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論　文　名	Molecular and pathologic characterization of avian adenovirus isolated from the oviducts of laying hens in eastern Japan （東日本における産卵鶏の卵管から分離されたトリアデノウイルスの分子的生物学及び病理学解析に関する研究）
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論文要旨

Introduction

Avian adenoviruses (AAVs) affect wild and domestic birds. Under the AAVs, are Fowl adenoviruses (FAdVs). The pathogenicity is complex and unclear, because FAdVs are reported in both healthy and sick birds. Also, non-pathogenic and pathogenic strains exist, of which the latter are implicated to disease.

Pathogenic FAdV4 mainly causes hydropericardium syndrome (HPS). Lesions include fluid accumulation in the pericardium and hepatitis. Viral load is highest in the liver, followed by the kidneys, spleen, and lungs. HPS can cause production losses due to decreased weight, depression, immune-suppression, morbidity, and mortality. Cardiac failure is the main cause of death, and mortality can reach 30% to 80%. Broilers and young birds, up to 5-week-old, have a higher risk. Older birds are resistant, but may harbor latent infection. Coinfection and synergism with other pathogens also occurs.

It appears that there are no previous reports of FAdV4 from the oviduct. In this study, two strains were isolated from the oviducts of layers. The results of genetic analysis, the biochemical properties, and the pathogenicity are described.

Chapter 1: Detection, isolation, and AAV genetic analysis

Two layer farms reported poor laying performance. Birds from M and Y farms, aged 44 and 26 weeks respectively, were at 82% egg production; lower than the ideal of 90-95%. Sampled

birds were apparently healthy, bacteria and *Mycoplasma spp.* isolation were negative. Histopathological examination of oviducts from Y farm showed mild inflammatory reaction and epithelial desquamation. PCR assays for infectious bronchitis virus (IBV) and egg-drop syndrome virus (EDSV) were negative. In M farm, out of 15 oviduct samples taken at 378 days and 381 days, 10 (66.67%) were AAV-positive. In Y farm, out of 30 oviduct samples at 199 days, 209 days, 212 days, 223 days, and 290 days, 13 (43.33%) were AAV-positive. Six bone marrow samples at 199 days, 209 days, and 212 days were chicken anaemia virus (CAV) -positive.

Primary chick kidney cell (CKC) was prepared from 3 to 7-days-old SPF chicks. Homogenized AAV-positive oviducts were inoculated into CKC, these were incubated in 5% CO_2 at 40˚C. The cells were observed 3 days post-inoculation (dpi) for CPE; passage and AAV-PCR were also performed. A non-pathogenic KR5 strain was used as positive control. At 5^{th} passage, the inoculated CKC cultures were AAV-PCR positive and CPE negative. At 6^{th} passage, these were both PCR and CPE positive. At 8^{th} passage, AAV-PCR products were used for nucleic acid sequencing of the hexon gene. The virus isolated from Y farm (Japan/Ibaraki/Y-H6/2016) showed 99.15% similarity with KR5. The virus isolated from M farm (Japan/ Ibaraki/ M-HB2/2016) was 99.57% similar to KR5. The two strains were 99.29% similar with each other. FAdV4-PCR assay for detection and sequencing of the fiber2 gene was also performed. The results also showed close homology with non-pathogenic FAdV4. Multiple alignment was performed on both hexon and fiber2 amino acid sequences. The results showed few mutations in the hexon and multiple mutations in fiber2.

M and Y farms have a common pullet source. Historically, the flocks in that pullet farm were monitored for CAV by PCR. Selected CAV-positive samples from the last 3 years were tested by AAV-PCR. The results indicated CAV-AAV coinfection at the pullet stage. PCR detection, isolation and nucleic acid sequencing confirmed FAdV4 from the oviduct of layers. Although the impact on performance is unconfirmed, isolation from the organ appears to be novel.

Chapter 2: Biochemical properties

Knowledge of viral properties can be useful. Possibly, viral sensitivity can be exploited for control. FAdVs can propagate in CKC, cultivation in chick embryo fibroblast (CEF) is also reported. Immunosuppression, in HPS cases, is linked to a negative impact on lymphocytes. The MDCC-MSB1 cell line is derived from lymphocytes. Perhaps, the use of CEF or MSB1 can eliminate the need for SPF chicks.

After 10 passages in CKC, viral fluids from each of the strains were diluted from 10^{-1} to 10^{-10} in Hanks solution. 6-well plates were prepared for each dilution. After inoculation, plates were incubated and checked for CPE 5 dpi. Computed titers of KR5, M strain, and Y strain were: $10^{5.25}TCID_{50}$/ml, $10^{5.40}TCID_{50}$/ mL, and $10^{6.75}TCID_{50}$/ml respectively.

The sensitivity and stability of the field strains were assessed. Viral fluids were subjected to different conditions such as: 100%, 70%, and 50% ethanol; 58˚C, 54˚C, 52˚C, and 50˚C heat; chloroform; ether; and diluted formaldehyde (1:2000), followed by titration. A decrease of 1 $\log_{10}$ in titer was considered sensitivity. The strains were sensitive to 100% ethanol, 52˚C and higher, and formaldehyde. These were stable against chloroform, ether, and 50˚C. Stability to 70% ethanol was variable. HA-test was performed using 0.5% chicken RBC in PBS. In a 96-well

microtiter plate, 50 µL of 2-fold serial dilutions of the strains was prepared. 50 µL RBC was added and allowed to react for 30 min. All of the three strains were HA-negative.

CEF was processed from 10-12 days old SPF eggs. Four 6-well plates were prepared, 1 plate each for M strain, Y strain, KR5, and negative control. Plates were inoculated with virus of known titers, and were observed for CPE 5-6 dpi. Cells were passaged 6 times with AAV/FAdV4-PCR performed every time. Inoculated CEF were CPE and PCR-positive 5 dpi on the 1st passage until the 6th. The infectivity to MSB1 cells was also assessed. MSB1 cells were cultivated in RPMI 1640 medium with 10% FBS. Five 24-well plates were prepared; 3 were inoculated with M strain, Y strain, and KR5. One plate was inoculated with Hanks, and the last one with $10^{2.3}TCID_{50}$ CAV-live-vaccine. These were observed for color change and CPE and passaged every 2 days. AAV/ FAdV4-PCR were performed on the 1st, 2nd, 3rd, 5th, 8th, and 13th passages. After 13 passages, cells were alive and CPE-negative. These were PCR-positive at the 1st passage, weak-positive at the 2nd, and negative at the 3rd and succeeding. CAV-vaccine inoculated cells were wrinkled or swollen at the 3rd passage, and dead at the 4th. Even after 13 passages, KR5, M strain, and Y strain cannot infect MSB1 cells.

The strains are sensitive against diluted formaldehyde, 100% ethanol, and 52˚C. Perhaps, environmental-contamination control can be initiated. Possibly, hot water spray or formaldehyde disinfection can be used during cleanup. The strains can propagate in CEF, but may take 5 days to produce CPE. These appear to have no affinity for MSB1 cells.

Chapter 3: Pathogenicity trials

Pathogenic FAdV4 can cause HPS in SPF chicks, as well as mortality in SPF embryos. Although sequencing revealed that the strains are closely related to non-pathogenic strains, the possibility of any effect on SPF embryos and chicks was checked.

Chorioallantoic-membrane (CAM) inoculation was performed on 9 days old SPF chick embryos. 37 eggs were divided into 3 groups; 11 each for KR5, M farm, and Y farm strains. The remaining 4 were negative control. At 4 dpi, 1 embryo (9.09%) from each virus-inoculated group died. At 5 dpi, all eggs were opened, and the lengths (cm) and weights (g) were measured. The results are as follows: M strain, length: 4.44 ± 0.49, weight: 3.39 ± 1.06; Y strain, length: 4.46 ± 0.19, weight: 3.52 ± 0.57; and KR5, length: 4.38 ± 0.34, weight: 3.28 ± 0.85. Statistical analysis showed that inoculated-embryos were significantly smaller ($p \leq 0.05$), hemorrhaging was also observed. 5 embryos and CAMs were selected from each group for AAV-PCR. The results of embryos are as follows: M strain, 3 of 5 (60%); Y strain, 1 of 5 (20%); and KR5, 2 of 5 (40%). The results of CAMs are as follows: for M strain, 5 of 5 (100%) positive; for Y strain, 5 of 5 (100%) positive; and for KR5, 5 of 5 (100%) positive.

Sixty 1-day old SPF chicks were divided into 4 groups; 15 birds each for KR5, M farm strain, and Y farm strain. The birds were given 0.1 ml of diluted virus (10^{-1}) orally. The remaining group was given Hanks solution as negative control. At 14 dpi no birds developed HPS, disease-related lesions were also absent at necropsy. The livers, hearts, and kidneys from each group were pooled as well as the duodenum, jejunum, and cecum. AAV/FAdV4-PCR were performed on pooled samples. All tests were negative.

Eight 400-d-old non-vaccinated commercial hens were reared and separated into 2 groups. Six were given 0.2 ml Y-farm strain IV, and 2 were given KR5 IV. Birds were observed for signs of HPS. At 5dpi and 10 dpi, half of the birds from each group were sacrificed and necropsied. No signs of HPS were observed. AAV-PCR was positive in pooled GIT only.

Virus-inoculated chick embryos were significantly smaller in size and weight, hemorrhaging and death also occurred. Because of vertical transmission, infection may be a greater concern for breeder operations. Oral inoculation of SPF chicks and commercial hens confirm that the strains do not cause HPS.

Conclusion

- This could be the first report of FAdV4 detection and isolation of from the oviduct of layers. Also, the birds may have been infected as pullets.
- The FAdV4 isolates are sensitive to diluted formaldehyde, 100% ethanol, and 52˚C. These strains lack HA activity.
- The strains can propagate in CEF, but not in MSB1.
- The virus is non-pathogenic to SPF chicks and commercial hens.
- Although a link to poor egg production was not evaluated, an effect on embryos was observed. The strains can cause stunting or mortality in infected embryos, thus, could be a bigger concern in breeding operations.

審査結果の要旨

家禽や野鳥から分離されるトリアデノウイルス（FAdV）は、12 の血清型に分類される。代表疾病として、筋胃びらん、心膜水腫症候群、および封入体肝炎があげられる。FAdV は環境中や健康な鶏に常在しており、多くは不顕性感染であるが、ストレスや環境要因、伝染性ファブリキウス嚢病ウイルス（IBDV）や鶏貧血病ウイルス（CAV）といった免疫抑制を引き起こすウイルスの混合感染など､様々な要因により発症すると報告されているが、その病原性の発現機構は複雑で不明である｡FAdV の中でも FAdV 血清型 4 型（FAdV4）は、心膜水腫症候群（HPS）の原因と考えられており、肝不全による急激な心嚢水貯留により起こる細胞浸潤によって重篤化すると報告されている。ウイルスは、肝臓での検出率が最も高く、次に腎臓、脾臓、肺で検出される。 HPS は、体重の減少、沈鬱、免疫抑制、罹患率および死亡率が高いことによる生産損失を引き起こす。心不全は死亡の主な原因であり、死亡率は 30～80％に達する可能性があることが報告されている。本ウイルスに対する感染リスクは、5 週齢までのブロイラーと産卵鶏で高く、高齢の鳥は抵抗力があると報告されているが、潜在的なリスクが潜んでいる可能性がある。FAdV4 は、過去に卵管からの検出の報告がなく、申請者は東日本の 2 養鶏場において、卵管から本ウイルスを分離した

ことから、本研究では、ウイルスの分子生物学的特性、生化学的特性、および病原性について解析した。

第 1 章では、産卵率の低下した 2 養鶏場（M、Y 農場）から採材した検体を定法に従い検査し、卵管からアビアデノウイルス（AAV)、骨髄から CAV を分離した。検体からは、細菌、マイコプラズマ及びその他のウイルスは分離されなかった。組織病理学的検査において、Y 農場から採材した卵管に軽度の炎症反応と上皮剥離を確認した。ウイルス学的検査において、FAdV4 の Hexon 及び fiber2 蛋白領域を標的とした PCR 検査を、卵管抽出物を用いて実施した結果、陽性であった。Y 農場と M 農場から分離されたウイルスは、非病原性 FAdV4（KR5 株）のヘキソン遺伝子及び fiber2 遺伝子と高い相同性を示し、さらに、初生雛鶏腎細胞（CKO）に接種、40℃で静置培養を行った結果、培養細胞に細胞変性効果（CPE）をもたらすことが確認された。ヘキソンと fiber2 のアミノ酸配列でマルチプルアラインメントを実施し、 その結果、ヘキソンの突然変異はほとんどなく、fiber2 の複数の突然変異であることを明らかにした。

第 2 章では、分離したウイルス株の生物学的性状を検討した。検査項目として、各種消毒薬に対する感受性（エタノール、クロロボルムエーテル、0.05%ホルムアルデヒドおよびエーテル)、温度感受性、 および赤血球凝集能検査（HA)を実施した。両ウイルス株は、52℃以上、100％エタノールと 0.05%ホルムアルデヒドに感受性であり、HA は陰性であった。

本症の蔓延を防御するためには、52℃以上の温水スプレー、または、ホルムアルデヒドでの消毒が有効であることが示唆された。さらに、マレック病リンパ腫由来樹立細胞株（MSB1）および鶏胎児線維芽細胞（CEF）に分離した 2 株を接種し、増殖を確認したところ、CEF では増殖が認められたが、MSB1 細胞においては認められないことを明らかにしている。

第 3 章では、分離したウイルス株の病原性について検討した。分離した 2 株の FAdV と非病原性の KR5 株を発育鶏卵及び SPF 鶏に接種して、病原性について検討したところ、発育鶏卵においては 3 株共にウイルスの増殖が確認されたが、SPF 鶏では病原性の発現は認められなかった。この結果から、本症例は CAV との混合感染により発病した可能性が高いことを見出している。

本研究において、国内で産卵低下を主訴とした産卵鶏の卵巣から FAdV を初めて分離した。本研究で分離した 2 株は報告されている非病原性の FAdV と性状や病原性が類似しており、当該農場において、CAV との混合感染により本症が発症した可能性が高いことを明らかにした。国内外を含め、類似の発症が報告されており、本研究で得た成果が、本症の発症機構の解明の一助となり、獣医ウイルス学および家禽疾病学の分野に重要な知見を提供するのみならず、本疾病の防疫を通して、養鶏産業へも貢献するものと考えられる。そのため、本論文の審査ならびに最終試験の結果と併せて、博士（獣医学）の学位を授与することを適当と認める。