results in a very low dose of protective vaccine virus when mass vaccinating chickens and consequently poor protection [12]. Although ArkGA was found to have subpopulations, as evidenced by the SNPs observed within each passage, which is quite typical for IBV vaccines [15], those subpopulations were able to produce a protective immune response when mass applied.

The ArkGA vaccine at P1 and P60 was effective at protecting chickens from a pathogenic Ark IBV challenge. Clinical signs and viral loads post-challenge were significantly lower than non-vaccinated and challenged groups, and all vaccinated birds passed the ciliostasis test. Again, this stands in contrast to previous ArkDPI vaccine and challenge experiments that showed that chickens were clearly not protected from challenge after ArkDPI vaccination by spray [12].

The attenuated ArkGA vaccine described herein is a significant improvement over the current commercially available ArkDPI vaccine when comparing infection and replication following spray application and induction of protective immunity following homologous challenge. The ArkGA (P60) is also genetically distinct, making it possible to distinguish the ArkGA vaccine from the ArkDPI vaccine or pathogenic viruses. Further molecular investigation is needed to fully evaluate the amino acid changes seen in pass 60, but these changes do not seem to impact the effectiveness of the vaccine. In conclusion, the ArkGA vaccine developed herein is safe when given to 1-day old broilers by spray, and it induces an efficacious immune response against homologous challenge.

5. Declarations

5.1. Competing interests statement

The authors declare that there were no conflicts of interest.

6. Animal care

All experiments in this research were conducted in accordance with animal care and use protocols approved by the University of Georgia IACUC committee.

7. Author contributions

Grace A. Albanese participated in the research and article preparation. Dong-Hun Lee performed the complete genome sequencing and de novo and directed assembly of genome sequences and revised the article. I-Hsin N. Cheng and Deborah A. Hilt participated in the research. Mark W. Jackwood and Brian J. Jordan participated in the study design and in the research and article revision. All authors have approved the final article.

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