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NOTE

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Identification of specific serotypes of fowl adenoviruses isolated from diseased chickens by PCR

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ABSTRACT. We have developed a polymerase chain reaction (PCR) assay to facilitate detection of the major disease-associated serotypes of fowl adenovirus (FAdV) including serotypes 1, 2, 4, 8a and 8b; primers were designed based on serotype-specific sequences of the hexon gene. We tested field isolates from chickens diagnosed with inclusion body hepatitis, gizzard erosion and hydropericardium syndrome together with reference FAdV strains characterized in Japan. We found that the primers were serotype specific; appropriate amplification of serotype-specific hexon genes was confirmed by sequence analysis of the PCR products. This PCR assay will be useful for detection of FAdV and for differentiation between disease-associated serotypes.

KEY WORDS: detection, fowl adenovirus, polymerase chain reaction, serotype-specific

Fowl adenoviruses (FAdVs) are non-enveloped, double-stranded DNA viruses belonging to the genus Aviadenovirus. FAdVs are classified according to their serological relationships; 12 fowl serotypes (including 8a and 8b) have been recognized to date [2]. These 12 serotypes have been grouped into five species (FAdV A–E) [2]. FAdVs can be isolated from both sick and normal birds and are distributed widely in poultry farms throughout the world [2]. Gizzard erosion (GE), inclusion body hepatitis (IBH), and hydropericardium syndrome (HPS) are diseases of chickens that result from infection with FAdVs [2, 17]; serotypes 1, 8a and 8b were isolated from chickens with GE, serotypes 2, 8a and 8b from chickens with IBH, and serotype 4 from those with HPS [1, 3, 16]. Epidemiologic analyses focused on FAdVs isolated from chickens with clinical signs suggestive of IBH, GE and HPS have recently been reported [8–10]; tendencies similar to these have been confirmed in Japan.

Serotyping of FAdVs has been classically conducted by virus neutralization assays using antisera panels. This test is both labor-intensive and time-consuming. Moreover, many laboratories do not have a panel of reference strains and strain-specific antisera and are thus unable to characterize isolated viruses. To overcome this problem, the polymerase chain reaction (PCR)−based methods have been developed to facilitate detection of FAdV [4, 13, 15, 20]; the serotypes are identified by sequence analysis of the amplification products and/or by using restriction fragment length polymorphisms (RFLPs) [13]. However, RFLP analysis may yield different patterns for viruses of the same serotype, thus precluding accurate interpretation of the results [12]. As such, DNA sequence analysis has been recognized as a more reliable [7]. However, as very few local diagnostic facilities have sequence analyzers, identification and typing of FAdV would require access to specialized laboratories and research institutions.

To address this problem and in an effort to obtain simple and clear results using tools and methods available at local diagnostic laboratories, our goal in this study was to devise a method that relies on conventional PCR and gel-electrophoresis that will facilitate not only detection of FAdV, but would permit the laboratory to distinguish between the major disease-associated serotypes. For verification purposes, we tested our method on both reference FAdV strains used in Japan and field FAdVs isolated from chickens diagnosed with IBH, GE and HPS.

The reference strains of each of the serotypes of FAdV as defined by Kawamura [5] were used in this study, including Ote (serotype 1), SR-48 (serotype 2), SR-49 (serotype 3), KR-5 (serotype 4), TR-22 (serotype 5), CR-119 (serotype 6), YR-36 (serotype 7), and TR-59 (serotype 8a) [5]. The TR-630 strain, which was isolated from chickens with IBH in 1972 [11], was identified as serotype 8b by sequence analysis of the gene encoding the virus hexon protein [11]. We also tested our assay on field

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FAdV isolates that were collected as part of routine virological diagnosis between the years 1996 and 2019; these are listed in Table 1. All FAdVs were grown in primary chicken kidney cell (CKC) cultures. The culture supernatants were harvested, subjected to centrifugation, and divided into aliquots prior to frozen storage at −80°C. These aliquots were used in the experiments to follow.

Viral DNAs were extracted from infected culture fluids using the QIAamp DNA Micro Kit (Qiagen Inc., Valencia, CA, USA). Each primer was designed based on the reported sequences of genes encoding hexon proteins of each type of FAdV. Hexons are composed of virion capsomer with pentons and determinant of serotypes [2]. PCR was performed with 30 cycles at 94°C for 30 sec, 50°C for 30 sec, and 72°C for 30 sec. The primer sequences and the predicted size of each type-specific amplification product are shown in Table 2.

Our first set of experiments targeted the reference serotype strains from Japan. We confirmed that the primers amplified sequences of the expected length in each of the target strains without amplification of nonspecific products. To determine whether there was any cross-reactivity using these primer sets, we attempted to amplify non-target types with each primer pair. As anticipated, type-specific primer pairs did not amplify hexon gene sequences of non-target types (Fig. 1A–E).

We then applied this PCR assay to field FAdV strains derived from chickens diagnosed with IBH, GE and HPS in Japan. PCR amplification products were detected only when isolated were targeted with type-specific primer pairs (Fig. 2A–E). To confirm specific amplification of type-specific hexon genes with each of the primer pairs, all amplification products were subjected to DNA sequencing. Via this method, we confirmed that all amplification products were type-specific and derived from the targeted FAdVs.

As a further test of our method, we performed PCR on isolates in which mixed infection with multiple types of FAdV was suspected; mixed infections with multiple FAdV types have been identified in the field. We performed PCR on a sample derived

Table 1. Fowl adenoviruses isolated from diseased chickens used in this study

Virus	Clinical diagnosis	The results of typing by PCR					Reference
		1	2	4	8a	8b	
Strains identifid in previous studies*							
JP/Kagawa/2000GE (1)	Gizzard Erosion	+	-	-	-	-	Mase *et al.* [7]
S-PL1 (2)	Inclusion body hepatitis	-	+	-	-	-	Mase *et al.* [7]
JP/Gunma/2009IBH (2)	Inclusion body hepatitis	-	+	-	-	-	Mase *et al.* [9]
JP/Tokushima/2010IBH (2)	Inclusion body hepatitis	-	+	-	-	-	Mase *et al.* [9]
JP/Saga/97 (8a)	Inclusion body hepatitis	-	-	-	+	-	Mase *et al.* [7]
JP/LVP-1/96 (4)	Hydropericardium syndrome	-	-	+	-	-	Mase *et al.* [8]
H-1 (4)	Hydropericardium syndrome	-	-	+	-	-	Mase *et al.* [8]
PARC-97 (4)	Hydropericardium syndrome	-	-	+	-	-	Mase *et al.* [8]
PARC-98 (4)	Hydropericardium syndrome	-	-	+	-	-	Mase *et al.* [8]
JP/Oita/2018IBH (8b)	Inclusion body hepatitis	-	-	-	-	+	Mase *et al.* [11]
JP/Tokushima/2019IBH (8b)	Inclusion body hepatitis	-	-	-	-	+	Mase *et al.* [11]
Field isolates in this study							
JP/Mie/2009IBH	Inclusion body hepatitis	-	-	+	-	-	This study
JP/Kagoshima/2010IBH	Inclusion body hepatitis	-	+	-	-	-	This study
Mixed infected cases for this study							
JP/Sga/98	Inclusion body hepatitis	-	+	-	+	-	This study

*Serotypes shown in parenenses.

Table 2. PCR primers used in this study

Type	Direction	Sequence (5′-3′)	Genomic position	Length (bp)
1	Forward	ATTTTCAACACCTGGGTGGAGAGCA	18691–19518 (in CELO, GenBank Acc. No.U46933)	828
	Reverse	CACGTTGCCCTTATCTTGC		
2	Forward	CCCAATATGATTCTACAGTCCA	21670–22388 (in SR-48, GenBank Acc. No.KT862806)	719
	Reverse	GAGATGGGTATTGTGGGTTCGTATTCGG		
4	Forward	CCAACGCCACTACCAACT	21567–21856 (in KR-5, GenBank Acc. No.HE608152)	290
	Reverse	CCAGTTTCTGTGGTGGTTG		
8a	Forward	TAACCCCTATGAGAATACCACT	20682–21063 (in TR-59, GenBank Acc. No.KT862810)	382
	Reverse	ATTGACCGTTCCGTACTCGAT		
8b	Forward	AAGAACGAGGCGCAAAACACAGCTA	20631–20891 (in 764, GenBank Acc. No.KT862811)	261
	Reverse	GTCTAACACGTAGTAAGGCGTTGTTCCA		
All	Forward	GAYRGYHGGRTNBTGGAYATGGG	18571–19361 (in CELO, GenBank Acc. No.U46933)	~900 [7]
	Reverse	TACTTATCNACRGCYTGRTTCCA		

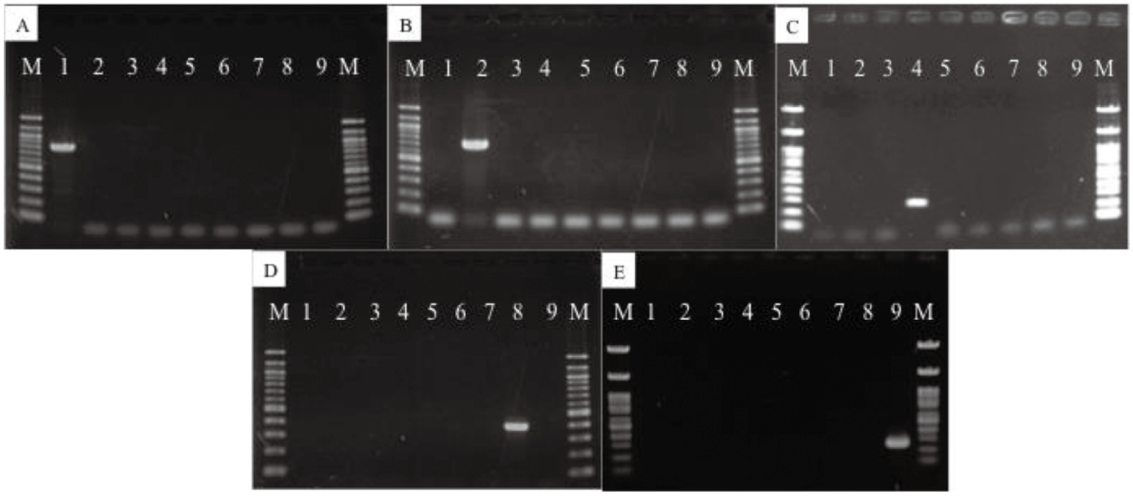

Fig. 1. Agarose gel electrophoresis of amplification products from the reference fowl adenovirus strains using type-specific primers. Panels A, B, C, D and E include the results of primer-specific PCR-amplification applied to types 1, 2, 4, 8a and 8b, respectively. Lane M, 100bp molecular weight marker DNA ladder; lane 1, Ote; lane 2, SR-48; lane 3, SR-49; lane 4, KR-5; lane 5, TR-22; lane 6, CR-119; lane 7, YR-36; lane 8, TR-59; lane 9, TR630.

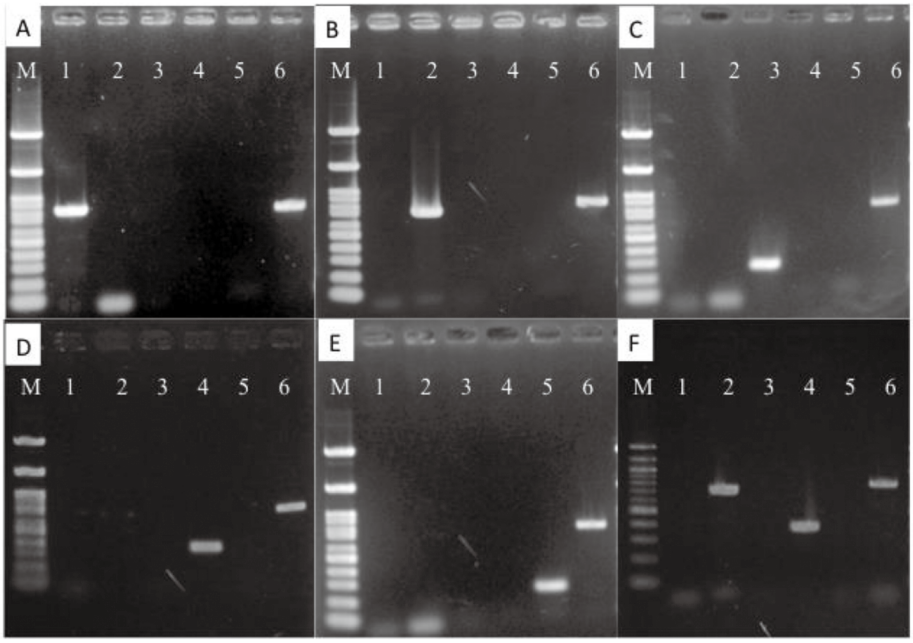

Fig. 2. Agarose gel electrophoresis of amplification products from field fowl adenovirus (FAdV) strains using serotype-specific primers. Field isolates include (A) JP/Kagawa/2000GE, (B) JP/Tokushima/2010IBH, (C) JP/LVP-1/96 (D) JP/Saga/97, (E) JP/Oita/2018IBH and (F) JP/Saga/98. Lane M, 100 bp molecular weight marker DNA ladder; lane 1, primers for type 1; lane 2, primers for type 2; lane 3, primers for type 4; lane 4, primers for type 8a, lane 5, primers for type 8b; lane 6, consensus primers for FAdV.

from the liver of a chicken diagnosed with IBH; this sample was collected in the Saga prefecture in 1998 and has been stored our laboratory since then. In this case, previous analysis using RFLP method suggested the existence of multiple FAdV types; this was later confirmed (types 2 and 8a) by cloning via plaque assays. As anticipated, these two types (2 and 8a) detected using our PCR assay (Fig. 2F). The nucleotide sequences of each PCR products were analyzed, and were confirmed to be 2, 8a type, respectively. This is a critical point, as this finding is not unusual in field cases of fowl adenovirus infection; we have shown clearly that this method can be used to detect individual components contributing to mixed type FAdV infection.

PCR assays have been developed that facilitate detection of many major disease-associated viruses. Among the best-known of these assays are those used for typing the hemagglutinin (HA) and neuraminidase (NA) proteins of avian influenza virus. In these cases, specific primers have been designed for unique amplification of all relevant virus types [6, 19]. Similarly, type-specific PCR primers that target the chicken pathogen, *Erysipelothrix rhusiopathiae* (ER), have also been developed [18]. ER has been divided

into numerous serotypes; those frequently isolated from clinical samples include serotypes 1a, 1b, 2 and 5. In this study, primers were designed that were capable of detection and specific serotype identification of using a conventional PCR assay.

Here, we designed PCR primers capable of distinguishing between the disease-associated types of FAdV. We used these primers successfully in our efforts to detect FAdV with type-specificity. FAdV is classified into 12 serotypes, but the serotype associated with the disease in chickens is limited recently. We previously described the specific types that were isolated in recent FAdV-associated outbreaks in chickens [8–10]. We found that type 1 FAdVs were isolated most frequently from chickens diagnosed with GE and type 2 FAdVs were predominantly associated with cases of IBH. In addition, types 8a and 8b FAdVs are sometimes isolated from these cases of GE or IBH. Similar trends have been observed in cases from abroad [1, 16, 17]. Appropriate diagnosis is critical, as IBH outbreaks continue to present problems in Japan [9, 11]. Type 2 (SR-48 type) viruses have been the major isolates from chickens diagnosed with IBH since 2009 in Japan [14]; however, since 2018, the isolation of type 8b (TR-630 type) in association with IBH has been increasing [11].

In conclusion, we have developed a conventional gel-based PCR assay that can be used for simultaneous detection of and differentiation between the major FAdV types (1, 2, 4, 8a and 8b) associated with disease in chickens. We are currently collecting additional field isolated as part of our ongoing efforts to evaluate this method as well as to advance our understanding of the epidemiology of FAdV.

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

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