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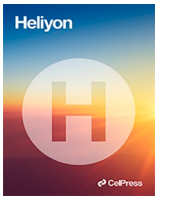

Genetic modification regulates pathogenicity of a fowl adenovirus 4 strain after cell line adaptation (genetic mutation in FAdV-4 lowered pathogenicity)

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ABSTRACT

Fowl adenovirus 4 (FAdV-4) is a major avian virus that induces fatal diseases in chicken such as, hydropericardium and hepatitis. The viral structure consists of hexon, penton, fiber-1, and fiber-2 which are associated with immunopathogenesis. In this study, we investigated the genetic modification of a FAdV-4 strain after continuous passages in a cell line and evaluated the pathogenicity associated with mutations.

We used the FadV-4 KNU14061 strain, which was isolated from layers in 2014. The virus went through 80 passages in the Leghorn male hepatoma (LMH) cell line. The full genetic sequence was identified, and we found a frameshift in the fiber-2 amino acid sequence after the initial thirty passages. To examine whether the frameshift in the fiber-2 gene affects the pathogenicity in chicken, we inoculated LMH80 (80 times passaged) and LMH10 (10 times passaged) into 3-day-old chickens and examined the pathogenesis. LMH10 infection via intramuscular route induced fatal pathology, but LMH80 did not. Furthermore, LHM80 pre-treatment protected hosts from the LMH10 challenge. Thus, the genetic modification isolated by serial passage lowered pathogenicity and the resulting virus acted as an attenuated vaccine that can be a FAdV-4 vaccine strain candidate.

1. Introduction

Fowl adenovirus (FAdV) belongs to the Aviadenovirus genus in Adenoviridae family. It is classified into five species (FAdV-A, B, C, D, and E) based on the DNA restriction enzyme fragmentation pattern, and the virus can be divided into 12 serotypes (FAdV 1–12) by serum cross-reaction [1–3]. Numerous FAdV strains have been isolated from poultry and wild birds, most of which are nonvirulent. However, some of them cause pathology in chickens, including inclusion body hepatitis (IBH), hepatitis-hydropericardium syndrome (HHS) and gizzard erosion (GE) [4,5].

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The FAdV-4 belongs to the FAdV-C species that developed hydropericardium symdrome (HPS), first reported in Pakistan, and since then outbreaks have been reported worldwide, including Middle east, Central and South America, Russia, Austria, India, Japan, and Korea [6–13]. The primary pathogenic lesions are clear or have straw-colored fluid in the pericardiac sac with an enlarged and discolored liver, hepatic hemorrhage, and necrosis. The mortality rate is between 20 and 80% in 4∼8 days post-infection and co-infection, with infectious bursal disease virus (IBDV) or chicken infectious anemia virus (CIAV) exacerbates the pathogenicity and mortality [10,11,14–19].

The FAdV-4 is a linear double-stranded DNA virus with a genome size of 43 kb–46 kb. The genome has genus-common genes in the relatively conserved central region, and different genes exist depending on the genus and species on both flaking sides. An inverted terminal repeat with complementary base sequences is located at both ends of the genome [20,21]. FAdV-4 has a nonenveloped-icosahedral structure consisting of 240 non-vertex capsomers, made of hexon, and 12 vertex capsomers made of penton base. The hexon is the major antigen that elicits host humoral and cellular immune responses [22,23]. The penton base is linked to two types of fiber proteins that react with host receptors and invade into host cells [8]. The fiber-1 protein attaches to a coxsackie and adenovirus receptor (CAR) in chicken. In addition, fiber-1 is the main structural protein attached to the leghorn male hepatoma (LMH) cells [24]. The fiber-2 has not been clearly associated to specific receptors, but it is assumed to be related to tissue affinity, interacting with host cells [20,25–27].

Recent studies have focused on major structural proteins for their pathogenetic activity during FAdV-4 infection. Entire genome sequences of nonvirulent and virulent FAdV-4 strains were analyzed, and it was found that hexon and fiber-2 proteins are related to pathogenicity while penton and fiber-1 participate in viral replication only [8,20,25,28–31]. Therefore, previous studies compared amino acid sequences between hexon and fiber-2 to examine pathogenicity. By comparing FAdV-4 with other adenoviruses, it is thought that the L1 loop domain and knob parts, which are highly mutated parts of hexon and fiber-2, encode amino acid sequences with an impact on pathogenicity. In addition, a nonstructural protein (up to 100 kDa), contributes to the translation of late mRNA, also changing the pathogenicity [25,30,32].

As virulent FAdV-4 strains emerged, efforts to develop vaccines have been made. The first inactivated vaccine was generated from the heat-inactivated liver suspension of infected animals, and then a formalin-treated form was developed [9,33]. However, improper viral inactivation prompted unwanted pathogenicity, and attenuated vaccines were subsequently generated that ameliorated viral pathogenicity via passages in cell lines or chicken embryos [34,35]. After fiber-2 was identified as a major factor for pathogenicity, studies on fiber-2-recombinant vaccines were reported [36–42]. However, issues regarding the vaccine efficacy and identification of antigen-specific antibodies were raised. In addition, the routes of vaccination that were shown to work seem to be limited to intravascular injections.

This study examined genetic modifications after consecutive passages of a virulent FAdV-4 strain in the LMH cell line. We focused on the analysis of a frameshift of amino acids in fiber-2 protein via complete genetic analysis. In addition, we showed that injection of the passaged virus did not induce pathology, and pre-treatment with this virus protected chickens from the fatal FAdV-4 challenge. These findings suggest a broaden understanding in genetic modification of FAdV-4, which regulates immunogenicity in the host.

2. Material and methods

2.1. Virus

FAdV-4 was isolated from 45-week-old layers infected with FAdV-4-induced hepatitis-hydropericardium syndrome (HHS) in commercial chicken flock (Yeoju-city, Gyeonggi province, Korea). The LMH cell line propagated the FAdV-4 strain. First, the LMH cells were cultured in Waymouth's medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and 1% antibiotic-antimycotic (Gibco, USA). Then, when the cells formed a monolayer in a T-25 flask (SPL, Korea), culture media was converted into a maintenance media supplemented with 1% antibiotic-antimycotic, and the viral media was inoculated at a ratio of 1:10 to the maintenance media. After 3–4 days, the process of freezing and thawing in the −70 °C freezer was repeated twice, and the supernatant was diluted to 1: 1,000–1: 10,000 and cultured in a T-25 flask.

2.2. Nucleic acid analysis

To investigate the genetic mutations of passaged virus, 3rd (LMH3), 10th (LMH10), and 80th (LMH80) passaged viruses were sent to GnCBio (Korea) for Next Generation Sequencing (NGS) analysis. The entire genome sequences were compared with other FAdV strains (Supplementary Data 1). Whole genome sequences of LMH10 and LMH80 are provided with GenBank accession numbers as follows; OR352897 for LMH10 and OR352898 for LMH80.

The nucleotide sequences were aligned via the Clustal W method using the DNAstar Lasergene MegAlign Pro software (DNAstar Inc., USA). To identify the timing of the fiber-2 amino acid mutation, fiber-2 nucleotides of FAdV-4 from different passages (10, 20, 30, 40, 50, and 60 times) were examined. The primer pair (Forward: 5′-GACCGCTACACCCTTCTATGCTAC-3′, Reverse: 5′-GGCGCTCCCCTGTCACTACT-3′) was used for polymerase chain reaction (PCR). The PCR was conducted with the following steps: predenaturation at 95 °C for 5 min, 35 cycles of 94 °C for 90 s, 45 °C for 30 s, and 72 °C for 90 s, and a post-polymerization step at 72 °C for 3 min. Finally, the amplified PCR product was sent to CosmoGenetech (Korea) for sequencing analysis. Using the Clustal W method and the DNASTAR 2.0 MegAlign program, mutations of the open reading frame (ORF) and the main structural proteins (hexon, penton base, fiber-1, and fiber-2) were identified.

2.3. PCR and viral titration

The viral gene was extracted using Maxwell RSC Viral Total Nucleic Acid Purification Kit (Promega). First, the FAdV-4 gene was amplified by Hexon A and Hexon B primers as described in a previous study [43]. Next, the virus was inoculated on a 96-well plate formed with monolayered-LMH cells for viral titration. And then, the medium was removed and replaced with a 10-fold serially diluted viral suspension in Waymouth's medium. After three days, cultured cells were frozen and thawed twice, viral DNA was extracted from the supernatant, and PCR was conducted. Viral DNA was detected until 10^{-6} dilution for LMH10 and 10^{-5} dilution for LMH80.

2.4. Evaluation of pathogenicity

Seventy-five 3-day-old SPF chicks (Namdeok ESPF, Korea) were divided in three groups of 15 or 45 animals each. Group 1 (15 animals) and, Group 2 (15 animals) were injected intramuscularly with LMH80. The same amount of LMH80 virus was injected in Group 3 (45 animals) by oral gavage, nasal spray, or eye drop. The virus was inoculated at 10^5 $TCID_{50}$ in each animal. Fecal samples were collected on the 3rd and 5th days after inoculation. At three days post-injection, three chicks per group were euthanized.

After serum collection at seven days post-injection of LMH80, LMH10 was intramuscularly injected at a concentration of 1×10^5 $TCID_{50}$. 100 μl of virus containing PBS was given in each animal. The fecal samples were collected at days three and five post-challenge. At three days after infection, three chickens per group were euthanized. The survival rate was observed up to day nine post-challenge. The liver and kidney tissues were homogenized with PBS and centrifuged at 3,000 rpm for 10 min, and PCR was conducted with the supernatant for viral detection.

Serum and feces were obtained from all surviving individuals on day nine post-challenge. The feces were examined for virus detection by PCR, and antibodies to FAdV-4 were examined in serum by the Fowl Adenovirus Group 1 Antibody test kit (Biochek, Netherlands) following the manufacturer's protocol. The animal experiment was approved by the Institutional Animal Care and Use Committee of Kangwon National University (No. KW-210401-1).

2.5. Statistical analysis

Statistical significance was analyzed by GraphPad Prism (version 9.0.0 for Windows). The survival rate was analyzed by Log-rank test and antibody titer by one-way ANOVA or Kruskal-Wallis test. The post hoc comparison was made with Dunn's multiple comparison test. P values < 0.05 were considered significant.

3. Results

3.1. FAdV-4 passages

FAdV-4 KNU14016 was passaged up to 100 times in LMH cells. The viral proliferation was confirmed in each passage using PCR, and the cultured media was stored at −70 °C. Cytopathic effect (CPE), such as spherization and swelling was observed three days after viral inoculation with same titer (Fig. 1A and C). However, after the 20th passage, the CPE was milder, and the CPE formation was delayed to four days post-inoculation (Fig. 1B) and no CPE was observed at 100th passage (data not shown).

3.2. Whole-genome analysis of passaged virus

Whole genome sequences of LMH3, LMH10, and LMH80 were examined. The entire genome of FAdV-4 KNU14061 (LMH3) has 45,669 bp and the G + C ratio is 54.71%. Nineteen ORFs found in the center, and 18 genus-common genes were identified. Ten ORFs were found on the left side and 14 ORFs on the right side (Table 1).

The LMH10 gene sequence was identical to LMH3 except for the insertion of a C-base at 39,197 bp, which is in the non-coding region. Comparing LMH10 to LMH80, besides the C-base insertion, genetic modifications were also observed at 24 different sites. The whole sequence of LMH80 was shortened by 26 bp, resulting in a total of 45,644 bp. Among the genes for IVa2, DNA polymerase,

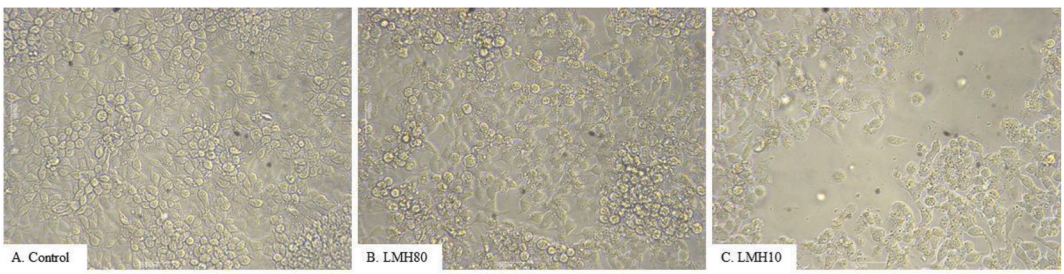

Fig. 1. Cytopathic effect in each LMH cell subculture of KNU14061 at 3 days post-infection. LMH cells had bipolar and elongated shapes (A). Cytoplasm was swollen (B). Cells had lost the specific shapes and were detached from bottom (C).

Table 1
ORF information of FAdV-4 (LMH3).

ORF	left	right	Strand	Length(bp)	ORF	left	right	Strand	Length(bp)	ORF	left	right	Strand	Length(bp)
ORF0	478	717	+	240	**Penton**	16,979	18,556	+	1578	ORF22	33,190	33,777	–	588
DUTP	790	1314	+	525	pVII	18,565	18,795	+	231	ORF20A	33,780	34,046	–	267
ORF1B	1485	1808	+	324	pX	18,967	19,506	+	540	ORF20	34,197	35,084	–	888
ORF2	1850	2665	+	816	pVI	19,600	20,283	+	684	lip 3	35,327	35,890	–	564
ORF24	2667	3203	–	537	**Hexon**	20,329	23,139	+	2811	lip 1	35,922	37,010	–	1089
ORF14A	3315	3902	–	588	Protease	23,157	23,786	+	630	ORF27	37,009	37,290	+	282
ORF14	3996	4682	–	687	DBP	23,909	25,390	–	1482	ORF43	37,712	38,377	+	666
ORF14B	4666	5295	–	630	11.7 kDa	25,429	25,737	–	309	ORF28	38,731	39,024	+	294
ORF13	5329	6129	–	801	100 kDa	25,769	28,933	+	3165	GAM-1	39,270	40,181	+	912
ORF12	6206	7075	–	870	33 kDa	28,551	29,138	+	588	ORF49	40,241	40,399	+	159
IVa2	7089	8273	–	1185	pVIII	29,453	30,196	+	744	ORF17	40,699	41,175	–	477
DNA polymerase	8257	12,015	–	3759	U exon	30,141	30,428	–	288	ORF16	41,168	41,578	–	411
pTP	12,020	13,828	–	1809	**Fiber-1**	30,427	31,731	+	1305	ORF19A	42,156	44,681	+	2526
52 kDa	13,958	15,148	+	1191	**Fiber-2**	31,715	33,139	+	1425	ORF4	44,775	45,272	+	498
pIIIa	15,135	16,907	+	1773										

pTP, pIIIa, penton, pVII, protease, DBP, and ORF19A, 15-point mutations were found. Although these 15 mutations changed amino acid sequences, no non-sense mutation was found. In LMH80, insertions and deletions were observed at 10 locations compared with LMH3. For example, two insertions were observed at the nucleotide sequences that encode 52 kDa and 100 kDa, but there was no frameshift. Two, four, and one bases were inserted among the non-coding genes, respectively. As a result, LMH80 has 129 nucleotides (from 45,425 bp to 45,553 bp) at the right terminal region, while LMH10 has 180 nucleotides (from 45,410 bp to 45,580 bp). Among those 129 nucleotides, the 53 nucleotides from 5′ side were identified as part of the inverted repeat region, and the 76 nucleotides from 3′ side were part of the ORF19A sequence. In the fiber-2 gene of LMH80, 'CCCGT' was inserted, which changed the terminal codon (TAA) into 'CCCGTAA', extending the fiber-2 protein by 14 amino acids (Fig. 2).

To verify the modification of the fiber-2 protein, the mutated region was amplified by PCR, and its sequence was analyzed. The original nucleotide sequence was maintained up to 20 passages (LMH20), but the intermittent mutation had appeared from by passage 30 (LMH30). After 40 passages, the frequency of the mutation was increased, and it became fully established from LMH50. The back mutation was not observed till LMH80 (Fig. 3).

3.3. The modification of amino acids in ORFs and main structural proteins by serial passages

Mutations in amino acids can affect the original function of proteins. Point mutations and insertions were observed at 17 sites in ORF regions from LMH80, and 13 of them were located at IVa2, DNA polymerase, pTP, 52 kDa, pIIIa, DNA-binding protein, and 100 kDa. These ORF regions are centrally located genus-common genes contributing to viral replication. In addition, we compared the amino acid sequences of those regions from virulent and nonvirulent strains. The nonvirulent strains showed no mutation in amino acids that are associated with pathogenicity (Table 2).

Next, we analyzed the amino acid sequences of structural proteins such as hexon, penton base, fiber-1, and fiber-2. We aligned our LHM10 and LMH80 strains with previously isolated FAdV-4 strains. In LMH80, the hexon sequence yielded a point mutation from thymine (T) to cytosine (C). This switched methionine (M) into threonine (T) at the 887th amino acid residue. Other than this, the hexon of LMH80 has the same amino acid sequence as LMH10. For penton base sequences, LMH80 showed point mutations at two sites: thymine (T) to cytosine (S) and adenine (A) to guanine (G); this replaced valine (V) with alanine (A) at the 324th and threonine (T) with alanine (A) at the 408th amino acid positions, respectively. Except for these changes, the penton amino acid of LMH80 was identical to LMH10.

The mutation at fiber-1 in LMH80 was not detected, but the insertion of five bases (CCCGT) was observed at fiber-2. The insertion inhibited termination at the 480th position, and 14 amino acids (SPKRPPHPVTNKES) were attached to the knob portion at the C-terminus. In other nonvirulent strains, modifications of fiber-2 were shown at four sites, including 219G, 261S, 378A, and 453S, which are not associated with mutations by serial passages (Table 3). Taken together, the amino acids for pathogenicity were not modified in hexon, penton base, and fiber-1. However, the mutation in fiber-2 was observed at the knob part, a viral binding site of host cell receptors [25–27]. Therefore, the mutation on the C-terminal of fiber-2 was assumed to modify the pathogenicity of LMH80.

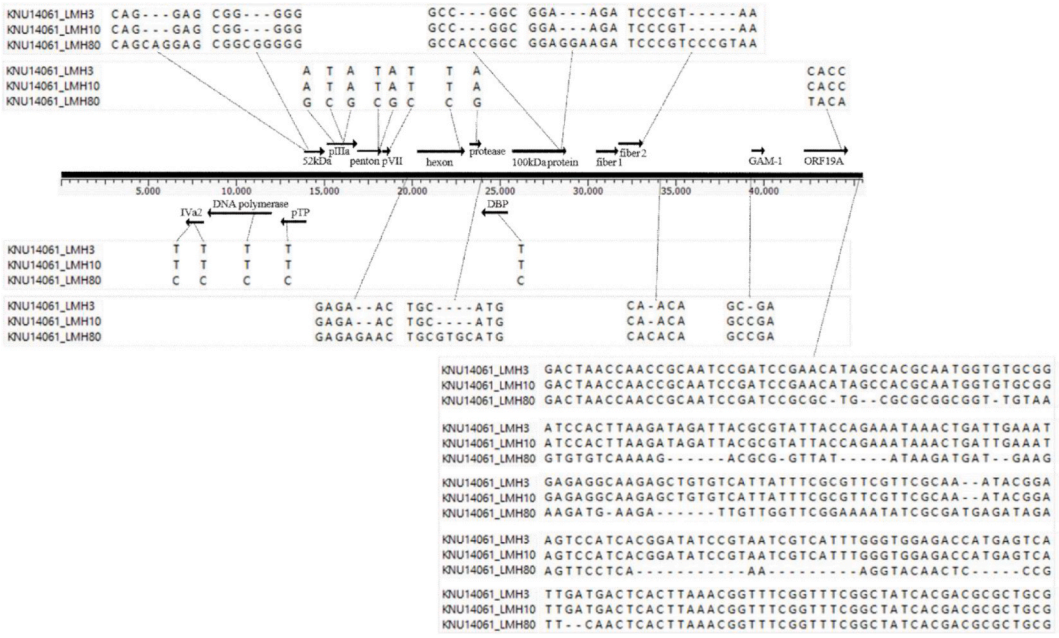

Fig. 2. Whole genome comparison after serial passages. Sequences of key proteins in LMH3, LMH10, and LMH80 were aligned. (IVa2: Encapsidation protein, pTP: terminal protein precursor, 52 kDa: Encapsidation protein, pIIIa: capsid protein precursor, pVII: core protein precursor, DBP: DNA-binding protein, 100 kDa: hexon assembly protein, ORF19A: lipase domain, type I membrane protein, GAM-1: Gallus anti-morte-1).

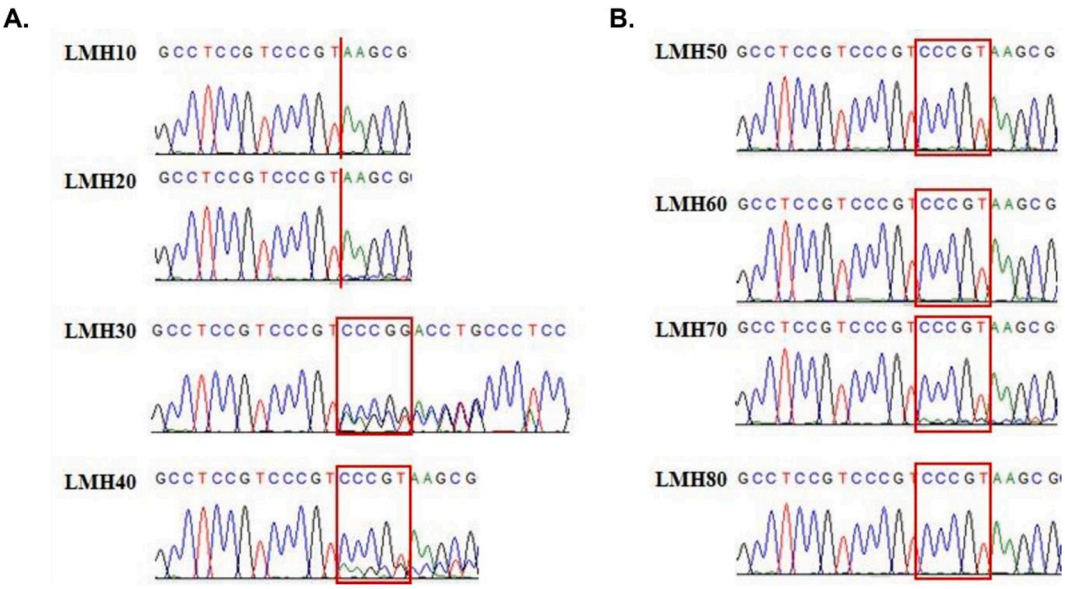

Fig. 3. Analysis of fiber-2 gene. Genetic mutations in the fiber-2 gene by each serial passage are compared. Nucleotide sequences from 10 to 40 passages (A) and 50 to 80 passages (B) are shown.

3.4. The pathogenicity of the FAdV-4 strain after serial passages

To test whether the mutations accumulated by serial passages were associated with increased pathogenicity, we administrated LMH80 and LMH10 in chickens. As previously mentioned, LMH10 has a single nucleotide difference compared with other virulent FAdV-4 strains, and LMH80 showed a frameshift in fiber-2 protein. Prior to infection, we determined the age of susceptibility to LMH10. The virus was not lethal to 4-week-old animals, but 80% mortality was seen in 3-day-old chickens. Therefore, we infected younger chickens (3-day-old) throughout the study. In the LMH10-injected group (intramuscularly), chickens began to die three days post-infection (dpi) and showed 89% mortality in five days. And majority of chickens revealed the virus in their feces. On the other hand, animals were not critically affected by LMH80 administration until nine dpi, and the viral load in the fecal sample was minimal (Table 4 and Fig. 4). This result indicates that serial passages mitigated the pathogenicity of the virulent FAdV-4 strain.

3.5. The protective action of LMH80 against a virulent FAdV-4 infection

LMH80 lost pathogenicity by passages, and we assumed that it could work as a vaccine candidate. Thus, we administrated LMH80 using multiple routes and the chickens were subsequently challenged with the pathogenic virus (LMH10). First, the LMH80 was administrated via muscle (I.M.), oral (P.O.), spray, and eye-drop (E.D.), and LMH10 was injected (I.M.) seven days later. Live attenuated vaccines induce stronger and faster immune responses than inactivated forms. Recent studies applied attenuated FAdV-4 as a vaccination at earlier than 3 weeks [44–46]. Moreover, considering the reduced pathogenicity of LMH10 in older animals, we decided to challenge earlier than other studies. Fecal samples were collected on the 3rd and 5th day after LMH80 administration. On

Table 2
Amino acids comparison of the mutated ORFs.

Pathogenicity	Strain	Country	IVa2		DNA pol	pTP	52 kDa		pIIIa			pVII	DBP	100 kDa	
			23	334	67	523	29	86–88	93	398	401	73	165	902-904	985-987
	LMH80	Korea	**R**	**A**	**R**	**R**	**Q**	**AGA**	**A**	**T**	**D**	**T**	**V**	**ATG**	**GG**
	LMH3	Korea	Q	T	Q	Q	- GA		T	M	N	M	I	AG	G
Non- pathogenic	KR5 ON1	Austria Canada	Q	T	Q	Q	- GA		T	M	N	M	I	TG	G
	B1-7	India	Q	T	Q	Q	- GG		T	M	N	M	I	AG	–
			Q	*	Q	Q	- GA		T	M	N	M	I	AG	GGG
	AH-F19	China	Q	T	Q	Q	Q	GA	T	M	N	M	I	AG	G
Pathogenic	SDSX1	China	Q	T	Q	Q	Q	GA	T	M	N	M	I	AG	G
	MX-SHP95	Mexico	Q	T	Q	Q	- GA		T	M	N	M	I	AG	G

Amino acid position.
*: Absent owing to previous frameshift.
DNApol: DNA polymerase, pTP: Preterminal protein, DBP: DNA-binding protein.

Table 3
Amino acids comparison of the major structure proteins. 1) Hexon, 2) Penton, 3) Fiber-1, 4) Fiber-2.

1) Hexon

Pathogenicity	Strain	Country	Hexon Amino acid positions															
			140	164	188	193	195	238	240	243	263	264	274	410	574	797	842	887
	LMH80	Korea	A	T	I	Q	-	N	A	E	M	I	D	T	V	A	G	**T**
	LMH10	Korea	A	T	I	Q	-	N	A	E	M	I	D	T	V	A	G	M
Non-pathogenic	KR5	Austria	S	T	I	Q	E	N	A	E	M	I	Y	T	I	P	G	M
	ON1	Canada	S	T	I	Q	E	N	A	E	M	I	Y	T	V	A	G	M
	B1-7	India	S	S	I	Q	E	D	T	N	I	V	Y	A	I	P	T	M
Pathogenic	AH-F19	China	S	S	R	R	Q	D	T	N	I	V	Y	A	I	P	A	M
	SDSX1	China	S	S	R	R	Q	D	T	N	I	V	Y	A	I	P	A	M
	MX-SHP95	Mexico	S	T	R	Q	E	N	A	E	M	I	Y	T	V	A	G	M

2) Penton

Pathogenicity	Strain	Country	Penton Amino acid positions											
			6	42	45	127	162	193	324	356	370	408	426	486
	LMH80	Korea	S	P	G	S	K	V	**A**	A	Q	**A**	I	S
	LMH10	Korea	S	P	G	S	K	V	V	A	Q	T	I	S
Non-pathogenic	KR5	Austria	P	S	G	N	K	V	V	A	Q	T	I	S
	ON1	Canada	P	S	G	N	K	V	V	A	Q	T	I	S
	B1-7	India	P	P	G	N	T	I	V	A	Q	T	I	S
Pathogenic	AH-F19	China	P	P	D	N	K	I	V	V	P	T	V	T
	SDSX1	China	P	P	D	N	K	I	V	V	P	T	V	T

3) Fiber-1

Pathogenicity	Strain	Country	Fiber-1 Amino acid positions									
			14	28	44	46	65-81	97	121	128	137	155	188
	LMH80	Korea	V	I	R	A	GGGGGSGGGGGGGGGGG	D	D	V	A	H	N
	LMH10	Korea	V	I	R	A	GGGGGSGGGGGGGGGGG	D	D	V	A	H	N
Non-pathogenic	KR5	Austria	A	I	P	A	-GGGGGSGGGGGGGGGG-	N	N	A	S	H	D
	ON1	Canada	V	I	P	A	-GGGGGSGGGGGGGGGG--	D	D	V	A	H	N
	B1-7	India	A	I	R	A	GGGGGGSGGGGGGGGGG	N	N	A	S	H	D
Pathogenic	AH-F19	China	A	S	R	A	-GGGGGSGGGGGGGGG--	D	N	A	A	R	D
	SDSX1	China	A	S	R	T	-GGGGGSGGGGGGGGG--	D	N	A	A	R	D
	MX-SHP95	Mexico	V	I	P	A	--GGGGSGGGGGGGGG-	D	D	V	A	H	N

Pathogenicity	Strain	Country	Fiber-1 Amino acid positions												
			198	206	253	264	265	312	331	333	376	385	403	430	433
	LMH80	Korea	T	G	L	H	D	H	H	R	S	I	N	N	G
	LMH10	Korea	T	G	L	H	D	H	H	R	S	I	N	N	G
Non-pathogenic	KR5	Austria	V	G	I	Q	E	R	Q	K	P	L	Y	H	S
	ON1	Canada	T	G	I	Q	E	R	Q	K	P	L	Y	H	S
	B1-7	India	V	G	I	H	D	R	H	R	P	L	N	-	G
Pathogenic	AH-F19	China	V	G	L	H	D	H	H	R	S	I	N	-	G
	SDSX1	China	V	G	L	H	D	H	H	R	S	I	N	-	G
	MX-SHP95	Mexico	V	A	I	Q	E	R	Q	K	P	L	Y	N	G

4) Fiber-2

Pathogenicity	Strain	Country	Fiber-2 Amino acid positions																		
			11-15	29	114	144	219	232	261	279	294	295	300	305	306	307	319	324	329	334	338
	LMH80	Korea	-	P	A	A	D	Q	N	I	D	A	T	A	H	P	V	F	L	T	T
	LMH10	Korea	-	P	A	A	D	Q	N	I	D	A	T	A	H	P	V	F	L	T	T
Non-pathogenic	KR5	Austria	ENGQP	A	D	S	G	E	S	V	N	T	I	S	H	P	V	F	V	T	T
	ON1	Canada	-	P	D	S	G	E	S	V	N	T	I	S	H	P	V	F	V	T	T
	B1-7	India	ENGKP	A	D	S	G	Q	S	T	N	T	T	A	N	A	I	V	L	A	N
Pathogenic	AH-F19	China	ENGKP	A	D	S	D	Q	T	V	N	T	T	A	N	A	I	V	L	A	N
	SDSX1	China	ENGKP	A	D	S	D	Q	T	V	N	T	T	A	N	A	I	V	L	A	N
	MX-SHP95	Mexico	-	P	A	A	D	Q	N	V	N	T	T	A	H	A	I	F	L	T	T

Pathogenicity	Strain	Country	Fiber-2 Amino acid positions																	
			343	344	346	372	378	380	391	393	400	403	406	413	427	435	439	453	459	478	480
	LMH80	Korea	W	N	V	L	T	A	S	S	A	E	S	T	V	S	D	A	A	V	**SRKRPPH PVTNKES**
	LMH10	Korea	W	N	V	L	T	A	S	S	A	E	S	T	V	S	D	A	A	V	stop codon
Non-pathogenic	KR5	Austria	N	S	A	V	A	A	S	P	A	Q	S	T	I	T	D	S	A	V	stop codon
	ON1	Canada	N	S	A	V	A	A	S	P	A	Q	S	T	I	T	D	S	A	V	stop codon
	B1-7	India	L	N	A	V	A	A	S	P	G	E	I	S	V	S	E	S	A	V	stop codon
Pathogenic	AH-F19	China	L	N	A	V	T	T	T	P	G	E	I	S	I	S	E	A	N	L	stop codon
	SDSX1	China	L	N	A	V	T	T	T	P	G	E	I	S	I	S	E	A	N	L	stop codon
	MX-SHP95	Mexico	N	S	V	V	T	T	S	S	A	Q	S	T	V	S	D	A	A	V	stop codon

Table 4
Detection of virus and mortality after infection of LMH10 and LMH80.

Groups	Virus positive from feces		Mortality (%)
	3 dpi	5 dpi	
A (Control)	0/9	0/9	0/9 (0)
B (LMH10)	5/6	1/2	8/9 (89)
C (LMH80)	0/9	1/9	0/9 (0)

day three, 33.3% of the animals in the I.M. group shed the virus, and the percentage increased to 58.3% on day 5. In the spray group, 8.3% of animals released the virus. FAdV-4 was detected in the liver and kidneys from I.M. and P.O. groups on day three. Animals in P.O. group did not show pathology (data not shown). Next, we detected virus after LMH80 immunization and the LMH10 challenge. In feces, livers, and kidneys, FAdV-4 was detected in each group but in the I.M. group both on day three and/or five post-challenge. Moreover, the animals from the I.M. and P.O. groups survived the LMH10 challenge (Fig. 4 and Table 5). On day nine, post challenge, chickens were euthanized, and an autopsy was performed. In each group, livers were discolored; some of the liver and kidney tissues were enlarged, and hemorrhage was observed. In addition, severe pericardial effusion was observed in some individuals (Fig. 5 A, B, D, and E). In contrast, the I.M. vaccine group showed a mild pathology. For example, kidney enlargement, bleeding and pale livers were not observed (Fig. 5C and F). The data implied that LMH80 invaded and proliferated in the host when intramuscularly injected but enabled protection against the virulent FAdV-4 infection.

At seven and nine dpi, serum was collected, and antibodies to FAdV-4 were examined by ELISA assay. On the samples from day

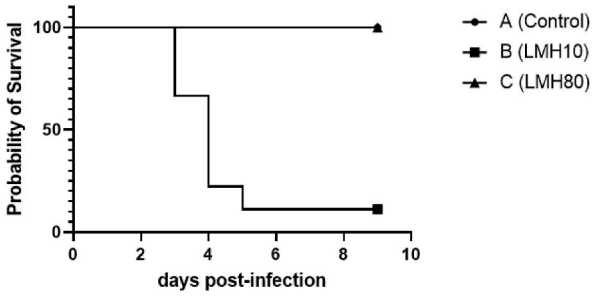

Fig. 4. Mortality by LMH10 or LMH80 inoculation. Survival rates were examined in LMH10 or LMH80 injected chickens.

Table 5
Detection of virus and mortality according to vaccination routes. I.M.: Intra-muscular, P.O.: Per-oral, E.D.: Eye-drop.

Groups	Vaccine virus positive from				Challenge virus positive from				Mortality (%)
	feces		liver	kidney	feces		liver	kidney	
	3dpv	5dpv	3dpv	3dpv	3dpc	5dpc	3dpc	3dpc	
A (Control)	0/15	0/12	0/3	0/3	11/12	7/7	3/3	1/3	3/9 (33)
B (I.M. vaccinated)	**5/15**	**7/12**	**2/3**	0/3	**0/11**	**0/8**	**0/3**	**0/3**	**0/8 (0)**
C (P.O. vaccinated)	0/15	0/12	**2/3**	0/3	8/12	4/9	3/3	2/3	**0/9 (0)**
D (Spray vaccinated)	0/15	1/12	0/3	0/3	12/12	5/7	3/3	3/3	3/9 (33)
E (E.D. vaccinated)	0/15	0/12	0/3	0/3	10/12	8/8	3/3	2/3	1/9 (11)

I.M.: Intra-muscular, P.O.: Per-oral, E.D.: Eye-drop

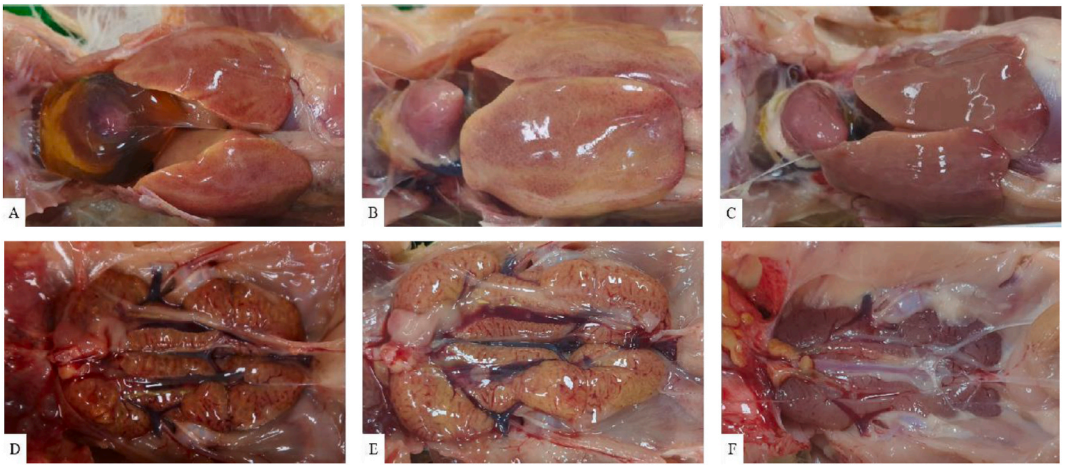

Fig. 5. Gross lesions of unvaccinated (A, B, D and E) or vaccinated (C and F) chickens after challenge with LMH10. Pericardiac sac is distended with clear straw-colored fluid (A). Livers are swollen and mottled with red foci (A and B). Kidneys are swollen and pale (D and E). Livers and kidneys were not affected in vaccinated animals (C and F).

seven, antibodies were not detected, but each group was positive for the antibodies on day nine post-challenge. Both antibody detection rate and titer were the lowest in the I.M. group (Table 6 and Fig. 6).

4. Discussion

The whole genome length of FAdV-4 is 45,640~45,670 bp, but a strain isolated in China showed 43,720 bp because of a deletion of 1,966 bp in the ORF19 and ORF27 region [8,20,29,30,47–49]. The G + C ratio of Chinese strains is 54.6% for ON1, 54.72% for MX-SHP95, and 54.88% for AH-F19 [20,29,30], whereas in our strains are 54.71% and 54.76% for LMH3 and LMH80 respectively, demonstrating that the nucleotide deficit leads to a higher G + C ratio [20,29,30].

ORFs for 10.3 kDa and 10.5 kDa exist in ON1 but not in the MX-SHHP95 and KR5 strains. In LMH3, the hypothetical proteins (10.3 kDa and 10.5 kDa) at the central part of the gene and ORF20B, ORF29, and ORF30 at the right genus-specific gene were absent compared with the ON1 strain. Thus, we speculate that these two proteins are non-functional for viral replication.

On the other hand, the hypothetical protein (11.7 kDa) was preserved in our LMH3 and LMH10 strains and other foreign strains. The ORF20B bound ORF20 in all other strains except ON1. ORF29 overlaps with the repetition sequence D. It was deleted in ON1, B1-7, and SDSX1 strains leading to a frameshift in LMH3, the length of ORF29 was only about 37 amino acids (AAs). One base was inserted in LMH10 and LMH80, extending ORF29 to 55 AA. The large number of deficits in each strain at the ORF29 are assumed to be derived from repetitive C bases. The ORF30 was identified only in ON1, KR5, and B1-7 [20,29].

V, VII, Iva2, and TP proteins bind to double-stranded DNA and IIIa, VI, VIII, and IX are minor structural proteins of the viral capsid [22]. These are genus-common genes found in FAdVs [20,35]. pTP, pIIIa, and pVII are TP, IIIa, and VII precursor proteins. The 52 kDa protein is important for the DNA encapsidation along with IVa2 [50,51] and the 100 kDa protein moves the hexon of monomer into the nucleus, which assembles a trimer [52,53]. Because they regulate gene expression in host cells or assemble viral particles [22],

Table 6
ELISA positive rate according to vaccination routes.
I.M.: Intra-Muscular, P.O.: Per-oral, E.D.: Eye-Drop.

Groups	Positive rate (%)	
	7dpv	9dpv
A (Control)	0.0	57.1
B (I.M. vaccinated)	0.0	12.5
C (P.O. vaccinated)	0.0	44.4
D (Spray vaccinated)	0.0	28.6
E (E.D. vaccinated)	0.0	75.0

mutation of amino acids in LMH80 does not affect the function of viral proteins.

The main structural proteins of FAdV-4 are hexon, penton base, fiber-1, and fiber-2. The hexon forms capsomers that surround the outside of the virus, the fiber-1 attaches to LMH cells, and the penton base acts as a link between fiber proteins and the virus [8,22,24]. The 188R amino acid in hexon was considered to be associated with pathogenicity [47]. However, the previous speculation needs to be corrected because the 188th amino acid was found to be 188I, a nonpathogenic viral strain amino acid in the pathogenic KNU14061 strain. The 42P in penton protein is assumed to be a pathogenic amino acid [47], but this may be wrong because the amino acid of B1-7, a nonpathogenic viral strain, appears to be 42P.

There are two fiber proteins in FAdV-1 and FAdV-4. FAdV-1 has a 'long fiber' extended from the penton base, which is connected to a 'short fiber' [54]. The 'long fiber' is known as fiber-1, and the 'short fiber' is for fiber-2 of FAdV-1. FAdV-4 consists of two types of fibers that have similar lengths. The length of fiber-1 is 432–435 AA, which varies by 1–3 AA depending on the number of amino acids in poly-G at the N-terminal side. The length of fiber-1 in both LMH3 and LMH80 is 435 AA with a longer poly-G than other strains [8,29,31].

Fiber-2 has 474 AA because LMH3 does not have 11∼15th amino acids, and fiber-2 in LMH80 has 488 AA because of the added amino acid to the C-terminus. Although fiber-2 has a longer amino acid in LMH80, phylogenetic analysis shows that fiber-2 proteins of FAdV-1 and FAdV-4 and other serotypes are classified into the same phylogeny. This indicates that they are derived from a common gene [8]. Therefore, we speculate that functions similarly to other fiber proteins in adenoviruses.

Fiber-1 is known for its interaction with the D2 domain of the CAR [24]. The interaction with those receptors is not essential for inflammation, but guides viral migration into LMH cells [55]. Genetic modification in fiber-1 has critical effects on the viral adhesion to LMH cells. However, we did not detect mutation in fiber-1 and hexon, and two amino acid changes in the penton base was observed during passages from LHM10 to LMH80. On the other hand, mutations in the fiber-2 gene, where five bases (CCCGT) were inserted, were stably maintained after 50 passages (LMH50). This implies that the mutation in fiber-2 is more associated with the viral pathogenicity. Moreover, tissue tropism might bear certain advantages and passages in LMH cells. This could act on the attenuation together with the frameshift of fiber-2.

Fiber-2 is a major controller for the pathogenicity of FAdV-4, and it has been shown that replacing the fiber-2 gene with a nonpathogenic strain lowered pathogenicity [25]. Accordingly, multiple reports tried to figure out the key amino acids for pathogenesis in the fiber-2 protein. Others found genetic modifications in 219G, 300I, and 380A [30,47,56,57], and we observed mutations

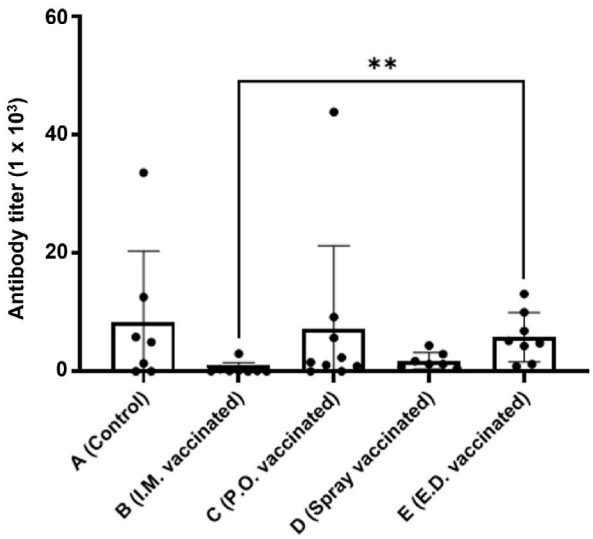

Fig. 6. Antibody titers by injection routes. Antibody titers to FAdV-4 were observed by ELISA assay on day nine post-challenge.

in 219G, 261S, 378A, and 453S from nonpathogenic strains. However, these amino acids were unrelated to the passaged virus' mutations.

Fiber proteins of adenovirus are critical for the formation of the stable trimeric complexes which directs viral assembly. For example, deletion or mutation in the N-terminus (tail region) hardly affects the assembly of the trimer, but the deletion or the insertion of amino acids in the C-terminus (knob region) can be critical. When short tags (6 AA) were added to the C-terminus, the assembly of the trimer was partially inhibited, but when longer tags (27 AA) were added, the assembly was completely inhibited by the trimer [58]. The mutation in fiber-2 LMH80 was also found to have added amino acids at the C terminus. Therefore, the knob part of fiber-2 is not likely to be functioning properly because the affected trimer formation inhibited the assembly.

The fiber proteins in adenovirus are thought to be associated with tissue affinity [59]. LMH80 had a mutation at the C-terminus of fiber-2. The reaction with host receptors was observed, but the virus was not isolated from the kidneys. We assume that the pathogenicity was ameliorated because of the variability of tissue affinity. FAdV-4 has an affinity to thymus, liver, intestines (feces), bursa of Fabricius, spleen, appendicitis, kidney, and proventriculus [29]. Further studies on organizational affinity and pathogenicity in different tissues are necessary.

Most FAdV-4 vaccines are in an inactivated form that is made using heat or formalin treatment. The oil-based inactivated vaccines have been commercially available in Korea, and the protective rate was 80–100%. The antibody titer and IL-4 level were maintained when the oil-based vaccine was intramuscularly injected. However, limited IFN-γ was produced because type 1 helper T cells are not activated, whereas type-2 helper T cells are activated [9,24,33]. Therefore, it is assumed that antibodies cleared viral antigens and could not interact with host immune cells.

In contrast to the inactivated form, the live attenuated vaccine showed a 95–100% protective ratio, and effector cytokines such as IFN-γ, and IL-10 were induced [34, 35, 60]. This indicates that the live attenuated vaccine improved immunity by supporting the cellular immunity. A fiber-2 protein-recombinant vaccine showed complete defense, but a few other studies did not show the protective effect against the pathogenic FAdV-4 challenge [33,36,38]. Insufficient antigens in recombinant vaccines might have induced lower protection than the live attenuated vaccines.

In this study, we used LMH80 as an attenuated form, and the virus was detected in I.M. and P.O. groups. After the challenge with LMH10, the virus was not detected in the I.M. group, and all animals survived. The result shows that LMH80 acts as an effective live attenuated vaccine when intramuscularly administrated. In spray and E.D. routes, the virus was detected in feces after the challenge showing the insufficient protection. The virus was only detected in feces in the P.O. group, and the protection was incomplete. This is inconsistent with previous studies that showed high efficiency by oral inoculation of the attenuated vaccine [34,35]. In addition, a high dose of a pathogenic viral challenge with MX-SHP95 showed 100% death but 40% mortality by low-dose infection [29]. Therefore, optimizing the route and dose of live attenuated vaccines is essential.

At day seven post-vaccination, antibodies to FAdV-4 were not detected, which is consistent with previous studies, but the antibody level was increased from day 14–21 [38,39]. After the challenge, the antibody titer was the lowest in the I.M. group after challenge, seemingly because the high antibodies at day seven were removed with the challenged virus. An attenuated virus by QT35 cells worked differently in two repeated trials. No antibody was detected in the first trial, but the hosts were protected from the challenge. It is possible that antibodies were formed and defended hosts faster than in the control group or cell-mediated immunity could be dominant to the antibody reaction [35]. In our study, the experiment might have terminated even before the antibody boost after the challenge. In addition, antigens could have been neutralized and removed which can be supported by observing the level of neutralizing antibodies. The antibody levels were compared between live attenuated and inactivated vaccines. Both antibody titers and type I immunity were enhanced by attenuated vaccination [60]. Further studies need to dissect the dynamics between cellular and humoral immunity.

In summary, the pathogenicity of FAdV-4 was lost through the serial passages in a cell line, which induced genetic mutations. The passaged FAdV-4 strain reliably protected the host from the virulent FAdV-4 challenge. Our results suggest the potential usage of the passaged virus as a live attenuated vaccine format. To support vaccine development, consecutive studies should investigate immune responses regulated by the vaccination.

Author contribution statement

Conceived and designed the experiments: Haan Woo Sung; Jeongho Park.
Performed the experiments: Ji-in Yeo; Rangyeon Lee.
Analyzed and interpreted the data: Haan Woo Sung; Jeongho Park; Ji-in Yeo; Rangyeon Lee; Haneul Kim; Somin Ahn.
Contributed reagents, materials, analysis tools or data: Haan Woo Sung; Jeongho Park.
Wrote the paper: Ji-in Yeo; Haan Woo Sung; Jeongho Park.

Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e19860.

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