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Molecular detection of canine respiratory pathogens between 2017 and 2018 in Japan

Aya MATSUU[1)]*, Mihoko YABUKI[1)], Emiko AOKI[2)] and Michio IWAHANA[2)]

[1)]Transboundary Animal Diseases Research Center, Joint Faculty of Veterinary Medicine, Kagoshima University, 1-21-24 Korimoto, Kagoshima 890-0065, Japan

[2)]Zoetis Japan Co., Ltd., 3-22-7 Yoyogi, Shibuya, Tokyo 151-0053, Japan

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ABSTRACT. A molecular survey was conducted to understand recent distribution of pathogens associated with canine infectious respiratory disease (CIRD) in Japan. Nasal and/or pharyngeal swabs were collected from asymptomatic dogs and those with CIRD, living in private house or in kennels. PCR-based examination was conducted for detecting nine pathogens. Among private household dogs, 50.8% with CIRD, 11.1% with respiratory disease other than CIRD, and 4.3% asymptomatic were positive for more than one pathogen, whereas in kennel-housed dogs, 42.9% with CIRD and 27.3% asymptomatic were positive. *Bordetella bronchiseptica* was most frequently detected, followed by canine herpesvirus 1, canine parainfluenza virus, canine pneumovirus, *Mycoplasma cynos,* and canine adenovirus type 2. In kennel environment, asymptomatic dogs might act as reservoirs carrying the respiratory pathogens.

KEY WORDS: asymptomatic, canine infectious respiratory disease, dog, kennel environment

Canine infectious respiratory disease (CIRD), classically known as “kennel cough”, is a common syndrome of dogs, especially young ones that are kept in large groups, as in shelters, pet shop, or breeding kennels. CIRD may occur even in pet dogs housed at home. Typical clinical signs include acute onset of coughing and nasal discharge, sometimes dyspnea with or without fever. CIRD is considered a complex infection with multifactorial etiology, associated with classical and newly emerging pathogens [10, 24].

Canine distemper virus (CDV), canine parainfluenza virus (CPiV), canine adenovirus type-2 (CAV-2), and *Bordetella bronchiseptica* are well known classical pathogens of CIRD [24] that are primarily associated with respiratory diseases in dogs. In addition, canine herpesvirus type 1 (CHV-1) [16], canine pneumovirus (CPnV) [20, 25, 26], canine respiratory coronavirus (CRCoV) [5, 21, 27, 34], *Mycoplasma cynos* [13], canine influenza virus (CIV) [11, 22], *Streptococcus equi* subsp. *zooepidemicus* [17, 23], canine bocavirus [15, 24], and canine hepacivirus [1, 7] are some recently identified emerging agents. In recent molecular epidemiological studies, the prevalence of these new and emerging respiratory pathogens in CIRD dogs have been reported. In Italy, it was reported that CPiV was mainly responsible for CIRD occurrences, followed by CRCoV, *B. bronchiseptica*, *M. cynos*, *M. canis*, and CPnV, whereas classical CIRD agents, such as CAV and CDV were not detected at all. In another study from New Zealand, CIRD dogs were found mostly positive for *M. cynos*, followed by CPiV, and *B. bronchiseptica*, and all dogs were found negative for CIV, CRCoV, CDV, and *S. equi* subsp. *zooepidemics*. Although CIRD is the most common, worldwide, its etiology or epidemiology may vary across countries [6, 12, 18, 19, 21, 28, 31].

In 2008, an etiologic study of upper respiratory infections of house dogs in Japan had reported *B. bronchiseptica* and CPiV as the principal etiologic agents [21]. Another study in 2009 reported *B. bronchiseptica*, CPiV, and CRCoV to possibly be the major pathogens in CIRD [29]. Prevalence of some pathogens, including CIV, CPnV, and *M. cynos* has not been elucidated till date. A more recent epidemiologic survey and the relative prevalence in Japanese dog population would be useful for veterinary practice and animal health sciences. Here, we conducted a molecular etiology-based study using samples from symptomatic and asymptomatic dogs living in two different environments.

Between 2017 and 2018, nasal and/or pharyngeal swab samples were collected from 167 household dogs, and submitted by 52 animal hospitals from 27 prefectures throughout Japan. Based on the information provided by the veterinarians, 61 dogs were suspected to have CIRD owing to acute onset (<1 week) of cough with or without fever, 83 dogs showed respiratory signs, although not CIRD (chronic cough since over a month, heart failure, or tracheal collapse), and 23 dogs were asymptomatic. Some dogs with respiratory symptoms had been already administered antibiotic products at the time of sampling. Other swab samples were collected from 80 dogs housed in 12 kennels from 12 prefectures. Among these dogs, 14 showed clinical signs of CIRD, 66 were asymptomatic, and no dogs showed chronic respiratory signs. Detailed animal information is summarized in Table 1. Swabs were placed immediately in BD universal viral transport medium (Becton, Dickinson and Co., Franklin Lake, NJ, USA). Total

*Correspondence to: Matsuu, A.: matsuu@vet.kagoshima-u.ac.jp

Table1. Numbers (%) of dogs with muptiple infection

Signalment	Private household dogs									Kennnel housed dogs					
	CIRD n=61			Respiratory disease, not CIRD n=83			Asymptomatic n=23			CIRD n=14			Asymptomatic n=66		
	No.	(%)	95%CI	No.	(%)	95%CI	No.	(%)	95%CI	No.	(%)	95%CI	No.	(%)	95%CI
Gender															
Male	20	(32.8)	21.3–46.0	30	(36.1)	25.9–47.4	1	(4.3)	0.1–21.9	5	(35.7)	12.8–64.9	15	(0.2)	13.3–34.7
Casted male	8	(13.1)	5.8–24.2	21	(25.3)	16.4–36.0	8	(34.8)	16.4–57.3	0	(0.0)	0–22.1	0	(0.0)	0–4.4
Female	19	(31.1)	19.9–4.3	5	(6.0)	2.0–13.5	3	(13.0)	2.8–33.6	4	(28.6)	8.4–58.1	22	(0.3)	22.2–46.0
Spayed female	6	(9.8)	3.7–20.2	20	(24.1)	15.4–34.7	10	(43.5)	23.2–65.5	0	(0.0)	0–22.1	0	(0.0)	0–4.4
Unknown	6	(9.8)	3.7–20.2	5	(6.0)	2.0–13.5	1	(4.3)	0.1–21.9	0	(0.0)	0–22.1	20	(0.3)	19.6–42.9
Age															
<6 month	43	(70.5)**	57.4–81.5	3	(3.6)	0.8–10.2	1	(4.3)	0.1–21.9	10	(71.4)	41.9–91.6	24	(36.4)	24.9–49.1
7 month-1year	5	(8.2)	2.7–18.1	7	(8.4)	3.5–16.6	1	(4.3)	0.1–21.9	1	(7.1)	0.2–33.9	3	(4.5)	0.9–12.7
2–5 year	5	(8.2)	2.7–18.1	6	(7.2)	2.7–15.1	4	(17.4)	5.0–38.8	1	(7.1)	0.2–33.9	10	(15.2)	7.5–26.1
6–10 year	3	(4.9)	1.0–13.7	26	(31.3)	21.6–42.4	12	(52.2)	30.6–73.2	1	(7.1)	0.2–33.9	18	(0.3)	17.0–39.6
>11 year	5	(8.2)	2.7–18.1	36	(43.4)	32.5–54.7	5	(21.7)	7.5–43.7	1	(7.1)	0.2–33.9	3	(4.5)	0.9–12.7
Unknown	0	(0.0)	0–4.8	5	(6.0)	2.0–13.5	0	(0.0)	0–12.2	0	(0.0)	0–22.1	8	(0.1)	5.4–22.5
Environment															
Indoor	58	(95.1)	86.3–99.0	74	(89.2)	80.4–94.9	22	(95.7)	78.1–99.9	6	(42.9)	17.7–71.1	16	(24.2)	14.5–36.4
Outdoor	0	(0.0)	0–4.8	1	(1.2)	0–6.5	1	(4.3)	0.1–21.9	0	(0.0)	0–22.1	1	(1.5)	0–8.2
Unknown	1	(1.6)	0–8.8	6	(7.2)	2.7–15.1	0	(0.0)	0–12.2	3	(21.4)	4.7–50.8	40	(60.6)	47.8–72.4
Inmate animals															
No	39	(63.9)	50.6–75.8	18	(21.7)	13.4–32.1	12	(52.2)	30.6–73.2	0	(0.0)	0–22.1	0	(0.0)	0–4.4
More than one animal	17	(27.9)	17.1–40.8	54	(65.1)	53.8–75.2	11	(47.8)	26.8–69.4	14	(100.0)	80.7–100	66	(100.0)	95.6–100
Unknown	5	(8.2)	2.7–18.1	11	(13.3)	6.8–22.5	0	(0.0)	0–12.2	0	(0.0)	0–22.1	0	(0.0)	0–4.4
Vaccination[a)]															
Vaccinated	44	(72.1)	59.2–82.9	46	(55.4)	44.1–66.3	21	(91.3)	72.0–98.9	3	(21.4)	4.7–50.8	11	(16.7)	8.6–27.9
None	2	(3.3)	0.4–11.3	5	(6.0)	2.0–13.5	0	(0.0)	0–12.2	0	(0.0)	0–22.1	0	(0.0)	0–4.4
Unknown	15	(24.6)	14.5–37.3	32	(38.6)	28.1–49.9	1	(4.3)	0.1–21.9	11	(78.6)	49.2–95.3	55	(83.3)	72.1–91.4

CIRD, canine infecious respiratory disease; 95%CI, 95% confidence interval. a) Vaccination defined as application including canine parainfluenza virus (CPiV) and canine adenovirus type-2 (CAV-2) with core vaccination. **Significantly higher than respiratory disese dogs and asymptomatic dogs (P<0.01).

nucleotide was extracted from the supernatant using an automatic extraction system magLEAD® with magLEAD® Dx SV reagent (Precision System Science Co., Ltd., Tokyo, Japan), according to the manufacturer's instructions. This study was approved by the Institutional Animal Care and Use committee, Kagoshima University (permission number: VM19045), and was carried out according to the guidelines of the committee.

For detection of CHV and *B. bronchiseptica* DNA, TaqMan-probe real-time PCR (THUNDERBIRD probe qPCR Mix, TOYOBO, Osaka, Japan) was conducted using the CHV-specific primer sets (Forward: 5′-ACAGAGTTGATTGATAGAAGAGGTATG-3′, Reverse: 5′-CTGGTGTATTAAACTTTGAAGGCTTTA-3′) and a probe (FAM-5′-TCTCTGGGGTCTTCATCCTTATCAAATGCG-3′-TAMRA) [4], and *B. bronchiseptica*-specific primer sets (Forward: 5′-AGGCTCCCAAGAGAGAAGGCTT-3′, Reverse: 5′-AAACCTGCCGTAATCCAGGC-3′) and a probe (5′-FAM-ACCGGGCAGCTAGGCCGC-TAMRA-3′) [32], respectively, according to previous studies. For CPnV detection, TaqMan-probe real-time RT-PCR (THUNDERBIRD probe one-step qRT-PCR Mix, TOYOBO) was conducted using the CPnV-specific primer sets (Forward: 5′- GACCTGTTTGAAAGGAAGCCTTATT-3′, Reverse: 5′- ACCAGAAAACAGCCCCTCAAC-3′) and a probe (5′-FAM-CTTCCATCACTTTTGGCCTGGCCCAG-TAMRA-3′) [20]. For detection of CPiV, CDV, and CIV RNA, SYBR green real-time RT-PCR (Brilliant III Ultra-Fast SYBR Green QPCR Master Mixes, Agilent Technologies, Santa Clara, CA, USA) was performed using specific primer sets for CaPiV (Forward: ATATGGCGGCGTGATTAAAG, and Reverse: TGAATCATTCGATTGCCAAA) [21], CDV (Forward: 5′-AGCTAGTTTCATCTTAACTATCAAATT-3′, Reverse: 5′-TTAACTCTCCAGAAAACTCATGC-3′) [8], and CIV (Forward: 5′-CCMAGGTCGAAACGTAYGTTCTCTCTATC-3′, Reverse: 5′-TGACAGRATYGGTCTTGTCTTTAGCCAYTCCA-3′) as in previous studies. For CRCoV detection, gel-based nested RT-PCR, using the first primer set (Forward: 5′-TATCGCAGCCTTACTTTTGT-3′, and Reverse: 5′-ACCGCCGTCATGTTATCAG-3′), followed by a second PCR, using another primer set (Forward: 5′-GCACAATCTACAGCTCTTTG-3′, and Reverse: 5′-AGACAGATTGCTTTCGTAGGA-3′) [34], was performed. For the detection of *M. cynos* DNA, gel-based conventional PCR was performed, using a specific primer set (Forward: 5′-CACCGCCCGTCACACCA-3′, and Reverse: 5′-GATACATAAACACAACATTATAATATTG-3′) as in a previous study [13].

In private household dogs, 70.5% with CIRD were young, under 6 months of age. The proportion of age was compared by χ^2

testing, and significant differences were seen among dogs with CIRD, without CIRD, and those asymptomatic ($P<0.01$). There was no significant difference in the proportions of gender, environment, presence of innate animals, and vaccination history.

In private household dogs, 31 out of 61 (50.8%) with CIRD, 9 out of 83 (10.8%) with respiratory diseases other than CIRD, and 1 out of 23 (4.3%) asymptomatic dogs were positive for more than one pathogen. On the other hand, in kennel-housed dogs, 4 out of 14 (28.6%) with CIRD and 18 out of 66 (27.3%) asymptomatic ones were positive for more than one pathogen. The number of positive mono- or co-infections is shown in Table 2, and the number of positive individual pathogens is shown in Table 3. Among the private household dogs, *B. bronchiseptica* was the most frequently detected (40.3%), followed by CHV1 (7.5%), CPiV (4.5%), *M. cynos* (4.5%), CaPnV (4.5%), and CAV-2 (1.5%) amongst the ones with CIRD. In dogs with respiratory diseases other than CIRD, *B. bronchiseptica* (9.6%) and CHV (1.2%) were detected. *B. bronchiseptica* was detected in an asymptomatic dog as well (4.3%). In dogs with CIRD, co-infection of more than two pathogens was detected in 11 cases (18.0%). Among kennel-housed dogs, *B. bronchiseptica* (28.6%), and *M. cynos* (14.3%) were detected in the ones with CIRD, whereas *B. bronchiseptica* (16.7%), CHV-1 (3.0%), CPnV (6.1%), and *M. cynos* (1.5%) were detected in asymptomatic ones. Co-infection of *B. bronchiseptica* and *M. cynos* was detected in 2 dogs (14.3%) with CIRD. In this study, CDV, CrCoV, and CIV were not detected in any grouped dog.

CIRD is a common disease of dogs worldwide. In the past decade, several studies have reported the prevalence of newly emerging as well as traditional CIRD pathogens [6, 18, 19], and the results vary widely across countries [24]. In general, *B. bronchiseptica*, CPiV, CDV, and CAV-2 have been considered the main agents of CIRD [2, 9, 21]. In our current study, CPiV and CAV-2 nucleic acids were detected in 4.5% and 1.5% of private household dogs with CIRD, whereas CDV nucleic acid was not. In a previous study in Japan, in 2008 [21], CPiV, CAV-2, and CDV nucleic acids were detected in 7.4%, 2.9%, and 1.5% of household dogs, respectively. The extensive vaccination programs might have maintained a low circulation of these viruses in the dog population in Japan. However, *B. bronchiseptica* was the most frequently detected agent in private household dogs with CIRD. This pathogen was detected not only in dogs with CIRD but also in those with respiratory diseases other than CIRD. This suggested that *B. bronchiseptica* circulates widely across Japan, and causes primary CIRD as well as secondary infection leading to other respiratory diseases. Dogs in kennel environment could be sub-clinical carriers, acting as a source of infection for susceptible dogs. *B. bronchiseptica* can act as the primary CIRD pathogen, and its pathogenic potential is increased with simultaneous viral and bacterial infections [24]. Ten out of 61 dogs with CIRD, among private house dogs, showed co-infection of *B. bronchiseptica* with other pathogens, and this frequency was significantly higher than in other groups by χ^2 testing ($P<0.01$). Although inactivated intranasal *B. bronchiseptica* vaccine, combined with CPiV and CAV-2, is commercially available in Japan, no vaccinated dog in this study population had been administered with this product. To control this pathogen more efficiently, more positive administration would be recommended.

In our knowledge, prevalence of *M. cynos* and CPnV among the dog population in Japan has not been described till date. In our present study, 4.5% private household dogs with CIRD were found positive for *M. cynos*, with the same prevalence of CPnV. These pathogens were detected from asymptomatic kennel-housed dogs, thereby indicating that they mainly circulate among kennel or sheltered environment just like *B. bronchiseptica*. Because some of the symptomatic private household dogs had been already administered antibiotics at the sampling time, the actual positive rate of *B. bronchiseptica* and *M. cynos* might be higher than this result.

CRCoV and CIV nucleic acids were not detected in this study. CRCoV infection had been confirmed in previous studies with Japanese household dogs, and the detection rate in dogs with CIRD was 1.5% [21] and 16.0% [29], respectively. Recent studies from European countries have also reported high prevalence of CCoRV [19, 28]. Reasons for the negative results of CRCoV in our study remain unclear; continuous study using bigger population might be necessary. Till date, several subtypes of type A influenza virus have been isolated from dogs, which have caused CIRD in them across USA, China, Korea, and European countries [3, 14, 18, 30, 33]. These viruses have not been detected in Japan till date; therefore, CIV seems to not be an important pathogen so far for dogs in Japan.

In this study, we collected swab samples from dogs, throughout Japan. We found that there was no obvious geographical distribution of pathogens. Additionally, we collected kennel samples from 80 dogs of 12 kennels, where *B. bronchiseptica* was detected from 15 dogs in 4 kennels, CaPnV was detected from 4 dogs in one kennel, and *M. cynos* was detected from 3 dogs in another kennel (data not shown). These pathogens seem to spread in a facility with a dense population.

In conclusion, we have demonstrated *B. bronchiseptica* to be the most frequently detected pathogen among dogs with CIRD in Japan, followed by CHV-1, CPiV, CPnV, *M. cynos*, and CAV-2. Considering the increasing association of emerging viral and bacterial pathogens, continuous and frequent surveillance studies, using a larger population, is needed, which might eventually be useful for specific vaccination and treatment programs.

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Table 2. Numbers of dogs with muptiple infection

Pathogens	Household dogs									Kennnel housed dogs					
	CIRD n=61			Respiratory disease, not CIRD n=83			Asymptomatic n=23			CIRD n=14			Asymptomatic n=66		
	Positive no.	(%)	95%CI	Positive no.	(%)	95%CI	Positive no.	(%)	95%CI	Positive no.	(%)	95%CI	Positive no.	(%)	95%CI
Bordetella bronchiseptica	17	(27.9)**	17.1–40.8	8	(9.6)	4.3–18.1	1	(4.3)	0.1–21.9	2	(14.3)	1.8–42.8	10	(15.2)	7.5–26.1
CHV-1	1	(1.6)	0–8.8	1	(1.2)	0–6.5	0	(0.0)	0–12.2	0	(0.0)	0–19.3	2	(3.0)	0.4–10.5
CPiV	1	(1.6)	0–8.8	0	(0.0)	0–3.5	0	(0.0)	0–12.2	0	(0.0)	0–19.3	0	(0.0)	0–4.4
CPnV	1	(1.6)	0–8.8	0	(0.0)	0–3.5	0	(0.0)	0–12.2	0	(0.0)	0–19.3	3	(4.5)	0.9–12.7
B. bronchiseptica+CHV	3	(4.9)	1.0–13.7	0	(0.0)	0–3.5	0	(0.0)	0–12.2	0	(0.0)	0–19.3	0	(0.0)	0–4.4
B. bronchiseptica+CPiV	2	(3.3)	0.4–11.3	0	(0.0)	0–3.5	0	(0.0)	0–12.2	0	(0.0)	0–19.3	0	(0.0)	0–4.4
B. bronchiseptica+CPnV	2	(3.3)	0.4–11.3	0	(0.0)	0–3.5	0	(0.0)	0–12.2	0	(0.0)	0–19.3	1	(1.5)	0–8.2
B. bronchiseptica+*Mycoplasma cynos*	2	(3.3)	0.4–11.3	0	(0.0)	0–3.5	0	(0.0)	0–12.2	2	(14.3)	1.8–42.8	2	(3.0)	0.4–10.5
B. bronchiseptica+CPiV+CAV-2	1	(1.6)	0–8.8	0	(0.0)	0–3.5	0	(0.0)	0–12.2	0	(0.0)	0–19.3	0	(0.0)	0–4.4
M. cynos+CHV	1	(1.6)	0–8.8	0	(0.0)	0–3.5	0	(0.0)	0–12.2	0	(0.0)	0–19.3	0	(0.0)	0–4.4

CIRD, canine infecious respiratory disease; 95%CI, 95% confidence interval; CHV-1, canine herpes virus type-1; CPiV, canine parainfluenza virus, CPnV, canine pneumovirus, CAV-2, canine adenovirus type-2. **Significantly higher than respiratory disese dogs and asymptomatic dogs ($P<0.01$).

Table 3. Numbers of dogs with positive individual Canine infecious respiratory disease (CIRD) pathogens

Pathogens	Housed dogs									Kennnel housed dogs					
	CIRD n=61			Respiratory disease, not CIRD n=83			Asymotomatic n=23			CIRD n=14			Asymptomatic n=66		
	Positive no.	(%)	95%CI	Positive no.	(%)	95%CI	Positive no.	(%)	95%CI	Positive no.	(%)	95%CI	Positive no.	(%)	95%CI
Bordetella bronchiseptica	27	(44.3)**	31.5–57.6	8	(9.6)	4.3–18.1	1	(4.3)	0.1–21.9	4	(28.6)	8.4–58.1	11	(16.7)	8.6–27.9
CAV-2	1	(1.6)	0–8.8	0	(0.0)	0–3.5	0	(0.0)	0–12.2	0	(0.0)	0–19.3	0	(0.0)	0–4.4
CDV	0	(0.0)	0–4.8	0	(0.0)	0–3.5	0	(0.0)	0–12.2	0	(0.0)	0–19.3	0	(0.0)	0–4.4
CHV-1	5	(8.2)	2.7–18.1	1	(1.2)	0–6.5	0	(0.0)	0–12.2	0	(0.0)	0–19.3	2	(3.0)	0.4–10.5
CIV	0	(0.0)	0–4.8	0	(0.0)	0–3.5	0	(0.0)	0–12.2	0	(0.0)	0–19.3	0	(0.0)	0–4.4
CPiV	3	(4.9)	1.0–13.7	0	(0.0)	0–3.5	0	(0.0)	0–12.2	0	(0.0)	0–19.3	0	(0.0)	0–4.4
CPnV	3	(4.9)	1.0–13.7	0	(0.0)	0–3.5	0	(0.0)	0–12.2	0	(0.0)	0–19.3	4	(6.1)	1.7–14.8
CRCoV	0	(0.0)	0–4.8	0	(0.0)	0–3.5	0	(0.0)	0–12.2	0	(0.0)	0–19.3	0	(0.0)	0–4.4
Mycoplasma cynos	3	(4.9)	1.0–13.7	0	(0.0)	0–3.5	0	(0.0)	0–12.2	2	(14.3)	0–19.3	1	(1.5)	0–4.4

95%CI, 95% confidence interval; CAV-2, canine adenovirus type-2; CDV, canine distempervirus; CHV-1, canine herpesvirus type-1; CIV, canine influenza virus; CPiV, canine parainfluenza virus, CPnV, canine pneumovirus; CRCoV, canine respiratory coronavirus. **Significantly higher than asymptomatic dogs ($P<0.01$).

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