10.2478/abm-2010-0071

Asian Biomedicine Vol. 4 No. 4 August 2010; 563-569

Original article

Prevalence of respiratory viruses isolated from dogs in Thailand during 2008-2009

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***Background*:** A highly contagious respiratory disease in canines is infectious tracheobronchitis or kennel cough characterized by inflammation of the upper respiratory tract. The cause of kennel cough has been associated with multiple or complex agents such as canine adeno virus (CAV), canine influenza virus (CIV), canine distemper virus (CDV), and canine para influenzavirus (CPIV).

***Objective*:** Study the prevalence of canine respiratory viruses detected from in Thailand during 2008-2009.

***Methods*:** Nasal swab samples collected from 102 healthy dogs and 109 dogs with respiratory diseases. Then CAV, CIV, CDV, and CPIV were detected by in-house nested PCR and further confirmed by nucleotide sequencing.

***Results*:** Nested PCR showed that primers designed and used in this study yielded high specificity without any non-specific amplification. The prevalence of CAV, CIV, CDV and CPIV in healthy dogs was 0%, 2.94%, 2.94%, and 0.98%, whereas that found in dogs with respiratory diseases was 9.17%, 1.83%, 2.75%, and 11.93%, respectively. In healthy dogs, co-infection with CPIV + CDV was detected in only 0.98%. On the other hand, dogs with respiratory symptoms showed multiple infections with CAV + CIV in 1.83%, CIV + CPIV in 0.92%, CAV + CPIV in 1.83%, and CAV + CDV + CPIV in 0.92%.

***Conclusion*:** The prevalence data obtained from this study may be useful for outbreak preventions and to raise awareness of potential transmission of the newly emerged canine influenza virus to humans.

Keywords: Canine, prevalence, respiratory viruses, Thailand

Respiratory disease in dogs called canine infectious tracheobronchitis (or kennel cough) is a highly contagious disease transmitted by aerosol from infected dogs especially in poorly ventilated kennels, animal shelters, and veterinary hospital [1]. This disease manifests as inflammation of the upper respiratory system. The clinical symptoms of respiratory disease in dogs can be divided into asymptomatic infection, mild illness, and severe illness. The mild illness is characterized by dry, hacking cough, increasing mucus formation and gagging whereas dogs with severe illness tend to be clinically ill, febrile, anorexic, depress, and die as a result of multiple infection, immuno-suppression, or lack of vaccination [2]. The potential etiological pathogens of respiratory disease in dogs are canine distemper virus, canine adeno virus and canine para-influenza-virus [3].

Canine adeno virus (CAV) is a double stranded DNA virus classified into the *Adenoviridae* family. It can be divided into two types, canine adenovirus type 1 (CAV-1) and canine adenovirus type 2 (CAV-2), which target different cell types. CAV-1 preferentially infects vascular endothelial cells, hepatic cells, and renal parenchymal cells, whereas the main targets of CAV-2 are epithelial cells in the respiratory tract and intestine [4, 5]. Therefore, CAV-2 is one of the causes of respiratory disease in dogs and has rapidly spread in dogs lacking immunity against CAV [6].

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Canine distemper virus (CDV) is a negative single stranded RNA virus belonging to the *paramyxoviridae* family, which is the causative agent for serious disease in dogs [7]. The main targets of CDA are mucous membranes and lymphoid tissues. CAD is transmitted by aerosol and then replicates in lymphatic tissues of the host's respiratory tract. Subsequently, the progeny viruses spread to other organs such as lower respiratory tract, gastrointestinal tract, lymphatic system, urinary bladder, and central nervous system because of subclinical infection or complex infection. The clinical signs were associated with respiratory and enteric diseases of infected dogs [8].

Canine parainfluenza virus (CPIV) is a negative single stranded RNA virus categorized into the *Paramyxoviridae* family, which is ubiquitous in canine populations. Usually, CPIV infects the upper respiratory tract but can sometimes be recovered from lungs, spleen, kidney, and liver of infected dogs. This virus replicates mainly in the epithelial cells of the nasal mucosa, large bronchi, pharynx trachea, and regional lymph nodes. It has been considered an important cause of kennel cough commonly isolated from outbreaks of the disease. The symptoms of the disease are mild or subclinical, but in some cases, CPIV infection may occur simultaneously with other viral or bacterial agents leading to more severe signs of kennel cough [9].

Recently, canine influenza A virus has been reported to infect and cause respiratory illness in dogs. In 2004, canine influenza (H3N8) has emerged in the United States because of direct transmission from equine influenza virus subtype H3N8 to greyhounds [10]. In 2005, CIV (H3N8) were isolated from non-greyhound dogs with respiratory disease in Florida [11]. Moreover, another subtype of canine influenza virus has recently emerged in Korea in 2007, which originated from avian influenza subtype H3N2 [12]. CIV is also the cause of respiratory diseases ranging from mild to severe illness. Mild illness includes fever, cough, nasal discharge, sneezing whereas severe illness consists of severe nasal discharge, and death resulting from hemorrhage in the respiratory tracts such as lungs, mediastinum, and pleural cavity [10].

At present, vaccines against CDV, CAV, and CPIV are readily available. However, infectious tracheobronchitis in dogs is still observed [13]. In 2009, pandemic influenza H1N1 2009 has been found in a 13-year-old dog in Bedford Hills, New York. The owner was ill with positive pandemic influenza H1N1 2009 virus after that the dog became sick. The clinical symptoms in dog were lethargic, coughing, refused feeding, and high grade fever. Investigation of the laboratory revealed that the dog was negative canine influenza virus (H3N8) but positive for pandemic influenza H1N1 2009 [14]. Moreover, there were two dogs in Beijing, China with detectable pandemic influenza H1N1 2009. It is possible that pandemic influenza H1N1 2009 can transmit the virus from human to dogs [15]. The pandemic influenza H1N1 2009 virus can infect cat, turkey, ferret, pig and human [14]. In Thailand, the pandemic influenza H1N1 2009 has also occurred since May 2009. The details have been reported elsewhere [16]. However, pandemic influenza H1N1 2009 virus in dogs has not been reported in Thailand. In this study, a sensitive nested reverse transcription-polymerase chain reaction (RT-PCR) was performed to detect respiratory virus including CDV (CDV), CPIV, CAV, and CIV in dogs with respiratory diseases as well as healthy dogs in Thailand between 2008 and 2009. The prevalence data obtained from this study may be useful for outbreak prevention and vaccine management as well as to raise awareness of potential transmission of the newly emerged CIV to humans.

Materials and methods

Clinical samples

Nasal swab specimens were collected from 102 healthy dogs and 109 dogs with respiratory disease in Thailand between 2008 and 2009. The specimens of healthy dogs were obtained from dogs subjected to sterilization at the Office of Veterinary Public Health, Bangkok, Thailand. Dogs with respiratory diseases were clinically diagnosed, symptoms recorded, and nasal swab specimens collected by veterinarians from the Small Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University, and the Thonglor Pet Hospital, Sirintorn-Pinkrao branch, Bangkok. The nasal swabs were collected in 1 mL of viral transport medium with antibiotics (penicillin G $2x10^6$ U/L and streptomycin 200mg/L), transported in a biohazard icebox and stored at -70°C until tested.

Nucleic acid extraction and cDNA synthesis

DNA and RNA were extracted by PureLink™ Viral RNA/DNA Mini kits (Invitrogen, Carlsbad, USA) according to the manufacturer's recommendation and final elution of the nucleic acids with 30 μL of elution buffer. The extracted RNAs were subsequently used

as templates for reverse transcription (RT) applying the M-MLV reverse transcription system (Promega, Madison, USA) following the producer's specification. Briefly, the reaction mixtures included 5 μL of 5x M-MLV reaction buffer, 5 μL of 10mM dNTP, 200 units of M-MLV reverse transcriptase, 25 units of RNasin® ribonuclease inhibitor, random hexamer (1 μM final concentration), 2 μg of the extracted RNA and DPEC-treated sterilized water to a final volume of 25 μL. The reaction mixtures were incubated at 37^{O}C for three hours and the reaction was terminated by heating at 70^{O}C for five minutes.

Specific primer designs

Nucleotide sequences of the M gene of influenza A virus, E3 gene of CAV, NP genes of CDV, NP gene of canine parainfluenza virus, and canine GAPDH gene (internal control) were obtained from the Gen Bank database. More than 40 sequences of each target gene were subjected to multiple alignments using the BioEdit Sequence Alignment Editor Version 7.0.9.0 [17]. The conserved regions were selected as candidate primers for the *in silico* specificity test by BLAST analysis (available online at http://blast.ncbi.nlm.nih.gov/Blast.cgi) to ensure the specificity of the selected primers without cross-amplification of canine genes. Subsequently, the candidate primers were evaluated by Oligos primer design software version 9.1 (Ruslan Kalendar, Institute of Biotechnology, University of Helsinki, Finland) to ensure absence of primer-dimers, self-pairing and secondary structure. Each primer pair was tested for specificity against viral DNA/RNA extracted from NOBIVAC DHPPi live vaccine (Intervet International BV, Boxmeer, Holland) containing CDV, VAV-2, canine parvovirus, and CPIV. All specific primers used in this study were synthesized by BioDesign Co, Ltd (Pathumthani, Thailand) and are summarized in **Table 1**.

Detection of canine respiratory viruses by nested PCR amplification

The reaction mixture for the first round of nested PCR included 10 μl of 2.5x Eppendorf MasterMix (Eppendorf, Hamburg, Germany), 1 μL of DNA or cDNA template, 0.2 μM final concentration of each primer and nuclease-free water to a final volume of 25 μL. The PCR reactions were performed in a thermal cycler (Eppendorf, Hamburg, Germany) under the following condition, initial denaturation at 94^{O}C for three minutes, four cycles of amplification consisting of 94^{O}C for 30 seconds, 55^{O}C for 30 seconds, and 72^{O}C for one minute, and final extension at 72^{O}C for seven minutes. The second round of nested PCR was performed using a similar reaction mixture and thermal cycling profile as described above but changing the primer pair and using the PCR product from the first round as a template for nested amplification. The PCR products obtained from the second round of amplification were analyzed by 2% agarose gel electropholysis at 100 volts, for 40 minutes. Subsequently, the agarose gel was stained with ethidium bromide and visualized under UV transillumination. The expected size of each amplified product is summarized in **Table 1** and displayed in **Fig. 1**.

DNA purification and nucleotide sequencing

The PCR products of interest were purified by using the Perfect Prep Gel Cleanup Kit (Eppendorf, Hamburg, Germany) following the company's protocol with the final elution performed in 40 μl of elution buffer. The purified DNA was subjected to nucleotide sequence determination by a commercially available sequencing service (1st BASE Laboratories, Selangor Darul Ehsan, Malaysia).

Sequencing analysis

Nucleotide sequences were analyzed by BLAST analysis tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi), BioEdit Sequence Alignment Editor version 7.0.9.0 [17] and Chromas Lite version 2.01 (Technelysium Pty, Ltd).

Result

Detection of canine respiratory viruses by nested PCR

Amplification of canine DNA/cDNA specimens by nested PCR yielded DNA bands of 350, 207, 220 and 180 bp indicating CAV, CIV, CDV, and CPIV, respectively (**Fig. 1**). The results showed that the bands of the positive samples were exactly the same size as those found in the positive control live vaccine from NOBIVAC DHPPi (Intervet International BV, Boxmeer, Holland) without any non-specific band indicating high specificity of the primers used for canine respiratory virus detection by nested PCR.

Table 1. Primers used for nested PCR amplification of canine respiratory viruses.

Virus	Primer name	Primer	Target gene	Position	Product size (bp)
Canine influenza virus	CIV_MF3'	5'-GATCGCTCTTCAGGGAGCGAAAGCAGGTAG-3'	M gene	1-30	1027
	CIV_MR3'	5'-ACTGGCTCTTCTATTAGTAGAAACAAGGTAGTTTTT-3'		1027-985	
	CIV_M_F56	5'-TCAGGCCCCCTCAAAGCCG-3'		49-67	207
	CIV_M_R276	5'-ACRCTCACCGTGCCCAGT-3'		256-233	
Canine distemper virus	CDV_F768	5'-AACAGRATTGCTGAGGACYTAT-3'	NP gene	768-782	289
	CDV_R1057	5'-TCCARRATAACCATGTAYGGTGC-3'		1057-1035	
	CDV_F838	5'-CAGGGAACAAGCCTAGAATTGC-3'		838-852	220
	CDV_R1057	5'-TCCARRATAACCATGTAYGGTGC-3'		1057-1035	
Canine adenovirus	CAV_F73	5'-TATTCCAGACTCTTACCAAGAGG-3'	E3 gene	25073-25095	525
	CAV_R629	5'-ATAGACAAGGTAGTARTGYTCAG-3'		25623-25601	
	CAV_F249	5'-AGGCRGCCTGTTCTATGTATAAC-3'		25249-25271	350
	CAV_R629	5'-ATAGACAAGGTAGTARTGYTCAG-3'		25623-25601	
Canine parainfluenzavirus	CPIV_F363	5'-GGGTAGAGATCGATGGCTTTGA-3'	NP gene	363-386	251
	CPIV_R538	5'-GCGCAGTCATGCACTTGCAAGT-3'		614-593	
	CPIV_F428	5'-GCCGTGGAGAGATCAATGCCTAT-3'		428-451	180
	CPIV_R538	5'-GCGCAGTCATGCACTTGCAAGT-3'		614-593	

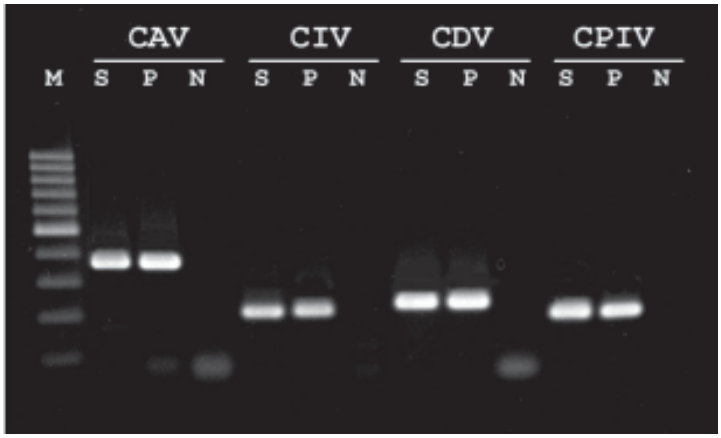

Fig. 1 Detection of canine respiratory viruses by nested PCR. A fragment of 350, 207, 220 and 180 base pair indicate the specific band of CAV, CIV, CDV, and CPIV, respectively. Lanes: M= 100-bp markers; S=sample, P= positive control, N= negative control.

Identification of positive DNA bands by nucleotide sequencing

After nested PCR amplification and agarose gel electrophoresis, all virus positive DNA bands obtained from clinical samples were subjected to purification and confirmed by nucleotide sequencing. Nucleotide sequences were corrected by Chromas Lite version 2.01, aligned and trimmed by BioEdit Sequence Alignment Editor version 7.0.9.0. The sequences were confirmed and identified by BLAST analysis. The sequencing results showed that most positive bands obtained from clinical samples were concordant with the results expected from nested PCR (data not shown). A few sequences neither matching CAV, CIV, CDV, or CPIV were interpreted as false positives**.**

Prevalence of canine respiratory virus infection in Thailand

Single infection with canine respiratory viruses. Based on amplification of canine DNA/cDNA specimens by nested PCR and confirmation by nucleotide sequencing, CAV was not detected in healthy dogs (0%) whereas it was found in 10 of 109 (9.17%) specimens obtained from dogs with respiratory symptoms. Interestingly, CIV was detected in three of 102 (2.94%) specimens from healthy dogs and 3 of 109 (2.75%) specimens obtained from dogs with respiratory disease. Surprisingly, CDV was detected in three of 102 (2.94%) and 2 of 109 (1.83%) clinical specimens obtained from healthy dogs and dogs with respiratory symptoms, respectively. Finally, CPIV had infected one of 102 (0.98%) healthy dogs and 13 of 109 (11.93%) dogs with respiratory diseases (**Table 2**).

Multiple infections with canine respiratory viruses. In healthy dogs, co-infection was detected in only one of 102 (0.98%) specimens infected with CPIV and CDV. On the other hand, dogs with respiratory symptoms displayed co-infection with CIV and CAV in two of 109 (1.83%) specimens, with CIV and CPIV in one of 109 (0.92%) cases, and with CAV and CPIV in two of 109 (1.83%) samples. Triple infection was found only in dogs with respiratory diseases in that one sample (0.92%) was infected with CAV, CDV and CPIV (**Table 2**).

Clinical symptoms observed in dogs with respiratory diseases. The clinical symptoms observed in dogs with respiratory diseases manifested as signs of illness including severe nasal discharge or upper respiratory tract infection in dogs infected with adenovirus. Dogs infected with CIV might display severe nasal discharge, mucous nasal discharge, sneezing, cough or respiratory tract infection. Dogs suffering from canine distemper virus infection might show severe nasal discharge, mucous nasal discharge, dyspnea, cough or sneezing. Dogs infected with CPIV might exhibit cough, dry cough, severe nasal discharge, purulent nasal discharge, or bronchitis. The clinical symptoms observed in the multiple-infected dogs were similar but the illness could potentially become more severe.

Table 2. Prevalence of canine respiratory virus infections in dogs.

	Normal dogs (n=102)	Dogs with respiratory disease (n=109)
Single infection		
CAV	0	10 (9.17%)
CIV	3 (2.94%)	3 (2.75%)
CDV	3 (2.94%)	2 (1.83%)
CPIV	1 (0.98%)	13 (11.93%)
Co-infection		
CIV+CAV	0	2 (1.83%)
CIV+CPIV	0	1 (0.92%)
CAV+CPIV	0	2 (1.83%)
CPIV+CDV	1 (0.98%)	0
Triple infection		
CPIV+CAV+CDV	0	1 (0.92%)

Discussion

This study investigated the prevalence of canine respiratory viruses including CIV, CAV, CDV, and CPIV in nasal swab samples of 102 healthy dogs and 109 dogs with respiratory diseases based on sensitive and specific nested PCR amplification and further confirmation and identification by nucleotide sequencing. The results of nested PCR showed that the primers designed and used in this study yielded high specificity without any cross-amplification of canine genes or unexpected non-specific amplification. Moreover, these primers yielded clearly defined DNA bands, which were also suitable for further direct nucleotide sequencing without requiring additional DNA cloning. Furthermore, the specific primers designed for each canine respiratory virus can be used with the same PCR reaction mixtures and thermo cycling profile. Therefore, the primers and conditions of nested PCR established in this study would be useful and suitable for further large-scale screening for canine respiratory viruses.

The prevalence of canine adenovirus in Thailand could not be determined in healthy dogs whereas the virus was detected in approximately 9.17% of dogs with respiratory diseases. CAV-2 positive cases in Thailand were more numerous than in Japan [18] where about 2.9% had been reported, indicating that Thai dogs may be at higher risk for infection with CAV than dogs in Japan. Approximately 2.94% of healthy dogs and 1.83% of dogs with respiratory diseases in Thailand were infected with CDV. This is comparable with previous study reported that the prevalence of canine distemper virus in Japan [18] was about 1.5% in dogs with respiratory signs. CPIV was found in 0.98% of healthy and 11.93% of dogs with respiratory disease. Based on pervious studies, the prevalence of CPIV in dogs with a history of respiratory disease was 7.4% in Japan [18] and 19.09% in USA [13]. Both publications reported that CPIV accounted for the highest prevalence of canine respiratory virus in dogs with respiratory disease, which is also concordant with the result obtained from this study.

In this study, the prevalence of CIV was approximately 2.94% in healthy dogs indicating that healthy dogs can be asymptomatically infected with CIV and might be able to act as a carrier of this virus. The virus was also found in 2.75% of dogs with respiratory symptoms including nasal discharge, mucous nasal discharge, sneezing, cough, or respiratory tract infection. The severity of symptoms observed in dogs with respiratory disease in Thailand appears to be less than that found in Florida, United States and South Korea [10-12] where infected dogs exhibited more severe respiratory diseases and died. This indicates that the sub-types of CIV detected in Thailand may be different from H3N8 or H3N2, which had been found in Florida and South Korea, respectively. Therefore, sub-types of the CIV isolated from both healthy dogs and dogs with respiratory symptoms in Thailand should be further investigated to predict the severity of infection and the potential for cross-infection to humans.

In conclusion, this study described the prevalence of canine respiratory viruses including CIV, CAV, CDV, and CPIV in nasal swab samples of 102 healthy dogs and 109 dogs with respiratory diseases based on the in-house nested PCR detection method and further confirmation by nucleotide sequencing. The prevalence data obtained from this study may be useful for outbreak prevention and vaccine management as well as to raise awareness of potential transmission of the newly emerged CIV to humans.

Acknowledgement

This study was supported by funding from the Thailand Research Fund (TRF), Chulalongkorn University (Ratchadapiseksompotch Fund CU Graduate School Thesis Grant), the Commission on Higher Education, Ministry of Education, Center of Excellence in Clinical Virology, Chulalongkorn University, CU Centenary Academic Development Project, and King Chulalongkorn Memorial Hospital. We thank Ms. Pinkphan Charoenphol (D.V.M.) and Ms. Suphasawatt Puranaveja (D.V.M.) for their kind assistance with specimen collection. We also thank Ms. Petra Hirsch for reviewing the manuscript. The authors have no conflict of interest to report.

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