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NOTE

Avian Pathology

Isolation and Molecular Characterization of Fowl Adenovirus and Avian Reovirus from Breeder Chickens in Japan in 2019–2021

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ABSTRACT. Fowl adenoviruses (FAdVs) and avian reoviruses (ARVs) are ubiquitous in poultry farms and most of them are not pathogenic, yet often cause damage to chicks. A total of 104 chicken fecal samples were collected from 7 farms of breeder chickens (layers and broilers) in Japan from 2019 to 2021, and yielded 26 FAdV plus 14 ARV isolates. By sequencing, FAdV isolates were classified as FAdV-1, 5 and 8b. ARV isolates were classified as genotype II, IV and V. These results suggest that FAdVs and ARVs are resident in the breeder chicken farms in Japan.

KEY WORDS: avian reovirus, fowl adenovirus, phylogenetic analysis, virus isolation

Fowl adenoviruses (FAdVs) and avian reoviruses (ARVs) are present in poultry farms worldwide, because they are transmitted vertically and horizontally, and are highly resistant to disinfectants [5, 15].

Although many isolates of FAdVs and ARVs have no apparent pathogenicity hence can be detected even in chickens without clinical symptoms [7, 15, 21], some of these pathogens cause significant economic loss. FAdVs cause inclusion body hepatitis (IBH), hepatitis-hydropericardium syndrome (HHS), and adenoviral gizzard erosion (AGE) [4, 19, 23]; while ARVs cause viral arthritis/tenosynovitis and are also associated in the development of malabsorption syndrome, and runting-stunting syndrome (RSS) [5, 9].

FAdVs and ARVs are non-enveloped viruses and are highly resistant to disinfectants. Under these circumstances, once these viruses invade poultry farms, it would be difficult to eliminate them. All in all, the viruses are regarded to be latent nationwide.

In the present experiment, the feces of reared chickens were investigated in order to clarify the viral contamination situation in breeder farms that require higher levels of biosecurity.

From 2019 to 2021, 104 fecal samples were collected from 7 breeder farms for layer or broiler chickens in Japan (Table 1). All were open houses (not windowless). Fresh fecal samples were collected from the floor of each compartment of different chicken houses housing symptom-free 5- to 64- week-old chickens and pooled for each compartment.

Fecal samples were homogenized in phosphate buffered saline (PBS, pH 7.4) to obtain a 10% suspension and centrifuged at 1,220 × *g* for 15 min. The supernatant was transferred to a sterilized polypropylene tube and ultra-centrifuged at 81,055 × *g* for 60 min to concentrate viruses. After centrifugation, the pellet was resuspended in 1.5 ml of PBS containing antibiotics 10,000 U/ml of penicillin, 10 mg/ml of streptomycin and 25 µg/ml of amphotericin B and incubated at room temperature for 1 hr. The suspension was transferred to a microtube, centrifuged at 17,400 × *g* for 3 min, and the resulting supernatant was taken in a screw-capped tube and stored at −80°C until use.

Primary chicken kidney cell (CKC) cultures were prepared according to the methods described by us [24]. The supernatants were inoculated into CKC, and the cytopathic effects (CPE) were observed. After harvesting, the cultures were frozen and thawed three times, then centrifuged at 1,220 × *g* for 15 min. The supernatants were divided into aliquots and stored at −80°C. Blind passages were performed three times or until apparent CPE appeared.

Samples which showed CPE were plaque cloned two times as described previously [24]. Two plaques were picked up from one sample at a time, so finally one sample consisted of four clones. After plaque cloning, they were passaged two to three times in CKC.

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PCR amplification was performed as described previously [13] using TaKaRa PCR Amplification Kit (Takara Bio Inc., Kusatsu, Japan). The harvested culture supernatant was used as a template DNA without extraction, and the following primer set for the hexon gene was used: HexF1, 5′-GAYRGYHGGRTNBTGGAYATGGG-3′ and HeXR1, 5′-TACTTATCNACRGCYTGRTTCCA-3′ [13]. The predicted size of the PCR products was approximately 800 bp. The PCR products were visualized on 1.5% agarose gels stained with ethidium bromide.

Viral RNA was extracted from the harvested culture using ISOGEN II (Nippon Gene Co., Ltd., Tokyo, Japan), following the manufacturer's instructions. Just before use, 2 µl of RNA was mixed with 1 µl of dimethyl sulfoxide (DMSO) (Wako Pure Chemical Industries, Co., Ltd., Osaka, Japan) together with 4.5 µl of redistilled water (dH_2O), and heated at 98°C for 5 min. Primer sets that have been previously described [1, 20, 26] were used to detect chicken astrovirus (CAstV), avian nephritis virus

Table 1. Number of samples collected from 2019 to 2021 and number of virus isolates

Broiler breeder farm (Prefecture name)	Year	Age (weeks)	Samples	FAdV	FAdV Serotype	ARV	ARV Genotype
A (Gifu)	2019	8	5 [a)]	5 [b)]	5 [c)]	0 [b)]	
		19	5	3	5	0	
		38	5	0		0	
		64	5	0		0	
B (Gifu)	2020	8	3	0		3	V [d)]
			3	0		3	II, V
		20	3	0		0	
			3	0		0	
		61	3	0		0	
			3	0		0	
	2021	13	3	1	5	0	
			3	1	5	0	
C (Nagano)	2020	30	3	0		0	
			3	0		0	
		39	3	0		1	II, V
			3	0		0	
		61	3	0		0	
			3	0		0	
	2021	35	3	0		0	
		56	3	0		0	
			3	0		0	
		45	1	0		0	
		60	1	0		0	
		64	1	0		0	
		Total	74	10		7	
Layer breeder farm (Prefecture name)	**Year**	**Age (weeks)**	**Samples**	**FAdV**	**FAdV Serotype**	**ARV**	**ARV Genotype**
D (Niigata)	2019	8	2	2	5	0	
			2	2	5	0	
	2020	30	2	0		1	IV
			2	0		2	IV
		50	2	2	8b	0	
			2	2	8b	0	
E (Niigata)	2019	10	2	2	5	0	
			2	2	5	0	
	2020	40	2	0		2	II
			2	0		2	II
F (Iwate)	2019	5	1	1	5	0	
G (Gifu)	2021	25	3	3	1	0	
			3	0		0	
		56	3	0		0	
		Total	30	16		7	

FAdV: fowl adenovirus; ARV: avian reovirus. a) The figures indicate the number of samples collected annually from each breeder farm, b) number of positive samples, c) FAdV serotype, d) ARV genotype.

(ANV), avian rotavirus, avian paramyxovirus (PAR) and ARV (S4 primer set) as described in Table 2. RT-PCR was carried out using PrimeScript One Step RT-PCR Kit ver.2 (Takara Bio Inc.). Briefly, 7.5 µl of DMSO-treated viral RNA was mixed with 1 µl of PrimeScript 1 step Enzyme Mix, 12.5 µl of 2 × 1-Step Buffer, 1 µl of the primer mixture, and 3 µl of RNase-Free dH_2O, totaling 25 µl and incubated at 44°C for 60 min, to make cDNA stop the reaction thereafter at 95°C for 15 min. Subsequently, the PCR reaction was performed with 35 cycles at 94°C for 45 sec, 48°C for 45 sec, and 72°C for 1 min, and a final incubation at 72°C for 10 min. The PCR products were visualized on 1.5% agarose gels stained with ethidium bromide.

For the sequencing of ARV, RT-PCR with primer set for S1 (P1 and P4) was performed as described previously [9]. The following primer set for the S1 gene was used: P1, 5′-AGTATTTGTGAGTACGATTG-3′ and P4, 5′-GGCGCCACACCTTAGGT-3′ [7]. The predicted size of the PCR products was approximately 1,088 bp.

The generated PCR products were sequenced directly. Two samples were selected from each flock and used for sequencing. The obtained PCR products were purified with QIAquick Gel Extraction Kit (Qiagen Inc., Tokyo, Japan), according to the manufacturer's instructions. The purified PCR products were sequenced from both directions using forward and reverse primers by Fasmac Corp. (Atsugi, Japan). The obtained sequence information was registered in the DNA Data Bank of Japan (DDBJ) of the National Institute of Genetics, and Accession numbers (FAdV: LC604651 to LC604666, LC637578 to LC637582, ARV: LC604637 to LC604650) were set. For comparison, ARV strains of Japanese prototype serotypes (CS-108, Uchida, OS-161, TS-17, TS-142) supplied kindly by National Institute of Animal Health (Tsukuba, Japan) and R-6 isolated in 1987 in Aomori prefecture in Japan were also used for RT-PCR and sequencing, and Accession numbers (LC604631 to LC604636) were set.

BLASTN searches were employed to investigate the sequence similarities between the isolated strains and reference strains in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic trees were constructed using MEGA-X software by the neighbor-joining (NJ) method with 1,000 bootstrap replicates.

In the present study, 26 FAdVs and 14 ARVs were isolated from 104 fecal samples of chickens as shown in Table 1. No viruses other than FAdVs and ARVs were detected or isolated. Infectious bronchitis virus (IBV) and infectious bursal disease virus (IBDV), which are commonly isolated in chicken farms, were not investigated in the present study. The virus isolations were conducted in CKC. FAdV isolates showed round type CPE, and ARV isolates showed fusion type CPE.

With the exception of samples collected from breeder farm C in 2021, either FAdV or ARV was isolated at the time of sample collection. Different viruses were isolated in one breeding farm, rather than the same serotype or genotype virus being isolated. The same serotype or genotype virus was often isolated in the same breeder farm at the same time. However, at the breeder farms B and C in 2020, two types, ARV-II and V, were isolated. When flocks in which the virus was isolated in even one sample were considered positive, FAdV was isolated at 31.6% and ARV at 18.4% (Table 3). In broilers, both FAdV and ARV were isolated at a relatively young age before the start of laying. In layers, FAdV was isolated at various ages and ARV was isolated after the onset of laying.

Table 2. Primers used in this study

Virus	Primer	Sequence (5′-3′)	Product size (bp)	Reference
Fowl adenovirus	HexF1	GAYRGYHGGRTNBTGGAYATGGG	800	[13]
	HeXR1	TACTTATCNACRGCYTGRTTCCA		
Chicken astrovirus	CASpol1F	GAYCARCGAATGCGRAGRTTG	362	[1]
	CASpol1R	TCAGTGGAAGTGGGKARTCTAC		
Avian nephritis virus	ANVpol1F	GYTGGGCGCYTCYTTYGAYAC	473	[1]
	ANVpol1R	CRTTTGCCCKRTARTCTTTRT		
Avian rotavirus	NSP4-F30	GTGCGGAAAGATGGAGAAC	630	[1]
	NSP4-R660	GTTGGGGTACCAGGGATTAA		
Avian paramyxovirus	PAR-F1	GAAGGITATTGTCAIAARNTNTGGAC	580	[20]
	PAR-R	GCTGAAGTTACIGGITCICCDATRTTNC		
Avian reovirus	S4-F13	GTGCGTGTTGGAGTTTCCCG	1,120	[26]
	S4-R1133	TACGCCATCCTAGCTGGA		
	P1	AGTATTTGTGAGTACGATTG	1,088	[7]
	P4	GGCGCCACACCTTAGGT		

International Union of Biochemistry codes used: Y=C or T, R=A or G, H=A, C, or T, N=A, C, G, or T, B=C, G, or T, K=G or T, D=A, G, or T.

Table 3. Number of positive flocks in relation to the age

Age (weeks)	Broiler breeder			Layer breeder		
	Flock	FAdV (%)	ARV (%)	Flock	FAdV (%)	ARV (%)
1–20	8 [a]	4 [b] (50.0)	2 (25.0)	5	5 (100)	0 (0.0)
21–40	6	0 (0.0)	1 (16.7)	6	1 (16.7)	4 (66.7)
40<	10	0 (0.0)	0 (0.0)	3	2 (66.7)	0 (0.0)
Total	24	4 (16.7)	3 (12.5)	14	8 (57.1)	4 (28.6)

FAdV: fowl adenovirus; ARV: avian reovirus. a) Number of flocks, b) number of flocks in which the virus was isolated from at least one sample.

Samples showing round type CPE were confirmed to be FAdV positive by PCR using the primer set for the hexon gene. The results of the phylogenetic analysis of FAdV isolated strains and reference strains are shown in Fig. 1. FAdV isolates were classified into three different clusters, FAdV-1 (FAdV-A), FAdV-5 (FAdV-B) and FAdV-8b (FAdV-E).

Two isolates were classified into FAdV-1. These isolates shared 99.9–100% sequence similarity with strain JM1/1 [25] which was isolated from broiler chickens with AGE in 2000 in Japan, and 98.9–99.1% similarity with strain Ote [8, 14].

As for FAdV-5, six isolates had a close relationship with the FAdV-5 reference strain 340. The other nine strains had a close relationship with strain 40440-M/2015, a variant isolated from diseased chickens in Hungary [6]. The former had 99.6% similarities with strain 340 and 92.9% similarities with strain 40440-M/2015. On the other hand, the latter had 92.2–92.9% similarities with strain 340 and 99.7–99.9% similarities with strain 40440-M/2015.

The remaining four isolates were classified into FAdV-8b, including the FAdV-8b reference strains 764 and HG. Japanese FAdV-8b strains preceding 2010 (ZK-4, TR630, and JP/Mie/2009IBH) [12] were also found affiliated with the same cluster. The isolates in the present study showed sequence similarity of more than 99% with the above strains.

Samples showing fusion type CPE were confirmed to be ARV positive by RT-PCR using the primer sets for the S4 and S1 genes. The results of the phylogenetic analysis of ARV isolated strains and reference strains are shown in Fig. 2. Phylogenetic analysis of the S1 gene revealed that ARV strains could be divided into six genotypes. Japanese prototype ARVs were each classified into different clusters, as Mase *et al.* described [11]. Strain R-6 was classified into genotype I with TS-142. Six strains were classified into genotype II. Four strains were clustered into genotype IV with strain OS-161. The remaining four strains were clustered into genotype V with strain Uchida.

FAdVs and ARVs are associated with important diseases in the poultry industry, but they are also isolated from chickens without any clinical symptoms [7, 15, 21]. In the present study as well, FAdVs and ARVs were isolated from the feces of chickens having no clinical symptoms. Except for the samples collected from breeder farm C in 2021, either FAdV or ARV was isolated at the time of sample collection, and it was considered that FAdVs and ARVs were resident in breeder farms. Since the same serotype or genotype viruses were not continuously isolated in one breeder farm, but different viruses were isolated, it was also suggested

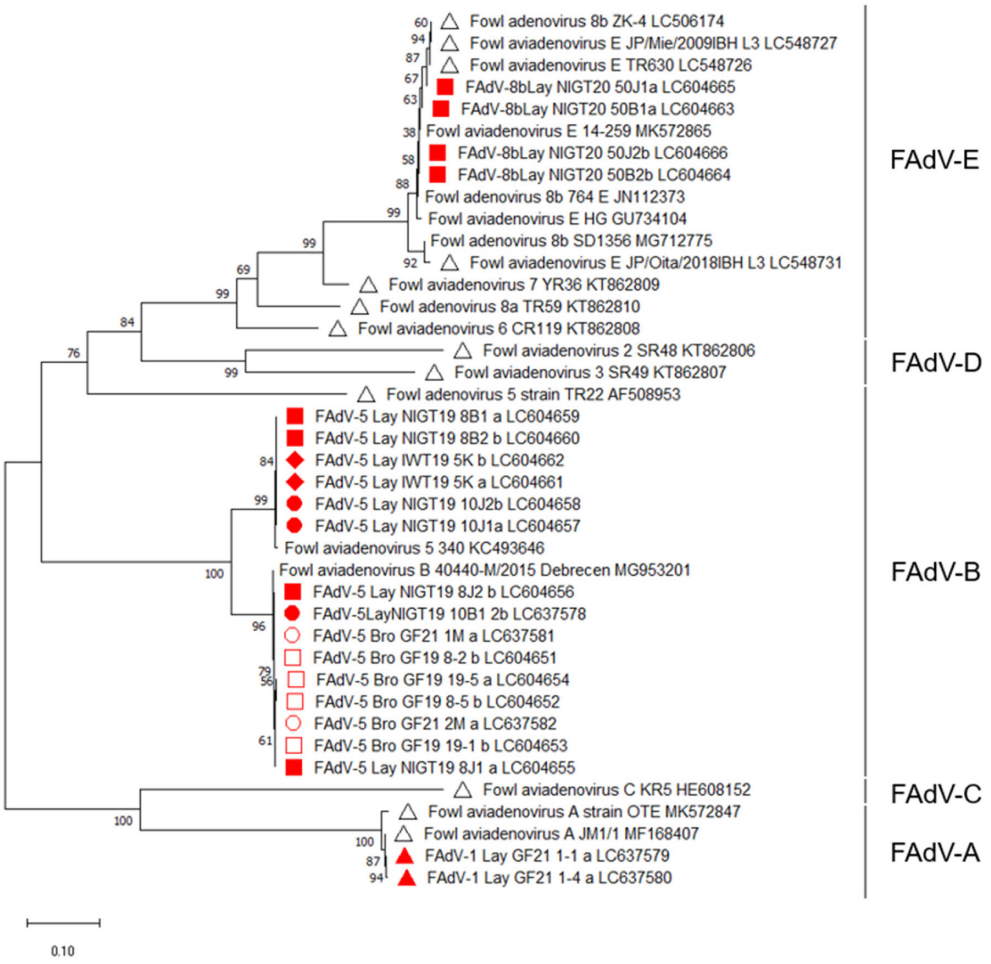

Fig. 1. Phylogenetic tree of fowl adenovirus (FAdV) strains based on the nucleotide sequence data of the hexon gene. Phylogenetic trees were constructed using MEGA-X software by the neighbor-joining (NJ) method with 1,000 bootstrap replicates. The marks are as follows; white color: broiler breeder farm (square: farm A, circle: farm B), red color: layer breeder farm (square: farm D, circle: farm E, diamond: farm F), white triangle: Japanese FAdV strains (GeneBank).

that new viruses were invading from outside of the farms. In many cases, viruses of the same serotype and genotype viruses were isolated at the same time in the same breeding grounds as shown in Table 1. It was described by Mirzazadeh *et al.* that FAdV serotypes are in most cases identical within the same farm [16]. In the samples collected from the breeder farms B and C in 2020, two types, ARV-II and V, were separated, and it was thereby confirmed that multiple isolates may exist at the same time in a given breeder farm.

In broilers, both FAdV and ARV were isolated at a relatively young age before the start of laying (Table 3). The virus isolation rate from adult chickens is lower than that of young chickens, and this is considered to be due to the higher proportion of adult chickens that have acquired immunity to FAdV in addition to age resistance [18]. Mirzazadeh *et al.* reported that a negative correlation was found between the viral load of fecal shedding and the level of antibodies [16]. In layers, FAdV was isolated even at an older age (Table 3), suggesting the possibility of FAdV invasion from the outside. ARV was isolated after the start of laying, and it was considered that viral shedding increased due to the stress of egg production [22].

Each of the 12 FAdV serotypes has been associated with outbreaks of IBH [21], especially FAdV-2, 8a, 8b and 11 [23]. In the present study, FAdV-1, 5 and 8b were isolated. Although FAdV-5 strains are not often isolated from sick chickens, they have been found in chickens with IBH, lameness or swelling of the tarsal joint [10, 17]. In Japan, FAdV-8b strains were isolated from chickens with IBH recently [12]. They were identical to SD1356 which was isolated in China [3]. However, FAdV-8b isolates in the present study were different from them and yet similar to strains ZK-4, TR630 and JP/Mie/2009IBH, which were isolated before 2010 in Japan. This suggests that FAdV-8b isolates in the present study are indigenous to Japan. FAdV-1 isolates are often obtained from chickens with AGE, although FAdV-8 has also been isolated on occasions [14]. FAdV-1 isolates in our study shared higher sequence similarity with strain JM1/1 [25].

Although the relationship between ARV genotypes and pathotypes is unknown, ARVs show a wide heterogeneity in pathogenicity [2, 7]. ARVs cause viral arthritis/tenosynovitis, malabsorption syndrome, and runting-stunting syndrome (RSS) [5, 9]. Moreover, even isolates classified into the same genotypic cluster have different pathogenicity, and these differences might

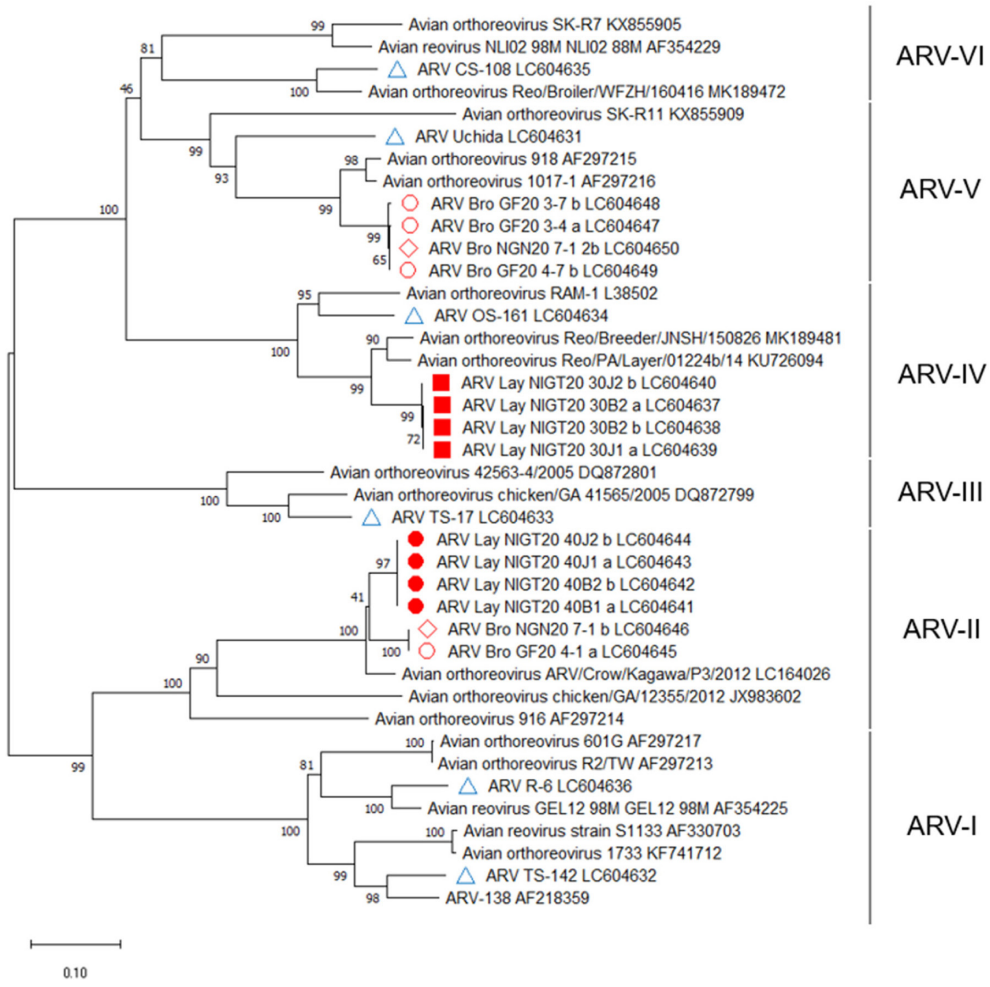

Fig. 2. Phylogenetic tree of avian reovirus (ARV) strains based on the nucleotide sequence data of the S1 gene. Phylogenetic trees were constructed using MEGA-X software by the neighbor-joining (NJ) method with 1,000 bootstrap replicates. The marks are as follows; white color: broiler breeder farm (circle: farm B, diamond: farm C), red color: layer breeder farm (square: farm D, circle: farm E), white triangle: Japanese prototype ARV strains and strain R-6 (these were sequenced in the present study).

be associated with genes other than S1 [2]. Further investigation is necessary to verify the relationship between genotype and pathogenicity.

In the present study, six isolates of genotype II were similar to the strain isolated from a crow in 2012 in Kagawa Prefecture in Japan. Therefore, it is important to avoid contact with wild birds and wild mammals. Since FAdVs and ARVs are transmitted vertically as well as horizontally [5, 15, 27], higher biosecurity is required at breeding chicken farms.

In conclusion, FAdVs and ARVs are significantly resident in poultry farms both clinically and sub-clinically, and it is hence required to strengthen biosecurity.

POTENTIAL CONFLICTS OF INTEREST. The authors have nothing to disclose.

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