Relevant clinical signs and risk factors associated with pathogens of canine infectious tracheobronchitis

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Abstract

Canine infectious tracheobronchitis(ITB) is usually caused by many pathogens such as canine adenovirus type II (CAV-2). It is difficult to confirm the diagnosis without help from molecular biologists. It is thus important to understand the risk factors and the important clinical signs associated with ITB. 78 nasal swabs of dogs were analyzed by (reverse transcriptase) polymerase chain reaction to detect the pathogen of ITB. The results showed 39.75% of dogs suffered from CAV-2. By logistic regression and chi-square analyses, the significant risk factors associated with CAV-2 infection were brachycephalic breed ($p<0.05$) and both inspiratory and expiratory abnormal lung sound ($OR = 7.00$, $p<0.05$). If a dog suffered from more than two pathogens, cough ($p=0.002$), sneeze ($p=0.002$) and induced cough by pressing trachea ($p=0.025$) became significant. Up to 99.2% of CAV-2 infections could be successfully predicted by a combination of cough induced by pressing trachea, abnormal lung sound and sneeze.

Keywords: dogs, canine infectious tracheobronchitis, risk factors, clinical signs

1.0 Introduction

Canine upper respiratory tract infection is frequently seen in veterinary clinics; however, the true cause is hard to establish quickly. Generally, the main causes of canine upper respiratory tract infection include fungi, parasites, bacteria and viruses. Both bacterial and viral infections have very similar clinical signs so they are all grouped as kennel cough, orcanine infectious tracheobronchitis (ITB). Traditionally, only one bacterial pathogen, *Bordetella bronchiseptica* (*B.b*), is considered as the primary pathogen of ITB. Those known viral pathogens are canine parainfluenza virus type 2 (CPiV-2), canine respiratory coronavirus (CRCoV), canine adenovirus type 2(CAV-2), and canine herpes virus (CHV)(Buonavoglia & Martella, 2007). Recently, canine peumovirus, canine bocavirus, canine hepacivirus, *Streptococcus zooepidemicus* and *Mycoplasma cynos* are all considered as the pathogens of ITB(Priestnall, Mitchell, Walker, Erles, & Brownlie, 2014).

Kennel cough is a highly contagious infectious disease. The main target sites of these viruses are canine pharynx, trachea, bronchus, nasal mucous, and lower respiratory tract. Mostly, outbreaks are associated with direct dog to dog contact or contact with infected dog's secretion (Buonavoglia & Martella, 2007). Normally a viral pathogen plays the primary role and infects alveolar epithelial cells within the upper respiratory tract. After the villi will lose their function, bacterial pathogens can cause secondary infection (Richard, 2006). Its clinical signs include vomit, fever, depression, anorexia, dyspnea, sudden cough with/out purulent or mucous eye and nasal discharge (Richard, 2006). Cough can be induced by manually pressing the trachea. The carrier may still shed viral particles even after 2 weeks of infection(Richard, 2006).

Reports points out that ITB can be caused by multiple pathogens simultaneously or separately (Richard, 2006).The morbidity and mortality vary according to many factors such as the pathogen causing the disease, multiple or single pathogen present, the age of infection, the vaccination status, the prevalence of the pathogen in an area, the season of the disease occurring, the humidity, the ventilation, and the density of animals in an area(Richard, 2006). For example, the prevalence can be higher than 50% in dog shelters(Appel, 1987). In Japan, 20 out of 68 dogs with respiratory problems can be found to have pathogens including *Bordetella* spp., *B.b.*, CpiV, CcoV, CAV, CRCoV and CDV. The detection rate of CDV was the lowest(Jacobs et al., 2005).A previous report conducted a survey in a shelter and found that CHV's prevalence was high but CDV and CAV-2 were not found in 149 dogs with respiratory problems(Erles et al., 2004). The low prevalence of CAV-2 might be related to the high vaccination rate in the shelter (Erles et al., 2004). The high detection rate of CHV might be related to the character of herpes virus, which can remain latent in ganglia. The different detective rate of CPiV-2 might relate to subclinical or mild infection so the virus was kept in reservoirs(Erles et al., 2004).

Up to date, there are many commercial kits and molecular methods to help clinical veterinary practices to find the possible pathogen causing ITB; however, all diagnostic kits are not cheap and the results based on molecular methods need time. There is no any simple predictive method based on the clinical findings and related factors. It is necessary to develop a faster diagnostic method to predict the most possible pathogen and

then to find the proper kits or to begin the proper treatment. The aim of this study is to find the factors related to clinical signs and other risk factors, and then clinical veterinary practitioners can use the results to generate a better diagnosis.

2.0 Materials and Methods

2.1 Samples

After physical examination and background information recorded, nasal swabs and cell morphology smear were collected from 78 dogs from shelters and veterinary hospitals around Southern Taiwan, mainly in Chiayi City. The nasal swab was maintained in 0.5X phosphate buffered saline of 1mL. After the solution was mixed and then centrifuged, the supernatant was collected for DNA/RNA purification or stored at -20^{o}C for further detection.

2.2 Primer and DNA extraction

DNA/RNA in the supernatant of 200 μL was extracted by EasyPure Viral Nucleic Acid Extraction Kit (Bioman Scientific Co., LTD, Taiwan), PCR or RT PCR was performed immediately afterwards. The primers selected were listed in Table 1 (Erles, Toomey, Brooks, & Brownlie, 2003; Hozbor, Fouque, & Guiso, 1999; Huang, 2006; Mochizuki, Yachi, Ohshima, Ohuchi, & Ishida, 2008).

2.3 Reverse transcriptase polymerase chain reaction

A standard protocol of reverse transcriptase polymerase chain reaction was adopted. The extracted RNA of five μLwere mixed with DDW of 5.5μL and pathogenic specific primers of three μL (10μM) for 65^{o}C for five min. The mixed solution was then put on ice immediately. The solution was mixed with 5X reaction buffer of four μL, dNTP (10μM) of two μL, M-MuLV reverse transcriptase (200U/μL) of 0.5μL at 42^{o}C for 60 mins. After heating at 70^{o}C for ten mins, the cDNA was collected for the following DNA reaction. The solution (cDNA) of two μL was mixed with Fast-Run^{TM}Taq Master Kit (Protech Technology Enterprise Co., LTD, Taiwan) of five μL, reverse primer (ten μM) of one μL, specific forward and reverse primer (10μM) of one μL each, sterile H_2O of 16μL. All PCR used the similar protocol (Table 1): first denaturation at 94^{o}C for three mins, followed by denaturation at 94^{o}C for 30 sec, the annealing temperature was 58^{o}C for CRCoV, 56^{o}C for CPiV-2, 51^{o}C for CHV, 56^{o}C for CAV, 57^{o}C for CDV, 53^{o}C for *B.b*, and 53^{o}C for *B.* spp, respectively, these samples were then placed in extension at 72^{o}C for 40 sec for 39cycles. After 39 cycles, the final product was set at 72^{o}C for the final extension. The product was analyzed using two percent agarose gel and 100V for electrophoresis.

Table 1. Primers used in the study for amplification of canine coronavirus (CRCoV), canine parainfluenza virus type 2 (CPiV-2), canine herpes virus (CHV), canine adenovirus type 2 (CAV-2), canine distemper virus (CDV), *Bordetella* spp. (*B*. spp.), and *Bordetella bronchiseptica* (*B.b.)*.

Pathogen	Primers	Sequences	Locations	Products (b.p.)
CRCoV	Sp1	5'-CTTATAAGTGCCCCCAAACTAAAT-3'	(1637-1660)	622
	Sp2	5'-CTTACTGTGAGATCACATGTTTG-3'	(2258-2236)	
CPiV-2	CPiVF1	5'-TGCAATCCATCCTGTTCTCC-3'	(570-589)	531
	CPiVR2	5'-TACTCTTCCCTCATCTTCGG-3'	(1100-1081)	
CHV	CHVF1	5'-CACCTAGAGCATGTCCTAAC-3'	(233-252)	627
	CHVR1	5'- CCAAGCTGTAGTCTAGTATC-3'	(857-838)	
CAV-2	CAVF1	5'-GTGTGTCCCTACGTGTAC-3'	(13972-13989)	605
	CAVR1	5'-CAGTTCCTGCTTGATGGC-3'	(1100-1081)	
CDV	PF4	5'-ACTGGGGATATTCTTTCG-3'	(2300-2317)	541
	PR4	5'- TCAGTCTCTCCTTTAAGC-3'	(2840-2823)	
B. spp.	B13615F	5'-ATCGCTGGGATTACCCCTAC-3'	(13615-13634)	316
	B13930R	5'-TACTTGAGCGGTTCGAAGGT-3'	(13911-13930)	
B.b.	Fla 2	5'-AGGCTCCCAAGAGAGAAAGGCTT-3'	(275-297)	237
	Fla 4	5'- TGGCGCCTGCCCTATC-3'	(61-76)	

2.4 Restriction fragment length polymorphism

According to the method used by (HUANG, 2006), in order to distinguish CAV type I and II, it is necessary to add *Pvu*II (BioLabs®, Taiwan) of 0.5 μL, DDW of 2.5 μL, 10x NBE buffer of 2.0 μL, PCR product of 15.0 μL at 37°C for one hour. Electrophoresis was then run again. In the case of CAV-1 infection, 597 bppcrproduct would be cut into 417 bp and 180 bp. But if the case was CAV-2 infection, the band will be 605 bp as well.

2.5 Statistical analysis

Chi-square, Fisher's exact test, one variable logistic regression analysis packages were used to find the risk factors. The *p* value less than 0.05 was considered as significant.

3.0 Results

In total, 78 dogs were collected. 52.56% (41 dogs) were found in shelters and 37 dogs were pets. 18 out of 26 vaccinated dogs were pets (Table 2). 49 of the dogs were older than seven months and were considered as mature. 40 of the dogs were female and 38 of them male. 12 dogs had medical history of respiratory symptoms.

The number of dogs reported in each season (spring, summer, autumn & winter) was 11 out of 17, ten out of 11, 18 out of 21, and 18 out of 29 dogs, respectively. Statistically there was no seasonal difference (p=0.26). Other information was listed in Table 3. During physical examination, 73.07% (57 dogs) of them displayed clinical signs associated with ITB (Table 4). The results of (reverse transcriptase) polymerase chain reaction of the following pathogens: canine coronavirus, canine parainfluenza virus type two, canine herpes virus, canine adenovirus type two, canine distemper virus, and *Bordetella bronchiseptica* are listed in Figures 1 and 2. PCR test showed that CAV-2 had the highest detection rate that was 39.75% (31 out of 78 dogs), followed by CDV (Table 4).

Brachycephalic breed had a higher rate of CAV-2 infection than dolichocephalic breed (p=0.02) (Tables 3 and 5). The odds ratio (OR) for dogs displaying clinical signs was 3.23, compared with the dogs without clinical signs (p=0.02). The CAV-2 detection rate was seven times higher in dogs having abnormal lung sounds, than dogs whose lung sounded normal (Table 4). Compared with dogs from shelters, pet dogs are more likely to get CDV (OR = 11, p<0.05). In addition, CDV were more easily detected in dogs with clinical signs of fast breathing and shadow and crackle (Table 5).*Bordetella bronchiseptica was* more easily detected in dogs found in shelters, compared with pet dogs (p=0.05).

Only seven dogs had multiple pathogen infection (Table 6). Three of them had CDV and CAV. One had CAV and CHV. One dog had CDV and CPiV. One had CDV, CAV and CHV. One dog suffered from CAV and CHV and *B.b.* Dogs suffered from more than one pathogen would have more symptoms of cough and sneeze (p=0.002) and cough induced by pressing bronchus would be more likely to be present (p=0.03)(Table 5).

Table 2.The vaccination history and breed of dogs from animal hospitals and a shelter.

Item	Percentage (samples/total)	
	Pets	shelter
Vaccination history		
Yes	48.65 % (18/37)	19.51 % (8/41)
No	51.35 % (19/37)	80.49 % (33/41)
Breed		
Purebred	56.76 % (21/37)	29.27 % (12/41)
Crossbreed	43.24 % (16/37)	70.73 % (29/41)

Table 3. The background information of 78 dogs examined.

Characters	Percentage(Numbers)	Characters	Percentage(Numbers)
Weight		Gender	
< 10 Kg	46.15 % (36)	Complete male dogs	41.03 % (32)
10~20 Kg	15.38 % (12)	Neutering male dogs	7.69 % (6)
> 20 Kg	5.13 % (4)	Complete female dogs	41.03 % (32)
Unknown	33.33 % (26)	Neutered female dogs	10.26 % (8)
Breed		Active area	
Purebred dogs	42.21 % (33)	Outdoor free	12.82 % (10)
Crossbreed dogs	57.69 % (45)	Outdoor restrictions	64.10 % (50)
Collected periods		Indoor	23.08 % (18)
Spring (2~4)	21.79 % (17)	Vaccination	
Summer (5~7)	14.10 % (11)	Yes	33.33 % (26)
Fall (8~10)	26.92 % (21)	No	66.67 % (52)
Winter (11~1)	37.18 % (29)	Heartworm medication	
Snout		Yes	11.54 % (9)
Dolichocephalic	94.87 % (74)	No	88.46 % (69)
Brachycephalic	5.13 % (4)	The medical history of respiratory symptoms	
Age		Yes	15.38 % (12)
< 7 months	37.18 % (29)	No	84.62 % (66)
> 7 months	62.82 % (49)		

Table 4. The different numbers of pathogens, including canine distemper virus (CDV), canine adenovirus type 2 (CAV2), canine parainfluenza virus (CPiV), canine coronavirus (CRCoV), canine herpes virus (CHV), *Bordetella bronchiseptica* (*B.b.*), and *Brodetella* spp. (*B.* spp.), detected from dogs with/out relevant respiratory symptoms.

	CDV	CAV2	CPiV	CRCoV	CHV	*B.b.*	*B.* spp.
With symptoms (n=57)	9	27	2	0	4	2	2
Without symptoms (n=21)	0	4	0	0	0	0	1
Total	9	31	2	0	4	2	3

Symptoms include sneeze, purulent discharge, abnormal lung sound, abnormal respiratory pattern, fever, cough, and cough induced by pressing trachea.

Table 5.Assessment of risk factors and clinical signs for canine adenovirus virus type 2 and canine distemper virus using one-variable logistic regression. (N=78)

Risk factors	CAV-2 Positive/total	CAV-2 **Odds ratio (95% CI)**	Risk factors	CDV Positive/total	CDV **Odds ratio (95% CI)**
Species			Source		
Dolichocephalic	27/74	1b*	Rehoming center	1/41	1b*
Brachycephalic	4/4	0	Animal hospital	8/37	11.03(1.31-93.13)*
Symptoms			Respiratory pattern		
No	5/23	1a*	Normal	2/52	1
Yes	26/55	3.23(1.05-9.93)*	Quick & Slight	5/16	11.36(1.95-66.38) *
Lung sounds			Slow & Deep	2/9	7.14(0.86-59.13)
Normal	19/57	1	Slow & Normal	0/1	-
Inspiration abnormal	4/9	1.60(0.38-6.66)	Lung sounds		
Expiratory abnormal	1/3	1.00(0.09-11.74)	Normal	3/57	1
All abnormal	7/9	7.00(1.32-37.01)*	Inspiration abnormal	2/9	5.14(0.73-36.32)
			Expiratory abnormal	13	9.00(0.63-129.60)
			All abnormal	3/9	9.00(1.47-54.94)*

*：Significantly different (p<0.05). a：Chi-square test b：Fisher's exact test

Table 6. The p values of Fisher's exact test between pathogen(s) and the relevant clinical symptoms.

Pathogens	*P* value a	b	C	d	e	f
1 (n=3)	0.478	0.222	0.222	1	1	0.146
2 (n=1)	0.192	0.307	0.307	1	1	1
3 (n=1)	1	0.307	0.307	0.371	0.282	0.245
4 (n=1)	1	0.307	0.307	0.371	1	0.245
5 (n=1)	1	0.307	1	1	0.282	1
6 (n=7)	0.614	0.0028*	0.025*	1	0.395	0.055

1.CDV+CAV 2.CAV+CHV 3.CDV+CPIV

4.CDV+CAV+CHV 5.CAV+CHV+B.b. 6.All mix inf.

a. Fever b. Cough and Sneeze

c. Pressure trachea to induced coughing

d. Purulent discharge e. Lymph node enlargement

f. Inspiration and expiratory abnormal

*：Significant difference (p<0.05).

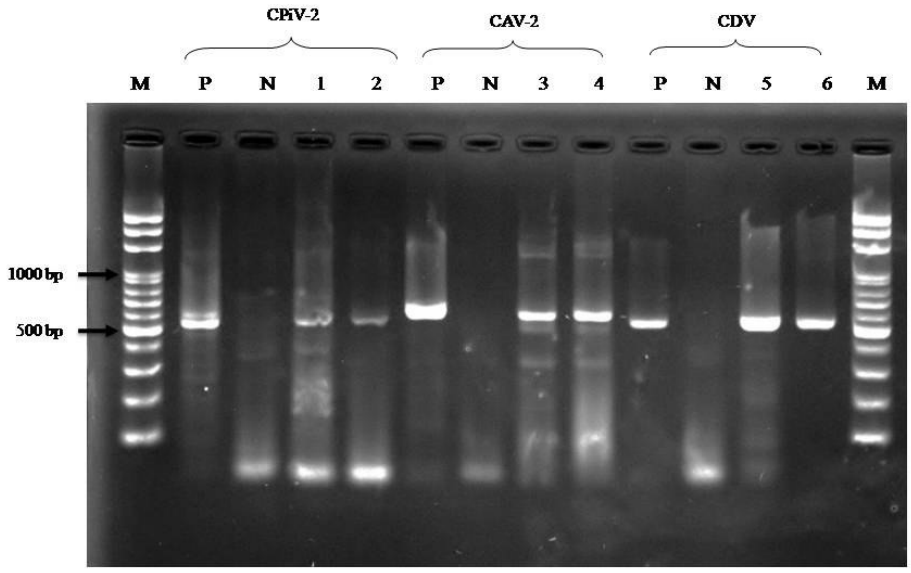

Fig. 1. PCR products of different specimens. Attenuated vaccine was used as the positive control of Canine Parainfluenza virus type 2 (CPiV-2), Canine adenovirus virus type 2 (CAV-2) and canine distemper virus (CDV).CAV-2 product has been processed with restriction Restriction fragment length polymorphism (RFLP) of *Pvu* II retriction enzyme. Lane M: 100 bp ladder marker, lane P: positive control, lane N: negative control, lane 1-2: CPiV-2 clinical specimens, lane 3-4: CAV-2 clinical specimens, lane 5-6: CDV clinical specimens.

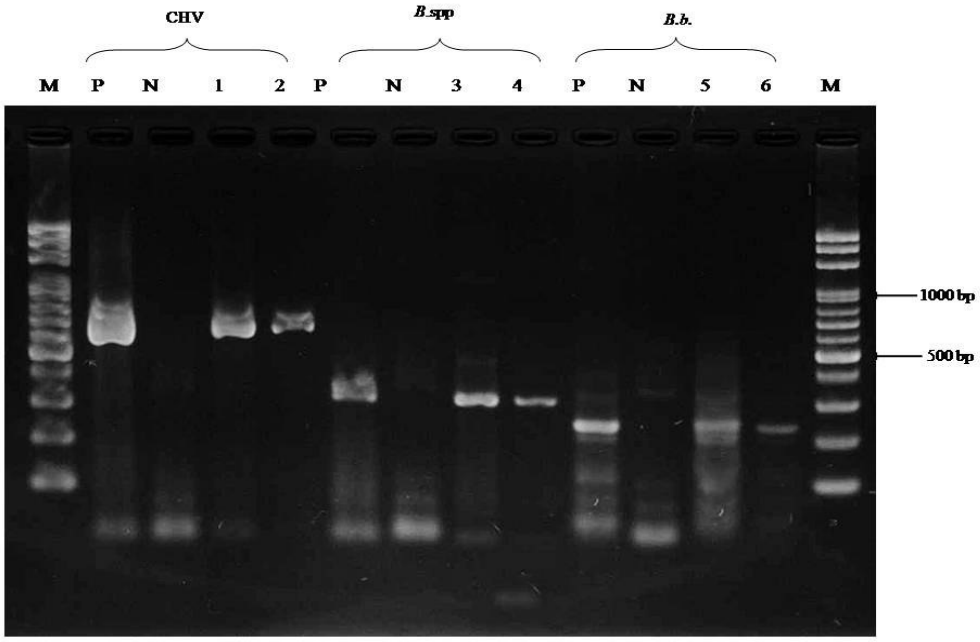

Fig. 2. PCR products of different specimens.No. 43 clinical specimens was used as the positive control of canine herpes virus (CHV).*Bordetellabronchiseptica*(*B.b*) was used as the positive control. Lane M: 100 bp ladder marker, lane P: positive control, lane N: negative control, lane 1-2: CHV clinical specimens, lane 3-4: *B*. spp. clinical specimens, lane 5-6: *B.b*. clinical specimens.

4.0 Discussion

One aim of this study was to investigate the risk factors and important clinical signs related to canine ITB, and then use the significant factors to predict the possible pathogen(s), which causes the disease and then a better treatment without further molecular biological test would be made as soon as possible.

It is generally believed that ITB has a seasonal pattern and mainly reported in summer and autumn (Richard, 2006); however, the result in this study did not show any season has a higher rate of ITB statistically. The temperature in Southern Taiwan is generally higher than 25^{o}C, and the temperature drops fast at night. The non-significant seasonal pattern but a significant drop in temperature every day might be a reason, which results in seasonal pattern is not significant.

The detective rate of different pathogen is not the same in this study. CAV-2 was detected in most of the cases. This was different from those studies conducted in Japan and UK where CPiV-2 was the main pathogen of ITB; it was considered that their low detection rate of CAV-2 was the result of vaccination (Erles et al., 2004; Mochizuki et al., 2008). However, results of this study showed that previous vaccination didn't affect the presence of pathogen. (p>0.05). CAV-2 was still detected in dogs, which had been vaccinated with CAV-2. This result was also supported by Edinboro's research where in private shelters, doses of vaccine did not relate to the presence of respiratory symptoms in dogs (Edinboro, Ward, & Glickman, 2004). The result of this study also supported the assumption that the current vaccine, which includes CDV, CPiV-2, and CAV-2, might not be enough to control the presence of upper respiratory symptoms (Willoughby & Dawson, 2001).

In this study, abnormal lung sound was significantly related to the presence of CAV-2 and CDV. The reason could be that lower respiratory system is the target site of CDV and CAV-2 and this symptom was related to lower respiratory system. Although Rodriguez-Tovar stated that CDV infection was related to CAV-2 infection (Rodriguez-Tovar et al., 2007) and simultaneous infections with CAV-2 and CDV would be possible, this relationship was not confirmed in this study. It might be the reason as well that the efficacy of the current vaccine might not be good enough (Willoughby & Dawson, 2001).

Although owners should have been aware of their pet's vaccination history, most owners were still unable to answer where, how long and what type of vaccines they have had. It was also possible that the protective ability of CAV-2 vaccine was low. This situation showed us that veterinarians should have explained more details to the owner.

Brachycephalic breed was a significant risk factor associated with CAV-2 infection (p=0.01). Brachycephalic airway syndrome usually results in upper airway obstruction (Lodato & Hedlund, 2012). Brachycephalic breed, such as bulldogs, and Pugs, have a short and narrow upper respiratory tract. In addition, their soft palates may be too long and may obstruct their respiratory tract. It remains a possibility that certain amount of pathogens could be retained in the airway and re-infect the target cells (Appel, 1987). Hence, more severe infection would be expected.

Canine distemper virus was the second highest detected virus. Pets from the animal hospital had a higher chance to be detected with the virus, compared with dogs from shelters (p=0.01). This was considered as a selection bias as that generally sick dogs would visit clinics. Pets which usually live in an open environment would have a higher chance of being attacked by pathogens (Murphy, 1999). However, stray dogs usually died because of different contagious diseases. After the sufferers went to shelters, they usually live in a closed environment where low prevalence of CDV was expected. Hence, the DNA detection of CDV was low in shelters. Thus, it is considered that the shelter is temporarily free of CDV at the sampling period.

In this current study, ITB is highly associated with cough induced by manually pressing trachea (p=0.025), cough and sneeze (p=0.002). This finding suggested that it is necessary for a veterinarian to use hands to manually pressing cough when a dog having clinical signs of cough and abnormal inspiratory/expiratory sound.

Environmental factor was not associated with *Bordetella bronchiseptica* infection in this study. However, generally it was believed that the infection rate would increase in crowded areas because the pathogen can survive up to 24 weeks, especially in high-humidity or wet areas (Appel, 1987; Goodnow, 1980) Cytology results showed that nasal swabs could still find a lot of bacteria (data not shown). However PCR showed that they were not *B.b*. Thus, cytology only offered limited information and were not used in this study (Meler, Dunn, & Lecuyer, 2008).

According to the results of uni-variable analysis, if a dog has symptoms of cough induced by pressing trachea, abnormal lung sound and sneeze, the detection rate of CAV-2 could go up to 99.2% and the observation could fit to the data published (Damian, Morales, Salas, & Trigo, 2005; Decaro et al., 2007; Huang, 2006).

5.0 Conclusion

In conclusion, this study found that CAV-2 is prevalent in Southern Taiwan. When a veterinarian faces a dog displaying clinical signs of cough induced by manual pressing trachea, sneeze, and abnormal inspiratory and respiratory sound, CAV-2 infection should be put on the first position.

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