研究成果の刊行に関する一覧表

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著者	タイトル	誌名	巻号	頁	発表年
Okuda H, **Ohya K**, Shiota Y, Kato H, **Fukushi H**	Detection of *Chlamydophila psittaci* by Using SYBR Green Real-Time PCR.	J. Vet. Med. Sci.	73 (2)	249-254	2011
Ohya K, Okuda H, Maeda S, Yamaguchi T, **Fukushi H**	Using CF0218-ELISA to distinguish *Chlamydophila felis*-infected cats from vaccinated and uninfected domestic cats.	Vet. Microbiol.	146 (3-4)	366-370	2010
Katoh H, Ogawa H, **Ohya K**, **Fukushi H**	A Review of DNA Virus Infections in Psittacine Birds.	J. Vet. Med. Sci.	72 (9)	1099-1106	2010
Katoh H, **Ohya K**, Ise K, **Fusushi H**	Genetic Analysis of Beak and Feather Disease Virus Derived from a Cockatiel (*Nymphicus hollandicus*) in Japan.	J. Vet. Med. Sci.	72 (2)	631-634	2010
Kasem S, Yu MH, Yamada S, Kodaira A, Matsumura T, Tsujimura K, Madbouly H,	The ORF37 (UL24) is a neuropathogennicity determinant of equine herpesvirus 1 (EHV-1) in the mouse	Virology	400 (2)	259-270	2010

Yamaguchi T, **Ohya K**, **Fukushi H**	encephalitis model.				
Ogawa H, Katoh H, Sanada N, Sanada Y, **Ohya K**, Yamaguchi T, **Fukushi H**	A novel genotype of beak and feather disease virus in budgerigars (Melopsittacus undulatus).	Virus Genes	41 (2)	231-235	2010
Murao W, Wada K, Matsumoto A, Fujiwara M, **Fukushi H**, Kishimoto T, Monden K, Kariyama R, Kumon H	Epidemiology of *Chlamydophila caviae*-like *Chlamydia* Isolated from Urethra and Uterine Cervix.	Acta Med Okayama	64 (1)	1-9	2010
福士秀人	鳥類のクラミジア感染症	INK+	8	8-9	2010

<u>NOTE</u> ***Bacteriology***

Detection of *Chlamydophila psittaci* by Using SYBR Green Real-Time PCR

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(Received 25 May 2010/Accepted 24 September 2010/Published online in J-STAGE 8 October 2010)

ABSTRACT. *Chlamydophila psittaci* is the causative agent of human psittacosis and avian chlamydiosis. This zoonotic pathogen is frequently transmitted from infected birds to humans. Therefore proper and rapid detection of *C. psittaci* in birds is important to control this disease. We developed a method for detecting *C. psittaci* by using SYBR Green Real-time PCR based on targeting the cysteine-rich protein gene (*envB*) of *C. psittaci*. This one step procedure was highly sensitive and rapid for detection and quantification of *C. psittaci* from fecal samples. This assay was also able to detect other zoonotic *Chlamydophila* species such as *C. abortus* and *C. felis*. The assay is well suited for use as a routine detection method in veterinary medicine.
KEY WORDS: avian chlamydiosis, *Chlamydophila psittaci*, real-time PCR, SYBR Green dye.

J. Vet. Med. Sci. 73(2): 249–254, 2011

Bacteria of the family Chlamydiaceae are obligate intracellular bacterial pathogens that cause various diseases in a wide range of animals including human. Among them, *Chlamydophila psittaci*, the causative agent of psittacosis in human and avian chlamydiosis, is the most important zoonotic pathogen, which is frequently transmitted by aerosols from infected birds. *C. psittaci* has been isolated from more than 460 avian species of 30 orders [15]. Parrot, parakeet and feral pigeon are the best known as representative natural hosts. Recently, we reported that 6.0% of pet birds (11 avian orders) in Japan were positive for *C. psittaci* [3]. Wild birds, especially pigeon, can serve as a source of human psittacosis [18, 27]. Avian chlamydiosis can exist as an inapparent infection. Hence birds can be carriers to human.

The clinical signs of psittacosis in human are influenza-like symptoms. Without appropriate treatment, this infection occasionally leads to severe respiratory disease and fatal systemic disease [28]. Therefore, proper and rapid detection of *C. psittaci* in human and birds is important to control psittacosis and avian chlamydiosis. Psittacosis is a notifiable infectious disease in Australia, the U.S., a number of European countries [13], and Japan. In Japan, since 1999, all physicians have been obliged to report psittacosis cases because it is classified as category IV under the Infectious Diseases Control Law, Japan. Hence, psittacosis should be differentiated from chlamydial pneumonia caused by *C. pneumoniae* (listed under the category V notifiable infectious diseases in Japan).

C. psittaci infection can be diagnosed by isolation of the pathogen, serological detection, or DNA detection [25]. Due to the contagiousness of this pathogen, direct isolation of the pathogen or serological test by using the purified elementary body (EB) or the *C. psittaci*-infected cells as antigens are hazardous and require specialized laboratory expertise and facilities. Therefore, microbiological diagnosis of psittacosis and avian chlamydiosis can be performed only in well-equipped laboratories. Various DNA amplification methods have been developed to detect *C. psittaci* such as conventional PCR and real-time PCR [25]. The real-time PCR assay is useful as a diagnostic test for *C. psittaci*, and has simultaneously enabled the identification and/or quantification of *Chlamydia* spp. and *Chlamydophila* spp. In addition, unlike conventional PCR, this assay can detect the pathogen in just one step, making post-PCR procedures such as electrophoresis unnecessary. That means it can be diagnosed rapidly and reduces the risk of carryover contamination.

Several studies have used real-time PCR to detect *C. psittaci*. The target genes in these studies are the major outer membrane protein (MOMP) gene (*ompA*) [10, 14, 22], 23S rRNA gene [1, 5, 7] and inclusion membrane protein A gene (*incA*) [19]. However, almost of these reports aimed at developing *C. psittaci*-specific diagnostic tests.

In this study, we chose a molecular cysteine-rich protein (*envB*) of *C. psittaci* as a target gene. We designed primers that could broadly detect animal-derived *Chlamydophila* including *C. psittaci*, *C. abortus* and *C. felis*, but that did not detect other species of *Chlamydia* or *Chlamydophila*. We also evaluated the potential of our real-time PCR system to be used as a clinical diagnostic system for *C. psittaci* and other related *Chlamydophila* spp. infections.

The chlamydial species and strains used in this study are listed in Table 1. All strains were cultivated in HeLa cells or L cells in suspension form. HeLa cells were pretreated with 30 $\mu g/ml$ DEAE-dextran in minimal essential medium α (Wako Pure Chemical Ltd., Osaka, Japan) at room temperature for 30 min before inoculation. After inoculation of each chlamydia at a multiplicity of infection of up to 10, the infected cells were incubated in the presence of 5% CO_2 at 37°C for 60 min. The inocula were exchanged into minimal essential medium α supplemented with 5% fetal bovine

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Table 1. Chlamydia species and strains used in this study

Species	Strain	Host	Reference
C. psittaci	6BC	parakeet	[11]
	Cal-10	human	[23]
	Daruma	parakeet	[8]
	GCP-1	parrot	[23]
	Mat116	psittacine	this study
	Nose	budgerigar	this study
C. abortus	B577	sheep	[23]
C. felis	Fe/C-56	feline	[21]
C. caviae	GPIC	guinea pig	[20]
C. pecorum	Maeda	cattle	[8]
C. pneumoniae	TW183	human	[12]
C. muridarum	Nigg	mouse	[8]
C. suis	S45	swine	[16]
C. trachomatis	L2/434/Bu	human	[8]
	D/UW-3/CX	human	[2]
	E/UW-5/CX	human	[4]

serum (Invitrogen, Carlsbad, CA, U.S.A.) and 1 μg/m*l* of cycloheximide in the presence of 5% CO_2 at 37°C until formation of the mature inclusion body. *C. psittaci* elemental body (EB) was purified from infected L cells in suspension form by sucrose gradient centrifugation as described previously [9, 21]. The purified EB was diluted at 2.0 mg/m*l* protein concentration in 0.01 M Tris-HCl (pH7.2) and stored at –80°C until use.

Bacteria commonly detected in fecal samples from birds were used for testing the specificity of the real-time PCR. The bacterial species were *Proteus* sp., *Pasteurella multocida*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Serratia marcescens*, *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus agalactiae*, *Salmonella enterica* biovar Pullorum, and *Yersinia enterocolitica* from the culture stock of our laboratory.

Two sets of oligonucleotide primers were designed based on the *envB* gene sequence of the *C. psittaci* 6BC with accession number M61116 (National Center of Biotechnology Information: NCBI). The entire *envB* region was amplified using a pair of primers (Clone-F: 5'-GTTCATTTGCC AGCGGGAAGATAGAGG-3'; and Clone-R: 5'-AGAAC-CACGGTTGGTTACACAAATACGG-3') for making plasmid DNA to generate a standard curve. Alignment of the *envB* gene of *C. psittaci* 6BC, *C. abortus* B577 (accession#AF111200), *C. felis* Fe/C-56 (accession#AP006861), *C caviae* GPIC (accession#U41579), *C. pecorum* W73 (accession#U76761), *C. pneumoniae* TW-183 (accession#AE009440), *C. muridarum* Nigg (accession#AE002160) and *C. trachomatis* L2/434/Bu (accession#AM884176), revealed that the region encompassing nucleotide positions 997 to 2,670 of *envB* gene was the most conserved among them (Fig. 1). Accordingly, for the real-time PCR analysis, another set of primers (Env-F: 5'-AACCTCGGATAGCAAATTAATCTGG-3'; and Env-R: 5'-ATTTGGTATAAGAGCGAAGTTCTGG-3') was designed to amplify the region of the *envB* gene (152 bp), which showed high similarity among *C. psittaci* and related *Chlamydophila* such as *C. abortus* and *C. felis* but not in other *Chlamydia* and *Chlamydophila* (Fig. 1).

To generate a standard curve for the real-time PCR assay, a PCR product containing 1,358 bp covering the part of *envB* was cloned into a pGEM T easy vector (Promega Corporation, Madison, WI, U.S.A.), resulting in pEnvB. The pEnvB was purified using a commercial kit (illustra plasmidPrep Mini Spin Kit, GE Healthcare UK Ltd., Little Chalfont, Buckinghamshine, U.K.) according to the manufacturer's instructions. The concentration of pEnvB solution was calculated from the absorbance at 260 nm measured with a spectrophotometer (GeneQuant II, Pharmacia Biotech, Piscataway, NJ, U.S.A.). Serial 10-fold dilutions of pEnvB were used in the amplification reactions.

In order to determine the detection limit of the real-time PCR analysis and to verify the accuracy of DNA extraction in field samples, feces of birds containing a known titer of *C. psittaci* EB were used as templates. Emulsion birds feces [20% (w/v) in PBS] were mixed with an equivalent amount of PBS containing 10-fold serial dilutions of EB, resulting in a 10% emulsion bird feces containing known titers of EB [10 to 10^6 inclusion forming units (IFU)].

DNA was extracted from chlamydia-infected cells, bird feces containing chlamydial EB and bacterial cultures by using a DNA extraction kit (SepaGene; Sankojunyaku Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions. The DNA was dissolved in 20 μ*l* of TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA) and stored at –30°C until used.

Real-time PCR was performed with the Thermal Cycler Dice Real Time System TP800 (Takara Bio Inc., Otsu, Japan). The reaction was carried out in 12.5 μ*l* of SYBR *Premix EX Taq* II (Perfect Real Time: Takara Bio Inc.), 10 μM of each primer, and 2 μ*l* of DNA extract in a final volume of 25 μ*l*. Cycling conditions were as follows: an initial cycle of heating at 95°C for 10 sec, 40 cycles of 95°C for 5 sec and 60°C for 30 sec with data acquired at the annealing and extension. After completion of the PCR reaction, a melting curve was constructed by 95°C for 15 sec, 60°C for 30 sec, then temperature was ramped up from 60 to 95°C. The identity of PCR product was confirmed by DNA sequence analysis.

Conventional nested PCR was examined using the same samples as described previously [3].

The sensitivity and the linearity of the real-time PCR assay were assessed using serially 10-fold diluted pEnvB DNAs containing the primer-spanning region of the *envB* gene as templates in triplicate. As shown in Fig. 2a, the sensitivity of detection was linear from 1×10 to 1×10^8 copies of pEnvB per reaction by real-time PCR. Negative controls did not provide any amplification. The standard curve covered a linear range of 8 orders of magnitude [regression coefficient (R^2) was 0.99], providing an accurate measurement of starting target amount (Fig. 2b). The amplification products were verified by the melting curves analysis after PCR procedures (Fig. 2c), showing that only one melting peak (at 81.75°C) was observed. These results show that a

```
C. psittaci      1   AACCTCGGATAGCAAATTAATCTGGACAATTGATCGCTTAGGTCAAGGTGAAAAATGCAA  60
C. abortus           ------A---------------------------T-------------------------
C. felis             ------C--------------T-----T--A----------------------G------
C. caviae            T--T--C-----T--------T-------------------------------G------
C. pecorum           ----G-A---G-G-----G--G----A---C-----T-----A-----C----G------
C. pneumoniae        T--AAGT---G-G------G------A---C------C-G---GC---A--T--------
C. muridarum         T--TG-T---G-T--GC--G-T----A---------G-----A--G--C-----GA-T--
C. trachomatis       T--TG-T---G-T--GC--G-T----A------C--------A-----C-----GA-T--

C. psittaci      61  AATTACCGTTTGGGTAAAACCTCTTAAAGAAGGTTGTTGCTTCACCGCGGCTACTGTATG  120
C. abortus           ------------------------------------------------------------
C. felis             ------------------------------------C--------A--T-----------
C. caviae            ------T-----------------------------C--------A--T-----C-----
C. pecorum           ------T-----------G--AA-A-----G--C--C--T--T-----A--G--A-----
C. pneumoniae        ------T--A--------------------------C--------A--T-----------
C. muridarum         ------T--A--------------------------C-----T--A--T--A--G--T--
C. trachomatis       ------T--A--------------------------C-----T--A--T--A--A-----

C. psittaci      121 TGCTTGCCCAGAACTTCGCTCTTATACCAAAT  152
C. abortus           C-------------------------------
C. felis             ------------G-------------------
C. caviae            --------------------------------
C. pecorum           ---A--T------A-C-----C--C--A----
C. pneumoniae        ------------G--C--T--------T----
C. muridarum         ------T-----GA-C--T--GGT---G----
C. trachomatis       C-----T-----GA-C--T--GGT---A----
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Fig. 1. Nucleotide sequence alignment of representative variant strains of *C. psittaci* 6BC, *C. abortus* B577, *C. felis* Fe/C-56, *C. caviae* GPIC, *C. pneumoniae* TW-183, *C. pecorum* W73, *C. trachomatis* L2/434/Bu, *C. muridarum* Nigg. Underscored and bold portions of the sequences are primer-binding locations.

minimum of 10 copies of the pEnvB was consistently detectable in the real-time PCR assay.

Since *C. psittaci* EB are shed in feces from infected birds, fecal samples of birds are routinely used as a source of DNA for laboratory diagnosis of *C. psittaci* infection [24]. We prepared bird feces containing known amount of EB in order to simulate clinical samples, adding with EB suspensions in PBS as a control. DNAs were extracted from the feces and PBS containing *C. psittaci* EB. The real-time PCR indicated that the cycle threshold (Ct) values of these samples were correlated with the amount of EB in both feces and PBS (data not shown).

The sensitivity of the real-time PCR was compared with that of the nested PCR routinely used in our laboratory for clinical diagnosis [3]. Template DNA samples extracted from feces and PBS containing known titers of EB as described above were analyzed by nested PCR. The detection limit of the nested PCR assay was 10^4 IFU per reaction (data not shown). In an experiment using same samples, the lower detection limit of the SYBR Green real-time PCR was only 10 IFU (data not shown).

The specificity of the real-time PCR assay was evaluated with 7 strains of *C. psittaci*, 10 strains of *Chlamydophila* and *Chlamydia* species, and bacterial culture. As a result, *C. psittaci* strains including 6BC, Borg, Cal-10, Daruma, GCP-1, Mat116, Nose, and closely related other *Chlamydophila* species such as *C. felis* and *C. abortus* were amplified by real-time PCR. All of these reactions showed a single melting peak at 81.75 ± 0.5°C (data not shown). No signal was detected from *C. pneumoniae* and *C. trachomatis* (Table 1), and also from none of bacterial culture that we examined.

In this study, we established a real-time PCR assay based on SYBR Green dye for the detection of *C. psittaci* and related *Chlamydophila* species. This assay is an effective alternative for the conventional nested PCR.

Although there are many assays for detecting *C. psittaci* [25], a simple and efficient analysis is still required by veterinarians and clinicians. Recently, real-time PCR assay has

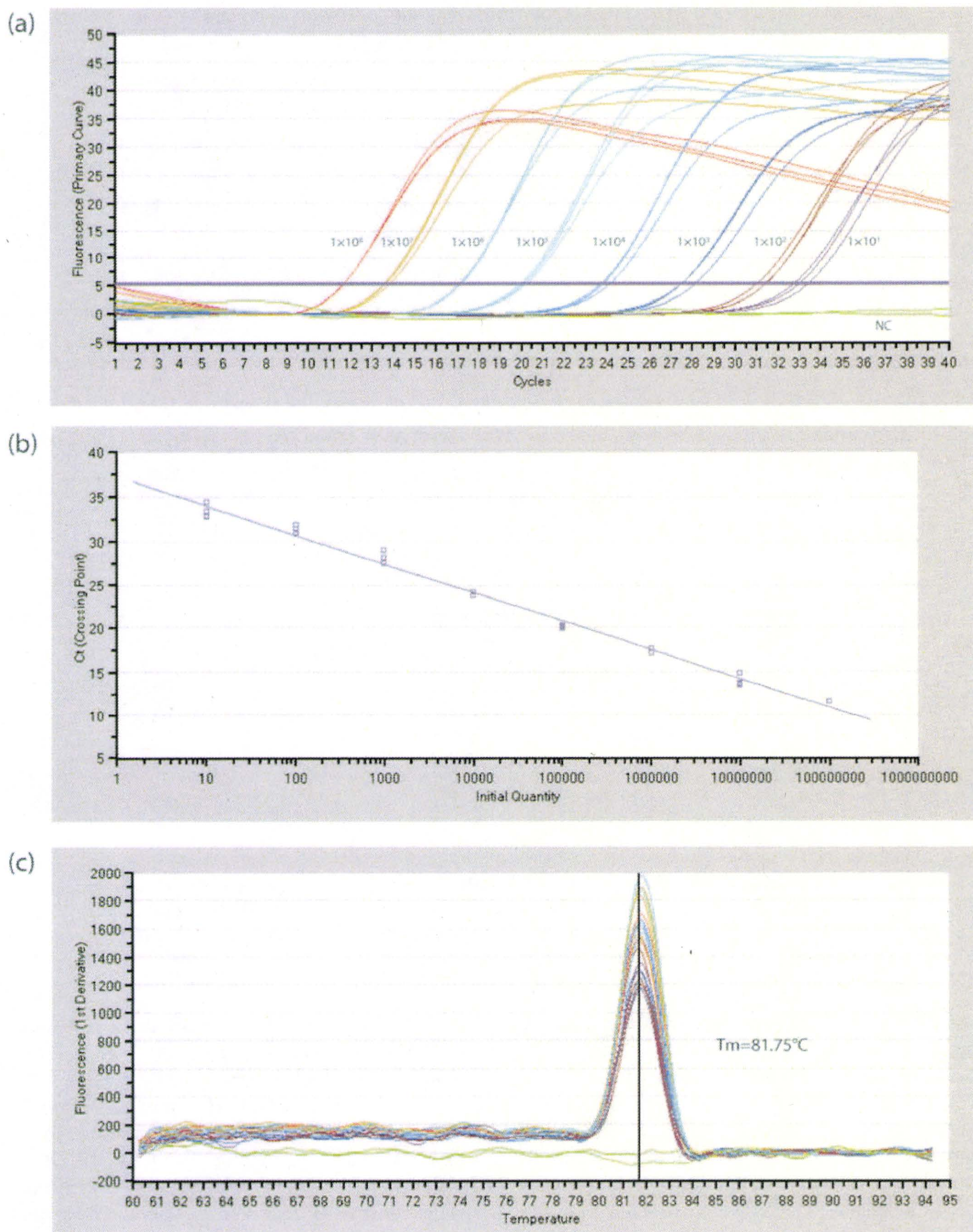

Fig. 2. Amplification curves, standard curves and melting curves of the real-time PCR assay. (a) Amplification curves were generated by fluorescence data collected at each cycle during the extension phase of the PCR. Values are triplicates of different dilutions of the pEnvB used as standard. pEnvB copy number per sample were 1×10^1, 1×10^2, 1×10^3, 1×10^4, 1×10^5, 1×10^6, 1×10^7, 1×10^8 and negative control (NC). (b) Standard curves based on serial dilutions of plasmid DNA (copy numbers are 1×10^1 to 1×10^8). The R^2 linearity value from linear regression is 0.99. (c) Melting curve analysis from the same experiment as in (a).

become more widely used as a major diagnostic test. Conventional PCR analysis including nested PCR [3] and PCR-restriction fragment length polymorphism (RFLP) [17, 26] confirm only the presence of a pathogen and take a long time for detection, because they require gel electrophoresis to confirm the presence of PCR products after PCR [25]. On

the other hand, present real-time PCR techniques can quantify and detect a pathogen more rapidly than conventional PCR. The real-time PCR method described here took only about half the time of conventional nested PCR.

Our assay uses primers that target a conserved region of the *envB* gene in *chlamydophila* spp. This *envB* region is suitable for differential diagnosis, that is, this method can distinguish from *C. psittaci* to *C. pneumoniae* or *C. trachomatis*. This primer pair amplifies closely related species such as *C. abortus* and *C. felis*. Pantchev *et al.* reported that *C. psittaci* and *C. abortus* have possibility to cause dual infections in pigs and cattle [22], and we speculate that it could also cause dual infection in avian species. Therefore, the real-time PCR established in this study which can simultaneously detect *C. psittaci* and *C. abortus*, may be a useful tool in veterinary medicine.

Fecal samples of birds are routinely used as a source of DNA for laboratory diagnosis of *C. psittaci* infection [24]. Fecal samples contain the number of different types of inhibitors of PCR [26]. These impurities might have influenced the amplification. However, it was confirmed that fecal impurities did not affect the results in this detection method. One of a general problem of PCR assay is that inhibitory substances can give a false-negative result. An internal control system may be needed to improve the accuracy of our real-time PCR [14].

Conventional nested PCR was able to detect 10^4 IFU, whereas the lower detection limit of the SYBR Green real-time PCR was only 10 IFU. Therefore, the SYBR Green PCR assay is 1,000 times more sensitive than conventional nested PCR. The short amplicons in the real-time PCR assays used in this study likely resulted in more efficient amplification and higher sensitivity. Our SYBR Green real-time PCR assay also achieved the same sensitivity as other TaqMan PCR assays compared with IFU based on Ct value [19, 22]. Ehricht *et al.* pointed out that the actual detection sensitivity depends on the integrity of the target DNA [6, 25]. The real-time PCR established in this study is applicable even to DNA samples that were extracted from avian feces.

In conclusion, the real-time PCR assay based on SYBR Green dye defined high sensitivity and rapidity and quantification for detection of *C. psittaci* from fecal samples. Early diagnostics and treatment are of importance in psittacosis. The format should emphasize use as a routine study.

ACKNOWLEDGMENTS. This work was supported in part by grants-in-aid for scientific research from the Japan Society for the Promotion of Science (20780218) and from Health Sciences Research Grant for Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health, Labour and Welfare (H19-shinkou-ippan-010, H21-shinkou-wakate-014).

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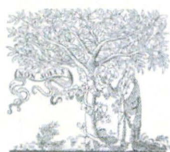

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Veterinary Microbiology

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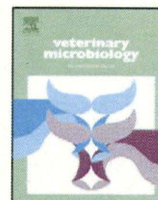

Short communication

Using CF0218-ELISA to distinguish *Chlamydophila felis*-infected cats from vaccinated and uninfected domestic cats

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ARTICLE INFO

Article history:
Received 25 February 2010
Received in revised form 14 May 2010
Accepted 19 May 2010

Keywords:
Chlamydial infection
Antigen
ELISA
Diagnosis

ABSTRACT

Chlamydophila felis is a causative agent of acute and chronic conjunctivitis and pneumonia in cats. Cats can be vaccinated with killed or attenuated *C. felis*. However, current serodiagnostics cannot distinguish these cats from naturally infected cats. This causes difficulty of early diagnosis and seroepidemiological survey for *C. felis*. We previously reported that *C. felis* CF0218 can be used as a *C. felis*-infection-specific diagnostic antigen in experimentally infected and/or vaccinated cats. In this study, we evaluated an enzyme-linked immunosorbent assay using recombinant CF0218 as antigen (CF0218-ELISA) to detect anti-*C. felis* antibody in 714 sera of domestic cats whose histories of vaccination against *C. felis* are known. The 44 vaccinated cats were 93% negative using CF0218-ELISA; half of these scored positive by immunofluorescence assay (IFA) using *C. felis*-infected cells as antigen. The 670 non-vaccinated cats had CF0218-ELISA positivity rates that were statistically in agreement with IFA (18% vs. 21%). These results show that CF0218, which was identified as a *C. felis*-infection-specific antigen, is a useful serodiagnostic antigen to distinguish naturally *C. felis*-infected cats from vaccinated and non-infected cats.

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1. Introduction

Chlamydia spp. and *Chlamydophila* spp. are obligate intracellular bacterial pathogens of a wide range of hosts and cause various diseases ranging from persistent latent infection to conjunctivitis, pneumonia and miscarriage in a variety of animals, including humans (Everett et al., 1999). In terms of veterinary medicine for companion animals, *Chlamydophila felis* causes conjunctivitis and upper respiratory signs in cats all over the world (Sykes, 2005). The clinical signs of feline chlamydiosis (conjunctivitis and upper respiratory signs) are quite similar to those caused by feline herpesvirus type 1, and differential diagnosis is clinically difficult (Sykes, 2005). In Western countries, inactivated and live vaccines for cats are commercially available, and in Japan inactivated vaccines have been available since 2002. These vaccines are recommended because they reduce the clinical signs (Sykes, 2005; Wills et al., 1987).

Early diagnosis of *C. felis* infection is important in order to provide an appropriate treatment and prevent the spread of the disease. In general, it is difficult to diagnose chlamydial infection by isolating pathogens because it usually takes over 2 weeks for isolation (Sachse et al., 2009). Similarly, in the case of *C. felis*, it is often difficult to isolate the pathogen. Therefore, feline chlamydiosis is

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doi:10.1016/j.vetmic.2010.05.026

diagnosed serologically by immunofluorescence assay (IFA) using the infected cells or elementary bodies (EBs; chlamydial infectious form) as antigens, or diagnosed etiologically by conventional PCR and real-time PCR (Helps et al., 2001, 2003; Sykes, 2005). IFA, is not widely used in the clinical laboratory, mainly because it requires culturing cells and live-organism to prepare antigens. PCR and real-time PCR assays are more rapid and more sensitive than other methods. However, PCR and real-time PCR assays often give false positive results due to inadequate handling of samples. Also, these tests (IFA, PCR and real-time PCR) can be conducted only in well-equipped laboratories, making it impracticable to conduct the test in clinical settings. Another problem is that with vaccines for *C. felis*, it is difficult to distinguish between *C. felis*-infected and -vaccinated cats by means of the current serological tests (IFA and ELISA) as positive reactions are observed in these tests.

In order to develop a simple serological diagnostic test for *C. felis* infection, we previously obtained a gene encoding an antigen that is specific to *C. felis* infection, termed CF0218 (also named *mhcB2*), which might be a useful antigen for diagnosing *C. felis* infection (Ohya et al., 2008). In this study, we used CF0218-ELISA (using recombinant CF0218 as antigen) to examine the prevalence of antibodies specific to *C. felis* infection in domestic cats. Our results show that CF0218-ELISA can differentiate *C. felis*-infected and -vaccinated cats, and should therefore be useful for conducting epidemiological surveys of *C. felis*.

2. Materials and methods

2.1. Serum samples

Serum samples were obtained from 714 domestic cats at veterinary clinics in 43 prefectures in Japan between 2005 and 2006. A cat was considered to have been vaccinated against *C. felis* if it had been vaccinated with Fel-O-Vax 5 (Kyoritsu Seiyaku, Tsukuba, Japan) or Feline-7 (Kyoto Biken Laboratories, Kyoto, Japan). To ensure antibody development, 44 cats were vaccinated for two or more weeks prior to collection of the sera (Ohya et al., 2008). The sera were stored at −30 °C until use.

2.2. Culturing cells, C. felis and immunofluorescence assay

HeLa cells were used for propagation of *C. felis* Fe/C-56 as described previously (Ohya et al., 2008). HeLa cells were cultured in Eagle's minimum essential medium (MEM)-1 (Nissui, Tokyo, Japan) with 5% fetal bovine serum (FBS) at 37 °C in 5% CO_2. Antibodies against *C. felis* were detected by IFA and results were evaluated as described elsewhere with a slight modification (Tapia et al., 2002; Yan et al., 2000). HeLa cells were placed on a 12-well assay slide (Matsunami Glass, Osaka, Japan). When the cells formed a monolayer, the cells were treated with MEM containing 0.03 mg/mL DEAE-dextran for 30 min at room temperature. The cells were inoculated with *C. felis* diluted with MEM containing 1 μg/mL cycloheximide. The negative control cells (non-infected cells) were incubated in MEM containing 1 μg/mL cycloheximide following DEAE-dextran treatment. Seventy-two hours after *C. felis* inoculation, the slides were air-dried, and fixed with chilled-acetone for 10 min. Two-fold dilutions of serum samples with phosphate-buffered saline (PBS), starting with an initial dilution of 1:8–1:512, were added onto each well, and the slides were incubated for 30 min at 37 °C. The slides were washed with PBS, and fluorescein isothiocyanate (FITC)-conjugated anti-cat IgG (H&L) antibody (Bethyl Laboratories, Montgomery, TX) that had been diluted at 1:50 with PBS containing 0.02% Evans blue (for counterstain) was added as the secondary antibody. After the slides were washed and air-dried, the plates were observed with a fluorescence microscope (BX50; Olympus, Tokyo, Japan). Infection with *C. felis* in HeLa cells on the slide was checked using the FITC-conjugated anti-*Chlamydia* antibody (Denka Seiken, Tokyo, Japan) in accordance with the manufacturer's instructions. The infected cells and non-infected cells were compared for each serum sample. The samples in which chlamydial inclusions were clearly observed in the infected cells but not in the non-infected cells at a dilution of ≥1:16 were assessed as *C. felis* antibody positive as described previously (Tapia et al., 2002; Yan et al., 2000).

2.3. ELISA

The amount of antibodies against CF0218 in feline sera was measured using CF0218-ELISA as described previously (Ohya et al., 2008). Briefly, purified glutathione S-transferase (GST)-conjugated CF0218 (GST-CF0218) or GST diluted at 1 μg/mL were used as antigens. The serum samples (diluted at 1:50 with PBS) were added and incubated for 1 h at room temperature. Horseradish peroxidase (HRP)-conjugated anti-cat light chain antibody (Bethyl Laboratories) was used as the secondary antibody. Samples were developed and absorbance at 405 nm (A_{405}) was measured using a Microplate Reader Model 550 (Bio-Rad, Hercules, CA). PBS (instead of a serum sample) was used as the negative control, and sera obtained from cats experimentally infected with *C. felis* (Ohya et al., 2008) were used as the positive control. A_{405} was measured in duplicate for each serum sample, and net absorbance was calculated as described previously (Livingstone et al., 2005; Ohya et al., 2008). To determine the cutoff value between positive and negative serum in CF0218-ELISA, IFA was used as a gold standard of *C. felis* infection. The mean net A_{405} of CF0218-ELISA for IFA-negative samples was 0.003 and the standard deviation (SD) was 0.103. The cutoff value of CF0218-ELISA was calculated as the mean net A_{405} of IFA-negative samples plus $3 \times$ SD of IFA-negative samples (0.311) as described elsewhere (Livingstone et al., 2005).

2.4. Statistical analysis

The correlation of IFA titers and CF0218-ELISA absorbance was determined by a nonparametric Spearman rank correlation test. P values of <0.01 were considered statistically significant. The sensitivity, specificity, and positive and negative predictive values (with 95% confidence intervals) of CF0218-ELISA were calculated on the basis of IFA by the chi-square test.

3. Results

3.1. Comparison of CF0218-ELISA and IFA

We examined the prevalence of anti-CF0218 in 714 feline serum samples using CF0218-ELISA. Of 714 serum samples examined, 164 (23.0%) were positive for anti-chlamydial antibodies by IFA. One hundred and twenty of the 714 samples (16.8%) were assessed as CF0218-ELISA-positive. The efficacy of CF0218-ELISA to detect *C. felis* infection was evaluated by comparing with IFA results. Vaccines against *C. felis* have been used in Japan. As IFA testing uses *C. felis*-infected cells as an antigen, both vaccinated cats and cats infected with *C. felis* should be assessed as positive in IFA. Therefore, to evaluate whether CF0218-ELISA can detect *C. felis* infection correctly, we excluded 44 serum samples, which were collected from cats inoculated with vaccines against *C. felis*, from a total of 714 samples. Among these 670 samples, the absorbance value of CF0218-ELISA was compared with the endpoint titer of IFA (Fig. 1). The absorbance value of CF0218-ELISA correlated with the endpoint titer of IFA results ($r_s = 0.607$, $P < 0.01$). Among these samples as shown in Table 1, 143 samples (21.3%) were assessed as positive for anti-chlamydial antibodies by IFA, and of these, 112 (16.7%) were also CF0218-ELISA-positive and 31 (4.6%) were CF0218-ELISA-negative. These results show that the sensitivity and specificity of CF0218-ELISA to detect *C. felis* infection are 78.3% (95% confidence interval [CI], 74.6–80.3%) and 99.1% (95% CI, 98.0–99.6%), respectively.

3.2. CF0218-ELISA and IFA results in vaccinated cats

Results obtained by IFA and CF0218-ELISA in 44 serum samples of vaccinated cats were compared as described

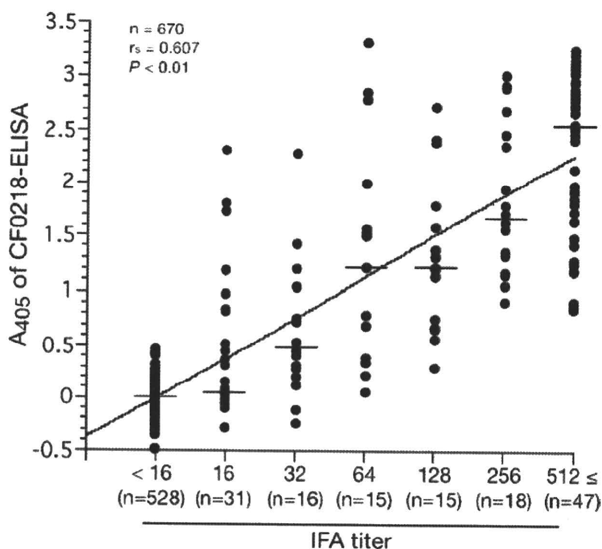

Fig. 1. Correlation of the absorbance value of CF0218-ELISA and IFA endpoint titer for 670 sera of non-vaccinated cats: the scatter plots indicate the direct comparison between the absorbance value of CF0218-ELISA and IFA endpoint titer. Spearman rank correlation coefficient (r_s) is shown (assessed as statically significant correlation; $P < 0.01$). Horizontal bars represent the median value of the absorbance of CF0218-ELISA at each IFA endpoint titer. The solid line is the regression line.

Table 1
CF0218-ELISA compared with IFA results for naturally infected and uninfected cats.

	# of serum	CF0218-ELISA[a]	
		Positive (%)	Negative (%)
IFA positive	143	112 (16.7%)	31 (4.6%)
IFA negative	527	5 (0.7%)	522 (77.9%)
Total	670	117 (17.5%)	553 (82.5%)

[a] Sensitivity (95% CI), 78.3% (74.6–80.3%); specificity (95% CI), 99.1% (98.0–99.6%); positive predictive value (95% CI), 95.7% (91.2–98.1%); negative predictive value (95% CI), 94.4% (93.4–94.9%).

Table 2
CF0218-ELISA and IFA for vaccinated cats.

	# of serum	CF0218-ELISA	
		Positive (%)	Negative (%)
IFA positive	21	2 (4.5%)	19 (43.2%)
IFA negative	23	1 (2.3%)	22 (50.0%)
Total	44	3 (6.8%)	41 (93.2%)

above. As expected, among these samples, the absorbance value of CF0218-ELISA and the endpoint titer of IFA did not show a significant correlation ($r_s = 0.239$, $P = 0.119$, data not shown). In Table 2, 21 (47.7%) of the vaccinated cats were assessed as IFA-positive. On the other hand, 41 (93.2%) of the vaccinated cats were assessed as CF0218-ELISA-negative. These results suggest that CF0218-ELISA can distinguish *C. felis*-infected and -vaccinated cats in field samples.

4. Discussion

In this study, we evaluated CF0218-ELISA that we had previously established to detect antibodies for *C. felis* infection. We found that serum from 93% of the 44 cats vaccinated with inactivated *C. felis* were not detected in CF0218-ELISA. In contrast, sera from 670 non-vaccinated cats had CF0218-ELISA positivity rates that were statistically in agreement with IFA (18% vs. 21%).

Generally, the microimmunofluorescence (MIF) test (Wang and Grayston, 1970), which uses purified chlamydial EB as antigen, is employed as the 'gold standard' for the serological diagnosis and the classification of any *Chlamydia* including *C. trachomatis*, *C. pneumoniae* and *C. psittaci* (Sachse et al., 2009). However, Tapia et al. (2002) showed that IFA titers using the *C. pneumoniae*-infected Hep2 cells as antigen (inclusion IFA) represented 97% sensitivity with the *C. pneumoniae* MIF titers of ≥16, and concluded that the inclusion IFA can be used as a screening assay for detection of anti-*C. pnumoniae* antibodies. Therefore, to determine the cutoff value of CF0218-ELISA, we performed IFA using *C. felis*-infected HeLa cells as a 'gold standard' in the presence of anti-*C. felis* antibodies. Among the 670 non-vaccinated cats, the correlation between the absorbance value of CF0218-ELISA and the endpoint titers of IFA exhibited statistical significance. These results suggest that CF0218-ELISA is an alternative way to detect anti-*C. felis* antibodies, although its sensitivity was slightly inferior

than that of IFA. This is probably because IFA uses *C. felis*-infected cells as antigen, and therefore it detects various types of antibodies that are induced at the time of *C. felis* infection. On the other hand, because CF0218-ELISA detects antibodies against a single antigen, the quantity of anti-CF0218 antibodies may not have increased enough or may have already decreased, depending on the timing of *C. felis* infection in each cat. In fact, our previous study showed that anti-CF0218 antibodies increased 2 weeks following infection (Ohya et al., 2008).

Among the vaccinated cats, the correlation between the absorbance value of CF0218-ELISA and the endpoint titer of IFA was not statistically significant. Results obtained in this study indicate that CF0218-ELISA is a useful method for the detection of antibodies specific to *C. felis* infection in field samples.

The overall prevalence rate of *C. felis* infection by CF0218-ELISA and IFA in the non-vaccinated cats was 16.7%, which was almost the same as the results obtained by the MIF test (17.3%) in domestic cats in Japan before the vaccines became practically available (Yan et al., 2000). The results of this study strongly indicate the usefulness of CF0218-ELISA for the epidemiological survey of *C. felis*, and that a significant percentage of domestic cats throughout Japan have histories of *C. felis* infection.

In the case of ovine enzootic abortion (OEA) caused by *C. abortus*, another chlamydiosis commercial vaccine is available (Longbottom and Livingstone, 2006). A method is thus needed to differentiate between vaccinated and infected animals (Gerber et al., 2007; Sachse et al., 2009). CF0218 is a TMH (transmembrane head)-family protein located on the TMH locus in the *C. felis* genome (Ohya et al., 2008). The *C. pneumoniae* and *C. trachomatis* genome do not have a TMH locus (and the TMH-family proteins). However, the *C. abortus* genome encodes several TMH-family proteins (e.g., CAB764 and CAB766) (Thomson et al., 2005). The TMH-family proteins of *C. abortus* may have the potential to be diagnostic antigens that can distinguish vaccinated and infected animals. A major problem with detecting antibodies in animal and human chlamydial infections is cross-reactivity of antigens such as LPS among strains and *Chlamydiaceae* (Brade et al., 1987; Sachse et al., 2009). We previously showed that the similarity of TMH-family proteins among *Chlamydiaceae* (*C. felis*, *C. abortus* and *C. caviae*) was low at the amino acid level (around 25–35%), that recombinant CF0218 was not recognized by antisera of *C. trachomatis* and *C. abortus*, and that there was no diversity of CF0218 among *C. felis* strains (Ohya et al., 2008). In addition, *C. psittaci*, the agent for psittacosis, also possesses the TMH-family proteins (K. Ohya and H. Fukushi, personal communication). These results raise the possibility that the TMH-family protein can be used as novel diagnostic antigens of animal chlamydiosis, which is species-specific and can differentiate infected and vaccinated animals, in spite of other conventional antigens such as whole EB and LPS.

Although live and inactivated vaccines for *C. felis* are available (Sykes, 2005), only the inactivated-form is approved in Japan. Therefore, in this study, we examined the cats that were inoculated formalin-inactivated form, but not with the live-form. Inactivated *C. felis* vaccine accounts for about 50% of total sales of *C. felis* vaccine in the United States (Ohya et al., 2008). Further CF0218-ELISA studies are needed to examine cats inoculated with live *C. felis* vaccine.

Conflict of interest

None declared.

Acknowledgments

We are grateful to veterinarians in private animal hospitals in Japan who supplied serum samples used in this study. We also would thank Y. Cai and M. Itoh for technical advice and help. This work was supported in part by grants-in-aid for the Zoonosis Control Project from the Ministry of Agriculture, Forestry and Fisheries of Japan (to H.F.); for scientific research from the Japan Society for the Promotion of Science (to K.O.); from Health Sciences Research Grant for Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health, Labor, and Welfare (to K.O.); and from Fort Dodge Animal Health (Overland Park, KS) (to H.F.).

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REVIEW *Virology*

A Review of DNA Viral Infections in Psittacine Birds

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(Received 12 January 2010/Accepted 2 April 2010/Published online in J-STAGE 16 April 2010)

ABSTRACT. To date, several DNA viral infections have been reported in psittacine birds. Psittacine beak and feather disease (PBFD) is characterized by symmetric feather dystrophy and loss and development of beak deformities. PBFD is caused by beak and feather virus, which belongs to the *Circoviridae*, and is the most important infection in psittacine birds worldwide. Avian polyomavirus infection causes acute death, abdominal distention, and feather abnormalities. Pacheco's disease (PD), which is caused by psittacid herpesvirus type 1, is an acute lethal disease without a prodrome. Psittacine adenovirus infections are described as having a clinical progression similar to PD. The clinical changes in psittacine poxvirus-infected birds include serious ocular discharge, rhinitis, and conjunctivitis, followed by the appearance of ulcerations on the medial canthi of the eyes. Internal papillomatosis of parrots (IPP) is a tumor disease characterized by progressive development of papillomas in the oral and cloacal mucosa. IPP has been suggested to caused by papillomavirus or herpesvirus. However, information about these diseases is limited. Here we review the etiology, clinical features, pathology, epidemiology, and diagnosis of these DNA viruses.
KEY WORDS: DNA virus, psittacine bird, review.

J. Vet. Med. Sci. 72(9): 1099–1106, 2010

In recent years, various psittacine birds have been popular and kept as pet animals in Japan as well as other countries. Avian medicine for poultry has made significant progress over the last several decades. However, there are few studies with regard to avian medicine for psittacine birds. Today, infectious diseases, especially viral infectious diseases, are the most common clinical problems in captive psittacine birds because of their association with acute death and difficulties in treatment and control. Many viral infections in psittacine birds have been reported, including DNA virus: psittacine beak and feather disease (PBFD) [73], avian polyomavirus infection (APV) [5], psittacid herpesvirus infection (PsHV) [92], psittacine adenovirus infection (PsAdV) [81], poxvirus infection [58], and papillomavirus infection [14] and RNA virus: reovirus infection [106], coronavirus infection [26], paramyxovirus infection [29], influenzavirus infection [76], and bornavirus infection [50]. However, information about these diseases is limited.

The objectives of this review are DNA viral infections in psittacine birds with respect to (I) etiology, (II) clinical features, (III) pathology, (IV) epidemiology, and (V) diagnosis about each DNA virus disease in psittacine birds. Table 1 is summary of each viral infection in this review.

DNA VIRUS INFECTIONS IN PSITTACINE BIRDS

Psittacine beak and feather disease (Circovirus infection): This chronic disease is characterized by symmetric feather dystrophy and loss, development of beak deformities, and eventual death. It was first observed in various species of Australian cockatoos in the early 1970s [73]. Because of the characteristic feather loss and abnormal beak associated with this disease, the disease was named Psittacine beak and feather disease (PBFD).

PBFD is found in many countries, including Australia [48], Germany [78], Italy [6], New Zealand [86], South Africa [31], Taiwan [18, 36], Thailand [49], and the U.S.A. [18]. Reported viral DNA positive rates in recent years are 23.0% in Australia [48], 40.4% in Germany [78], 8.0% in Italy [6], 41.2% in Taiwan [36], and 3.5 to 4.0% in the U.S.A. [18]. In Japan, PBFD has been reported in many psittacine species [70], with an overall positive rate of 18.5% [89]. At present, PBFD is the most important viral infection in psittacine birds in Japan as well as other countries.

Beak and feather disease virus (BFDV), the causative agent of PBFD, belongs to the family *Circoviridae*. The BFDV virion is icosahedral and nonenveloped with a diameter of 14–17 nm [84]. It carries a single-stranded ambisense circular DNA with a complete genome size of approximately 2 kb [84]. BFDV genome DNA has two major open reading frames (ORFs), which encode a replication associated protein (ORF V1 or Rep) and a capsid protein (ORF C1 or CP) [2, 3, 65]. A third ORF (ORF V2) has been suggested, although its function is unknown.

PBFD has been confirmed in over 60 species of both free-ranging and captive psittacine birds [48, 101]. This disease has peracute, acute, and chronic forms. Sudden death occurs in peracute and acute forms. The chronic form is characterized by progressive symmetric feather dystrophy and loss and by beak deformities [73]. Feather dystrophy is due to necrosis and hyperplasia of epidermal cells [6, 104]. Beak deformities are not always present and seem to occur in specific species or are dependent on other factors [84].

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Table 1. Summary of DNA virus infections in psittacine birds

Virus	Genome	Disease	Clinical features	Diagnosis	Reports in Japan
Beak and feather disease virus (*Circoviridae*)	ssDNA, circular 2 kbp	Psittacine beak and feather disease	Feather dystrophy, Beak deformity Immunosuppression	HA, HI, EM, PCR	+
Avian polyomavirus (*Polyomaviridae*)	dsDNA, circular 5 kbp	Avian polyomavirus infection	Acute death, Subcutaneous hemorrhage Feather abnormality	IFA, EM, NT, PCR	+
Psittacid herpesvirus type 1 (*Herpesviridae*)	dsDNA, linear 160 kbp	Pacheco's disease	Acute death, Depression, Anorexia Diarrhea, Tremor, Instability	EM, PCR	+
Psittacine adenovirus (*Adenoviridae*)	dsDNA, linear 45 kbp[a)]	Psittacine adenovirus infection	Depression, Anorexia Diarrhea, Cloacal hemorrhage	EM, PCR	+
Psittacine poxvirus (*Poxviridae*)	dsDNA, linear 300 kbp[b)]	Psittacine poxvirus infection	Ocular discharge, Rhinitis Conjunctivitis	ELISA, HI, NT, PCR	−
Psittacine papillomavirus (*Papillomaviridae*)	dsDNA, circular 7.8 kbp	Internal papillomatosis of parrot	Papillomas of oral and cloacal mucosa	PCR	−

HA: Hemagglutination test. HI: Hemagglutination inhibition test. EM: Electron microsopy. NT: Virus-neutralization test.
Plus indicates that this disease has been reported in Japan. Minus indicates that this disease has not been reported in Japan.
a) This is the genome size of fowl adenovirus, b) This is the genome size of fowl poxvirus.

Histopathological examinations have revealed basophilic intranuclear and intracytoplasmic inclusion bodies within infected feather epithelial cells or macrophages (Fig. 1A) [37, 73]. This disease does not always cause death. BFDV-induced immunosuppression causes secondary infections, which are the cause of death in most infected birds [84].

In the case of progressive feather loss, PBFD should be suspected. However, visible feather changes grossly similar to those caused by BFDV can be induced by any number of factors, including APV, PsAdV, trauma, bacterial folliculitis, malnutrition, endocrine abnormalities, and drug reactions to penicillins and cephalosporins. Specific assays for BFDV infection have been developed. These include *in situ* hybridization [79], hemagglutinating and hemagglutination inhibition [83], electron microscopy [84], PCR [70, 109], and real-time PCR [45, 82]. Among them, PCR is a very sensitive, specific, and rapid tool for detection of viruses.

Avian polyomavirus infection: APV infection causes acute death, abdominal distention, and feather abnormalities known as "French molt" in fledgling and young budgerigars [34]. It also causes a loss of down feathers on the back and abdomen, filoplumes on the head and neck, and subcutaneous hemorrhage of nesting budgerigars [5, 16]. APV infections have also been detected in other psittacine bird species and in other avian orders. In these cases, APV causes clinical signs similar to those observed in budgerigars. However, the degree of susceptibility for and severity of the diseases seems to be dependent on the species infected [19]. Pathologic examinations have found hydropericardium, enlarged heart, swollen liver, congested kidneys, and hemorrhage within the body cavities [5, 16]. Histopathological findings have revealed large and slightly basophilic nuclear inclusion bodies in various tissues, especially in the spleen, liver, and kidneys (Fig. 1B) [5, 16].

This disease has been observed in Canada [5], China [51], Australia [71], Germany [95], Slovakia [55], Italy [6], and Taiwan [36]. In Italy and Taiwan, the viral DNA positive rates were 0.8% and 15.2%, respectively. In Japan, APV infections have been reported in several bird species, such as budgerigar, black-headed caique (*Pionites melanocephala*) and eclectus parrot (*Eclectus roratus*) [34, 47], with an overall DNA positive rate of 2.7% [69].

Polyomaviruses are widely distributed among mammalian and avian species. To date, four polyomaviruses in birds are known, namely avian polyomavirus (APV), goose hemorrhagic polyomavirus (GHPV), finch polyomavirus (FPyV) and crow polyomavirus (CPyV) [9, 30, 43]. In the early 1980s, the first bird polyomavirus, which was isolated from budgerigars (*Melopsittacus undulatus*) [16], was then designated as budgerigar fledgling disease polyomavirus by the International Committee on Taxonomy of Viruses (ICTV) [5, 9]. However, it is now termed APV because of its broad host range [40]. The other three bird polyomaviruses have not been reported in psittacine birds.

The APV virion is icosahedral and nonenveloped with a diameter 45–50 nm [9]. The APV genome is a circular double-stranded DNA, 4,981 bp in size, and forms a chromosome-like structure with cellular histones [88]. Functionally, the APV genome can be divided into early and late gene-coding regions. The early region encodes both a large tumor antigen and a small tumor antigen by analogy with mammalian polyomaviruses. The late region in APV encodes a major structural protein, VP1, and three minor structural proteins, VP2, VP3, and VP4 [41]. The outer shell of the virion is composed of VP1. The three minor proteins are also present in the viral capsid [41]. A single amino acid at position 221 in VP2 has been reported to be a key element for propagating in cell cultures derived from several avian species [47, 94]. VP4 is a structural protein specific to polyomaviruses isolated from avian species and has multiple functions such as interaction with VP1 and double-stranded DNA and induction of apoptosis [42].

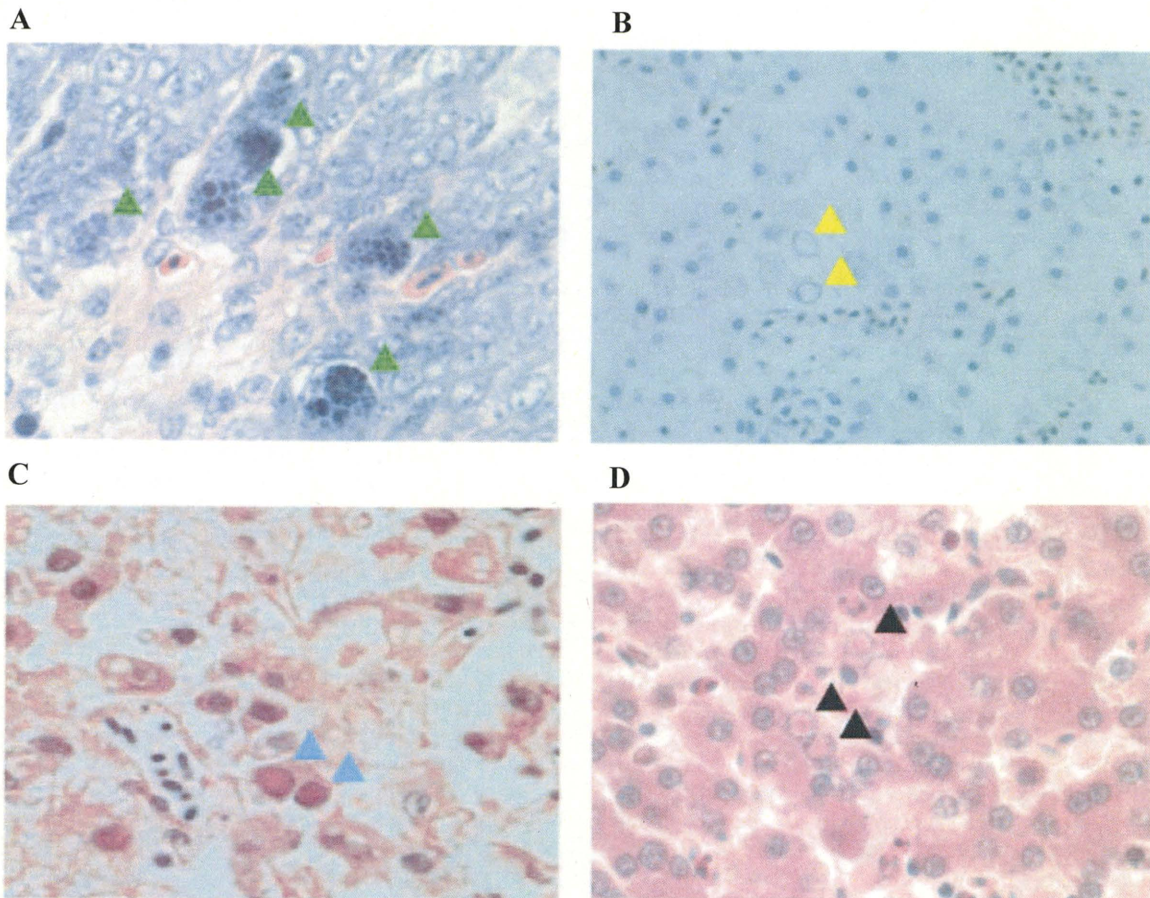

Fig. 1. (A) Basophilic intracytoplasmic inclusion bodies are located within BFDV-infected feather epithelial cells (green arrowheads). Inclusion bodies are shown as characteristic various sizes of multiple globules. (B) Basophilic intranuclear inclusion bodies are located within the APV-infected epithelial cells of the renal tubules (yellow arrowheads). Opaque inclusion bodies are observed with enlarged nuclei. (C) Eosinophilic intranuclear inclusion bodies are located within the hepatocytes of PD-affected birds (blue arrowheads). (D) Basophilic intranuclear inclusion bodies are located in the hepatocytes of PsAdV-affected birds (black arrowheads).

Clinical changes that are suggestive of APV infections include acute death or development of feather abnormalities. However, these changes can also be associated with other infectious and non-infectious diseases. Recently, APV has been identified by immunofluorescent antibody staining [28], *in situ* hybridization [79], electron microscopy [34], virus-neutralization test [74], PCR [70, 75], and real-time PCR [45].

Psittacid herpesvirus infection: In 1929, Brazilian veterinarian Pacheco encountered an outbreak of acute, fatal hepatitis with intranuclear inclusion bodies in psittacine birds in Brazil. This syndrome became known as Pacheco's disease (PD). In 1975, an avian herpesvirus, later named psittacid herpesvirus type 1 (PsHV-1), was confirmed to cause PD [92]. The usual clinical history for a bird of normal appearance is to be found dead in its enclosure without a prodrome. If still alive, clinical signs may include depression, anorexia, diarrhea, tremors, and instability [91, 92]. Because the virus kills the birds very rapidly, affected birds may show no gross lesion but may show abnormal changes in the liver, spleen, kidneys, and intestines. Histopathological findings associated with PD include necrotizing lesions in many organs, as well as hemorrhage and congestion of the liver, spleen, and kidneys. Intranuclear inclusion bodies (Cowdry type A) are most commonly found in the liver, but have also been demonstrated in the kidneys, spleen, pancreas, and small intestines (Fig. 1C) [105]. PD has been observed in the U.S.A. [60, 92], UK [25], Spain [22], Kenya [44], South Africa [35], and Japan [105].

PsHV-1 has also been suggested to have an etiological role in the development of tumors, as specific PsHV-1 genome sequences have been repeatedly detected in mucosal papillomas from parrots [39, 64, 96]. Recently, another type of psittacid herpesvirus, PsHV-2, has been identified in three African grey parrots. However, the prevalence and pathogenicity of PsHV-2 is, as yet, unclear [97,

103].

The PsHV-1 genome is 163,025 bp in length and contains 73 predicted ORFs [100]. PsHV-1 is closely related to infectious laryngotracheitis virus but distinct from Marek's disease virus and herpesvirus of turkey. Thus it is proposed to belong to the *Iltovirus* genus. PsHV-1 has been classified into 4 genotypes on the basis of the UL16 gene sequence. These genotypes show distinct biological characteristics and the potential to cause PD [102]. On the other hand, PsHV-1 genotypes 1, 2, and 3, but not 4 have been found in mucosal papillomas [96]. The genotypes detected in PD-affected birds are biased according to bird species and their geographic origins. Therefore, specific PsHV-1 genotypes may be the cause of PD in certain species. Amazon parrots are those most commonly diagnosed with PD and have been identified with all 4 PsHV-1 genotypes. The 4 PsHV-1 genotypes were also found among birds from the Pacific region such as cockatiels and cockatoos. PsHV-1 genotypes found in African grey parrots include genotypes 2, 3, and 4, but not genotype 1. In the case of macaws, genotype 4 has been commonly found, while genotype 3 is rarely found and genotypes 1 and 2 have not been found [102].

PD should be suspected in any bird that dies suddenly without clinical signs. However, bacterial hepatitis, lead poisoning, APV, PsAdV, and reoviruses can all cause clinical and gross changes similar to those noted with PD. Additionally, the intranuclear inclusion bodies induced by PsHV-1 can appear under the microscope similar to those caused by APV and PsAdV. Thus, confirmation that a bird has died from PD requires the demonstration of a viral infection by electron microscopy [92], cell culture [33], PCR [102], or real-time PCR [45].

Psittacine adenovirus infection: Adenovirus infections in psittacine birds have been associated with depression, anorexia, diarrhea, and cloacal hemorrhage [56, 62, 72]. Adenovirus outbreaks have also been described as having a clinical progression similar to PsHV-1 infection, in which normal-appearing birds are found dead in their enclosures [59, 90]. Gross lesions associated with adenovirus infections in various birds include hepatomegaly, splenomegaly, dilatation of the duodenum and proventriculus, swollen kidneys as well as edema, congestion, and hemorrhage of the lungs [23, 79, 80]. Enlarged friable livers may be hemorrhagic, pale or mottled. Liver lesions are the most consistent gross change in infected birds. Basophilic intranuclear inclusion bodies are routinely seen in association with necrosis in the liver and spleen (Fig. 1D) [62].

Adenoviruses (AdVs) have been isolated from a wide range of vertebrates including mammals, birds, reptiles, amphibians, and fishes [4]. The classification of adenoviruses has been recently revised [4] and avian adenoviruses are now classified into three genera: *Aviadenovirus, Atadenovirus*, and *Siadenovirus*. Most of the avian adenoviruses that have been characterized are classified into the genus *Aviadenovirus.*

The adenovirus genome is a linear, double-stranded DNA and is estimated to be 25–45 kbp in length. The *Aviadenovirus* fowl adenovirus CELO strain, is 43,804 bp in length, TAdV-3 26,263 bp in length, and EDSV 33,213 bp in length [11, 32, 77]. The adenovirus is nonenveloped and has an icosahedral capsid with a diameter of 70 nm. The capsid is made up of 2 types of capsomeres: 12 vertex capsomeres composed of a fiber attachment protein and its penton base, and 240 hexons [15]. The hexon protein is the major viral capsid protein of the adenovirus [66] and consists of 2 functional components: the conserved pedestal regions P1 and P2, and the variable loops L1-L4 [1, 87]. L1, L2, and L4 are located at the surface of the hexon protein and interact with the immune response of the host. The hexon protein is known, therefore, to possess family-, genus-, species-, and type-specific determinants [15, 54]. The hexon gene has been used for the phylogenetic study of adenoviruses [17, 52].

Adenovirus infections in psittacine birds have been identified on the basis of microscopic studies but not by clinical diagnosis. Infections with the adenovirus [10] or adenovirus-like particles [21, 57, 62, 72] have been described in a variety of psittacine birds including budgerigars (*Melopsittacus undulatus*), macaws (*Ara* spp.), Amazon parrots (*Amazona* spp.) and cockatoos (*Cacatua* spp.). However, little is known about the genomic organization of adenoviruses infecting psittacine birds. Recently, an adenovirus was detected by PCR in Senegal parrots (*Poicephalus senegalus*) showing clinical and pathological signs of adenovirus infection. The adenovirus was subsequently identified by its hexon gene sequence as a new avian adenovirus belonging to *Aviadenovirus* [81]. Designation of this virus as psittacine adenovirus (PsAdV) was approved by the ICTV [81].

Very recently, we identified a new adenovirus belonging to *Siadenovirus* in budgerigars showing ruffled feathers, named as Budgerigar Adenovirus type 1 (BuAdV-1) [46]. However, the prevalence and pathogenicity of BuAdV-1 is, as yet, unclear.

Psittacine poxvirus infection: Avian poxvirus infections have been observed in more than 230 of the known 9,000 species of birds, spanning 23 orders [38]. Avian poxvirus infections in psittacine birds (psittacine poxvirus (PsPoV) infections) have been reported in several psittacine species, especially Amazon and pionus parrots [8, 58], although this disease has never been reported in Japan. Avian poxviruses are all classified in the genus *Avipoxvirus* of the family *Poxviridae* with the subfamily *Chordopoxvirinae*. In common with other poxviruses, they contain a double stranded DNA genome, ranging from 230 to > 300 kbp. Based on phylogenetic studies of the *fpv167* locus, which encodes orthologues of vaccinia virus core protein P4b, PsPoV located in a distinct cluster from the avian poxvirus derived from other avian species [38].

The earliest clinical changes in affected birds are characterized by a serous ocular discharge, rhinitis, and conjunctivitis, followed by the appearance of ulcerations on the eyelid margins [8, 58]. Dry crusty lesions are noted on the lid margins and the lateral and medial canthi of the eyes.

PsPoV has been shown to vary in virulence in different hosts. Amazon parrots appear to be most susceptible to PsPoV infections. Infected Amazon parrots may also develop a severe upper respiratory tract disease. Mortality rates are highest when diphtheritic lesions cause defects in the mucosal barrier of the alimentary and respiratory tract, allowing secondary bacterial, fungal, or chlamydial organisms unrestricted access to the affected birds [8, 27, 58]. Histopathological lesions include necrosis of the heart and liver, as well as air sacculitis, pneumonia, peritonitis, and accumulation of necrotic debris on the surface of the alimentary tract. Characteristic intracytoplasmic inclusion bodies (Bollinger bodies) may be noted in lesions in the mucosa of the sinuses, trachea, crop, esophagus or throat [24].

The clinical changes associated with the cutaneous form of PsPoV are often suggestive. However, trauma and fugal, bacterial, and papillomavirus infection can cause similar lesions. Thus, recently, PsPoV infection was confirmed by PCR [38]. In other bird species, virus neutralization, ELISA, and hemagglutination-inhibition test are also used to diagnose of poxvirus infection [12, 63].

Psittacine papillomavirus infection: Internal papillomatosis of parrots (IPP) is a tumor disease characterized by progressive development of papillomas in the oral and cloacal mucosa [98]. Papillomatosis of the cloaca has been described as the appearance of large, raised, distinct masses or small, coalescing bumps that cover much of the cloacal mucosa. These lesions may cause a local inflammatory response and secondary infections of bacteria or fungi. In addition, papillomatosis of the oral cavity or esophagus may mechanically obstruct the movement of food, causing anorexia, chronic weight loss or vomiting. Birds with papillomatosis tend to develop neoplasias of the pancreas or liver [13]. Papillomatosis lesions are characterized by proliferation of epithelial cells on thin fibrovascular stalks [98]. Histopathological and microbiological studies suggest that papillomaviruses are one of the etiologic agents of IPP [68, 98]. Papillomaviruses are a large group of pathogens that cause epithelial proliferations in a wide spectrum of vertebrate species. Several genetic analyses of avian papillomaviruses have been reported [61, 99, 107]. The papillomavirus isolated from a cutaneous lesions of an African grey parrot (*Psittacus erithacus*) was suggested to be phylogenetically related to another papillomavirus derived from the chaffinch (*Fringilla coelebs*) [99]. A herpesvirus also has been suggested to be involved in the etiology of IPP [39].

Papillomatosis is diagnosed by histological examination of biopsy samples. The etiological agents of papillomatosis have been identified by PCR and *in situ* hybridization [39, 53].

Problems and proposals: A common problem of these DNA virus infections is that there are few etiological treatments and no efficient vaccines available at present. Thus, the main care for these diseases is symptomatic treatment and preventive therapy for bacterial secondary infection.

Although only acyclovir has been shown to reduce the sickness and death of PD-affected birds, it is also associated with kidney damage in some species and it can not prevent acute death [67]. Chicken interferon-γ has been reported to alleviate manifestations of PBFD-affected African grey parrots [93].

The baculovirus-expressed recombinant BFDV capsid protein was recently reported to be immunogenic and might be a suitable candidate vaccine to prevent PBFD in psittacine birds [7]. However, an optimal vaccination regime needs to be determined to protect against PBFD. Poxvirus vaccines are available for use in several avian species, including chickens, pigeons, turkeys, quail, canaries, and psittacine birds. However, poxvirus that infects psittacine birds is serologically unrelated to other poxviruses [108]. Thus, effective prevention against PsPoV requires species-specific vaccines. Commercial vaccines for APV and PsHV-1 infection are available in U.S.A., but not in Japan [20, 85].

The goal of maintaining any bird in captivity is to keep the birds in best possible condition. In veterinary medicine, vaccination plays a major role in preventing viral infections. Therefore, effective and safe vaccines for these virus infections are urgently needed.

ACKNOWLEDGMENTS. We thank Dr. Toshiaki Masegi, Dr. Tokuma Yanai, and Dr. Hiroki Sakai, Laboratory of Veterinary pathology, Gifu University, for providing the histopathological photographs.

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