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# Biochemical Properties and Cell Culture Affinity of Fowl Adenovirus Serotype-4 Strains Isolated from the Oviducts of Layer Hens in East Japan

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## ABSTRACT

In the present study, the biochemical properties of two fowl adenovirus serotype-4 (FAdV4) sample strains were determined. These were previously isolated from the oviducts of laying chickens from two layer operations in East Japan, namely M and Y farms. Tests for stability and sensitivity, hemagglutinating (HA) activity, and growth in two different cell lines were performed. The results showed that the M farm strain, (Japan/Ibaraki/M-HB2/2016) was sensitive to 100% ethanol, 52°C and higher temperature, and formaldehyde. The Y farm strain (Japan/Ibaraki/Y-H6/2016) was sensitive to 70% ethanol, 100% ethanol, 52°C and higher temperature, and formaldehyde. Both strains were stable against ether and chloroform, and lacked HA activity. To the best of the author's knowledge, these FAdV4 strains were the first to be detected and isolated from laying chicken's oviduct. Their biochemical characteristics; specifically, sensitivy to heat and formaldehyde, can be included in farm cleanup and disinfection protocol. This could help in reducing environmental contamination. The strains propagated well in chick embryo fibroblast (CEF) as indicated by cytopathic effect (CPE) observation with positive AAV-PCR and FAdV4-PCR results. The strains failed to propagate in MDCC-MSB1 cells as indicated by the negative results in both CPE and PCR. It appears that MDCC-MSB1 cells are not suitable for FAdV4 cultivation. However, only non-pathogenic FAdV4 strains were used in this work. It was not confirmed if pathogenic strains have the same behavior, perhaps, further trials are advisable. Future studies may benefit from the reduction of use of primary cells from live animals. This information contributes to the current understanding of FAdV4 characteristics.

**Keywords:** Biochemical properties, Cell culture, Fowl adenovirus serotype 4, Laying hen, Oviduct.

## INTRODUCTION

The family *Adenoviridae* is comprised of middle-sized, non-enveloped, icosahedral, double-stranded DNA viruses. There are five genera, namely *Mastadenovirus*, *Aviadenovirus*, *Siadenovirus*, *Atadenovirus*, and *Ichtadenovirus* (ICTV, 2018). Virions are 70-90 nm in size, hexagonal in shape, and have three exposed structural proteins. The hexon forms the capsid, the penton base anchors the fiber, and the fiber interacts with the cellular receptor. Many Adenoviruses (AdVs) bind with the coxsackie and adenovirus receptor (CAR) for cellular entry (Fujino et al., 2016). Some have single fiber protein, such as the case for most mammalian AdVs while others have two, including avian adenoviruses (AAVs) (Harrach et al., 2011).

The Fowl aviadenoviruses (FAdVs), also known as group-1 AAVs, are pathogens associated with important avian disease syndromes (Knowles, 2011). They are classified into five species (A to E) with 12 serotypes (1-8a, 8b-11), wherein members are associated with certain diseases (Hess, 2017). Although implicated, the pathogenicity of FAdVs is not well-defined (Niczyporuk et al., 2012). These viruses may act as primary or secondary pathogens (Toro et al., 2001; Niczyporuk et al., 2013), and there are pathogenic and non-pathogenic strains. Additionally, FAdVs can be isolated from both

healthy and sick chickens (McFerran and Smyth, 2000; Hess, 2017).

Serotypes are linked to poultry diseases. These include pathogenic FAdV1 to adenoviral gizzard erosion (GE, Grafl et al., 2012), pathogenic FAdV2, 8a, 8b, and 11, to inclusion-body hepatitis (IBH, Nakamura et al., 2011), and pathogenic FAdV4 to hydropericardium-hepatitis syndrome (HPS, Mc Ferran and Smyth, 2000; Joubert et al., 2014). These diseases have been replicated under controlled conditions (Okuda et al., 2006; Mase et al., 2010). At present, the genetic determinant of pathogenicity is still unclear. This is true for FAdV1 (Matczuk et al., 2017), FAdV11 (Absalón et al., 2017), and FAdV4 (Liu et al., 2016; Mo et al., 2019). Reported FAdV infections in Japan are mainly connected with increased mortality and this was observed in IBH and HPS cases (Nakamura et al., 2000; Mase et al., 2009; Nakamura et al., 2011). On the other hand, some GE cases did not have clinical signs (Ono et al., 2001).

The biochemical properties of viruses can be used to differentiate one from the other (Rovozzo and Burke, 1973) or to characterize different strains. There are numerous studies on the biochemical properties of FAdVs (Otsuki et al, 1976; Cook, 1983; Park et al., 2011). Integral to these studies is the cultivation of virus on cell cultures. FAdVs can propagate in both primary and transformed cell lines. Perhaps the most commonly used primary cells are chick kidney cells (CKC, Kawamura et al., 1964; Mase et al., 2010) chick embryo liver cells (CEL, McFerran and Smyth, 2000; Park et al., 2011), and chick embryo fibroblasts (CEF, Bauer et al., 1986; Niczyporuk et al., 2013). Transformed cell lines are also suitable for propagation, these include the QT35 (Schonewille et al., 2008) and LMH (Zhao et al., 2015). Other cell lines that have been used for FAdV affinity studies include the Crandel-Rees Feline Kidney (CRFK) cell and Vero cells (Taharaguchi et al., 2012).

Another viral property used for characterization is hemagglutinating activity (HA) (Rovozzo and Burke, 1973). Some viruses adsorb red blood cells (RBCs) through cellular receptors. This results in agglutination, otherwise known as hemagglutination (Rovozzo and Burke, 1973). Of the three structural proteins in AAVs, the fiber is the one responsible for HA activity (Louis et al., 1994). Some AAVs are known to have HA activity (Knowles et al., 2011) and some others do not. FAdVs from the field has been reported to be HA-negative (Otsuki et al., 1976).

Two FAdV4 strains were previously isolated from laying hens in two different farms, namely M and Y farms. Both are located in Ibaraki prefecture Japan and previously suffered from poor egg production. The M farm strain was named Japan/Ibaraki/M-HB2/2016, and the Y farm strain was entitled Japan/Ibaraki/Y-H6/2016. The hexon and fiber2 genes of both strains have been sequenced, and they have been reported as non-pathogenic (Del Valle et al., 2020a). Both M and Y farms receive their replacement pullets from the same replacement-pullet farm. That particular farm had a history of chick anemia virus (CAV) infections (Del Valle et al., 2020b). Retrospective testing of some CAV-positive samples has indicated avian adenovirus (AAV) coinfection as well (Del Valle, 2019, Table 5).

The present study aimed to investigate the biochemical properties and propagation of M farm and Y farm strains in cell culture. More specifically, the study was designed to determine their stability and sensitivity, HA activity, and affinity to two different cell lines. In this regard, CEF and MSB1 cells were used for cell culture growth comparison.

## MATERIALS AND METHODS

### Viruses

The FAdV4 strains used in the present study were Japan/Ibaraki/M-HB2/2016 and Japan/Ibaraki/Y-H6/2016, previously isolated from the oviducts of laying hens (Del Valle et al., 2020a). The KR5 strain was used as a positive control.

### Biochemical properties

Previously described methods for determining viral titer and biochemical properties were performed. Ten heads of 5- to 7-day-old specific-pathogen-free (SPF) chicks were sacrificed by cervical dislocation, then, the kidneys were collected for CKC cultivation following previously described methods with slight modifications (Rovozzo and Burke, 1973). Titration was done in 6-well plates with 1 plate assigned to each dilution from $10^{-1}$ to $10^{-10}$. In every plate, each well served as a replicate and was seeded with 900 µl EMEM containing CKC, and later, 100 µl of virus solution was added. All plates were incubated at 40°C in 5% $CO_2$ and observed for Cytopathic Effect (CPE) until day 5. The endpoint dilution was computed using the Reed and Muench method (Reed and Muench, 1938). The Y farm titer in CKC was $10^{6.75}TCID_{50}$/ml, and that of M farm strain was $10^{5.40}TCID_{50}$/ml. The viral fluids were exposed to various chemical and physical agents (Rovozzo and Burke, 1973). The titer was computed using the Reed and Muench method (Reed and Muench, 1938), and a drop of 1 $\log_{10}$ from the baseline $TCID_{50}$ indicated sensitivity (Rovozzo and Burke, 1973).

**Sensitivity to chloroform**

Virus suspensions were centrifuged to remove cellular debris and 500 µl chloroform was added to 1 ml undiluted virus suspension. These were mixed manually for 10 minutes at room temperature, followed by centrifugation at 33 x g for 5 minutes. The uppermost clear layer was diluted 10-fold and used for titration in CKC.

**Sensitivity to ether**

In this phase, 200 µl ether was mixed with 800 µl virus suspension to form a 20% solution. These were shaken manually and kept at 4˚C for 18-24 hours with intermittent shaking. The suspensions were transferred to sterile Petri dishes and the ether was allowed to evaporate for 1 hour. After evaporation of ether, the fluid was diluted 10-fold followed by titration in CKC.

**Sensitivity to ethanol**

Viral isolates were mixed with ethanol at varying concentrations of 50%, 70%, and 100%. Then, 0.5 ml of virus fluid was mixed with 0.25 ml 50% ethanol solution in a micro centrifuge tube, and was incubated at room temperature for 1 hour. The mixture was placed in a petri dish and allowed to air-dry for 30 minutes; after which it was diluted 10-fold, and titrated in CKC. The same method was used for 75% and 100% ethanol.

**Sensitivity to formaldehyde**

Formaldehyde and distilled water were mixed to form 1:2000 solution. In this regard, 1ml of solution and 1ml virus fluid were mixed and incubated at 37˚C for 1 hour. The mixture was diluted 10-fold and titrated in CKC.

**Sensitivity to heat**

At this stage, 500 µl undiluted viral suspensions were placed in microcentrifuge tubes. These were immersed in water baths with temperatures of: 50˚C, 52˚C, 54˚C, and 58˚C for 30 minutes. The solutions were cooled in the ice-cold water bath, diluted 10-fold, and titrated in CKC.

**Hemagglutinating activity**

Hemagglutination (HA) test was performed using previously described methods by Rovozzo and Burke (1973) with slight modifications. Chicken RBCs were collected by centrifugation at 205 x g for 10 minutes as described by Rovozzo and Burke (1973) and used to prepare 0.5% suspension on phosphate buffered saline (PBS). In a 96-well microtiter plate, 50 µl of 2-fold serial dilutions of the virus was prepared in PBS. A DAdV-A strain was used as a positive control. In the next step, 50 µl of 0.5% RBC was added to all wells and was kept at room temperature for 30 minutes, afterwards, these were checked for agglutination reaction.

**Growth comparison in two cell cultures**

Chick embryonic fibroblasts (CEF) cells were cultivated and inoculated using previously described methods with slight modifications (Rovozzo and Burke, 1973). The MDCC-MSB1 cells were cultivated using previously described methods with slight modifications (Simeonov et al., 2014). After inoculation with the FAdV4 strains, the cell cultures were incubated at 40°C in 5% $CO_2$ and observed for 2 to 5 days, and then passaged until CPE was observed. For confirmation, AAV and FAdV4-PCR (Mase et al., 2009; Mase et al., 2010) were performed at different passages, meaning that first to sixth for CEF and first, second, third, fifth, eighth, and thirteenth for MSB1 cell (Table 1).

## RESULTS

**Biochemical properties**

Both M-farm and Y-farm strains were sensitive to 100% ethanol, 52˚C and higher temperature, and formaldehyde. These were stable against chloroform, ether, and 50˚C. Stability to 70% ethanol was variable, the M-farm strain was stable and the Y-farm strain was sensitive (Table 2).

**Hemagglutination**

The KR5, M-farm, and Y-farm strains lacked HA activity. The DAdV-A positive control had 4096 HA units (Table 3).

**Growth comparison in two cell cultures**

***Chicken embryo fibroblast***

After 5-6 days of incubation, CPE was observed on infected CEF inoculated with KR5, M-farm strain, and Y-farm strain. Cellular swelling, death, and monolayer destruction were evident 5-6 days post-infection. All three strains produced the same effect from the first passage until the sixth (Figure 1). The viral fluids also had positive results in AAV and FAdV-4 PCR in every generation (Figure 4 and Table 4).

***MDCC MSB1 cells***

After 2-4 days of incubation, no color change in the media was observed on virus-inoculated plates (Figure 2). From passages 1 to 13, the results were the same for all three strains (Figures 2 and 3, and Table 4). The AAV and FAdV-4 PCR assays were positive only at the first passage, weak positive at the second, and negative on the third, fifth, eighth, and thirteenth passages. The CAV-live-vaccine produced cell swelling and crenation in MSB1 cells starting at the third passage, with cellular death at the fourth (Figure 3 and Table 4).

**Table 1**. Primer and PCR conditions used for viral genome detection

| Assay | Target virus | Primer (5'-3') | | Initial Denaturation | Denaturation | Annealing | Extension | Final Extension | Cycles | Expected size | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|
| PCR | Avian Adenovirus | Hex F1 | GAYRGYHGGRTNBTGGAYATGGG | 94˚C, 5 minutes | 94˚C, 1 minutes | 55˚C, 1 minute | 72˚C, 30 seconds | 72˚C, 5 minutes | 35 | 800 bp | Mase et al. (2009) |
| | | Hex R1 | TACTTATCNACRGCYTGRTTCCA | | | | | | | | |
| | Fowl Adenovirus Serotype-4 | Fib F1 | CAGGGTTACGTCTACTCCCC | 94˚C, 5 minutes | 94˚C, 1 minutes | 55˚C, 1 minute | 72˚C, 30 seconds | 72˚C, 5 minutes | 35 | 1500 bp | Mase et al. (2010) |
| | | Fib R1 | TTTGTCACGCGGTGGGGAGG | | | | | | | | |

**Table 2.** Biochemical properties of the FAdV4 strains from the oviducts of layer chicken farms located in Ibaraki Prefecture, Japan

| Physical/chemical agents | Strain titer | |
|---|---|---|
| | M-farm strain (Japan/Ibaraki/M-HB2/2016) | Y-farm strain (Japan/Ibaraki/Y-H6/2016) |
| Baseline Titer | $10^{5.40}$TCID$_{50}$/ml | $10^{6.75}$TCID$_{50}$/ml |
| 100% ethanol | $10^{4.16}$TCID$_{50}$/ml* | $10^{4.3}$ TCID$_{50}$/ml* |
| 70% ethanol | $10^{5.30}$TCID$_{50}$/ml | $10^{5.25}$ TCID$_{50}$/ml* |
| 50% ethanol | $10^{5.84}$TCID$_{50}$/ml | $10^{7.5}$ TCID$_{50}$/ml |
| 58˚C | $10^{3.16}$TCID$_{50}$/ml* | $10^{4.69}$ TCID$_{50}$/ml* |
| 54˚C | $10^{3.60}$ TCID$_{50}$/ml* | $10^{3.59}$ TCID$_{50}$/ml* |
| 52˚C | $10^{3.75}$ TCID$_{50}$/ml* | $10^{4.5}$ TCID$_{50}$/ml* |
| 50˚C | $10^{5.75}$ TCID$_{50}$/ml | $10^{6.50}$ TCID$_{50}$/ml |
| formaldehyde | $10^{4.16}$ TCID$_{50}$/ ml* | $10^{5.25}$ TCID$_{50}$/ml* |
| ether | $10^{5.40}$ TCID$_{50}$/ml | $10^{6.40}$ TCID$_{50}$/ml |
| chloroform | $10^{5.50}$ TCID$_{50}$/ml | $10^{6.36}$ TCID$_{50}$/ml |

*At least 1log10 decrease in titer indicates sensitivity.

**Table 3.** Hemagglutinating Activity test results of the FAdV4 strains from the oviducts of layer chicken farms located in Ibaraki Prefecture, Japan

| Strain | Hemagglutinating Activity Titer |
|---|---|
| KR5 | Negative |
| M-farm strain (Japan/Ibaraki/M-HB2/2016) | Negative |
| Y-farm strain (Japan/Ibaraki/Y-H6/2016) | Negative |
| PBS* (negative control) | Negative |
| DadV-A (positive control) | 4096 |

PBS: Phosphate buffered saline

**Table 4.** Summary of cell culture cultivation results of FAdV4 strains from the oviducts of layer chicken farms located in Ibaraki Prefecture, Japan in CEF and MSB1 cells

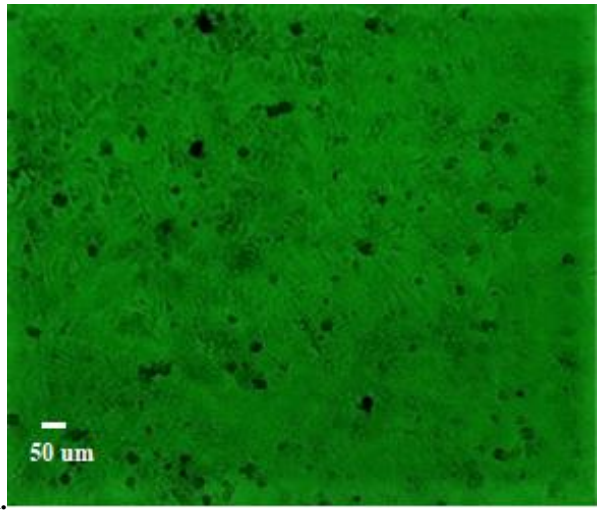
| Passage | | KR5 | | | M-farm strain (Japan/Ibaraki/M-HB2/2016) | | | Y-farm strain (Japan/Ibaraki/Y-H6/2016) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | CPE | AAV-PCR | FAdV4-PCR | CPE | AAV-PCR | FAdV4-PCR | CPE | AAV-PCR | FAdV4-PCR |
| CEF | 1 | + | + | + | + | + | + | + | + | + |
| | 2 | + | + | + | + | + | + | + | + | + |
| | 3 | + | + | + | + | + | + | + | + | + |
| | 4 | + | + | + | + | + | + | + | + | + |
| | 5 | + | + | + | + | + | + | + | + | + |
| | 6 | + | + | + | + | + | + | + | + | + |
| MSB1 | 1 | - | + | + | - | + | + | - | + | + |
| | 2 | - | + | + | - | + | + | - | + | + |
| | 3 | - | - | - | - | - | - | - | - | - |
| | 4 | - | not tested | not tested | - | not tested | not tested | - | not tested | not tested |
| | 5 | - | - | - | - | - | - | - | - | - |
| | 6 | - | not tested | not tested | - | not tested | not tested | - | not tested | not tested |
| | 7 | - | not tested | not tested | - | not tested | not tested | - | not tested | not tested |
| | 8 | - | - | - | - | - | - | - | - | - |
| | 9 | - | not tested | not tested | - | not tested | not tested | - | not tested | not tested |
| | 10 | - | not tested | not tested | - | not tested | not tested | - | not tested | not tested |
| | 11 | - | - | - | - | - | - | - | - | - |
| | 12 | - | not tested | not tested | - | not tested | not tested | - | not tested | not tested |
| | 13 | - | - | - | - | - | - | - | - | - |

CPE: Cytopathic effect, AAV-PCR: Avian adenovirus polymerase chain reaction, FAdV4-PCR: Fowl adenovirus serotype-4 polymerase chain reaction

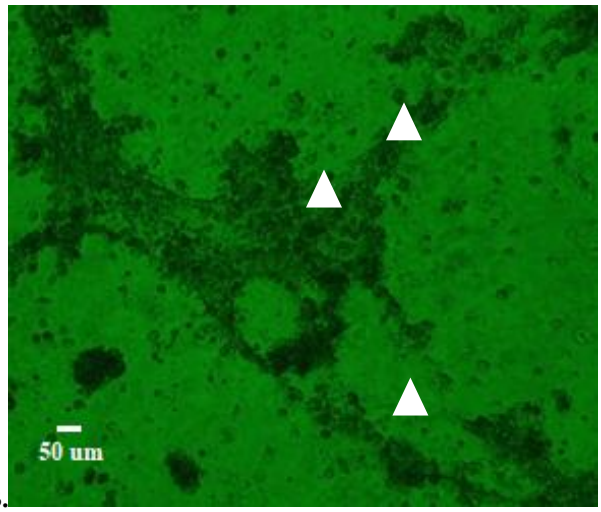
**Table 5.** Retrospective avian adenovirus-polymerase chain reaction testing of CAV-positive samples collected from layer chicken farms in Ibaraki Prefecture, Japan during 2017-2019

| Year | Sampling (months) | Laying Chickens (total) | CAV-PCR* positive | AAV-PCR** | |
|---|---|---|---|---|---|
| | | | | Tested | Positive |
| 2017 | January to December | 168 | 81 | 40 | 5 |
| 2018 | January to December | 154 | 68 | 25 | 4 |
| 2019 | January to August | 110 | 26 | 18 | 3 |

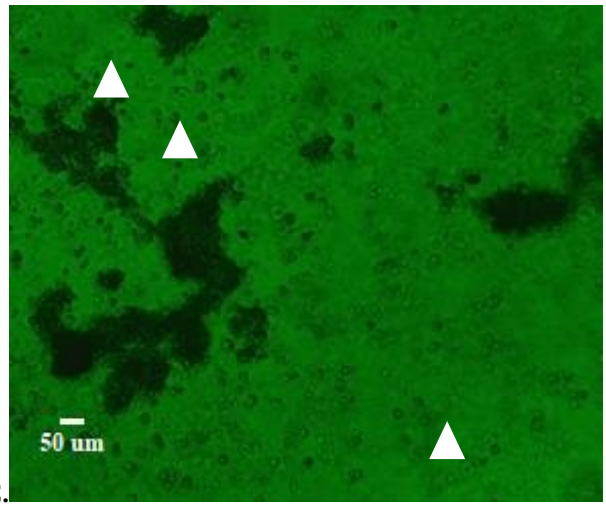
CAV-PCR: Chick anemia virus-polymerase chain reaction. AAV-PCR: Avian adenovirus-polymerase chain reaction



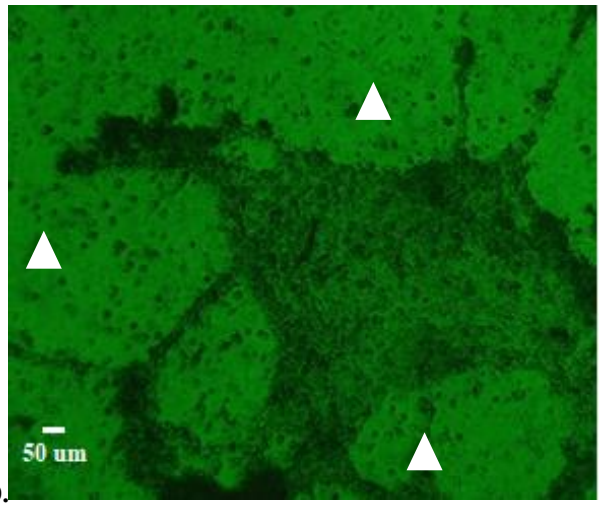

1A.




1B.

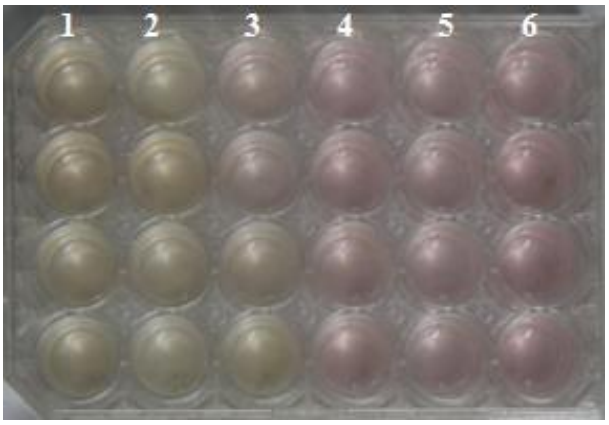



1C.

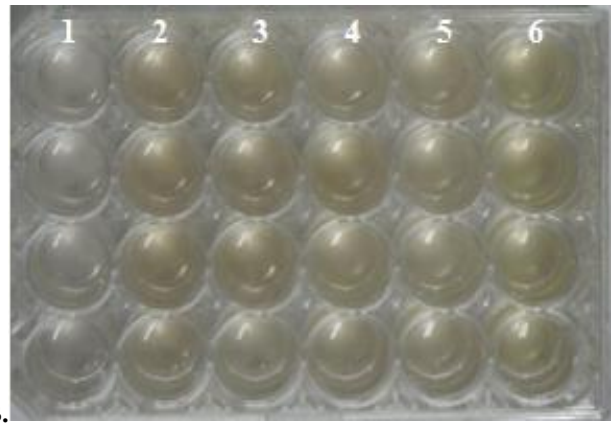



1D.

**Figure 1.** Representative chick embryo fibroblast observations, first passage, 2 dpi, LPO. **1A:** Negative control, **1B:** KR5 Positive control, cell swelling, rounding, and death (pointers), **1C:** M farm sample-inoculated, cell swelling, rounding and death (pointers), **1D:** Y farm sample-inoculated, cell swelling, rounding, and death (pointers)




2A.




2B.

**Figure 2.** Representative MDCC-MSB1 gross observations, first to sixth passages. **2A:** Live vaccine, color changes in replicates at the third passage indicate cell death, **2B:** M-farm strain, no color change

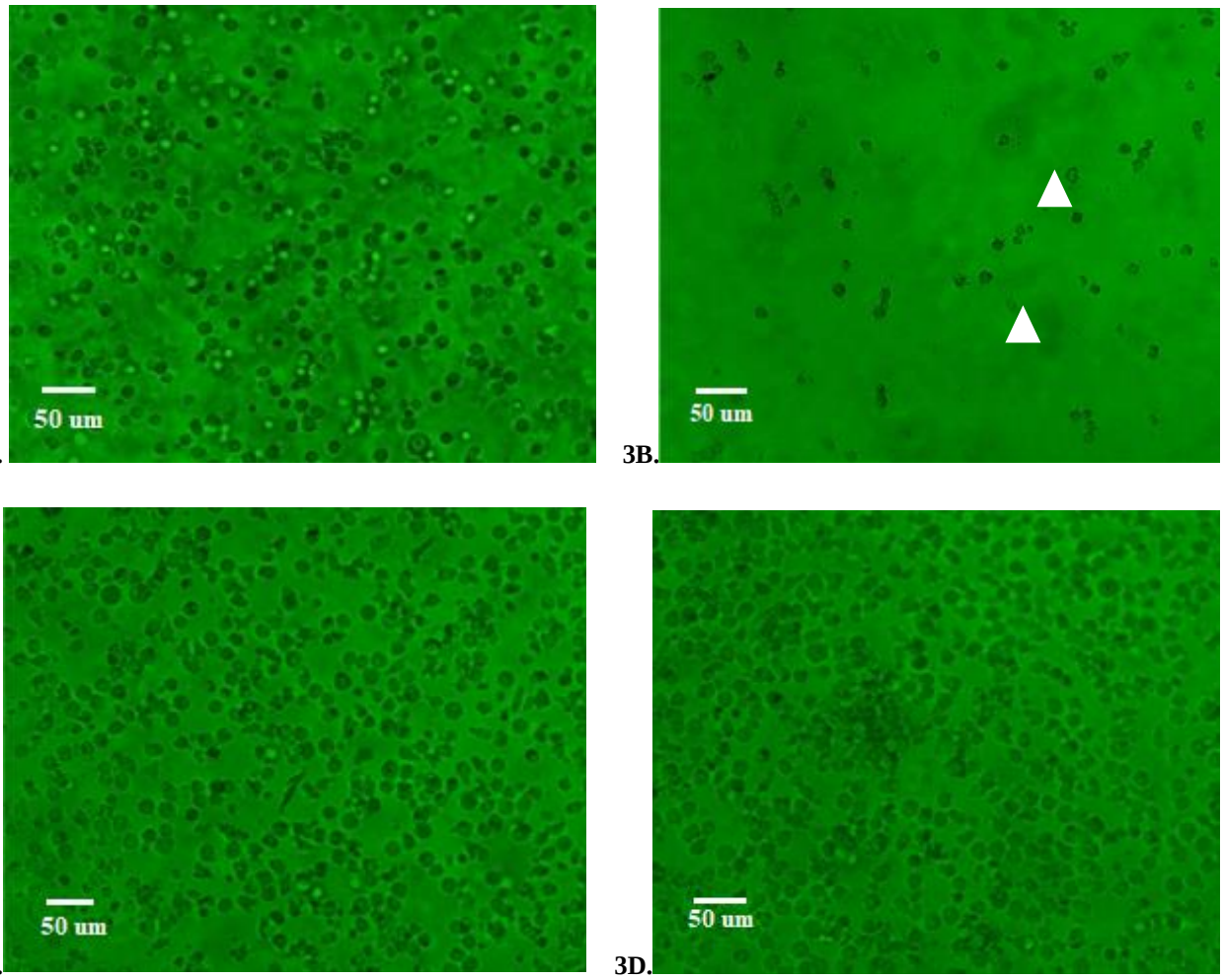
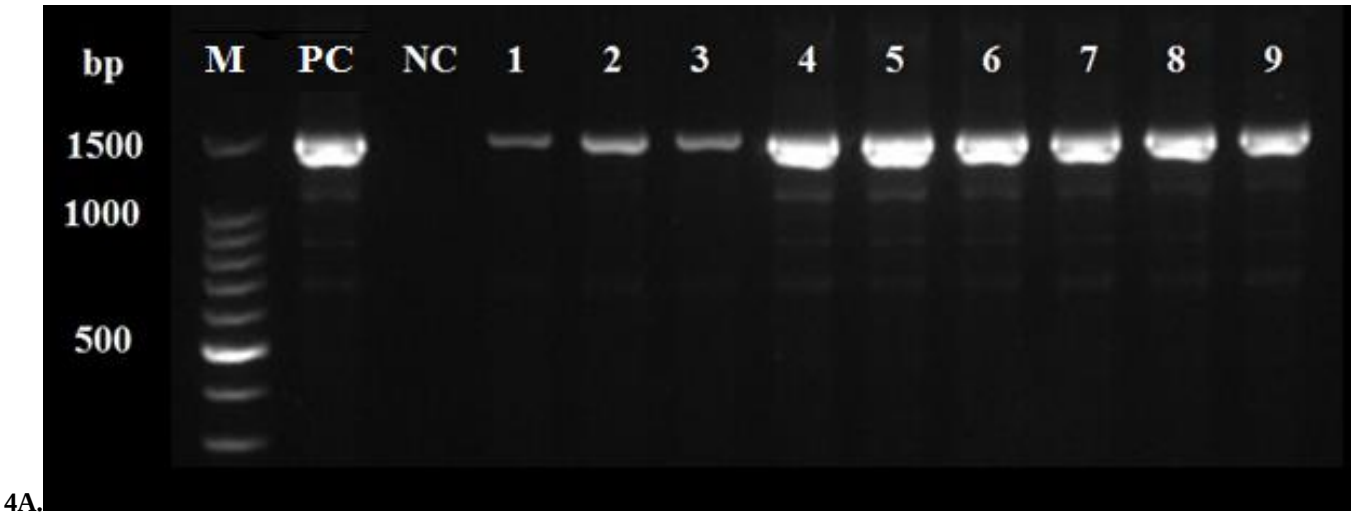



**Figure 3.** Representative MSB1cell cultivation microscopic observations at the 3rd passage. **3A:** Negative control- rounded and numerous cells, **3B:** Live-vaccine positive control, cellular swelling or wrinkling (pointers), apparently fewer cells, **3C:** M farm strain- rounded and numerous cells, **3D:** Y farm strain- rounded and numerous cells



**4B.**

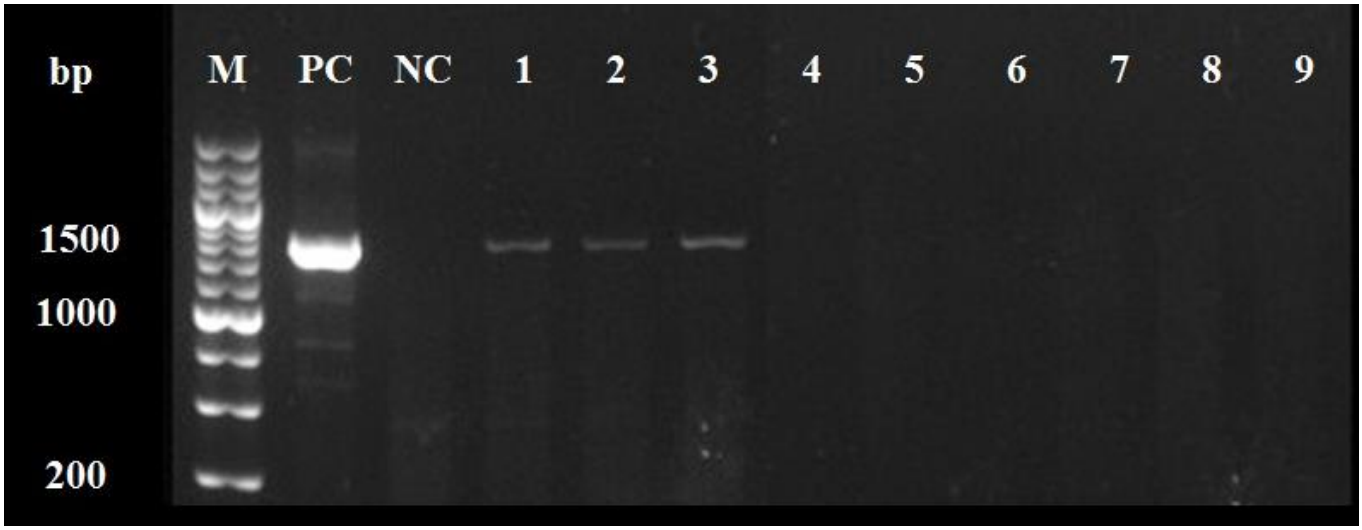



**Figure 4.** Representative FAdV-4 PCR results of CEF and MSB1 cultivation. 4A: CEF cultivation. Lane-M: 100bp ladder, Lane PC: positive control, Lane-NC: negative control, Lanes 1 to 3: KR5, M farm strain, and Y farm strain at 4th passage, Lanes 4 to 6: KR5, M farm strain, and Y farm strain at 5th passage, Lanes 7 to 9: KR5, M farm strain, and Y farm strain at 6th passage. 4B: MSB1 cultivation. Lane-M: 200bp ladder, Lane PC: positive control, Lane-NC: Negative control, Lanes 1 to 3: KR5, M farm strain, and Y farm strain at 2nd passage; Lanes 4 to 6: KR5, M farm strain, and Y farm strain at 8th passage, Lanes 7 to 9: KR5, M farm strain, and Y farm strain at 13th passage.

## DISCUSSION

Before the advent of molecular methods, virus identification and characterization relied mostly on biochemical properties as described in earlier studies (Kawamura et al., 1964; Otsuki et al., 1976). However, even today, these basic techniques have remained useful for a couple of reasons. They do not require too many technical skills, thus, are less challenging to perform. Moreover, not all laboratories are equipped with the necessary resources and equipment for molecular-based diagnosis. Finally, the results of biochemical tests can supplement molecular data.

Both M-farm and Y-farm strains are sensitive to 100% ethanol, 52˚C or higher, diluted formaldehyde, and lack HA activity. These observations are consistent with the properties of FAdVs from Japan (Otsuki et al., 1976), Korea (Park et al., 2011), and the prototype strains (Kawamura et al., 1964). Although the strains in the current study were already confirmed as FAdV4 (Del Valle et al., 2020a), the presented data adds to the characterization of these strains and provides a wider picture. The virus sensitivity is now known which might be helpful in controlling virus environmental contamination. In both M and Y farms, AAV infection was confirmed. Perhaps, hot water sprays and formaldehyde disinfection can be included in the farm's cleanup and disinfection protocol. Ruano et al. (2001) mention disinfectants that can be effective against FAdVs. Although the role of the M and Y farm strains in poor egg production is not yet proven, it may be useful to start environmental control measures. Ideally, this should be applied in both layer and replacement pullet houses. In another study, it was found that pullets from the farm supplying M and Y farms were positive for CAV (Del Valle et al., 2020b). Some of these CAV-positive pullets were also AAV-positive (Table 5). This indicated that the replacement pullets received by M and Y farms could already be infected by FAdV4. It would also be ideal to check the parent stock for FAdV infection. All control measures would be useless if the progeny are infected from the start.

Initially, it was unknown if the M and Y farm strains had any HA activity; a characteristic present in DAdV-A which is known as a viral pathogen with affinity to the chicken oviduct. Now, it is clear that they do not share this characteristic. The latter agglutinates avian RBC, which can aid in EDS diagnosis. The fiber protein is responsible for hemagglutination (Louis et al., 1994). Apparently, the FAdV4 isolates and DAdV-A fiber proteins have different properties. The negative HA results also confirmed the absence *Orthomyxovirus* and *Paramyxovirus* which can

also produce round cell CPE in CEF or CKC (McFerran and Smyth, 2000).

The field strains and KR5 readily produced characteristic CPE on CEF, as well as good PCR results throughout cultivation. This may be attributed to the fact that the strains underwent multiple passages in CKC prior to titration and inoculation. One observation was that CPE in CEF appeared 5-6 days post-inoculation while CPE in CKC was visible as early as 2-3 days. This was consistent from the first passage throughout the study, meaning that strains replicate faster in CKC. Although it takes a longer time for CPE to appear, the use of CEF seems to be adequate for cultivating the strains. Chick embryo fibroblast is easier to prepare, compared to CKC or CEL. Moreover, the use of 10 to 14-day-old SPF chick embryos is more humane, compared to sacrificing day-old SPF chicks. Chick embryos are considered non-sentient until 17 days of incubation (Ribatti, 2016).

The field strains and KR5 did not produce CPE on MDCC-MSB1 cells. Viral DNA in MSB1 fluid was detectable only until the second passage after which results were all negative. MSB1 cells are derived from an MD lymphoma (Akiyama and Kato, 1974) and have the characteristics of helper T-lymphocytes (Adair et al., 1993). Since pathogenic FAdV4 can induce apoptosis in T-cells (Niu et al., 2019), it was hypothesized that the strains would affect MSB1 cells. CPE in MSB1 is characterized by cell death and failure to passage. Microscopically, the normally round cells become wrinkled or swollen, and the growth will stop (Simeonov et al., 2014). Gross color change in growth media also occurs. RPMI-1640, which is the usual media, will turn pink upon cell death. This CPE was observed only in the CAV-live vaccine-inoculated wells. Taharaguchi et al. (2012) observed adsorption of FAdV1 on the non-susceptible cell lines Vero cell and Crandell-Rees Feline Kidney (CRFK) cell. However, the virus was only bound to the cellular surface without successful entry. Although no tests were performed to confirm it, perhaps the same occurred in the present study. FAdV4 may have adsorbed to the MSB1 cell surface only, but failed to induce endocytosis. The virus probably persisted in the media until it gradually disappeared.

The MSB1 cell line is used for the cultivation of CAV (Noteborn et al., 1994). Simeonov et al. (2014) cultivated CAV in MSB1 and observed CPE after three passages, and Yamaguchi et al. (2001) in 12. The FAdV4 strains in the current study cannot infect MSB1 even after 13 generations. The strains may not be virulent enough to cause CPE, or the cell line is not ideal for cultivation. To the best of the authors' knowledge, it is unknown if pathogenic FAdV4 strains can affect MSB1 cells which require future studies for further investigations. Cultivation of the virus in transformed cell lines may reduce the need for live animals. Some authors describe the use of QT35 (Schonewille et al., 2008) and LMH (Zhao et al., 2015) for cultivation. Unfortunately, these were unavailable at the time of experimentation.

## CONCLUSION

The field strain's behavior and properties are similar to other fowl adenovirus strains. These are sensitive to pure and diluted ethanol, 52˚C or higher, and diluted formaldehyde, they also lack hemagglutinating activity. Potentially, it is time to initiate environmental control measures in the farms, which could be supplemented with the knowledge of the viruses' sensitivity. The field strains, which are non-pathogenic fowl adenovirus serotype-4, cannot infect MSB1 cells in spite of multiple passages. However, there appear to be no reports on the effect of pathogenic fowl adenovirus serotype-4 on that cell line. Considering the fact that the effect on T-cells has been reported by other researchers, perhaps the interaction between pathogenic fowl adenovirus serotype-4 and MSB1 cells could be studied in the future.

## DECLARATIONS

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### Competing interests

The authors declare that they have no competing interests.

### Consent to publish

All authors contributed equally to the accomplishment of this work, and gave their informed consent prior to inclusion.

### Authors' contribution

All authors participated in the conceptualization, experimentation, interpretation of results, and preparation of this paper.

**Ethical considerations**

The authors confirm that all of the ethical issues and rules concerning: plagiarism, consent to publish, misconduct, data fabrication, and/or falsification, double publication and/or submission, and redundancy, have all been checked and adhered to.

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